Dako

PATHOLOGY

Education Guide | Immunohistochemical Staining Methods
Fifth Edition

Education Guide | Immunohistochemical (IHC) Staining Methods

Updated and Expanded Fifth Edition

Editors

George L Kumar PhD
Managing Editor / Scientific Communications Manager
Dako North America, Inc. | Carpinteria, CA 93013, USA

Lars Rudbeck PhD
Scientific Editor
Dako Denmark A/S | Glostrup, Denmark

Contributors

Thomas Boenisch MS
Retired Dako Consultant | Santa Barbara, CA, USA

Clive R. Taylor MD, DPhil, FRCPath, FRCP (Ir)
Department of Pathology and Laboratory Medicine
University of Southern California | Los Angeles, CA, USA

A.J. Farmilo PhD
Independent Consultant | Canada
(Formerly with Dako Canada)

Ronald H. Stead PhD
The Holburn Group of Companies
Bowmanville, Ontario, Canada

James F. Happel DLM (ASCP), HTL
Massachusetts General Hospital | Boston, MA, USA

Rashmil Saxena BFA, HT (ASCP)^{CM} and Sunil Badve MD, FRCPath
Clarian Pathology Laboratory
Department of Pathology & Laboratory Medicine
Indiana University | Indianapolis, IN, USA

Marc Key PhD
Celerus Diagnostics, Inc. | Carpinteria, CA, USA
(Formerly with Dako)

J. Paul Robinson PhD and Jennifer Sturgis BS
Purdue University Cytometry Laboratory
Purdue University | West Lafayette, IN, USA

Lars Rudbeck PhD, Henrik Winther PhD, Sussie Steen Jensen MS, Sven Müller PhD, Steen H. Matthiesen PhD, Kirsten V. Nielsen MS, Ulla Henriksen PhD, Andreas Schönau MS, EBA, Nanna K. Christensen PhD, Helle Grann Wendelboe MS and Ole Feldballe Rasmussen PhD
Dako Denmark A/S | Glostrup, Denmark

Lars Winther PhD
Independent consultant | Copenhagen Area, Denmark
(Formerly with Dako Denmark)

Dan Osborn
Omega Optical, Inc | Brattleboro, VT, USA

Robert M. Zucker PhD
U.S. Environmental Protection Agency
National Health and Environmental Effects Research Laboratory | Research Triangle Park, NC, USA

Kirsten Bisgaard BSc
Statens Serum Institut | Copenhagen, Denmark
(Formerly with Dako Denmark)

George L Kumar PhD, Gale E. Pace BS, Joachim Schmid PhD and Mark Verardo PhD,
Dako North America | Carpinteria, CA, USA

David C. Spaulding, PhD
Stellar Biotechnologies | Port Hueneme, CA, USA

Karen N. Atwood BS, MT (ASCP), CLS
Retired, Dako North America | Carpinteria, CA, USA

© Copyright 2009 Dako North America, Carpinteria, California.
All rights reserved. No part of this book may be reproduced, copied or transmitted without written permission. US $50

Table of Contents

Preface

Chapter 1 | Antibodies 1

Thomas Boenisch MS

(Revised by Betsy Spaulding, Henrik Winther PhD and Sussie Steen Jensen)

Immunoglobulins | IgG | IgM | Polyclonal Antibodies | Monoclonal Antibodies | Polyclonal Antibodies vs. Monoclonal Antibodies | Antibody Affinity | Antibody Cross-Reactivity | Antibody Reaction Rates | Antibody Stability | Handling of Antibodies | Receiving | Storage | Storage Containers | Storage Temperature | Use and Care

Chapter 2 | Basic Immunochemistry 11

Thomas Boenisch MS

Antibody Titer | Antibody Dilution | Antibody Incubation | Incubation Time | Incubation Temperature

Chapter 3 | Basic Enzymology 15

Thomas Boenisch MS

Enzymes | Suggested Procedures for Substrate-Cromogen Reagents

Chapter 4 | Immunohistochemical Standardization and Ready-to-Use Antibodies 21

Clive R. Taylor MD, DPhil, FRCPath, FRCP (Ir)

Introduction and Background: The Growing Consensus for 'Standardization' | Ready-to-Use (RTU) Reagents | Reagent Validation | Primary Antibody, Selection, Validation, and Optimization | Secondary or Labeling Reagents; Protocols | Controls | Impact of RTUs on Other Aspects of the Total Test | Summary

Chapter 5 | Fixation and Processing 29

A.J. Farmilo PhD and Ronald H. Stead PhD (Revised by A.J. Farmilo PhD)

Fixation | Tissue Handling | Alcoholic Fixatives | Specialized Tissue Preparations | Tissue and Slide Processing | Detailed Dewaxing Protocol

Chapter 6 | Tissue Identification in Immunohistochemistry 35

James F. Happel DLM (ASCP) HTL

Specimen Collection and Identification | Specimen Tracking at the Collection Site | Specimen Accessioning | Grossing and Prosection | Histology—Tissue Processing and Microtomy | Routine Staining and Coverslipping | Slide and Block Reconciliation with Specimen Requisition | Laboratory Asset Tracking and Work Flow Management | Do's and Don'ts

Chapter 7 | Tissue Microarray—Construction and Quality Assurance 43

Rashmil Saxena BFA, HT(ASCP)^{CM} and Sunil Badve MD, FRCPath

Advantages and Disadvantages | When Not to Use TMA's | Types of TMAs | Team Required for TMA Construction | Steps Involved in TMA Construction | TMA Analysis | Data Analysis

Chapter 8 | Demasking of Antigens 51
*eds George L Kumar PhD and Lars Rudbeck PhD**
**Incorporated from the Dako Education Guidebook—Demasking of Antigens (2008)*
Water Bath Methods | Pressure Cooker Heating | Autoclave Heating | Proteolytic Pre-treatment | Combined Proteolytic Pre-treatment and HIER | Combined Deparaffinization and Target Retrieval

Chapter 9 | Immunohistochemistry Staining Methods 57
Marc Key PhD
Immunohistochemistry: In the Beginning | Avidin-Biotin Immunohistochemistry | Polymer-Based Immunohistochemistry | Tyramide Amplification | Cycled Tyramide Amplification | Fluorescyl-Tyramide Amplification | Rolling Circle Amplification | Conclusion

Chapter 10 | Immunofluorescence 61
J. Paul Robinson PhD, Jennifer Sturgis BS and George L. Kumar PhD
Principle of Fluorescence | Limitations of IF Techniques | Applications of Immunofluorescence in Pathology

Chapter 11 | Preparation of FFPE Tissue Slides for Solid Tumor FISH Analysis 67
Sven Müller PhD, Steen H. Matthiesen PhD and Kirsten V. Nielsen MS
Composition of a Solid Tumor FISH Probe Mixture | Tissue Sample Preparation | FFPE Slide Preparation | Probe Hybridization and Stringency Wash | Mounting and Visualization | Conclusion

Chapter 12 | Filters for FISH Imaging 75
Dan Osborn
Filters and Fluorescent Imaging | Filter descriptions | Specialized filters for FISH and mFISH | Conclusion

Chapter 13 | Fluorescence In Situ Hybridization (FISH) Imaging 81
George L. Kumar PhD and Robert M. Zucker PhD
Optimization of the Microscope for Obtaining Good FISH Images | Numerical Aperture (NA) of the Objective | Köhler Illumination or Field Illumination of the Lens | The Coverslip | The Stability of the Light Source | Use of Laser Scanning Confocal Microscopes (LSCM) for FISH Imaging | Spectral Registration | FISH Colocalization | Standards | XY and Z Stage Drift | Other Factors | CCD Camera as a Detector for FISH Imaging | Improving FISH Images by 3D Deconvolution Microscopy | Deconvolution | Conclusion | Glossary and Appendix | Resolution | Sampling Frequency | Laser Power Meter

Chapter 14 | Dual Color CISH and FISH to CISH Conversion 97
Ulla Henriksen PhD, Sven Müller PhD and Andreas Schönau MS, EBA
Chromogenic In Situ Hybridization | The Principle of the CISH Procedure and FISH to CISH Conversion | Added Value of CISH | Applications of Dako DuoCISH™ | Conclusion and Future Perspectives for CISH

Chapter 15 | Multi-Staining Immunohistochemistry 103

Nanna K. Christensen PhD and Lars Winther PhD

Advantages of Multiple Staining | Examples of Multiple Staining | Technical Challenges Pre-treatment | Multi-Staining Method Selection | Selection of Dyes | Automated Image Acquisition and Analysis in Multiple Staining | Conclusion

Chapter 16 | Optimization of Immunohistochemical Reactions 109

Gale E. Pace (Revised and Updated)

Tissue Digestion Using Proteolytic Enzymes | Endogenous Enzyme Blockers | Protein Blocking Reagent | Antibody Diluents | Important Points to Consider Regarding Antibody Diluents | Wash Buffers | Tris-Buffered Saline | Phosphate-Buffered Saline (PBS) | Suggestions for Making Wash Buffers | Chromogen Enhancers for DAB

Chapter 17 | Background 115

Helle Grann Wendelboe MS and Kirsten Bisgaard BSc

Background Associated With Detection Methods | Double Staining | Biotin/Streptavidin-Based Detection Methods | Polymer-Based Detection Methods | Antigen Retrieval | General Factors | Natural and Contaminating Antibodies | Cross-Reactivity | Hydrophobic Interaction | Tissue Proteins | Antibodies | Ionic and Electrostatic Interactions | Complement-Mediated Binding | Miscellaneous Sources | General Aspects

Chapter 18 | Peptide Nucleic Acids: Robust Probe Hybridization Technology 123

David C. Spaulding PhD

Chapter 19 | Controls 127

Ole Feldballe Rasmussen PhD

Reagent Controls | Negative Controls | Tissue Controls | Processing Control Indicator | Cell Line Controls | Control Programs | Future Aspects

Chapter 20 | Virtual Microscopy and Image Analysis 131

Kenneth J. Bloom MD (Revised by Joachim Schmid PhD and Mark Verardo PhD)

The History | Scanning the Slide | Viewing the Virtual Slide | Capturing Images for Image Analysis | Image Analysis | Applications for Image Analysis in Pathology

Chapter 21 | Troubleshooting 137

Karen N. Atwood and Dako Technical Support Group

Section One: Inadequate Staining | General Background | Limited Background | Undesired Specific Staining | Miscellaneous | Section Two: Background Staining | Negative Control Reagent | Section Three: Tissue Specimen | Endogenous Blocks Section | Four: Using a Typical Specification Sheet for an IVD Antibody

Glossary 155

Index 160

Preface

Dako is pleased to introduce this fifth edition of "Immunohistochemical Staining Methods". The fifth edition builds upon a solid foundation of previous editions by integrating new chapters and techniques that further enhance the learning experience in immunohistochemistry (IHC). The fifth edition also introduces fluorescence in situ hybridization (FISH), a technique that is becoming widespread in the field of anatomic pathology. The elements of previous editions that have proved successful have been kept, while necessary changes, some obvious, others more subtle, have been made. The articles published in this updated edition have been carefully chosen to provide current information on IHC and FISH methods pertaining to preparation, staining and image analysis. Virtually every change to this edition is the result of thoughtful comments and suggestions made by colleagues who have used previous editions. Within the pages of this guide, you will find articles that address IHC theory, practical tips, step-by-step protocols and applications in a wide range of areas. New topics that have been added to this edition are: IHC Standardization; Tissue Identification; Peptide Nucleic Acids; Tissue Microarrays; Preparation of Tissue Slides for FISH Analysis; Dual Color CISH; Filters for FISH Imaging. The chapter on Antigen Retrieval has been completely revised and updated, giving the reader access to validated and standardized protocols. Other chapters that have been updated are on Antibodies; Multi-Staining IHC, Immunofluorescence and Virtual Microscopy & Image Analysis. We hope this edition will be as useful as the previous editions, and we ask that you share your experiences with us and with your colleagues.

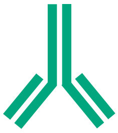
The task of bringing a new edition requires the talents and efforts of many dedicated people. On behalf of Dako, we would like to thank all the participating authors for making this new edition possible. We would like to specially thank the following individuals for contributions and suggestions:

Clive R. Taylor MD, Dr. Phil. FRCPath, FRCP (Ir). University of Southern California, Los Angeles, CA, USA. | James F. Happel DLM (ASCP) HTL. Massachusetts General Hospital, Boston, MA, USA. | Dan Osborn. Omega Optical, Inc., Brattleboro, VT, USA. | J. Paul Robinson PhD and Jennifer Sturgis, BS. Purdue University Cytometry Laboratory (PUCL), Purdue University, West Lafayette, IN, USA. | Sunil S. Badve MD. FRCPath, Rashmil Saxena, BFA, HT(ASCP)^{CM}. Clarian Pathology Laboratory, Indiana University, Indianapolis, IN, USA. | Robert M. Zucker PhD. US Environmental Protection Agency National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC, USA. | David C. Spaulding PhD. Stellar Biotechnologies, Port Hueneme, CA, USA. | Thomas Boenisch. Retired Dako Consultant and Researcher, Santa Barbara, CA, USA. | Betsy Spaulding MSc, Joachim Schmid PhD and Mark Verardo PhD. Dako R&D, Carpinteria, CA, USA. | Jon-Sverre Schanche PhD, Dave Stanforth MS, Ulla Henriksen PhD, Sven Müller PhD, Steen H. Matthiesen PhD, Andreas Schønau MSc, Nanna K. Christensen PhD and Henrik Winther PhD. Dako Global R&D, Glostrup, Denmark. | Helene Metz and Peter Staben. Dako Denmark A/S, Glostrup, Denmark. | Jaime Sullivan BS. Dako North America, Inc., Carpinteria, CA, USA. | Technical Support Group. Dako North America, Inc., Carpinteria, CA, USA.

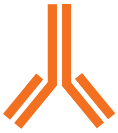
George L. Kumar, PhD, Managing Editor
Dako North America, Inc. | *Carpinteria, CA, USA.*

Lars Rudbeck, PhD., Scientific Editor
Dako Denmark A/S., Produktionsvej | *Glostrup, Denmark.*

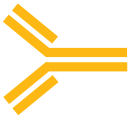
Legend for Schematics



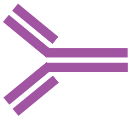
primary
antibody



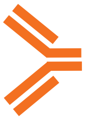
antibody



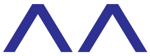
secondary
antibody



antibody



F(ab^1)^2
secondary
antibody



tissue
antigen

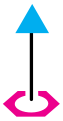
HRP
enzyme

AP
enzyme

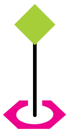
biotin
label

streptavidin

DBA



biotinyl
tyramide



fluorescein
label

Fast Red

polymer

Chapter 1 | Antibodies

Thomas Boenisch MS (Revised by Betsy Spaulding, Henrik Winther PhD, Sussie Steen Jensen MS)

The pivotal reagent common to all immunohistochemical* techniques is the antibody. The availability of new antisera, their immunoglobulin fractions and monoclonal antibodies to an ever-increasing number of clinically useful tissue antigens has enormously expanded the quantity and quality of the immunohistologic repertoire. To better comprehend the potential of immunohistochemical staining methods as well as associated problems, it is necessary to have a basic knowledge of antibodies, their potentials and their limitations.

Immunoglobulins

Antibodies belong to a group of proteins called immunoglobulins (Ig) that are present in the blood of immunized animals. The removal of cells and fibrin from blood is used to collect the serum fraction frequently referred to as antiserum. Listed in order of decreasing quantity found in plasma or serum, immunoglobulins comprise five major classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. Each is composed of two identical heavy chains (H) and two identical light chains (L). The H chains differ in antigenic and structural properties, and determine the class and subclass of the molecule. The two L chains are either of type kappa (κ) or lambda (λ). Distribution of κ and λ chains differs in all Ig classes and subclasses, as well as between different species. Covalent interchain disulfide bridges join L to H and H to H chains. By participating in the tertiary structure, they confer greater stability to the immunoglobulin molecule.

Of the five classes of immunoglobulins, IgG and IgM will be considered in more detail here, as these are by far the most frequently utilized antibodies in immunohistochemistry. Unless otherwise noted, most of what is described of the IgG structure in this text was learned from studies with human IgG of subclass IgG$_1$.

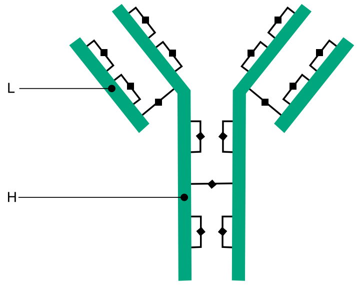


Figure 1. Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. Inter- and intrachain disulfide bonds (|—◆—|) contribute to the structure and stability of the molecule.

IgG

The heavy chains of IgG are denoted as gamma (γ) chains. IgG has the general formula of $\gamma_2\ \kappa_2$ or $\gamma_2\ \lambda_2$, which denotes that one molecule of IgG (MW = 150 kDa) is composed of two γ heavy chains, and two light chains of either type κ or type λ (Figure 1). The structure of the IgG molecule has been determined in part by proteolytic digestions and reductive dissociation of the molecule (Figure 2). Digestion by papain results in the cleavage of a susceptible bond on the N-terminal side of the inter-heavy chain disulfide bridges. This yields two monovalent antigen-binding fragments (Fab) and one crystalline fragment (Fc). Pepsin cleaves the γ chains on the C-terminal side of the inter-heavy chain disulfide bridges, resulting in one bivalent antigen-binding fragment, F(ab')$_2$. In this case, the Fc fragments are destroyed. Reductive dissociation of an IgG molecule splits the interchain disulfide bridges, and if the free sulfhydryl groups are blocked, results in the formation of two H chains (molecular weight 50 kDa each) and two L chains (25 kDa each).

---

*It should be understood that the term “immunohistochemistry”, as used in this chapter, denotes and includes the term “immunocytochemistry” also.

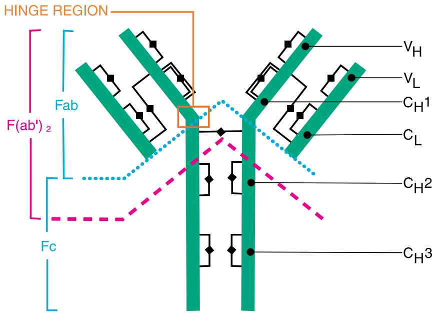


Figure 2. Diagram showing the structure of rabbit IgG (which exists as a single major subclass). The heavy (H) and light (L) chains are composed of variable (V) and constant (C) domains and are linked by inter- and intrachain disulfide bonds (|—◆—|). Proteolytic digestion with papain (•••••) yields two antigen-binding fragments (Fab) and one crystalline fragment (Fc), whereas digestion with pepsin (– – – –) yields one F(ab')$_2$ fragment.

The IgG molecule can be further divided into so-called domains, namely the variable domains (V) and the constant domains (C). Each domain contains 110 to 120 amino acids and one intrachain disulfide bond. On the variable domain of the light chain (V_L), and on the variable domain of the heavy chain (V_H), the amino terminals of the immunoglobulin molecule are located. Together, V_L and V_H form the antigen-combining site. Several hypervariable (HV) regions are located within the V_L and V_H domains of the antibody. During their reaction with antigens, HV regions are brought into close proximity to the antigenic determinant (epitope). The distance between the antigen and HV regions of the antibody is approximately 0.2 to 0.3 nm.

In this region, unique structural specificities called idiotypic determinants are located. Each antibody clone expresses its own idiotype. Each L chain also has one constant domain (C_L) in addition to the V_L domain. The H chain also has three constant domains (C_H1, C_H2 and C_H3) and carries the carboxyl terminal portion of the immunoglobulin. Located on the C_H2 domain is the carbohydrate moiety of the IgG molecule and several strongly hydrophobic neutral aromatic amino acids. The hinge regions are located between the C_H1 and C_H2 domains of the H chains. Minor differences within these hinge regions contribute to the subclass specificity of immunoglobulin G. The same are designated by subscripts as in IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$ and IgG$_4$. Whereas in human IgG the overall ratio of κ to λ is 2:1, in the subclasses IgG$_2$ and IgG$_4$, for example, the ratios are 1:1 and 8:1, respectively. Mice have approximately 95 percent κ chains, and therefore most monoclonal IgG antibodies from this species have κ chains. The number of disulfide bridges linking the heavy chains also varies among the IgG subclasses. IgG$_1$ and IgG$_4$ each have two, while IgG$_2$ and IgG$_3$ have four and five, respectively. Because of the flexibility of the hinge region, the angle that both Fab fragments form can vary to accommodate varying distances between identical antigenic determinants.

IgM

IgM is a pentamer (MW approximately 900 kDa) consisting of five subunits of approximately 180 kDa each (Figure 3). The general formula can be expressed as $(\mu_2\ \kappa_2)^5$ or $(\mu_2\ \lambda_2)^5$. Each subunit is linked by a sulfhydryl-rich peptide, the J chain (15 kDa), and consists of two heavy chains μ and two light chains of type κ or λ. The J-chains contribute to the integrity and stability of the pentamer. As with IgG, IgM subunits can be fragmented by enzymatic and reductive cleavage into F(ab')$_2$, Fab and Fc portions, as well as heavy and light chains, respectively. The Fc fragment of IgM is a cyclic pentamer (molecular weight approximately 340 kDa). Treatment of pentameric IgM with 0.1% mercaptoethanol cleaves the disulfide bridges between the subunits to yield five monomers. Subclasses of IgM$_1$ and IgM$_2$ have been reported.

Whereas IgG is the most abundant antibody in the hyperimmunized host, in the newly immunized animal, IgM is the first humoral antibody detectable. The primary antibody formation proceeds in several major stages. Injected immunogen first reaches equilibrium between extra- and intravascular spaces, then undergoes catabolism resulting in smaller fragments, and finally is eliminated from the intravascular spaces by the newly formed antibodies. The period from the introduction of an immunogen until the first appearance of humoral IgM antibodies is called the latent period and may last approximately one week. Within two weeks, or in response to a second injection, IgG class antibodies usually predominate. Like all proteins, antibodies are subject to catabolism. Whereas antibodies of class IgM have a relatively short half-life of only four to six days, IgG antibodies have a mean survival of approximately three weeks. Unless repeated booster injections with the immunogen are given, the serum antibody level will decrease after this period.

Antibody formation on the molecular level is a complex process, and a detailed account of it is beyond the scope of this guidebook. The interested reader is referred to the textbook Molecular Immunology by Atassi et al (1).

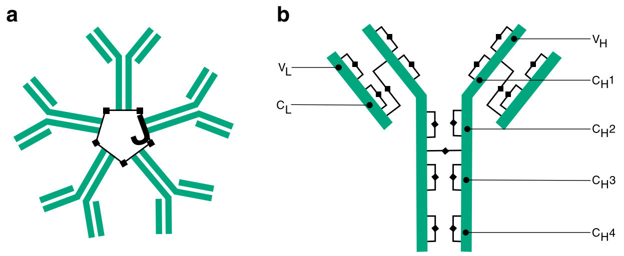


Figure 3. Diagram showing **(a)** the five subunits of mouse IgM linked by disulfide bridges (|—◆—|) and the J chain to form a pentameric ring structure. Each subunit **(b)** comprises two mu heavy (H) chains and two light (L) chains each composed of constant (C) and variable (V) domains.

Polyclonal Antibodies

Polyclonal antibodies are a heterogeneous mixture of antibodies directed against various epitopes of the same antigen. The antibodies are generated by different B-cell clones of the animal and as a consequence are immunochemically dissimilar. The antibodies in a polyclonal mixture can have slightly different specificities and affinities (see Antibody Affinity). Polyclonal antibodies are most frequently produced in rabbits but also made in other mammals including goat, swine, guinea pig and cow. Rabbits are frequently the species of choice in polyclonal antibody production due to the ease in maintenance of the animals and relative rarity of human antibodies to rabbit proteins when compared to other species such as goats. Additionally, rabbit antibodies precipitate human proteins over a wider range of either antigen or antibody excess. A long history of selective breeding for optimal immune response has made the New Zealand White rabbit the most frequently used animal in the production of polyclonal antibodies.

Polyclonal antibodies are produced in rabbits by immunizing with antigen (also known as immunogen) using doses ranging from 10 ug to 200 ug. Immunization is typically performed intradermally or subcutaneously, but can also be made into the footpad, muscle or peritoneal cavity. Antigen can be prepared with or without an adjuvant such as Freund's Complete or Incomplete Adjuvant which can boost the immune response. For smaller or less immunogenic proteins or peptides, the immunogen can also be coupled to carrier proteins such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA) and purified protein derivative of tuberculin (PPD). The immunization period lasts from 3 to 8 months and the animal is usually boosted with injections of immunogen on a biweekly basis. Blood is collected from the ear of rabbits, jugular vein of larger animals or from the heart through sacrifice of the animal. Serum is prepared by removing cells from the blood via centrifugation and the polyclonal antibody preparation can be used in the form of stabilized antisera or further purified. Purification of the immunoglobulin; to eliminate other serum proteins can be performed through ammonium sulfate precipitation and ion exchange chromatography, Protein A or G purification or affinity isolation.

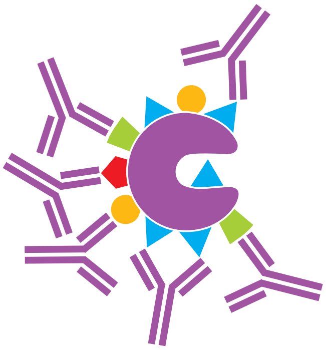


Figure 4. Schematic diagram of polyclonal antibodies binding to various epitopes on an antigen.

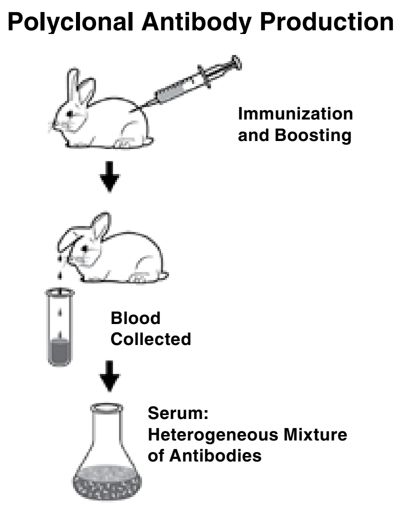

Polyclonal Antibody Production
Immunization and Boosting
Blood Collected
Serum: Heterogeneous Mixture of Antibodies

Figure 5. The process of polyclonal antibody production.

Monoclonal Antibodies

Monoclonal antibodies are a homogeneous population of immunoglobulin directed against a single epitope. The antibodies are generated by a single B-cell clone from one animal and are therefore immunochemically similar. Monoclonal antibodies are most commonly produced in mice and rabbits, but may also be produced in other species such as rat and camel. The animals are immunized using intraperitoneal injections of an immunogen and boosted every two weeks over a period of two months for mice and two-four month for rabbits. The animal's immune response is monitored through periodic testing of the serum. Upon achieving an acceptable immune response, the animal is sacrificed and the B-lymphocytes are isolated from the spleen and fused with an immortal cell line (myeloma cell line/fusion partner). The B-lymphocytes confer the capacity to produce specific immunoglobulin while the fusion partner cell line enables immortality and indefinite growth in culture. The fused and immortalized cell line is called a hybridoma. The hybridoma cell line is cultured, selected and sub-cultured by limiting dilution to isolate a stable clone with a high antibody production capacity. For production of tissue culture supernatant, the hybridoma cell line is cultured in multiple tissue culture flasks. Bioreactors can be used for large scale hybridoma growth and antibody generation. A bioreactor is a system that continually replenishes cells with fresh media and promotes growth for cultivation of concentrated amounts of antibody. Ascites fluid is produced by injecting the hybridoma into the peritoneal cavity of an animal (usually of a mouse) which forms a tumor. Immunoglobulin is secreted into the peritoneal cavity and ascites fluid is extracted. Ascites fluid has a very high concentration of antibody compared to tissue culture supernatant; however contaminating antibodies will be present in ascites preparations. Ethical considerations involving the treatment of animals lead some to avoid ascites fluid production in favor of alternative methodologies. Monoclonal antibody preparations can be used as either stabilized tissue culture supernatant or ascites fluid. Further purification of immunoglobulin can be performed through the use of protein A or G chromatography or affinity purification.

While the vast majority of monoclonal antibodies are produced in mice, a growing number of rabbit monoclonal antibodies are being manufactured. The use of rabbits for monoclonal antibody production confers some advantages over mouse monoclonals. These advantages are also shared by rabbit polyclonals. Rabbits are reported to have more diverse epitope recognition than mice (less immunodominance) and an improved immune response to small-sized epitopes. There is also a tendency with rabbits to generate antibodies with higher affinity and overall avidity. The resulting rabbit antibodies also have enhanced binding properties due to heavy glycosylation. Mouse hybridomas however tend to generate a higher yield of immunoglobulin than rabbit hybridomas and the mouse hybridoma cell lines are typically more stable in culture.

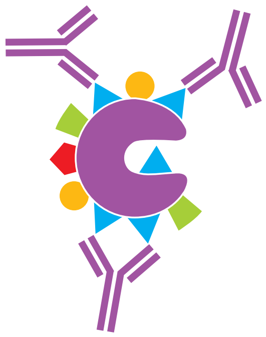


Figure 6. A given clone of monoclonal antibodies reacts with a specific epitope on an antigen.

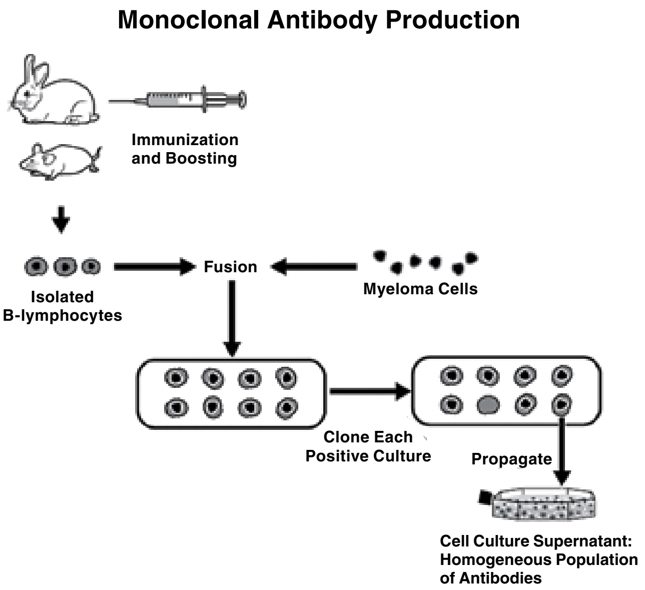

Monoclonal Antibody Production
Immunization and Boosting
Isolated B-lymphocytes
Fusion
Myeloma Cells
Clone Each Positive Culture
Propagate
Cell Culture Supernatant: Homogeneous Population of Antibodies

Figure 7. The process of monoclonal antibody production.

Polyclonal Antibodies vs. Monoclonal Antibodies

When comparing the advantages and disadvantages of polyclonal and monoclonal antibody preparations, there are benefits to both. Due to their multiclonality, polyclonal antibodies are typically more robust reagents when used on routinely-processed tissue specimens. The ability of a polyclonal reagent to recognize multiple epitopes on a single molecule means that the reagent is not as subject to the deleterious effects of pre-analytic processing of patient specimens, as are monoclonal antibodies which are directed against a single epitope. The presence of antibodies to multiple epitopes however can increase the chance for cross-reactivity (see Antibody Cross-Reactivity) with other proteins.

Monoclonal antibodies have the advantage of lot-to-lot consistency and lack the inherent variability of polyclonal antibodies due to the immunological state of the animal. The use of a hybridoma in monoclonal antibody production enables a sustained production of antibody and is not dependent on the life of the animal as with polyclonal antibody production.

Antibody Affinity

Antibodies from hyperimmunized animals not only differ with regard to the determinants they recognize on multivalent antigens, but also differ in their affinities for the same. The term “affinity” has been used to describe both intrinsic and functional affinities (4).

The intrinsic affinity of an antibody resides in the HV region and is determined by the same sequence of amino acids that determines specificity. Primarily ionic (electrostatic) interactions, but also hydrogen bonding and van der Waals forces are the major contributors to the intrinsic affinity between the paratope on the antibody and the epitope on the antigen. Hydrophobicity forms last and has a stabilizing effect on the formed immune complex, and with soluble reactants usually leads to its precipitation. Covalent binding between antibody and antigen does not occur. The association constant (Ka) of the binding between an antibody and its antigenic determinant is a measure of the antibody’s affinity. It can range from 10^3 to 10^{10} liters per mole and is the reciprocal of concentration in moles per liter. The higher the intrinsic affinity of the antibody, the lower the concentration of the antigen needed for the available binding sites of the antibody to become saturated (reach equilibrium). Just as the quantity (titer) of an antibody increases with time during immunization, so does its quality (affinity). This has been called “affinity maturation” (5). Lower doses of immunogen increase the rate of affinity maturation, but may result in lower titers of antibody, and vice versa.

In immunohistochemistry, the functional affinity of an antibody or an antiserum can be very loosely defined by the time required to reach equilibrium with the tissue antigen. If equal aliquots of two antibodies or antisera of identical titer are incubated for increasing periods of time with the antigen on the tissue, the antibody that reaches a plateau of maximum staining intensity first is of a higher functional affinity. The term “avidity” has been used synonymously to describe functional affinity (5), but has also been used to denote the strength of the binding reached between antibody and its antigen (6). The term avidity has also been used to describe the sum total of all intrinsic affinities found in a polyclonal antibody population.

Because antigen-antibody reactions are reversible, the simple immune complexes formed on tissue may occasionally dissociate during the washing cycles used in immunohistochemistry. The ease and extent of this dissociation vary from antibody to antibody, and low salt concentrations as well as low temperatures will reduce the likelihood of weak staining due to dissociation of an already formed immune complex. Thus, high affinity antibodies are desirable and have the advantage that during washing, dissociation is less likely to occur than with low-affinity antibodies. As mentioned before, a polyclonal population of antibodies contains a more or less continuous spectrum of low to high affinities against several epitopes on a given antigen. Therefore after incubation with a primary antibody of this type, excessive washing is unlikely to result in any appreciable loss of staining.

On the other hand, monoclonal antibodies are of uniform affinity and, if the same is low, loss of staining may be due to the dissociation of the antibody from its epitope. Therefore monoclonal antibodies of high affinity should be selected, if possible. As indicated above, factors that weaken the antigen-antibody bond such as high salt concentrations, high temperature and very low pH during the washing of the specimens should be avoided. Experience in the handling of antibodies in immunohistochemistry has shown that the washing and incubation in buffer baths can be safely reduced and that gentle agitation helps to reduce background staining (7).

The affinity of antibodies is also related to their capacity to form insoluble immune complexes. Generally, the higher the affinity of an antibody, the greater its tendency to form a precipitate. Precipitation proceeds through a rapid stage in which soluble antigen-antibody complexes form, followed by slower aggregation and, eventually, precipitation. Non-precipitating antibodies are mostly of lower affinity and are incapable of forming the lattice required for precipitation to occur.

Monoclonal antibodies, regardless of whether they are of high or low affinity, do not form a lattice with antigen, and, hence only rarely form insoluble precipitates. However, in immunohistochemistry, the capability of a primary antibody to form precipitating immune complexes is of little importance because reaction with immobilized tissue antigen entails antibody capture onto tissue rather than precipitation.

Prozone is a property which was first noted in antibody-induced agglutinations. It is the observation that some antibodies, when insufficiently diluted, fail to agglutinate cells even though higher dilutions will do so. While prozone can also be observed in precipitin reactions, in immunohistochemistry, it is a rare event (7).

As most antibodies carry a net positive electrostatic charge, the strength of the antibody's affinity for the targeted tissue antigen also depends on the availability and abundance of the net negative electrostatic charges present on the latter. Excessive formalin-fixation times of many tissues were largely held responsible for alteration of these charges, and as a consequence, for the unpredictably erratic immune reactivity with the primary antibody. Lost affinities were, however, largely restored by the routine use of heat-induced retrieval for all antigens (8).

Antibody Cross-Reactivity

The term "cross-reactivity" denotes an immunochemical activity that can occur either between an antibody and two or more antigens or vice versa, when an antigen reacts with several different antibodies. Typical examples are when anti-L (or -K) chain antibodies interact with all five Ig classes or when carcinoembryonic antigen (CEA) reacts with antibodies against CEA, blood group antigens and normal tissue proteins, respectively. The common denominator in each case is the sharing of at least one common epitope between several antigens.

Another valid use of the term cross-reactivity denotes the experimentally or accidentally induced changes within one or several epitopes, through antigen retrieval (9), leading to a possible loss of specificity by a given monoclonal antibody for this antigen. The term cross-reactivity also describes the interaction of an antibody with similar or dissimilar epitopes on unrelated antigens. This latter phenomenon however is frequently a property of low affinity antibodies, and is usually subject to change because of affinity maturation during immunization.

Cross-reactivity of antibodies to human antigens with identical or similar antigens of other species, or "cross-species cross-reactivity," can be of interest to the researcher and veterinarian because of the scarcity of animal-specific antibodies. To overcome this, two publications reported the results of cross-species reactivity studies using commercially available antihuman polyclonal and monoclonal antibodies (10, 11). It was demonstrated that the majority of animal antigens selected showed strong reactivity with antihuman antibodies. However, for more technical detail on the use of a given mouse primary antibody on animal tissues, the reader is referred to animal research kit products.

The terminology of cross-reactivity however is misplaced when describing any observed staining by the same antibody of different cells or tissue components, regardless whether they contain common antigens, as this would distort the strict immunochemical definition of the term.

Antibody Reaction Rates

Although under ideal conditions antibodies react with their antigens very rapidly, in immunohistochemistry the conditions are rarely ideal. Depending on length of tissue fixation, antibody concentration, ambient temperature and other variables, primary antibody incubation times of up to 48 hours may be required for maximum reactivity (12). It is not surprising therefore, that as immunohistochemical procedures have become increasingly useful in surgical pathology, the need for shortened processing times has also been voiced. Very short incubation periods are made feasible by the relatively rapid reaction rates that occur when higher concentrations of high-affinity primary and link antibodies are used.

In these situations equilibrium between antigen-bound and free antibody is rarely achieved. To achieve equilibrium, very long incubation periods with more dilute antibody preparations are required. It is not known whether shorter incubations with more concentrated antibody preparations would establish equilibrium sooner, because as a rule nonspecific background staining may result under these conditions, preventing unambiguous interpretation. Incubates of primary antibody have been salvaged experimentally after their first use by aspiration from one section, and transferred to additional sections (7). With some antibodies, up to seven identical tissue specimens could be stained with equal quality when the primary antibody was used in concentrations required for routine 10-minute incubations. This suggests that only a very small fraction of the available antibody is actually utilized during these relatively short incubation times. Needless to say, once an incubation time has been selected, it must be maintained uniformly, or staining will not be consistently reproducible.

Generally, the size and shape of the antibody molecule and its conjugates or complexes appear to be of little consequence in immunohistochemistry. Insufficient tissue penetration, even when staining intranuclear or cytoplasmic antigens, has never been observed, regardless of whether primary antibodies of class IgM (900 kDa), large complexes like PAP (400–430 kDa) or APAAP (approximately 560 kDa) or dextran-linked reagents were used (see Chapter 10, Immunohistochemistry Staining Methods). However, it is reasonable to assume that gross overfixation of tissue may make penetration more difficult for antibodies and their complexes.

Antibody Stability

Polyclonal antibodies, when stored unfrozen and used subsequently in immunohistochemistry, are somewhat less stable as immunoglobulin fraction compared to whole antiserum (7). However, this reduced stability was found to depend largely on the method of purification and storage as well as on the method of application. Exposure of antibodies to extreme pH, as well as high or very low concentrations of salts during purification tends to decrease their stability more than does exposure to mild conditions such as ion exchange chromatography. Formation of soluble aggregates, and subsequently precipitated polymers, are the most frequently resulting changes noted after prolonged storage. These changes are probably the result of hydrophobic interaction between the IgG molecules in solution. While the presence of soluble aggregates may enhance their performance as precipitating antibodies, their increased hydrophobicity has been shown to cause increased nonspecific binding in immunohistochemistry (see Chapter 16, Background) (7). Removal of these aggregates and polymers from IgG fractions is therefore prudent prior to their application for immunohistochemistry.

Just as storage of purified antibodies may augment their hydrophobicity due to aggregation and polymerization, so may their conjugation to other molecules (13). Conjugation with glutaraldehyde involves the epsilon-amino groups of lysine and alpha-amino groups of the N-terminal amino acids resulting in their cross-linking. Because there are many glutaraldehyde-reactive sites in IgG molecules, the hydrophobicity of the conjugated antibodies may increase significantly, resulting in augmented attraction to hydrophobic sites in the fixed tissue and increased background.

Monoclonal antibodies also have been shown to be influenced in their performance by methods of purification and storage; 42 percent of monoclonal antibodies investigated by Underwood and Bean showed changes in specificity, affinity and cross-reactivity (14). Antibodies of class IgM and subclass IgG_{2b} were especially sensitive.

It must be noted that actual-time testing of proteinaceous reagents is not feasible. While commonly practiced in the pharmaceutical field (15, 16), high-temperature accelerated degradation testing when applied to immunochemicals such as antisera and antibodies can be irrelevant or even misleading (17, 18).

Antibody stability in commercially produced reagents is determined best by real-time and real-temperature testing by each manufacturer. Most manufacturers demonstrate stability by testing during a pre-determined period of time, ie, the “shelf life”.

In addition, it is this writer’s experience that the conditions for the storage of reagents in the user’s laboratory are frequently not identical to those that prevailed during the manufacturer’s shelf life studies. Because of the possibility of adverse storage conditions after the purchase of the product, the manufacturer can only offer a limited liability instead of predicting the actual demise of a reagent.

The only possible corollary to these requirements is to allow laboratories to document the activity of the product until the loss of the same. Alternatively, laboratories may aliquot and freeze undiluted antibody at –20 °C for later use. At this time, laboratories must confirm activity prior to the use of the antibody in any test.

Finally, expiration dating as practiced today also serves the purpose of conforming to regulatory requirements. Regulatory guidelines in place in the United States for clinical laboratories have been mandated by the Clinical Laboratory Improvement Act of 1988 and by the College of American Pathologists. These regulations mandate that expired reagents cannot be used in the clinical diagnostic laboratory on human tissue.

Handling of Antibodies

In order to achieve optimal performance from reagents used in immunohistochemistry, it is imperative to observe basic rules for their handling and storage. If properly maintained, most reagents will remain stable for months or even years. Recommendations given by the manufacturer on specification sheets and on vial labels should always be heeded.

Receiving

Although many commercially produced immunochemicals are guaranteed to be stable for up to several years, ready-to-use (RTU) antibodies have a shorter shelf life (see Antibody Stability). Upon receipt, immunochemicals should be promptly stored according to the manufacturer’s recommendations. Log reagents by entering the manufacturer’s lot numbers, expiration date, date of receipt and invoice number. These entries provide valuable information for the user, especially if later reclamations should become necessary.

Storage

Perhaps the two most important considerations when storing antibodies are the storage container and the temperature.

Storage Containers

Ideally, preferred materials for storage containers of protein solutions should have negligible protein adsorptivity. Polypropylene, polycarbonate or borosilicate glass are recommended and are used widely. Solutions containing very low concentrations of protein (ie, less than 10-100 µg/mL), should receive an addition of immunochemically inert protein. Generally, 0.1% to 1.0% bovine albumin is used to reduce loss through polymerization and adsorption onto the container. Containers made of clear and colorless materials are preferred, as these will allow ready inspection of contents. Container labels also should allow access for inspection.

Storage Temperature

Probably more than any other factor, observe proper storage temperature as recommended by the manufacturer. Monitor refrigerators and freezers used for storage of immunochemicals for accurate and consistent temperatures. Store valuable or large quantities of immunochemical reagents in equipment with temperature alarm and emergency back-up power systems.

Store most RTU antibodies and their conjugates solutions at 2-8 °C, because freezing and thawing is known to have a deleterious effect on their performance. This also applies to entire kits that contain ready-to-use reagents, including monoclonal antibodies. Store concentrated protein solutions such as antisera and immunoglobulin fractions in aliquots and frozen at –20 °C or below, in order to prevent cycles of repeated freezing and thawing. Bring frozen protein solutions to room temperature slowly, and avoid temperatures above 25 °C.

Use and Care

Proper reagent care can reduce problems stemming from contamination, heat or excessive light exposure. Reagent contamination can be avoided by the use of clean pipet tips. Prompt return of reagents to proper storage conditions will prolong their shelf life.

The appearance of immunochemical reagents, particularly undiluted antisera, is not always indicative of their performance. Although beta-lipoproteins have a strong hydrophobic property, neither lipemia nor lipolysis in antisera has been studied systematically for interference with immunohistochemical staining. Where obvious lipemia is encountered in an antiserum and thought to be the cause of interference with successful staining, removal of the lipids by use of dextran sulfate and calcium (19), or by extraction with organic solvents is recommended. Alternatively, the addition of 2 g Aerosil (Degussa, NY) to 100 mL antiserum followed by incubation for four hours at 37 °C has proven useful.

Mild to moderate hemolysis in antiserum resulting from sub-optimal bleeding techniques probably does not interfere with most immunohistochemical staining procedures, but excessive hemolysis should be avoided. If excessive hemolysis or lipemia is encountered, isolation of the immunoglobulin fraction from the antiserum may be necessary. Such isolates will usually appear colorless and clear. Discard all immunochemicals, including antisera and normal non-immune sera contaminated with bacterial growth. Their use in immunohistochemical procedures most likely will introduce artifacts and nonspecific staining.

Familiarity with the nature of antibodies, their capabilities and limitations, will allow the user to better utilize these reagents and to more efficiently solve problems, if they occur. The following chapters will further contribute to the understanding of antibodies and also provide detailed information about the ancillary reagents and procedures used in immunohistochemistry.

References

1. Atassi MZ et al. Molecular Immunology. Marcel Decker, Inc. New York, 1984.
2. Harboe NMG and Ingild A. Scand J Immunol 1983;17:345-51.
3. Boenisch T. Appl. Immunohistochem 1999;7(4):300-6.
4. Hornick CL and Karush F. Immunochemistry 1979;9:325-40.
5. Steward MW and Steensgaard J. Antibody Affinity: Thermodynamic Aspects and Biological Significance. Boca Raton: CRC Press, 1983.
6. Herschowitz HID Immunophysiology: Cell function and cellular interactions in antibody formation. In Bellanti JA. Immunology III. Philadelphia: Saunders, 1985.
7. Boenisch T. Appl Immunohistochem 2001;9(2):176-9.
8. Boenisch T. Appl Immunohistochem 2005;13(3):283-6.
9. Alexander J and Dayal Y. Appl Immunohistochem 1997;5(4):252-3.
10. Smith RA. J Histotech 1990;13(4):255-69.
11. Martin CA and Badrán AF. Appl Immunohistochem 1998;6(2):84-8.
12. Boenisch T. Appl Immunohistochem 2002;10(4):363-7.
13. Sternberger LA. Immunocytochemistry (2nd ed). New York: Wiley, 1979.
14. Underwood PA and Bean PA. J Immunol Meth 1985;80:89-97.
15. Kirkwood TBL et al. Biometrics 1977;3:38-48.
16. Tydeman MS and Kirkwood TBL. J Biol Standard 1984;12:195-206.
17. Jackson CM. IVD Technology 1997;3:38-48.
18. van Erp R et al. J Biotech 1991;20:249-62.
19. Kim YC and Nishida TJ. Biol Chem 1979;254:9621-26. 64.

Chapter 2 | Basic Immunochemistry

Thomas Boenisch MS

In immunohistochemistry (IHC), antibody titer and dilutions as well as incubation time and temperature are tightly interwoven in their effect on staining quality. These factors can be changed independently, or as is more often the case, in complementary fashion to bring about positive differences. Generally, when making changes the overriding goal should be to achieve optimal specific staining accompanied by minimal interference from background staining. This chapter will highlight these variables.

Antibody Titer

Optimum antibody titer may be defined as the highest dilution of an antiserum (or monoclonal antibody) that results in maximum specific staining with the least amount of background under specific test conditions. This highest dilution is determined primarily by the absolute amount of specific antibodies present.

With polyclonal antisera, antibody titers have traditionally been expressed as micrograms of antigen precipitated per milliliter of antiserum. While this is of interest, it is not necessary information to the immunohistochemist. Augmenting polyclonal antisera titers by isolating and enriching immunoglobulin fractions produces little benefit for immunohistochemical applications, because nonspecific antibodies and soluble aggregates — frequent sources of nonspecific background become enriched also (see Chapter 16, Background). For monoclonal antibody preparations, the absolute concentration of specific antibodies can be readily determined, and frequently forms the basis for making required dilutions.

An optimal antibody dilution is also governed by the intrinsic affinity of an antibody. If the titer is held constant, a high-affinity antibody is likely to react faster with the tissue antigen and give more intense staining within the same incubation period than an antibody of low affinity.

In more practical terms, titers may vary from 1:100 to 1:2000 for polyclonal antisera, from 1:10 to 1:1,000 for monoclonal antibodies in cell culture supernatants, and up to 1:1,000,000 for monoclonal antibodies in ascites fluid. These dilutions may likely be exceeded in the future due to ever-increasing sensitivities of newer detection methods, including the use of an appropriate antigen retrieval procedure.

Antibody Dilution

Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. Often a manufacturer offers ready-to-use (RTU) reagents ready for use, or recommends dilution ranges compatible with other variables such as method, incubation time and temperature. If this information is not provided, optimal working dilutions of immunochemical reagents must be determined by titration. Correct dilutions are best determined by first selecting a fixed incubation time and then by making small volumes of a series of experimental dilutions. Depending on specimen size, applications of 0.1 0.4 mL of solution per section is generally adequate. It should be noted that at least on paraffin sections optimal dilutions of primary antibodies are not only signaled by a peak in staining intensity, but also by the presence of minimal background (maximal signal to noise ratios). Once the optimal working dilution has been found, larger volumes can be prepared according to need and stability.

The extent to which monoclonal antibodies can be diluted is subject to additional criteria. Because of their restricted molecular conformation and well defined pI, monoclonal antibodies are more sensitive to the pH and ions of the diluent buffer (1). Indeed, it has been demonstrated that with the exception of the relatively rare IgG_3 isotype, all monoclonal antibodies could be diluted higher and stained more intensely at pH 6.0, especially after the use of heat-induced antigen retrieval (HIAR) (1). IgG_3 isotype antibodies retained a preference for a more alkaline pH both before and after HIAR. Almost all monoclonal antibodies stained more intensely in the absence of NaCl. Of several diluents used in this investigation, phosphate buffered saline (PBS), although still widely used as a diluent for primary antibodies, was found to suppress the reactivity of all monoclonal antibodies tested (1). Differences in the net negative electrostatic charges of the target antigen are likely the explanation for these pH- and ion-related observations (1,3).

Dilutions are usually expressed as the ratio of the more concentrated stock solution to the total volume of the desired dilution. For example, a 1:10 dilution is made by mixing one part of stock solution with nine parts diluent. Two fold serial dilutions are made by successive 1:2 dilutions of the previous dilution. In order to make a very small volume of a highly diluted solution, it may be necessary to make it in two steps. For example, to prepare 1.0 mL of a 1:1000 dilution, first make 100 µL of a 1:10 dilution (10 µL + 90 µL), and then 1000 µL of a 1:100 dilution using 10 µL of the intermediate dilution (10 µL + 990 µL).

The use of adjustable pipets for preparing dilutions allows for greater flexibility and more accurate delivery. To measure volumes in excess of 1.0 mL, serological or volumetric pipets can be used. Table 1 indicates the volumes of stock reagents and diluents necessary to obtain dilutions ranging from 1:50 to 1:200. Checkerboard titrations are used to determine the optimal dilution of more than one reagent simultaneously. In the following example of a checkerboard titration, the optimal dilutions of the primary antibody and the streptavidin-HRP reagent are found, while the dilution of the biotinylated link antibody is held constant. Nine tissue sections are required for testing three dilutions.

Table 1.

Streptavidin-Peroxidase	Primary Antibody Dilutions		
1:50	1:50	1:100	1:200
1:100	1:50	1:100	1:200
1:200	1:50	1:100	1:200

If results achieved by use of several different dilutions are identical or similar, reagent costs may become an additional factor in selecting optimal dilutions.

Precise definition of the optimal signal-to noise ratio as a function of the primary antibody dilution is likely to be more critical with some methods. For example, it has been found to be more restricted with the use of unlabeled enzyme antienzyme complexes (PAP, APAAP), than with methods utilizing the streptavidin biotin technology (2). This is probably consistent with the observation that as opposed to the PAP method, the avidin-biotin method cannot distinguish between high and low concentrations of tissue antigens (4). For additional information on immunohistochemistry staining methods the reader is referred to Chapter 10, Immunohistochemistry Staining Methods.

Antibody Incubation

As mentioned above, incubation time, temperature and antibody titers are interdependent. A change in one factor may affect the others.

Incubation Time

There is an inverse relationship between incubation time and antibody titer: the higher the antibody titer, the shorter the incubation time required for optimal results. In practice however, it is expedient to first set a suitable incubation time before determining the optimal antibody dilution.

Incubation times for the primary antibody may vary within up to 24 hours, with 10-30 minutes probably being the most widely used incubation time. For an antibody to react sufficiently strongly with the bound antigen in a short period of time, it must be of high affinity and concentration, as well as have the optimal reaction milieu (pH and diluent ions). Variables believed to contribute to increased nonspecific background staining should be kept to a minimum (see Chapter 16, Background). Primary antibody incubations with a 24-hour duration allow for greater economy, because higher dilutions of the same may be used. Low affinity and/or low titer antibodies must be incubated for long periods in order to reach equilibrium*. But nothing can be gained by prolonging primary antibody incubation beyond the time at which the tissue antigen is saturated with antibody.

---

*The term “equilibrium” here denotes saturation of antigen with antibody.

Equilibrium is usually not reached during primary antibody incubations of less than 20 minutes. Consistent timing of this step is therefore important. Inconsistent incubation times can cause variations in overall stain quality and intensity, and may lead to incorrect interpretation of results. These criteria are particularly essential in efforts that attempt to assess the degree of tumor differentiation.

Incubation Temperature

Because antigen antibody reactions reach equilibrium more quickly at 37 °C compared to room temperature, some workers prefer to incubate at the higher temperature. However, while increases in incubation temperature allow for greater dilution of the antibody and/or a shortened incubation time, consistency in incubation time becomes even more critical. It is not known whether an increased temperature promotes the antigen antibody reaction selectively, rather than the various reactions that give rise to background.

A temperature of 4 °C is frequently used in combination with overnight or longer incubations. Slides incubated for extended periods, or at 37 °C should be placed in a humidity chamber to prevent evaporation and drying of tissue sections. Similarly, tissue incubated at room temperature in a very dry or drafty environment will require the use of a humidity chamber.

References

1. Boenisch T. Appl Immunohistochem & Mol Morph 1999;7(4):300-6.
2. Boenisch T. Personal observations.
3. Boenisch T. Appl Immunohistochem & Mol Morph 2002;10(4):363-7.
4. Sternberger LA and Sternberger NH. J Histochem Cytochem 1986;34:599 605.

Chapter 3 | Basic Enzymology

Thomas Boenisch MS

Immunoenzymatic staining methods utilize enzyme substrate reactions to convert colorless chromogens into colored end products. Of the enzymes used in these applications, only horseradish peroxidase and calf intestine alkaline phosphatase will be considered in some detail. Because of its low sensitivity, glucose oxidase *(Aspergillus niger)* is only rarely used today.

This chapter will also discuss the various chromogens and substrates that can be used in conjunction with peroxidase and phosphatase, together with suggested procedures for the preparation of some substrate solutions.

Enzymes

Enzymes are proteinaceous catalysts peculiar to living matter. Hundreds have been obtained in purified and crystalline form. Their catalytic efficiency is extremely high—one mole of a pure enzyme may catalyze the transformation of as many as 10,000 to 1,000,000 moles of substrate per minute. While some enzymes are highly specific for only one substrate, others can attack many related substrates. A very broad classification of enzymes would include hydrolytic enzymes (esterases, proteases), phosphorylases, oxidoreductive enzymes (dehydrogenases, oxidases, peroxidases), transferring enzymes, decarboxylases and others.

Enzymatic activity is dependent upon several variables, such as enzyme and substrate concentrations, pH, salt concentration of the buffer milieu, temperature and light. Many enzymes also possess non proteinaceous chemical portions termed prosthetic groups. Typical prosthetic groups are the iron protoporphyrin of peroxidase, and biotin of carboxy transferases. In addition, many enzymes require the presence of metal ions such as Mg^{++}, Mn^{++}, and Zn^{++}, which function as electrophilic (electron attracting) agents.

The general formula which describes the reactions of an enzyme with its substrate may be written as follows:

1. **Enzyme (E) + Substrate (S) = ES complex**
2. **ES → E + Products (P)**

Thus before formation of the product, a transient enzyme-substrate complex is formed at the "active site" (prosthetic group) of the enzyme.

Substances which interfere with the specific binding of the substrate to the prosthetic group are "specific inhibitors," and differ significantly from agents, which cause nonspecific denaturation of an enzyme (or any protein). Two basic types of inhibitions are recognized: competitive inhibition and noncompetitive inhibition. Competitive inhibition is the result of a reversible formation of an enzyme inhibitor complex (EI):

$$\mathbf{E + Inhibitor\ (I) + S = EI + S}$$

The formation of the complex EI can be reversed by a change in the concentration of either the substrate or the inhibitor, unless the affinity of I for E is greater than of S for E. The action of carbon monoxide or azides on the heavy metals of respiratory enzymes is a typical example of competitive inhibition.

In noncompetitive inhibition, the inhibition depends solely on the concentration of the inhibitor and generally is not reversible. Noncompetitive inhibition may or may not involve the prosthetic group of the enzyme, and manifests itself by slowing down or halting the velocity of the enzyme's reaction upon the substrate:

$$\mathbf{E + I + S \rightarrow E\,I\,S}$$

Selecting the enzyme most suitable for a particular immunohistochemical application depends on a number of criteria:

1. The enzyme should be available in highly purified form and be relatively inexpensive.
2. Conjugation (covalent binding to antibody or avidin, for example) or noncovalent binding should not abolish enzyme activity, although it may diminish it.
3. The bound enzyme should be stable in solution.
4. Endogenous enzyme activity should interfere only minimally with specific antigen related staining.
5. Products of the enzyme reactions should be readily detectable and stable.

Horseradish peroxidase and calf intestine alkaline phosphatase meet most of these criteria, and the following will list their properties in more detail.

Horseradish Peroxidase (HRP)

This enzyme (molecular weight 40 kDa) is isolated from the root of the horseradish plant (Cochlearia armoracia). HRP has an iron containing heme group (hematin) as its active site, and is colored brown in solution. The hematin of HRP first forms a complex with hydrogen peroxide (H_20_2), and then causes it to decompose, resulting in water and atomic oxygen. HRP oxidizes several substances, two of which are polyphenols and nitrates. It should be noted that similar to many other enzymes, HRP and some HRP like activities can be inhibited by excess substrate. The complex formed between HRP and excess hydrogen peroxide is catalytically inactive, and in the absence of an electron donor (eg, chromogenic substance), is reversibly inhibited. It is the excess hydrogen peroxide and the absence of an electron donor that brings about quenching of endogenous peroxidase activities. Cyanide and azide are two other strong (reversible) inhibitors of peroxidase.

HRP can be attached to other proteins either covalently or noncovalently. Covalent binding of HRP to other proteins can be performed using either one step or two step procedures and glutaraldehyde. The chemical 4,4’ difluoro 3,3’ dinitrophenyl sulfone (FNPS) is less commonly used for this purpose. In all cases, the epsilon amino groups of lysine and N terminal amino groups of both proteins are involved in this reaction. The two step conjugation procedure is preferred, because relative to the antibody molecule the HRP molecule has a paucity of reactive groups. As a consequence, adding glutaraldehyde to a solution containing an admixture of HRP and antibody will result in more antibody molecules being conjugated to each other, than to the enzyme. In the two step procedure, HRP reacts with the bifunctional reagents first. In the second stage, only activated HRP is admixed with the antibody, resulting in much more efficient labeling and no polymerization. The resulting conjugates are predominantly of 200,000 – 240,000 kDa.

HRP is also conjugated to (strept)avidin using the two step glutaraldehyde procedure and is used in this form in the Labeled Streptavidin Biotin (LSAB) procedure for example. Conjugation with biotin also involves two steps, as biotin must first be derivatized to the biotinyl N hydroxysuccinimide ester or to biotin hydrazide before it can be reacted with the epsilonamino groups of the enzyme.

Noncovalent binding of HRP to antibody, also known as unlabeled antibody binding, is described in great detail by Sternberger (1). Instead of the use of bifunctional reagents, IgG class antibodies to HRP are used to form a soluble semicyclic immune complex consisting of two antibody and three enzyme molecules. The molecular weight of the peroxidase antiperoxidase, “PAP” complex is 400 – 430 kDa.

Calf Intestine Alkaline Phosphatase (AP)

Calf intestine alkaline phosphatase (molecular weight 100 kDa) removes (by hydrolysis) and transfers phosphate groups from organic esters by breaking the P 0 bond; an intermediate enzyme-substrate bond is briefly formed. The chief metal activators for AP are Mg^{++}, Mn^{++} and Ca^{++}.

AP had not been used extensively in immunohistochemistry until publication of the unlabeled alkaline phosphatase-antialkaline phosphatase (APAAP) procedure (2, 3). The soluble immune complexes utilized in this procedure have molecular weights of approximately 560 kDa. The major advantage of the APAAP procedure compared to the earlier peroxidase techniques was the lack of interference posed by endogenous peroxidase activity. Because of the potential distraction of endogenous peroxidase activity, the alkaline phosphatase techniques were particularly recommended for use on blood and bone marrow smears. Endogenous alkaline phosphatase activity from bone, kidney, liver and some white cells can be inhibited by the addition of 1 mM levamisole to the substrate solution (4), although 5 mM has been found to be more effective (5). Intestinal alkaline phosphatases are not adequately inhibited by levamisole.

Substrates and Chromogens

Peroxidase

As described above, HRP activity in the presence of an electron donor first results in the formation of an enzyme substrate complex, and then in the oxidation of the electron donor. The electron donor provides the driving force in the continuing catalysis of H_20_2, while its absence effectively stops the reaction.

There are several electron donors, which upon being oxidized become colored products and are therefore called chromogens. This, along with the property of becoming insoluble upon oxidation, make such electron donors useful in immunohistochemistry.

3,3' diaminobenzidinetrahydrochloride (DAB)

This produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization, resulting in the ability to react with osmium tetroxide, and thus increasing its staining intensity and electron density. Of the several metals and methods used to intensify the optical density of polymerized DAB, gold chloride in combination with silver sulfide appears to be the most successful (6). DAB has been classified as a potential carcinogen and therefore should be handled and disposed of with appropriate care.

3 amino-9-ethylcarbazole (AEC)

Upon oxidation, AEC forms a rose red end product which is alcohol soluble. Therefore, specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (for example, Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is unfortunately susceptible to further oxidation and when exposed to excessive light will fade in intensity. Storage in the dark is therefore recommended.

4 chloro 11 naphthol (CN)

CN precipitates as a blue end product. Because it is soluble in alcohol and other organic solvents, the specimen must not be dehydrated, exposed to alcoholic counterstains, or coverslipped with mounting media containing organic solvents. Unlike DAB, CN tends to diffuse from the site of precipitation.

p phenylenediamine dihydrochloride/pyrocatechol (Hanker Yates reagent)

This gives a blue black reaction product which is insoluble in alcohol and other organic solvents. Like polymerized DAB, this reaction product can be osmicated. Varying results have been achieved with Hanker Yates reagent in immunoperoxidase techniques.

Alkaline Phosphatase

In the immunoalkaline phosphatase staining method, the enzyme hydrolyzes naphthol phosphate esters (substrate) to phenolic compounds and phosphates.

The phenols couple to colorless diazonium salts (chromogen) to produce insoluble, colored azo dyes. Several different combinations of substrates and chromogens have been used successfully.

Naphthol AS-MX Phosphate

This can be used in its acid form or as the sodium salt. The chromogens Fast Red TR and Fast Blue BB produce a bright red or blue end product, respectively. Both are soluble in alcoholic and other organic solvents, so aqueous mounting media must be used. Fast Red TR is preferred when staining cell smears.

New Fuchsin

This also gives a red end product. Unlike Fast Red TR and Fast Blue BB, the color produced by New Fuchsin is insoluble in alcohol and other organic solvents, allowing for the specimens to be dehydrated before coverslipping. The staining intensity obtained by use of New Fuchsin is greater than that obtained with Fast Red TR or Fast Blue BB.

Additional substrates include naphthol AS-BI phosphate, naphthol AS-TR phosphate and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP). Other possible chromogens include Fast Red LB, Fast Garnet GBC, Nitro Blue Tetrazolium (NBT) and Iodonitrotetrazolium Violet (INT).

Detailed descriptions and information for the preparation of the most commonly used substrate-chromogen mixtures for HRP (7) and AP (8) as well as their appropriate use and advantages or disadvantages are available (9-12).

Suggested Procedures for Substrate-Chromogen Reagents

Peroxidase

AEC Substrate Solution (recommended for cell smears)

1. Dissolve 4 mg AEC in 1 mL N,N dimethylformamide.
2. Add 14 mL 0.1 M acetate buffer, pH 5.2 and 0.15 mL 3% hydrogen peroxide.
3. Mix, and filter if precipitate forms.
4. Add solution to tissue and incubate for five to 15 minutes at room temperature.
5. Rinse with distilled water.
6. Counterstain and coverslip with aqueous based medium.

DAB Substrate Solution

1. Dissolve 6 mg DAB in 10 mL 0.05 M Tris buffer, pH 7.6.
2. Add 0.1 mL 3% hydrogen peroxide. Mix, and filter if precipitate forms.
3. Add solution to tissue and incubate for three to 10 minutes at room temperature.
4. Rinse with distilled water.
5. Counterstain and coverslip with either organic- or aqueous-based medium.

Alkaline Phosphatase

Fast Red Substrate Solution (recommended for cell smears)

1. Dissolve 2 mg naphthol AS-MX phosphate, free acid (Sigma N 4875) in 0.2 mL N,N-dimethylformamide in a glass tube.
2. Add 9.8 mL 0.1 M Tris buffer, pH 8.2.
3. Add 0.01 mL of 1 M levamisole (Sigma L 9756) to block endogenous alkaline phosphatase. (Solution can be stored at 4 ºC for several weeks, or longer at -20 °C.)
4. Immediately before staining, dissolve 10 mg Fast Red TR salt (Sigma F 1500) in above solution and filter onto slides.
5. Incubate for 10-20 minutes at room temperature.
6. Rinse with distilled water.
7. Counterstain and coverslip with aqueous based medium.

New Fuchsin Substrate Solution (recommended for tissue sections)

1. Solution A: Mix 18 mL of 0.2 M 2-amino-2-methyl-1,3-propanediol (Merck 801464) with 50 mL 0.05 M Tris buffer, pH 9.7 and 600 mg sodium chloride. Add 28 mg levamisole (Sigma L 9756).
2. Solution B: Dissolve 35 mg naphthol AS-BI phosphate (Sigma N 2250) in 0.42 mL N,N-dimethylformamide.
3. Solution C: Under fume hood, mix 0.14 mL 5% New Fuchsin (Sigma N 0638, 5 g in 100 mL 2 N HCl) with 0.35 mL of freshly prepared 4% sodium nitrite (Sigma S 2252, 40 mg in 1 mL distilled water). Stir for 60 seconds.
4. Mix Solutions A and B, then add Solution C; adjust to pH 8.7 with HCl. Mix well and filter onto slides.
5. Incubate for 10-20 minutes at room temperature.
6. Rinse with distilled water.
7. Counterstain and coverslip with either organic or aqueous-based medium.

New Fuchsin Substrate Solution (alternative procedure)

1. Solution A: In fume hood add 0.2 mL of 5% New Fuchsin (Merck 4041, in 2 N HCl) to 0.5 mL of fresh 4% sodium nitrite. Agitate for 30-60 sec. Add 100 mL of 0.05 M Tris buffer, pH 8.7, and 100 µL of 1 M levamisole to block endogenous alkaline phosphatase.
2. Solution B: Dissolve 50 mg naphthol AS-BI phosphate (Sigma N 2250) in 0.6 mL N,N-dimethylformamide.
3. Add Solution B to Solution A and mix well. Filter directly onto slides.
4. Incubate for 10-20 minutes at room temperature.
5. Rinse with distilled water.
6. Counterstain and coverslip with either organic or aqueous-based medium.

References

1. Stemberger LA. Immunocytochemistry (2nd ed). New York: Wiley, 1979.
2. Mason DY et al. J Cancer Res Clin Oncol 1981;101:13-22.
3. Cordell JL et al. J Histochem Cytochem 1984;32:219-29.
4. Ponder BA and Wilkinson MM J. Histochem Cytochem 1981;29:981-4.
5. Gown AM In DeLellis RA (ed) Advances in Immunohistochemistry. New York: Raven Press, 1988, pp 31-45.
6. Newman GR et al. J Microscopy 1983;132:RPl-2.
7. Dako Specifications, numbers K0597, K0598, K0599, K0624 and K0698.
8. Dako Specifications numbers K3461, K3465 and K3467.
9. Newman GR et al. J Microscopy 1983; 132; RP1-2.
10. Clark CA et al. J. J Histochem Cytochem 1982;30:27-34.
11. Gay et al. J Histochem Cytochem 1984;32:447-51.
12. Bondi A et al. Histochemistry 1982;76:153-8.

Chapter 4 | Immunohistochemical Standardization and Ready-to-Use Antibodies

Clive R. Taylor MD, DPhil, FRCPath, FRCP (Ir)

Introduction and Background: The Growing Consensus for 'Standardization'

From the beginning there has been concern relating to the relatively poor reproducibility of immunohistochemical (IHC) methods as applied to formalin-fixed paraffin-embedded (FFPE) tissue sections, reproducibility day to day within a single laboratory, and reproducibility among different laboratories. In recent years these concerns have, if anything, increased and lack of 'standardization' is now recognized as a major impediment to basic research, clinical trials, and direct patient care. Over the past three decades a number of conferences have been held to address this topic and to seek constructive resolutions. Among the most productive were a series of meetings sponsored by the Biological Stain Commission and the FDA in the early 1990s, that led to recommendations for manufacturers concerning the precise description and validation of IHC reagents (1), and also highlighted the necessity to pay attention to all aspects of the IHC test procedure. The latter recommendation, borrowed from the much more rigorous protocols applied to immunologic assays in clinical laboratories, became known as the 'Total Test' approach (Table 1) (2, 3). A more recent meeting attended by invited experts, the FDA and NIST (National Institute of Standards and technology) focused upon standardization of Her 2 IHC assays, and the need for universal control materials (reference standards) (4).

Table 1. The Total Test; An IHC stain managed in the same rigorous manner as a clinical laboratory analysis.

Pre-analytic
Test selection
Specimen type
Acquisition, pre-fixation/transport time
Fixation, type and total time
Processing, temperature
Analytic
Antigen retrieval procedure
Selection of 'primary' antibodies
Protocol; labeling reagents
Reagent validation
Control selection
Technician training/certification
Laboratory certification / QA programs
Post-analytic
Assessment of control performance
Description of results
Interpretation/reporting
Pathologist, experience and CME specific to IHC

Modified from Taylor (2, 3, 5).

The consensus arising from these meetings was that the translation of IHC methods from 'bench to bedside', in the sense of use in routine surgical pathology, has been greatly hindered by two key factors. While decades have passed these issues have not been satisfactorily addressed and have not been ameliorated.

1. The reagents available for IHC, have increased in quality, but even more so in number of sources and variety of 'staining' methods. This plentitude of reagents contributes to lack of standardization in significant ways, that in theory are manageable by good technique and use of proper controls, but in practice have led to requirements of such excellence in technical process, that many laboratories cannot find sufficient, or sufficiently skilled, staff to comply.

2. The usual method of sample preparation for tissue remains as formalin fixation and paraffin embedment (FFPE). This venerable approach may be satisfactory for the preservation of morphologic detail, but does adversely affect the antigenicity of many target molecules in the tissue, to degrees that are unknown. The enormous variation in protocols (including fixation times) employed for FFPE among different laboratories, or within the same laboratory from specimen to specimen, compounds the problem, and contributes to the current poor reproducibility.

Legions of investigators, and many manufacturers, have addressed different aspects ofthe problem, focusing upon better sample preparation (fixation), more effective methods of antigen retrieval, improved reagents, more sensitive detection methods, and the development of reference standards or controls (2-8) . To date, these approaches have failed to produce an overall system of IHC that assures uniform high quality, with a level of reproducibility and reliability sufficient to allow robust comparison of IHC results across laboratories, especially where semi-quantitative results are sought (as for Her 2 , or ER) (4, 7, 8).

Some broad conclusions are possible:

- Resolution of the problem of sample preparation is not imminent; the scientific aspects of developing a new fixative to replace formalin are challenging and more importantly the logistical and economic obstacles to replacing formalin worldwide are formidable.
- High-quality reagents are available, with highly sensitive detection methods, but they must be employed properly in controlled fashion, and currently often are not.
- There is a pressing need for tissue-based IHC controls (or ‘reference standards’)(4,7,8) that can be made available to all laboratories performing IHC assays, somewhat analogous to the ‘universal reference or calibration standards’ that are available to clinical laboratories performing ELISA testing, which in principle is a strictly analogous technique (the same reagents that are employed in an ELISA test to measure the insulin level in serum, may be employed to perform an IHC stain for insulin in a paraffin section) (ELISA — enzyme linked immunosorbent assay).

From this brief discussion it follows that for ‘maximum standardization’ all laboratories would carry out the IHC in identical fashion for every phase of the ‘Total Test’, namely use the same fixative and fixation time, the same AR process, the same primary antibodies and detection systems, from a single vendor, with the same automated stainer, and common controls. Clearly this perfect option will never happen, and we therefore must do what we can to ameliorate the consequences of the variables in the process.

Ready-to-Use (RTU) Reagents

The present chapter has as its focus the use of ‘ready to use’ (RTU) reagents, sometimes included under the terms ‘pre-diluted reagents’ or IHC kits. The potential utility of RTUs will be examined with respect to improved standardization of IHC, including indirect benefits that relate to ‘sample preparation’ and the use and availability of control tissues.

Within the context of the Total Test (Table 1) those areas listed under the rubric of ‘Analytic’ are of particular relevance to the IHC laboratory, for their proper performance is the direct responsibility of the Laboratory Director and staff, including technologists and pathologists. Obviously, ‘Pre-analytic’ and ‘Post-analytic’ issues are of concern, and must be considered in the context of overall performance, but the IHC laboratory is properly and directly responsible for the actual performance of the IHC stain or assay.

While it may appear to be a matter of semantics, the author has become convinced that whether IHC is viewed as a ‘stain’ or as an ‘assay’, can play an important role in establishing the proper mind set of the laboratory staff and the pathologists. The IHC method is regarded by many as simply a ‘stain’; it produces a visible tinctorial reaction within the tissue section. However, IHC should not be regarded as simply another ’special stain’, like a PAS stain or a silver stain. As already noted, IHC is essentially an ELISA method applied to a tissue section. In this respect, when correctly performed, IHC has the potential to perform as a reproducible and quantitative tissue based ELISA assay; much more than a simple stain. That the IHC method mostly does not perform to this level, reflects faults in the application of the method, specifically inconsistent sample preparation, lack of reference or calibration standards, and inadequate validation of reagents (7, 8). The use of RTUs does not finally solve these problems, but for reasons that will be discussed below, can lead to increased reproducibility and consistency in a practical sense, in large part by forcing the use of external standards, external reagent validation, and defined, extensively tested protocols upon the user laboratory.

Reagent Validation

Selection and validation of reagents for use in the IHC method is not straightforward. Most of the IHC procedures in current use for diagnostic pathology employ multiple steps. The primary antibody is the essential first component, imparting target specificity to the 'test'. The primary antibody is itself not labeled, and localization in the section is visualized indirectly, through the subsequent application of secondary labeling reagents, requiring that multiple reagents be optimized together in a rational and documented manner that fosters control and reproducibility.

Primary Antibody, Selection, Validation and Optimization

The primary antibody imparts specificity to the IHC method, and may be obtained either as an antibody concentrate, or as an RTU reagent. If obtained as a concentrate it must be adjusted to an optimal working dilution for use with a secondary labeling system selected by the laboratory. If obtained as an RTU reagent, it typically is purchased along with an optimized labeling system and defined protocol, for use with an automated stainer, or sometimes by manual methods.

In setting out to perform and IHC test (assay, stain) selection of an antibody having the desired specificity, unless prepared *de novo* in house, must rely upon descriptions by the manufacturer(s) in catalogues, package inserts or sales materials. The experience of the author strongly supports the notion that the best assurance of appropriate specificity and good performance rests with using product(s) from manufacturers who are 'tried and true'; that is purchasing reagents from suppliers who are reputable and from whom the laboratory has a history of obtaining consistently reliable reagents. Even so, it is the responsibility of the performing laboratory to confirm functional specificity of each and every reagent, using appropriate positive and negative control materials *(vide infra)*. While this statement is directed first at assuring the specificity of the primary antibody, it applies equally to all reagents utilized in the protocol (Total test — Analytic phase; see Table 1). Also the requirement to validate specificity applies to all antibodies, whether purchased in concentrated form and subsequently diluted by the laboratory, or obtained as RTUs; it just is easier to validate specificity of RTUs, as discussed below.

The huge variety of reagents available for IHC, and the large number of different vendors, in a way contributes to lack of standardization; almost there is too much choice. As described elsewhere (Chapter 1), the primary antibody may be polyclonal or monoclonal. A polyclonal antibody or antiserum is produced by traditional immunization techniques, with 'booster' injections to maximize reactivity against the target antigen. Such antisera in fact contain many different antibody 'species', having varying specificity for many antigens, but are effectively enriched for high affinity antibody molecules that target the antigen of interest (while antibodies to other antigens, not of interest, are present at low levels, or are of low affinity, or may be selectively depleted by absorption methods). Monoclonal antibodies, prepared by hybridoma methods, or by molecular engineering, contain a single 'species' of antibody molecule, where every antibody molecule is identical by idiotype, with a single specificity and single affinity.

The concentration of the active specific antibody molecule may thus be known and measured accurately for a monoclonal antibody preparation, but cannot be more than estimated in a polyclonal antibody (antiserum), because of the variety of different molecules present in the latter. The concentration of a monoclonal antibody may be accurately given in terms of µg/ml or mg/L by the manufacturer, and working dilutions (in the laboratory) may then be given as, for example, µg/L; although often a simple dilution fraction is given, as 1/50, 1/100 etc. For polyclonal antibodies (antisera) an accurate concentration of the 'active specific antibody molecule', by weight, cannot be given, and working dilutions are expressed as dilution fractions, 1/50, 1/100 etc. of the primary antiserum, as supplied by the manufacturer. Polyclonal antibodies of the same claimed specificity obviously differ greatly among manufacturers, as may monoclonal reagents in terms of concentration, even if from the same clone, and in terms of absolute specificity as defined by the epitope with which they bind. The process of choosing the working dilution results in significant differences in actual concentration of specific antibody among laboratories, varying by vendor source, and varying also by the precision, or lack thereof, of the serial titrations used to achieve optimal concentration *(vide infra)*. RTU reagents may be polyclonal or monoclonal, and are pre-diluted by the manufacturer, under rigorous quality control using a defined labeling system and defined protocol, to demonstrate consistent performance upon selected positive (and negative) control cells and tissues. The concentration by weight of antibody usually is not given for RTUs (even when monoclonal) for the intention is that the reagent should be used directly, without further dilution. In fact, by definition, RTU reagents should not be subject to additional dilution by the performing laboratory, neither should the recommended detection system be changed, or any step in the staining or labeling protocol. Any change mandates that the performing laboratory conduct extensive re-validation of the whole system.

As noted above, both polyclonal and monoclonal antibody reagents must be added to the tissue section at an optimal concentration or working dilution, defined as that giving the highest intensity of specific reaction, with the lowest level of non-specific background staining: i.e. the highest 'signal to noise ratio'. For RTUs the operative dilution is pre-determined by the manufacturer on the basis of extensive in house studies, and the performing laboratory is 'spared' this effort. For antibodies that are purchased in concentrated form, the optimal concentration (or working dilution) is determined experimentally within each laboratory by serial titration experiments (see Chapter 2 and reference 5 for detailed discussion). Because the primary antibody is usually not itself labeled, it is necessary to explore different working dilutions of the primary antibody, with different working dilutions of the labeling reagent or secondary antibody, a so called 'chequer board titration' (see Chapter 2 and reference 5). This is a complex process that requires skill in performing dilutions of protein solutions that *ab initio* have very low concentrations of protein (in the order of mg/L), where minor errors in dilution technique may result in manifold differences in actual protein concentration after serial dilution, translating to great differences in observed performance. For reasons already alluded to, if the performing laboratory cannot be assured of accuracy and consistency in performing complex serial dilutions and titrations, then RTU reagents provide a viable alternative, allowing the laboratory to achieve consistent performance day to day, once there has been initial validation of specific staining on appropriate FFPE control tissue processed in a manner similar to (ideally identical to) the test tissues.

Secondary or Labeling Reagents: Protocols

One of the early consequences of attempts to apply antibody based methods to FFPE tissue sections was a pressure to develop increasingly 'sensitive' labeling methods, an unspoken rationale being that if only small amounts of 'antigenically intact' protein remain after formalin fixation, then the greater the sensitivity of the detection method, the greater the chance of a successful 'stain' on FFPE tissue sections. Thus the simple indirect method, employing conjugated or labeled secondary antibody, was succeeded in turn by the PAP method, ABC method, tyramide amplification and polymer based methods (see Chapter 10 and reference 5), all seeking amplification of some faint, but specific, initial signal, without compromising the specificity of that initial reaction. In this context the term 'sensitivity' is used in relation to the ability to detect ever smaller amounts of antigen in the tissue section, or to detect the same amount of antigen at ever higher working dilutions of antibody. This usage resembles analytical sensitivity in the clinical laboratory, but is different from the more common application of the term sensitivity in diagnostic practice, where it refers to the ability of a test to detect true positive cases in a population of patients known to have the disease (diagnostic sensitivity).

In general terms the greater the sensitivity or signal amplification of the detection method, the more complicated it is likely to be, and the more steps are involved; the corollary being that establishing the optimal protocol, and then reproducing performance, is more difficult by several orders of magnitude. As referenced above in determining the working dilution of a concentrated primary antibody (monoclonal or polyclonal) optimization must be performed in conjunction with optimization of the entire detection system. The one depends upon the other. Each laboratory must conduct 'chequer board titration' studies that must be performed for all new reagents, both primary and secondary, and reproduced subsequently for every new batch of familiar reagents (see Chapter 2 and reference 5). The use of RTUs, if accompanied by detection reagents and defined protocols as designated by the manufacturer, obviates the need for complex titration procedures, and thereby avoids the variation among laboratories that results from inconsistencies in establishing the optimal dilutions of primary antibodies and the various labeling reagents that are employed. It is important to note, however, that if an RTU is used with any detection system or protocol other than that validated by the manufacturer, then full optimization studies must be performed.

The above discussion has focused upon selecting the optimal concentration (or working dilution) of primary and secondary reagents, or electing instead to use RTUs with defined detection systems and protocols. It must, however, not be forgotten that other aspects of the IHC protocol are also of critical importance in achieving consistency, including selection and validation of any antigen retrieval procedure, precision in volume of reagents applied to the slide, choice and pH of dilution buffers, optimized incubation times (at defined temperature), number and effectiveness of wash cycles, and concentration, temperature and incubation time of the chromogen of choice. There are at least 25 separate steps in a typical IHC assay. All must be optimized and performed in an identical manner, run after run, day after day; and ideally from laboratory to laboratory. In this respect use of an automated system can greatly enhance reproducibility within a laboratory, whether using concentrated reagents and self optimized detection systems, or RTUs. The use of RTUs has an additional benefit for many laboratories in that it forces standardization of reagents, dilutions, detection systems, and overall protocol among different laboratories using the same system

As noted previously, for ultimate standardization of IHC all laboratories would perform the ‘Total Test’ in identical manner with common reagents and identical protocols, or alternatively all would adopt the same universal reference materials (calibration standards) against which each laboratory would establish its IHC assay(s) and validate performance on a daily basis. Failing these options, if all laboratories adopt a similar level of internal consistency (albeit with different reagents and protocols) then standardization across different laboratories will be enhanced. Internal consistency is more readily achieved with RTUs than with use of concentrated reagents, because of the inherent variability in optimization of the latter among different laboratories. Furthermore as several laboratories adopt the same RTUs, detection systems and protocols, improved standardization will result, against the remaining major variable of sample preparation.

Controls

The proper use of appropriate controls is vital to all IHC assays, and is discussed in Chapter 19, and at length in reference 5.

As a minimum each IHC run should include a positive control, and a negative control. In practice the positive control is usually a tissue section fixed and processed in a similar manner to the test section and known to contain the target molecule, ideally based upon independent assay, but more often based upon presumptive presence of the target molecule derived from knowledge of the literature and prior experience (e.g. normal breast for ER, normal prostate tissue for PSA, etc.). Positive controls ideally should be selected for giving a range of intensity of reaction, from strong to intermediate to weak; use of tissue containing very high amounts of antigen may lead to selection of optimal working dilutions that fail to detect lower levels of the target antigen found in other (pathological) tissues. The term ‘negative’ control, covers two control concepts; a ‘negative reagent control’, typically omitting the primary antibody on a parallel tissue section that otherwise is treated identically, and a ‘negative tissue or cell control’. In practice the latter requirement is in most cases fulfilled by identification of non-staining cell types within the test section, that is cells that do not contain the target molecule, and do not, therefore, give a positive stain reaction.

Laboratories electing to use concentrated reagents as the primary antibody must employ positive and negative controls through all of the titration and validation procedures, to achieve optimal working dilutions of primary antibody and detection system reagents. Single tissue blocks or micro-tissue arrays may be used. For some tests sources of appropriate documented control tissues may be readily exhausted in performing the extensive titrations necessary to establish the protocol and to validate its ongoing use. With use of RTU reagents the approach is simpler, albeit somewhat pragmatic. The selected positive control tissue (fixed in a manner similar to anticipated test specimens and selected on the expectation that it contains the antigen of interest) is ‘stained’ strictly according to the manufacturer’s protocol for the RTU, and either gives a satisfactory specific pattern of staining, or does not. If it does, then the assay is established pending further validation on additional cases, and reproducibility assessment by repeated runs. If it gives no staining, or non-specific staining, then the only possible recourse is to attempt to vary and optimize the antigen retrieval process (or to improve sample preparation). As noted previously, changing concentration of the RTU or adjusting the staining protocol outside of the manufacturer's guidelines places the entire burden of validation back on the performing laboratory.

The RTU primary antibody, together with the detection system and protocol, is usually set up by the manufacturer to produce satisfactory results on a range representative tissues (usually FFPE tissues), and is validated for run to run reproducibility on a variety of FFPE tissues containing differing levels of the target antigen. The goal of the manufacturer is that the RTU, with its associated detection system, will therefore perform well with the range of FFPE tissues that may be encountered in many different laboratories. The ideal outcome for a performing laboratory in evaluating an RTU is that the RTU, with its detection system and protocol, gives an acceptable result on FFPE tissues that are processed in the performing laboratory (institution), 'straight out of the box'. If it does not then it may be necessary to go to concentrated reagents and set up the particular IHC test by detailed titration studies, with attention to fixation, antigen retrieval and all aspects of the Total Test (3, 5).

Impact of RTUs on Other Aspects of the Total Test

To this point discussion has properly focused upon reagents and the Analytic Phase of the Total Test, this being the phase where RTUs are employed and have most impact.

While choice and validation of reagents are critical issues, in my opinion, by far the most important contributory factor to variable performance of IHC worldwide, as well as in individual laboratories, relates to the 'pre-analytic' phase (6-10). Most important is the almost complete absence of consistency in tissue fixation, and other aspects of specimen handling. While formalin is by far the most widely employed fixative, there is no consistency in its preparation, whether freshly prepared or not, or in time for which tissues are exposed to the fixative. Indeed almost always the 'fixation time' is unknown and undocumented. Similarly 'pre-fixation' time (sometimes referred to as 'ischemic time' — post resection, but prior to immersion in fixative) is not documented and is unknown in duration or impact. Similarly the other stages of processing, dehydration, impregnation, storage, de-paraffinization and rehydration are ill understood and also undocumented. Finally antigen retrieval, while acting to restore antigenicity for many proteins, also is inconsistently applied. It is the clear consensus of those experienced in the field of IHC (4, 6-18) that all of these steps taken together, under the rubric of sample preparation, represent the largest single obstacle to standardization, and one that will not easily be overcome.

In this respect the more widespread use of RTUs may have benefit, not solving the problem of standardization, but perhaps contributing to some practical improvement. As noted above, typically those manufacturers who market RTU reagents conduct extensive in house studies that establish successful performance of the RTU on a wide range FFPE tissues, that have been subject to different times and conditions of fixation, and contain varying concentrations of antigen. To some extent, therefore, use of a carefully validated RTU, with associated detection system and protocol, including defined conditions for AR (antigen retrieval), may compensate for lack of standardization of fixation and processing, and thus contribute to overall standardization of IHC results. This approach is admittedly empirical, and in no sense argues against the notion that the best strategy, in skilled hands, is to obtain separate concentrated primary and secondary labeling reagents and to perform the necessary titrations to establish the optimal protocol for the FFPE tissues available to each laboratory performing IHC. The skill set and experience necessary to conduct optimization studies are considerable and may not be readily available within the laboratory, particularly where IHC assays are performed in smaller institutions at relatively low volume. Even in large academic centers the availability of skilled histotechnologists, who also are trained in basic immunologic methods may be limited. RTUs may be then be an option for some less commonly performed tests.

In the 'post-analytic' phase (Table 1), interpretation of results is also not well standardized among pathologists (3, 10), especially where semi-quantitative assessments are attempted (7, 8). Interpretation of an IHC slide should always be accompanied by review of the relevant controls by a trained pathologist, experienced with the IHC assay in question. Ideally this same pathologist should contribute to the final pathology report. However, such an ideal approach is not practical where surgical pathology sub-specialization is extensive and where IHC is centralized into a single laboratory facility that may serve many dispersed pathologists. In these instances the controls should be reviewed by experienced technologists and pathologists and appropriate performance should be recorded before release of the stained slides to surgical pathology. Manufacturers of RTUs, including Dako, may provide atlases of representative staining results that are useful to surgical pathologists at the bench, in viewing expected patterns of staining, especially for the less common tests. Finally interpretation, assisted by image analysis, is a growing trend that will eventually become essential for IHC as it is used in a quantitative mode (7, 8). But image analysis and quantification are pointless unless first the IHC method is standardized to the greatest degree possible, which in return requires integration and control of as many components of the Total Test as possible. This large task can be approached by manufacturers for all phases, with the current exception of sample preparation, because they have the resources to develop and integrated complete systems, from reagents, to processors, to stainers, to image analysis, whereas few single laboratories can do this. In this context RTUs are likely to play a growing role because manufacturers will find it difficult to produce reliable integrated systems unless they also control reagent optimization and protocols to the greatest degree possible, and routine laboratories are likely to welcome this outcome, it does, after all, mirror the course of events that occurred over the past four decades as clinical laboratories underwent automation and standardization.

Summary

There have been several schools of thought as to the reason why IHC ‘stains’ are difficult to run in a reproducible or consistent manner. If there is a consensus as to the cause of lack of standardization, it is that several reasons conspire together. These may conveniently be grouped into three general areas as presented in Table 1, that summarizes those factors that must come together to produce the ‘Total Test’ (10, 16).

Resolution of ‘pre-analytic’ issues (sample preparation, fixation) will require an order of general collaboration hither to unseen, even should there be agreement as to which new and better fixative to use. This author believes that replacement of formalin on a large scale is unlikely in the next decade; therefore we must seek to improve reproducibility within that limitation.

Similarly the scientific and logistical problems that must be overcome in developing and establishing a set of universal IHC tissue reference materials are daunting. The problem is not, however, insurmountable and a number of proposals for external reference standards based on cell lines, faux tissues, and peptide deposits are under consideration (4, 5), together with the notion of developing Internal Reference Standards, including ultimately Quantifiable Internal Reference Standards (QIRS) (7, 8) that would permit calibration of IHC assays across laboratories. Again time and resources are required, but the outcome is likely to be similar to that experienced already in the clinical laboratory.

This chapter has, therefore, focused on a more immediate and pragmatic approach, namely the use of RTUs, that if widely employed are likely to lead to improved outcomes for IHC assays, in terms of reproducibility of results from day to day, and laboratory to laboratory. The use of RTUs is empirical, and in no sense argues against the notion that the best approach, in skilled hands, is to obtain separate concentrated primary antibody and secondary labeling reagents, and to perform the necessary titrations to establish to the optimal protocol for each different antibody using the FFPE tissues available to each laboratory performing IHC. The latter is a huge task, beyond the expertise on many laboratories, and expensive in terms of reagents and use of control tissues.

Ultimately the overriding factor in effecting significant change must be to transform the mindset of pathologists, at least of the next generation, to the view that the end result of an IHC protocol is not as just a ‘stain’, but rather is a precise immunoassay that is strictly quantifiable, and must be performed only with a degree of technical rigor and control that matches any other immunologically based assay of like principle (namely ELISA). ELISA is a ‘gold standard’ method for quantitative assays in the clinical laboratory. ELISA reagents are purchased in prepared form, with all of the necessary reagents, defined protocols, and reference or calibration standards, for use with specified instrumentation. RTUs, coupled with proven detection systems, fixed protocols, recommended controls and automation, represent an analogous pathway that can lead to improved levels of reliability and performance for IHC.

References

1. Taylor CR. Quality assurance and standardization in the immunohistochemistry. A proposal for the annual meeting of the Biological Stain Commission, June 1991. Biotech Histotech 1992; 67:110-17.
2. Taylor CR. Report from the biological stain commission: FDA issues final rule for classification/reclassification of immunochemistry (IHC) reagents and kits. Biotech Histochem 1998;73:175-7.
3. Taylor CR. An exaltation of experts: concerted efforts in the standardization of immunohistochemistry. Appl Immunohistochem 1993;1:232-43.
4. Hammond MEH, Barker P, Taube S, et al. Standard Reference Material for Her2 testing: report of a national institute of standards and technology sponsored consensus workshop. Appl Immunohist Mol Morph 2003; 11; 103-6.
5. Taylor CR, Cote RJ. Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist. Saunders Elsevier. 3rd edition. 2006.
6. Taylor CR. Standardization in Immunohistochemistry: the Role of Antigen Retrieval in Molecular Morphology. Biotechnic & Histochemistry 2006; 81:3-12.
7. Taylor CR. Quantifiable Internal Reference Standards for Immunohistochemistry; the measurement of quantity by weight. Applied Immunohisto Mol Morph 2006; 14:253-59.
8. Taylor CR, Levenson RM. Quantification of Immunohistochemistry—issues concerning methods, utility and semiquantitative assessment. Histopathology 2006; 49:411-24.
9. Miller RT, Swanson PE, Wick MR. Fixation and epitope retrieval in diagnostic immunohistochemistry: A concise review with practical considerations. Appl Immunohistochem Mol Morphol 2000; 8:228-35.
10. Taylor CR. The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124:945-51.
11. Shi S-R, Gu J, Cote RJ, et al. Standardization of routine immunohistochemistry: where to begin? In: Shi S-R, Gu J, Taylor CR, eds. Antigen retrieval technique: immunohistochemistry and molecular morphology. Natick, MA: Eaton; 2000:255–272.
12. Rhodes A, Jasani B, Balaton AJ, et al. Study of interlaboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe. Documentation of poor reliability and identification of insufficient microwave antigen retrieval time as a major contributory element of unreliable assays. Am J Clin Pathol 2001;115:44-58.
13. Rait VK, O'Leary TJ, Mason JT. Modeling formalin fixatin and antigen retrieval with bovine pancreatic ribonuclease A: I-Structural and functional alterations. Lab Invest 2004; 84:292-9.
14. Rait VK, Xu L, O'Leary TJ, Mason JT. Modeling formalin fixation and antigen retrieval with bovine pancreatic RBase A II. Interrelationship of cross-linking, immunoreactivity, and heat treatment. Lab Invest 2004; 84:300-6.
15. Wolff AC, H Wolff AC, Hammond EH, Schwartz JN, et al. ASCO/CAP Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 testing in Breast Cancer. Arch Pathol Lab Med 2007; 131:18-43.
16. Goldstein NS, Hewitt SM, Taylor CR, Yaziji H, Hicks DG and Members of Ad-Hoc Committee on Immunohiostochemistry Standardization. Recommendations for Improved Standardization of Immunohistochemistry. Appl Immunohistochem Mol Morphol 2007:15:124-33.
17. Yaziji, H, Taylor C, Goldestein N, Dabbs D, Hammond E, Hewlett B, Floyd A, Barry TS, Martin A, Badve S, Baehner F Cartun R, Eisen R, Swanson P, Hewitt S, Vyberg M, Hicks D. and Members of the Standardization Ad-Hoc Consensus Committee. Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer By Immunohistochemistry. Appl Immunohistochem Mol Morphol 2008;16:513-20.
18. Yaziji H, Taylor CR. Begin at the beginning, with the tissue! The key message underlying the ASCO/CAP Task-Force Guideline Recommendations for HER2 Testing. Appl Immunohistochem Mol Morphol 2007; 15:239-41.

Chapter 5 | Fixation and Processing

A.J. Farmilo PhD and Ronald H. Stead PhD (Revised by A. J. Farmilo PhD)

Immunohistochemistry (IHC) has advanced considerably since the first edition of this handbook was published in 1983 (1), and the driving force behind that change has been the need for standardization. If tissue staining is to provide consistent, reproducible diagnostic information, it must continue to evolve from an "art form" to a science. That evolution demands quantitation and reproducibility of methodology, and extending from that, consistency of results.

One of the last of these IHC "art forms" is tissue fixation and processing. Laboratory professionals are little closer to uniformity in this part of the process, and achieving that uniformity, or "standardization," remains one of the true unknowns in diagnostic interpretation.

Fixation

Part of the challenge is the finite amount of antigen in each tissue sample, and the fact that most steps in the IHC process destroy some of this antigen. This is especially problematic at the critical step of tissue fixation, because it is at this step that we intentionally try to change protein structure in order to preserve them from elution, degradation, or other modifications that occur in normal, unfixed tissue samples.

In addition to preventing antigen elution or degradation, fixation also should preserve the position of the antigen, whether nuclear, cytoplasmic or membrane-bound, and preserve as much antigenic secondary and tertiary structure as possible, to provide a target for antibodies that will be used to detect the antigen.

Many examples exist of situations that have led to incorrect interpretation of staining patterns, as a result of poor or inadequate fixation. One example is elution of estrogen receptor protein from nucleus to cytoplasm. In this situation, the antigen is detected in the cytoplasm and therefore the cell stains "positive". But in fact the antigen should be primarily localized in the nucleus, and therefore diagnostically the stain is useless.

The same antigen can be used to demonstrate the importance of fixation and antibody-antigen reactions. Fixation in neutral buffered formalin will result in the destruction of an epitope against which some monoclonal antibodies react. Use of those antibodies would indicate a "negative" reaction for estrogen receptor, while the use of antibodies for a different epitope, one that is not destroyed by the fixation, would indicate a "positive" reaction.

What is the solution to this complex issue? Standardization of fixative and fixation protocols would be an ideal start. Many fixatives have been developed over the years and at least two fairly recent ones have been promoted as possible "standards." But so far no single fixative has proven ideal for all markers, antibodies and applications. Therefore standardization and validation will have to focus on particular antibodies and their corresponding staining protocols.

The acceptance of a common procedure for fixation is also extremely important and essential to achieving reproducible results. This means that reagent preparation must be done exactly the same way each time a particular staining protocol is performed. Reagents and protocols need validation, which would include determining the limits of the reagent's shelf life, optimal fixation time and conditions such as temperature and humidity. Many fixation reagents are concoctions of reactive and moderately toxic chemicals, and often little is known about the exact reactions that occur within them. For example, formalin preparations vary greatly, and concentrations of aldehydes, acids, and other by-products in each preparation may change with time and storage, and those changes will vary from product to product.

Validation is an initial step for two reasons: first, to ensure that a certain standardized procedure will give consistent and diagnostically useful results. Second, to test the limits of changes in the procedure that will continue to provide those results. For example, users can validate fixation time by running a series of tests using fixation times of 0, 4, 8, 12, 24 and 36 hours; plus times of 5, 15 and 30 days. For a given antigen and antibody combination, users might find that the 0-, 4- and 8-hour fixations gave sub-optimal results, perhaps because the antigen was not fixed completely, and diffused through the cell or tissue. They might then determine that a range of 12 hours to five days is optimal, and that the 15- and 30-day results are sub-optimal due to over-fixation. They have therefore validated their procedure with respect to fixation time, and now know that the tissue requires a minimum fixation of 12 hours and a maximum fixation of five days.

Practically speaking, that would mean that overnight fixation would be required, that weekend fixation would be OK, but a longer fixation time would not be useful. With this information, users would be able to evaluate the results obtained from tissues received from outside sources by comparing fixation procedures.

For smaller laboratories, the work involved in validation is often difficult, but there are two alternatives. Users can choose a system with an existing standardized and validated protocol and validated interpretation system. Commercially available kits generally provide these, and when utilized exactly as described in the kit insert, are guaranteed to provide diagnostically useful results. A second option would be to use one of the more common “standard” systems of fixatives with known antibodies, in which publication data has provided some evidence of functionality. As an example, a laboratory could use a 10% neutral buffered formalin fixation with a standard protocol, followed by a biotin-streptavidin HRP system, using a monoclonal antibody combination called AE1/AE3. This has been proven to be a reliable measure of cytokeratin in tissue sections.

Tissue Handling

The computer-related adage, “garbage-in, garbage-out” can apply to IHC as well, because the first steps of tissue handling arguably dictate the quality of results, more than do any steps that follow. Therefore a good foundation is to remember that the “first steps” start the very moment that tissue becomes a sample. Necrotic degradation begins immediately once the tissue is separated from its source of nutrients, so the time to processing is quite often critical. For most IHC procedures, it is imperative that tissue not dry out. Collection from the surgical arena should be onto moist absorbent paper, in a covered container, followed by rapid delivery to the pathology lab for processing.

Tissue should then be trimmed and cut for fixation. The area of interest should be cut into blocks no more than two cm square by four mm thick. Thickness is important. The fixative must penetrate tissue in order to be effective. Fast penetration is desirable — the thinner the tissue, the faster fixation can begin. The most common formalin fixatives penetrate quickly, then fix tissue slowly.

The most frequently used fixative is a solution of 10% neutral buffered formalin. Due to its cross-linking characteristic, it is an especially good fixative for small molecules such as hormones (2). Optimum fixation time is critical and will vary from one antigen-antibody combination to another. Generally, 6-12 hours is acceptable, but longer fixation is occasionally needed. Over-fixation can pose problems, in that the cross linking can mask epitopes needed to react with the antibody. A frequently used method of repairing this damage involves heating the fixed tissue in distilled water to a temperature of 95 °C for 15 to 20 minutes. This will be discussed later in this chapter, as part of the overall staining procedure.

Many other fixatives are available and a considerable body of literature exists that describes situations in which one of these performs better than others. Some of these fixatives will be discussed later in this chapter. Other specific applications exist in which tissue is frozen and cut, rather than fixed.

Formalin should always be fresh (see above reference to formaldehyde and formic acid formation with time), and buffered to a pH of 7.0-7.6. As this is a slow reacting fixative, acidic mixtures may induce structural or antigenic changes resulting in poor morphology and low detection.

Table 1. Ten percent neutral buffered formalin, pH 7 (10 percent NBF).

Formalin (40 percent formaldehyde)	100 mL
Dibasic sodium phosphate, anhydrous	6.5 g
Monobasic sodium phosphate, monohydrate	4.0 g
Distilled water	900 mL

There are other aldehyde-based fixatives, such as those using glutaraldehyde as a base, but they all act similarly to 10% NBF and are used much less frequently.

Another class of fixatives used significantly in the past is mercuric-chloride fixatives. These do not initiate aldehyde linkages, but react with a number of amino acid residues such as thiols, amino groups, imidazole, phosphate and hydroxyl groups. On the positive side, fixation times are short, in the order of five to eight hours. On the negative side, it should be noted that mercuric chloride is highly toxic, and special disposal procedures are required. For these reasons and because of the prevalence of viable alternatives, these fixatives are used less and less in laboratories today.

Table 2. B5 Fixative.

Reagent A:	Mercuric chloride	60 g
	Sodium acetate	12.5 g
	Distilled water	1000 mL
Reagent B:	10 percent neutral buffered formalin	
Working solution is 90 mL of Reagent A with 10 mL of Reagent B		

Table 3. Zenker's Fixative.

Distilled water	900 mL
Potassium dichromate	25 g
Mercuric chloride	50 g
Glacial acetic acid	50 g
NOTE: Fixation times are four to 24 hours, with an overnight wash or removal of mercuric chloride crystals required. This can be accomplished by one wash in 0.5 percent iodine in 70 percent ethanol, and a second wash in five percent sodium thiosuphate in water.	

Alcoholic Fixatives

This class includes Carnoy's, Methacarn and others. They have been used for IHC purposes primarily in order to try to avoid the loss of antigenicity caused by excessive formalin fixation, or for monoclonal antibodies that reacted against an epitope destroyed by formalin. These fixatives typically found most of the application in looking at lymphocytes using CD-specific markers, and in looking for immunoglobulins such as IgG, A, and M.

Specialty fixatives, such as Osmium tetroxide, which is used primarily in electron micrography and acetone, is used in fixation of frozen sections. Others used for research purposes on specific tissue, organs or even whole organisms are not discussed in this publication.

The final fixative class that is becoming more significant is the "combination fixative." These often combine alcohol with formalin, calcium or other heavy metals, and also with some kind of buffering mixture. Many are commercial, and as such their exact formulations are typically not disclosed by their manufacturers. Most are designed to address the search for a universal fixative that can standardize this element of IHC. To be truly universal, most of these fixatives also address RNA and DNA fixation, for genetic studies in fixed tissue (3, 4, 5).

Many of these fixatives, especially the commercial ones (for instance Omnifix II formerly from AnCon Genetics), have not become universally accepted as standard fixatives.

Specialized Tissue Preparations

No discussion of fixation would be complete without mentioning a few specialized tissue preparations that have been popular in the past. Frozen sections used to be required for some studies, particularly lymph node studies. Their use was due primarily to antigen destruction caused by formalin fixation, or for example, to the need to study a thicker (10 µm) section to study axons in nerve tissue. The introduction of antigen unmasking methods using heated water has largely reduced the need for frozen sections.

The primary remaining motive for using frozen sections in routine practice is the need for a quick examination that eliminates the time required for fixation, processing and de-waxing. Frozen tissue sections are also used when direct or indirect immunofluorescence is the detection method, in which case formalin fixation can produce weaker results. Frozen sections should be fixed with acetone (room temperature, five seconds) before storing. They are then re-processed in acetone (4 °C, 10 minutes) and then re-hydrated in buffer for five minutes before immunostaining.

Blood smears, tissue imprints, cell cultures and purified cells may be examined as fresh tissue or as fixed tissue. These cells can be centrifuged to make a pellet that is then fixed just as in tissue fixation. Alternatively, a fresh smear may be made on the slide, and the cells fixed either with acetone or 10% NBF for 10 minutes. It is important to incubate the slide with an endogenous peroxidase blocking solution prior to staining if there are a large number of erythrocytes present, as these will stain due to endogenous peroxidase.

Finally, many of the newer fixation methods incorporate microwave treatments, either for the fixation itself or to speed fixation of other reagents (6). Direct microwave fixation is probably fixation due to heat, and is primarily a coagulation of the proteins. In conjunction with fixatives, microwaving probably speeds the reaction by heating the solution. It also perhaps speeds the penetration of the solution due to the relaxing of the cell structure.

Tissue and Slide Processing

Once the tissue is well-fixed, subsequent steps seem to have little effect on antigen detection. Variations in xylol processing, alcohol re-hydration, wax temperature, time or formulation, instrumentation used etc., provide satisfactory results in most cases. Some basic processing principles are:

- No processes should raise tissue temperature to higher than 60 °C, as this will cause severe loss of antigenicity that may not be recoverable.
- Tissue fixation medium must be replaced by wax, generally done through a series of incubations in increasing alcohol concentrations to 100%, followed by xylene and then hot wax. This is to provide stability of the tissue (wax) in order to make the cutting of the sections easier.
- The tissue sections should be cut at three or four microns or so in thickness, and certainly no thicker than five microns. Thick sections have multiple layers of cells, and make interpretation extremely difficult.
- When cut, sections are floated on water and picked up on slides that are coated with some adherent material. Some commercially available slides come with a positive charge that attracts the negative charges of tissue proteins. Slides can also be bought or prepared with a coating of albumin or lysine, either of which will provide a sticky surface for creating flat, adherent sections. Sections that are not flat and that have non-adherent ridges will likely be digested or torn off of the slide during immunostaining.
- Once on the slide, wax must be removed completely, in order that the aqueous antibody solution can properly adhere to and penetrate the tissue. This is usually done by heating the slides to about 60 °C to soften the wax, and then reversing the procedure described in A above. The slide is immersed in xylene, 100% alcohol and then diminishing concentrations of alcohol until the final buffer is fully aqueous. Note that 50 slides per 250 mL of xylene is the limit before the xylene is no longer effective, and residual wax begins causing artifacts in the final stained tissue.

Detailed Dewaxing Protocol

1. Circle and label the specimen with a diamond pencil
2. Place in 60 °C oven for 30 minutes
3. Transfer immediately to a fresh xylene bath for three minutes
4. Repeat step C above with a second xylene bath
5. Place in a fresh bath of absolute alcohol for three minutes
6. Repeat step E above with a second bath of absolute alcohol
7. Place in a bath with 95% ethanol for three minutes
8. Repeat step G with a second 95% ethanol bath
9. Rinse under gently running water
10. Do not let dry, store in buffer, begin required antigen treatment (see previous section) or immunostaining

References

1. Immunochemical Staining Methods, 4th Edition, Key, M. editor, Dako Corporation, Carpinteria, 2001.
2. Mies, C. Molecular biological analysis of paraffin-embedded tissues; Hum Pathol. 1994;25;555-60.
3. Gillespie, J.W., et al. Evaluation of Non-Formalin Tissue Fixation for Molecular Profiling Studies, Am J. Pathol. 2002;60:449-57.
4. Herman GE, Chlipala E, Bochenski G, Sabin L, Elfont E. Zinc formalin fixative for automated tissue processing. J Histotechnol 1988;11:85-9.
5. Morales, AR, et al. Continuous-specimen-flow, high-throughput, 1-hour tissue processing. A system for rapid diagnostic tissue preparation. Arch Pathol lab Med 2002;126(5):583-90.
6. Ni C, Chang TC, Searl SS, Coughlin-Wilkinson E, Albert DM. Rapid paraffin fixation for use in histological examination. Ophthalmology (Rochester) 1981;88:1372-4.

Bibliography

Pearse AGE. Histochemistry. Theory and applied. 4th ed. Edinburgh: Churchill Livingstone, 1980.

Leong, Anthony. Extract from Woods and Ellis, Laboratory Histopathology: AComplete Reference, 1994, Churchill Livingstone.

Chapter 6 | Tissue Identification in Immunohistochemistry

James F. Happel DLM (ASCP) HTL

Maintaining identification of patient specimens during the processing, embedding, microtomy, labeling of slides, drying, staining, cover slipping and delivery to the pathologists remains one of the more daunting challenges facing histologists today. The number of steps from processing to delivery that are still manually performed in most laboratories today allows more than ample opportunity for mislabeling and misidentification of cassettes, slides and requisitions to occur.

The importance of a comprehensive specimen identification system throughout the clinical laboratory cannot be understated. Both the College of American Pathologists (CAP) (1) and The Joint Commission (JCAHO) (2) have addressed this important subject with directives that address the pre-analytical, analytical and post-analytical phases of specimen processing. These patient safety initiatives date back to the 1990's and the formation of the National Patient Safety Foundation (3). These mandates have become the backbone of most laboratories' Quality and Safety Programs and offer an excellent foundation for procedures that assure the safe and accurate identification of patient specimens throughout the analysis and reporting of critical laboratory tests.

Specimen Collection and Identification

Correct patient and specimen identification is recognized as being of paramount importance in every hospital. Despite the best efforts of the admitting, nursing and laboratory staff, incorrect patient identification remains a difficult challenge to arrest, contributing to 13% of surgical errors and 67% of transfusion errors (4). In order to help mitigate this persistent perennial problem, a comprehensive specimen tracking system must be developed and strictly adhered to. This system should be built on a foundation of established and recognized "best laboratory practice" procedures that begin at the time of specimen collection. Whether specimens are collected and processed in the same location or if they are transported to a laboratory at a different location for processing, it is essential that every staff member responsible for the labeling and handling the specimen from the time of collection through sign out is certain that each container is labeled with at least two patient identifiers which can include the patient name, date of birth, and/or medical record number among others (1, 2).

Once the staff collecting the specimen has appropriately labeled the container, it is then transported to the laboratory where it is received into the histology laboratory, accessioned into the Histology Laboratory Information System (HLIS) and given a unique identification moniker. This identifier remains with the specimen throughout its prosection, processing, embedding, microtomy, hematoxylin and eosin (H & E) staining, coverslipping and subsequent delivery to the pathologist for interpretation and diagnosis. Occasionally, a specimen may require additional processing in another clinical laboratory, i.e. microbiology. When this is necessitated, the specimen collection staff should be notified of the request as soon as is practical, ideally prior to specimen collection. Every attempt should be made to collect separate specimens for each laboratory in order to allow for the simultaneous processing of the specimen in each laboratory thereby minimizing specimen turnaround-time (TAT). Specimens received in the Clinical Laboratory are traditionally assigned different identification moniker from the HLIS number, typically generated by the Clinical Laboratory Information System (CLIS).

If the pathologist requires more information in order to render a diagnosis than can be gathered from the initial review of the H & E stained slides, a variety of special stains are available that are designed to elucidate specific histological features in the tissue. Chromophilic special stains ranging from Acid Fast to Ziehl-Neelsen are used routinely in most histology laboratories today. Immunohistochemical staining is available as is fluorescent in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH) which are quickly becoming standard in many laboratories. Whatever stains or methods are used, positive patient identification must be maintained throughout the entire process in order to assure that every result is attributed to the correct patient, thereby insuring that the patient receives the proper diagnosis and subsequent treatment.

Specimen Tracking at the Collection Site

It is critical that correct specimen identification is maintained at every step of laboratory testing process including all pre-analytical, analytical and post-analytical stage of testing. Labels to be used for specimen container identification should only be printed at the time of specimen collection and, once printed, placed immediately on the container. Any and all extraneous labels should be discarded. Being certain that the staff responsible for placing the collected specimen into the fixative or transport container is the same staff member responsible for labeling that container, will help minimize labeling errors at the site of origination (5). It is a good practice to have a second member of the staff verify and sign off indicating that they have checked the requisition and specimen containers for accuracy and that they match. Acknowledgment that a discernible tissue specimen is visible in the container at this point is suggested and should likewise be indicated on the requisition form or, perhaps, in a separate log.

Once the specimens are collected and labeled, they should be sent to the laboratory as quickly as possible. Many a specimen has "vanished" into the abyss of "I-Put-It-In-The-Basket-For-The-Courier-To-Take-It-To-The-Lab", never to be seen again. Procedural protocols should be designed in such a way as to help minimize potential sources of distraction that might result in a specimen being misplaced or lost. For instance, requiring that all paperwork and specimens are immediately delivered to the laboratory will greatly reduce the potential for losing or misplacing a specimen.

Even the most exacting and properly followed procedures can fall prey to an unfortunate mishap such as a specimen accidentally falling into an open waste basket. Therefore, the removal of waste baskets from the specimen/requisition assembly area will greatly diminish the possibility of losing a specimen in this manner. Specimens can and have been discovered in waste baskets along with extraneous pre-printed labels that were tossed away in an attempt to prevent them from being used on a subsequent case. Additionally, it is good practice to retain all trash containers for a minimum of three working days. This practice gives you the opportunity to check the refuse for any tissue specimen that may have been accidentally discarded with embedding materials like gauze, sponges or lens paper that was used for processing small pieces of tissue. Most laboratories have a turnaround-time where a missing specimen will be discovered within that 72 hour timeframe.

Once the specimens are ready for transportation to the laboratory, they should be delivered with all practical haste. Frequent specimen pickup and/or delivery to the laboratory greatly reduce the risk of losing or misplacing a specimen. Whichever procedure your facility utilizes, a "chain of custody" should be maintained by utilizing a log or record so that each specimen transported has been accounted for at the time and location of pickup and delivery. The more explicit the record, the better. For instance, having a transporter sign that he or she picked up and delivered specimens at an exact time with no mention of the number of specimens attributed to each case is helpful but not specific enough. Each specimen must be accounted for at every step along the way. A record indicating the quantity and type of specimens transported is preferable.

Upon reaching the laboratory, the requisition and specimens should be checked at once to assure that they match. The transporter presenting the specimens and staff receiving them into the laboratory should communicate and agree that every specimen has been accounted for. Any discrepancies should be identified and documented immediately. Specimens that are in discordance with their associated paperwork should be barred from entering the laboratory, with limited exception. One such exception is when delaying the processing of a specimen will result in a concomitant delay in diagnosis that is critical to the survival of the patient or specimen. Any other specimen should be turned away.

Specimens that are accepted into the laboratory should be triaged immediately to determine if there are any special handling requirements necessary for that specimen, i.e. fresh tissue for frozen sectioning, time sensitive processing or staining runs, fixation needs like glutaraldehyde for specimens destined for electron microscopy, etc. Formalin to tissue ratio should be assessed at this time (the suggested formalin to tissue ratio is 10:1). The specimen is now ready for accession into the LIS.

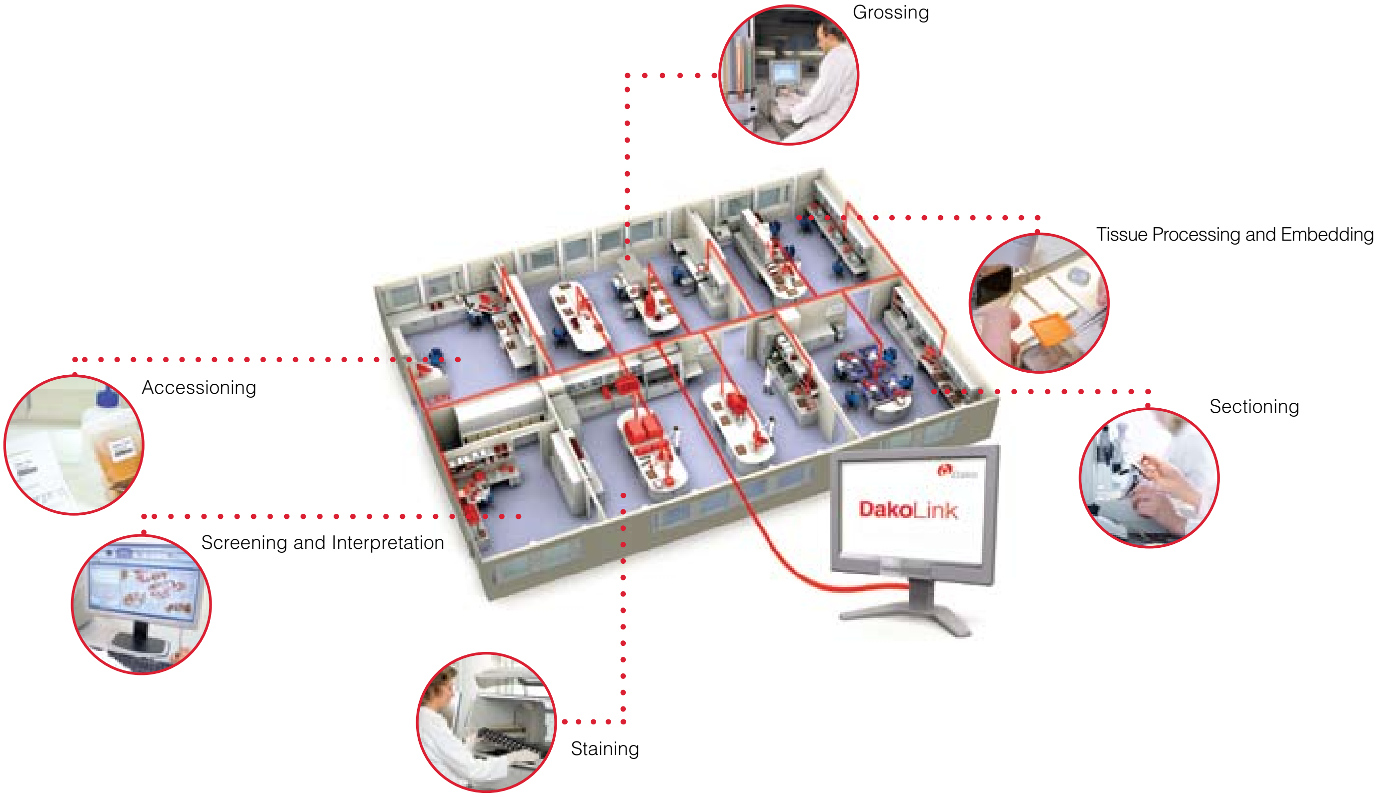


Figure 1. The workflow from accessioning to interpretation.

Specimen Accessioning

Most laboratories use LIS software programs specifically designed to streamline work flow in the pathology laboratory. These systems readily communicate with client and hospital databases used at the time of patient admission when valuable demographic and medical histories are recorded. It is of vital importance, therefore, that the correct patient (and subsequently their demographic information and medical record) is identified. Skilled accessioners are responsible for entering the pertinent data for a particular case, including, but not limited to the patient's name and insurance information, clinical history, surgical procedure performed, specimens generated, and post operative findings. Most LIS's traditionally assign the case number to the patient at this time. In many facilities, the requisition and specimen containers are labeled with that number at this time. It is still common practice for the accessioning staff to write the case number on both the requisition and individual specimen containers. Specimen processing cassettes can be hand written or machine printed at this time as well. Certain "rules of thumb" are universally applied at this critical procedural step in order to reduce the potential of mixing up or mislabeling specimens. These would be: whenever possible, do not accession two similar specimen types in sequence (i.e. two prostate needle core biopsies or two breast lumpectomies, etc.). As with the site of specimen generation and its redundant review of the number of specimens generated, it is a good idea to have a review step as part of the accession work flow. Several facilities I have been associated with require that a second accessioner or supervisor review and initial the accession for completeness and accuracy.

Grossing and Prosection

With the case number assigned, the requisition form and specimen containers correctly labeled and tissue cassettes printed, it's time for the grossing of the specimen.

The grossing of a surgical specimen involves the description and dissection of the tissue for the purpose of rendering a formal diagnosis. From the standpoint of the laboratory, the process involves a pathologist, resident, pathologists' assistant or specimen grossing technologist correctly and accurately associating the case number of the specimen and patient's name with the tissue submitted (6). Ultimately, the prosector is responsible for placing the appropriate tissue section in the correctly numbered tissue cassette.

Here again, there are certain "rules of thumb" that can help minimize the chance of mislabeling errors; have only the requisition and cassettes for the case being grossed and dictated on the cutting bench until the cassettes have been filled and closed. Remove all cassettes containing the dissected tissue sections by placing them in formalin until formal histology processing can be performed. Discard any unused cassettes immediately to eliminate the possibility of accidentally using them with a subsequent case (6).

Histology – Tissue Processing and Microtomy

Even the most careful histology technologist can inadvertently reverse numbers when transferring the number on a tissue cassette to a glass slide. It is therefore essential to adopt and integrate procedures that minimize potential opportunities for errors. For instance, the steadfast rule of not opening more than one cassette at a time when embedding is a well established and practiced policy designed to minimize the accidental transfer of tissue from one cassette to another. Similarly, the wiping off the forceps used for removing processed tissue from cassettes after every use will remove any residual tissue that may have affixed itself to the ridges on the tip of the instrument.

When it's time for microtomy, there are several processes that can, if followed, greatly reduce errors. There are several schools of thought regarding slide labeling that continue to evoke strong preference and discussion. Namely, pre-labeling versus "just-in-time" labeling of glass slides.

Many laboratories choose to pre-label slides using either an automated slide etching system or the traditional hand writing method. Both systems have distinct advantages and disadvantages. The advantage to batch printing glass slides for histology sectioning is that it can be performed at anytime, usually during laboratory downtime. Printing slides when the work load is at a minimum allows for increased productivity by the histologists as they do not have to spend extensive time hand writing every slide. Batch printing has certain inherent dangers, namely that if the slides are labeled in numerical sequence matching the embedded tissue blocks and for any reason the slides and blocks become out of synchrony, there is the likely possibility that a mislabeling event will occur. In labs that pre-label slides, an additional slide/block verification step must take place and is often performed at the histologist's work station via exhaustive visual verification or by a laser scanner and recognition software. This software is designed to scan a bar coded cassette and a similarly bar coded glass slide and, when the block and slide match, a visual and audible confirmation is given to the histologist. Many of these systems are independent from the LIS and thus do not allow for this information to transfer into useful specimen tracking data that would provide insight as to where a specific block or slide is at any given moment. The True Positive Identification (TPID) system offers an outstanding software tool that integrates the various histology processes from accession through sign out. In addition TPID offers a variety of reports and tracking data that can provide managers with information on TAT, that can be used to track individual productivity and error reduction in the laboratory.

Handwritten slides present an additional set of potential identification pitfalls. If the slides are written in batch (pre-writing the slides based on predetermined protocols with expected numbers of slides to be cut for that case) the same potential sequencing problem exists as discussed previously. In situations where slides are written at the microtome when the paraffin block is in the histologist's hand, it is imperative that the "no-more-than-one-at-a-time" rule come into play again. Slides are only written when the tissue has been sectioned and is picked up on the slide, thereby eliminating the risk of selecting a different slide than that assigned to that block.

One of the best ways to minimize the risk of incorrectly labeling a slide by attributing the wrong section to a case is to be certain to clean the water floatation bath of all unused paraffin ribbons after the block has been cut. Vigilantly keeping the floatation bath clean of debris eliminates the possibility of cross contaminating a section with tissue from a different case.

Once the sections have been picked up on the glass slide and labeled correctly, they are traditionally air dried for several minutes to allow excess water to drain from the section. The prepared slides are then placed in a rack and progressed to the drying step where they are placed in an oven. The heating of the glass slides in the oven provides a twofold benefit. Excess paraffin is removed from the section while simultaneously adhering the tissue section to the slide. At the completion of this step the slides are ready for routine staining.

Routine Staining and Coverslipping

H & E staining is the lifeblood of the histology laboratory. Virtually every one of the cases received in the histology laboratory will receive this ubiquitous pathology stain.

The process of H & E staining has been automated in most laboratories today. Whether you use automation or rely on manual staining processes, the chance of mislabeling a slide during this step is minimal due to the fact that the slides are traditionally processed in a rack and very little, if any, disruption to the order of the slides is likely. Nonetheless, there remains the chance that small pieces of may lift from a slide and remain in one of the reagent vessels. There are several precautions that can and should be practiced in order to minimize possible floater cross contamination; be certain that the slides have had ample time to dry at an appropriate temperature. This will give you the best chance of securing the tissue to the slide thereby lessening the potential lifting-off of a section and having it remain behind in the reagent as a “floater” that can be picked up by a subsequent slide.

Once the slides have been stained, a cover slip will be placed over the section and secured with mounting medium in order to protect the tissue and allow for greater clarity when looking at the slide under the microscope.

Slide and Block Reconciliation with Specimen Requisition

The final step in the processing of surgical specimens in the histology laboratory involves the reconciliation of the requested work at the time of accessioning with the produced blocks and slides from the laboratory, thus insuring that all necessary work has been completed and accounted for. This labor intensive yet necessary process involves the accurate interpretation of the requisition’s data as well as an understanding of the protocols required for processing. The ability to determine whether a case is complete falls to the individual who reviews the slides just prior to delivering them to the pathologist.

In order to perform this task the staff must first retrieve the requisition for the case and be able to confirm that the appropriate procedure was requested at the time of accession. When the identity of the patient (and/or case number) is confirmed, the next assessment required is to confirm that the number of tissue blocks submitted has produced the appropriate number of slides and stains. This task requires a well trained technician who is attentive to details and has a thorough understanding of the operations in the histology laboratory. If the case is incomplete the technician must troubleshoot the problem in order to locate the missing materials.

Laboratory Asset Tracking and Work Flow Management

The goal of every laboratory director is to develop safe procedures that benefit the patient, facilitate diagnoses, maximize productivity and decrease errors. These challenges are proving to be more difficult to meet with a paucity of qualified staff and decreasing operational budgets. Laboratories are all being asked to do more with less and to do so more accurately than ever before. The need for assistance has never been greater.

As previously noted, TPID offers a work flow solution that helps to manage the work flow throughout the histology laboratory from specimen receipt and accession, through cassette assignment and printing, grossing, histological processing, microtomy, staining, cover slipping and delivery to the pathologist.

The TPID system provides a precise and accurate method for maintaining specimen identity throughout specimen handling and processing. Both CAP and The Joint Commission require that specimens be identified by not less than two patient identifiers (1, 2). TIPID achieves this with a system that accurately identifies the patient and prints bar coded labels with the two patient identifiers CAP and The Joint Commission require for patient identification for each requisition.specimen container, cassettes, and slide. Each of aforementioned tasks are performed at their respective work stations thereby reducing the errors that can arise when transporting paperwork, cassettes or slides from the printer to the work station. Greater accuracy in identification translates into less time having to be spent on locating mislabeled or "lost" blocks thereby reducing the TAT for each case and giving the technologists a greater feeling of security when processing each specimen.

This demonstrative TAT data, along with a multitude of other process management information, is readily available from the TPID database. This information allows the user to monitor work load and productivity trending that can be used to adjust staffing and instrument usage to meet the demands of the day's work. When instruments are interfaced with the TPID software, locating a particular case of interest in any step of the histological process is made easier. This flexibility allows you to minimize the time required to locate and "move to the front of the line" a STAT case, whenever it is determined to be so.

Do's and Don'ts

Do's

- Have clear and concise procedures based on CAP and JCAHO specimen identification recommendations
- Remove waste baskets from the specimen collection areas
- Retain all trash for a minimum of 3 working days
- Label only one case at a time
- Keep all materials from a single case together (requisition, specimen containers, cassettes, etc.)
- Have the staff responsible for placing the specimen in the transport vessel (i.e. specimens collected during a surgical procedure, cultures to be sent for microbiological analysis, tubes of blood, etc.) be the same individual who labels all materials generated for that case
- Have a second staff member review and verify what was sent to the laboratory
- Have only the tissue processing cassettes for the case being grossed at the prosection station during that cases dissection. Immediately remove from the work bench and discard all cassettes that are not utilized for the processing of the case
- When grossing, only have the tissue cassettes for the current case on the cutting board at the time of dissection. All other tissue cassettes should be with their appropriate specimen and not on or near the prosection station.
- When embedding, be certain to wipe off forceps after every use to insure that any residual tissue is removed thereby preventing carryover from one cassette to another
- Work in tandem with all those who come in contact with the specimen including the Operating Room Team, Transporters, Specimen Accessioning staff, Pathologists' Assistants, Residents, Pathologists, Histologists, Secretaries, Transcriptionists, slide and block Filing Room Staff, etc.

Don’ts

- Do not pre-label requisitions, specimen containers or tissue cassettes
- Do not accession similar case types (tissue) in sequence
- When embedding, do not open more than one cassette at one time
- When cutting on the microtome, never cut a second paraffin block until the first block has been cut to completion
- Do not leave ribbons on the water bath after picking up sections on slides
- Do not leave printed labels in the work area. Discard all unused labels as soon as it is identified that they are not to be used

References:

1. CAP Website: www.cap.org. Laboratory Patient Safety Plan.http://www.cap.org/apps/cap.portal?_nfpb=true&cntvwrPtlt_actionOverride=%2Fportlets%2FcontentViewer%2Fshow&_windowLabel=cntvwrPtlt&cntvwrPtlt%7BactionForm.contentReference%7D=patient_safety%2Flaboratory_patient_safety_plan.html&_state=maximized&_pageLabel=cntvwr
2. The Joint Commission Website: http://www.jointcommission.org. National Patient Safety Goals document.
3. The National Patient Safety Foundation Website: http://www.npsf.org/
4. Ohio University Center for Automatic Identification: Executive Summary: Datamatrix and PDF417 Data Integrity Test, Russ, Fritz J, Russ, Dolores H.
5. Clinical and Laboratory Standards Institute: Collection, Transport, Preparation and Storage off Specimens for Molecular Methods; Approved Guidelines; MM12-A, Vol. 25 No.31
6. Allen, Derek C., Histology Specimens: Clinical, Pathological and Laboratory Aspects, Springer; 2004

Chapter 7 | Tissue Microarray — Construction and Quality Assurance

Rashmil Saxena BFA, HT(ASCP)^{CM} and Sunil Badve MD, FRCPath

Recent times have seen the advent of high throughput assays such as array comparative genomic hybridization and cDNA microarray, which have lead to the rapid discovery of thousands of potential biomarkers. However, these need to be validated in tissue-based studies in large datasets to prove their potential utility. As these datasets are typically present in the form of formalin fixed paraffin-embedded tissue blocks, immunohistochemical (IHC) methods are ideal for validation. However, performing whole-section IHC on hundreds to thousands of blocks requires lot of resources in terms of reagents and time. In addition, an average block will yield less than 300 slides of 5 µm each. The tissue microarray (TMA) technique circumvents some of these problems.

The origin of TMAs can be attributed to Dr Hector Battifora's humble "sausage" blocks (1) in which a number of tissues, typically from different organs, where thrown together in the same block and tissue distribution of a particular antigen/protein was assessed. A significant disadvantage of this technique was that when tumors or tissues from the same site were put together it was difficult if not impossible to trace them back to the patient. This prevented meaningful analysis of prognostic markers. However, many laboratories small and large (including our own) adopted this technology to generate multi-tissue or multi-organ tissue blocks. The next step in the development of TMA was described by Wan et al. (2) who used a 16-gauge needle to manually bore cores from tissue blocks and array them in a multi-tissue straw in a recognizable pattern. This method was further modified by Kononen et al. (3) using a 4 mm skin biopsy punch. They used a cast of a small amount of melted paraffin to record the position of each punch specimen. This landmark study lead to the development of a TMA precision microarray instrument with an x-y guide by Beecher Instruments, Sun Prairie, WI. This enabled real high-throughput analysis with arraying of up to 1000 cores in the same block.

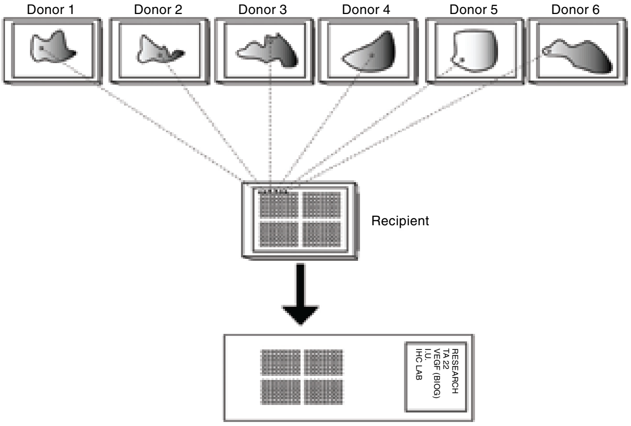


Figure 1a. Principle of tissue microarray (TMA) analysis. Cylindrical cores are obtained from a number (up to 1,000) of individual formalin-fixed, paraffin-embedded tissue blocks. These are transferred to recipient TMA. Each TMA block can be sectioned up to 300 times. All resulting TMA slides have the same tissues in the same coordinate positions. The individual slides can be used for a variety of analyses saving labor and reagent costs while maintaining uniformity of assay.

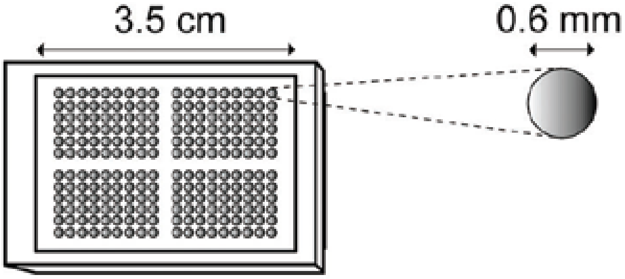


Figure 1b. Typically a minimum of 3 cores for each case are used for 0.6 mm cores.

Advantages and Disadvantages

Advantage to TMA: The major advantage of TMAs is that they allow the performance of tissue-based assays (immunohistochemistry, histochemistry, in situ hybridization, etc.) on a large number of patient samples in an efficient and cost-effective manner. With TMA technology, several hundred representative cores from several hundred patients may be included on a single glass slide for assay. Thus, significantly more tissue can be conserved than if the blocks were to be sectioned serially. TMAs have been generated from all tissue types including decalcified bone and core biopsies. The latter are usually rotated 90 degrees and embedded vertically to ensure presence of tissue of interest in multiple cuts. In addition, methods for generating TMAs from fresh frozen tissue using blocks made from either optimal cutting temperature (OCT) compound or from a mix of gelatin- sucrose have been described.

Disadvantage to TMA: The major disadvantage of TMAs is that each core (or set of cores) represents a fraction of the lesion. This was considered a major weakness, particularly in the early days of the TMA. However, multiple studies in different organ systems have now demonstrated that consistent and comparable results can be obtained using TMA cores as with whole sections. In order to obtain comparable results two main strategies have been used. The first is increasing the number of cores from each case. It is typical to use cores at least two cores from each case when using a 1 mm core; a minimum of 3 cores from each case for 0.6 mm cores. Although a 2 mm core might be theoretically considered better than multiple smaller cores, in practice this usually not true in practice. Smaller cores permit sampling of different tumor areas and are therefore more likely to be representative of the entire tumor. In addition, smaller cores tend to inflict lesser degree of damage on the original tissue blocks. The second strategy consists of increasing the number of tumors included in the study. This method averages out the errors associated with tumor heterogeneity associated with the use of tissue cores.

When Not to Use TMAs

TMAs are not recommended for certain types of studies. In certain tumors such as glioblastoma, there is marked heterogeneity within tumors; this cannot be captured in TMA studies. In addition, TMAs are also not very useful to study rare or focal events such as number of immune cells in tumors. It is also difficult to study facets of tumor biology such as interactions between the tumor and it's stromal as these stromal components may not be adequately represented in the cores. The use of large cores (2 mm) has been advocated for these types of studies.

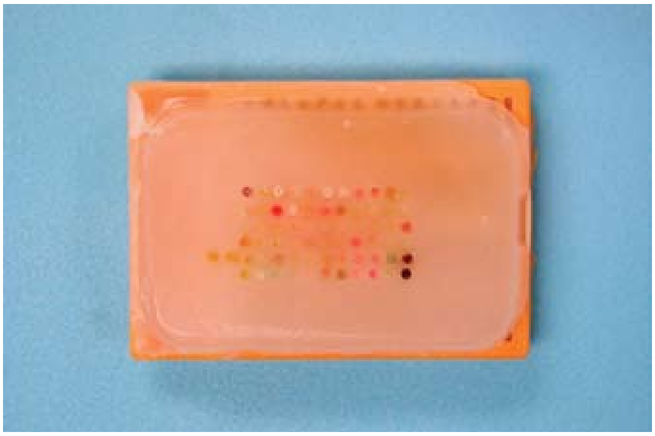


Figure 2. Photograph of low density TMA block.

Types of TMAs

The type of TMA to be generated depends on the question being asked within the study. The following are the commonly used types

Cell line arrays: These arrays consist of normal or cancer cell lines that are grown in culture. The major function of these arrays is survey the presence of proteins that are known to be present in one or more the cell lines. In addition, they can be used to analyze the utility (and specificity) of an antibody in detecting the proteins. The commonest example of this type of array is the 3 cell line control that is used with HER2/neu testing in breast cancer.

Random tissue/tumor arrays: These arrays contain tissues from multiple sites and contain tumor and or non-tumor tissues. Small arrays of this kind can be used for quality control measures such as monitoring of existing reagents/antibodies as well as work-up of novel reagents. In addition they can also be used as discovery tools. For example a survey of CD10 in tumors arising in multiple tissue sites lead to the discovery of its utility in diagnosing uterine stromal tumors (4).

Consecutive case array: This type of array is constructed using consecutive cases belonging to a single tissue site. These types of arrays are extremely useful for quality control purposes including identifying shifts and drifts in reagent quality. They are also useful in studying the prevalence of a protein/antigen in a given tumor type and analyzing the relationships between different biomarkers.

Tumor characteristic-based array: This is a special type constructed solely on the basis of a given characteristic such as patient age or tumor grade. The latter is useful for evaluating the frequency of a marker throughout the spectrum of tumor differentiation. Similarly TMAs can be generated based on the expression of a biomarker such as estrogen receptor or HER2/neu positive or triple negative breast cancers. These types of biomarkers are useful in analyzing interrelationships between different cellular pathways.

Progression arrays: These types of arrays are used to analyze the role of protein(s) in cancer progression and consist of normal tissues from patients without cancer, normal tissue from patients with cancer, pre-invasive lesions and tumor (from local and metastatic sites). The addition of normal tissue from close to the tumor and those much further away from the tumor site might enable study of “field effect”.

Outcome based arrays: These are the most valuable and most difficult to generate as they involve collation of tissues from patients that have the same disease and have been more or less similarly treated and followed up for a significant period of time. The period of follow-up depends on the type of disease or tumor being studied. These types of arrays are mostly used to evaluate prognostic or predictive biomarkers. The presence of biomarkers in tumor subtypes might then be used to design novel therapeutic strategies.

Other special types: TMAs can be generated based on specific question being asked whether it be race (Caucasians versus African Americans), sex (male versus female) or more tissue oriented questions such as center of the tumor versus invasive edge of the tumor.

Team Required for TMA Construction

Construction of TMA is a team effort. The first and foremost question that needs to be answered is why is the TMA being constructed? This will decide the composition of the team. For the generation of the simplest of TMA, a technologist might be all that is required. However, most TMA synthesis will require close collaboration with the pathologist, who will identify the areas of interest and mark them for the technologists to obtain cores. It is a good idea to involve biostatisticians from the onset rather than just asking them to do the data analysis. They can help with deciding the number of cores from each case, total number of cases needed, how they should be distributed across the array to avoid bias. In general the number of cases required in a TMA is dependent on the size of the difference expected in the outcome and the degree of variance in the samples; this sample size calculation is best done with the assistance of a statistician. Outcome based TMAs may need input from or participation of treating physicians/oncologists.

Steps Involved in TMA Construction

The following steps are recommended for breast TMA construction:

Step 1. Define the question

As described above TMAs are created to answer specific questions. It is important to clearly define this question at the outset. The question will help define the number of cases and cores that need to be used in the generation of the TMA. For example a TMA containing 20 cases might be sufficient for routine quality control/ assessment but is not enough for biomarker assessment.

Step 2. Review the cases to be included in the TMA

Pull all the cases to be included in the TMA together. If the blocks have been previously cut into for other clinical or research purposes, a fresh H&E slide may be obtained to ensure that the slide is representative of the block. Review all the slides and mark areas of interest. It is useful to mark multiple areas from more than one block, as blocks may be depleted or misplaced. Areas to be sampled (tumor, normal, and pre-malignant tissues) should be identified.

Step 3. TMA core size and number of cores

Size of the cores: The typical core sizes used for TMA constructs are 0.6 mm, 1 mm, 1.5 mm and 2 mm. Many workers consider the small 0.6 mm cores as the standard of practice. Use of smaller core diameters, however, allows for a greater number of cores to be extracted from the lesion and a greater number of cores that can fit into the TMA block. In addition, they tend to inflict little damage on the donor and recipient blocks and the cores are easier to remove and replace from these blocks.

The larger core sizes have the advantages of being more robust and the cores are more difficult to damage during handling. However, these larger sizes can lead to increased likelihood of difficulty in extracting the cores from the blocks as well as greater chance of the blocks being broken or cracked during the TMA generation process.

Number of cores: The optimal number of cores, to be included in the TMA, is marker dependent and can vary depending on the degree of tumor heterogeneity. In general, the greater the degree of intratumoral heterogeneity for any given marker, the greater the number of cores that will be required. When using 0.6 mm sized cores, it is typical to use a minimum of 3 cores per case. Three 0.6 mm cores are still better than one 1.0 mm core, even though the tissue surface area is essentially identical. Three 1.0 mm cores could result in destruction of the donor block; so tumor size would also drive number/size of cores to be taken. Studies that have used 1 mm core punches have tended to use two cores (5).

Density: The maximum number of cores that should be placed on a single block will vary depending on core size, block size, and IHC methodology, among other factors. It is best to avoid placing so many cores on a TMA that the surface section of cores becomes larger than the antibody coverage area on the slide programmed by the Autostainer (Dako A/S). Similarly, it diminished the amount of paraffin at the edge of the block creating difficulties in sectioning. Cores should start at least *3 mm away from the block edges*, to prevent the paraffin from cracking. Max number of cores per block should therefore depend on the comfort level of the technician as well as the pathologist, who is ultimately going to read the slides. For these reason, it is typical for most workers to put somewhere between 100 and 300 of 0.6 mm cores in a TMA block.

Distance: The distance between cores should NOT exceed the core diameter. It is easier for the microscopist to follow the rows and columns if he/she can "lead" from one core to another. If the distance between cores is large, it difficult to follow the chain of cores and may result in skipping of lanes and false recording of data when performing manual interpretation.

Step 4. Identify control tissues to be included in the block

Controls should be placed on each TMA block, for quality control and to address tumor heterogeneity. Three types of control tissues may be used:

A. **Tissue-specific controls:** Normal tissues and cell lines from the organ site can help in comparative analysis of the marker expression status in addition to help ensure standardization.

B. **Biology-associated controls:** It is useful to insert pathway associated controls to ensure that the reagents are working well. These serve as good internal controls within the TMA block. Common examples include endometrium for hormone receptor, testes or lymph node or tonsils for proliferation.

C. **Organ system controls:** adrenal gland, brain, breast, colon, kidney, liver, lung, pancreas, placenta, prostate, testes, salivary gland, uterine myometrium (smooth muscle). These are particularly useful when the TMA is being used to analyze novel markers, as one or more of these tissues can serve as internal controls. Normal tissue, at a minimum, should contain: liver, kidney, endometrium, lymph node, colon, and testis.

Step 5. Make a TMA map depicting the layout

The TMA map may consist of a simple Excel sheet or may be a more sophisticated datasheet made using one of the TMA generation programs. This will serve as a guideline to in order arrange blocks and sequence in which they need to be arrayed. Thus the TMA map will contain the exact location of each case, including the duplicate samples, and controls are located. Mini-arrays ("City Blocks") of the cores (3x5, 4x5, 5x5, 6x5) can be spaced for easy orientation, with control tissue in the rows between the mini-arrays.

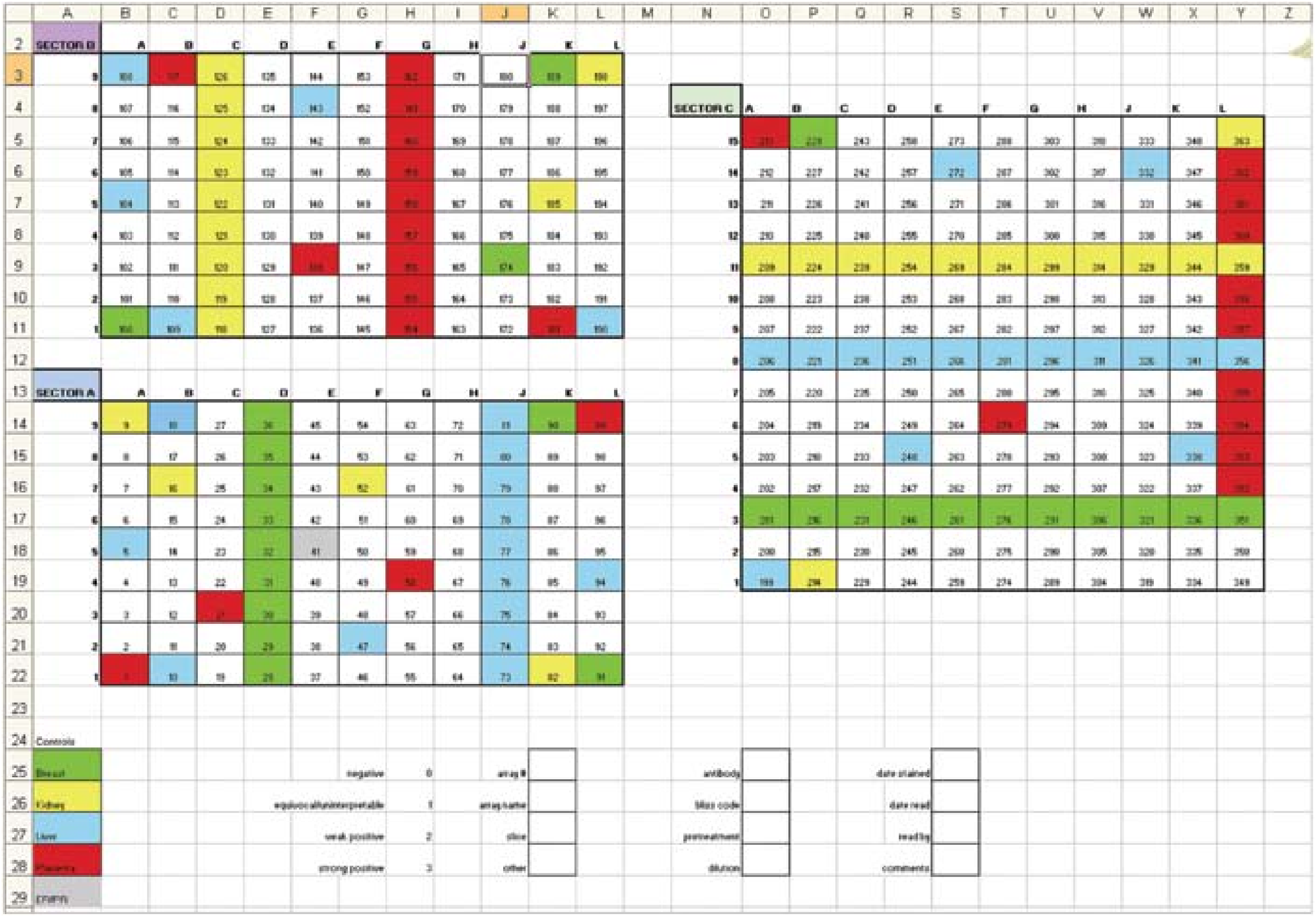

SECTOR B
SECTOR A
SECTOR C
Controls
Breast
Kidney
Liver
Placenta
negative 0
equivocal/uninterpretable 1
weak positive 2
strong positive 3
array #
array name
slice
other
antibody
Bliss code
pretreatment
dilution
date stained
date read
read by
comments

Figure 3. TMA Map and block design: TMA layout should be asymmetric and irregular so that it is relatively easy to orient the TMA block. This irregularity should be obvious to the histo-technician who is cutting the block so that all the cuts from the block are taken on the slides in an identical manner. In addition, locating the controls in an asymmetric manner is also helpful when reading the slides. For example, the following features may be included:

1. Blank rows and columns that do not run down the center lines of the TMA blocks but to one side so that the block is cut into two-third and one-third grids.
2. A blank corner for orientation or a tail coming out from close to one of the corners.
3. Asymmetric distribution of control cell lines and tissue controls. Placing stained cores of “control” tissues at the edge of the grid can be useful to mark orientation.

Issues related to layout:

1. TMA layout should be *asymmetric and irregular* (see figure 3).
2. If multiple TMA blocks are being made from the same project, one consideration is to carry a small proportion of cases onto other blocks (e.g., 10%).
3. Cores from the same case: Ideally, if *same-patient cores* are to be placed on the same block, they should be *dispersed on the block.* This will decrease the risk of interpretation bias. However, some workers prefer this arrangement so that they can immediately "normalize" or "confirm" the results between the cores from the same patient. If same-patient cores are to be dispersed across multiple blocks, it is better to place them in different regions of the array (outer and inner) (outer in one block and inner in the other), with random placement, rather than placing them in the same location in each block.

Step 6. Creating the TMA itself

Instrumentation: The need for specialized instrumentation for creating TMAs is entirely based on the number of cores and value of the tissue being inserted in these TMAs. For TMAs being constructed for quality control/quality assessment or work-up of new reagents, the number of cores being inserted is relatively low. This enables use of larger cores and diminishes the need for specialized instrumentation. However, for TMAs to be made from valuable cases with scant materials, it is necessary to use these instruments. The simplest of these consist of hand-held punches and are generally not very useful for a serious TMA project, where it is necessary to use at least an intermediate grade device. These intermediate grade devices consist of a stand in addition to a positioning apparatus and ensure vertical punching of the blocks and proper placement within the grid. Fully automated devices additionally have integrated computers that can be programmed to select the donor sites from different blocks and transfer them in the recipient block.

Donor block: The block from which a core will be taken is referred to as the donor block. The area of the donor block to be cored for TMA should be selected by a pathologist. Although it is intuitive, it must be stated that the donor blocks should be optimally processed and should not contain any poorly processed areas. Similarly, cores should be obtained from the block before the block gets depleted. The thicker the donor blocks the more the number of useful sections can obtained from the TMA. Core punches should be pushed gently into the TMA block, and not too deeply as this can damage the needle as well as the block. When using semi-automated devices it is easier to mark the depth of the punch to the level of the plastic of the cassette.

Recipient block: The block in which the cores are placed is referred to as the recipient block. It is best to place the cores towards the center of this block in order to prevent cracking of the block. After the cores are inserted, place the TMA in 37 °C overnight, and then on the cold plate of the tissue embedding station with subsequent two to three 1-hour cycles of hot/cold to temper the array. Multiple sections from the block should be cut at the same time to prevent wastage of tissue. Incomplete sections should not be discarded; these can be used for standardization of staining technique (see below).

Staining TMAs: When performing staining of the TMA, the step is to ensure that the staining procedure actually works in the laboratory and the procedure has been standardized. If the TMA has been obtained from an outside institution, it is useful to get the tissue processed in that laboratory or poor-quality sections from the TMA for practice and standardization; following standardization good quality TMA sections should be used for analysis. As TMA sections are usually larger, they might require care to ensure that the entire section is covered with reagents otherwise uneven staining will be observed.

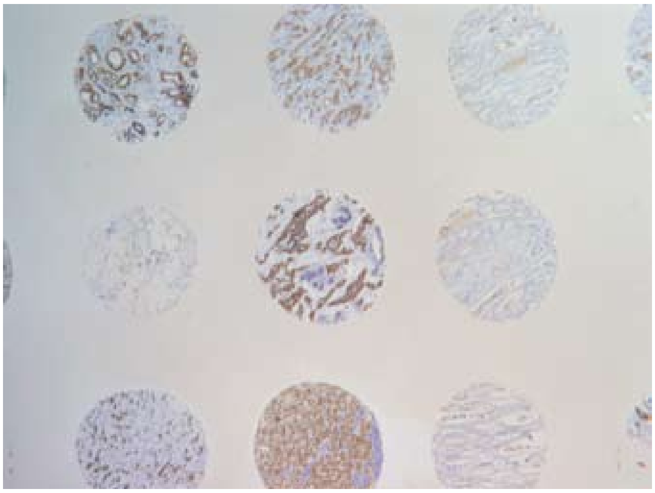


Figure 4a. Low power photograph of TMA stain.

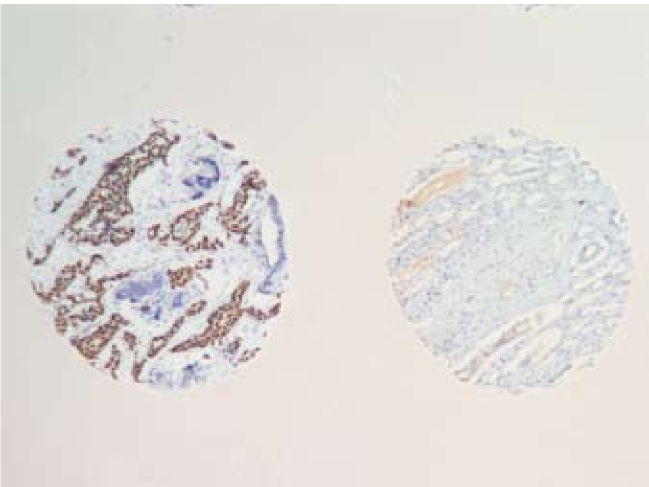


Figure 4b. High power photograph of TMA stain.

One of the limitations of the TMA is that the tissues in the tumor cores have been processed at different types and often with different protocols. This will lead to optimal staining of some tumors but also sub-optimal staining (over or under-staining) of quite a few tumor cores. However, the large number of cases included in the TMA can to some extent compensate for the tissue heterogeneity.

Step 7. Validation and quality assurance

Measures for the TMA should include the following:

Validation: The use of TMAs enables analysis of large data sets, however this does not by any means suggest that the data set is not skewed. This skewing may be the result of the institution's location (population distributions with regards to race, ethnicity, access to health care), type of practice (community hospital versus referral center). These collectively might influence the tumor size, grade and subtype composition of the cases in the dataset. Such abnormalities of the dataset need to be compensated; the involvement of a biostatistician from the start (i.e from case selection) helps to prevent the creation of biased TMAs. It is useful to perform common biomarker analysis on sections from the created TMA to confirm the "normal" distribution of known parameters. Comparison of this data with prior clinical data (e.g. ER analysis) obtained from whole section analysis is particularly useful to validate utility of the TMA. Alternatively the incidence of expression of a number of biomarkers in the TMA should be compared to that in published literature (using whole sections).

Quality assurance measures: It is critical to perform and analyze H&E sections from the TMA to confirm the presence of tissue of interest (usually tumor) in the TMA sections. In addition, H&E should be performed at regular interval (e.g. on every 25th slide) on cuts from the TMA blocks. The above tests should be reviewed by a pathologist.

TMA Analysis

The analysis of the TMA has 2 components. The first involves analysis of the slides and recording of the data. The second involves data analysis.

Slide analysis: The TMA slides can be analyzed manually; alternatively automated image analysis programs that can assist with the analysis are also available. The need for these programs is based on the work volume as well as density of the TMAs. Some programs generate a virtual slide of the TMA and further analysis can be done using a computer screen. This has the advantage of avoiding "burn' to the slides and all the cores can be analyzed at the same "optical and illumination" conditions. It additionally permits electronic storage of the fresh images and later re-analysis if required. This is particularly true for FISH sections which typically fade with time.

Data Analysis

Step 1. Data cleaning

Given the large number of samples in a typical TMA study, analysis of the data can become quite a challenge. One needs to exclude the cases that are not informative; it is not unusual to lose up to 10% of cases due to insufficient representation of tissue of interest. On the informative cases, strategies for conversion of 3 values (one per core) per each case into a single data point have to be devised. The commonly used strategies include using the highest value or a numerical mean of the values obtained (for review see Bentzen et al.) (6). Each method used for normalization has its own value as well as limitation.

Step 2. Statistical analysis

The tests used to determine the p value will be dependent on the type of data (i.e. nominal or categorical) as well as the degree of variance within the data. For simple analyses of relationships, contingency tables, and chi-squared tests are used. For demonstration of survival distributions, most researchers use the Kaplan-Meir plot and then apply Log-rank analysis to test survival differences between groups. The most frequently used analytical strategy is to subdivide patient material into high- and low-risk group based on the expression of novel biomarkers. Some commercially available computer programs such as X-tile program (7) may assist the selection of best cut-off point. This cut-off point needs to be confirmed in a separate series of cases to validate its utility. The NCI-EORTC group has developed the REMARK (Reporting recommendations for Tumor Marker prognostic studies) (8) guidelines which should be followed whenever possible

References:

1. Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest 1986;55:244-8.
2. Wan WH, et al. A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. J Immunol Methods 1987;103:121-9.
3. Kononen, J., et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998; 4:844-7.
4. Chu P and Arber DA. Paraffin-section detection of CD10 in 505 nonhematopoietic neoplasms. Frequent expression in renal cell carcinoma and endometrial stromal sarcoma. Am J Clin Pathol 2000;113: 374-82.
5. Badve SS, et al. Estrogen- and progesterone-receptor status in ECOG 2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. J Clin Oncol 2008;26:2473-81.
6. Bentzen SM, et al. Multiple biomarker tissue microarrays: bioinformatics and practical approaches. Cancer Metastasis Rev 2008; 27:481-94.
7. Camp RL, et al. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Cancer Res 2004;10:7252-9.
8. McShane LM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005; 97: p. 1180-4.

Chapter 8 | Demasking of Antigens

eds George L Kumar PhD and Lars Rudbeck PhD*

Tissue specimens are fixed in formalin and subsequently embedded in paraffin in the majority of cases. While formalin fixation preserves tissue morphology, it also alters the three-dimensional structure of tissue proteins. This alteration can result in a modification of the antigen's epitopes and/or of its electrostatic charges (EC). The loss of an epitope results in an antigen's inability to react with the paratope of the antibody and can only be corrected by the restoration (retrieval) of the epitope. The reduction of net negative ECs of antigens decreases the avidity of the immune reaction and may be compensated for by prolonging the incubation time with the primary antibody (1) or the use of different antibody diluents (2).

The development of primary antibodies reacting with fixation-resistant epitopes has somewhat improved this situation. However, the introduction of enzymatic pre-treatments for tissue sections — and particularly the introduction of methods for the heat retrieval of tissue antigenicity — were aimed at restoring the affinity and avidity of the immune reaction and have become very important pre-treatment tools in immunohistochemistry (IHC) (3).

Before proceeding, proper definitions of terms are required. Some terms such as "heat-induced epitope retrieval" (HIER) and "demasking of epitopes" have been used to describe epitopes being destroyed during formalin fixation. "Heat-induced target retrieval" or HITR is another commonly used term, which allows for the inclusion of nucleic acids as retrieved targets prior to in situ hybridization. Finally, the term "heat-induced antigen retrieval" (HIAR) has been coined to describe the retrieval of antigenicity lost during formalin fixation (3). In this guide, the term HIER for retrieval of antigenicity (e.g. epitopes and ECs) will be used due to the prevalence of its use in IHC practice.

The routine use of HIER following the formalin fixation of antigens has been shown to minimize the inconsistency of IHC staining well-known to be the consequence of variable lengths of fixation (3-5). The use of a uniform size of tissue blocks for formalin fixation is also recommended. Both will make important contributions to the standardization of IHC. Unfortunately, the proliferation of new HIER methods during the last 10-15 years has led to some confusion and made the ongoing efforts of standardization more difficult. It should be noted that HIER requires increased tissue adherence to the slides as compared to H&E staining; thus appropriate baking (60-70 °C) is necessary.

Dako continually evaluates and compares published retrieval procedures and has updated this Guide in order to help immunohistochemists obtain optimal and reproducible staining results when using Dako's primary antibodies and visualization systems (6-9).

This chapter will describe the following retrieval protocols:

- I. Water Bath Methods
  - A. Dako **PT**Link
  - B. Conventional Water Bath Heating
- II. Pressure Cooker Heating
- III. Autoclave Heating
- IV. Microwave Oven Heating
- V. Proteolytic Pre-treatment
- VI. Combined Proteolytic Pre-treatment and HIER
- VII. Combined Deparaffinization and Target Retrieval

The composition and the pH of retrieval buffers are crucial for optimal retrieval. Dako offers the following retrieval buffers for HIER.

Table 1.

Product Name	Code
Target Retrieval Solution, pH 6.1, Ready-to-Use	S1700
Target Retrieval Solution, pH 6.1, 10x Concentrated	S1699
Target Retrieval Solution, pH 9, Ready-to-Use	S2368
Target Retrieval Solution, pH 9, 10x Concentrated	S2367
FLEX Target Retrieval Solution, Low pH	K8005
FLEX Target Retrieval Solution, High pH	K8004
Target Retrieval Solution, pH 9, 10x Concentrated, (3-in-1)	S2375

Although citrate buffers of pH 6 are widely used retrieval solutions, high pH buffers have been shown to be widely applicable for many antibodies (10-11). It is the responsibility of the individual laboratory to determine which of the listed buffers perform optimally for each antigen/antibody and then to use them consistently. Although 0.01 M citrate buffers of pH 6 (e.g. Codes K8005/S1700/S1699) have historically been probably the most widely used retrieval solutions, high pH buffers (e.g. Codes K8004/S2368/S2367) have started being implemented for many cases showing improved end results. Again, it is the individual laboratory's responsibility to determine which of the listed buffers perform optimally for each antigen/antibody and then to use them consistently.

---

*Incorporated from the Dako Education Guidebook — Demasking of Antigens (2008)

The following protocol descriptions should serve as guidelines only. It is the responsibility of the individual laboratory to compare methods and select the optimal protocol for consistent use. It is recommended for the HIER methods to control temperature settings as well as the factual temperature at regular intervals.

Water Bath Methods

A. Dako **PT**Link

Dako **PT**Link instrument simplifies the water bath antigen retrieval process by performing automated retrieval using specified protocols, which incorporate preheat temperature, antigen retrieval temperature, and time as well as cool down settings. Typically, antigen retrieval is performed for 20 minutes at 97 °C.

Materials Required

Dako **PT**Link (Code PT100/PT101)

Dako Autostainer Slide Rack (Code S3704)

Retrieval solution

FLEX IHC Microscope Slides (Code K8020) or slides coated with other suitable adhesives

Personal protective equipment

Protocol

Wear chemical-protective gloves when handling parts immersed in any reagent used in **PT**Link.

1. Deparaffinize and rehydrate tissue sections.
2. Prepare a working solution of the selected target retrieval solution according to specifications.
3. Fill tanks with 1.5 L of desired target retrieval solution.
4. Place tank lids on tanks. Close and lock main lid with external latch.
5. See Operator's Manual for instrument set-up details.
   a. Recommended time is 20-40 minutes.
   b. Set antigen retrieval temperature to 97 °C.
   c Set preheat temperature to 65 °C (allows up to 85 °C).
6. Press [RUN] button for each tank and the CYCLE will show PREHEAT. Allow solution to reach the selected preheat temperature.
7. Open the **PT**Link and immerse the Autostainer Slide Rack with deparaffinized tissue sections into the preheated target retrieval solution.*
8. Place tank lids on tanks. Close and lock main lid with external latch.
9. Press [RUN] button for each tank to start run. CYCLE will show WARM-UP and the lid lock will engage.
10. **PT**Link will warm up to preset temperature and then start the countdown clock for target retrieval cycle.
11. When target retrieval cycle is finished, CYCLE will show COOL. The COOL cycle is finished when temperature reaches Preheat SET temperature, even if Preheat is disabled.
12. When COOL cycle is finished, CYCLE will show DONE and lid will unlock automatically.
13. Open the **PT**Link and remove each Autostainer Slide Rack with the slides from the **PT**Link Tank and immediately immerse slides into the **PT**Link Rinse Station (Code PT109) containing diluted, room temperature Dako Wash Buffer (10x), (Code S3006).
14. Leave slides in the diluted, room temperature Dako Wash Buffer for 1-5 minutes.
15. Place slides on a Dako Autostainer instrument and proceed with staining.

B. Conventional Water Bath Heating

One of several advantages of the water bath heating method is the ready availablility of water baths in most clinical laboratories. Temperature settings just below the boiling point of water (95-99 °C) are most commonly used.

*As an alternative, a 3-in-1 solution can be used for both deparaffinization and target retrieval. See Section VII. Combined Deparaffinization and Water Bath Heating.

Materials Required

Temperature-controlled water bath

Dako Autostainer Slide Rack (Code S3704)

Incubation container and cover

Retrieval solution

Tris-Buffered Saline (Code S3001)

Silanized Slides (Code S3003) or slides coated with other suitable adhesives

Thermometer

Personal protective equipment

Protocol

It is recommended to wear insulated gloves when handling parts immersed in any reagent used in a water bath.

1. Deparaffinize and rehydrate tissue sections.
2. Fill container with enough retrieval solution to cover slides and equilibrate to 95-99 °C in water bath.
3. Immerse racked slides in preheated retrieval solution, cover container with lid, and incubate for specified time within the 20-40 minutes range after the set temperature has been reached.
4. Remove the container from the water bath and cool the contents with the lid in place for 20 minutes at room temperature.
5. Rinse with Tris-Buffered Saline (TBS) or distilled water at room temperature. When removing the slides from the container it is very important that the slides do not dry out.
6. Transfer slides to TBS.
7. Proceed with IHC staining.

Pressure Cooker Heating

Pressure cookers set to approximately 103 kPa/15 psi will achieve a temperature of approximately 120 °C at full pressure. Alternatively, setting at 125 °C can be used for antigen retrieval. Stainless steel pressure cookers are recommended as the aluminium models are susceptible to corrosion by some retrieval solutions. As an alternative, individual plastic container(s) can be filled with retrieval solution and placed in reagent grade water in the pressure cooker pan.

Materials Required

Stainless steel pressure cooker, preferably electrically programmable

Metal or plastic slide racks

Retrieval solution

Silanized Slides (Code S3003) or slides coated with other suitable adhesives

Tris-Buffered Saline (Code S3001)

Incubation container (optional)

Personal protective equipment

Protocol

It is recommended to wear a face safety shield and insulated gloves.

1. Deparaffinize and rehydrate tissue sections.
2. Fill the pressure cooker with enough retrieval solution to cover slides. Alternatively, fill individual plastic container(s) with retrieval solution and add at least 500 mL of reagent grade water to pressure cooker chamber.
3. Bring contents to near boiling point, place racked slides into retrieval solution, seal the pressure cooker, and again bring the solution to a boil. For programmable pressure cookers, set target temperature and heating time, place racked slides in retrieval solution, seal the pressure cooker, and begin antigen retrieval procedure from room temperature.
4. Boil for 30 seconds to 5 minutes, allow the pressure cooker to cool for 20 minutes prior to opening. (Note: Vent any residual pressure before opening). Open programmable pressure cooker when antigen retrieval procedure is completed.
5. Transfer slides to room temperature Tris-Buffered Saline. When removing the slides from the container it is very important that the slides do not dry out.
6. Proceed with IHC staining procedure.

Autoclave Heating

When set to 15 psi, an autoclave, like a pressure cooker, will achieve a temperature of about 120 °C at full pressure (12-13).

Materials Required

Bench top autoclave

Plastic or metal slide rack

Incubation container

Retrieval solution

Silanized Slides (Code S3003) or slides coated with other suitable adhesives

Tris-Buffered Saline (Code S3001)

Personal protective equipment

Protocol

It is recommended to wear face safety shield and insulated gloves.

1. Deparaffinize and rehydrate tissue sections.
2. Place slides in plastic or metal slide rack.
3. Fill the incubation container with enough retrieval buffer (typically 250 mL) to cover slides. Insert the slide rack and cover.
4. Place the container in the autoclave and follow Autoclave Manufacturer's Operating Instructions.
5. Set the temperature to 120 °C/15 psi and the time to 10-20 minutes. Start operation.
6. After venting pressure, open the lid and remove the slide container from the autoclave.
7. Rinse slides in Tris-Buffered Saline (TBS) or reagent grade water. When removing the slides from the container it is very important that the slides do not dry out.
8. Transfer slides to TBS.
9. Proceed with IHC staining procedure.

Proteolytic Pre-treatment

As with other pre-treatment methods, procedures for proteolytic pre-treatment vary due to laboratory-specific differences in formalin fixation. Proteolytic pre-treatment must be optimized (dilution and time — specific elevated temperature may also be selected) according to the particular fixation process used in each laboratory. Examples of antigens most often treated with proteolytic enzymes include cytokeratins and immunoglobulins.

Proteolytic Enzymes Available from Dako:

Proteinase K, (Concentrated), (Code S3004)
Proteinase K, Ready-to-Use, (Code S3020)
Pepsin (Code S3002)
Proteolytic Enzyme, Ready-to-Use, (Code S3007).

Materials Required

Humidity chamber

Silanized Slides (Code S3003) or slides coated with other suitable adhesives

Proteolytic Enzyme, Ready-to-Use (Code S3007)

Tris-Buffered Saline (Code S3001)

Protocol

1. Deparaffinize and rehydrate tissue sections.
2. Place slides horizontally and apply enough enzyme working solution to cover tissue section(s), typically 200-300 µL.
3. Incubate for defined time, typically 5-15 minutes.
4. Stop enzymatic reaction by rinsing with distilled water or Tris-Buffered Saline.

It is recommended that enzyme digestion is included in the relevant Autostainer protocols. For the RTU series antibodies, enzyme digestion is included.

For Dako Proteolytic Enzymes, the following guidelines apply:

Proteinase K, (Concentrated), (Code S3004) and Ready-to-Use (Code S3020):
Digestion for 6 minutes at room temperature is generally sufficient, but may be prolonged to 15 minutes.

Pepsin (Code S3002):
Digestion for 10 minutes at room temperature is generally sufficient, but may be prolonged to 15 minutes.

Proteolytic Enzyme, Ready-to-Use (Code S3007):
Digestion for 5-10 minutes at room temperature is sufficient.

For details, please refer to the product specification sheets.

Combined Proteolytic Pre-treatment and HIER

Some antigens are more efficiently retrieved by a combination of heating and enzyme digestion, e.g. some cytokeratins and immunoglobulin light chains. The protocol below describes a method of first treating with Proteinase K and then HIER by either water bath or microwave method.

Materials Required*

Silanized Slides (Code S3003) or slides coated with other suitable adhesives

Target Retrieval Solution, pH 6 (Code S1700, S1699 or K8005)

Tris-Buffered Saline (Code S3001)

Tris-buffered NaCl Solution with Tween 20 (TBST), pH 7.6 (Code S3006)

Protocol

1. Deparaffinize and rehydrate tissue sections.
2. Cover tissue sections with Proteinase K and incubate for 5-10 minutes.
3. Rinse with distilled water and place in Tris-Buffered Saline.
4. Proceed to antigen retrieval using either **PT**Link, another water bath or microwave method below.

HIER — Water Bath

5. Fill container with enough retrieval solution (200 mL) to cover slides and equilibrate to 95-99 °C in water bath. Place the incubation container into the water bath and incubate for 20-40 minutes.
6. Remove the container from the water bath and cool the contents with the lid removed for 20 minutes at room temperature.
7. Rinse with Tris-Buffered Saline (TBS) or distilled water at room temperature.
8. Transfer slides to Tris-Buffered NaCl Solution with Tween 20 (TBST), pH 7.6 Wash Buffer.
9. Proceed with IHC staining.

HIER — Microwave

5. Fill incubation container with enough retrieval solution (200 mL) to cover slides and insert slide holder. Insert slides in holder and cover.
6. Place the incubation container into microwave oven and incubate for 2 x 5 minutes.
7. In between steps 4 and 5, fill up the container with enough distilled water (50 mL) to cover slides.
8. After the second treatment, leave the sections in the retrieval solution at room temperature to cool for 15-20 minutes.
9. Rinse with distilled water.
10. Proceed with IHC staining.

Combined Deparaffinization and Target Retrieval

Combining deparaffinization and HIER reduces slide handling time significantly and provides added convenience without sacrificing staining quality. Using Dako **PT**Link instrument simplifies the combined deparaffinization and target retrieval process by performing automated deparaffinization and retrieval in a single step.

---

*Other target retrieval solutions will work with a similar protocol optimized according to individual laboratory requirements.

Materials Required

PTLink (Code PT100/PT101) and **PT**Link Rinse Station (Code PT109)

Silanized Slides (Code S3003) or slides coated with other suitable adhesives

Target Retrieval Solution, pH 9, 10x Concentrated, (3-in-1) (Code S2375)*

Dako Wash Buffer (10x) (Code S3006)

Protocol

Wear chemical-protective gloves when handling parts immersed in any reagent used in **PT**Link. Recommended 3-in-1 specimen preparation procedure using **PT**Link (Code PT100/PT101) and above target retrieval solution:

1. Prepare a working solution of the selected target retrieval solution according to the specifications.
2. Fill **PT**Link Tanks with sufficient quantity (1.5 L) of working solution to cover the tissue sections.
3. Set **PT**Link to preheat the solution to 65 °C.
4. Immerse the mounted, formalin-fixed, paraffin-embedded tissue sections into the preheated target retrieval solution (working solution) in **PT**Link Tanks and incubate for 20-40 minutes at 97 °C. The optimal incubation time should be determined by the user.
5. Leave the sections to cool in **PT**Link to 65 °C.
6. Remove each Autostainer Slide Rack with the slides from the **PT**Link Tank and immediately dip slides into a jar/tank (**PT**Link Rinse Station) containing diluted, room temperature Dako Wash Buffer (10x).
7. Leave slides in the diluted, room temperature Wash Buffer for 1-5 minutes.
8. Place slides on a Dako Autostainer instrument and proceed with staining. The sections should not dry out during the treatment or during the immunohistochemical staining procedure.
9. After staining, it is recommended to perform dehydration, clearing and permanent mounting.

---

*When used in **PT**Link for 3-in-1 specimen preparation procedure, the diluted deparaffinization/target retrieval solution can be used three times within a five day period, if stored at room temperature.

Acknowledgement

We would like to thank the following people for their contributions to this chapter: Thomas Boenisch, Ole Feldballe Rasmussen, Kenlyn Crosby, Gitte Nielsen, Katherine Ellison, Tony Knoll. Marianne Knudsen, Stefan Cuoni Teilmann, Helle Grahn Wendelboe, Majken Nielsen and Helene Metz (for proof-reading).

References

1. Boenisch T. Heat-induced antigen retrieval: What are we retrieving? J Histochem and Cytochem 2006;54:961-4.
2. Boenisch T. Diluent buffer ions and pH: Their influence on the performance of monoclonal antibodies in immunohistochemistry. Appl Immunohistochem Mol Morphology 1999;7:300-6.
3. Shi S-R, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 1991;39:741-8.
4. Shi S-R, Cote RJ, Chaiwun B, Young LL, Shi Y, Hawes D, et al.. Standardization of immunohistochemistry based on antigen retrieval technique for routine formalin-fixed tissue sections. Appl Immunohistochem Mol Morphol 1998;6:89-96.
5. Boenisch T., Effect of heat-induced antigen retrieval following inconsistent formalin fixation. Appl Immunohistochem Mol Morphol 2005;13:283-6.
6. Taylor CR, Shi SR, Chen C, Young L, Yang C: Comparative study of antigen retrieval heating methods: Microwave, microwave and pressure cooker, autoclave, and steamer. Biotech Histochem 1996;71:263-70.
7. Pileri SA, Roncador G, Ceccarelli, C, Piccioli M, Briskomatis A, Sabattini E, et al: Antigen retrieval techniques in immunohistochemistry: A comparison of different methods. J Pathol 1997;183:16-23.
8. Tacha D and Teixeira M. History and overview of antigen retrieval: Methodologies and critical aspects. J Histotechnol 2002;25:237-42.
9. Larsson LI. Tissue preparation methods for light microscopic immunohistochemistry. Appl Immunohistochem Mol Morphol 1993;1:2-16.
10. Shi S-R, Imam SA, Young L, Cote RJ, Taylor CR. Antigen retrieval under the influence of pH using monoclonal antibodies. J. Histochem Cytochem 1995;43:193-201.
11. Bankfalvi A, Navabi H, Bier B, Böcker W, Jasani B, Schmid KW. Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. J Pathol 1994;174:223-228.
12. Shin RW, Iwaki T, Kitamoto T, Tateishi J. Hydrated Autoclave pretreatment enhances tau immunoreactivity in formalin-fixed normal and Alzheimer's disease brain tissues. Lab Invest 1991;64:693-702.
13. Shin, R.-W, Iwaki T, Kitamoto T, Sato Y, Tateishi J. Massive accumulation of modified tau and severe depletion of normal tau characterize the cerebral cortex and white matter of Alzheimer's disease. Am J Pathol 1992;140;937-45.

Chapter 9 | Immunohistochemistry Staining Methods

Marc Key PhD

Immunohistochemistry has emerged as a powerful investigative tool that can provide supplemental information to the routine morphological assessment of tissues. The use of immunohistochemistry to study cellular markers that define specific phenotypes has provided important diagnostic, prognostic, and predictive information relative to disease status and biology. The application of antibodies to the molecular study of tissue pathology has required adaptation and refinement of immunohistochemical techniques, particularly for use in fixed tissues. In contrast to solution-based immunoassays that detect relatively abundant native proteins, in fixed tissues the preservation of antigen is variable and unpredictable. Thus, the history of immunohistochemistry has been a constant effort to improve sensitivity for detection of rare surviving antigenic targets with the ultimate goal of integrating tissue-based analysis with proteomic information.

Immunohistochemistry: In the Beginning

Because of the superior morphology provided by formalin-fixed paraffin-embedded tissues, this has become the medium of choice for most clinical and research studies. The peroxidase-labeled antibody method, introduced in 1968, was the first practical application of antibodies to paraffin-embedded tissues and overcame some of the limitations of earlier fluorescence antibody methods (1). These pioneering studies using enzyme labels instead of fluorescent dyes opened the door to the development of modern methods of immuohistochemistry.

The successful application of immunohistochemical methods to formalin-fixed surgical pathology specimens stimulated rapid progress in this newly emerging field, and in quick succession came the introduction of the immunoperoxidase bridge method (2) and the peroxidase anti-peroxidase (PAP) complex method (3).

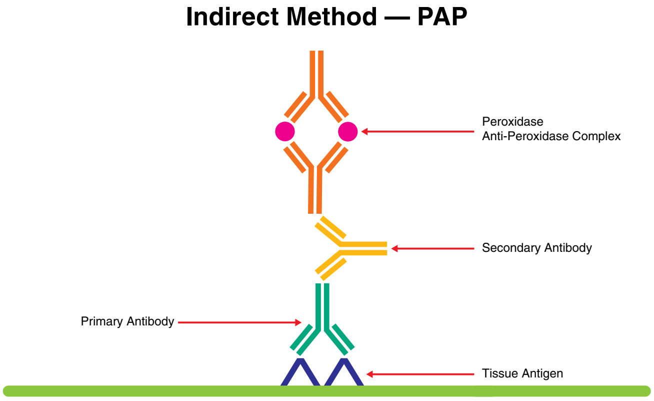


Figure 1. Peroxidase Anti-Peroxidase (PAP) Complex Method.

Avidin-Biotin Immunohistochemistry

In 1981 a new generation of immunohistochemical methods emerged with the advent of the avidin-biotin methods, which remains widely used today (4). All avidin-biotin methods rely on the strong affinity of avidin or streptavidin for the vitamin biotin.

Streptavidin (from Streptomyces avidinii) and avidin (from chicken egg) both possess four binding sites for biotin. The biotin molecule is easily conjugated to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (5).

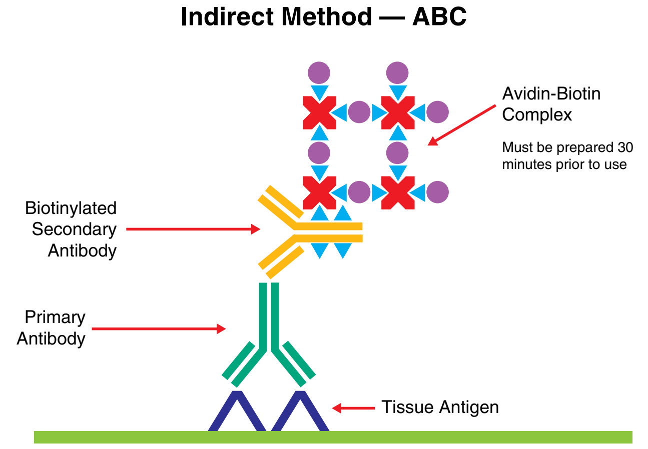


Figure 2. Avidin-Biotin Complex (ABC) Method.

In a similar method the labeled streptavidin-biotin (LSAB) method also utilizes a biotinylated secondary antibody that links primary antibodies to a streptavidin-peroxidase conjugate (6). In both methods a single primary antibody is subsequently associated with multiple peroxidase molecules, and because of the large enzyme-to-antibody ratio, a considerable increase in sensitivity is achieved compared to direct peroxidase-conjugate methods.

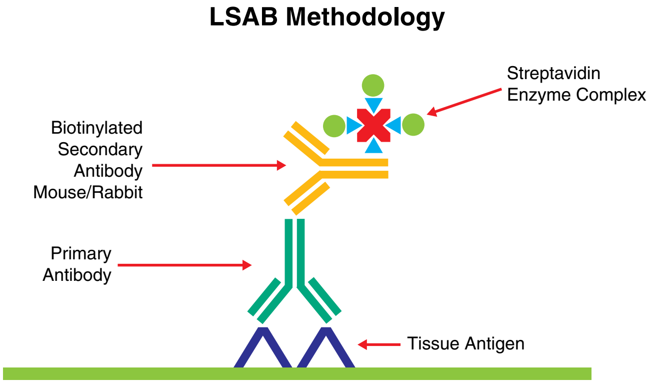

LSAB Methodology
Streptavidin Enzyme Complex
Biotinylated Secondary Antibody Mouse/Rabbit
Primary Antibody
Tissue Antigen

Figure 3. Labeled Streptavidin-Biotin (LSAB) Method.

Because avidin is a glycoprotein and has an isoelectric point (pI) of 10, it has a propensity to bind non-specifically to lectin-like and negatively charged tissue components at physiological pH. In contrast to avidin, streptavidin has a more neutral isoelectric point and lacks the carbohydrate moieties. These differences result in less nonspecific tissue binding.

Polymer-Based Immunohistochemistry

Although many of these streptavidin-biotin methods are still in widespread use, there are certain limitations characteristic of these methods. The presence of endogenous biotin in tissues can lead to significant background staining in certain circumstances. Formalin fixation and paraffin embedding has been shown to significantly reduce the expression of endogenous biotin, but residual activity can still be observed in tissues such as liver and kidney. Furthermore, with the advent of heat-induced antigen retrieval, the recovery of endogenous biotin can appear as an unwanted side effect. Methods to block endogenous biotin are partially effective, but add another layer of complexity to an already complex procedure. These limitations are further exacerbated by the use of frozen tissue sections, in which levels of endogenous biotin are usually even higher than those encountered in paraffin-embedded specimens.

Because of these limitations, polymer-based immunohistochemical methods that do not rely on biotin have been introduced and are gaining popularity (5). These methods utilize a unique technology based on a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. In the EPOS (Enhanced Polymer One Step)* system, as many as 70 enzyme molecules and about 10 primary antibodies were conjugated to a dextran backbone. This allowed the entire immunohistochemical staining procedure, from primary antibody to enzyme, to be accomplished in a single step (6). On the other hand, one limitation of this method was its restriction to a select group of primary antibodies provided by the manufacturer, and not suitable for user-supplied primary antibodies.

To overcome this limitation a new type of dextran polymer, EnVision™ *, was introduced. This polymer system contained a dextran backbone to which multiple enzyme molecules were attached. However, unlike

---

* A proprietary methodology developed by Dako.

EPOS, which contained primary antibodies, the EnVision™ system contained secondary antibodies with anti-mouse Ig and anti-rabbit Ig specificity. This universal reagent could be used to detect any tissue-bound primary antibody of mouse or rabbit origin. The utility of this method opened the door to a new family of polymer-based immunohistochemical methods. The sensitivity of these methods compared to LSAB and ABC methods was comparable or even slightly greater in most cases (7). With the latest development of EnVision™ FLEX+ the sensitivity has been improved even further. However, because of the large molecular size of the polymer conjugates, accessibility to certain epitopes was restricted, presumably due to steric hindrance, in a minority of cases.

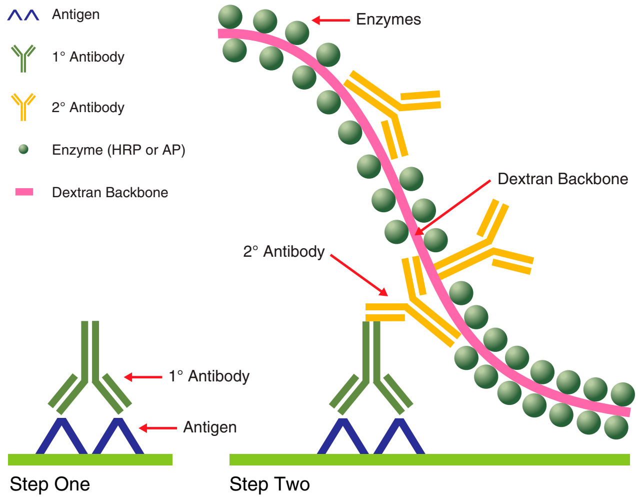


Figure 4. Two-Step Polymer Method (EnVision™).

Tyramide Amplification

The tyramide amplification technique is based on the ability of phenolic compounds to become oxidized to highly reactive and unstable intermediates (8). When biotinyl tyramide is oxidized, dimerization with electron-rich aromatic compounds, such as those found in protein molecules, occurs (9). This reaction can be harnessed in immunohistochemistry to generate highly reactive biotinyl-tyramide intermediates that bind rapidly to protein molecules in the immediate vicinity of peroxidase enzymes. This reaction results in the deposition of numerous biotin signals. In a typical immunohistochemistry procedure, peroxidase enzymes are first associated with primary antibodies by any of the standard immunohistochemical methods, for example by the ABC or LSAB methods. Biotinyl tyramide and hydrogen peroxide are applied as a substrate to generate numerous biotin (biotinyl tyramide) signals. These biotin molecules can then be used to capture subsequent streptavidin-peroxidase enzymes that are converted to a chromogenic endpoint via diaminobenzidine or similar chromogenic substrates (10).

Cycled Tyramide Amplification

The sequence of streptavidin-peroxidase and biotinyl-tyraminde can be alternately applied to perform a cycled tyramide amplification procedure. In practicality, however, cycling usually cannot exceed two or three cycles before background staining limits the utility of this approach. Commercial tyramide amplification products are available and include Tyramide Signal Amplification (TSA, DuPont NEN Life Sciences, Boston, MA) and Catalyzed Signal Amplification (CSA)*.

Fluorescyl-Tyramide Amplification

In keeping with current trends in immunohistochemistry to develop alternatives to biotin-streptavidin detection methods, a fluorescyl-tyramide amplification system has recently been introduced (FT-CSA)*. In this procedure peroxidase is associated with a tissue-bound primary antibody by application of a secondary anti-mouse Ig antibody to which peroxidase has been conjugated. The peroxidase catalyzes the conversion and deposition of fluorescyl-tyramide onto the tissue section. At this point the reaction can be terminated and viewed by fluorescence microscopy, or the signal can be converted to a colorimetric reaction by the sequential application of an anti-fluorsecein antibody conjugated to peroxidase followed by a diaminobenzidine-hydrogen peroxide substrate.

In comparison to standard IHC methods, tyramide amplification methods have typically increased sensitivity by at least 50 fold or greater (11). As with any amplification method, background tends to increase along with signal. Therefore it is essential to run appropriate positive and negative controls and interpret any positive staining within the context of the negative control.

---

* A proprietary methodology developed by Dako.

Rolling Circle Amplification

Rolling Circle Amplification (RCA) is a signal amplification system that generates a local signal via extension and amplification of an oligonucleotide tail. Although initially developed for nucleic acid detection, this method can also be applied to immunohistochemistry. RCA-mediated immunohistochemistry has been successfully applied to the detection of a variety of cell surface and intracellular molecules (12). The method utilizes a short oligonucleotide sequence coupled to a primary or secondary antibody molecule. After binding to the tissue, a circularized nucleic acid probe with a complementary sequence is hybridized to the oligonucleotide. The oligonucleotide then acts as a primer and is linearly extended using a DNA polymerase and the rolling circle. The extended DNA is then hybridized with labeled oligonucleotide probes. These labels may include for example biotin, which can then be visualized by any one of the many avidin-biotin detection methods. RCA derives its specificity from an antigen-antibody reaction and its sensitivity from nucleic acid synthesis. RCA has been reported to generate a 10^5-fold increase in signal (13).

Conclusion

As immunohistochemical techniques continue to evolve, their application to surgical and research pathology is becoming increasingly valuable. Various amplification methods have made significant improvements to this technology such that many antigens, previously believed to have been lost to the process of fixation and embedding, can now be routinely demonstrated. However, as the sensitivity of immunohistochemistry continues to increase, accepted staining criteria and clinical interpretation may require re-evaluation.

New signal amplification methods continue to be developed, each with their own unique strengths and weaknesses, and this can present a bewildering assortment of choices to the investigator or clinician that profoundly influence the practice of immunohistochemistry. As technology marches forward, new arrays of tissue markers are emerging that are providing the tools to generate important new discoveries. As new markers are added to this list, our knowledge of the underlying biology and pathogenesis of disease is increased. The full impact is still many years away.

References

1. Nakene PK. Simultaneous localization of multiple tissue antigens using the peroxidase labeled antibody method: A study of pituitary glands of the rat. J Histochem Cytochem 1968;16:557-60.
2. Mason TE, Phifer, RF, Spicer SS. An immunoglobulin-enzyme bridge method for localizing tissue antigens. J Histochem Cytochem 1969;17:563-9.
3. Sternberger LA, Hardy PH Jr., Cuculis JJ, Meyer HG. The unlabeled antibody-enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorse-radish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 1970;18:315.
4. Hsu SM, Raine L, and Fanger H. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577-80.
5. Heras A, Roach CM, Key ME. Enhanced polymer detection system for immunohistochemistry. Lab Invest 1995;72:165 (Abstract).
6. Chilosi M, Lestani M, Pedron S, Montagna L, Benedetti A, Pizzolo G, Menestrina F. A rapid immunostaining method for frozen sections. Biotech Histochem 1994;69:235.
7. Sabattini E, Bisgaard K, Ascani S, Poggi S, Piccioli M, Ceccarelli C. The EnVision™ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMateTM, CSA, LABC, and SABC techniques. J Clin Pathol 1998;51:506-11.
8. Gross AJ, Sizer IW. The oxidation of tyramine, tyrosine, and related compounds by peroxidase. J Biol Chem 1959;234:1622-1614.
9. Bobrow MN, Harris TD, Shaughnessy KJ, Litt GJ. Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. J Immunol Methods 1989;125:279-285.
10. Adams JC. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. J Histochem Cytochem 1992;40:1457-63.
11. Merz H, Malisius R, Mann-Weiler S, Zhjow R, Hartmann W, Orscheschek K, Moubayed P, Feller AC. Methods in laboratory investigation immunoMax. A maximized immunohistochemical method for the retrieval and enhancement of hidden antigens. Lab Invest 1995;73:149-56.
12. Gusev Y, Sparkowski J, Raghunathan J, Ferguson H, Montano J, Bogdan N, Schweitzer B, Wiltshire S, Kingsmore SF, Maltzman W. Weeler V. Rolling circle amplification. A new approach to increase sensitivity for immunohistochemistry and flow cytometry. Am J Pathol 1002;159:63-75.
13. Wiltshire S, O'Malley S, Lambert J, Kukanshis K, Edgar D, Kikngsmore S, Schweitzer B. Detection of multiple allergen-specific IgE on microarrays by immunoassay with rolling circle amplification. Clin Chem 2000; 46:1990-3.

Chapter 10 | Immunofluorescence

J. Paul Robinson PhD, Jennifer Sturgis BS and George L. Kumar PhD

Immunofluorescence (IF) is a common laboratory technique used in almost all aspects of biology. This technique based on pioneering work by Coons and Kaplan (1, 2), and later by Mary Osborne (3), has been widely used both in research and clinical diagnostics. Applications include the evaluation of cells in suspension, cultured cells, tissue, beads and microarrays for the detection of specific proteins. IF techniques can be used on both fresh and fixed samples. In IF techniques, antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). These labeled antibodies bind (directly or indirectly) to the antigen of interest which allows for antigen detection through fluorescence techniques. The fluorescence can then be quantified using a flow cytometer, array scanner or automated imaging instrument, or visualized using fluorescence or confocal microscopy.

The two main methods of immunofluorescent labeling are direct and indirect. Less frequently used is direct immunofluorescence whereby the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect immunofluorescence, the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye (Figure 1).

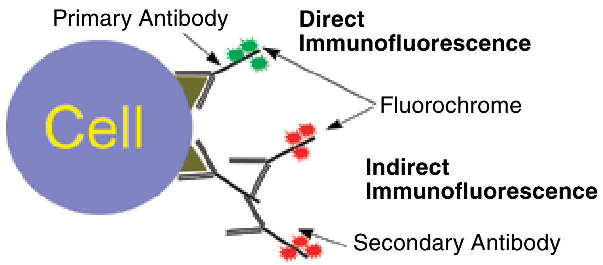


Figure 1. Schematic of direct and indirect immunofluorescence.

Advantages of direct immunofluorescence include shorter sample staining times and simpler dual and triple labeling procedures. In cases where one has multiple antibodies raised in the same species, for example two mouse monoclonals, a direct labeling may be necessary.

Disadvantages of direct immunofluorescence include lower signal, generally higher cost, less flexibility and difficulties with the labeling procedure when commercially labeled direct conjugates are unavailable.

Advantages of indirect immunofluorescence include greater sensitivity than direct immunofluorescence. There is amplification of the signal in indirect immunofluorescence because more than one secondary antibody can attach to each primary (see Figure 1). Commercially produced secondary antibodies are relatively inexpensive, available in an array of colors, and quality controlled.

Disadvantages of indirect immunofluorescence include the potential for cross-reactivity and the need to find primary antibodies that are not raised in the same species or of different isotypes when performing multiple-labeling experiments. Samples with endogenous immunoglobulin may exhibit a high background.

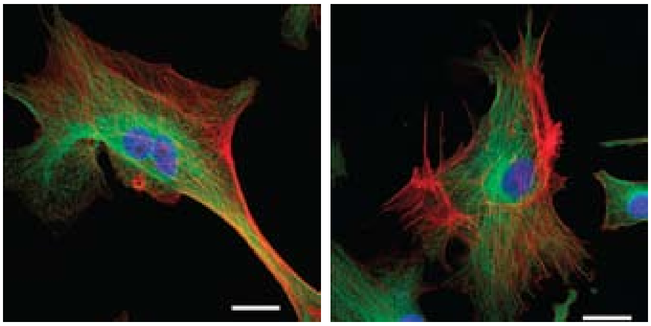


Figure 2. Cultured pulmonary artery endothelial cells stained for tubulin (red), actin (green) and DNA (blue). The dual immunofluorescence procedure used rabbit anti-actin IgG and mouse anti-alpha tubulin IgG as primary antibodies. The secondary antibodies used were Texas Red-conjugated goat, anti-rabbit IgG and FITC-conjugated goat, anti-mouse IgG. The sample was also stained with the DNA-specific dye Hoechst 33342. Scale bar is equal to 20 microns.

Principle of Fluorescence

Fluorescence and phosphorescence are both types of luminescence. When molecules with luminescent properties absorb light, they emit light of a different wavelength. With fluorescence the emission of light occurs extremely rapidly after the absorption of excitation light, whereas with phosphorescence emission continues for milliseconds to minutes after the energy source has been removed. Fluorescent materials give off light because of their atomic structure. Electrons are arranged in discrete energy levels surrounding the atom's nucleus with each level having a predetermined amount of energy. When an electron absorbs the energy from a photon of light (Figure 3) it becomes "excited" and jumps to a higher, less stable energy level. The excited state does not last long. The half-life of the excited state is generally less than 10 (8) seconds. The electron loses a small amount of energy as heat and the remainder of the extra energy is given off in the form of a photon. The emitted fluorescence has a lower energy than the absorbed light, so the wavelength of the emitted light is longer than that of the excitation light (except in the case of multi-photon excitation).

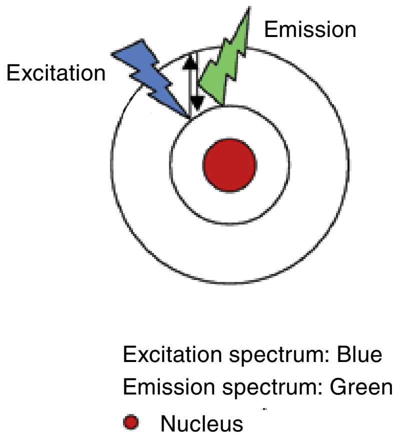


Figure 3. Principle of fluorescence.

A range of wavelengths of light can excite the electrons of a fluorochrome. For example, fluorescein will fluoresce when hit by light with any wavelength between 450 nm and 520 nm. However, the closer the excitation wavelength is to 495 nm, the more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly, the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein ranges from 490 nm to 630 nm, and the emission peak is approximately 515 nm. Since the phenomenon of fluorescence was first explained by a British scientist, Sir George Stokes, in 1852, the shift in wavelength from short to long during fluorescence is called "Stokes shift" (Figure 4).

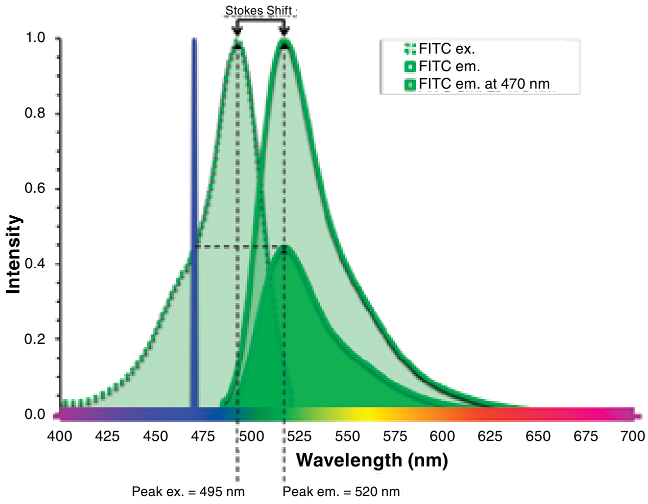


Figure 4. Excitation and emission spectrum of fluorescein. When fluorescein is excited at a wavelength other than its peak excitation (470 nm in this example), the shape of the emission curve (darker green) remains the same, but the relative intensity is reduced. The efficiency of the excitation at 470 nm is 45% of peak excitation.

Some fluorochromes have a small Stokes shift while other fluorescent compounds have large Stokes shifts. For example, the fluorochrome fluorescein can be excited by blue-green light, and its Stokes shift is only about 20 nm, which means that the light emitted is green. This contrasts with another fluorochrome, phycoerythrin, which also can be excited by blue-green light, but has a large Stokes shift. Thus, the light emitted is yellow-orange. In immunofluorescence, a single wavelength can be used to excite several fluorochromes with different Stokes shifts and thereby produce a variety of fluorescent colors as shown in Figure 5.

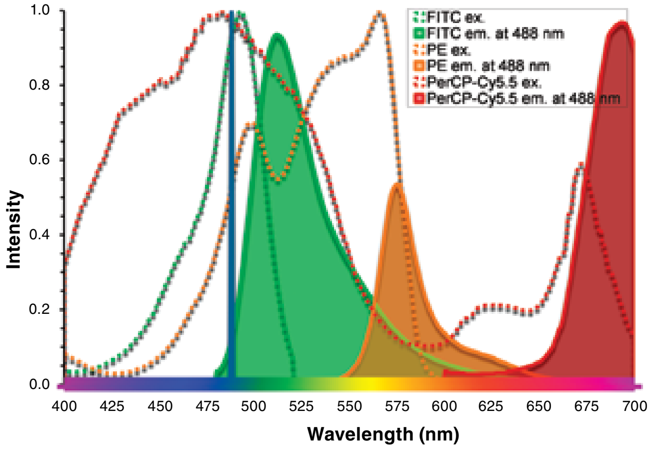


Figure 5. Excitation of three spectrally distinct fluorochromes using a single laser line.

The example in Figure 5 shows a single wavelength at 488 nm (blue line) exciting three different fluorochromes identified by their absorption curves on the left of the figure (blue line). Each fluorochrome is excited at a different efficiency and, therefore, the resulting emission will be at different intensities for equivalent fluorochrome concentrations. Knowing the excitation and emission properties of fluorescent compounds makes it possible to select combinations of fluorochromes that will work together. However, for a fluorochrome to be useful in a biological application it must attach to or be contained within a structure of biological significance.

Fluorochromes can be attached to antibodies which will then bind to specific chemical structures on or inside cells. Many other chemical and physical properties of fluorochromes determine when and where these dyes are useful in various biological assays. For example, some of the fluorochromes that bind to DNA, such as Hoechst 33342, can get into living cells, but most DNA-binding fluorochromes cannot get past the cell membrane. Those fluorescent dyes that cannot get past an intact cell membrane, such as propidium iodide (PI), are often used to distinguish live from dead and dying cells.

The ideal fluorochrome would be a molecule with the following properties:

- An absorption peak at an excitation wavelength available on the fluorescence detection instrument (large extinction coefficient at the wavelength of excitation)
- Bright fluorescence (high quantum yield)
- A narrow emission spectrum that falls within one of the instrument's detection bands
- Good photostability and
- Fluorescence properties that are not significantly altered by conjugation to an antibody or by the local environment of the sample

Limitations of IF Techniques

Photobleaching

As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching (and phototoxicity). Photobleaching is the photochemical destruction of a fluorophore due to the generation of reactive oxygen species in the specimen as a byproduct of fluorescence excitation (Figure 6). Although the exact mechanism of photobleaching is unknown, it is thought that the primary causative mechanism appears to be photosensitization of singlet oxygen ($^1O_2$) (see glossary for details) generation by the dye triplet-excited state and reference (4) for details). Photobleaching can be minimized by: (a) decreasing the excitation light in both intensity and duration, (b) reducing the availability of singlet oxygen ($^1O_2$) by the addition of singlet oxygen scavengers (= antifade reagents), and (c) using a low concentration of a fluorochrome with high-quantum efficiency.

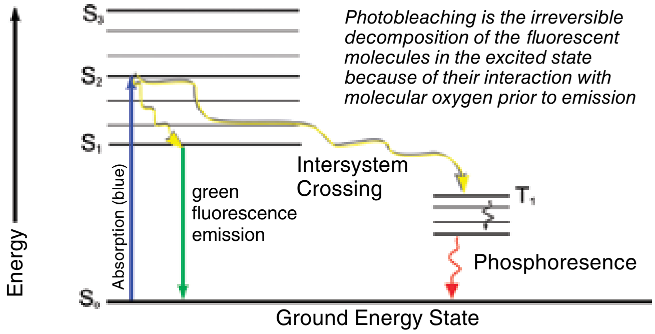

Energy
S_3
S_2
S_1
S_0
Absorption (blue)
green fluorescence emission
Intersystem Crossing
T_1
Phosphoresence
Ground Energy State
Photobleaching is the irreversible decomposition of the fluorescent molecules in the excited state because of their interaction with molecular oxygen prior to emission

Figure 6. Illustration of how a singlet-excited state can convert to a triplet-excited state.

Autofluorescence

Biological autofluorescence in mammalian cells due to flavin coenzymes (FAD and FMN: absorption, 450 nm; emission, 515 nm) and reduced pyridine nucleotides (NADH: absorption, 340 nm; emission, 460 nm) can be problematic in the detection of fluorescence probes in tissues and cells. Fixation with aldehydes, particularly glutaraldehyde, can result in high levels of autofluorescence. This can be minimized in fixed cells by washing with 0.1% sodium borohydride in phosphate-buffered saline (5) prior to antibody incubation. Problems due to autofluorescence can be minimized by selecting probes and optical filters that maximize the fluorescence signal relative to the autofluorescence. Other factors that limit IF include the performance of the detection instrument (i.e. how well the microscope has been calibrated and set), the specificity of the antibodies, and the specimen preparation.

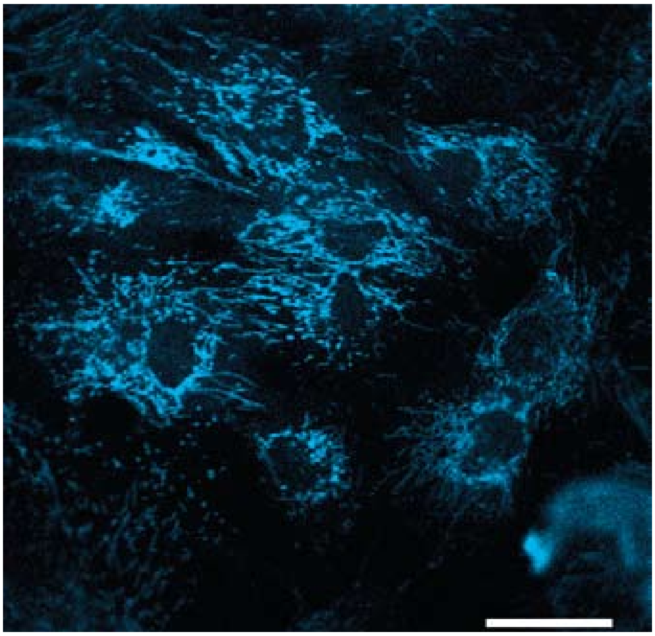


Figure 7. NADH autofluorescence in a human colon carcinoma cell line (HCT116). Ultra-violet excitation at 363 nm was used and the emitted fluorescence greater than 440 nm was collected. Scale bar is 10 microns. Credit: Giselle M. Knudsen, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA.

Fluorescence Overlap

One of the problems that must be dealt with when measuring fluorescence of more than one color is the possibility that the emission signals overlap. It is necessary to remove the overlapping signal or it will give a false level for one or more colors. For example, as shown in figure 8, there is significant overlap when using FITC and PE. A range of wavelengths will be collected for each detection channel. In the figure, these are identified as the fluorescein detector bandwidth and the PE detector bandwidth. These band-pass optical filters will allow photons within this wavelength range to reach the detector. However, as can be seen in figure 8, there is a very small amount of PE fluorescence which is within the FITC band and similarly a small amount of FITC fluorescence within the PE band. These must be electronically removed or the measurement for each detector will overestimate the actual signal. This process is called fluorescence compensation and can be automatically calculated in many detection systems using single positive controls.

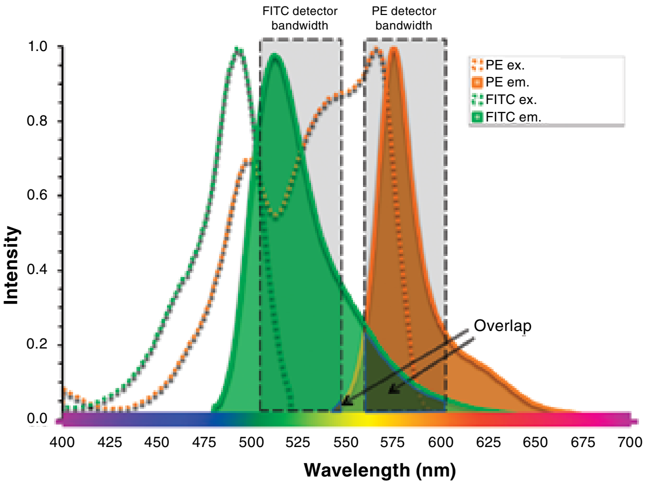


Figure 8. Fluorescence overlap of FITC and PE.

Applications of Immunofluorescence in Pathology

Some practical applications of immunofluorescence in diagnostic pathology are:

- Analysis of antigens in fresh, frozen or fixed tissues; sub-cellular localization of antigens in tissue culture monolayers; observation of bacterial or parasitic specimens;
- Detection and localization of the presence or absence of specific DNA sequences on chromosomes; and
- Defining the spatial-temporal patterns of gene expression within cells/tissues.

The most widely used method of IF in pathology is indirect IF. However, in some very specialized applications direct IF has been used for localization of IgG in immune complexes along the dermal-epidermal junction of skin biopsies from patients suffering from systemic lupus erythematosus (6).

In summary, immunofluorescence is the visualization of antigens within cells using antibodies as fluorescent probes. The benefits of immunofluorescence are numerous, and the technique has proven to be a powerful tool for determining the cellular distribution of known antigens in frozen tissues or in the localization of specific DNA sequences on chromosomes. The method has achieved the status of combining high sensitivity with high resolution in the visualization of antigens and will be a major tool for many years to come that any pathologist studying cells or molecules cannot afford to ignore.

For a methodology article on immunofluorescence labeling of formalin-fixed, paraffin-embedded tissue, see reference 7.

References

1. Coons AH, et al. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol Med 1941;47,200-2.
2. Coons AH and Kaplan MH () Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. Journal of Exp Med 1950; 91,1.
3. Weber K, et al. Specific visualization of tubulin-containing structures in tissue culture cells by immunofluorescence. Cytoplasmic microtubules, vinblastine-induced paracrystals, and mitotic figures. Exp Cell Res 1975;95,111-20.
4. Johnson ID. Practical considerations in the selection and application of fluorescent probes. In: Handbook of biological confocal microscopy, 3rd ed. (J.B. Pawley. Ed), Plenum Press. New York. 2006. pp. 362-3.
5. Bacallao R, et al. Guiding principles of specimen preservation for confocal fluorescence microscopy. In: Handbook of biological confocal microscopy,3rd ed. (J.B. Pawley. Ed), Plenum Press. New York. 2006. pp. 368-80.
6. Jennette JC and Wick MR. Immunohischemical techniques. In “Immunohistology in diagnostic pathology”, Ed. Jennette JC. CRC Press. Boca Raton, FL. USA. 1988. Pp. 1-29.
7. Robertson D, et al. Multiple immunofluorescence labelling of formaqlin-fixed paraffin-embedded (FFPE) tissue. BMC Cell Biology 2008; 9:13.

Chapter 11 | Preparation of FFPE Tissue Slides for Solid Tumor FISH Analysis

Sven Müller PhD, Steen H. Matthiesen PhD and Kirsten V. Nielsen MS

Tumor tissue analysis by fluorescence in situ hybridization (FISH) is a well-established method for identification of genetic aberrations like gene copy number changes and translocations. The method has been used as a research tool for more than 20 years and due to the prognostic and predictive value of some FISH markers, e.g. *TOP2A* and *HER2*, the method has been implemented in the clinic. In clinical pathology, the quantitative nature of analysis results has made FISH an attractive supplement or alternative to IHC-based analysis. Although promising substitutes have been identified (1, 2), tissue preservation practice is still largely dominated by the classic method of fixation in neutral-buffered formalin, followed by paraffin embedding (FFPE). The FFPE tissue preservation method is fully compatible with FISH, but technically valid FISH results require that tissue preparation match the fixation history of the individual tissue sample. This article focuses on preparation of FFPE tissue slides for FISH analysis and how sub-optimal stains can be identified.

Composition of a Solid Tumor FISH Probe Mixture

The optimal composition of a FISH probe mixture for detection of changes in gene copy number in solid tumors includes two labeled probes. One probe directed towards the gene of interest and one probe directed towards a reference sequence for the chromosome on which the target gene is located. Traditionally, the reference probes are directed towards the non-coding centromere sequences.

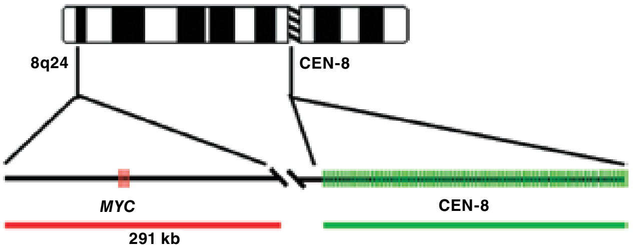


Figure 1. Example of a FISH probe mixture. A 291 kb long Texas Red-labeled probe directed towards *MYC* and a fluorescein-labeled probe towards CEN-8 reference sequences on the same chromosome.

Gene-directed probes are based on genomic clones — typically COS or BAC clones — and cover the genomic area where the coding sequences of the gene of interest are located. Labeling of the genomic clones, e.g. by nick translation, includes labeling of introns and gene flanking sequences. Thereby the repetitive sequences interspersed in the human genome are labeled as well. The resulting unspecific staining can be blocked by addition of unlabeled competitive sequences. The first consistently efficient blocker identified was the Cot-1 DNA fraction (3). It has, subsequently, been demonstrated that a mixture of unlabeled peptide nucleic acid (PNA) oligonucleotides directed towards the dominating Alu repeat sequences is comparable to or better than Cot-1 DNA fraction for blocking (4).

Tissue Sample Preparation

Specimens from biopsies, excisions or resections must be handled as soon as possible to preserve the tissue for FISH. Specimens should be preserved in 10% neutral-buffered formalin (NBF), preferably as 3-4 mm blocks fixed for 18-24 hours followed by dehydration and embedment in paraffin. Sections should be cut into 4-6 µm, mounted on positively charged slides (e.g. SuperFrost Plus, Mentel-Gläser, Thermo Scientific) and adhered to the slide by baking at 60°C for approximately 1 hour.

Fixation in formalin is suitable because the induced protein-protein and protein-nucleic acid cross-links preserve the tissue efficiently while retaining morphology relatively intact. However, the macromolecular network introduced by formalin significantly reduces the access of FISH probes to target DNA. Consequently, the initial steps in a FISH staining must address suitable breakdown of this network.

FFPE Slide Preparation

Deparaffinization

The need for deparaffinization of FFPE slides for FISH analysis does not differ from standard preparation of slides for histological staining methods like H&E, special stains or IHC. Following mounting by baking, the slides are deparaffinized in xylene (or substitutes) and rehydrated in a series of ethanol.

Pre-treatment

The aim of pre-treatment is to ease the subsequent protease digestion by breaking the formalin-induced disulphide bonds (6). Correct pre-treatment, therefore, has a huge impact on final FISH staining quality. Inadequate pre-treatment results in increased autofluorescence level due to intact proteins and ultimately reduced FISH probe signal intensity, whereas excessive pre-treatment distorts tissue morphology. It is important to adjust pre-treatment methods to tissues that have been fixed in weaker fixatives than NBF, otherwise pre-treatment may induce significant harm to the nuclear membrane. Degraded nuclear membranes will show as a blurred membrane demarcation (Figure 3.D). Degradation of the nuclear membrane and areas close to the membrane may reduce the presence of the non-coding (centromere) reference target sequences as non-coding sequences have a preference for localization at the nuclear envelope (7). Some of the more intense pre-treatment methods combine incubation in acidic solution (e.g. HCl) with exposure to chaotropic agents (e.g. sodium thiocyanate). A pre-treatment which can be harsh for both morphology and tissue slide attachment. However, very efficient results can be obtained solely by incubation in 2-(N-morpholino) ethanesulfonic acid (MES) at 95-99 °C for 10 minutes. The MES-based pre-treatment is relatively tolerant to variations in NBF fixation times, but in order for this simple approach to bring consistent results, it is crucial that incubation is performed at no less than 95 °C.

Enzymatic digestion

Proper protease digestion is by far the most decisive step in order to obtain technically valid FISH results. The breaking of peptide bindings by protease digestion directly affects signal quality as it eases access of the FISH probes to the genomic target DNA and reduces autofluorescence generated by intact proteins. Some protocols are based on enzymatic digestion by the very active serine protease, proteinase K, but gentler and still adequate digestion can be achieved by use of a carboxyl protease called pepsin.

Enzymatic digestion time must be adapted to the tissue fixation time. For tissues that have been fixed in NBF for 6-72 hours, the enzymatic digestion time can be between 30 seconds and 12 minutes at 37 °C using Dako Ready-to-Use Pepsin. The digestion can also be performed at room temperature (20-25 °C), but will require 3-4 times longer incubation time.

In general terms, longer fixation time requires longer enzymatic digestion time. Typically, 2-3 minutes at 37 °C will be an optimal enzymatic digestion time for the majority of routine FFPE samples fixed in NFB for 12- 24 hours. However, present hospital routines, in accordance with which fixation time can vary significantly or even be unknown to the FISH laboratory, present a challenge for proper digestion. It is important that the FISH laboratory recognizes when a sample is unsuitable for analysis and accordingly reprocess the sample using a digestion intensity-adapted protocol.

Identification of Sub-optimal Digestion

To verify the digestion quality of a stained slide several evaluation parameters must be examined, the most important being inspection of:

- Autofluorescence level
- DAPI staining pattern
- Signal distribution

Autofluorescence

Heavily under-digested samples may be identified by inspection of the sample in the relevant double filter (e.g. Texas Red/FITC), see Figure 2.A. Under-digested samples are often characterized by apparent green autofluorescence in the cytosol as well as the extracellular matrix (Figure 2.A and C).

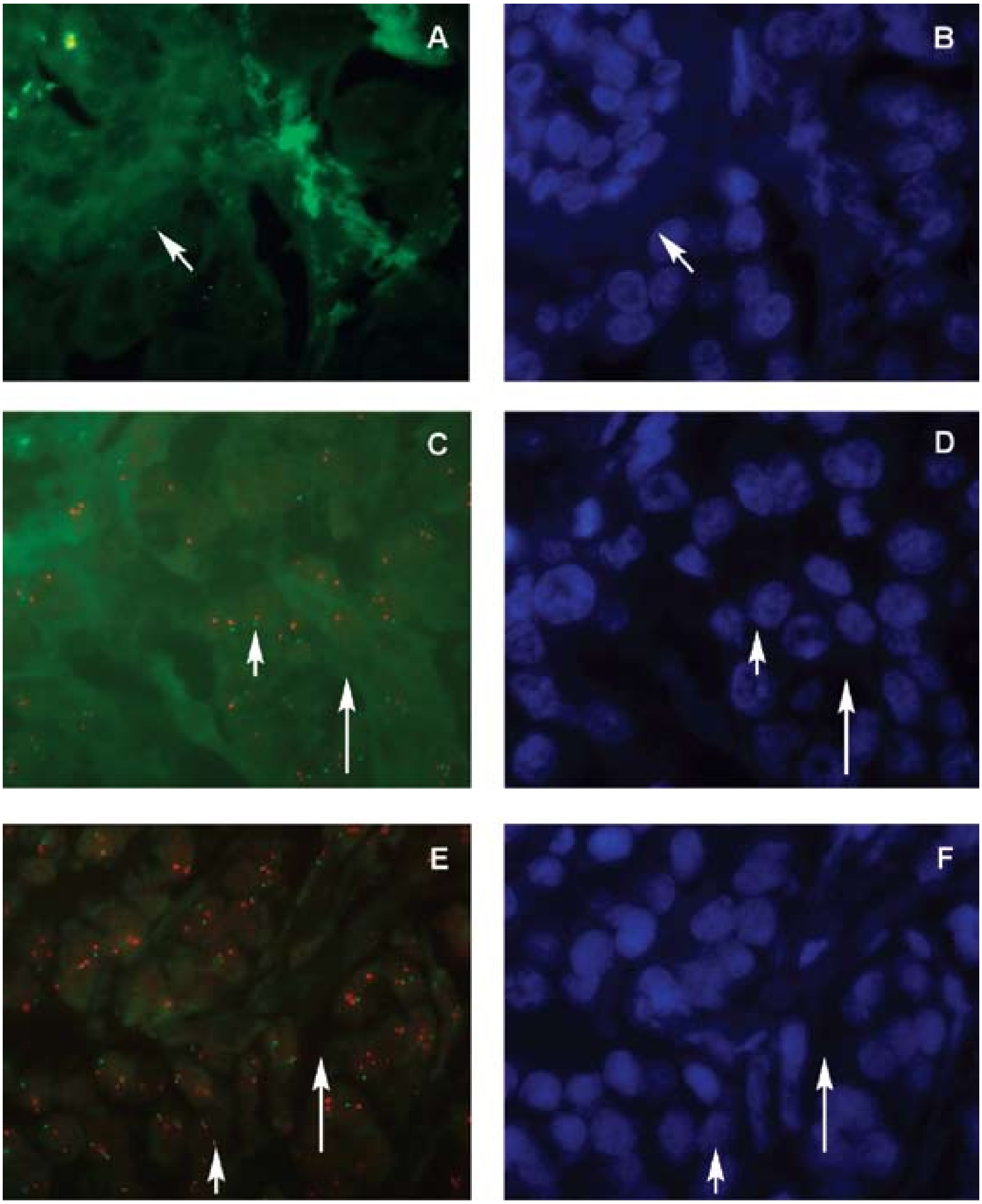


Figure 2. FFPE breast tissue stained with *MYC*/CEN-8 (Y5504, Dako). **(a)** Under-digested with excessive green cytosolic and extracellular autofluorescence background staining. No red gene signals (*MYC*) and some green reference signals (CEN-8). The *arrow* points to a green signal. **(b)** DAPI stain of A. **(c)** Slightly under-digested with green cytosolic and extracellular autofluorescence background staining *(long arrow).* Some red gene signals and green reference signals are seen *(short arrow).* **(d)** DAPI stain of C. **(e)** Acceptable digestion time. *Short arrow:* Green and red signals; *long arrow*: Low level of cytosolic and extracellular autofluorescence. **(f)** DAPI stain of E.

When under-digestion is pronounced, probe hybridization is hampered by the high presence of proteins and peptide chains. This reduces signal intensity as a consequence of the higher autofluorescence and lower probe hybridization, thereby reducing the signal to noise ratio. The use of labeled PNA oligos or DNA as reference probes towards highly repetitive sequences (e.g. centromeric sequences) may result in the presence of green signals despite sub-optimal (under-) digestion of the sample (Figure 2.A).

FISH stains with under-digestion of tissue can be recognized due to autofluorescence in the cytosol and extracellular matrix and potential lack or reduced presence and intensity of red signals from the gene of interest (single locus).

In properly digested cells, the autofluorescence is mainly restricted to the nuclei, and the level does not disturb red and green signals (Figure 2.E). Visual inspection of autofluorescence level may help identify under-digested samples, but for over-digested samples the autofluorescence level does not differentiate properly-digested samples from over-digested samples.

DAPI staining pattern

DAPI (4',-6-diamidino-2-phenylindole) forms fluorescent complexes with double-stranded DNA and this ability makes it possible to use the DAPI staining pattern as a tool to evaluate digestion status. Insufficient enzymatic digestion impedes the DNA DAPI complex formation and makes the nuclei staining appear heterogeneous. This is seen when the staining pattern of an optimally digested sample (Figure 3.A) is compared to an under-digested sample (Figure 3.B). Over-digestion can also be seen in the DAPI staining pattern as doughnut formation or damaged nuclei membranes (Figure 3.C and Figure 3.D, respectively).

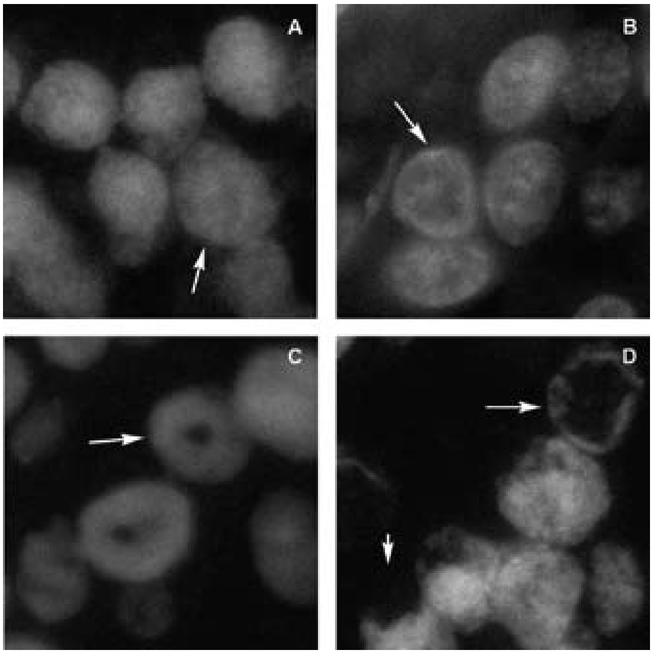


Figure 3. Nuclei DAPI counter stains of FFPE breast tissue at different digestion times. **(a)** Optimal digestion time, homogenous staining (*arrow*). **(b)** Under-digested, heterogenous staining (*arrow*). **(c)** Over digested, some nuclei with doughnut formation (*arrow*). **(d)** Heavily over-digested, “ghost” nuclei (*long arrow*) and damaged nuclei membranes (*short arrow*).

However, using the DAPI staining pattern for evaluation of under-digested samples is very coarse as many lightly fixed tissues appear homogenously stained despite insufficient digestion. A well digested tissue will often morphologically, depending of tissue type, result in a more spherical nucleus with a light swollen appearance compared to an under-digested tissue.

Over-digestion of a tissue section results in destroyed tissue, cell and nuclei morphology as well as loss of DNA. As binding of DAPI to double-stranded DNA results in an approximately 20-fold fluorescence enhancement (8), the reduced presence or absence of DNA is reflected in the DAPI staining pattern. Partly over-digested nuclei may be identified by the absence of DAPI staining in the center of the nucleus, giving rise to a doughnut-like appearance (Figure 3.C). In case of intense over-digestion, degradation of the nuclei becomes quite easy to spot with almost empty “ghost” nuclei and destroyed nuclear morphology (Figure 3.D).

Caution should be taken not to conclude cases of under-digestion as over-digested based solely on a DAPI staining pattern. In under-digested samples, the DAPI molecule will only slowly enter the core of the nuclei (Figure 2.B and D) and this effect may result in lack of core staining and the doughnut-like appearance seen for over-digested samples.

Signal distribution

The digestion pattern as shown by DAPI staining (Figure 3.A-D) indicates that enzymatic digestion of material in the nucleus is heterogeneous. According to the DAPI staining pattern, the center of the nucleus is the most affected area. Loss of genomic DNA as a consequence of over-digestion compromises the technical validity of a FISH staining as the number of probe target sequences may be reduced. A study by Bolzer, et al. (7) showed that localization of genomic DNA in human nuclei correlates to gene density. Gene-poor domains form a layer beneath the nuclear envelope and gene-dense domains are enriched in the nuclear interior. The centromere-based reference probes used in solid tumor FISH probe mixes are per definition targeted to gene-poor domains and should — according to the observations by Bolzer, et al. — have a preference for localization close to the nuclear envelope. This is supported by the signal distribution that can be seen when centromere sequence-based probes are used for interphase stains, see Figure 4.

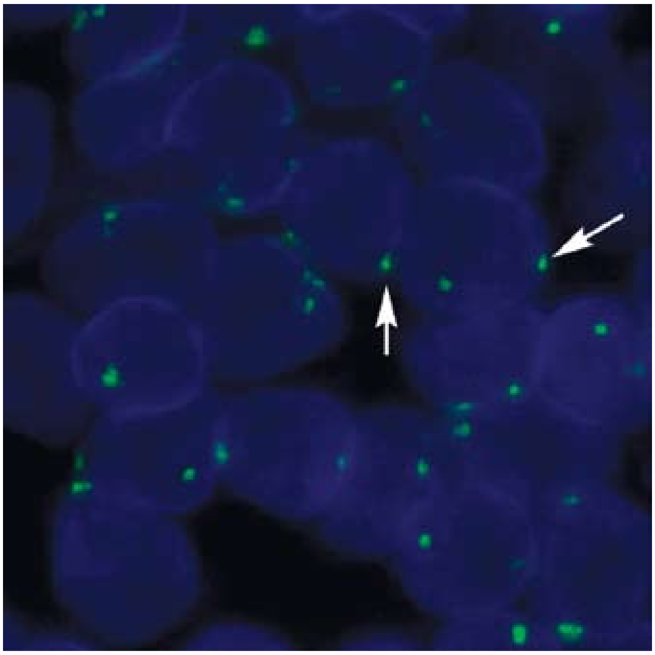


Figure 4. Staining with CEN-18 FITC-labeled PNA probe (green signals). Arrows illustrate the localization preference close to the membrane of gene-poor DNA domains.

FISH probe target genes are often located in gene-dense domains enriched in the nuclear interior, thus a heterogeneous enzymatic degradation affecting the center of the nucleus may bias probe signals towards a reduced presence of gene targeted signals versus centromere probes closer to the envelope. A possible outcome of over-digestion is shown in Figure 5.

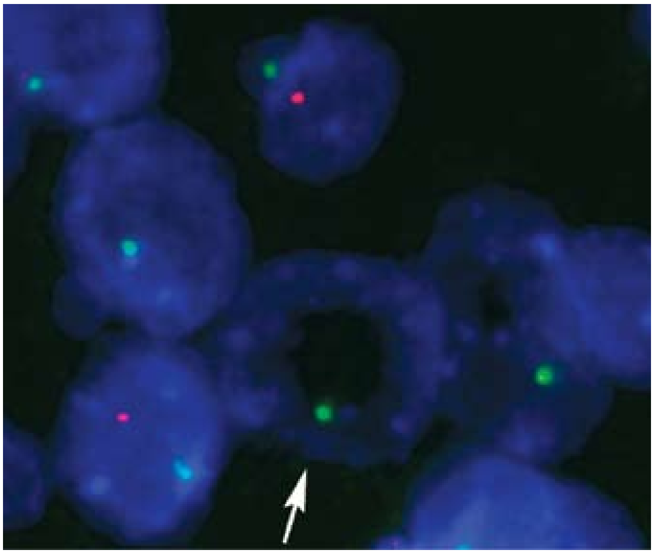


Figure 5. Over-digested (8-minute enzymatic digestion) FFPE breast tissue stained with gene probe (red), a centromere reference PNA probe (green) and DAPI (blue). The image shows doughnut formation and uneven DNA counter stain (blue). The arrow points to a nuclei with loss of red signal. The tissue specimen has a normal 1:1 gene to reference ratio at its optimal digestion time (3-minute enzymatic digestion — data not shown).

Probe Hybridization and Stringency Wash

Following pre-treatment and digestion, a two-step process is required to ensure efficient marking of the target sequences. First the genomic target DNA and the labeled probes (when double-stranded) must be denaturated by heating. Then the temperature is lowered to start a hybridization competition in which unlabeled repetitive sequences in the probe mix hybridize to complimentary sequences in the target DNA to block unspecific signals. Simultaneously, the labeled probe sequences hybridize to complimentary sequences in the target DNA and thereby mark the target sequences. The denaturation and hybridization events take place in the presence of formamide, and the optimal temperature profile depends mainly on the concentration of this chaotrope. In a 45% formamide solution, optimal results are obtained by denaturation at 82 °C for 5 minutes followed by hybridization at 45 °C over night (14-20 hours). The reaction should be covered and sealed and the humidity kept at saturation (100% relative humidity) as evaporation during hybridization can change the probe composition or even dry out the solution thereby compromising signal quality.

Unspecific hybridization events are washed away at stringent conditions prior to mounting. Optimal temperature of the post-hybridization wash depends mainly on the salt concentration in the buffer. High salt concentration decreases DNA backbone repulsion and thereby increases the DNA duplex melting temperature. Wash using a saline-sodium citrate (SSC) buffer with a detergent for 10 minutes at 65 °C efficiently removes mismatch hybrids, but if this temperature is exceeded, it may result in a reduction of signal intensity or loss of the specific signals.

Mounting and Visualization

After mounting, the slides should be left in the dark for at least 15 minutes for DAPI to stain the nuclear material. Slides are inspected in a fluorescence microscope. It is important to use the recommended filter sets since use of sub-optimal filters significantly reduces signal intensity (see Chapter 12, Filters for FISH Imaging).

Table 1. Possible Staining Patterns*

Preparation	Autofluorescence	DAPI Pattern	Possible Effects
Under-digestion	▪ excessive ▪ autofluorescence	▪ heterogeneous staining ▪ some doughnut nuclei	▪ reduced target gene signal in some cells
Over-digestion	▪ autofluorescence level does not differentiate properly digested samples from over-digested samples	▪ doughnut nuclei ▪ "ghost" nuclei ▪ destroyed tissue morphology	▪ loss of target gene signal in some cells
Optimal digestion	▪ non-disturbing autofluorescence	▪ intact nuclei ▪ intact tissue morphology ▪ homogeneous DAPI staining	▪ all signals will show ▪ reference signals, typically, near membrane ▪ target gene signals, typically, in interior

*These effects may not be seen in all cells or in all slides.

Conclusion

Correct pre-treatment of FFPE slides is very important to obtain valid FISH staining results. The key factor is to adjust the enzymatic digestion to the fixation history of the tissue. Inspection of autofluorescence level, DAPI staining pattern and nuclear morphology are informative tools for an initial evaluation of sample digestion. The signal distribution pattern may add valuable information to assess, if digestion has been optimal. An optimally processed sample is characterized by intact nuclear membranes, non-disturbing autofluorescence and homogeneous DAPI staining. Reference signals are expected to be present near the nuclear membrane, whereas the gene targets are predominantly expected in the nuclear interior when arising from gene-dense domains. Finally, areas of normal cells may serve as a control for proper tissue treatment. In normal cells, the target/reference signal ratio should match the ratio which is expected in a normal diploid cell. A prerequisite for use of normal cell areas as tissue treatment control is that the impact of fixation is similar in the normal and the malignant areas.

Acknowledgement

We wish to thank Dr. Martina Schmidt, Scientist, and Dr. Oliver Stoss, Project Manager, Biomarker Analytics and Bioinformatics, Targos Molecular Pathology Gmbh, Kassel, Germany, for providing the FISH images used in Figure 2-3.

References

1. Morales A R, Nassiri M, Kanhoush R, Vicek V, Nadji M. Experience with an automated microwave-assisted rapid tissue processing method. Validation of histologic quality and impact on the timeliness of diagnostic surgical pathology. Am J Clin Pathol 121: 528-36, 2004.
2. Nassiri M, Ramos S, Zohourian H, Vinvek V, Morales A R, Nadji M. Preservation of biomolecules in breast cancer tissue by a formalin-free histology system. BMC Clinical Pathology 8:1, 2008.
3. Landegent J E, Jansen in de Wal N, Dirks R W, Baas F and van der Ploeg M. Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive in situ hybridization. Hum Genet 77: 366-70, 1987.
4. Nielsen K V, Müller S, Poulsen T S, Gabs S and Schønau A. Combined Use of PNA and DNA for Fluorescence *In Situ* Hybridization (FISH). In PEPTIDE NUCLEIC ACIDS — Protocols and Applications. 2nd Edition. Nielsen P E, eds. Horizon Bioscience, 2004.
5. Nielsen K V, Müller S, Agerholm I, Poulsen T S, Matthiesen S H, Witton C J, Schønau A. PNA suppression method combined with fluorescence in situ hybridization (FISH) technique. In PRINS and PNA Technologies in Chromosomal Investigation. Frank Pellestor, eds. NOVA Publishers, 2007.
6. Hopman A H N, Poddighe P, Moesker O and Ramaekers F C S. Interphase cytogenetics: an approach to the detection of genetic aberration in tumours. In Diagnostic molecular pathology: A practical approach. M J O and H C S, eds. IRL Press, NY, 1991.
7. Bolzer A, Kreth G, Solovei I, Koehler D, Saracoglu K, Fauth C, Müller S, Eils R, Cremer C, Speicher M R, Cremer T. Three-Dimensional Maps of All Chromosomes in Human Male Fibroblast Nuclei and Prometaphase Rosettes. PLoS Biology 3, 826-842. 2005.
8. Barcellona M L, Cardiel G, Gratton E. Time-resolved fluorescence of DAPI in solution and bound to polydeoxynucleotides. Biochem Biophys Res Commun. 170: 270-80, 1990.

Chapter 12 | Filters for FISH Imaging

Dan Osborn

The application of in situ hybridization (ISH) has advanced from short lived, non-specific isotopic methods, to very specific, long lived, multiple color fluorescent-ISH probe assays (FISH). Improvements in the optics, filter technology, microscopes, cameras, and data handling by software, have allowed for a cost effective FISH setup to be within reach of most researchers. The application of mFISH (multiplex-FISH), coupled to the advances in digital imaging microscopy, have vastly improved the capabilities for non-isotopic detection and analysis of multiple nucleic acid sequences in chromosomes and genes (1).

Filters and Fluorescent Imaging

In an upright microscope, the fluorescence illuminator follows an epi-fluorescent path (illumination from above) to the specimen. In the pathway is housed the filter blocks containing the dichroic mirror, excitation, and emission filters, which work to greatly improve the brightness and contrast of the imaged specimens, even when multiple fluorochromes are being used. Figure 1 illustrates the basic setup of the fluorescence illuminator on an upright microscope.

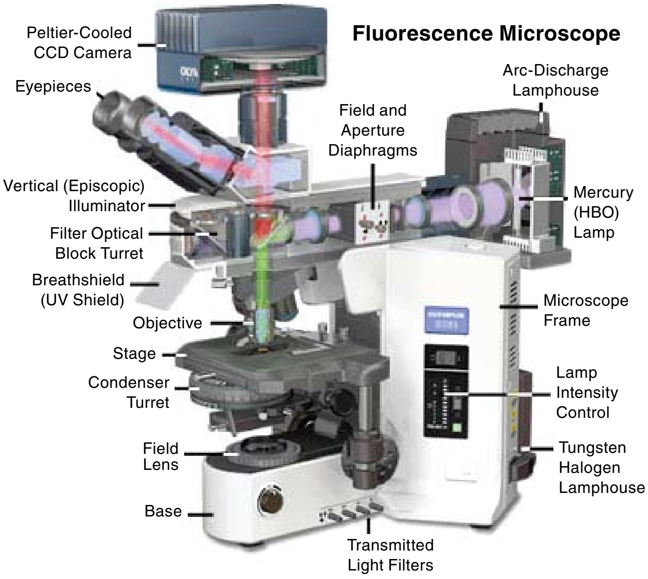
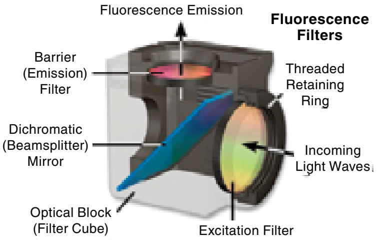

Fluorescence Microscope
Peltier-Cooled CCD Camera
Eyepieces
Field and Aperture Diaphragms
Arc-Discharge Lamphouse
Vertical (Episcopic) Illuminator
Filter Optical Block Turret
Mercury (HBO) Lamp
Breathshield (UV Shield)
Objective
Microscope Frame
Stage
Condenser Turret
Lamp Intensity Control
Field Lens
Tungsten Halogen Lamphouse
Base
Transmitted Light Filters

Figure 1. Anatomy of a fluorescence microscope.
Figure Courtesy: http://www.olympusmicro.com/primer/techniques/fluorescence/anatomy/fluoromicroanatomy.html

The principle components in the episcopic (reflected illumination) pathway consist of the light source (here depicted as a Mercury arc lamp), a series of lenses that serve to focus the light and correct for optical aberrations as the beam travels towards the filters, diaphragms which act to establish proper and even illumination of the specimen, and the filter turret, which houses the filter sets. In the diagram it can be seen schematically how the broad band excitation light from the light source is selectively filtered to transmit only the green component by the excitation filter in the turret, which is in turn reflected by the dichromatic mirror to the specimen. The red fluorescence emission is then transmitted back through the objective lens, through the mirror and is further filtered by the emission filter before visualization by eye or camera.

An exploded view of the filter cube is shown in Figure 2. The excitation filter is shown in yellow and the emission filter in red to describe a typical bandpass Texas Red filter set.


Fluorescence Emission
Fluorescence Filters
Barrier (Emission) Filter
Threaded Retaining Ring
Dichromatic (Beamsplitter) Mirror
Incoming Light Waves
Optical Block (Filter Cube)
Excitation Filter

Omega XF207 Texas Red filter set:
XF1207 Exciter 580/20
XF3028 Emitter: 630/30
XF2020 Dichroic 600DRLP

Figure 2. Anatomy of a typical fluorescence filter cube.
Figure Courtesy: http://www.microscopyu.com/articles/fluorescence/fluorescenceintro.html

Filter descriptions

Bandpass filters can be described in several ways. Most common is the Center Wavelength (CWL) and Full Width Half Maximum (FWHM) nomenclature, or alternatively, by nominal Cut-on and Cut-off wavelengths. In the former, the exciter in Fig. 2 is described as a 580/20 or, a filter with nominal CWL of 580 nm and a FWHM of 20 nm. The half maximum value is taken at the transmission value where the filter has reached 50% of its maximum value (Figure 3). In the latter scheme, the filter would be described as having a Cut-on of 570 nm and a Cut-off of 590 nm, no CWL is declared. The Cut-on describes the transition from attenuation to transmission of the filter along an axis of increasing wavelengths. The Cut-off describes the transition from transmission back to attenuation. Both values indicate the 50% point of full transmission.

Cut-on and Cut-off values are also used to describe two types of filters known as Longpass (or highpass) filters (Figure 4) and Shortpass (or lowpass) filters (Figure 5). A longpass filter is designed to reflect and/or absorb light in a specific spectral region, to go into transmission at the Cut-on value (here 570 nm) and transmit light above this over a broad wavelength range. A shortpass filter does the reverse, blocking the wavelengths of light longer than the Cut-off value for a specific distance, and transmitting the shorter wavelengths. It should be noted that these reflection and transmission zones do not continue indefinitely, but are limited by properties of the coating chemicals, coating design, and the physical properties of light.

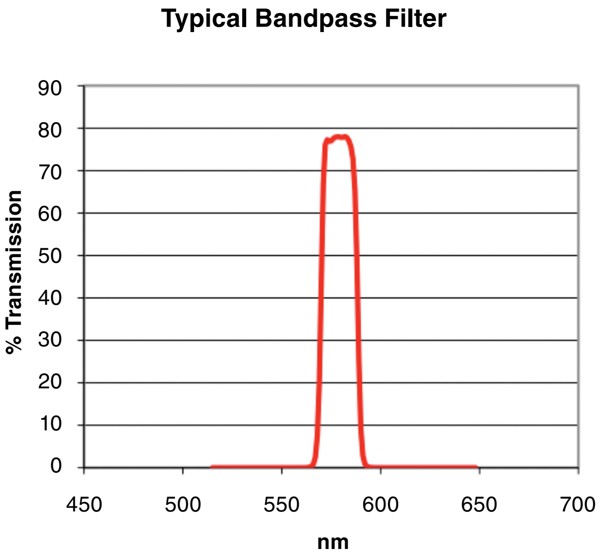


Figure 3. Typical bandpass filter.

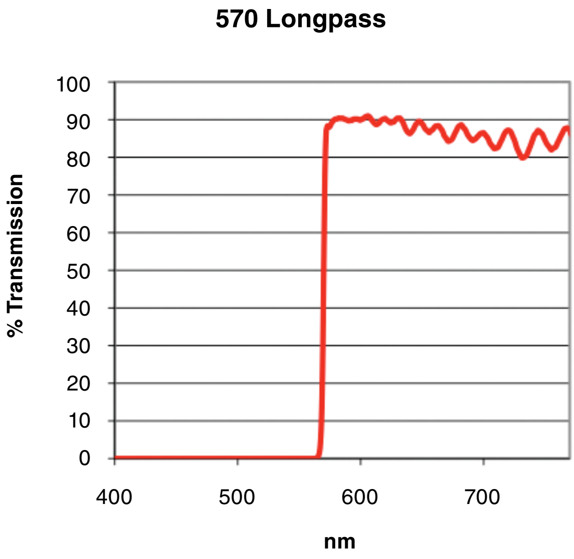


Figure 4. Longpass (or highpass) filter.

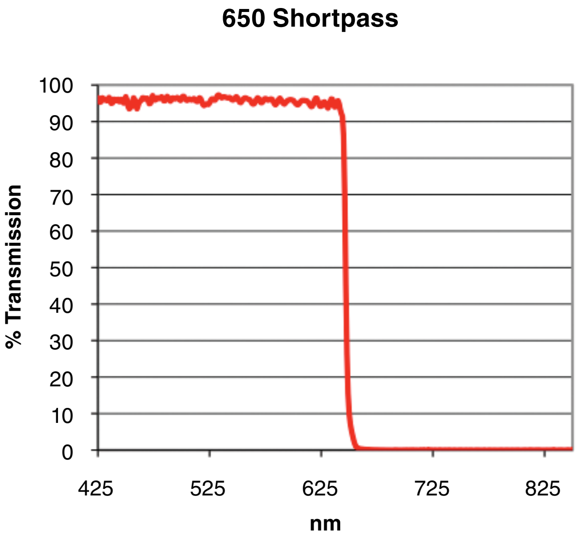


Figure 5. Shortpass (or lowpass) filter.

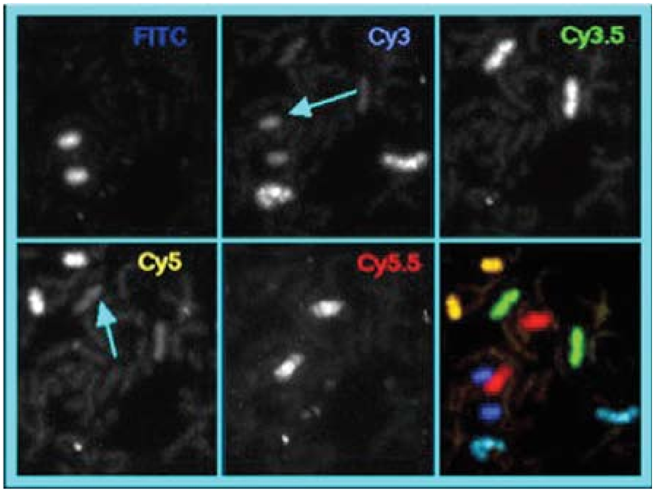
Specialized Filters for FISH and mFISH

The imaging of multiple fluorescent probes requires special considerations towards the set up of the filter blocks in the microscope turret. One strategy is to use individual filter cubes for each probe in the specimen. This is an effective strategy for 6 color viewing (six being the standard number of filter positions in most upright research microscopes), as good spectral isolation of the different probe species can be obtained through careful filter design. This setup also reduces the potential bleaching of the probes by illuminating only one fluorescent species at a time. A potential drawback to this setup is image registration shifts caused by slight misalignments of the filters, producing a minor beam deviation that can be detected when switching between several different filter cubes. The dichroic mirror and the emission filter are the imaging elements of the filter cube and are the two components which can contribute to this effect.

Another strategy is to utilize single multiband dichroic mirrors and emission filters and separate exciter filters either in an external slider or filter wheel. This will preserve the image registration and reduce mechanical vibrations, but the trade offs are a reduced brightness of the fluorescence, limitations on how many different probes can be separated, and reduced dynamic range and sensitivity due to the necessary color CCD camera.

Fluorescent microscopes typically come equipped with standard filter cubes for the common DAPI stain, FITC, TRITC, and Texas Red fluors. Standard filter sets have mostly wideband excitation and emission filters (sometimes using longpass emission filters) in order to provide maximal brightness. When employing FISH, these standard sets can work well for 2, 3 and 4 color labeling, but spectral bleedthrough can rapidly become a problem. For instance, FITC is partially visualized through the Cy3 filter, and Cy 3.5 can be seen through the Cy 5 filter.[2]

Figure 6 depicts five different labeled chromosome pairs, the crosstalk between channels is shown by the arrows in the top middle and bottom left images. Bottom right panel is a overlaid and pseudocolored image of the series.



(Image courtesy of Octavian Henegariu, Yale University)

Figure 6. Multiple color FISH.

In order to minimize the spectral bleedthrough of very closely spaced fluors in multicolor labeling schemes, specialized narrow band filter sets are needed. Exciter filters of 10-20 nm in bandwidth and emission filters of 20-40 nm provided the specificity necessary to achieve the degree of sensitivity and spectral resolution required in mFISH. Figure 7 shows a typical wide band FITC filter set overlaid on the excitation and emission peaks of FITC and CY 3. Although the filters are designed for covering a substantial area under the absorption and emission curves, there is a significant overlap with both the excitation and emission curves of Cy3, thus resulting in FITC channel contamination by Cy3. A solution is seen in Figure 8, where excitation and emission bands have been narrowed to improve the spectral resolution of FITC from Cy3, especially in the emission band. By limiting the red edge of the emission filter, a reduction in the area under the emission curve of the Cy3 dye of about 4-fold is achieved.

FITC and Cy3: Standard Wideband Filter Set

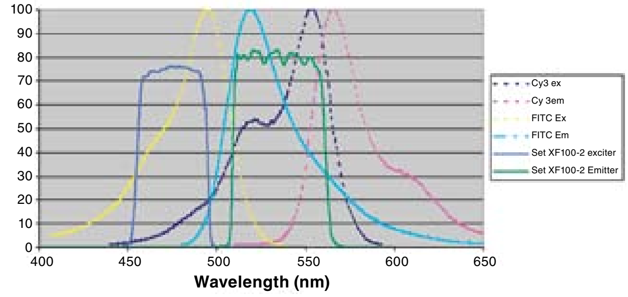


Figure 7. FITC and Cy3. Standard wideband filter set.

FITC and Cy3: Narrow Band mFISH Set for FITC

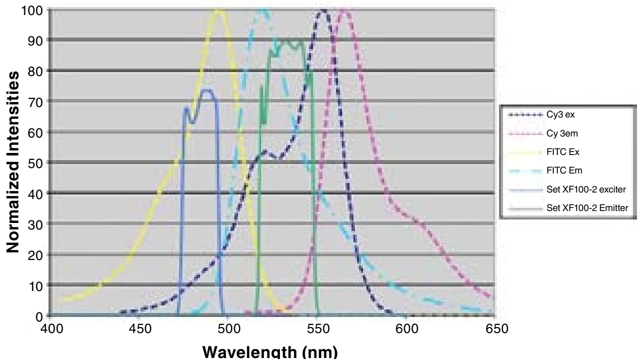


Figure 8. FITC and Cy3. Narrow band M-FISH filter set for FISH.

By incorporating the design strategy of narrow band, steep-edged filters, the spectral window for adding multiple fluorescent probes widens without the cost of adding emission bleedthough between fluors. This can be seen in Figure 9 where three fluors are effectively separated within a spectral window of less than 300 nm.

A fourth dye, such as Cy 3.5, could easily be incorporated in this scheme as well in the 570-620 nm region, but is left off to reduce congestion.

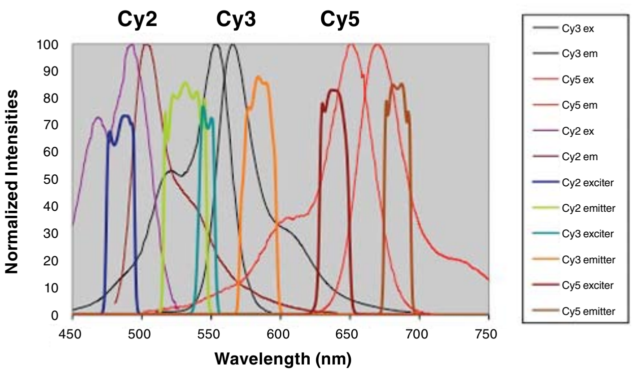


Figure 9. Narrow band, steep-edged filters showing three fluors effectively separated within a spectral window of less than 300 nm.

The demands on the filters used for mFISH are such that it is necessary to provide a specific category of products which are matched together to make optimal use of the available bandwidth for each mFISH fluor.

Table 1. shows the Omega Optical series of filter sets for the more prevalent fluors used in mFISH, along with excitation and emission filter bandwidths. Note all are single fluor sets except XF231 and 232 which used single exciters for each fluor and triple band dichroics and emission filters. This setup minimizes registration shift and stage movement by requiring only that an external filter slider or wheel be moved to excite the different dyes while the multiband dichroics and emission filters are kept stationary in the microscope turret.

Table 1.

Set Name	Fluorophores	Filters
XF06	DAPI, AMCA	Exciter: 365/50 Dichroic: 400DCLP Emitter: 450/65
XF201	DEAC	Exciter: 436/8 Dichroic: 455DRLP Emitter: 480/30
XF202	FITC, Cy2	Exciter: 485/20 Dichroic: 505DRLP Emitter: 530/30
XF203	Alexa 532	Exciter: 520/18 Dichroic: 545DRLP Emitter: 565/20
XF204	Cy3, TRITC, Alexa 546	Exciter: 546/10 Dichroic: 555DRLP Emitter: 580/30
XF206	Cy3.5	Exciter: 572/15 Dichroic: 590DRLP Emitter: 620/35
XF207	Texas Red, Alexa 594	Exciter: 580/20 Dichroic: 600DRLP Emitter: 630/30
XF208	Cy5, Alexa 647	Exciter: 640/20 Dichroic: 650DRLP Emitter: 682/22
XF210	Cy5.5	Exciter: 665/32 Dichroic: 692DRLP Emitter: 710/40
XF231	DAPI/ FITC/ TRITC	Single exciters for each dye can be housed in external filter wheel, triple band dichroics and emitters housed in filter holder
XF232	DAPI/FITC/ Texas Red	

In addition to the use of specialized filter sets for mFISH protocols, other features of the filters which have improved image quality are broad band AR(anti-reflection) coatings and polished substrates.

The AR coating can provide transmission enhancements by reducing secondary surface reflections at glass interfaces. This improvement can be realized by up to a 7% increase in filter throughput (3). AR coatings on emission filters and dichroic mirrors also reduce the “ghost image” sometimes seen in optical pathways with multiple reflective surfaces.

Polished substrates are features also found predominantly on the emission filters and dichroics. By exposing the glass substrate on which the filter is manufactured upon to a two-sided polishing procedure, the substrate achieves a high degree of “parallelism”, or even thickness. This process has the effect of reducing significant beam deviation of the transmitted image, thus allowing for minimal registration shifts when rapidly switching between filter sets.

Conclusion

The techniques of FISH and mFISH used in conjunction with the resolving power and automated digital imaging capabilities of the fluorescence microscope offer a powerful combination of advantages that stand to benefit many areas of biology, from basic research to prenatal disease detection, cancer research, pathology, and cytogenetics.

In the fluorescence microscope, careful consideration of the sample and system components is necessary to specify the correct filters for probe detection. Use of multiband dichroics and emission filters in a stationary turret with single exciters in an external slider or filter wheel can give near simultaneous probe detection with no registration shift, but there are likely compromises in overall brightness, color balance difficulty, and reduced resolution of the color CCD camera.

If sensitivity, spectral resolution, and minimal photobleaching are primary concerns, single narrow band filters sets with black and white CCD camera detection is the best option. Image registration shifts are minimized in today’s filters by the use of polished substrates and virtually eliminated by using filter sets made to “zero shift” specifications.

The type and number of fluorescent probes also plays a role in the optimizing of the filters. For a small number of probes with adequate spectral separation it is possible to use traditional wide bandpass filter sets. In protocols where 5 or 6 probes are being used, it is necessary to use dye-specific narrow band filter sets to reduce spectral bleedthrough.

As methodologies in FISH and mFISH on the fluorescent microscope evolve, so must the software and hardware used to unravel the information contained in the specimen. A proper combination of filters, dyes, imaging hardware, and software is desirous for obtaining the resolution and contrast necessary for accurate image capture and analysis.

References

1. Brenner, M., Dunlay, T., & Davidson, M. (n.d.). Fluorescence in situ hybridization: Hardware and software implications in the research laboratory. Retrieved October 7, 2008, from Molecular Expressions Microscopy Primer Web site: http://www.microscopyu.com/articles/fluorescence/insitu/brennerinsitu.html
2. Henegariu, O. 2001. Multi-color FISH. Retrieved October 8, 2008, from Tavi's Multicolor FISH Page: http://info.med.yale.edu/genetics/ward/tavi/fi12.html
3. Johnson, B. 2006. Anti-Reflection Coatings. Omega Optical Application Note.

Troubleshooting

If there is no image:

- check that fluorescence light source is on and the light path is clear. Light can usually be seen illuminating the sample unless it is below 400 nm (DAPI excitation).
- check that the image is being sent to correct port, camera or eyepiece.
- correct filter block is in place for the desired fluor.
- if desired fluor emission is > than approx. 670 nm (Cy5) it is not visible by most eyes. If not visible by camera, check that there is no IR blocking filter in camera.

If image has high bleedthrough from other fluors:

- make sure filter set is correct for single dye usage, not using longpass emission filter or wide bandpass filter set.

Chapter 13 | Fluorescence In Situ Hybridization (FISH) Imaging

George L. Kumar PhD and Robert M. Zucker PhD

Fluorescence In Situ Hybridization (FISH) is a powerful technique for detecting chromosomal changes in tumor cells and is one of the most frequently used techniques in the study of structural cytology of the cell nucleus. It provides a reliable means for studying the genetic composition of cells in mitosis as well as in interphase. As of today, the technique has reached high detection sensitivity, (i.e. individual genes can be detected), and high multiplicity (i.e. several probes can be applied to the same nucleus) (1-3). The most common methods for FISH visualization are: Flow cytometry systems and slide-based systems. In flow systems, FISH-stained cells are prepared in suspension, and the suspension flows in a narrow stream across a laser beam wherein the detector records their fluorescent intensities (4). In slide-based systems, FISH-stained cells are fixed to a conventional wide-field or a confocal fluorescence microscope slide and observed as a static image. Depending on the sophistication of both systems, a laser or a non-laser light source is used. In this technical note, we will discuss the most important microscope and instrument associated factors that are crucial for obtaining good FISH images- assuming that the sample has been well-prepared and a highly efficient fluorophore with high quantum yield is selected. Since there is not enough room in this short technical note to fully describe each term, where appropriate, we will provide hyperlinks that will guide the reader to detailed explanations, animations and other useful material.

Optimization of the Microscope for Obtaining Good FISH Images

Obtaining good FISH images depends on various factors, such as the "Numerical Aperture (NA) of the objective, Köhler illumination or field illumination of the lens, the refractive index of the embedding medium, the coverslip thickness, the stability of the light source, depth of the objects observed below the coverslip, and the ratio between emission and excitation wavelengths. These factors influence the basic features of the optical system, such as the "Point Spread Function (PSF)", the resolution (see appendix), optical sectioning (or confocality in case of a confocal microscope, please refer to http://www.olympusmicro.com/primer/techniques/confocal/index.html), the light throughput, and the degree of monochromatic and chromatic aberrations. In addition, it is critical to have a lens that colocalizes the fluorescence pixels. In both wide field and confocal systems, this is highly dependent on the quality of the lens and the proper alignment of the system (5-11).

Before the advent of digital imaging, lenses were evaluated subjectively,i.e. the image looks good or the image looks bad. So from these pretty pictures, it was very difficult to access the quality of these lenses from the point of lens aberrations (i.e. chromatic or spherical) so it was not clear, if one had a good lens or an imperfect lens. With the advent of sophisticated microscope XYZ stages, digital cameras and confocal microscopy, it has become easier to check the quality of the lenses by way of measuring PSF, spectral registration and colocalization (i.e. the degree of overlap between two different fluorescent labels, each with a separate emission wavelength and with the two "targets" located in the same area or very near to one another). The later three factors are especially critical in order to resolve hybridization dots (i.e. FISH-stained genes), taking into consideration that hybridization dots are quite small in size and occupy miniscule volumes inside the nuclei (e.g. 103 nm with a volume of 5.7 × 10^{-4} µm^3 for c-myc, 119 nm with a volume of 8.9 × 10^{-4} µm^3 for p53 and 123 nm with a volume of 9.7 × 10-4 µm3 for p58, respectively) (12). In this technical note, we shall discuss all of the above issues in brief.

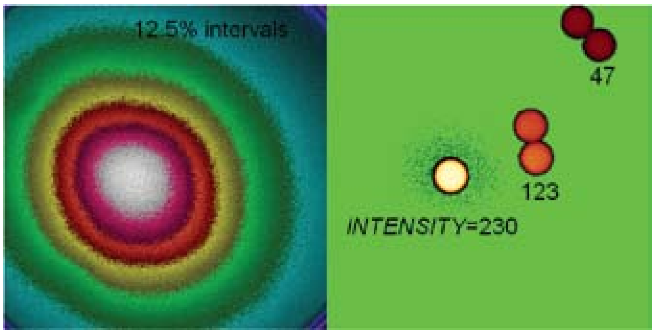
Numerical Aperture (NA) of the Objective

In its simplest form, the NA of an objective is its light gathering capacity. The higher the NA, the higher its light gathering capacity. As a result, an objective that is marked to have a high NA will collect more emission wavelength coming out of the FISH (dots or sample). A 60x Plan Apochromat objective with a 1.4 NA will produce better results (depending on the detector) in contrast to a 40x objective with a 0.95 NA. In terms of resolution, a 100x 1.4 NA objective will produce the same results as a 60x 1.4 NA objective, except that it will be dimmer, because Image Brightness (Fluorescence) α (NA^4/M^2). However, a 100x objective is a more complex lens and will provide higher magnification of the FISH dot (when a digital camera is used) which may be desirable in some specialized applications (e.g. microtubule dynamics in yeast or bacterial cell division). The higher NA lens will also have provide more resolution than the lower NA lens which will be essential to distinguish FISH pixels. A higher NA lens will also have less field depth so objects of slightly different planes may be out of focus inciting the need for a confocal microscope or an epifluorescence microscope capable of obtaining optical sections. For a Java tutorial of NA, please refer to: http://www.microscopyu.com/tutorials/java/objectives/nuaperture/index.html.

Köhler Illumination or Field Illumination of the Lens

A first demand for analysis of FISH objects or ratio-labeled objects is Köhler illumination (named after the German inventor August Köhler) or also called field illumination. This is a technique that provides optimum resolution and contrast in a light microscope by aligning and focusing the illumination light source and critically setting the apertures of the microscope to best match the numerical aperture of the objective lens. This type of lighting provides even illumination on the object (i.e. FISH spots) also when there are irregularities in the brightness of the light-emitting surface. Improper setting of this illumination makes it impossible to use the full resolving power of the objectives thus resulting in non-optimal FISH images (Fig. 1, 2). In addition to optimal FISH imaging, homogeneous illumination is critical for relative quantitation, such as comparing the signal intensity between one region of interest to another and absolute quantitation, i.e. knowing the exact number of target molecules present in the sample of interest by the use of linear detectors such as cooled CCD cameras and PhotoMultiplier Tubes (PMT).

UV Field Illumination



100x Objective (1.4 NA)

Figure 1. UV field illumination of a Plan Apo 100x lens (1.4 NA) derived with a fluorescent plastic slide and the intensity measurement of 10-micron Spherotech beads (obtained from Spherotech, Libertyville, IL, USA). This illustrates the problem of using a lens with improper field illumination to make comparative measurements on a sample. The field illumination pattern shows a bull's eye intensity pattern slightly off-center and the five beads located in different parts of the field to illustrate the variation in intensity occurring by using a lens that has improper field illumination. The intensity of beads was derived by a small Region of Interest (ROI) inside the bead. The five beads show a decrease in intensity relative to the bead in the center of the illumination. Although this figure was obtained with UV optics, it represents the type of field illumination that can also occur with visible light excitation. This pattern is also unacceptable, if a confocal laser scanning microscope optical system is used for a FISH study, as the maximum intensity should be in the center of the objective and not in the corner.

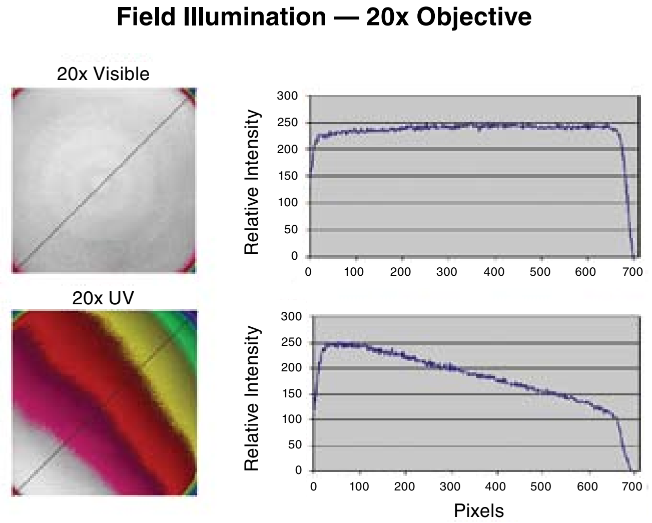


Figure 2. Field illumination. Field illumination patterns of visible (A) and UV (B) excitation using a 20x (Plan Apo, NA 0.7) lens. The visible field illumination shows uniform illumination with the brightest intensity located in the center of the objective. The line running diagonally in panels A and B measures the histogram intensity of the field illumination graphically represented in Figures C and D. The variation in intensity from the left to right side of the field is less than 10% for visible excitation and over 150% for UV excitation. Acceptable field illumination has brightest intensity in the center of the objective decreasing less than 25% across the field. The intensity regions were prepared by using Image Pro Plus to divide the Grey Scale Value (GSV) into 10 equal regions and a median filter was used for additional processing. The field illumination test slides used in this study were from Applied Precision Inc., Issaquah, WA, USA, and consists of three fluorescent plastic slides with excitation peak wavelengths of 408 nm (blue), 488 nm (orange), and 590 nm (red), and emission peak wavelengths of 440 nm, 519 nm, and 650 nm, respectively. The blue slides (408 nm) were used for UV field illumination and alignment while the orange slides were used for 488/568 nm field illumination and alignment. (For a Java tutorial of Köhler illumination, please refer to: http://micro.magnet.fsu.edu/primer/anatomy/kohler.html).

The refractive index of the medium between the objective and coverslip: There is more light collected with oil immersion optics than with dry optics (Fig. 3).

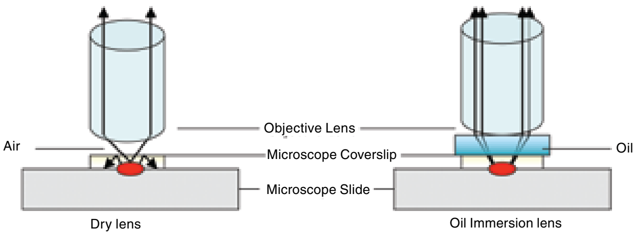


Figure 3. Paths of light rays through a dry and oil-immersion objective lenses. The red circle at the origin of the light rays is the sample. If the refractive index of the immersion oil (1.52) is matched to the glass coverslip and the objective lens, then an optically homogenous light path is created, preventing distortion. Figure modified after Bruce Alberts et.al. Molecular Biology of the Cell. Garland Publishers 2008.

Oil objectives usually have a higher numerical aperture, a sharper airy disk (see http://www.microscopyu.com/tutorials/java/imageformation/airyna/ and appendix) and higher resolution than an air lens. In other words, the light path of the optic changes the light gathering power. Oil immersion objectives improve light gathering power as less light is lost. For example, the refractive index (η) of the glass that makes a microscope objective and a glass coverslip that is over the specimen sample is ~ 1.5. If the η of oil that goes between the objective and coverslip is also ~ 1.5, then there is no refractive index mismatch. In this case, most of the light is collected. On the other hand, if oil that has a wrong refractive index (η) is used (e.g. 1.36) or air that has a η of ~ 1.00 is used between the objective and coverslip, then there is a refractive index mismatch. This can lead to emission light loss, spherical aberration and a reduced resolution of the FISH spot. In addition to these precautions, it is also important to make sure that there are no air bubbles in the immersion oil that could act as a "lens". For a Java tutorial, please refer to: http://www.microscopyu.com/tutorials/java/objectives/immersion/index.html. In a nutshell, the best FISH images are obtained when there is minimal or no refractive index mismatch between the objective, coverslip and the sample.

The Coverslip

This is the least expensive optical component, but very important and the most likely to be carelessly chosen. Many objectives are designed and marked to be used with coverslips of a certain thickness, usually 0.17 mm (or ~ 170 ±5 µm), which corresponds to a thickness grade of 1.5. Ideally, for obtaining the best FISH images, it is recommended that an oil immersion lens is used and these lenses almost always require a 1.5 coverslip for optimal resolution. Any deviation from this thickness leads to spherical aberration, loss in resolution and results in larger and dimmer FISH spots.

The Stability of the Light Source

Usually, the illumination light source consists of a mercury arc or a proprietary pre-aligned high-efficiency light source such as Exfo http://www.exfo-xcite.com/, xenon arc or lasers.

Mercury/Xenon arc lamp: If the arc is not focused sharply on the back aperture, the specimen plane will be unevenly illuminated. In addition, fluctuations in the illumination intensity of the lamp will also result in variable intensity over the whole field of view. In order to avoid this, it is better to consider a fluorescence microscope wherein the light coming from the mercury/xenon lamp goes through a quartz optical fiber for light scrambling, resulting in an even illumination and less fluctuations (Fig. 4).

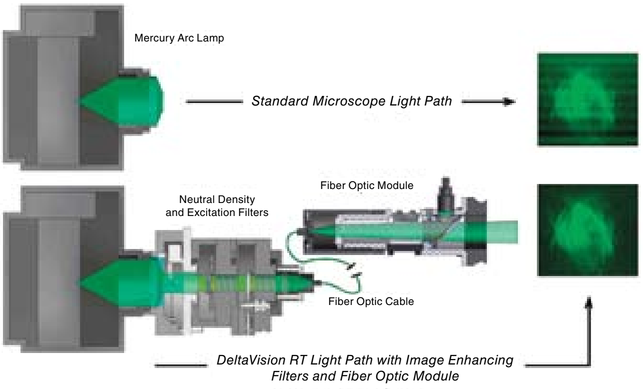

Mercury Arc Lamp
Standard Microscope Light Path
Fiber Optic Module
Neutral Density and Excitation Filters
Fiber Optic Cable
DeltaVision RT Light Path with Image Enhancing Filters and Fiber Optic Module

Figure 4. The integrated photosensor measures light intensity delivered to each individual image. All images acquired in a series, 2D or 3D, are then normalized and balanced. Courtesy: DeltaVision® High Resolution Imaging System of Applied Precision, Inc. Issaquah, WA, USA.

Mercury arc light sources have a lifetime of 200-300 hours while proprietary pre-aligned high-efficiency light sources such as EXFO has a life time of 2000 hours. For FISH imaging, it is better to use light sources when they are more stable and at their peak, i.e. at the beginning or in the middle of their lifetime rather than using them at the end of the lifetime when there are more power fluctuations and more instability in the bulb and decreases in power.

Laser unit: Argon gas, helium neon gas and diode lasers are usually very stable with less than 1% power fluctuations. However, if the heat is not dissipated adequately or there are other forms of inadequate cooling, the result can be large laser power fluctuations (Fig. 5).

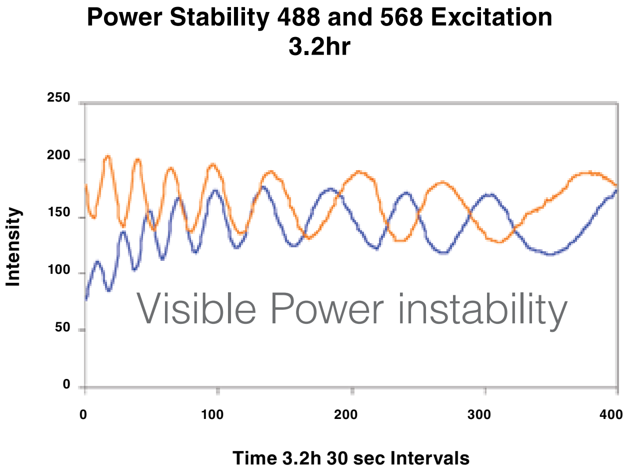


Figure 5. Visible laser stability. The laser power fluctuations using a 488 nm (blue) and 568 nm (red) lasers were determined using a 10x objective and a Chroma red slide. The fluorescence was sequentially measured every 30 sec (400 times) for total time duration of 3.33 hrs. The variation of the peak to peak using 488 nm or 568 nm excitation was approximately 25%. The fluctuating power intensity line suggests that the system scanning and detection devices are yielding large power fluctuations that will affect the illumination of the sample. The Acousto Optical Transmission Filter (AOTF) is probably contributing to this 488-568 nm sinusoidal pattern.

These fluctuations also occur when there are temperature or instabilty problems in the Acousto Optical Transmissions Filter (AOTF) of Laser Scanning Confocal Microscopes (LSCM), or when there is incorrect alignment of the fiber (8-11). When such fluctuations occur, weakly fluorescent structures (such as FISH spots) cannot be properly acquired or quantified (13).

Use of Laser Scanning Confocal Microscopes (LSCM) for FISH Imaging

LSCM (see glossary) equipped with PMT are becoming common in FISH imaging (14). In general, good FISH images are obtained with LSCM that are properly aligned and calibrated. Furthermore, FISH images can also be improved by: (a) Adjusting the pinhole to maximize photon capture; (b) Decreasing the laser power and thereby reducing photobleaching; (c) Choosing optimum filters and dichroics to get the maximum excitation and emission; (d) Reducing the scan speed for acquiring more photons and (e) frame averaging (see Fig. 6). The latter not only reduces photobleaching of faint FISH spots, but also reduces the noise in the system.

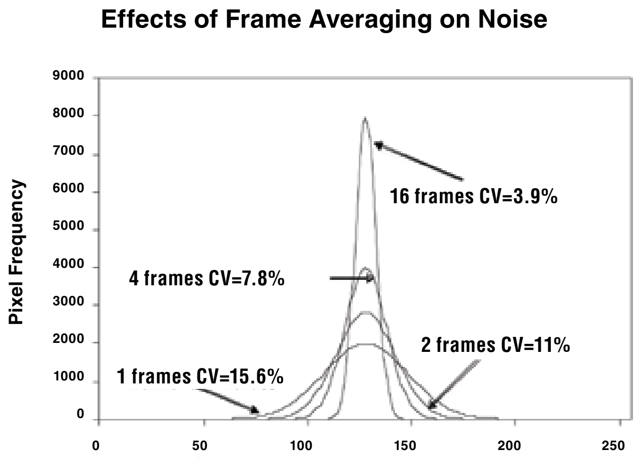


Figure 6. A confocal image was acquired with and without averaging the mean intensity at channel 135. The coefficient of variation or CV (CV = Mean/Standard Deviation) was calculated for the images acquired with averaging of 2, 4, and 16 frames, respectively. The distribution seen here is actually a pixel distribution of intensities from the image. It shows a distribution of different pixel intensities that decrease as the averaging increases. The narrow distributions are from more averaging and result in better image quality. In other words, the figure shows that for obtaining ideal image quality it is better to average more times so as to increase the signal to noise ratio.

Spectral Registration

The ability of light from different lasers and different fluorochromes to colocalize given points in an X, Y and Z plane is defined as spectral registration. For FISH it is crucial that this occurs so one can determine, if the fluorochromes representing FISH probes are colocalized or, if they are separated. Confocal systems that do not have spectral registration between the blue light and the other visible lines can provide inaccurate results (Fig. 7). Therefore, it is important for a FISH imaging setup that the system of lasers and lenses be checked with FocalCheck™ microspheres (Invitrogen, Carlsbad, California) to insure the system shows the correct spectral registration at all wavelengths (Fig. 8).

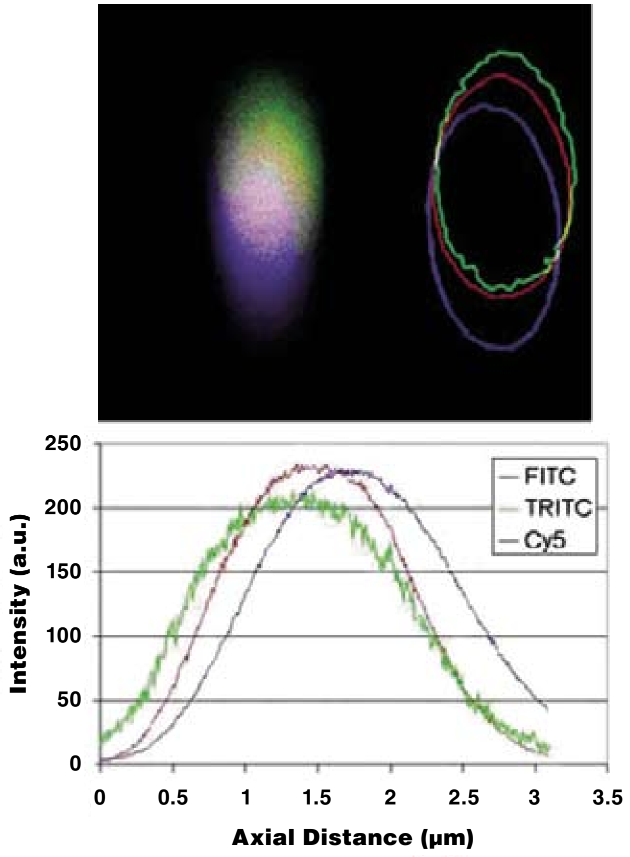


Figure 7. Spectral and axial resolution of a 1 µm bead obtained at three different wavelengths. The figure shows an image of a bead taken using three wavelengths of light — 488 nm (FITC), 568 nm (TRITC) and 647 nm (Cy5). The XZ image was converted into an outline of the bead using Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). The distribution of intensities can be made in the XZ (long axis) direction and this value can be compared to the XY (short axis) value to determine, if all the three colors register correctly and, if they show a good axial resolution. The more circular the bead image is and the more they are registered, the better the spectral registration and axial resolution.

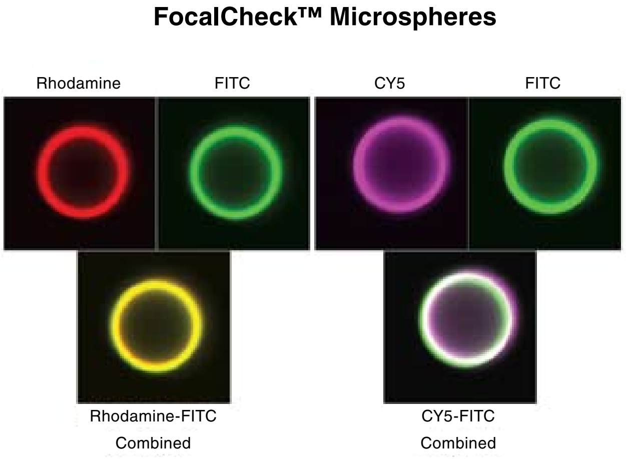


Figure 8. FocalCheck™ microspheres (Invitrogen, Carlsbad, CA, USA) were used to monitor the laser alignment for the Zeiss 510 laser scanning confocal microscope. FocalCheck™ microspheres have rings in three different fluorescent colors that can be excited by the 488 nm, 543 nm and 633 nm lasers contained in the confocal system. If the lasers are aligned properly, the emission fluorescence will be superimposed and only one mixed color (yellow) for Rhodamine and FITC (543 nm and 488 nm) will be observed (left). However, if the lasers and the system is misaligned (right), then the colors will not be superimposed and individual colors will be observed in the image. This was observed in the alignment of the 633 nm and 488 nm lasers by the emission of Cy5 and FITC, respectively. The misaligned system shows reduced spectral registration and, therefore, one can observe green, pink and the overlap of the two dyes designated as white instead of only white superimposed colors.

FISH Colocalization

Colocalization can only be ascertained in the absence of “cross-talk” between or “bleed-through” from selected fluorochromes. Accurate colocalization can only occur, if emission spectra are sufficiently separated between fluorochromes and, if correct filter sets are used during the acquisition step. Choosing fluorochromes with well-separated excitation and emission spectra is, therefore, critical for multiple labeling (multiple fluorochromes) and colocalization (15, 16). In addition, it is important to consider the microscope setup because in an improperly aligned microscope moving different filter cubes into the image path can cause lateral and focus shifts, thereby leading to misregistration of the colocalization images. As a note of caution, colocalization never shows that two fluorescent probes are interacting. Colocalization only indicates that two probes are located within close proximity. It is the technique of "Förster Resonance Energy Transfer (abbreviated FRET) that actually shows protein/protein or protein/nucleic acid or nucleic acid/nucleic acid interaction.

Resolution affects colocalization analysis. If the distance separating two fluorochromes is below the resolution of the imaging system (by the use of low NA objectives, e.g. 10x magnification/0.25 NA objective), they may appear colocalized. On the other hand, if a high resolution system is used (e.g. 100x magnification/1.45 NA objective), what did appear as colocalized may now be actually separate spots and not actually colocalized. Therefore, the NA of the objective lens, good refractive index match, and appropriate sampling frequency (or intervals) (i.e. small pixel sizes of the camera matched with magnification of the objective lenses and high NA lenses) will all affect resolution and, consequently, colocalization analysis.

Standards

The epifluorescence and confocal laser-scanning microscopes have enormous potential in many biological fields apart from FISH imaging. When tests are made to evaluate the performance of these microscopes, the usual subjective assessment is accomplished by using a histological test slide to create an image. However, without the use of functional tests and standards to measure a microscope performance, many of the machines may be working at sub-optimal performance levels, delivering misleading colocalization and FISH data. In order to replace the subjectivity in evaluating these microscopes, standards are necessary for evaluating machine performance. Depending on the sophistication of the user, different parameters are usually used in evaluating microscope performance, such as spectral registration, axial resolution or field illumination (Fig. 7. 8). One such standard is a microscopic tool box that includes slides that can be used for various tests. For field illumination, it is better to use a set of four fluorescence slides provided by microscope filter companies (e.g. Omega Optical Fluorescence Reference Slide Set: https://www.omegafilters.com/index.php?page=omega/Prod/prod_rslides_chart or Chroma Technology Corporation Slides: http://www.chroma.com (8-11). The red slide from such a set can be excited at all wavelengths and is used to measure field illumination (see Fig. 2). For "Point Spread Function" (PSF) generation (see Appendix) to facilitate the calibration of epifluorescence microscopes and LSCM for multicolor applications and associated image-processing equipment, kits from Invitrogen: http://probes.invitrogen.com/media/pis/mp07279.pdf or from PolyScience (Warrington, PA, USA):

http://www.polysciences.com/Catalog/40/categoryId__4/ can be used. For more details, please refer to references 5-11.

XY and Z Stage Drift

Mechanical drift at the microscope stage can cause the plane of the object image to change with time (Fig. 9). This can lead to false artifacts in colocalization and/or FISH, and such artifacts can be avoided by using a high-speed servo or piezoelectric-driven objective or stage insert, or with a built-in motorized z-axis controller housed within the microscope stand. Stage drifts can also be avoided by using a high-quality vibration isolation table and by temperature regulation around the table.

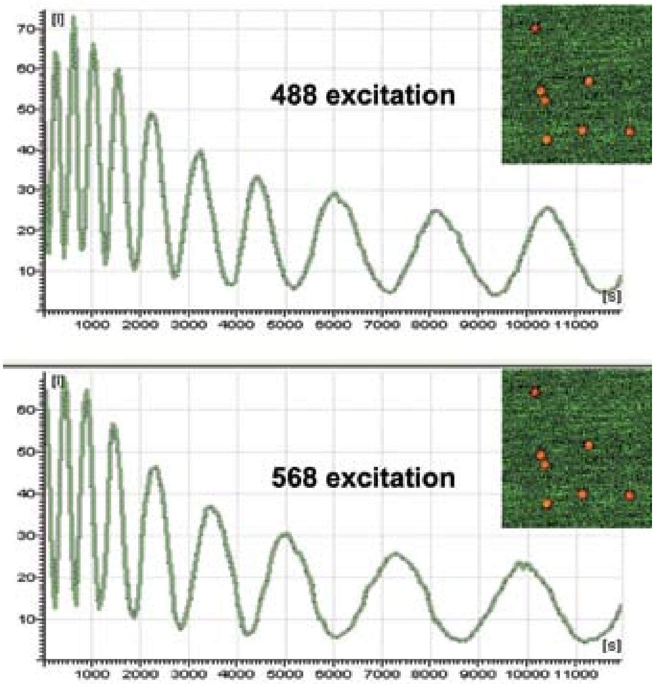


Figure 9. Beads (similar to FISH spots) were excited every minute sequentially with a 488 nm and a 568 nm laser. The mean intensity of the bead was measured every minute for 3.3 hours (200 sample times). The pattern shows periodic fluctuations in intensity over time indicating an unstable system (6-11). The mean intensity also drops over time, which implies that the sample is either going out of focus, is emitting less light or is bleaching and emitting less light. Newer microscopes have controls that can correct such a stage drift.

Other Factors

A number of other factors will affect the image quality and the amount of photons recorded by the detectors. These include:

- efficiency of the optical coupling
- alignment and reflection characteristics of laser mirrors and
- temperature of the detector (i.e. cooled PMT or a cooled CCD camera).

All of these factors can be synthesized into the following principle: More photons will hit the detector in aligned systems that have the correct filters. With dim FISH signals it is necessary to increase the exposure time and averaging. It is important not to bleach the sample with excessive light. The use of possible antifade reagents (i.e. Prolong Vectoshield) on fixed samples will reduce the bleaching and preserve the probe (8-11).

CCD Camera as a Detector for FISH Imaging

Many users use CCD cameras for FISH (and H&E) imaging (17-19). These are widely used in wide field and laser scanning confocal microscopes and can usually be purchased separately from the microscope. The user has the discretion to choose from a large number of models provided by a variety of manufacturers. Although we will not describe in detail the operation of a CCD camera, nor other details such as sources of noise, gain, linearity, offset gamma, full-well capacity and image time (please see JB Pawley book for more details or please refer to: http://www.microscopyu.com/tutorials/java/digitalimaging/signaltonoise/index.html. In the next section we will provide some useful hints on how to select a suitable camera and use it for FISH imaging.

Quantum Efficiency (QE): FISH spots are often faint. It is, therefore, best to select a camera with a high QE corresponding to the wavelength of interest. What is QE? QE is defined as the proportion of the photons arriving at the detector that actually contribute to the output signal that is linearly proportional to the photon input. QE is often a strong function of the wavelength λ of the detected photons (see the book by Pawley 2006). In very simple terms, if 100 photons of a certain λ entering the imaging pixel generate 100 electrons and, if 100 units of electrons are detected, then the CCD camera has a 100%QE (which is an ideal situation). Although it is technically impossible to achieve a 100% QE, there are newer cameras available that have a high QE (80-90% range). Highly recommended are Electron Multiplying CCD (EMCCD) cameras which are highly sensitive and use an on-chip amplification technology to amplify the signal above the read noise floor. The advantage of the EMCCD camera is twofold: (1) Its flexibility to operate as a high standard Quantum Efficiency (QE) CCD or as a single photon-sensitive powerhorse when the signal is weak and/or exposure times need to be reduced; and (2) Its ability to preserve labeled FISH specimens from the effects of photo-bleaching by filtering the power of the illumination light and compensating with higher Electron Multiplying gain, please refer to: http://www.andor.com/learn/applications/?docid=95 and http://www.emccd.com/what_ is_emccd/emccd_tutorial/Q3_In%20Practice_How_Is_EM_Gain_Applied_to_Multiply_The_Signal.

Pixel size: If a precise gene position determination is to be performed then, in general, a high powered objective (60x or 100x magnification, 1.4 NA) combined with a small pixel size (6-10 µm) camera should be used. This not only helps to improve the spatial resolution, but also increases the sampling frequency (see appendix), dynamic range and signal-to-noise ratio. However, if the FISH spots are faint, then an EMCCD camera is recommended. For technical details on sampling frequency and pixel size, please refer to the book of Pawley (2006) and to reference Chen et al. (2000) (17).

Dynamic range: This refers to the ability of a CCD camera to detect fine differences in the intensity levels of the signal. The dynamic range is useful for determining the digitization requirements for a given signal. In general, for FISH imaging, a 12-bit (or 2^{12} = 4,096 grey levels) CCD camera is sufficient. Furthermore, cameras with lower values (8-bit or 2^8 = 256 grey levels or 10-bit = 1,024 grey levels) are usually insufficient. As a note of caution, the dynamic range should not be higher than the total gray levels for optimum representation of the different intensity levels. Higher values do not improve the image quality anymore due to low light levels from the sample. For low light levels, photon shot noise caused by statistical fluctuations of the acquired signal levels does not allow accurate determination of fluorescence level.

Readout speed (in units of MHz): This refers to how fast the accumulated electrons in each pixel can be transferred and digitized into a signal. The readout speed affects the readout noise. In general, the higher the readout speed the higher the readout noise. Since all of the FISH imaging is static with pathology samples, a slower readout speed is better suited for this imaging. For instance, if a camera has two readout speeds, 10 MHz and 20 MHz, it is better to use a 10 MHz speed than to use a 20 MHz speed.

Binning: CCD are versatile devices and their readout pattern can be manipulated to achieve various effects. Binning allows charges from adjacent pixels to be combined and thus can offer benefits in faster readout speeds and improved signal to noise ratios albeit at the expense of a reduced special resolution. Binning also increases sensitivity for quantitation and imaging at very low light levels. Depending on the intensity and resolution requirement of the FISH spot, one can try binning twice. (Hint: The sample will look brighter at the expense of a reduced special resolution). For more details on this topic, please visit the "Microscopy Resource Center":

http://www.olympusmicro.com/primer/digitalimaging/digitalimagingdetectors.html and http://www.microscopyu.com/tutorials/java/digitalimaging/pixelcalculator/index.html.

The problem of spherical aberration: Wavelengths of light have different colors and focus through a lens at different positions. If the lens is carefully made, it is possible to make blue, green and red light focus at the same point. If the lens is of a cheaper quality or, if there is a defect, then chromatic aberrations occur. In simple terms, this means the red blue and green light does not focus at the same point or pixel. It is a very serious problem as interpretations of molecules represented by fluorochromes may be misinterpreted due to their proximity in a sample (see also Fig. 7, 8). As shown below (Fig. 10), the 170 nm point spread Tetraspec beads (Invitrogen) should show wavelengths of emitted light that colocalize to the same point, if the system is aligned and functioning properly. If the system is misaligned, the light from the different laser lines will occur at different points in the X, Y and Z planes.

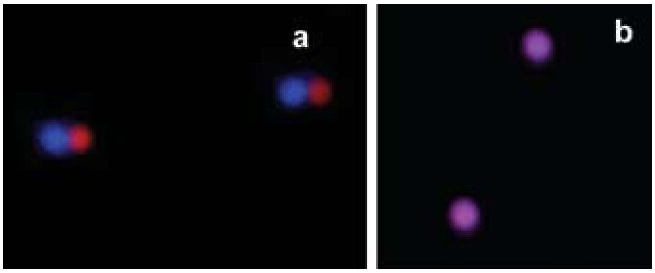


Figure 10. The figure shows the Tetraspec beads excited with a 365 nm UV and 568 nm visible laser. The emitted fluorescence does not appear at the same point in a misaligned system that shows two distinct images (blue and red) (Fig. 10a) representing the emission from the two beads. However, in an aligned system only one point of colocalized emitted light is observed (purple) (Fig. 10b).

Importance of the wavelength of exciting light and emission filters: One way to increase the intensity of FISH fluorescence is to use a wideband exciter. This way, more photons capable of exciting the fluorophore are captured. However, the drawback is noise or crosstalk with another fluorophore. In these situations, narrowband exciters become useful. But again, the disadvantage is that there are less photons available for the detector which increases the noise in the system. The only way to overcome the problem of using a wideband exciter vs. narrowband exciter is by experimenting by trial and error, i.e. try to estimate how much fluorescence is available from the sample, how sensitive is the detector (camera vs. PMT), objective magnifications, etc.

Emission filters: Broadband emission will produce a larger signal and should always be used except when there is crosstalk with the emission of another fluorophore in the same sample. For more details, please refer to: http://www.chroma.com, http://www.omegafilters.com/ or http://www.semrock.com/Catalog/BrightlineCatalog.htm.

Practical tip: The order of imaging dim objects such as FISH hybridization spots: Shorter wavelengths of light have more energy compared to longer wavelengths of light. Therefore, it is always advisable to image FISH spots in the following order: Longer excitation wavelengths first followed by shorter excitation wavelengths (e.g. Rhodamine followed by FITC and DAPI). This reduces the possibility of bleaching the farther red samples.

Improving FISH Images by 3D Deconvolution Microscopy

The use of an epifluorescence or a confocal microscope that uses a Z stage to create a stack of 2D images in a dataset and/or 3D images is suggested for FISH imaging. For more details, please refer to: http://www.olympusfluoview.com/theory/index.html and http://www.olympusconfocal.com/java/confocalvswidefield/index.html.

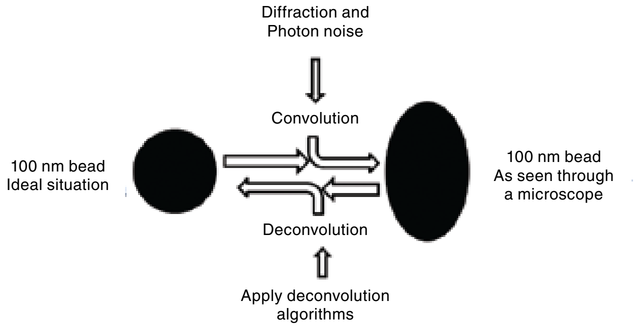
Because it can be difficult to interpret the data from a single 2D image due to the lack of continuity of the labels that may not be easy to assess in other planes, it is better to construct a 3D dataset from 2D FISH images for refined analysis. Why 3D imaging? 3D imaging technology is far superior to conventional 2D imaging. It enables practitioners to better identify potential problems in FISH images, such as artifacts in the probe localization (see Fig. 10 and the section "Science in Pictures" for an example of 3D FISH images). Please also refer to: http://www.olympusmicro.com/primer/digitalimaging/deconvolution/deconintro.html.

In addition, 3D imaging also allows the trained eye to identify other important parameters such as e.g. the distance between the probes and whether the probes are colocalized (2D systems simply cannot reveal this). Furthermore, the documented improved accuracy and precision of 3D images help avoid potential mistakes such as image distortion and magnification common with 2D imaging technology.

Deconvolution

Stacks of three-dimensional FISH images obtained by optical sectioning can undergo an improvement in contrast and reduction in blur by the method of image deconvolution (see cartoon below and Fig. 11 and 12). Image deconvolution is an algorithm-based process used to reverse the effects of convolution on recorded data. In non-mathematical terms, deconvolution is the converse of convolution. For instance, if a perfectly circular subdiffraction (100 nanometer) fluorescent bead is viewed under a microscope, it will not appear as a circular structure, but rather as an elongated structure (see cartoon below). The circular structure appears elongated (or convoluted) due to the diffraction of light within the microscope and photon noise, i.e. inherent natural variation of the incident photon flux. The process of deconvolution is the converse of convolution or making the elongated (convoluted) structure into a spherical (deconvoluted) structure using deconvolution computational algorithms. (For more details, please refer to: http://www.olympusmicro.com/primer/digitalimaging/deconvolution/

Schematic Concept of Convolution/Deconvolution


Diffraction and Photon noise
Convolution
100 nm bead Ideal situation
100 nm bead As seen through a microscope
Deconvolution
Apply deconvolution algorithms

deconvolutionhome.html. You may also refer to the section "Science in Pictures" in this issue).

In the last few years, deconvolution computational algorithms have been extended and developed by a number of commercial software vendors (please refer to: http://www.olympusmicro.com/primer/resources/deconvolutionweb.html and http://www.olympusmicro.com/primer/digitalimaging/deconvolution/deconalgorithms.html. Although most of these methods remain proprietary and the differences between various algorithms can be difficult to understand at times, they all work to improve the contrast of the image (see Fig. 12a and b). As a user the best approach is to test different deconvolution algorithms on images that are familiar to you and to assess the performance for yourself.

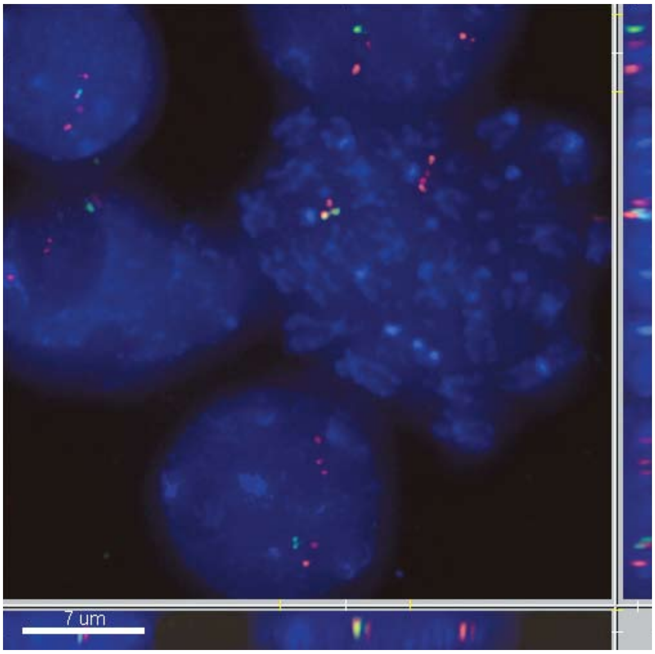


Figure 11. Analysis of FISH signals using a widefield deconvolution microscope. Seen in this image are xy (top), xz (bottom), and yz (right) projections of deconvolved data. The colocalized FISH signals (yellow) are easily seen in XY and XZ images. Image courtesy. Peter Franklin. Applied Precision Inc, Issaquah, WA, USA.

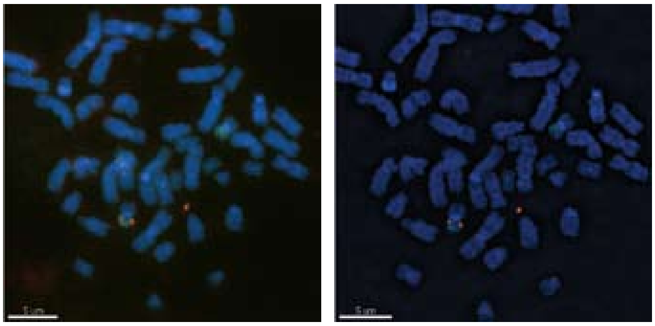


Figure 12. Metaphase cell hybridized with FISH probes. (a) Widefield fluorescence microscopy without deconvolution and (b) Widefield fluorescence microscopy with deconvolution. The chromosomes are seen in blue. The chromosome that is labeled with FISH probes shows green and red spots which are sharper in the deconvolved image (right) compared to the image without deconvolution (left). Image courtesy, Peter Franklin, Applied Precision Inc., Issaquah, WA, USA.

Conclusion

The fluorescent and confocal microscopes are sophisticated instruments with enormous potential in solving biological questions. They are fast becoming the foundation for 21st century biology research. In order to utilize the great potential of these fantastic tools, it is important for the user to be knowledgeable of some of the components that constitute the microscope as well as understand how it works. We feel that it is important to understand the characteristics of objectives, detectors, illumination sources, XYZ stages and other imaging equipment in order to collect optimal FISH images. It is important for the scientists to understand the components that comprise this technology so that they can resolve the cause of poor image quality. We have found that when the equipment does not function optimally one of the components often is not working correctly. Utilization of software remains one of the most important tools that must be mastered to use this technology for optimum result.

The importance of proper instrument and experimental standards to evaluate the machine performance and experimental variables should not be underestimated. We recommend to check for even illumination, PSF, system/laser stability and correct deviations, when necessary, using proper procedures such as fluorescence slides and triple or double-labeled fluorescence beads containing defined amounts of fluorophores.

There is a wealth of information resources online about microscopes and confocal microscopes that has been assembled by Mike Davidson of Florida State University. It can be accessed on the Web sites of Nikon or Olympus. Basic microscopy and confocal microscopy concepts are described on the following Web site: http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html. In addition, the following Web site offers basic and advanced microscopy courses: http://www.olympusfluoview.com/resources/courses.html. Central imaging facilities can provide more advanced information required for FISH imaging and other specific applications.

Glossary and Appendix

Laser scanning confocal microscope: A fluorescence microscope achieving improved depth discrimination and contrast by blocking fluorescence that originates outside the plane of focus by use of a 'confocal' pinhole.

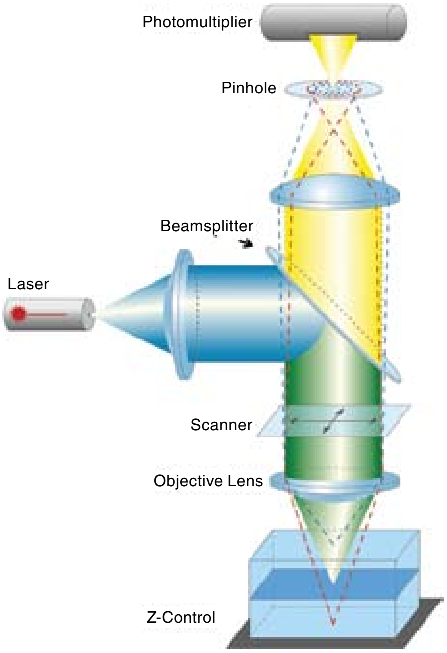
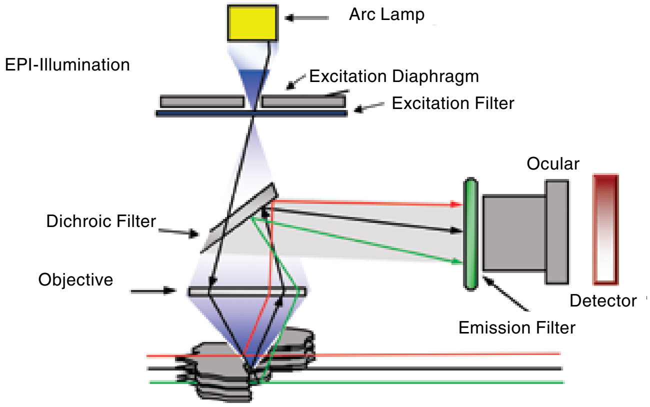


Figure Courtesy: Dr. Jochen Tham. Carl Zeiss MicroImaging, Inc.

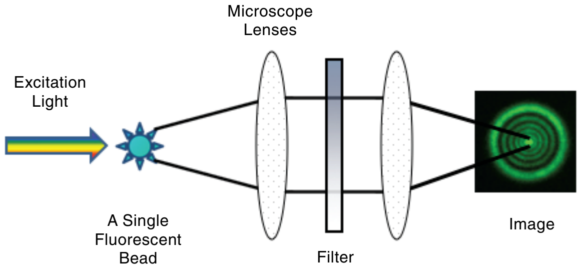
Epifluorescence microscope: An epifluorescent microscope (below) using an objective lens to perform two tasks: To focus light upon the specimen being observed and to collect light being emitted by that specimen, which is fluorescent.

Working of an Epifluorescent Microscope



Epifluorescence Microscope. The diagram shows the working of an upright epifluorescent microscope. The excitation beam (black ray) from the arc lamp passes through the excitation filter and dichroic mirror and directed toward the specimen. The return beam of emitted fluorescence wavelength (red, green and black rays) is reflected of the dichroic filter, emission filter, ocular and goes to the detector (eye or camera). Courtesy of Prof. J. Paul Robinson, Ph.D., Director of Purdue University Cytometry Laboratory, Purdue University, West Lafayette, IN, USA.

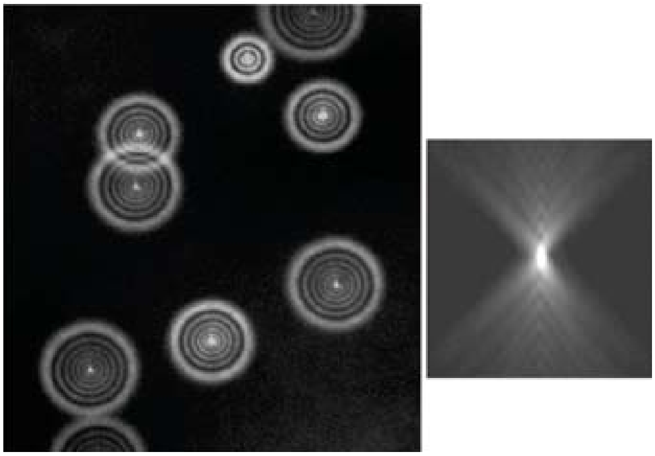
Point spread function (PSF): If a tiny population of 100 nm fluorescent beads sandwiched between a coverslip and a microscope slide are examined at high resolution (i.e. at 100x objective magnification, 1.4 NA. and in a correctly matched refractive index of oil), it can actually show a tiny set of rings in the horizontal (XY) view (also called an airy disk (see Fig. below). This airy disk cannot be avoided due to diffraction and the wave nature of light. If a specimen is optically sectioned and projected in a vertical (XZ) view (see Fig. xx), a set of concentric rings will flare from the center. When a three-dimensional image of this specimen is collected, a complete point spread function is said to be recorded for each bead. The (PSF)



From left to right. Excitation light beam excites a fluorescent bead. The excited bead emits fluorescence in all directions. A fraction of the emitted light is collected by microscope lens, filtered and focussed into an image plane where it appears as an airy disk. Figure modified after Dr. E.H.K.Stelzer. EMBL, Heidelberg Germany. Practical Limits to Resolution in Fluorescence Light Microscopy; Imaging Neurons: A Laboratory Manual. Cold Spring Harbor Laboratory Press. 2000.

is based on an infinitely small point source of light originating in the specimen (i.e. from each fluorescent bead) in object space. The PSF is a description of what happens to each point source of light from that single bead after it passes through the imaging system. This concept is of fundamental importance to the process of deconvolution and should be clearly understood in order to avoid imaging artifacts. For more details, please refer to: http://www.olympusmicro.com/primer/digitalimaging/deconvolution/deconintro.html.

In this figure airy disks of fluorescent beads can be seen in the XY (horizontal) plane (left) and the XZ (vertical) plane (right). Courtesy of Applied Precision, Inc., Issaquah, WA, USA.



An Airy disk (named after George Biddell Airy) is the central bright circular region of the pattern produced by light diffracted when passing through a small circular aperture. The central disk is surrounded by less intense concentric rings.

Resolution: Resolution is defined by the ability to demonstrate contrast between two points. For an animation, please see: http://micro.magnet.fsu.edu/primer/java/imageformation/rayleighdisks.

The lateral (XY) and axial (Z) resolutions in a fluorescence microscope are calculated as: $d_{xy} = 0.61\ \lambda\ /\ NA$ and $d_z = 2\ (\lambda\ n/NA^2)$, respectively.

For example,

if λ = 488 nm and NA = 1.4 NA, then the lateral resolution is ~ 212 nm or 0.212 μm.

if λ = 488 nm, NA = 1.4 NA and *n* = 1.515 = refractive index, then the axial resolution is ~ 754.40 nm or 0.755 μm.

Sampling Frequency: A perfect reconstruction of a signal is possible when the sampling frequency is greater than twice (~ 2.3x) the maximum frequency of the signal being sampled.

Laser Power Meter: A laser power meter is used to measure the power coming form the individual lines using a 10x lens. The power detector is placed on the stage, the zoom is set to 10x or higher and the system is set for bidirectional scanning. The value obtained allows you to determine, if the system is performing properly as power transmission is related to the alignment and function of the system.

366
LaserMate-Q

Laser Power Measurement

Government Disclaimer

Although the research described in this article is supported by the United States Environmental Protection Agency, it has not been subjected to Agency review and, therefore, does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. This report does not necessarily reflect EPA policy.

References

1. T. Cremer, P. Lichter, J. Borden, D. C. Ward, and L. Manuelidis (1988). Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. *Hum Genet* 80: 235-246.
2. Kozubek Michal. Confocal and two-photon microscopy: Foundations, applications, and advances, *FISH Imaging*, pages 389-429. New York: Wiley-Liss, 2001. ISBN: 0-471-40920-0.
3. Walter J, Joffe B, Bolzer A, Albiez H, Benedetti PA, Müller S, Speicher MR, Cremer T, Cremer M, Solovei I (2006) Towards many colors in FISH on 3D-preserved interphase nuclei. *Cytogenet Genome Res* 114: 367-378.
4. Baerlocher GM, Vulto I, Gary de Jong & Peter M Lansdorp PM (2006). Flow cytometry and FISH to measure the average length of telomeres (flow FISH). *Nature Protocols* 1, 2365 – 2376.
5. Zucker, R.M. Rigby P, Clements I; Salmon W, Chua, P. Reliability of Confocal Spectral Imaging Systems: Use of Multispectral Beads. *Cytometry* V71A: 174-189 2007 (COVER).
6. Zucker, R.M.Technical note: Whole insects and Mammalian Embryo Imaging with Confocal Microscopy: Morphology and Apoptosis. *Cytometry* 2006 69A: 1143- 1152 (COVER).
7. Zucker, R.M. and Jeffay SC. Confocal Laser Scanning Microscopy of Whole Mouse Ovaries: Excellent Morphology with Apoptosis Detection and Spectroscopy. *Cytometry* 2006 69A August 2006 930-939 (COVER).
8. Zucker RM Confocal Slide Based System: Performance. *Cytometry* 2006 69 A 659-676.
9. Zucker RM Confocal Microscopy Slide Based Systems: Instability. *Cytometry* 2006 69A 677-690.
10. Zucker RM and Lerner J: Wavelength and Alignment tests for Confocal Imaging systems *Microscopy Research and Technique.* 68:5 307-319 (2005).
11. Zucker, R.M. Evaluation of Confocal Microscopy System Performance. Cell Imaging Techniques. Douglas Taajets editor Humana Press Chapter 5 77-135 2005.
12. Hildenbrand G, Rapp A, Spori U, Wagner C, Cremer C, Hausmann M (2005). Nano-Sizing of Specific Gene Domains in Intact Human Cell Nuclei by Spatially Modulated Illumination Light Microscopy. Biophysical Journal 88; 4312–4318.
13. Swedlow JR, Hu K, Andrews PD, Roos DS, Murray JM (2002). Measuring tubulin content in toxoplasma gondii: A comparison of laser-scanning confocal and wide-field fluorescence microscopy. *Proc. Natl. Acad.* Sci 99: 2014–2019 (2002).

14. Itoh H, Miyajima Y, Umemura S, Osamura RY (2008). Lower HER-2/Chromosome Enumeration Probe 17 Ratio in Cytologic HER-2 Fluorescence in Situ Hybridization for Breast Cancers. Three-dimensional Analysis of Intranuclear Localization of Centromere 17 and HER-2 Signals. *Cancer (Cancer Cytopathol)* 114:134–40.
15. Smallcombe, A. (2001). Multicolor imaging: the important question of co-localization. *Biotechniques*. 30:1240– 1242, 1244–1246.
16. North AJ (2006). Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. *J. Cell. Biol.*172 (1), 9-18.
17. Chen, T.; Catryssee, P.; El Gamal, A.; Wandell, B. (2000): How small should pixel size be? *Proc. SPIE*; vol. 3965; pp. 451-459. Sensors and Camera Systems for Scientific, Industrial, and Digital Photography Applications, Morley M. Blouke; Nitin Sampat; George M. Williams; Thomas Yeh; Eds.
18. J Konsti, J Lundin, M Jumppanen, M Lundin, A Viitanen and J Isola (2008). A public-domain image processing tool for automated quantification of fluorescence in situ hybridization signals. *J. Clin. Pathol*. 2008; 61;278-282.
19. Rauser S, Weis R, Braselmann H, Feith M, Stein HJ, Langer R, Hutzler P, Hausmann M, Lassmann S, Siewert JR, Hofler H, Werner M, Walch A (2007). Significance of HER2 Low-Level Copy Gain in Barrett's Cancer: Implications for Fluorescence In situ Hybridization Testing in Tissues. (Imaging, Diagnosis, Prognosis). *Clin Cancer Res* 2007;5115 13(17).

Microscopy Books

1. Douglas B. Murphy. *Fundamentals of Light Microscopy and Electronic Imaging. Wiley-Liss*. 2001.
2. Greenfield Sluder and David E. Wolf (Editors). *Digital Microscopy.* A second edition of video microscopy. Elsevier- Academic Press. 2003.
3. Alan R. Hibbs. *Confocal Microscopy for Biologists*. Kluwer Academic Publishers. 2004.
4. Robert D. Goldman and David L. Spector (Editors). *Live Cell Imaging*. A Laboratory Manual. 2005.
5. Julian P. Heath. *Dictionary of Microscopy.* John Wiley & Sons Ltd. 2005.
6. James Pawley (Editor). *Handbook of Biological Confocal Microscopy*. Academic Publishers. 2006.
7. D. Stephens (Editor). *Cell Imaging*. Scion Publishing Ltd. 2006.

Chapter 14 | Dual Color CISH and FISH to CISH Conversion

Ulla Henriksen PhD, Sven Müller PhD and Andreas Schønau MS, EBA

Fluorescence in situ hybridization (FISH) is a sensitive yet reliable technique for evaluating gene status on a cell-to-cell basis. The technique has been utilized as a research tool for more than 20 years to evaluate the presence or absence of specific DNA sequences on chromosomes (1). The method has become popular as a diagnostic tool in clinical oncology. In the field of pathology, FISH has been applied as an alternative or adjunct to IHC testing, and the method has been shown to have a prognostic and a predictive value with regard to certain cancer markers (2, 3). Recently, a new ISH technique based on chromogenic signals instead of fluorescent signals has evolved. The technique, chromogenic in situ hybridization (CISH), has proven qualities with respect to ease of use that might explain the recent growth in popularity:

1. CISH results are easily interpreted by the use of a bright-field microscope which is generally used in diagnostic laboratories.
2. CISH enables visualization of the nucleus and is also able to distinguish invasive from in situ carcinomas.
3. CISH signals do not generally fade over time allowing the tissue samples to be archived and reviewed later.
4. CISH resembles IHC to a large extent (as opposed to FISH) due to the use of conventional counterstains, e.g. hematoxylin, for visualization of tissue morphology.

Because CISH combines the genetic information from FISH with the visualization and interpretation resembling IHC, the CISH technique is a practical and user friendly alternative to FISH.

Chromogenic In Situ Hybridization

The first description of the CISH procedure as a practical alternative to FISH for the detection of genetic alterations was published in 2000 (4). Since then, many publications comparing FISH and CISH have emerged and all have reached the conclusion that CISH is an accurate, reproducible technique with high concordance to FISH (5-9). The technique is as sensitive as FISH with reference to borderline and low amplification samples (9). Chromogenic visualization (colorimetric method) is based on enzyme-conjugated antibodies that recognize the target of interest. Reaction of substrates with enzymes such as horseradish peroxidase (HRP) and/or alkaline phosphatase (AP) leads to chromogen precipitates, which then can be detected with a bright-field microscope. Another approach for bright-field evaluation of the colorimetric method is metallographic in situ hybridization by which a target is visualized using a probe or antibody that deposits metal selectively at its binding site. One metallographic method includes silver-enhanced in situ hybridization (or SISH). SISH is based on the same basic principle as CISH, but with SISH the signal appears as a black coloration due to silver precipitation (10, 11). Although both CISH and SISH have their advantages, they suffer from one drawback. In the traditional CISH/SISH procedures described so far, only signals of one color can be detected on each slide. As a consequence, the target gene and the reference signals are detected on two separate slides, which sometimes can be cumbersome. DuoCISH™, a dual color CISH kit developed by Dako Denmark A/S, overcomes this barrier by simplifying the interpretation of cases with borderline gene copy number and making it easier to visualize two probes simultaneously (e.g. the ability to distinguish between true gene amplification/deletion and chromosomal aneuploidy) on the same slide.

At present, only a few procedures enabling detection of target and reference gene on the same slide apart from DuoCISH™ have been described. One is the bright field double in situ hybridization (BDISH) for *HER2* gene and chromosome 17 centromere (CEN 17) detection and the other is the dual color chromogenic in situ hybridization for testing of *HER2* oncogene amplification in archival breast tumors (12, 13). Dual color CISH kit (Dako DuoCISH™) converts Texas Red and FITC-labeled signals to red and blue chromogenic signals (See Fig. 1, 2 and 3). Dako DuoCISH™ is based on Fast Red as a substrate of alkaline phosphatase (AP) and a blue cyanine dye as a substrate for horseradish peroxidase (HRP). Both precipitates are water and/or organic solvents insoluble which enables mounting of slides in both water-based and permanent-mounting media (14). The chromogenic signals developed in DuoCISH™ are clear and distinct and are supported by a hematoxylin counterstain to enhance morphological features. The signals emanating from a DuoCISH™ staining are readily apparent using low-power objectives (10x and 20x), but can be clearly visualized and counted using high-power objectives (40x, 60x or 100x).

The Principle of the CISH Procedure and FISH to CISH Conversion

Dako DuoCISH™ Kit includes reagents that are necessary to convert fluorescent signals into chromogenic signals in an immunohistochemical reaction on top of a FISH reaction. Instead of mounting and developing the FISH signals, the fluorescent signals are converted into chromogenic signals prior to interpretation. The kit includes peroxidase block, antibody mix, red and blue chromogens and their respective substrate buffers. The antibody mix includes anti-Texas Red conjugated with AP which converts the red fluorescent signals into red chromogenic signals, and anti-FITC conjugated with HRP which converts the green fluorescent signals into blue chromogenic signals. Figure 1 is a schematic representation of the main steps in the FISH and CISH procedures.

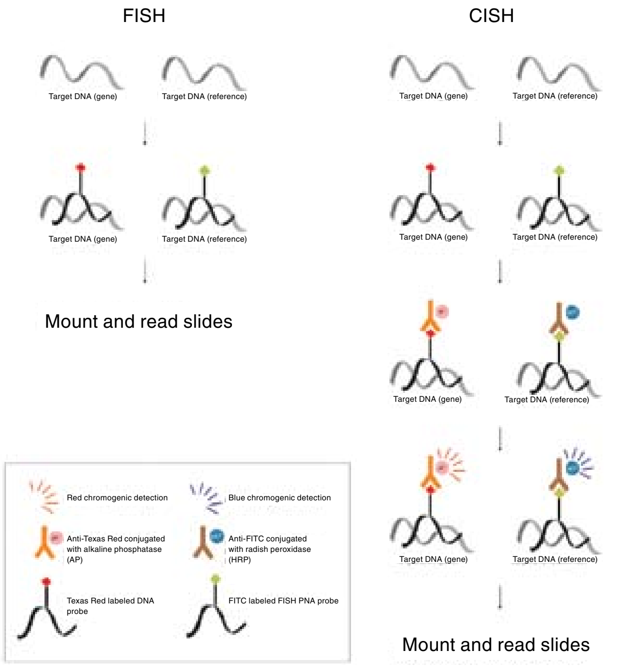

FISH
CISH
Target DNA (gene)
Target DNA (reference)
Target DNA (gene)
Target DNA (reference)
Mount and read slides
Target DNA (gene)
Target DNA (reference)
Target DNA (gene)
Target DNA (reference)
Target DNA (gene)
Target DNA (reference)
Target DNA (gene)
Target DNA (reference)
Mount and read slides
Red chromogenic detection
Blue chromogenic detection
Anti-Texas Red conjugated with alkaline phosphatase (AP)
Anti-FITC conjugated with radish peroxidase (HRP)
Texas Red labeled DNA probe
FITC labeled FISH PNA probe

Figure 1. A schematic illustration of the procedure for FISH and CISH.

In practice, the FISH procedure should be followed until just prior to the final dehydration step. Instead of proceeding with dehydration, the slides are immersed in CISH wash buffer and are ready for CISH staining. A CISH staining is a standard IHC staining that involves the initial blocking of endogenous peroxidase activity in cells/tissues followed by the addition of a primary antibody mix for both fluorescent labels and visualization of the signals by chromogens. Finally, counterstaining with hematoxylin is performed to enhance and visualize the nuclear borders. Normal cells within the tissue may serve as an internal control for success of the staining procedure since properly processed nuclei should expose the red to blue signal ratio expected of normal diploid cells. The results of a CISH test is an almost complete 1:1 conversion ratio of FISH signals to CISH signals resulting in a high level of concordance between the two analyses (figure 2).

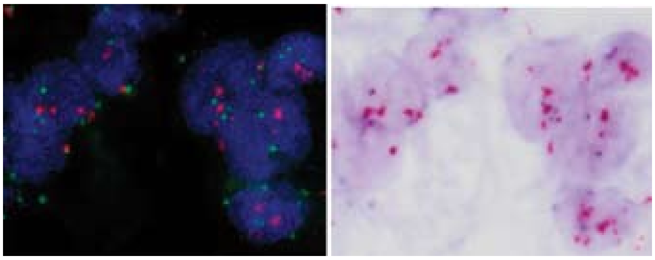


Figure 2. The images illustrate a "dot-to-dot" conversion of FISH signals (left) to CISH signals (right). The Dako DuoCISH™ Kit converts the red florescent signals to red chromogenic signals and the green fluorescent signals to blue chromogenic signals. In praxis the sample is stained in the FISH procedure, evaluated by fluorescence microscopy and, subsequently, converted to CISH. The same nuclei were evaluated both by FISH and CISH. As seen when comparing a "normal" CISH slide with a slide that has been converted from an already mounted FISH slide, the quality of the CISH staining is compromised.

Added Value of CISH

The gain of applying a CISH reaction on top of a FISH reaction is the bright field evaluation of the sample. This is an attractive feature because it results in the easy interpretation of the staining for the pathologist without the use of specialized equipment. Furthermore, it supports the parallel assessment of IHC staining of the same sample. As a consequence of the easy access to tumor morphology information (invasive vs. in situ), the scoring time for a CISH sample is considerably shorter than for the identical FISH sample (9). In addition, studies have shown that interlaboratory and observer-to-observer variability is much less for interpretation of CISH compared to FISH. This also applies when it comes to inexperienced interpreters (15-17). Another advantage of using the CISH technique is that compared to a FISH slide, which is stable for a limited period due to fading of the fluorescent signals, the storage ability of chromogenic signals is unlimited which enables re-evaluation without additional tests. Finally, since CISH is an add-on to the FISH procedure, it is possible to redevelop an already mounted and developed FISH slide for reinterpretation (see figure 2).

Applications of Dako DuoCISH™

Dako DuoCISH™ can be applied to samples hybridized with a Texas Red-labeled probe and a FITC-labeled probe. Although the kit has not yet been validated against any specific probe, the kit reagents can be used to visualize genetic alterations, including amplification, deletion and translocation on formalin-fixed paraffin-embedded specimens. The staining procedure is a simple IHC staining and can be performed either manually or on automated instruments (such as Dako Autostainer or **Autostainer**Link platforms). Visualization with DuoCISH™ results in signals visible at low- and high-objective magnifications (see figure 3).

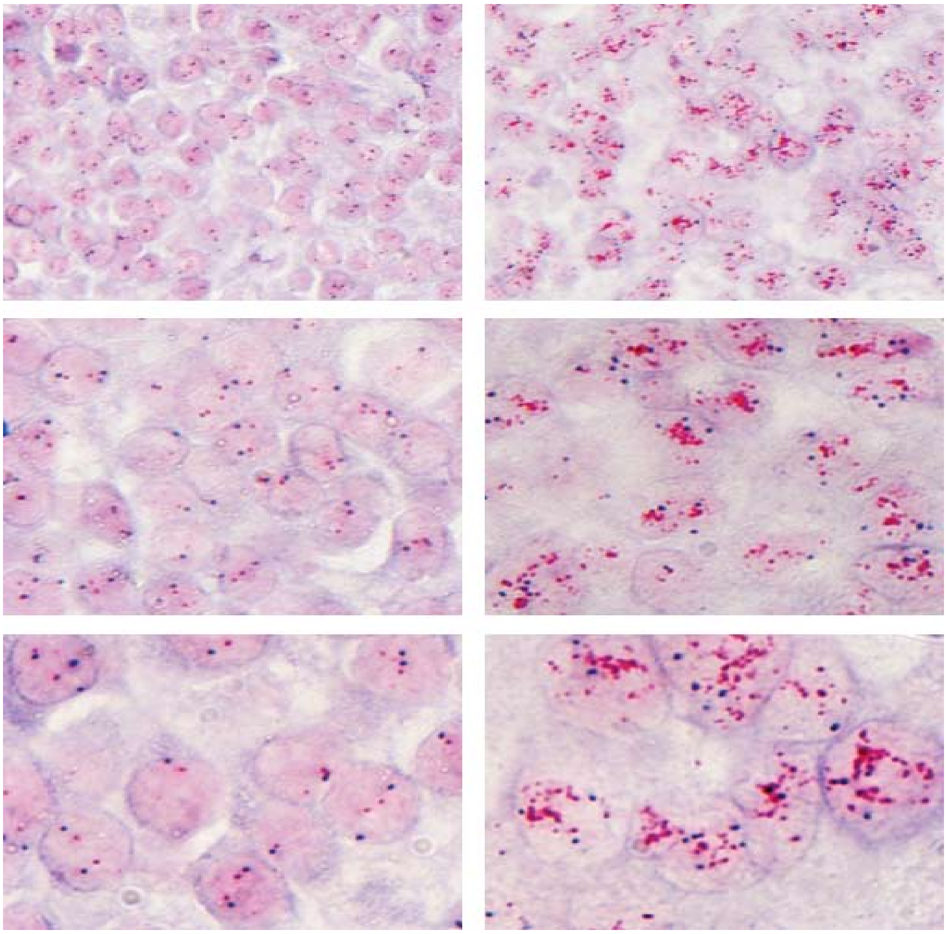


Figure 3. Examples of FFPE human mamma carcinoma specimens with (right) and without (left) amplified gene status stained with Dako DuoCISH™ and visualized with a 20x (top), 40x (middle) and 60x magnifying objective (bottom).

Conclusion and Future Perspectives for CISH

Many studies have addressed the validity of CISH compared to FISH — mostly as related to detection of *HER2* gene alterations. Most studies report a concordance between the two techniques above 90% and consider CISH a viable alternative to FISH (6, 18, 19). Differences in interpretation between FISH and CISH have primarily been identified in cases where the sample exhibits borderline/low amplification, specific gene alterations, including polysomy and aneusomy, or in samples that are heterogeneous. Consequently, most of the concerns surrounding CISH have been aimed at the use of single color CISH techniques that rely on the evaluation of target gene and reference on two different slides stained in parallel. In contrast to this, as described previously, the dual color CISH kit allows the detection of two different signals on the same slide. Based on these results and observations, it is thought that the future of the CISH technology most likely lies with the dual color technique(s) compared to the single color method because the former is less prone to interpretation errors as opposed to the latter. In spite of the advantages, a cause for concern has been an inefficient signal conversion leading to alterations in the signal to noise ratio when an additional layer is added to the FISH procedure. However, analytical studies on Dako DuoCISH™ have shown that there is no unspecific binding or coloring of unrelated tissue elements and that the CISH reaction does not add any signals that are not already detected by FISH. This implies that there is a high level of analytical concordance between CISH and FISH.

The results obtained by a number of studies thus far suggest that CISH is a viable alternative to FISH and may complement IHC in the determination of *HER2* status in breast cancer. *HER2* testing is performed according to the ASCO-CAP Guidelines (20), and the trend now is to do more *HER2* FISH at the expense of IHC. This would indicate that with the emergence of *HER2* CISH tests with predictive and prognostic claims, a future testing guideline might well include CISH staining for *HER2*. Whether this is an effective or an ineffective strategy remains to be determined.

Reference

1. Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci U S A 1986;83(9):2934-8.
2. Knoop AS, Knudsen H, Balslev E, Rasmussen BB, Overgaard J, Nielsen KV, et al. retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. J Clin Oncol 2005;23(30):7483-90.
3. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235(4785):177-82.
4. Tanner M, Gancberg D, Di Leo A, Larsimont D, Rouas G, Piccart MJ, et al. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. Am J Pathol 2000;157(5):1467-72.
5. Di Palma S, Collins N, Faulkes C, Ping B, Ferns G, Haagsma B, et al. Chromogenic in situ hybridisation (CISH) should be an accepted method in the routine diagnostic evaluation of HER2 status in breast cancer. J Clin Pathol 2007;60(9):1067-8.
6. Gong Y, Gilcrease M, Sneige N. Reliability of chromogenic in situ hybridization for detecting HER-2 gene status in breast cancer: comparison with fluorescence in situ hybridization and assessment of interobserver reproducibility. Mod Pathol 2005;18(8):1015-21.
7. Hauser-Kronberger C, Dandachi N. Comparison of chromogenic in situ hybridization with other methodologies for HER2 status assessment in breast cancer. J Mol Histol 2004;35(6):647-53.
8. Saez A, Andreu FJ, Segui MA, Bare ML, Fernandez S, Dinares C, et al. HER-2 gene amplification by chromogenic in situ hybridisation (CISH) compared with fluorescence in situ hybridisation (FISH) in breast cancer-A study of two hundred cases. Breast 2006;15(4):519-27.
9. Bhargava R, Lal P, Chen B. Chromogenic in situ hybridization for the detection of HER-2/neu gene amplification in breast cancer with an emphasis on tumors with borderline and low-level amplification: does it measure up to fluorescence in situ hybridization? Am J Clin Pathol 2005;123(2):237-43.
10. Powell RD, Pettay JD, Powell WC, Roche PC, Grogan TM, Hainfeld JF, et al. Metallographic in situ hybridization. Hum Pathol 2007;38(8):1145-59.
11. Dietel M, Ellis IO, Hofler H, Kreipe H, Moch H, Dankof A, et al. Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists. Virchows Arch 2007;451(1):19-25.
12. Nitta H, Hauss-Wegrzyniak B, Lehrkamp M, Murillo AE, Gaire F, Farrell M, et al. Development of automated brightfield double In Situ hybridization (BDISH) application for HER2 gene and chromosome 17 centromere (CEN 17) for breast carcinomas and an assay performance comparison to manual dual color HER2 fluorescence In Situ hybridization (FISH). Diagn Pathol 2008;3:41.

13. Laakso M, Tanner M, Isola J. Dual-colour chromogenic in situ hybridization for testing of HER-2 oncogene amplification in archival breast tumours. J Pathol 2006;210(1):3-9.
14. Petersen KH. Novel horseradish peroxidase substrates for use in immunohistochemistry. J Immunol Methods 2009;340(1):86-9.
15. van de Vijver M, Bilous M, Hanna W, Hofmann M, Kristel P, Penault-Llorca F, et al. Chromogenic in situ hybridisation for the assessment of HER2 status in breast cancer: an international validation ring study. Breast Cancer Res 2007;9(5):R68.
16. Bilous M, Morey A, Armes J, Cummings M, Francis G. Chromogenic in situ hybridisation testing for HER2 gene amplification in breast cancer produces highly reproducible results concordant with fluorescence in situ hybridisation and immunohistochemistry. Pathology 2006;38(2):120-4.
17. Di Palma S, Collins N, Bilous M, Sapino A, Mottolese M, Kapranos N, et al. A quality assurance exercise to evaluate the accuracy and reproducibility of chromogenic in situ hybridisation for HER2 analysis in breast cancer. J Clin Pathol 2008;61(6):757-60.
18. Isola J, Tanner M, Forsyth A, Cooke TG, Watters AD, Bartlett JM. Interlaboratory comparison of HER-2 oncogene amplification as detected by chromogenic and fluorescence in situ hybridization. Clin Cancer Res 2004;10(14):4793-8.
19. Arnould L, Denoux Y, MacGrogan G, Penault-Llorca F, Fiche M, Treilleux I, et al. Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer. Br J Cancer 2003;88(10):1587-91.
20. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007;25(1):118-45.

Chapter 15 | Multi-Staining Immunohistochemistry

Nanna K. Christensen PhD and Lars Winther PhD

Immunohistochemistry (IHC) has become an established tool for both research and diagnostic purposes. However, in some cases there is a need for knowledge about the relative localizations of targets, which can only be obtained by visualizing all relevant targets in one slide. This chapter describes the advantages of multi-staining IHC and various considerations that have to be made to ensure successful staining. It also discusses the choice of appropriate protocols and visualization systems.

Advantages of Multiple Staining

Multiple staining can be defined as the detection of two or more targets on one slide, thus increasing the information obtained from each slide and reducing turnaround-time compared to single staining or sequential staining (see definition below). This technique also makes it possible to assess the topographic relationship of the targets, e.g. to determine whether targets are present in different cells, in the same cell or even in the same cellular compartment. In addition, multiple staining allows the combination of in situ hybridization (ISH) and IHC, giving information about a particular target both at protein level and DNA/mRNA level. Information can also be obtained on possible cell-to-cell spatial contacts of different cell types. Furthermore, with an increasing demand for less invasive sampling techniques and smaller and fewer specimens available, multiple staining has an additional advantage of saving time and reagents.

Examples of Multiple Staining

The diagnosis of prostatic intra-epithelial neoplasia (PIN) is just one example of the clinical importance of multiple staining. Prostate needle biopsy is the preferred method for diagnosing early prostate cancer, but in some cases an ambiguous diagnosis can be made due to the fact that the biopsy has identified only a few malignant glands or several histological benign mimics of cancer (1). Since basal cells are present in the benign cancer mimics, but absent in the malignant glands, the basal cells can be used to distinguish between the two cases. Basal cells are labeled using high molecular weight cytokeratin, cytokeratin 5/6 or p63 immunostaining. In addition, the gene product of p504s, alpha-methylacyl-CoA-racemase is expressed in a high percentage of prostate carcinomas, but is negative or only weakly expressed in benign prostate tissue. Thus, it is used as a positive cancer marker (see example in figure 1). If single-stainings are done on serial sections, ambiguous lesions may disappear, especially when dealing with small foci, causing suspected malignancies to remain undiagnosed. A multiple staining protocol significantly improves the ability to distinguish between benign and malign lesions. This reduces the percentage of residual ambiguous lesions and the need for additional biopsies.

ISH routinely uses multiple staining on slides to determine gene amplification from the ratio of the signals or from the gene probe of interest to a reference probe. In addition to the traditional fluorescence in situ hybridization (FISH), chromogenic versions of the signals in red and blue colors can also be produced enabling the results to be evaluated in bright field microscopy. This adds morphological information to the ratio of signals (Please find additional information on this subject in Chapter 13, Dual-Color CISH).

Technical Challenges

Before embarking on a multi-staining project, some important issues should be considered:

- Since most primary antibodies used today originate from either mouse or rabbit and are visualized using systems based on anti-mouse and anti-rabbit antibodies, the challenge of distinguishing between primary antibodies has to be addressed. This can require quite elaborate protocols.
- Spectral differentiation of stain colors may be difficult, especially if the targets are co-localized leading to a mix of colors (2). The mixed color should be well contrasted with the two basic colors. In the case where a rare target is co-localized with a more abundant target one color will tend to dominate the other.
- Even if targets are not co-localized, it is difficult to balance signals enabling rare targets to be visible in the same slide as highly abundant targets. An adjustment in concentration of the primary antibodies may solve this problem.
- If different targets are viewed under different magnifications, it may be difficult to get the topographic information desired.

Pre-treatment

Multiple staining, like single staining, can be performed on both formalin-fixed, paraffin-embedded tissue sections, frozen sections, cell smears and cytospin preparations. Multiple staining is constrained by the fact that it may not be possible to find one tissue pre-treatment protocol that is optimal for all targets. Often protocols optimized for individual stainings differ from one target to the other, e.g. different target retrieval methods may be used. In this case, it may be necessary to determine a method that allows all targets to be stained, although the method may be sub-optimal for some targets.

In cases where targets of different abundance are to be stained, a method must be selected to best balance the signals. Combining ISH and IHC on one slide is particularly challenging because targets require very different pre-treatment protocols. Since ISH processes such as DNA denaturing are not compatible with the presence of the antibodies for IHC, the ISH protocol is normally performed first.

Multi-Staining Method Selection

To ensure success, IHC staining must be carefully planned. This is even more important with multi–staining. If primary antibodies, both directly-labeled and unlabeled and from different host-species, are commercially available, there are several different staining methods that one can choose. However, very often the choice may be limited by the reagents available (3). Care must be taken to avoid cross-reactivity between reagents. A flow chart or similar aid might prove useful in selecting the best method.

In general, staining methods can be divided into the following classes:

Sequential staining: By this method, one staining procedure succeeds another. For example, the first primary antibody is applied to the tissue section followed by a labeled detection system such as streptavidin-biotin horseradish peroxidase (HRP), with a chromogen such as DAB. The second primary antibody is applied only after the excess DAB is rinsed off, followed by labeling with a streptavidin-biotin alkaline phosphatase (AP) detection system and a colored chromogen. The biggest advantage of sequential staining is that by this procedure problems related to cross-reactivity are avoided.

A sequential staining is shown in Figure 1. Here, the primary and secondary antibodies from the first staining were eluted before the staining of the next target was performed. The disadvantages of sequential staining are: The method cannot be used for co-localized targets, the technique often leads to a long staining protocol and carries an inherent risk of incorrect double staining due to insufficient elution of one set of reagents before application of the next reagent.

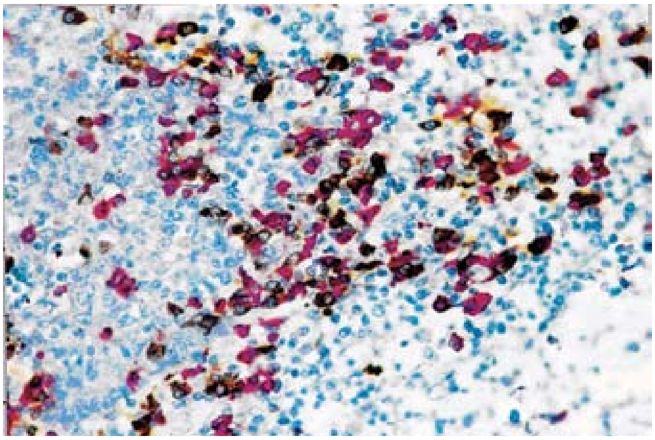


Figure 1. Sequential double staining method performed with the EnVision^{TM} G|2 Doublestain Kit using polyclonal anti-kappa light chains (red) and polyclonal anti-lambda light chains (brown) as primary antibodies. Formalin-fixed, paraffin-embedded tissue sections from tonsils.

Elution may become an issue with some high-affinity primary antibodies as these may remain at their binding site, leading to spurious double stained structures. Elution also risks denaturing epitopes of antigens to be visualized subsequently. Furthermore, for some chromogens there is a risk that the first chromogen (DAB in particular) may shield other targets. This technique is, therefore, not recommended for evaluation of mixed colors at sites of co-localization, because not all reaction products are capable of surviving the rigorous washing required to remove the antibodies. To avoid such problems and blurry staining results, it is recommended to use the most “robust” dyes such as DAB, Fast Red, AEC and X-Gal first followed by other less “robust” dyes.

Simultaneous staining: In a simultaneous double stain, the primary antibodies can be applied simultaneously. The advantage of this method is that it is less time-consuming because the reagents can be mixed together. However, the technique can only be used, if suitable primary antibodies are available. Two methods can be adopted: A direct method with directly-labeled primary antibodies, or an indirect method based on unlabeled primary antibodies raised in different host species, or of different Ig isotype or IgG subclass (4).

A simple example of the direct method is when the primary antibodies are fluorescence-labeled to allow direct visualization. This avoids cross-reactivity, but is rarely practical since some form of amplification is necessary to get sufficient signal. Alternatively, the primary antibodies may be conjugated directly with enzymes, biotin, haptens or fluorochromes, subsequently employing the corresponding secondary antibody or streptavidin reagent. This is less time-consuming than the sequential method, since primary and secondary antibodies can be mixed together in two incubation steps. However, it requires avoiding all cross-reactivity.

With the indirect method it is also possible to apply time-saving antibody cocktails since the primary antibodies are recognized by different secondary antibodies (for an example, see Figure 2). Generally, it is advantageous to use secondary antibodies raised in the same host in order to prevent any unexpected interspecies cross-reactivity. One example of such a system is the new EnVision DuoFLEX from Dako. This system applies a mixture of primary antibodies of mouse and rabbit origin, followed by a mixture of the secondary goat-anti mouse and goat-anti-rabbit antibodies labeled with HRP and AP, respectively. Finally, the chromogens are applied sequentially. The result is a double stain where the primary mouse antibodies are stained brown with DAB and the primary rabbit antibodies are stained red with Permanent Red (for an example, see Figure 2). The system has been developed for Dako's new line of RTU cocktails of primary antibodies, but may also be used with other antibody cocktails or individual antibodies that are sequentially incubated on a single slide.

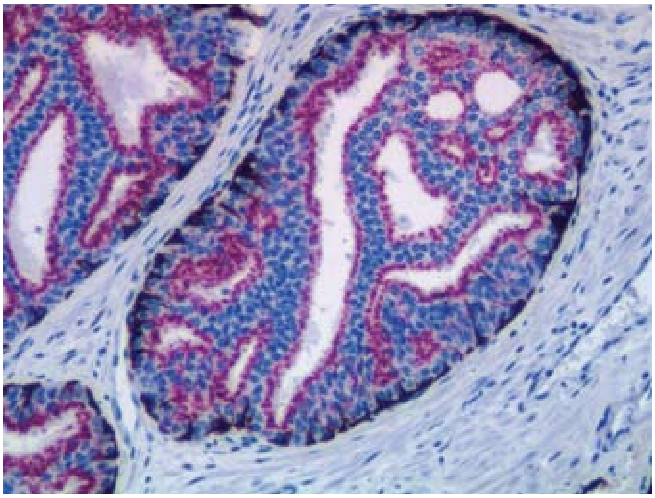


Figure 2. Simultaneous double staining performed with EnVision^{TM} DuoFLEX using an antibody cocktail containing monoclonal rabbit anti-AMACR (red), monoclonal mouse anti-HMWCK and monoclonal mouse anti-CK 5/6 (brown/black). Formalin-fixed, paraffin-embedded tissue sections from prostate.

Multi-step technique (3): This is an indirect/direct method combining unlabeled primary antibodies with directly-conjugated antibodies. The method starts with staining the unlabeled antibody/antibodies with the appropriate detection system, but without performing the final enzymatic staining reaction. The tissue is blocked with normal serum from the host of the first primary antibody before the second, directly-labeled primary antibody is added. The staining ends with the two enzymatic reactions being performed sequentially.

Multi-step staining can be used when the selection of primary antibodies is limited. However, when using this method it is not possible to mix reagents.

Users will often find that the choice of staining method is limited by the availability of the primary antibodies with respect to species origin or label.

Difficulties arise when targets are known or suspected to be co-localized and the only available primary antibodies are unlabeled monoclonal mouse antibodies of the same IgG subclass. In that case, none of the techniques described above are applicable.

One solution is the Dako Animal Research Kit (ARK™), which contains reagents for labeling mouse primary antibodies with a biotinylated anti-mouse Fab fragment, followed by blocking of the remaining reagent with normal mouse serum. This can be applied to the tissue as part of the multi-step technique (5). The kit gives a non-covalently labeled antibody, thus avoiding the risk of reducing the affinity. In addition, only small amounts of primary antibody are needed and the kit does not require time-consuming purification steps.

Another solution is Zenon Technology (Invitrogen) developed for flow cytometry. It essentially uses the same technique and offers labeling kits for mouse primary antibodies available as enzyme conjugates or conjugated to one of a wide variety of fluorescent dyes.

Finally, it is important to be aware of the fact that visualization systems with dual recognition such as the EnVision™+ Dual Link System do not discriminate between species, and thus are only suitable for multiple staining when using the sequential method. Visualization kits with amplification layers that are not well specified should be avoided since possible cross-reactivity cannot be predicted.

Selection of Dyes

The primary choice to make when deciding how to make the targets visible is whether to use immunoenzyme staining or fluorescence. Both have advantages and disadvantages and in the end, decisions should be made based on conditions of the individual experiment.

Chromogenic Dyes

Examples of enzyme/chromogen pairs suitable for triple staining are:

- Gal/X-Gal/Turquoise, AP/Fast blue, HRP/AEC/Red
- HRP/DAP/Brown, Gal/X-Gal/Turquoise, AP/Fast red
- HRP/DAP/Brown, AP/New Fucsin/Red, HRP/TMB/Green

When selecting color combinations for multiple staining with chromogenic dyes, it is advisable to choose opposing colors in the color spectrum such as red and green to facilitate spectral differentiation. If using a counterstain, this must also be included in the considerations. When working with co-localized targets, dyes must be chosen so that it is possible to distinguish the mixed color from the individual colors. Double staining using chromogenic dyes is well-established, but if the targets are co-localized, the percentage of the single colors cannot be easily identified (6). For a triple staining, it is naturally more difficult to choose colors that can be unambiguously differentiated and even more so, if targets are co-localized. In such cases, a technique known as spectral imaging may be applied (2). Spectral imaging allows images of the single stains to be scanned and by using specialized software algorithms the colors are unmixed displaying the distribution and abundance of the individual chromogens.

Visualizing Rare Targets

A narrow, dynamic range is a disadvantage for immunoenzymatic staining. The precipitation process, which is crucial for this method, is only triggered at a certain concentration of substrate and product. On the other hand, at high concentrations the precipitated product may inhibit further reaction. Therefore, it is difficult to visualize rare targets and highly abundant targets in the same slide. To ease this problem, catalyzed signal amplification — an extremely sensitive IHC staining procedure (such as e.g. CSA from Dako) can be used. The method can bring rare targets within the same dynamic range as highly expressed targets.

Fluorescent Dyes

Double immunofluorescence labeling is quite well established (7). Some of the same considerations as with chromogenic dyes apply when working with immunofluorescence. It is equally necessary to select dyes with distinguishable spectral properties. However, there are more colors available and the emissions spectra of the fluorescent molecules are narrower than the spectra of the chromogenic dyes. The use of multiple-fluorescent colors is also well established in FISH and flow cytometry, where dichroic and excitation/emission filters are employed to separate different fluorescent signals. The spectral separation can be aided by digital compensation for overlapping emission spectra. In addition, new fluorescent microscope systems such as e.g. Laser Scanning Confocal Microscope can unmix the spectral signatures of up to eight fluorochromes without any problems using multi-spectral imaging techniques such as e.g. emission fingerprinting (8).

When staining targets that are co-localized, fluorescent dyes allow separate identification of targets. This makes it possible to discern targets even in very different concentrations, whereas subtly mixed colors from chromogenic dyes may easily pass unnoticed with immunoenzyme staining.

Immunofluorescence potentially has a wider, dynamic range than immunoenzyme staining (9). Using this method, there is no enzymatic amplification involved and thus the dynamic range is determined solely by the sensitivity of the detectors.

On the other hand, there are some inherent problems with the use of immunofluorescence or fluorescence in general:

- A fluorescent signal is quenched when the fluorochromes are in close proximity (10)
- Dyes undergo photobleaching when subjected to light and will thus only fluoresce for a limited time.
- Even when stored away from light, fluorochromes will slowly deteriorate at room temperature.
- The morphology viewed in slides is different from what is observed in an immunoenzyme staining with counterstains.
- Increased background staining due to autofluorescence can pose a problem when working with some formalin-fixed tissues.

In spite of these drawbacks, immunofluorescence gives clear, sharp localization of targets and has advantages over chromogenic dyes when working with co-localized targets. Some chromogenic dyes fluoresce as well, such as e.g. Fast Red — an AP-substrate which is brighter in fluorescence microscopy than in bright field microscopy. (for a detailed review of the immunofluorescence technique, see Chapter 11, Immunofluorescence).

Alternatives to the conventional chromogenic dyes are colloidal, gold-labeled antibodies that can be used with normal light microscopy with silver-enhancement, Green Fluorescent Proteins (GFP and their variants) and Quantum dots. The latter, especially, has been found to be superior to traditional organic dyes on several counts such as brightness (owing to the high-quantum yield) as well as their higher stability (owing to less photodestruction). They can be linked to antibodies or streptavidin as an alternative to fluorochromes (11, 12). However, the size of these conjugates pose diffusion problems in terms of getting these inorganic particles into cells or organelles.

Automated Image Acquisition and Analysis in Multiple Staining

Digital image analysis will increase the number of usable dyes since it does not rely on the human eye for detection and differentiation. A digital image is acquired at excitation wavelengths relevant for the dyes applied, and separate detectors record individual colors. Thus, digital image analysis e.g. will allow the combination of fluorescent and immunoenzyme dyes.

Detectors, however, have biased color vision. They amplify colors differently than does the human eye. Therefore, dyes used in image analysis should be optimized for the best fit possible with the detector's filter properties.

Image analysis systems contain algorithms that allow compensation for overlapping emission spectra comparable to flow cytometry. They also allow signal gating within an interesting range of wavelengths, enabling users to see only signals within the desired range. Visualizing a combination of several gates with color selected independently of the dyes used for staining may clarify pictures and make conclusions easier to reach. This also makes it possible to determine signal intensity to exclude unspecific staining or background staining from final images.

Another advantage of digital image analysis is that it allows signal quantitation. Through software algorithms users can count how many signal clusters exceed a certain level of intensity and, potentially, calculate the ratio of different cell types. E.g. an image analysis algorithm can calculate the percentage of cells that stain positive for a certain target, combine that percentage with information of another stained target and, based on this, highlight diagnosis. A more thorough discussion of image acquisition and analysis can be found in Chapter 18, Virtual Microscopy and Image Analysis.

Conclusion

Multiple-target staining will one day be a routine procedure just as single-target staining is today. Use of the technique will extend, since it offers reduced turnaround-time and information not obtainable from single-target staining. Availability of reagents that give a wider range of possibilities when it comes to choice of technique, such as e.g. labeled primary antibodies and antibodies raised in different host species, will likely increase. In addition, some suppliers now offer complete kits with clinically-relevant antibody cocktails and visualization systems optimized to give the correct, balanced stain, thus significantly reducing the workload for the user.

Software for automated image acquisition and analysis will play a key role in this evolution since the limit to how many colors the human eye is capable of distinguishing is limited. Analysis algorithms will never entirely replace a skilled pathologist, but algorithms will gradually improve as the amount of information loaded into underlying databases increase. Eventually, algorithms will become sufficiently "experienced" to be able in many cases to suggest a diagnosis, and only the final decision will be left for the pathologist.

References

1. Molinié V et al. Modern Pathology 2004; 17:1180-90.
2. Van der Loos CM, J Histochem Cytochem 2008; 56:313-28.
3. Van der Loos CM. Immunoenzyme Multiple Staining Methods, BIOS Scientific Publishers Ltd. 1999.
4. Chaubert P et al. Modern Pathology 1997; 10:585-91.
5. Van der Loos CM and Göbel H J Histochem Cytochem 2000; 48:1431-7.
6. Merryn V.E. Macville Analytical Cellular Pathology 2001; 22: 133-42.
7. Mason D Y et al. J Pathol 2000; 191:452-61.
8. Dickinson ME et al. Bio Techniques 2001; 31: 1272-8.
9. Landon A. et al. The Journal of Pathology, 2003, 200: 577-88(12).
10. Förster Th., Fluoreszenz organischer verbindungen, Vandenhoeck & Ruprecht, 1951.
11. Wu X et al. Nat Biotechnol 2003; 21:41-6.
12. The small, small world of quantum dots. CAP Today.December 2008.

Chapter 16 | Optimization of Immunohistochemical Reactions

Gale E. Pace (Revised and Updated)

The process of implementing immunohistochemical tests in the diagnostic laboratory has been greatly simplified by the availability of standardized reagents, instruments, and assay protocols from commercial manufacturers. However, researchers and diagnosticians who wish to develop new immunohistochemical assays or to explore new applications for existing tests must carefully consider the methods of tissue preparation and the reaction conditions for each assay step in order to obtain clear, specific antigen signals and to minimize non-specific (background) reactions.

The complex nature of tissue specimens presents both an aid and a challenge to the development of any new immunohistochemical test. Tissue morphology can be tremendously helpful for interpreting the specificity of staining signals in a new immunohistochemical reaction; on the other hand, tissues are notoriously prone to non-specific binding artifacts, and are also sources of enzymatic and other biochemical activities that can generate false signals from assay reagents and confound even the most expert interpretation. The goal of assay optimization is to enhance the strength and specificity of the signal generated by the immunological and enzymatic staining reactions while suppressing noise and artifacts. This chapter will discuss the formulation and use of several basic immunohistochemical reagents, including proteolytic enzymes for tissue pre-treatment, antibody diluents, blocking and enhancing solutions, and wash solutions as they relate to assay optimization. Complementary information on the topics of tissue preparation, antigen retrieval, background suppression, and other aspects of assay optimization may also be found in the related chapters in this Guide.

Tissue Digestion Using Proteolytic Enzymes

Proteolytic digestion is commonly used to overcome the effects of covalent cross-links that are formed in tissues during formalin fixation. Controlled proteolysis can improve the penetration of reagents into the tissue structures and restore the immunodominant conformation of epitopes of interest, thus allowing the primary antibody to reach and bind its intended target. If the enzymatic cleavage points for the protease are in proximity to a formalin cross-link, digestion may induce a relaxation of the rigid protein structure and facilitate contact between the primary antibody and the corresponding antigenic determinant.

Proteolytic enzymes cleave specific amino acid sequences within the peptide chain of the digested protein; since proteases differ in their cleavage specificities, they can have markedly different effects upon tissues depending upon the type of fixative used for processing the tissue, the antigenic target, and the epitope recognized by the antibody.

Because the effectiveness of proteolytic digestion is dependent upon multiple factors, optimal digestion conditions must be determined empirically for each tissue and antigen/antibody combination. Carbohydrate epitopes, for instance, being non-proteinaceous, may be expected to be unaffected by proteolytic digestion; however, glycoproteins that contain carbohydrate epitopes may benefit from proteolysis if the epitope is “unmasked” by cleaving and de-constraining the peptide backbone, allowing the antibody access to the epitope for binding. Non-protein antigens may also benefit from the improved reagent penetration that results from tissue digestion.

Preliminary experiments should be conducted to determine the appropriate choice of enzyme, incubation time, temperature, and concentration for optimal digestion. Most of the proteolytic enzymes commonly used for IHC display their highest activities near 37 °C, and many protocols use this reaction temperature in order to achieve the digestive effect in the shortest possible time. In some cases, however, the use of a lower temperature may be preferable. By reducing the rate of the digestion reaction, one may extend the reaction time and thus exert more control over the digestion process — a method that is especially useful for situations requiring very mild digestion.

Table 1 lists several commonly used enzymatic reagents and the typical incubation conditions used in IHC.

Table 1. Proteolytic enzymes and typical incubation conditions.

Enzyme	Approximate activation temperature	Incubation time
Proteinase K	25-37 °C	5 min.
Trypsin	37 °C	10 min.
Pepsin	37 °C	5–20 min.
Protease XXIV	37 °C	5–10 min.
Pronase	25-37 °C	30 min.

NOTE: Formalin does not preserve tissue proteins by coagulation but it is thought to form cross links with basic amino acids. Ethanol and mercuric chloride-based fixatives are based on coagulation. With few exceptions retrieval should not be performed on ethanol fixed tissues. It should only be conducted with limited controlled protocols in mercuric–chloride-based fixatives.

Endogenous Enzyme Blockers

The two most common enzyme activities that are used to generate chromogenic signals in immunohistochemistry, horseradish peroxidase and alkaline phosphatase, are also both encountered as endogenous activities in a variety of cellular and tissue specimens. If the endogenous enzymes are similar in specificity to the enzymes used in the immunohistochemical detection system, the endogenous activity can produce false-positive signals that interfere with, and even overwhelm, the signals from the immunohistochemical reactions. Enzyme blockers are used to inhibit the activity of such endogenous enzymes within cells and tissue specimens.

Table 2 lists some of the common sources of these endogenous activities. Simple reagents may be used to completely block these endogenous enzymes by either competitive inhibition or acid inactivation (see Chapter 17, Background for further information on enzyme inhibitors)

Table 2. Endogenous enzymes found in a variety of cells and tissue types.

Enzyme: Peroxidase	Enzyme: Alkaline Phosphatase*
Red Blood Cells	Placenta Intestine — situated between cellular components of mucosa
Granulocytes	Proximal tubules of kidney
Eosinophils	Osteoblast in bone
Hepatocytes	Arterial & capillary endothelial cell surfaces
Muscle	Stromal reticulum cells
Kidney	Neutrophils
Monocytes	Follicle and mantle zones in most lymphoid tissue
*Alkaline Phosphatase is destroyed by routine fixation and paraffin-embedding procedures	

Table 3. Common endogenous enzyme blocking reagents for horseradish peroxidase and alkaline phosphatase systems.

Dual endogenous enzyme block	Horseradish peroxidase and alkaline phosphatase labels
Hydrogen peroxide	Horseradish peroxidase label
Levamisole + chromogen except intestinal alkaline phosphatase	Alkaline phosphatase label
Weak acid (0.3 N HCl), including intestinal alkaline phosphatase	Alkaline phosphatase label

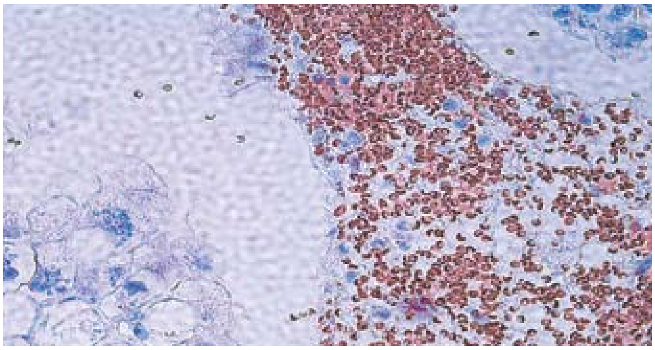


Figure 1. Example of endogenous peroxidase in red blood cells of kidney stained with DAB.

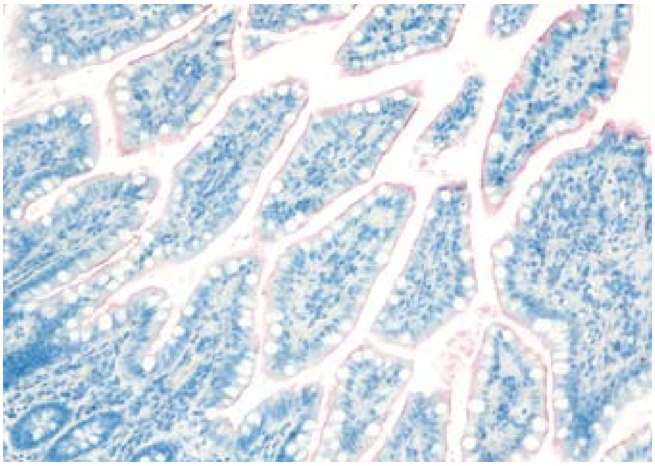


Figure 2. Example of endogenous alkaline phosphatase in ileum stained with Permanent Red.

Generally speaking, enzyme blockers are applied prior to the addition of antibody reagents in the staining protocol. However, in rare instances, the enzyme blocking reagent may interfere with the immunohistochemical reaction; mild acid treatment, for example, which is effective at abolishing endogenous alkaline phosphatase activity, may also alter sensitive epitopes to the extent that they are no longer recognized by their cognate antibody. In such cases the blocking reaction should be attempted after the primary antibody has been applied, but *before* the application of the enzyme-labeled immunohistochemical detection reagent.

Protein Blocking Reagent

These protein-containing reagents are used to reduce non-specific reactions that may result from the binding of antibodies and other assay reagents to various tissue components. Antibodies and detection systems used in immunohistochemistry are primarily protein-based reagents, and they are susceptible to non-specific binding. Protein blockers act to minimize non-specific protein adsorbance by competing for the non-specific protein binding sites on the specimen. By occupying the tissue binding sites with blocking protein prior to (or during) the incubations with the other immunohistochemical reagents, the non-specific signals that would otherwise develop can be greatly reduced. A more thorough discussion of the various non-specific protein-protein interactions that occur between immunohistochemical reagents and tissues, and the protein blockers that may be used to prevent such interactions, is given in Chapter 16, Background.

Antibody Diluents

Antibody diluents are buffered solutions that are used to formulate working solutions of antibodies for use in IHC assays. Because antibody conformations are highly dependent upon the aqueous environment, diluent formulations can significantly alter the stability and binding properties of antibodies, affecting both epitope specificity and “non-specific” interactions with the Fc region of the antibody molecule. Variations in charge, hydrophobicity, glycosylation, and other physico-chemical properties between antibodies can make it difficult to accurately predict the behavior of antibody-diluent combinations, but the availability of several effective commercial diluents has simplified the empirical process of diluent selection.

Ionic interactions are one of the primary forces controlling the immunochemical binding of antigens with antibodies, and these interactions are pH-dependent. Generally speaking, buffers that approximate pH (7.3-7.4) are often suitable for dilution of primary antibodies, and are a good starting point for optimization. The isoelectric point, or pI (the pH at which the net electric charge of a molecule is zero), for immunoglobulins can range from 5.8 to 8.5 for a given antibody. If the pH of the diluent is too close to the pI of the antibody, solubility can be diminished, with negative effects upon both signal and background. If low signal and high background are observed at the intial pH tested, try raising or lowering the pH of the diluents buffer by 0.5 pH units. Antibody interactions are also affected by ionic strength; most diluents contain millimolar quantities of sodium chloride or other salts which serve to reduce non-specific interactions among charged molecules. Excessive ionic strength should be avoided, since it may overcome the specific charge interactions required for high avidity antibody-epitope binding.

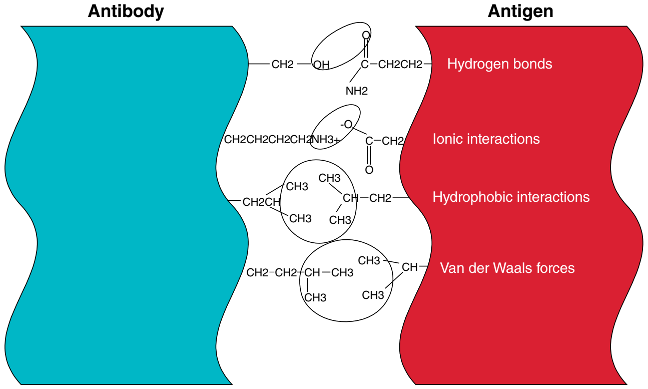


Figure 3. Antibodies are attracted to antigens initially through electrostatic interactions, and subsequently through Van der Waals and hydrophobic interactions.

Several pre-formulated antibody diluents are now available from commercial manufacturers; these diluents can simplify the optimization process when selecting diluents for a new antibody or assay, and may also confer increased stability to the working primary antibody solution. Many of the commercially available antibody diluents are based upon Tris-HCl buffers containing a detergent and proprietary stabilizers. Some diluents also contain protein-based background-reducing components such as bovine serum albumin or serum proteins. Serum-containing diluents should be used with caution; serum binding to the primary antibody can cause a reduction in sensitivity, while binding to the secondary antibodies used in IHC detection systems can result in false positive results.

Antibody stability in solution cannot be predicted without thorough stability studies; technicians are advised to follow proper quality control procedures for stability validation if primary antibodies are to be diluted in the laboratory and utilized for extended periods of time. An advantage to using commercially diluted primary antibodies is the built-in customer protection provided by the regulatory mandates that govern reagent manufacturers. Manufacturers must demonstrate the stability of commercially produced reagents for defined periods to establish a predictable shelf life for their antibody products.

Important Points to Consider Regarding Antibody Diluents

- Diluent pH can be an important determinant of effectiveness.
- High concentrations of sodium chloride and azides are used frequently as preservatives in commercial preparations, but these components can reduce antibody reactivity. Excessive ionic strength can decrease specific staining by interfering with antibody-antigen binding.
- Generally speaking, the more dilute the antibody, the less stable the working solution.
- Highly diluted antibody solutions should not be used for more than a few days unless validated stability data is available.
- The pH's of Tris-based buffers are sensitive to temperature changes. Always prepare buffers at the same temperature in which they will be used, and be aware that refrigeration and heating can cause changes in the pH of temperature-sensitive buffers. Always allow reagents to equilibrate to room temperature before use.
- Phosphate buffered saline (PBS) should generally not be used as a diluent unless specifically recommended by the manufacturer for a particular antibody.

Wash Buffers

Wash buffers are used to remove excess reagents from the specimen after each incubation step in the assay protocol. Commonly used wash buffers, including those that are commercially available, include Tris-buffered saline (TBS) and phosphate-buffered saline (PBS).

Tris-Buffered Saline

Tris(hydroxymethyl)aminomethane-based wash buffers are often utilized in combination with the non-ionic determent Tween 20. Commercial wash buffer preparations may also include a preservative, such as 0.01% sodium azide, to prevent the growth of microorganisms and extend the shelf life of the reagent.

Tris-buffered solutions are pH-sensitive; as the solution decreases in temperature, pH increases at a rate of approximately 0.03 units per degree centigrade, so care should be taken to equilibrate buffer solutions to the proper working temperature in order to maintain consistent pH. This effect is illustrated in Table 4

Table 4. Effects of temperature on pH of Tris-buffered saline.

Temperature	pH
5 °C	8.18
25 °C	7.6
37 °C	7.30

Wash buffers can be employed to counteract the effects of non-specific reagent binding to tissue specimens. When conditions require very high specificity reactions, such as when using highly sensitive detection methods, an increase in the saline and detergent content of the wash buffer can be used to minimize non-specific binding. The addition of high concentrations of salts (e.g. sodium chloride) and detergents (e.g. Tween 20) to wash buffers will significantly reduce the non-specific binding of many immunohistochemical reagents, including antibodies and detection reagents such as labeled polymers.

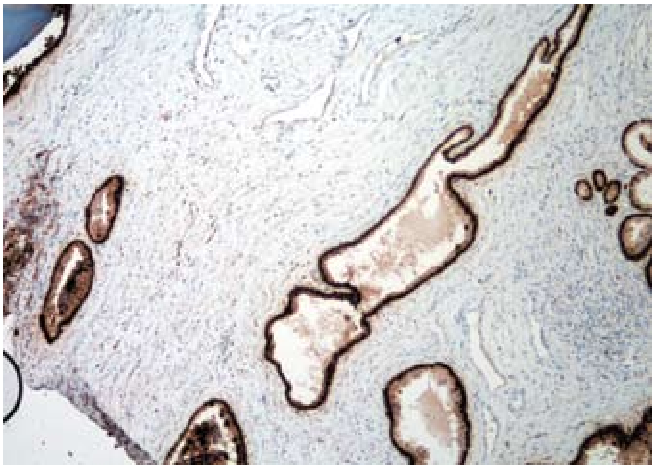


Figure 4a. Anti-Cytokeratin, Clone 34BE12, Dako Code M0630, on human prostate tissue, rinsed with 150 mM NaCl, 0.05% Tween 20, Tris-Buffered Saline, Dako Code S3006, pH 7.6 at 25 °C. Non-specific staining is evident in the lumen and the connective tissue.

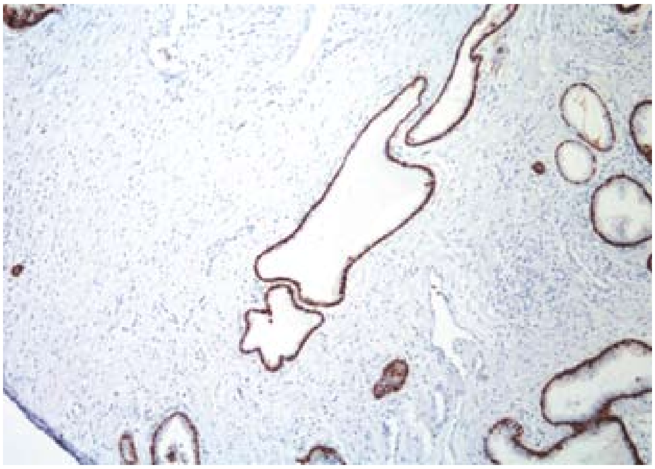


Figure 4b. Anti-Cytokeratin, Clone 34BE12, Dako Code M0630, on human prostate tissue, rinsed with 300 mM NaCl 0.05% Tween 20 Tris-Buffered Saline, Dako Code S3306, pH 7.6 at 25 °C. Staining is confined to specific signals in the cells of the prostatic epithelium.

Phosphate-Buffered Saline (PBS)

PBS is also commonly used as a wash buffer for IHC. PBS's advantages are reduced auto fluorescence in immunofluorescent assays, and it is relatively inexpensive compared to Tris-based buffers. However, in some cases PBS can cause higher levels of nonspecific staining, and it has been observed to reduce the specific binding abilities of certain monoclonal antibodies (Anti-CD30, for example).

Suggestions for Making Wash Buffers

- Always replace buffers after expiry, and do not combine old buffers with new.
- Do not dilute reagents beyond the manufacturer's recommendations; buffering ability may be compromised, and ionic strength will no longer be optimal if the recommended dilution is exceeded.
- Clearly label all new solutions with the date when they are opened or made.
- Do not mix different types of buffers with one another.

- Use distilled, organically filtered deionized, or high quality reagent grade water only to make and dilute buffers. Do not use tap water, as the presence of trace quantities of metals, inorganic ions, and other contaminants in tap water may interfere with immunohistochemical reactions.

Chromogen Enhancers for DAB

The DAB chromogen is a popular choice for signal generation with peroxidase-based immunohistochemical detection systems. The DAB reaction product, which normally appears as a light brown precipitate, may be enhanced by reaction with any one or a combination of several types of metals. The metals enhance the reduction of the DAB reaction product and darken its appearance. Copper, silver, nickel, gold and cobalt have all been used as enhancers.

Metal enhancers may be added directly to the chromogenic DAB reaction, or they may be applied in a separate incubation step following the development of the DAB reaction product and a rinse in high-purity water. The latter approach is recommended when it is desirable to fine-tune the degree of enhancement; the optimal incubation time for the enhancement reaction may be determined empirically by monitoring the development of the enhanced chromogen under the microscope. The hue and intensity of the final DAB reaction product will vary depending upon the type and concentration of the metal(s) used in the enhancing reaction, but in general the enhanced product will appear darker and more intense than the non-enhanced DAB product. Since the enhancer metals work only upon the reduced chromogen that has been deposited by the peroxidase reaction, the enhancer reaction generally does not contribute significantly to background. Incubation times for enhancement should be determined by the individual laboratory, based on the desired hue of the chromogen.

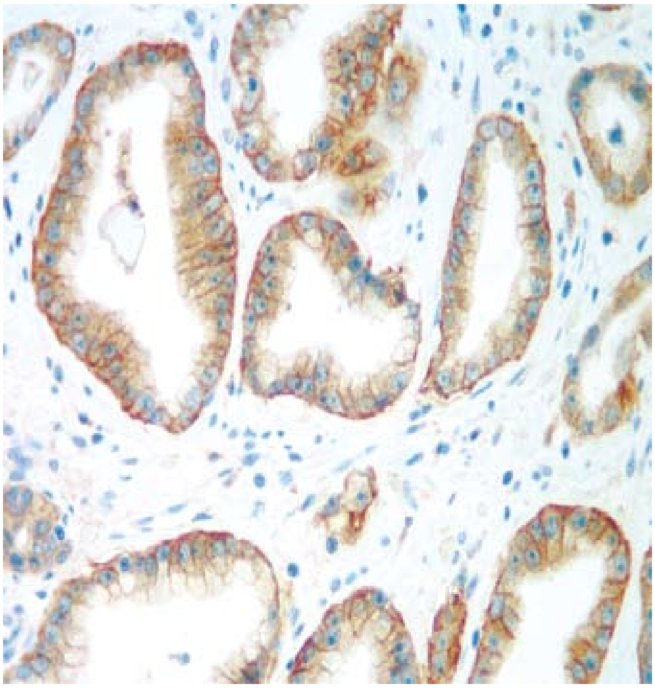


Figure 5a. Standard DAB chromogen without enhancement.

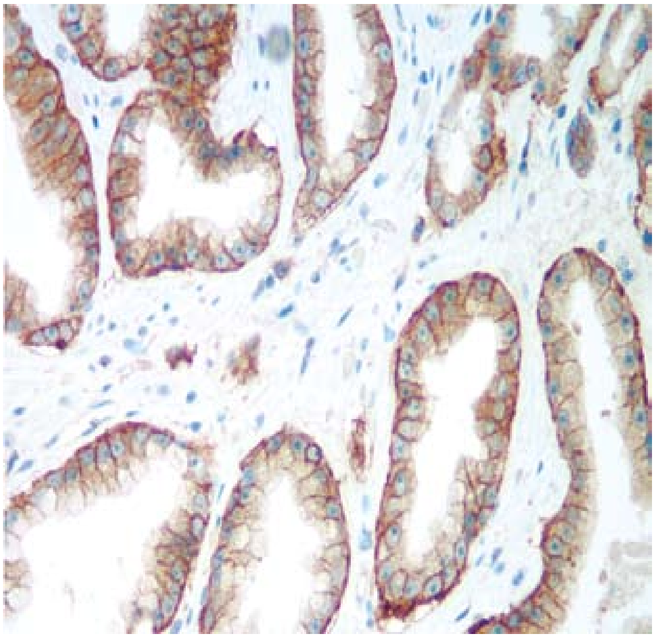


Figure 5b. DAB enhanced with Dako DAB Enhancer.

Chapter 17 | Background

Helle Grann Wendelboe MS and Kirsten Bisgaard BSc

As immunostaining of histological tissue specimens becomes more diversified in methodology and more sensitive in detection, background staining has develop into one of the most common problems in immunohistochemistry. Background staining in tissue sections may be due to several factors, some of which are specific to the antigen and antibody reaction or detection method, and others which are of a more general character. The terminology used in this chapter uses the term unwanted specific staining if the staining is mediated by interactions between any antibodies and their respective epitopes and nonspecific staining for all other interactions. The following description will cover the major causes of background staining related to antibodies, detection methods, and other general factors and will offer possible solutions to these problems.

Background Associated With Detection Methods

Horseradish Peroxidase-Based Detection Methods

Endogenous Peroxidase Activity

For practical purposes in immunohistochemistry, endogenous peroxidase activity can be defined as any activity that results in the decomposition of H_20_2. Such activity is a common property of all hemoproteins such as hemoglobin (red cells), myoglobin (muscle cells), cytochrome (granulocytes, monocytes) and catalases (liver and kidney). Peroxidase activity may also be encountered in tissue areas adjacent to vascularized areas due to the diffusion of blood prior to fixation.

The most commonly used procedure for suppressing endogenous peroxidase activity in formalin-fixed tissue is the incubation of sections in three percent H_2O_2 for five to 10 minutes (Figure 1). Methanolic H_2O_2 treatment (11 parts three percent H_2O_2 plus 4 parts absolute methanol) for 20 minutes is also used, but is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment may also detach frozen sections from the carrier glass. Endogenous peroxidase activity can also be suppressed by a mixture of sodium azide and H_2O_2 (1). However, in most work with formalin-fixed tissue sections, the interpretation of specific staining is not impaired by any endogenous peroxidase activity. If the formalin-fixed tissue is rich in blood-containing elements then it will be a good idea to quench endogenous peroxidase activity. In cell preparations and frozen sections, routine quenching of endogenous peroxidase is also advisable.

Specimens rich in endogenous peroxidase activity may be processed using an alkaline phosphatase detection method instead of a peroxidase method, eliminating the background.

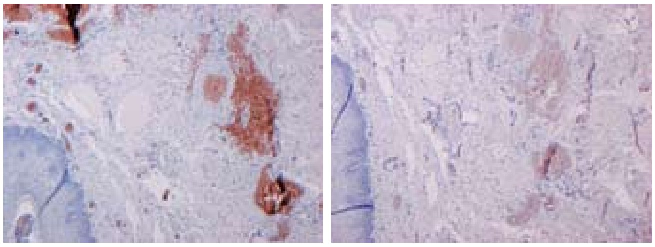
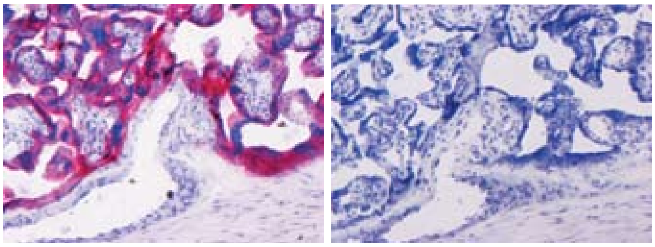

(a) Before (b) After

Figure 1. Red blood cells showing endogenous peroxidase activity **(a)** before, and after blocking with three percent hydrogen peroxide. **(b)** Alkaline phosphatase-based detection methods.

Endogenous Alkaline Phosphatase

Endogenous alkaline phosphatase activity is frequently encountered in intestine, kidney, osteoblasts, endothelial cell surfaces, neutrophis, stromal reticulum cells, lymphoid tissues, and placenta. In frozen tissue, where endogenous alkaline phosphatase activity is most pronounced, routine quenching of the endogenous alkaline phosphatase is recommended. In most formalin-fixed tissue sections, interpretation is not usually impaired by endogenous alkaline phosphatase which makes quenching an optional choice. Most forms of endogenous alkaline phosphatase can be quenched by including five mM levamisole in the chromogen substrate solution (Figure 2). The intestinal form of alkaline phosphatase is the exception and resists this treatment, but it can be quenched by treating the tissue sections with a weak acid wash prior to the application of the primary antibody.



(a) Before (b) After

Figure 2. Placenta showing endogenous alkaline phosphatase activity **(a)** before, and **(b)** after blocking with levamisole.

Double Staining

Combined Endogenous Peroxidase and Alkaline Phosphatase

Double staining using these enzymes requires quenching of both endogenous activities. To achieve this, use the H_2O_2 method for endogenous peroxidase and the weak acid method for endogenous alkaline phosphatase. The sequence of blocking endogenous activities is optional: the procedure will work effectively conducting either step first. Also, reagents that block both endogenous peroxidase and alkaline phosphatase in one step are available.

Biotin/Streptavidin-Based Detection Methods

Endogenous avidin-binding activity (EABA) has been observed with all biotin-based techniques, due to its presence in a wide variety of tissues. Biotin is bound to enzymes and other proteins especially in the liver (hepatic nodules), kidney (tubular epithelia) and lymphoid tissue (paracortical histiocytes) (Figure 3). EABA is usually observed within the cytoplasm and is most pronounced when using frozen tissue sections. Paraffin-embedded tissues also hold substantial endogenous biotin. Other examples of EABA include the nonimmunochemical staining of myelin (2) and mast cells (Figure 4) in both frozen and paraffin-embedded tissue (3). Guesdon et al (4) found EABA in granulocytes from mouse spleen.

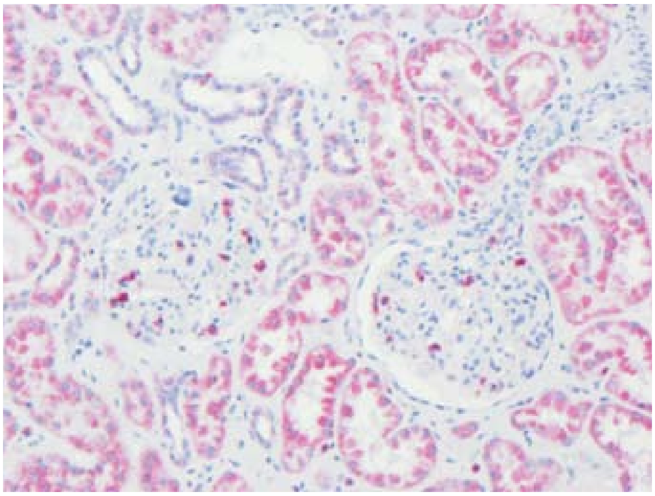


Figure 3. Endogenous biotin expression in the kidney.

EABA is best suppressed by sequential incubations of 10 to 20 minutes of tissue sections, first with 0.01 percent to 0.1 percent avidin followed by 0.001 percent to 0.01 percent biotin prior to the staining protocol (5). Avidin has four binding sites for biotin while each biotin molecule can bind to only one avidin molecule. The first incubation with avidin effectively blocks endogenous biotin but simultaneously adds three more potential biotin-binding sites to the specimen. This means there are extra biotin binding sites open to link antibodies or detection systems that can give background staining. Therefore it is important to block these extra biotin binding sites with a subsequent biotin incubation.

Because avidin is a glycoprotein containing 10 percent carbohydrates and has a pI of 10, it tends to bind nonspecifically to lectin-like and negatively charged tissue components at physiological pH. Streptavidin contains no carbohydrates and has a pI of five. Its introduction to IHC has largely eliminated these problems. A sugar solution can block the lectin-like elements. Many commercially available Avidin detection systems contain modified avidin to minimize nonspecific avidin background.

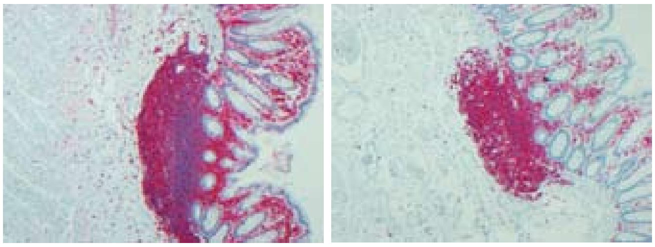

(a) Before (b) After

Figure 4. Avidin-biotin-complex (ABC) binding to mast cells in submucosa **(a)** before, and **(b)** after blocking for endogenous avidin binding activity (EABA).

Polymer-Based Detection Methods

Use of polymer detection systems avoids endogenous avidin/biotin background completely. General overall background staining may occur if insufficient washing is performed after polymer application. Due to the large size of polymer conjugates, the diffusion rate of these molecules is lower than for low-molecular weight conjugates. In addition, polymer conjugates based on a hydrophobic backbone have a tendency to be sticky. This can be resolved by applying multiple wash steps, adding detergent to the wash buffer and by prolonging washing time.

Antigen Retrieval

This has been reported both to eliminate and introduce cytoplasmic and nuclear background in immunohistochemical procedures (6). A possible explanation is that antigen retrieval influences antigen-antibody binding activity, and thereby affects binding of the antibody to tissue proteins. Different types of antigen retrieval solutions with different buffer compositions, pH and chelating abilities exist, Citrate pH 6.0, TRIS/EDTA pH 9.0, and TRIS pH 6.0 retrieval solutions vary in the way they influence antigen-antibody binding. Retrieval time can also influence antigen-antibody binding so for new antibodies it is advisable to investigate which antigen retrieval solution and time are best to optimize signal and minimize background.

General Factors

Antigen Diffusion

Unwanted specific background staining may occur when the tissue marker to be stained has diffused from its site of synthesis or storage into the surrounding tissue. Because many fixatives penetrate tissues slowly, it is important to keep tissue specimens as small as possible and to fix immediately. Otherwise the antigens may not be adequately fixed and may be extracted or displaced by the subsequent tissue processing steps. Extracellular antigens or those of low molecular weight are more likely to diffuse than high-molecular-weight antigens.

A typical example is the diffusion of thyroglobulin from thyroid follicular epithelium and colloid-containing lumen into surrounding stromal tissue. Similarly, specific background may result when the tissue marker is present in high concentrations in blood plasma and has diffused in the tissue prior to fixation. This can be seen when tonsil tissue is stained for immunoglobulin heavy and light chains (Figure 5), particularly when fixation was not performed promptly and when antisera were not diluted sufficiently. Ingestion of target antigens by phagocytes may also produce specific background staining, resulting in stain patterns not normally seen in such cells.

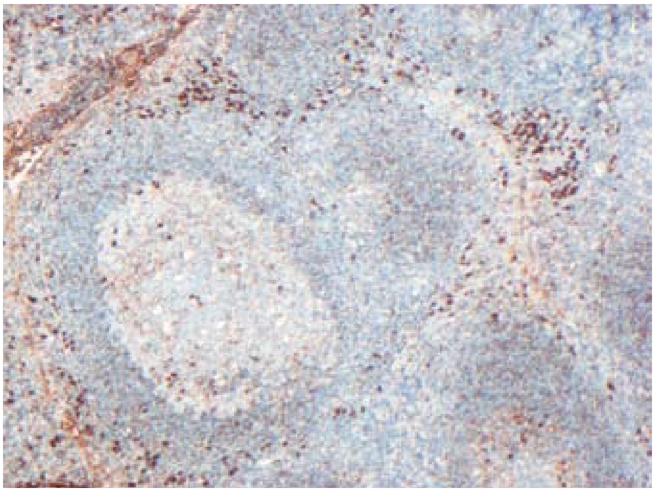


Figure 5. Undesirable staining of plasma proteins with antibody to kappa light chain. Plasma cells stain specifically.

Natural and Contaminating Antibodies

Natural Antibodies

Low-level natural antibodies present in the antiserum as a result of prior environmental antigenic stimulation may increase in titer during immunization with use of adjuvants. As a consequence, they can give rise to nonspecific staining. In 1979, Osborn et al (7) reported that sera from non-immunized rabbits and goats, but not from guinea pigs, contained environmental antibodies to keratins. This may be an example of specific epithelial background staining caused by natural antibodies. Although also observed by others, attempts to isolate or remove these antibodies from the antiserum were not successful (8).

Most natural antibodies are of the nonprecipitating type and occur only in relatively low concentrations. These antibodies are usually rendered non-reactive on tissue if the antiserum is used at a sufficiently high dilution or by shortening the incubation periods.

Contaminating Antibodies

Isolated antigens used for immunization are rarely pure. If a host's immune system reacts to impurities, contaminated antibodies will result. Usually these contaminating antibodies are present in low concentration and will not detract from the immunohistochemical specificity of high-titered antisera provided they are diluted sufficiently.

Contaminating antibodies may be related to infectious agents, other animal species kept in the same facilities, or carrier proteins used for immunization. These antibodies may be of special concern when dealing with antisera against synthetic peptide. Small peptides are not antigenic, and must therefore be coupled to carrier proteins prior immunization. The antisera produced will therefore contain antibodies against the carrier protein and the peptide.

However, if contaminating antibodies do interfere with specificity, affinity absorption of the antiserum is usually performed. "Batch-absorbed" antisera almost always contain residual levels of contaminating antibodies (mostly of the non-precipitating type) and will cause nonspecific staining of tissue if used at excessively high concentration (8).

Monitoring and evaluating the results of absorption by use of such techniques as immunodiffusion, immunoelectrophoresis and rocket immunoelectrophoresis can only be used to determine non-specificity. This monitoring cannot establish the specificity of an antiserum. Ultimate mono-specificity must be demonstrated by use of the designated technique and by extensive use of tissues.

Problems stemming from natural and contaminating antibodies, of course, do not occur with monoclonal antibodies produced in tissue culture, but may be present in monoclonal antibodies prepared from ascites fluid.

Cross-Reactivity

Background staining due to antibody cross-reactivity may result when epitopes of the target tissue antigens are shared with other proteins. A typical example is the use of unabsorbed antiserum to carcinoembryonic antigen (CEA). Because CEA shares epitopes with some normal tissue proteins and blood group antigens, non-specific staining may result. Careful absorption of such antisera or in the case of monoclonal antibodies careful screening of clones, will eliminate this type of background staining.

Nonspecific antibody cross-reactivity with similar or dissimilar epitopes on different antigens may also be the cause of confusing background staining. This is rare however, and can be avoided by using antibodies from hyper-immunized animals or carefully selected clones.

Cross-reactivity of antigens from related species is a common problem in multi-staining. This can often be avoided by using affinity purified antibodies, sub-type specific antibodies or site/region specific antibodies. For more detail on cross-reactivity, see Chapter 1, Antibodies.

Fc Receptors

Fc receptors (FcR) are a family of detergent-soluble membrane glycoproteins with approximate molecular weights of 50–70 kD. They comprise less than one percent of the total membrane proteins and are most frequently present on macrophages and granulocytes. They have also been reported on B cells and some T cells. The intrinsic affinity of the FcR for monomeric IgG is approximately 1x106 to 1x108

M–1, but is higher for polymers and immune complexes of IgG. There is considerable class/subclass and species specificity among different FcR's. For example, the FcR on some human cells was found to bind mouse monoclonal IgG2a and IgG3 but not other IgG subclasses (9). Goat sera do not react with FcR's of human leucocytes (10).

Background staining due to FcR is more common in frozen sections, smears and in lightly fixed than in tissues fixed by harsher procedures. It can be avoided by use of F(ab')2 fragments instead of whole IgG molecules and by careful screening of monoclonal antibodies.

Hydrophobic Interaction

In aqueous media, hydrophobic interactions between macromolecules occur when surface tensions are lower than that of water (called van der Waals forces). These interactions can be interatomic as well as intermolecular, and originate through the fluctuating dipolar structure within these macromolecules.

Hydrophobicity is a property shared to varying degrees by most proteins and is imparted primarily through the side chains of neutral aromatic amino acids phenylalanine, tyrosine and tryptophan. By their lower attraction for water molecules, these amino acids tend to link to one another, thus expelling water from the molecule. While hydrophobicity is one of the natural forces that confer stability on the tertiary structure of peptides, it also imparts stability to formed immune complexes and depending on environmental factors, can exist also between different protein molecules.

Tissue Proteins

In tissue, proteins are rendered more hydrophobic by fixation with aldehyde-containing reagents such as formalin and glutaraldehyde. Increased hydrophobicity often results from cross-linking reactive epsilon- and alpha-amino acids within and between adjacent tissue proteins. The extent of this hydrophobic cross-linking during fixation is primarily a function of time, temperature and pH. Changes in these factors will likely result in variable hydrophobicity due to variable cross-linking of tissue proteins. Therefore once optimized fixation procedures must be maintained and controlled. Tissues that commonly have the most background staining as a result of hydrophobic, as well as ionic, interactions are connective tissue: collagen laminin, elastin, proteoglycans and others and squamous epithelium (keratins) and adipocytes (lipoids) if incompletely removed during processing with xylene. Excessive background staining due to overfixation with formalin may be remedied by postfixation with Bouin's, Zenker's or B5 fixative (11).

Antibodies

Of the major serum proteins, immunoglobulins unfortunately are particularly hydrophobic. In general, mouse antibodies of subclass IgG_3 and IgG_1 are more hydrophobic than those belonging to subclasses IgG_2 and IgG_4. Furthermore, some isolation procedures for IgG class antibodies promote the formation of aggregates, thereby further increasing their hydrophobicity. Storage of immunoglobulins may also increase their hydrophobicity and lead to aggregation and polymerization. This frequently leads to a diminution in, or loss of, immune reactivity. Attendant increase in non-specific background staining by use of a polyclonal IgG fraction when compared to that obtained by use of the original whole antiserum has been demonstrated (12).

The diluent buffer's formulation can also influence hydrophobic binding between monoclonal IgG and tissue proteins: the greater the proximity of diluent pH and the isoelectric point (pI) of antibodies, the stronger hydrophobic interaction will be. The lower the ionic strength of the diluent, the weaker will be the strength of hydrophobic attraction. The following anions and cations are arranged in order of their diminishing effect on hydrophobicity:

Anions: **PO4-3, SO4-2, Cl-, NO3-, SCN-**
Cations: **NH4+, K+, Na+, Ca2+**

Other possible methods to reduce hydrophobic interactions between tissue and reagent proteins include adding detergent, for example Tween 20, or ethylene glycol to the diluent, or by raising the pH of the diluent used for polyclonal antibodies only.

The most widely practiced measure to reduce background due to hydrophobic interaction is to use a protein blocking solution either in a separate step, or by adding it to the antibody diluent. However this will only be successful if the blocking protein is a type that can compete effectively with IgG or its aggregates or conjugates, for hydrophobic binding sites. Separate incubation with a solution containing blocking protein is best carried out immediately prior to application of the primary antibody. The solution should contain proteins identical to those present in the secondary link or labeled antibody, but not to those in the primary antibody, in order to prevent nonspecific binding of the secondary antibody.

The addition to the primary antibody diluent of one percent bovine serum albumin (BSA) is probably the most widely practiced step for reducing non-specific binding due to hydrophobic interaction. Use of non-fat dry milk (13) or of casein (14) for reducing background staining is also recommended. Casein, when used as a blocking agent, an antibody diluent and in the wash buffer, was found to result in significantly less background staining compared to normal swine and sheep sera (14).

Because of the different uses of biotinylated antibodies today, it should be of interest to note that biotinylation can change the pI of the antibody in excess of three units, for example from a pI of eight for the antibody to less than five for the conjugate (15). This may have a marked effect on the solubility of these conjugates, possibly due to increased hydrophobicity.

Ionic and Electrostatic Interactions

Ionic interactions are one of the prime forces that control immunochemical interaction between antigens and their corresponding antibodies. They may however also contribute to non-specific background.

The pI of the majority of polyclonal IgG ranges from approximately 5.8 to 8.5. At physiological pH and at the pH commonly used for diluents, antibodies can have either net negative or positive surface charges. Ionic interaction of some antibodies with tissue proteins can be expected if the latter possess opposite net surface charges. Negatively charged sites on endothelia and collagen fibers have been reported to interact with cationic conjugates composed of rabbit Fab fragments and horseradish peroxidase type VI (pI 10.0) (16). In general, interactions of the ionic type can be reduced by use of diluent buffers with higher ionic strength. Addition of NaCl to the diluent buffer can reduce background staining stemming from ionic interactions but its routine use in diluents for monoclonal antibodies is not recommended (17).

Unfortunately most diffuse background staining results from a combination of ionic and hydrophobic interactions. Remedies for one type of interaction may aggravate the other.

Complement-Mediated Binding

Complement-mediated binding may occasionally be a cause of background in frozen tissue when whole antisera are used. However by the time large pools of antisera have been prepared for use, several of the complement factors are usually inactivated.

Miscellaneous Sources

Physical injury to tissue, drying out prior to fixation or incomplete penetration of fixative may cause diffuse staining of all or most tissue elements within an affected area. Similar diffuse background staining of both the section and the glass slide, usually limited to the area of antibody incubate, has been observed and may be due to residual embedding medium. Sections mounted routinely in water baths containing protein additives such as Knox gelatin or Elmer's glue may also show this type of diffuse background, especially in procedures of high staining sensitivity. Water baths should be free of bacterial or yeast contamination.

Nonspecific staining due to undissolved chromogen granules may on occasion also be encountered.

Nonimmunologic binding of horseradish peroxidase (either in free form or as a conjugate) to HbsAg in hepatocytes was reported by Omata et al. (18). The precise nature of this binding was not known.

Necrotic areas of tissue may stain with all reagents. Nadji and Morales (19) provide an excellent collection of color plates illustrating background staining and accompanying explanations.

Excessive counterstaining may compromise the specific staining signal.

General Aspects

While it is clear that background staining can be caused by the factors outlined above, it is also important to work with well-characterized reagents and established protocols in order to avoid background or to troubleshoot background staining. Many IHC reagent providers offer “system solutions,” which are IHC product lines with carefully optimized buffers, target retrieval reagents, primary antibodies, detection reagents and substrates to be run on an automated platform. These are designed to provide users with consistent, optimal staining. Several countries have established national quality programs, such as United Kingdom National External Quality Assessment Service (UK NEQAS) (20), and Nordic Immunohistochemical Quality Control (NoridQC) (21), formed to raise awareness of the need for quality and best practices in IHC laboratories, including improving the reduction of background staining. See Chapter 15, Controls, for further discussion. Information on individual national programs can be found on each program’s Web site.

References

1. Li C-Y et al. J Histochem Cytochem 1987;35:1457-60.
2. Sternberger LA and Sternberger NH. J Histochem Cytochem 1986; 34:599-605.
3. Hsu S-M and Raine L. In DeLellis RA(ed) Advances in Immunohistochemistry. New York: Masson 1984:31-42.
4. Guesdon J-L et al. J Histochem Cytochem 1979;27:1131-9.
5. Wood GS and Warnke R. J Histochem Cytochem 1981;29:1196-204.
6. Wieczorek R et al. J Histotech 1997;20:139-43.
7. Osborn M et al. Proc Natl Acad Sci (USA) 1977;74:2490-4.
8. Conway de Macario E et al. J Immunol Meth 1986;90:137-41.
9. Gadd J and Ashman LK. Clin Exp Immunol 1983;54:811-8.
10. Alexander EL and Sanders SK.J Immunol 1977;119:1084-8.
11. Caron BL and Banks PM. Lab Investig 1979;40:244-5.
12. Boenisch T. Unpublished observations.
13. Duhamel RC and Johnson DA. J Histochem Cytochem 1985;7:711-4.
14. Tacha DE and McKinney LA. J Histotech 1992;15:127-2.
15. Wadsley JJ and Watt RM. J Immunol Meth 1987;103:1-7.
16. Pino RM. J Histochem Cytochem 1985;33:55-8.
17. Boenisch T. Appl Immunohistochem 1999;7(4):300-6.
18. Omata et al. Am J Clin Pathol 1980;5:626-32.
19. Nadji M and Morales AR Immunoperoxidase Techniques: A Practical Approach to Tumor Diagnosis. Chicago: American Society of Clinical Pathologists, 1986.
20. United Kingdom National External Quality Assessment Service (UK NEQAS): www.ukneqas.org.uk.
21. Nordic Immunohistochemical Quality Control (NordiQC): www.nordiqc.org.

Chapter 18 | Peptide Nucleic Acids: Robust Probe Hybridization Technology

David C. Spaulding PhD

Peptide nucleic acid (PNA) is an artificially synthesized polymer that is capable of binding DNA and RNA in a sequence-specific manner. Since the discovery of its unique binding properties, PNA has been employed in a wide variety of biomedical applications, including genetic research, diagnostics, and experimental therapeutics (1). This article will focus on the diagnostic PNA assays that have gained widespread use in the pathology setting and briefly touch upon other promising applications of this technology.

Unlike DNA and RNA, which have backbones of repeating sugar-phosphate units, the PNA molecule is built upon a pseudo-peptide backbone of N-(2-aminoethyglycine) units linked by peptide bonds, to which purine and pyrimidine bases (the specific base-pairing units of nucleic acids) are linked via methylene carbonyl bonds.

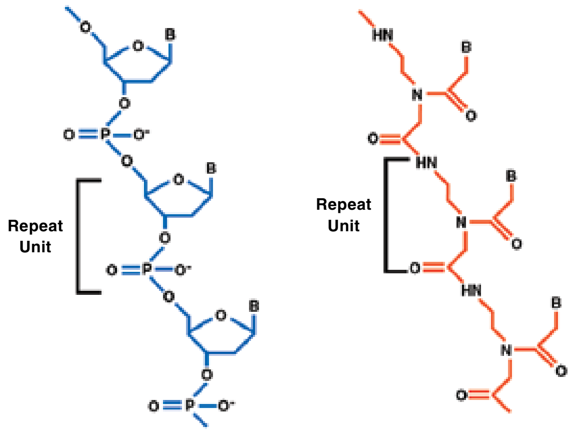


Figure 1. Basic structures of DNA and PNA. In contrast to the negatively charged backbones of naturally occurring nucleic acids (DNA and RNA), the synthetic pseudo-peptide backbone of PNA is uncharged. PNA's electrostatically neutral backbone is responsible for the higher binding energies (and correspondingly higher melting temperatures) of complementary PNA-DNA and PNA-RNA duplexes relative to similar DNA-DNA, DNA-RNA, or RNA-RNAs. The same bases that are responsible for the sequence-specific base-pairing of nucleic acid duplexes may also be incorporated into PNA. B = pyrimidine (T, C) or purine (A,G) base; H = hydrogen; N = nitrogen; O= oxygen; P= phosphate.

The most common usage for PNA molecules are as probes of complementary nucleic acid sequences. As with other nucleic acid probes, the sequences of bases on PNA probes dictate the specificity of binding to complementary DNA and RNA sequences, but the uncharged PNA backbone confers a key advantage to PNA probes. By eliminating the repulsive electrostatic force between traditional nucleic acid probes and their complementary target strands, the neutral PNA backbone confers increased probe affinity and thermal stability to the probe-target duplex.

Specificity of probe binding is a critical aspect of probe assay design, and the physico-chemical properties of PNA probes offer significant advantages for controlling assay specificity. In assays that use traditional DNA or RNA probes, the selectivity conferred by hydrogen bonding between the complementary base pairs on the probe and target strands is offset by the repulsive ionic forces between the strands' negatively charged backbones. Optimization of assay specificity requires a delicate balance between parameters such as hybridization temperature, probe concentration, length, and G-C content, and the concentrations of organic solvents and ions, making the design of a robust assay challenging even for experienced diagnosticians. The higher binding energies of PNA probe-target duplexes contributed by the uncharged PNA backbone offer several practical advantages for diagnostic probe assay development. The higher melting temperatures of PNA-DNA duplexes allow PNA probes to "invade" and overcome many problematic secondary structures in target sequences, and permit very stringent hybridization and wash conditions to be used to increase binding specificity. The higher binding affinities of PNA probes also permit shorter probe sequences and lower probe concentrations to be used in assays, lowering costs and reducing potential non-specific interactions with assay substrates and biological sample components. Mismatches in PNA-DNA duplexes are more de-stabilizing than in corresponding DNA-DNA duplexes, a characteristic which allows PNAs probes to distinguish single base sequence discrepancies such as point mutations and single nucleotide polymorphisms with higher selectivity than DNA or RNA probes.

Another clear benefit of PNA probe chemistry is its exceptional stability. PNA molecules are highly resistant to both nuclease and protease enzymes, and are stable over a wider pH range than DNA or RNA molecules. Probe stability is especially important in diagnostic settings with potentially high amounts of contaminating enzymes, such as assays of minimally processed biological specimens or point-of-use field applications. PNA's stability can also be used to advantage in the design of simplified, rapid diagnostic tests which incorporate PNA probes with other assay components, such as sample preparation reagents, in order to consolidate and reduce steps in the assay procedure.

The majority of the commercial PNA probe products available today are designed for fluorescent in *situ* hybridization (FISH) assays. Dako was an early pioneer in the development of PNA-based tests, and in keeping with its pathology focus is using PNAs to enable novel cancer diagnostics. The first PNA probe diagnostic products on the market were Telomere PNA FISH Kit. These assays, originally conceived and developed by Peter Lansdorp's group at the Terry Fox laboratory of the British Columbia Cancer Reseach Center, use PNA probes to quickly and quantitatively visualize the lengths of the telomeric repeat sequences at the ends of each chromosome (2). The kits can be used to assess telomeres in humans and other vertebrate species using interphase nuclei, metaphase spreads, or flow cytometry preparations. Telomere length has been implicated as a critical regulator of a cell's capacity for division, and the PNA telomere assays have proven to be valuable tools for studying the relationship between telomere length and cancer, senescence, and other events that influence genetic longevity.

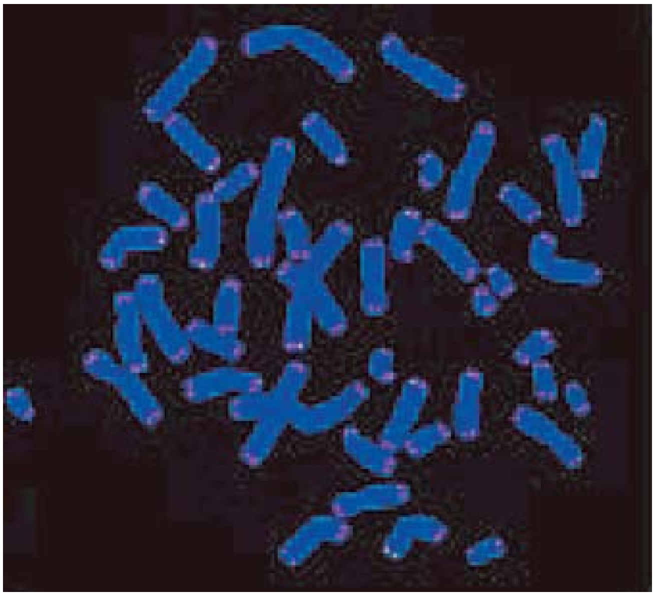


Figure 2. Metaphase spread of human lymphocyte stained with Telomere PNA FISH Kit/Cy3, Dako Code K5326.

More recently, PNAs have been incorporated into a line of cancer cytogenetic FISH probes, where they are used to enhance assay performance. Each of the FISH products, which include both the Split Signal and Sub-Deletion Signal categories of FISH probes, consists of two DNA fragments (labeled with green and red fluorophores, respectively) complementary to adjacent chromosomal regions that are susceptible to re-arrangement in hematological cancers.

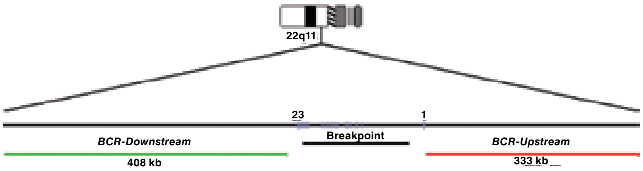


Figure 3. The human *BCR* gene consists of 23 exons spanning a region of 135 kb on chromosome 22 band q11. Y5403 is a probe mix is based on a combination of DNA and PNA technology, and contains two FISH DNA probes and unlabeled PNA blocking probes. The FISH DNA probes are a mixture of a Texas Red-labeled DNA probe (*BCR-Upstream*) covering 333 kb centromeric to the *BCR* breakpoint cluster region and a fluorescein-labeled DNA probe (*BCR-Downstream*) covering 408 kb telomeric to the *BCR* breakpoint cluster region.

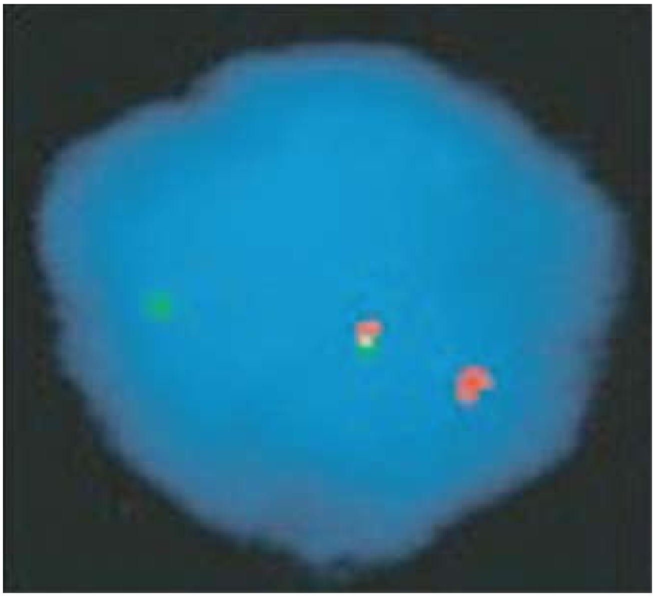


Figure 4. Precursor B-ALL with BCR gene translocation.

This line of FISH probes is used for the diagnosis of specific hematologic malignancies — the juxtaposition or separation of the red and green DNA probe signals in the interphase nuclei of cancer cells are indicative of gene re-arrangements that are characteristic of particular leukemia and lymphoma phenotypes. Gene-based testing in this setting can resolve diagnostic ambiguities that often confound traditional immuno-phenotyping methods, but early FISH assays required difficult, lengthy procedures which only a few highly specialized laboratories were able to perform. By incorporating PNA technology into the FISH hybridization probe mixture, it has been possible to simplify and shorten the FISH methodology and broaden its use among pathology labs. These unique probe mixtures contain several unlabelled PNAs that hybridize to the highly abundant repetitive (non-coding) DNA sequences found on human chromosomes. By quenching the non-specific signals from labeled probe binding that ordinarily plague such assays, the PNAs increase the signal-to-noise ratios of the assays and improve the specificity and sensitivity of these tests over what has previously been possible using traditional FISH technology, all in a single hybridization step. A comprehensive product line of two dozen FISH probe mixtures is available, covering the major hematological malignancies.

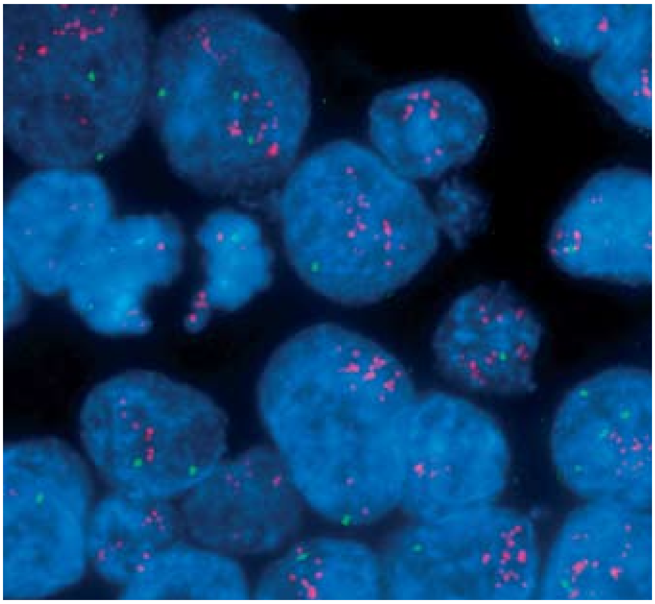


Figure 5. Breast carcinoma (FFPE) stained with HER2 FISH pharmDxTM Kit, Dako Code K5331. Tumor cells show HER2 gene amplification (HER2/ CEN-17 ratio ≥ 2).

PNA probe technologies are also employed in the pharmacoDiagnostic® line of FISH kits for determining *HER2* and *TOP2A* gene status in formalin-fixed, paraffin-embedded breast tumor sections. These assays are part of a growing arsenal of highly specific genetic tests which help guide pathologists and oncologists in the diagnosis, prognosis, and selection of treatment for breast cancer. Marketed as the *HER2* FISH pharmDx™ and *TOP2A* FISH pharmDx™ Kits, these assays employ PNAs as both unlabeled and labeled probes in a complex hybridization mixture. The unlabeled PNAs block repetitive sequences, while the fluorescein-labeled PNAs generate green signals that identify centromeric sequences on chromosome 17. The number of green PNA signals in each nucleus determines the copy number of chromosome 17 in the cells. Copy number is likewise obtained for the *HER2* or *TOP2A* gene regions using the red signals from the corresponding *HER2* or *TOP2A* DNA probe, and from the ratio of gene-to-chromosome 17 signals, a numerical value for gene amplification or deletion is derived. The *HER2* FISH pharmDx™ Kit is approved by the FDA as an aid in the assessment of breast cancer patients that are being considered for Herceptin® (trastuzumab) therapy. The *TOP2A* FISH pharmDx™ Kit is approved by the FDA as a marker of poor prognosis in high-risk breast cancer patients.

PNA probes have also been developed to RNA targets specifically for chromogenic ISH detection, with an aim toward improving upon immunohistochemical methods of detection on routinely processed histological sections. PNAs to the Epstein-Barr virusEBER RNAs and the human immunoglobulin light-chain Kappa and Lambda gene mRNAs are the most prominent examples of probes that used for routine bright-field ISH. The EBV EBER probe is a mixture PNAs that detect two nuclear RNA transcripts, EBER1 and EBER2, that are produced by the Epstein-Barr virus in latent infections, including conditions such as Burkitt's and Hodgkin's lymphomas, nasopharyngeal carcinomas, and mononucleosis. Since the EBER transcripts are not translated into protein, these unique analytical targets cannot be detected by antibody tests.

The detection of only one of either the kappa or lambda light chain mRNAs in the lymphoid cells of a suspected lymphoma tissue (light chain restriction) is indicative of the monoclonal populations that typify lymphoid malignancies. The use of PNA ISH technology for this application allows the light chain mRNAs to be visualized where they are synthesized in the cytoplasm — an improvement over immunophenotyping by IHC, which can suffer from high levels of background signal contributed by secreted antibodies in serum and interstitial fluids.

Significant advances in the implementation of PNA technology have also been made in other areas of medicine, including:

- Microbiology — Commercial assays are now available for identification of *Candida, Psuedomonas, Staphylococcus*, and *Enterococcus* sp. in smears made from blood cultures, and several tests for detection of specific genes associated with drug resistance in *Staphylococcus* aureus isolates are available in microwell format (3, 4).
- Genetic disease testing and research — The scientific literature contains descriptions of the uses and advantages of PNAs in nearly every molecular testing format, as applied to many different genetic diseases (5).
- Gene therapy — PNA has been used to manipulate gene expression in disease models by a variety of techniques including anti-sense, anti-gene, and transcription factor decoy approaches (6).

PNA technology has enabled several complex diagnostic methodologies to be greatly simplified and accelerated, resulting in robust and rapid tests that can be routinely performed in laboratories for many critical diagnostic situations. The compelling practical benefits of PNA probes are continuing to drive the development of new assays that overcome difficult diagnostic challenges and open new paradigms for patient diagnosis and treatment. The rigorous optimization and commercialization of standardized PNA assays will ensure that this promising technology continues to facilitate new diagnostic tests.

References

1. Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, et al. PNA Hybridizes to Complementary Oligonucleotides Obeying the Watson-Crick Hydrogen Bonding Rules. Nature 1993; 365:566-8.
2. Lansdorp P, Verwoerd N, van de Rijke F, Dragowska V, Little MT, Dirks R, et al. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet 1996; 5: 685-91.
3. Forrest GN. PNA FISH: present and future impact on patient management. Expert Rev Mol Diag 2007; 7:231-6.
4. Marketed by AdvanDx Inc.
5. Pellestor F, Paulasova P, Hamamah S. Peptide nucleic acids (PNAs) as diagnostic devices for genetic and cytogenetic analysis. Curr Pharm Des. 2008; 14:2439-44.
6. Karkare S, Bhatnagar D. Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. Appl Microbiol Biotechnol. 2006 ;71:575-86.

Chapter 19 | Controls

Ole Feldballe Rasmussen PhD

Many factors may introduce variations in immunohistochemistry: differences in tissue fixative and fixation time, day-to-day variations due to temperature, variations due to different workers' interpretations of protocol steps or in the conditions of reagents applied on a particular day.

Most diagnostic reagents suppliers have implemented measures to safeguard the quality of their reagents. However, many factors may influence an immunohistochemical staining, so it is not always sufficient to assume that any given staining is correct. It is therefore important to include reagent and tissue controls for verification of immunohistochemical staining results for in vitro diagnostic use. It is also important to understand what information a given control can provide or not provide. This chapter will describe the range of controls that should be adapted in a diagnostic laboratory.

Reagent Controls

The most important reagent in immunohistochemistry is the primary antibody. Without good specificity of the primary antibody the IHC stain will be jeopardized. In addition to the manufacturers' quality guarantee, it is important for the user to ensure the quality of the primary antibody prior to its use.

During development, most manufacturers ensure specificity using a range of immunochemical techniques. These may include immunoelectrophoresis, Western Blot, double diffusion, rocket immunoelectrophoresis and ELISA. Testing on transgenic cells expressing the specific as well as closely related antigens may also be performed. It is, however, imperative to test the primary antibody in immunohistochemistry. In general, manufacturers first test antibodies on a range of positive tissues to identify optimal antibody dilution in combination with chosen staining protocols. Next, immunohistochemistry testing is extended to an expanded panel of additional tissues known to either contain or not contain targeted antigens. For new antibody lots, manufacturers typically perform quality control to ensure specificity and sensitivity documented during development.

Users must control reagents within routine quality programs, documenting reagents, dilutions, diluents, incubation times and dates to which any procedural changes are introduced by proper record keeping. In laboratories with changing environmental conditions, it is also advisable to keep track of the relative humidity and temperature.

Negative Controls

For monoclonal primary antibodies, nonspecific negative reagent controls may be developed by different methods. The optimal method is an antibody of the same isotype, present in the same immunoglobulin concentration, using the same diluent and exhibiting no specific reactivity with the given human tissues tested. A less optimal alternative is to use mixtures of antibodies representing all or most relevant IgG subtypes. Finally, the diluent itself may also be used as an alternative which, however, is neither efficient nor desirable.

For polyclonal antibodies, negative reagent controls should be a dilution of immunoglobulin fractions or whole serum of normal/non-immune serum of the same animal source. Again, the negative reagent control should be applied in the same concentration as the test antibody, and the same diluent should be used.

Using the same protocol as the primary antibody, the negative reagent control should be applied to a sequential section of each patient specimen, to evaluate nonspecific staining in that particular tissue.

NOTE: A special situation to be aware of is when two or more antibodies are applied to serial sections. In this case, negative stain areas of one slide may serve as the negative/nonspecific binding background control for other antibodies.

In cases where it is necessary to evaluate non-specific binding potentially caused by sources other than the primary antibody, additional patient tissue sections may be stained with selected reagents. For example, tissues may be stained with just the secondary antibody and/or the enzyme followed by application of the substrate/chromogen. In cases where the suspected non-specific staining may be the result of endogenous enzyme present within the tissue, this can be confirmed by application of the substrate/enzyme only.

Tissue Controls

Tissue controls can be negative, positive, or internal. Each serves a different purpose.

Positive Tissue Controls

These are indicative of proper staining techniques and provide a measure of whether the target retrieval procedure has been carried out correctly. They should assess correct temperature and incubation period of water baths or other retrieval methods. Likewise, positive tissue controls verify that all reagents were applied, that they performed correctly, and the proper incubation time and temperature were used.

These controls are also indicative of properly prepared tissue. To be as accurate as possible, positive tissue controls should be prepared in the same manner as patient samples. Optimally autopsy/biopsy/surgical specimens should be fixed, processed and embedded as soon as possible for best preservation of antigens. Please see the section, Processing Control Indicator below.

One positive tissue control should be included for each set of tests. Ideally this control should contain a spectrum of weak to strongly positive reactivity. If such tissue is not available, another option is to select a weakly positive tissue, as this provides the best basis to evaluate whether a particular staining reaction is too weak or too strong.

During a staining run, positive tissue controls may be run on a separate slide, or included on the same slide as the test specimen. If this second option is chosen, one method is to use small arrays with selected tissue or cell lines to serve as a positive control for a range of stains. In this method, one tissue may serve as a positive control, a different tissue may serve as a negative control (see below).

If positive tissue controls do not perform as expected, results, from test specimens should be considered invalid.

Positive controls cut and stored in bulk with cut surfaces exposed for extended periods should be tested to determine if the antigens are stable under these storage conditions.

Internal Tissue Controls

Internal controls, also known as “built-in” or intrinsic controls, contain the target antigen within normal tissue elements, in addition to the tissue elements to be evaluated. Thus, they can replace external positive controls. This is ideal, as the tissue elements to be evaluated have been treated exactly as the positive control. One example of a “built-in” control is the presence of S-100 protein in both melanoma and normal tissue such as peripheral nerves and dendritic reticulum cells. Other examples include vimentin which is distributed ubiquitously, and desmin which is present in blood vessel musculature.

Negative Tissue Controls

Positive staining of negative controls could indicate a lack of specificity of the antibody or nonspecific background staining. Just as in positive controls, tissue used for negative controls should be prepared in the same manner as the patient sample. Additionally, the selected tissue should not contain the specific antigen to be tested. One example would be use of normal liver tissue s as a control for hepatitis B surface antigen, or a HER2/*neu*-negative tissue for testing with Dako HercepTest™ Kit.

If positive staining occurs in the negative tissue control, consider test specimen results invalid.

Processing Control Indicator

There is currently no optimal way to evaluate whether tissue processing has occurred satisfactorily. Battifora (1) has suggested using vimentin, a substance present in virtually every tissue specimen. Furthermore, the vimentin V9 clone recognizes an epitope that is partially susceptible to fixation with formaldehyde and can function as a “reporter” for the quality of fixation. Proper processing should give uniform distribution of vimentin reactivity in tissue vessels and stromal cells. Good, uniform vimentin staining demonstrates adequate fixation, while heterogeneous staining indicates variable and sub-optimal fixation. In these cases only fields demonstrating the best staining intensity and homogeneity should be used in the patient sample analysis.

Cell Line Controls

Several FDA-approved predictive IHC assays contain cell line controls as part of the diagnostic kit or are sold separately. These are specifically developed to monitor staining of the antigen of interest, and should be included in all stain runs as an additional protocol control.

Just as with tissue controls, cell line controls may be positive or negative. Positive cell line controls monitor staining performance by assessing target retrieval, blocking, antibody incubation and visualization. Negative cell line controls assess specificity and, depending on the characteristics of the chosen cell line, may also provide information on performance.

An ideal negative cell line control will contain an amount of target antigen, sufficiently low to produce no staining if the procedure has been performed correctly. At the same time, the amount should be sufficiently high to produce a weakly positive stain if the run has been performed under conditions that produce an excessively strong stain.

An ideal positive cell line control would contain a number of target antigens producing staining of medium intensity. This would allow the control to assess both stains that are too weak and stains that are too strong.

An example of the way in which cell line controls can be used is illustrated by Dako's HercepTest™ kit, which contains three cell line controls: a negative, a 1+ (weak staining) and a 3+ (strong staining). All are designed to be placed on the same microscope slide.

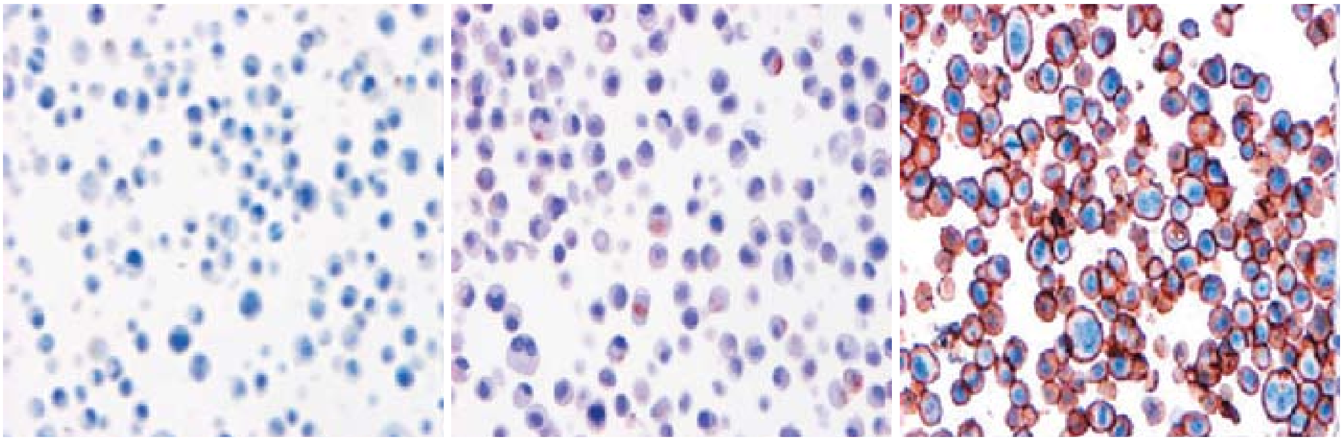
Figure 1 below shows examples of staining HercepTest™ control cells. Fluorescent in situ hybridization kits such as *HER2* FISH pharmDx™ do not contain cell line controls because they include probes against both the target gene and the respective centromere, in order to evaluate the amplification ratio (or deletion) of the target gene. In this way, the centromere probe also serves as internal control.

Control Programs

Immunohistochemical stain test results have no common quantitative measures. Instead, results are typically based on subjective interpretation by microscopists of varying experience (2-4). Quality control and assurance therefore remain crucial, and need high attention by both manufacturers and laboratory users.

A number of scientific bodies have quality programs or quality assessment services. These programs should be seen as an aid or external assistance and should never replace national requirements for internal quality control.

One highly regarded program is the non-profit United Kingdom National External Quality Assessment Service (UK NEQAS), established in 1995 to "advance education and promote the presentation of good health by providing external quality assessment services for clinical laboratories." UK NEQAS includes a range of specific external quality assessment (EQA) services, each focusing on areas such as breast screening pathology.



Negative Cell Line

1+ Cell Line

3+ Cell Line

Figure 1. Cell line controls for Dako HercepTest™.

Likewise, in the United States the College of American Pathologists (CAP) provides a similar proficiency testing service for its member laboratories.

Also the external quality assessment program run by Nordic Immunohistochemical Quality Control (NordiQC), established in 2003, should be mentioned. It now has more than 100 laboratories participating.

Each organization provides proficiency testing in IHC for participating laboratories by sending out tissue samples to be included as part of a laboratory's routine IHC staining procedures. Laboratories then return their results, which are compared with all other participating laboratories and summarized in a final report.

Contact information for CAP can be found at the Web site, www.cap.org, for UK NEQAS at the Web site, www.ukneqas.org.uk, and for NordiQC at the Web site, www.nordiqc.org.

Future Aspects

To ensure quality diagnosis, immunohistochemistry quality control will become even more important. It is expected that the next five years will see increased participation in proficiency testing, as an increase in the number of laboratories that become accredited. New technologies on the horizon will also likely facilitate more efficient means of quality control.

References

1. Battifora H. Am J Clin Pathol 1991;96:669-71.
2. Taylor CR. Applied Immunohistochem 1993;1:232-43.
3. Elias JM et al. Am J Clin Pathol 1989;92:836-43.
4. Taylor CR. Arch Pathol Lab Med 2000;124:945-51.

Chapter 20 | Virtual Microscopy and Image Analysis

Kenneth J. Bloom MD (Revised by Joachim Schmid PhD and Mark Verardo PhD)

The History

Automating manual microscopy has been evolving since the first demonstration of telepathology in 1968. The concept has evolved along two distinct pathways, one based on technology, the other on need. The technology field focused on adapting new knowledge developed for other disciplines in an effort to prove that automated microscopy was possible. Improvements in digital camera resolution, speed and fidelity, the invention of the robotic microscope and stage, the development of the internet and the exponential advancements in computer technology including processor speed, memory and storage have contributed to eliminating the hurdles that prevented a viable automated microscopy system. The second group focused on resolving problems in anatomic pathology, such as rapid second opinion, pathology staffing of remote frozen sections, cost reduction for reference laboratories, medical student and resident teaching, continuing medical education, and improved storage and retrieval of slides — to name just a few (1-7).

The concept of developing a functional robotic telepathology network, with the aim of providing real-time expert opinions for frozen sections and difficult cases was put forth by Ronald Weinstein in the mid 1980s (8, 9). All of the performance studies supported the feasibility of telepathology, and a demonstration project for the U.S. Department of Defense showed how slides prepared in El Paso, Texas could be read remotely via satellite, at the Armed Forces Institute of Pathology in Washington, D.C. Although the prototype was successful, there were many limitations to commercializing a telepathology system, most involving a lack of telecommunication infrastructure and standards. But just as importantly, there was little perceived need for the technology. Since it was not cost effective to purchase a satellite, cheaper alternatives were sought, and the concept of "static telepathology" was introduced as an alternative. In static telepathology, a pathologist captures and saves a digital image or series of images from a camera mounted on a microscope, and then forwards the images to a remote computer, where they may be reviewed by a second pathologist. E-mail and servers were readily available to facilitate the transfer of images and standards in image formats were evolving rapidly; however, the static nature of the images severely limited clinical use. To fully represent a standard pathology slide, it would be necessary to acquire thousands of static images, making routine use impractical. Although few pathologists used these systems diagnostically, many pathologists use them for tumor boards, teaching and other educational purposes.

The next step in the evolution was the creation of "stitching software." This technology allowed the digital representation of an entire microscopic slide by digitizing individual microscopic fields and then stitching them together to create a virtual slide. This process was laborious and time consuming, and the computer processing and storage requirements pushed the limits of available technology. Because of these limitations, the next systems that were created were hybrids, containing elements of both static and dynamic systems. These systems would digitize an entire slide at low power magnification creating a tissue map for the pathologist to select areas of interest, which would then be re-digitized at higher magnification and forwarded as a series of static images.

Scanning the Slide

The first step in virtual microscopy is to obtain a digital representation of a pathology slide. Before scanning the slide, the system must first establish a focal plane. The scanned image or images are saved as two-dimensional digital files, however, pathology slides and the tissue on pathology slides are three-dimensional structures. With a low power magnification lens (for instance a 5X lens), scanning a single focal plane is often sufficient, for capturing this three-dimensional tissue, but with higher magnification (for example a 40X) lenses, the depth of focus is shallow, necessitating that the scanning system have the ability to automatically focus on, select and capture one focal plane. All systems have the ability to autofocus; however, they all do it in a slightly different manner. A second camera can be used to monitor and adjust the focus continuously as the slide is scanned. In addition, a map of the tissue can be made available on the slide to be scanned and "points of focus" can be created. Then as the slide is scanned the system performs an autofocus at each calculated focus point. In this way, systems may overcome problems such as variations in thickness of the tissue or tissue folds.

The process of scanning a slide also differs between various systems. All processes involve acquiring images in some fashion and then stitching them together to create a representation of the slide. Some systems acquire images as tiles, while other systems use other methods such as “linear scanning,” or using an array of lens (10).

Scanning systems generally are judged by two criteria: speed and resolution. The total speed of acquisition involves not only acquiring the image, but can also involve stitching the images together and storing the resulting image on a computer. Since the purpose of acquiring a virtual slide is to view it in the future, some additional processing is often implemented to facilitate this viewing process. For example, to implement the full functionality of a standard light microscope, the scanned image must be viewable at the same objective magnifications found on a standard microscope, namely, 1x, 2.5x, 5x, 10x, 20x and/or 40x. Assuming the slide was scanned, stitched and stored at the equivalent of a 40x objective, viewing the 40x image would not require additional processing. However, to view the image at the equivalent of a 5x objective, the 5x image would have to be derived from the 40x image before it was displayed. This would result in a significant delay between the time an area was selected and the time it was displayed on a monitor. To overcome this delay, the acquired image is processed to a format where the intermediate magnification levels have already been calculated and stored. While this format, known as a pyramidal format, decreases the time to load intermediate magnification views, it increases the image processing necessary following image acquisition and produces a larger file to be stored (11).

Figure 1. Automated Cellular Imaging System (ACIS® III), an example of an image acquisition and image analysis instrument.

The ability of the image acquisition system to resolve features present in the microscopic slide is known as the absolute or, “point-to-point resolution” and is dependent on the microscope objectives, the camera lens and the analog to digital conversion process. What is more important is the actual resolution, which is also dependent on hardware and software compression techniques, the video card and monitor used to display the image. Many commercial digital cameras, for example, have chips that automatically “clean” the image by performing tasks such as edge sharpening.

Viewing the Virtual Slide

Virtual slides are a digital representation of a glass slide which can be viewed remotely. There is currently no accepted standard for viewing virtual slides. Each hardware vendor has created software for viewing the image files that they create. All software programs perform similar tasks such as changing the apparent objective, moving the slide in any direction, saving screenshots as image files, and annotating specific areas of the virtual slide.

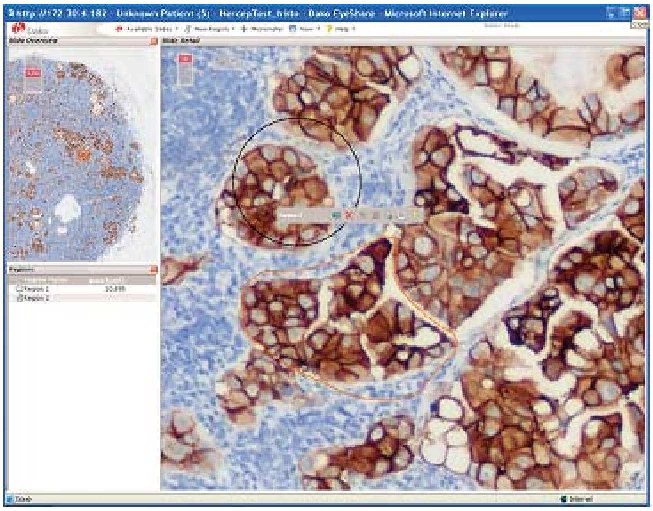

http://172.30.4.182 - Unknown Patient (5) - HercepTest_histo - Dako EyeShare - Microsoft Internet Explorer

Figure 2. An example of a virtual slide shown in a web interface. Specifically, this image is from the EyeShare software.

Applications for Virtual Microscopy in immunohistochemistry include, but are not limited to:

- Image analysis
- Telepathology
- Obtaining a rapid second opinion
- Education and
- Quality assurance

Capturing Images for Image Analysis

Collecting an image for subsequent analysis has a few more requirements than systems that obtain images for viewing only. Since the image is going to be analyzed, procedures must be put in place to ensure that the image is captured reproducibly, and that the system is operating in the detectable range for the image analyzed. For example, if an image is acquired for quantifying the amount of HER2/*neu* protein, then the same image must be produced whether the slide is scanned today, tomorrow or six months from now. The reproducibility of such an image depends on a number of factors such as stage and camera drift over time, fluctuation/variation in the light source and variation in the analog-to-digital conversion process. In addition, complexity may be introduced by using different objective magnifications. To avoid these problems calibration needs to be performed on a regular basis.

All light sources can drift, and the rate of drift can be significant enough to require daily calibration. Care must be taken to perform this calibration at an intensity that will not saturate any channel, because saturation of the channels can give rise to a false appearance of uniformity.

Systems used for image analysis therefore must undergo calibration prior to use. At a minimum, the calibration must adjust for all of the aforementioned variables, and also include a set of calibrators that help determine the minimal amount of chromogen that can be detected; the maximal amount of chromogen that can be detected before saturation of the signal occurs; and help establish that the system can detect the specific chromogen in a linear fashion over the reportable range. This is identical to the sort of calibration routinely performed on equipment in a clinical pathology laboratory.

One way to calibrate a system is to use a set of slides that test several of these variables. An example of a set of calibration slides is shown below.

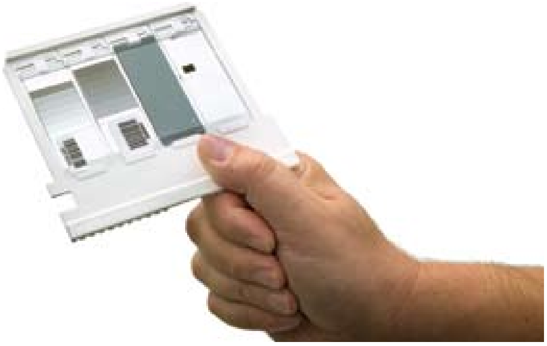


Figure 3. An example set of calibration slides.

The systems for illuminating a glass slide do not provide truly uniform illumination. Unless corrected for, this will cause algorithms to give different results in different parts of the field of view. A system must collect calibration images of known-to-be-stable, dust-free, and blank fields to have enough data to correct the captured image to that of a flat field. Since the calibration will be different at each level of magnification, this data must be collected for each objective.

Image Analysis

Image analysis represents a significant step in standardizing the interpretation of slides in pathology (12). The creation of an image analysis application is not an easy task. Just as it takes considerable time and effort to create and validate a pharmacodiagnostic assay, so too does it take significant effort to create and validate a clinically useful image analysis system. These systems are meant to complement pathologists, not replace them. Accordingly they should not attempt to emulate what pathologists can do well, but rather they should aid pathologists in tasks that the human eye does not do well. The pathologist is very good at selecting relevant areas on the slide using morphology; the image analysis system is very good at quantifying intensities and percentages.

Additionally, the principle of garbage-in, garbage-out cannot be overstated. There is no point in attempting to quantify IHC slides unless one is sure that the immunohistochemical procedure has been produced in a reproducible fashion. Automation and standardization in the process of preparing the IHC slides are very important components for successful image analysis.

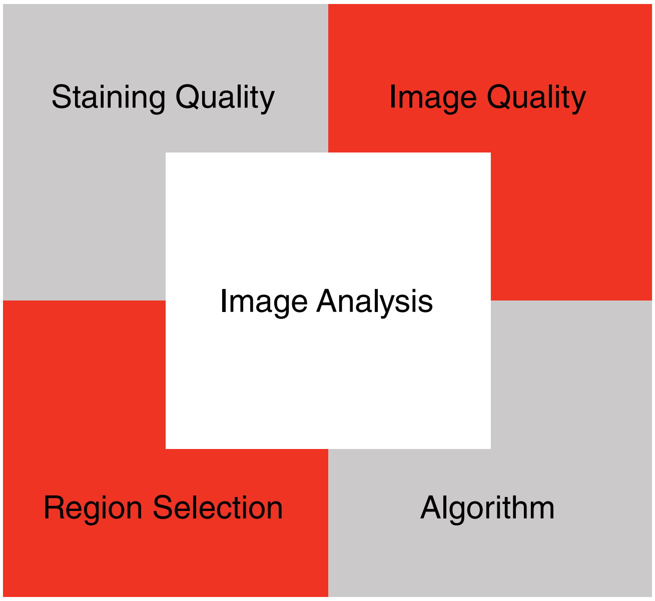


Figure 4. A diagram showing the importance of staining quality, image quality, the algorithm, and region selection in image analysis. See text for explanation.

As shown in the figure above, the staining quality, image quality, algorithm and region selection are all necessary and important pieces for image analysis. Staining quality can be standardized through the use of automated staining with defined staining protocols. Additionally, linking specific pre-treatment, antibody, and staining protocols to an image analysis is key for a consistent result. Having consistent, standardized staining is necessary for successful image analysis because differences in staining lead to differences in acquired digital images thereby affecting image analysis. The importance of image quality is described above. The algorithm needs to be developed to produce results that are appropriate for the stained slide that is imaged. The region selection relies on the Pathologist’s expertise. Areas that are to be evaluated (such as the tumor regions) need to be selected for the image analysis to be relevant. Thus, image analysis can be considered an aid to the Pathologist.

In summary, virtual microscopy is an import new technology that already has penetrated pathology education significantly. With recent improvements in the technology, other uses are sure to follow quickly. If performing image analysis is an important consideration, care must be taken to select acquisition systems that have calibrators appropriate for the type of analysis to be performed (13).

Applications for Image Analysis in Pathology

- Her2 quantification
- ER/PR quantification
- DNA Ploidy quantification
- Ki67 quantification
- p53 quantification

References

1. Demichelis F, Della Mea V, Forti S, Dalla Palma P, Beltrami CA. Digital storage of glass slides for quality assurance in histopathology and cytopathology. J Telemed Telecare 2002;8:138-42.
2. Harris T, Leaven T, Heidger P, Kreiter C, Duncan J, Dick F. Comparison of a virtual microscope laboratory to a regular microscope laboratory for teaching histology. Anat Rec 2001;26 5:10-4.
3. Heidger PM Jr, Dee F, Consoer D, Leaven T, Duncan J, Kreiter C. Integrated approach to teaching and testing in histology with real and virtual imaging. Anat Rec 2002;26 9:107-12.
4. Helin H, Lundin M, Lundin J, Martikainen P, Tammela T, van der Kwast T, Isola J. Webbased virtual microscopy in teaching and standardizing Gleason grading. Hum Pathol 2005;36 :381-6.
5. Kumar RK, Velan GM, Korell SO, Kandara M, Dee FR, Wakefield D. Virtual microscopy for learning and assessment in pathology. J Pathol 2004;204:613-8.
6. Marchevsky AM, Wan Y, Thomas P, Krishnan L, Evans-Simon H, Haber H. Virtual microscopy as a tool for proficiency testing in cytopathology: a model using multiple digital images of Papanicolaou tests. Arch Pathol Lab Med 2003;127 :1320-4.
7. Okada DH, Binder SW, Felten CL, Strauss JS, Marchevsky AM. “Virtual microscopy” and the internet as telepathology consultation tools: diagnostic accuracy in evaluating melanocytic skin lesions. Am J Dermatopathol 1999;21 :525-31.
8. Weinstein RS: Prospects for telepathology. Hum Pathol 1986;17:433-4.
9. Weinstein RS, Bloom KJ, Rozek LS: Telepathology and the networking of pathology diagnostic services. Arch Pathol Lab Med 1987;111:646-52.
10. Weinstein RS, Descour MR, Liang C, Barker G, Scott KM, Richter L, et al. An array microscope for ultrarapid virtual slide processing and telepathology. Design, fabrication, and validation Study. Hum Pathol 2004;35:1303-14.
11. Glatz-Krieger K, Glatz D, Mihatsch MJ. Virtual slides: high-quality demand, physical limitations, and affordability. Hum Pathol 2003; 34:968-74.
12. Bloom K, Harrington D. Enhanced Accuracy and Reliability of HER-2/neu Immunohistochemical Scoring Using Digital Microscopy. Am. J. Clin Pathol. 2004; Vol. 121, 620-630.
13. Glatz-Krieger K, Spornitz U, Spatz A, Mihatsch M, Glatz D. Factors to keep in mind when introducing virtual microscopy. Virchows Arch 2006;448:248-55.

Chapter 21 | Troubleshooting

Karen N. Atwood and Dako Technical Support Group

Immunohistochemistry is a multi-step process that requires specialized training in the processing of tissue, the selection of appropriate reagents and interpretation of the stained tissue sections. In general, IHC staining techniques allow for the visualization of antigens by sequential application of a specific antibody to the antigen, a secondary antibody to the primary antibody, an enzyme complex and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Because of its highly complex nature, the causes of unexpected negative reactions, undesired specific staining or undesired background could be difficult to isolate. The information contained in this chapter should enable you to rapidly pinpoint and resolve problems encountered during the staining procedure.

Section One is a compilation of common problems encountered when using immunohistochemical-staining reagents, the underlying causes of staining failure and recommended corrective actions. The chart is divided into sections describing little or no staining, general background staining and limited background staining.

Section Two presents a method of systematically adding one IHC reagent at a time to determine at which stage non-specific or undesired staining may be occurring in a peroxidase, streptavidin-biotin staining system.

Section Three is a simple chart used to define the type of tissue specimen, the IHC staining and ancillary reagents already in place in the laboratory, and the staining protocol used by the laboratory personnel. You are encouraged to copy this chart and use it to help troubleshoot any problems you may encounter with your staining systems.

Section Four is a guide to reading a manufacturers' specification sheet for IVD antibodies. This includes general information for use in immunohistochemistry including fixation, recommended visualization systems, recommended titer and diluent, pretreatment, and selection of required controls.

Section One

Inadequate Staining

Little or no staining of controls or specimen tissue, except for counterstain.

May show little or no background staining.

Possible Cause	Solution	See Page
Primary antibody or labeled reagent omitted. Reagent used in wrong order.	Repeat the procedure using the manufacturer's staining system specification sheet or standard operating procedure reagent checklist as established by the individual laboratory.	57-60
Excessively diluted or excessively concentrated reagents; inappropriate incubation time and temperature.	Determine correct concentration for each reagent. Depending on the degree of staining obtained, if any, a 2- to 5- fold change in concentration may be needed. Incubation temperature and incubation time are inversely proportional and will affect results. To determine optimal incubation protocol, vary either the time or temperature for each reagent in the IHC staining system. Generally, incubation times can be extended if little or no background was detected.	7, 11-12
Primary antibody diluted with inappropriate buffer. Use of PBS or TBS as an antibody diluent. Lack of stabilizing or carrier protein. Detergent in diluent.	Check formula and compatibility of antibody diluent. A change of ion content and/or pH of the antibody diluent can cause a diminution in the sensitivity of the antibody. Addition of NaCl should be avoided. This problem is primarily seen with monoclonal antibodies.	57-60
Primary antibody defective; one or several secondary or ancillary reagents defective. Do NOT use product after expiration date stamped on vial.	Replace defective or expired antibody; repeat staining protocol, replacing one reagent at a time with fresh, in-date reagents. ▪ Store products according to each product specification sheet or package insert. ▪ If using a neat or concentrated antibody, and directed by the manufacturer to store frozen, it may be aliquoted to avoid repeated freezing and thawing. ▪ For freezing, use a -70 °C to -80 °C freezer or a non frost free -20 °C freezer. A frost free freezer will cycle on and off which will cause damage to the antibody¬. ▪ Do not freeze ready-to-use or customer diluted products. ▪ Follow manufacturer recommendations on product specification sheets, package inserts and reagent labels.	7-8
Dissociation of primary antibody during washing or incubation with link antibodies	A feature of low affinity antibodies: ▪ Polyclonal primary antiserum: Attempt staining at low dilutions. ▪ Monoclonal primary antibody: Replace with higher affinity antibody of identical specificity. ▪ Re-optimize incubation times for washing buffer and link antibody.	5-6
Use of alcohol-based counterstain and/or alcohol-based mounting media with aqueous–based chromogens.	▪ Repeat staining, using water-based counterstain and mounting media. ▪ Use a permanent chromogen, such as DAB/DAB+, that is not affected by organic solvents.	16-17
Excessive counterstaining may compromise proper interpretation of results.	Use a counterstain that: ▪ Will not excessively stain tissue sections. ▪ Can be diluted so as not to obliterate the specific signal. ▪ Reduce incubation time of the counterstain.	115-121
Incorrect preparation of substrate-chromogen mixture.	▪ Repeat substrate-chromogen treatment with correctly prepared reagent. ▪ Staining intensity is decreased when excess DAB/DAB+ is present in the working reagent.	Specification Sheet
Incompatible buffer used for preparation of enzyme and substrate-chromogen reagents: Use of PBS wash buffer with an alkaline phosphatase staining system. Sodium azide in reagent diluent or buffer baths for immunoperoxidase methodologies.	Check compatibility of buffer ingredients with enzyme and substrate-chromogen reagents. Repeat staining. ▪ Commercial phosphate buffers may contain additives that will inhibit alkaline phosphates activity. ▪ Avoid sodium azide in diluents and buffers. A concentration of 15mM/L sodium azide, which is routinely added to IHC reagents to inhibit bacterial growth, will not impair HRP conjugated labels.	57-60

Possible Cause	Solution	See Page
Antigen levels are too low for detection by the employed visualization system. May be due to loss of antigenic differentiation in some tumors or loss of antigenicity due to sub-optimal tissue fixation.	▪ Utilize a higher sensitivity staining system. ▪ Prolong incubation time of primary antibody. ▪ Re-optimize incubation times and concentrations of ancillary reagents. ▪ Perform antigen retrieval, if applicable, using a range of pH buffers.	57-60
Steric hindrance due to high antigen level and possible prozone effect.	Re-optimize concentration of the primary antibody and ancillary reagents. Antibody concentration of the primary antibody may be too high.	57-60
Use of inappropriate fixative. Use of certain fixatives may damage or destroy antigens or epitopes in the tissue specimen. Use of non-cross linking fixatives may allow the elution of antigens soluble in IHC reagents.	Check manufacturer's specifications regarding recommended fixative.	29-33
Different fixatives may affect standardization		29-33, see also 21-28
Immunoreactivity diminished or destroyed during embedding process.	Use a paraffin wax with a melting temperature ~ 55-58 °C. Wax used for embedding should not exceed 60 °C.	29-33
Immunoreactivity diminished or destroyed during dewaxing at high oven temperature.	Oven temperature not to exceed 60 °C. NOTE: The intensity of immunostaining may be diminished when tissue is exposed to prolonged heat. Refer to the primary antibody specification sheet for additional information.	29-33
Immunoreactivity diminished or destroyed on pre-cut tissue sections.	The intensity of immunostaining may be diminished when pre-cut tissue sections are exposed to air. Use freshly cut sections and reseal paraffin-embedded blocks.	29-33
Immunoreactivity diminished or destroyed by the enzyme blocking reagent altering a specific epitope.	More common on frozen sections: apply the primary antibody prior to the enzymatic block to insure its reaction. In such cases the blocking reagent can be applied at any point after the primary and before the enzyme labeled components.	29-33, 115
Excessive wash buffer or blocking serum remaining on tissue section prior to application of IHC reagents.	Excess reagent will dilute the next consecutive reagent. Repeat staining, making sure to wipe away excess washing buffer and blocking serum.	11-13
Demasking protocol is inappropriate or has been omitted.	Some tissue antigens require proteolytic enzyme digestion or heat induced antigen retrieval performed prior to staining. The need for pretreatment depends on the type and extent of fixation, specific characteristics of the antigen and the type of antibody used. Use the pretreatment method recommended by the manufacturer. No single pretreatment is suitable for all applications.	29-33, 51-65
Repeated reuse of antigen retrieval buffer.	Do not reuse buffer.	Specification Sheet
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol, using fresh xylene or xylene substitute.	115-121
Failure to achieve the optimal temperature required for heat induced antigen retrieval.	▪ When using a waterbath or steamer, allow sufficient time for the retrieval buffer to equilibrate to a temperature range of 95-99 °C. ▪ At high altitude (greater than ~4,500 feet), the buffer will boil at less than 95 °C. ▪ Utilize a closed heating system such as a pressure cooker, autoclave or Pascal, or utilize a low temperature protocol if standardization of the validated procedure is not affected.	51-65
Excessive or incomplete counterstaining.	Re-optimize concentration of counterstain and incubation time.	51-65
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specifications.	Specification Sheet

Positive control tissue shows adequate specific staining with little or no background staining. Specimen tissue shows little or no specific staining with variable background staining of several tissue elements.

Possible Cause	Solution	See Page
Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants due to aldehydes cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.	51-65
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor.	Serum proteins diffuse through tissue and are fixed in place. Re-cut tissue using sharp blade.	115-121
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	51-65
Section portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration. Unfixed tissue tends to bind all reagents nonspecifically.	29-33, 115-121, see also 51-56

General Background

Background seen in all control tissue and specimen tissue. May see marked background staining in several tissue elements such as connective tissue, adipose tissue and epithelium.

Possible Cause	Solution	See Page
Excessive incubation with substrate-chromogen reagent.	Reduce incubation time.	Specification Sheet
Substrate-chromogen reagent prepared incorrectly.	Repeat incubation with correctly prepared chromogen reagent.	Specification Sheet
Secondary or link antibody cross-reacts with antigens from tissue specimen.	Absorb link antibody with tissue protein extract or species-specific normal serum from tissue donor.	57-60
Secondary or link antibody and /or tertiary reagents too concentrated.	Repeat staining. Determine correct concentration for each reagent. Incubation temperature and incubation time will affect results. To determine optimal incubation protocol, vary both the time and temperature for each reagent in the IHC staining protocol.	11-13
Slides inadequately rinsed.	Gently rinse slide with wash buffer bottle and place in wash bath for 5 minutes. Gentle agitation of the wash bath may increase effectiveness when used with cytoplasmic or nuclear staining protocols.	5-6
Insufficient saline or detergent in wash buffer.	High-sensitivity staining systems may require higher concentrations of saline or detergent in the wash buffer. Refer to the staining system specification sheet for optimal formulation.	115-121
Blocking serum or wrong blocking serum used.	Block with serum from the host of the secondary or link antibody. Avoid serum that contains auto-immune immunoglobulins. Alternatively, a serum-free protein block, lacking immunoglobulins, may be substituted for the serum block.	115-121
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol using fresh xylene or xylene substitute.	115-121
Non-specific binding of the secondary antibody with an animal tissue specimen.	Use a secondary antibody that has been absorbed against a species specimen, or use a secondary antibody produced in a host that exhibits little or no cross-reactivity with the tissue source.	57-60, see also 115-121
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.	Specification Sheet

Specimen tissue and negative reagent control slides show background staining. Positive and negative control tissue show appropriate specific staining. May involve several tissue elements such as connective tissue, adipose tissue and epithelium.

Possible Cause	Solution	See Page
Specimen held for too long in a cross-linking fixative, usually in formalin, causing “masking” of antigenic determinants due to aldehydes cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.	29-33
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue. Unfixed tissue tends to bind all reagents nonspecifically.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration.	29-33
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Serum proteins diffuse through tissue and are fixed in place. Re-cut tissue using sharp blade.	115-121
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	115-121
Excessive or unevenly applied subbing agent on poly-L-lysine, charged, or silanized slides.	Some IHC reagents may bind to these products, resulting in a light stain over the entire slide surface. Some slides may be unevenly coated, and will exhibit the above problems on only a portion of the tissue or glass.	115-121
Antigen diffusion prior to fixation causing specific background outside the expected antigen site.	Avoid delays in fixation of the tissue.	115-121
Tissue sections too thick.	Cut tissue sections thinner. Formalin-fixed paraffin-embedded tissue sections should be approximately 4-6 μm; cryostat section <μm.	29-33

Negative reagent control slide shows background. Positive control tissue, negative control tissue and specimen tissue show expected specific staining.

Possible Cause	Solution	See Page
Negative control serum insufficiently diluted.	Use properly diluted negative reagent control serum ▪ For polyclonal antibodies, dilute the negative reagent control serum until the protein concentration is equal to that of the primary antibody. ▪ For monoclonal antibodies, dilute the negative reagent control serum until the Ig concentration is equal to that of the primary antibody.	127-130
Contaminating antibodies in the negative control serum are cross-reacting with proteins from the specimen tissue.	Replace the negative reagent control serum; repeat staining protocol.	127-130
Negative reagent control serum contaminated with bacterial or fungal growth.	Replace product with non-contaminated serum.	8-9

Limited Background

Areas of inconsistent staining on controls, specimens and glass slides.

Possible Cause	Solution	See Page
Protein trapped beneath the tissue during the mounting process will allow partial lifting of the section. Pooling of IHC reagents beneath the section, or partial detachment of the tissue from the slide may occur.	Avoid the use of commercial adhesives, glue starch or gelatin in water baths when mounting tissue sections. Avoid allowing water from an initial section mounting to flow over an area where additional sections will be mounted. This is particularly important when using charged or silanized slides.	51-65, 115-121
Undissolved granules of chromogen.	Insure that chromogen in tablet or powder form is completely dissolved, or switch to a liquid chromogen.	115-121
Incomplete removal of embedding medium.	Remove embedding medium thoroughly, using fresh reagents	115-121
Incomplete dezenkerization* of tissue fixed with B5 or mercury containing reagents.	Perform dezenkerization with fresh reagents.	29-33
Bacterial or yeast contamination from mounting waterbath.	Clean and refill waterbath.	115-121
Partial drying of tissue prior to fixation. Unaffected areas show normal staining.	▪ Immerse tissue promptly in fixative or holding reagent. ▪ Keep moist during the entire staining process. ▪ Use a humidity or moist chamber during incubation steps. ▪ When using an automated staining instrument, addition of wet towels to the sink may prevent drying of slides.	115-121
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.	Specification Sheet

Adipose or connective tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Background in connective and epithelial tissue.

Possible Cause	Solution	See Page
Hydrophobic and ionic interactions between immunoglobulins and lipoid substances in fatty tissue.	Nonspecific staining of fatty tissue rarely interferes with interpretation of specific staining and can usually be disregarded.	115-121
Primary antibody and negative reagent control serum are insufficiently diluted.	Reoptimize the dilution of the primary antibody and negative control serum.	11-13

*"De-zenk" - "dezenkerization" - is the use of iodine to remove mercury pigment

Epithelial tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Staining is moderate to marked, especially in epidermal epithelium. Background in epithelia accompanies background in connective tissue.

Possible Cause	Solution	See Page
Both the primary antibody and negative control serum contain contaminating antibodies to epithelial elements, possibly cytokeratins.	▪ Use a higher dilution of the primary antibody and negative control serum. ▪ Increase the incubation time. ▪ Replace the antibody.	3-5, 115-121
Excessive formalin fixation of tissues may increase protein cross-linking, resulting in tissue hydrophobicity.	Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody and/or the negative reagent control specification sheet for appropriate pretreatment.	29-33, 115-121

Focal cytoplasmic staining observed in epithelium in the specimen tissue.

Possible Cause	Solution	See Page
Focal cytoplasmic staining is seen, particularly in intermediate and superficial layers of the epidermis. May be caused by passive absorption of plasma proteins into degenerating epidermal cells.	This observation is rare and should not interfere with interpretation of specific staining.	115-121

Background seen in all control and specimen tissue when using an immunoperoxidase staining system.

Possible Cause	Solution	See Page
Unquenched endogenous peroxidase activity may be seen in all hemoprotein-containing specimens, including hemoglobin in erythrocytes, myoglobin in muscle cells, cytochrome in granulocytes and monocytes and catalases in liver and kidney.	▪ Use alternate or prolonged peroxidase blocks or use another enzyme label such as alkaline phosphatase. ▪ Eosinophils and mast cells are particularly resistant to peroxidase quenching. Use a peroxidase blocker. ▪ Use special stains: eosin will stain eosinophils a bright red-orange.	115-121

Background seen in all control and specimen tissue when using an alkaline phosphatase staining system.

Possible Cause	Solution	See Page
Unquenched endogenous alkaline phosphatase activity may be seen in leucocytes, kidney, liver, bone, ovary bladder, salivary glands, placenta and gastro-intestinal tissue.	Add levamisole to the alkaline phosphatase chromogen reagent or use another enzyme label such as horseradish peroxidase. Intestinal alkaline phosphatase is not quenched by the addition of levamisole. Pretreat the tissue with 0.03 N HCl.	115-121

Background seen in all control and specimen tissue when using a biotin-streptavidin staining system.

Possible Cause	Solution	See Page
Endogenous protein-bound biotin (water-soluble B vitamin). High amounts of biotin are found in adrenal, liver, and kidney. Lesser amounts are found in the GI tract, lung, spleen, pancreas, brain, mammary gland, adipose tissue, lymphoid tissue, and cells grown in culture media containing biotin as a nutrient.	Use a biotin block or chose another non-biotin based staining system.	115-121

Background of skeletal or smooth muscle tissue in positive control tissue, negative control tissue, specimen tissue and negative reagent control.

Possible Cause	Solution	See Page
Cause is not understood. It is possibly due to antibodies to muscle antigens in primary and negative reagent control serum.	Should not interfere with interpretation of specific staining.	115-121

Undesired "Specific" Staining

Positive staining of leucocyte membranes in specimen tissue, positive control, negative tissue control and negative reagent control.

Possible Cause	Solution	See Page
Binding of the F_c portion of Ig by F_c receptors on the cell membrane of macrophages, monocytes, granulocytes and some lymphocytes.	▪ Use $F(ab')_2$ or F(ab) fragments for the primary and secondary antibodies rather than intact antibodies. ▪ Add detergent to the wash buffer.	115-121

Positive staining of histiocytes and granulocytes in the specimen tissue only, with a marker not normally reactive with these cells.

Possible Cause	Solution	See Page
Phagocytosis of antigens may render phagocytes positive for the same.	Rare. Should not interfere with interpretation of specific staining.	115-121

Positive membrane staining of specimen tissue and negative reagent control tissue when using a horseradish peroxidase staining system.

Possible Cause	Solution	See Page
Tissue from persons infected with Hepatitis B virus and expressing Hepatitis B surface antigen may exhibit undesired staining.	Utilize a non-peroxidase staining system.	115-121

Miscellaneous

Loss of viability of cell cultures.

Possible Cause	Solution	See Page
Some manufacturers produce antibodies and reagents for in vitro use only. These products may contain preservatives, usually sodium azide, which is a known poison.	Utilize an in vivo product for application on viable cells. For use on cell cultures only: sodium azide may be dialyzed out of some reagents. Contact Dako Technical Support for additional information.	115-121

Section Two

Troubleshooting flow chart: Use this flow chart to determine source(s) of non-specific staining when using an immunohistochemical protocol.

Background Staining Encountered with HRP-Peroxidase Reagents

Reagents	Result/Action
SLIDE #1 Positive Control Tissue: Counterstain with hematoxylin **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Brown endogenous pigment (such as melanin) observed: - To distinguish melanin pigment from DAB chromogen, Azure B can be used as a counterstain. The melanin stains blue-green, while the DAB remains brown. - An alternate method is to use AEC as the chromogen. However, if high levels of pigment exist in the tissue, the red chromogen may be partially obscured. Since bleaching protocols to remove melanin may compromise tissue antigenicity, it should be avoided if at all possible.
SLIDE #2 Positive Control Tissue: DAB/AEC + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Brown/Red color observed: - Indicates endogenous peroxidase activity in the tissue sections. It is present in all hemoprotein containing tissue including erythrocytes, muscle, liver, kidney, granulocytes and monocytes. - Block with three percent hydrogen peroxide or other peroxidase blocking reagent. Using a new bottle of hydrogen peroxide, perform a three percent H_20_2 peroxidase block, followed by DAB and an appropriate counterstain.
SLIDE #3 Positive Control Tissue: Peroxidase Block + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Brown/Red color observed: - Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cell grown in culture media containing biotin (RPMI, NCTC, MEME). - Block with a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction.

Reagents	Result/Action
SLIDE #4 Positive Control Tissue: Peroxidase Block + Biotin Block (if required) + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Brown/Red color observed: ■ Indicates non-specific or undesired binding of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. ■ To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody.
SLIDE #5 Positive Control Tissue: Peroxidase Block + Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-HRP + DAB/AEC **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Brown/Red color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.
SLIDE #6 Negative Control Tissue: Perform complete staining protocol. **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Brown/Red color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination. ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.

Background Staining Encountered with Alkaline Phosphatase

Reagents	**Result/Action**
SLIDE #1 Positive Control Tissue: Fast Red, Fuchsin or BCIP/NBT + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Red/Blue color observed: ▪ Indicates endogenous alkaline phosphatase activity in the tissue sections. It is present in liver, kidney, GI tract, bone, bladder, ovary, salivary gland, placenta, leukemic, necrotic or degenerated cells. ▪ Block with levamisole (Intestinal alkaline phosphatase may be quenched by the addition of 0.03 N HCl prior to the addition of the alkaline phosphatase).
SLIDE #2 Positive Control Tissue: Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Red/Blue color observed: ▪ Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cells grown in culture media containing biotin (RPMI, NCTC, MEME). ▪ Block with a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction.
SLIDE #3 Positive Control Tissue: Biotin Block (if required) + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Red/Blue color observed: ▪ Indicates non-specific or undesired binding of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. ▪ To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody.

Reagent	Result/Action
SLIDE #4 Positive Control Tissue: Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Red/Blue color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or a protein block; add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue or as specific staining in pancreatic sections.
SLIDE #5 Negative Control Tissue: Perform complete staining protocol **NO STAINING SEEN. GO TO NEXT STEP.** ▼	Red/Blue color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination. ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.

Negative Control Reagent

Reagent

Negative Control Reagent:
Perform complete staining protocol.

Result/Action

Red/Blue color observed:

- (Human tissue) Perform the peroxidase blocking protocol from Slide #2 under “Background Staining Encountered with HRP-Peroxidase Reagents.”
- Perform a biotin block if required, protein block if required, apply the appropriate negative reagent control (see below), apply biotinylated secondary antibody, apply streptavidin/HRP reagent and DAB.
- Prepare a negative reagent control
  - Polyclonal: non-immunized sera from the same species, diluted to the same protein concentration as the primary antibody.
  - Monoclonal: negative reagent control that matches the isotype as the primary antibody. Additionally, the diluent used to manufacture a monoclonal primary antibody and isotypic negative control should contain the same ions. Diluents containing sodium or phosphate ions may change the sensitivity of some monoclonal antibodies.
  - Calculation:
    - Ig or total protein concentration of primary antibody divided by dilution factor of primary antibody = x.
    - Ig or total protein concentration of negative reagent control divided by x = dilution factor of negative reagent control.

Section Three

Tissue Specimen

Tissue Specimen: Successful staining of tissue with an IHC marker is dependent on the type and preparation of the specimen. Record in the chart below, the species of the animal to be tested, the tissue source or organ from which it was collected, the collection method, how the specimen was fixed and tissue

Species: ____________________

Organ/tissue source: ____________________

Collection:

☐ Surgical specimen/biopsy
☐ Post-mortem specimen
☐ Fine needle aspirate
☐ Peripheral blood (include anti-coagulant)
☐ Brushing
☐ Biologic fluid
☐ Cell culture
☐ Other ____________________

Tissue preparation:

☐ Paraffin embedded
☐ Plastic embedded
☐ Cryostat section
☐ Cytospin
☐ Cell smear
☐ Mono-layer cultured cells
☐ Other ____________________

Tissue fixation:

Type of fixative ____________________
Length of time ____________________
Size of specimen ____________________

Tissue mounting:

☐ Slide mount
☐ Tissue thickness
☐ Gelatin, glue commercial adhesive or starch in the water bath
☐ Other ____________________

Endogenous Blocks

Background staining is defined as unexpected or undesirable staining seen on the test or control tissue, which does not represent the target antigen. Frequent causes of background staining are endogenous enzyme activity and endogenous biotin.

Peroxidase is an enzyme of the oxido-reductase class that reacts with a substrate containing hydrogen peroxide as the electron acceptor. To block this activity, a variety of hydrogen peroxide reagents can be applied to cells producing this enzyme.

Alkaline phosphatase is an enzyme having various isoforms, which are produced in the leukocytes, liver, bone, intestine, placenta and Regan (carcinoma). Addition of levamisole to the chromogen/substrate will inhibit endogenous alkaline phosphatase activity, with the exception of the intestinal isoform. If necessary, this can be blocked with a weak acid wash, such as 0.03-0.5 N HCl.

Biotin, a B vitamin, may be protein-bound to tissue and can interfere with proper interpretation of staining patterns when using a streptavidin or avidin reagent. To block this binding, a biotin/avidin block.

Peroxidase block:

☐ 3% H_2O_2
☐ Methanol/H_2O_2
☐ Sodium azide
☐ Peroxidase Block (S2001)
☐ Other ________________________________

Alkaline Phosphatase block:

☐ Levamisole
☐ 0.03 N HCl (not for use on cryostat tissue)
☐ Other ________________________________

Biotin block:

☐ Biotin Block (X0590)
☐ Other ________________________________

Protein block:

☐ Protein Block (X0909)
☐ Normal sera from host species of the secondary antibody
☐ Other ________________________________

Section Four

Using a Typical Specification Sheet for an IVD Antibody

Information You Need to Know	Information Located on the Specification Sheet	Comments
Regulatory Status of the Primary Antibody	Intended use For in vitro diagnostic use.	Indicates that a product meets the FDA requirements as a clinical diagnostic product. Likewise, a CE icon indicates the reagent meets European Union requirements. Patient test results do not require an FDA disclaimer.
Tissue Preparation	Specimen preparation Paraffin sections: The antibody can be used for labeling paraffin- embedded tissue sections fixed in formalin. Pre-treatment of tissues with heat-induced epitope retrieval is required. Optimal results are obtained with 10 mmol/L citrate buffer, pH 6.0. Less optimal results are obtained with 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0. The tissue sections should not dry out during the treatment or during the following immunocytochemical staining procedure. Frozen sections and cell preparations: The antibody can be used for labeling frozen sections or fixed cell smears.	Indicates the type of specimen that was used during validation studies. In many cases this would include formalin-fixed tissue and frozen sections. Use of other fixatives requires validation by each individual laboratory. This section also indicates the optimal epitope retrieval procedure and warns against procedures that may destroy the epitope. Specimen preparation and staining procedure sections can and will change periodically, to reflect changes in technology. So remember to retain copies of each version of the reagent specification sheet. Version numbers are usually found on each page.
Choosing the Visualization System	Staining procedure Visualization: This antibody can be used with an immunperoxidase staining method. Follow the procedure enclosed with the selected visualization kit. Automation: The antibody is well-suited for immunocytochemical staining using automated platforms.	Indicates the recommended visualization system to be used with the antibody. It also indicates that the antibody can be used for automated staining. NOTE: If your state regulatory agency requires written documentation that a reagent can be used for automated staining and this indication is not listed on the specification sheet, you may wish to contact the manufacturer's technical support group for further information.
Diluting the Primary Antibody	Staining procedure Dilution: Monoclonal Mouse Anti-Vimentin, may be used at a dilution range of 1:50-1:100 when applied on formalin-fixed, paraffin-embedded sections of human tonsil.	Includes a suggested dilution range for the antibody and the recommended diluent. The dilution range is merely a suggested starting point for an individual laboratory. Optimal conditions may vary depending on specimen, preparation method, temperature of the laboratory or automated instrumentation.
Negative Reagent Control	Reagent provided Isotype: IgG1, kappa. Staining procedure The recommended negative control is Mouse monoclonal IgG1, diluted to the same mouse IgG concentration as the primary antibody. Positive and negative controls should be run simultaneously with patient specimen.	Use of a negative reagent control is required by the College of American Pathologists (CAP), based on Clinical Laboratory Improvement Amendments (CLIA 2003), for each patient or patient block in a staining run.

Information You Need to Know	Information Located on the Specification Sheet	Comments
Positive Control Tissue	Performance characteristics Normal tissues: In general, most human mesenchymal cells are labeled by the antibody, including fibrocytes, lipocytes, smooth muscle cells, vascular endothelial cells, astrocytes, peripheral nerve (Schwann) cells, macrophages (including Kupffer cells), as well as myoepithelial cells of sweat and salivary glands and of breast, which are all labeled strongly. Also positive, with variable intensity and distribution, are the follicular cells of the thyroid, adrenal cortex, renal distal tubules, and mesangial and endothelial cells of the renal glomerulus, as well as pancreatic acinar cells (1,2). In the human eye, the antibody labels the pigmented posterior and the anterior epithelia of the human iris, including the muscle portion (dilator pupillae) of the anterior epithelium, as well as the nonpigmented and pigmented ciliary epithelia (4). In the ciliary epithelia, vimentin was coexpressed with cytokeratin (4). Abnormal tissues: The antibody labeled 17/20 sarcomas, 16/18 melanomas, 4/4 meningeomas, and 3/3 schwannomas, and was the sole intermediate filament present in these tumours. In addition, variable percentages (10 to 57 percent) of carcinomas, neuroendocrine carcinomas, neuroblastomas, thymomas and mesotheliomas were positive with the antibody. With the exception of the neuroblastomas, cytokeratin was coexpressed with vimentin in these tumours. Among adenocarcinomas, more than 50 percent of papillary carcinomas of the thyroid as well as renal, endometrial, ovarian and lung carcinomas were labeled by the antibody and coexpressed keratins and vimentin.	CLIA 2003 Sec. 493.1273 (3) Mandates that fluorescent and immunohistochemical stains must be checked for appropriate positive and negative reactivity each time they are used. Most IVD antibody specification sheet will list tissue that will exhibit positive and negative staining patterns in the Performance Characteristics section. NOTE: abnormal tissue will not necessarily be labeled. Both negative and positive tissue controls should be processed using the same fixation, embedding, mounting, drying, epitope retrieval and immunostaining protocols as the patient tissue.
Negative Control Tissue	Performance characteristics Normal tissues: Skeletal and cardiac muscle cells, epidermal, squamous, urothelial, colonic and gastric mucosal, and glial cells, as well as neurons are consistently negative with the antibody .	

References

1. Wood G, et al. Suppression of Endogenous Avidin-Binding Activity in Tissues and Its Relevance to Biotin-Avidin Detection Systems. Journal of Histochemistry and Cytochemistry 2981;29:1196-204.
2. Sayaki H, et al. Azure B as a Counterstain in the Immunohistological Evaluation of Heavily Pigmented Nevomelanocytic Lesions. Applied Immunohistochemistry 1995;3:268-71.
3. Federal Register: January 24, 2003;68 42CFR Part 493.
4. College of American Pathology; Anatomic Pathology Checklist, October 2005.

Glossary

This Glossary was not intended to be an all-encompassing list of terminology as used in immunochemical staining. Rather, it assumes a basic level of technical knowledge beyond which the included definitions were selected to help in clarifying the text of this Guide.

Adjuvant In immunology, any substance that enhances the immunogenicity of an antigen and results in a superior immune response. There are two types, those that possess the ability to enhance both cellular and humoral response to a large number of antigens (general potentiation), and those that strengthen specific response to only a few antigens (specific potentiation). Adjuvants work by several mechanisms including prolongation of antigen release, improving immunogenicity by antigen denaturation, recruitment of other immunocompetent cells and induction of inflammation.

Affinity Absorption A method of separation by affinity chromatography. It may be used, for example, to remove unwanted antibodies from an antibody preparation. The preparation is passed through a column matrix containing antigens against which the unwanted antibodies are directed. Thus, the unwanted antibodies remain bound to the column. The antibody solution leaving the column contains only the desired antibodies, purified by affinity absorption.

Affinity Isolation A method of separation by affinity chromatography. For example, affinity isolated antibodies may be prepared by passing the antibody solution through a column matrix to which antigens are coupled. Antibodies directed against the coupled antigens remain bound on the column and may then be eluted using a solution which disrupts antigen-antibody binding.

Agglutination The clumping of cells that are distributed diffusely in a fluid. It is caused by agglutinins, antibodies developed against that specific cell type, and is seen when a bacterial culture is treated with serum from an animal immunized against that particular organism or when a suspension of cells, particularly red blood cells, is exposed to antisera. This phenomenon commonly is employed in blood banking as an indicator of antigen-antibody reaction between red cells and specific antiserum or donor plasma.

Algorithm A set of rules specifying how to solve some imaging problem.

Antigen A molecule that is capable of binding to an antibody.

Antigenic Determinant See Epitope.

Antigen Retrieval (AR) Also known by the terms “epitope retrieval” or “target retrieval,” pertains to the restoration of antigenicity (immunoreactivity) to an immunogen.

Anti-reflection coating An optical thin film interference coating designed to minimize reflections that occur when light travels from one medium into another.

Antiserum A serum that contains antibodies.

Ascites or Ascitic Fluid An accumulation of fluid in the abdominal cavity.

Background Unless defined otherwise, background staining includes all nonspecific staining as a result of procedural artifacts. Occasionally, it may also include “undesirable” staining, eg, due to diffused antigen.

Chromogen One of a group of chemical species that can form a particular colored material or can be identified by such a reaction with an appropriate reagent.

Counterstain A second stain that provides a contrasting effect to another stain.

Cross-reactivity The ability of an antibody to react with antigens other than the immunogen. The term should not be used when referring to reactions occurring between an antibody and different cell or tissue components.

Dichroic Mirror Selectively transmitting and/or reflecting light according to its wavelength. A longpass dichroic transmits a broad spectral range while efficiently reflecting shorter wavelengths along a different optical channel.

Epitope The structural part of an antigen that reacts with an antibody. These are groupings of amino acids in globular proteins and sugar side-chains in polysaccharides. The most critical part is called the immunodominant point.

Epitope Retrieval See Antigen Retrieval.

Expiration Date This term signals the minimum expected shelf life of biological materials, including immunochemicals. (See Shelf Life).

Extinction coefficient (ε) is a measure of how efficiently a fluorochrome absorbs light, and unless specified, is stated for the peak excitation wavelength.

Hyperimmunization The practice of establishing a heightened state of the actively acquired immunity by the administration of repeated doses of antigen.

Idiotype Traditionally, antigenic determinants that relate to the specificity of the antibody. Idiotypic arrangement of several groups of amino acids in the hypervariable regions of light and heavy chains were thought to bestow unique antigenic determinants to the antibody molecule and, as a consequence, a high degree of specificity. However, anti-sera directed against these antigenic determinants have since been found to cross-react with other antibody molecules. The term idiotype has yet to be redefined.

Image Analysis Image analysis is the extraction of meaningful information from images.

Immunochemistry The branch of immunology concerned with the chemical substances and reactions of the immune system, the specific study of antigens and antibodies and their interactions with one another.

Immunocytochemistry Immunochemistry applied to the study of intracellular activities. (Now frequently used interchangeably with immunohistochemistry.)

Immunogen Any substance capable of generating an immune reaction, in contrast to any substance that binds to an antibody (ie, an antigen).

Immunogenicity The ability of an immunogen to elicit an immune response. Immunogenicity depends upon foreignness to the host, the size of the immunogen, the complexity of its molecular structure, the length of time it remains in the host and its ability to reach certain immunocompetent cells in order to generate immunity.

Immunohistochemistry Immunochemistry applied to the study of cells and tissues. (Now frequently used interchangeably with immunocytochemistry.)

In Situ Hybridization An assay for nucleic acids “on site” in fixed tissue sections by the use of heat to first denature and then to reanneal with specific DNA, RNA or PNA probes.

Internal Tissue Control A specimen from the patient donor, which contains the target marker, not only in the tumor to be identified, but also in adjacent normal tissue. Thus, no separate positive control sections are needed.

Ligand A molecule, ion or atom that is bound to the central atom (usually a metal atom) of a coordination compound or chelate.

Link Antibody See Secondary Antibody.

Monoclonal Antibodies Immunochemically identical antibodies produced by one clone of plasma cells that react with a specific epitope on a given antigen. Produced commercially using hybridomas.

Monospecific Having an effect only on a particular kind of cell or tissue, or reacting with a single antigen, as a monospecific antiserum.

Negative Tissue Control A tissue specimen from the same organ lacking the target antigen and processed by use of the primary antibody.

Nonimmune Serum Serum obtained from animals which have not been immunized.

Polyclonal Antibodies Immunochemically dissimilar antibodies produced by different cells and reacting with various epitopes on a given antigen.

Positive Tissue Control A specimen previously shown to stain specifically for the target antigen after exposure to primary antibody. Nonspecific background staining should be at a minimum. Note that, for some target antigens (e.g., prostate specific antigen), the staining intensity ideally should be less than maximal to allow monitoring not only for positivity, but also for variation in intensity.

Primary Antibody The first antibody used in a staining procedure.

Prozone Phenomenon The phenomenon exhibited by some sera, which give effective agglutination reactions when diluted several hundred- or thousand-fold, but do not visibly react with the antigen when undiluted or only slightly diluted. The phenomenon is not simply due to antibody excess, but often involves a special class of antibodies (blocking or incomplete) which react with the corresponding antigen in an anomalous manner. The bound antibody not only fails to elicit agglutination, but actively inhibits it. The phenomenon may also occur with precipitation or other immunologic reactions.

Quantum yield is a measure of how efficiently absorbed light is converted to emitted fluorescence by a fluorochrome. It is the ratio of the number of photons emitted to the number of photons absorbed.

Quenching Refers to the inactivation of a chemical activity by an excess of reactants or products. In enzymology, excess substrate or product may inhibit the enzymatic activity.

Scanner Automated microscope that scans a complete slide and stores a digital image of the slide as a virtual slide.

Secondary Antibody The second antibody used in a staining procedure; it reacts with the primary antibody, now the antigen, and forms a bridge between the primary antibody and a subsequent reagent, if any. Also known as “link” antibody.

Shelf Life This term refers to the expected duration of the functional stability of biological substances, including immunochemicals, and most commonly is assessed by experimental tests, statistical work and observation. Within the user’s laboratory, periodical comparisons of the working solution with aliquots kept frozen at –20 °C is recommended. The shelf life is terminated by an Expiration Date.

Singlet and Triplet states The electronic states of most organic molecules can be divided into *singlet* and *triplet* states. Singlet state molecules have an outermost pair of electrons with antiparallel spins (symbolized by↑↓). Triplet state molecules have an outermost pair of electrons with parallel spins (symbolized by ↑↑). A higher excited singlet state is generally formed by absorption of light. However, quite often the lifetime of this singlet state is sufficiently long to allow the spin of one of the two electrons to invert thereby producing a triplet.

Specific Staining Positive staining of tissue or cells by use of primary antiserum. Occasionally this includes diffused, absorbed or phagocytosed antigen, giving rise to “undesirable” staining. The staining seen due to contaminating antibodies in the primary antiserum should be considered as nonspecific.

Standardization Classically, to standardize means to compare with or conform an assay of unknowns to established standards. In quantitative analytical work numbers readily allow for conforming to such standards. In semi-quantitative or qualitative assays such as immunocyto- or immunohistochemistry, which frequently conclude with an opinion, only subjective comparisons to carefully selected tissue and reagent controls can be used to monitor and maintain excellence.

Target Retrieval See Antigen Retrieval.

Telepathology: sharing pathology information, including images, electronically across different locations.

Titer In immunohistochemistry, the highest dilution of an antiserum, which results in optimal specific staining with the least amount of background.

Virtual slide: A digital representation of a glass slide.

Index

ABC (Avidin-biotin-enzyme-complex) 24, 57-60, 117

Acetic acid 31

Acetone 31

Adipocytes 119

Adjuvant 3, 118, 155

AEC (3-Amino-9-ethylcarbazole) 17, 18, 145, 146

Aerosil 9

Autofluorescence 64, 68-73, 107,

DAPI staining pattern 68, 70

Signal distribution 68, 71, 73

Affinity Chapter 1, Chapter 2, Chapter 3, 23, 51, 57, 104, 106, 118, 123, 138, 155

Air 39, 83, 139

Albumin 3, 8, 32, 112, 120

Alkaline phosphatase 15-18, 97, 98, 104, 110, 111, 115, 116, 138, 143, 147, 151

Analytic 5, Chapter 4

Animal 2-7, 106, 118, 127, 140, 150, 155

Animal Research Kit (ARK™) 7, 106

Antibodies 1-9, 11, 12, 16, 21, 22-27, 29-31, 45, 50, 51, 54, 56-59, 61-65, 103-107, 111-113, 115-121, 127, 137, 138, 142, 144, 149, 155-157

- adsorption 8
- affinity 3-9, 11, 12, 15, 23, 51, 57, 104, 106, 118, 123, 138, 155; also see Chapter 1, Chapter 2, Chapter 3
- aggregates 7, 11, 119,
- association constant 5
- avidity 4, 5, 51, 111
- conjugates 7, 9, 16, 59, 61, 106, 107, 117, 119, 120,
- epitopes 36, 30, 51, 59, 104, 109, 111, 115, 118, 139, 156
- dilutions 6-12, 23-25, 127, 138
- incubation temperature 13, 138, 140
- monoclonal (See Monoclonal antibodies) 1, 2, 4-6, 8, 9, 11, 23, 24, 29, 31, 50, 56, 105, 113, 118-120, 127, 138, 142, 146, 148, 149, 152, 156
- polyclonal (See Polyclonal antibodies) 3-11, 23, 24, 104, 119, 120, 127, 138, 142, 146, 148, 149, 156
- polymerization 7, 8, 16, 17, 119

Antigens (See also **Epitope**) 1-7, 12, 23, 51, 53-55, 60, 65, 104, 109, 111, 112, 117, 118, 120, 127, 129, 137, 139, 140, 141, 143, 144, 155, 156

- Antigen retrieval See Chapter 8 (demasking of antigens), 156, 157

Avidin-Biotin 12, 16, 57, 58, 60, 117

avidity 4, 5, 51, 111

B5 fixative 31

Background 107, 109, 110-114, 127, 128, 137, 138, 140, 141, 142-144, 151, 155-157, See Chapter 17

Bacterial 9, 65, 81, 120, 138, 142, 155

BCR gene translocation 125

B cell(s) 3-5, 118

Beta-lipoproteins 9

Biotin (See also **Avidin-biotin**) 12, 15, 16, 30, 57, Also see Chapter 9, 58, 60, 104, 105, 116, 117, 143, 145-149, 151, 154,

Biotinylated 12, 58, 106, 120, 149

Blood smears 31

Bovine serum albumin (BSA) 3, 8, 112, 120

Breast 25, 28, 37, 44, 69, 72, 73, 95, 97, 100, 101, 125, 126, 130, 153, Also see Chapter 7

5-Bromo-4-chloro-3-indoxyl phosphate (BCIP) 17, 147, 148

Buffer 6, 11, 15, 18, 25, 31, 32, 52, 54-56, 72, 98, 111, 112, See Chapter 3, Chapter 8, Chapter 17 and 21

Calcium 9, 31

Carbohydrates 116

College of American Pathologists 8, 35, 130, 152

Clinical Laboratory Improvement Amendments 152

Catalyzed signal amplification (CSA) 59, 106

Cycled Tyramide Amplification 59

Labeled Streptavidin-Biotin (LSAB) Method 16, 58, 59,

Cell smears 17, 18, 104, 141, 152

Checkerboard titrations 12

4-Chloro-1-naphthol (CN) 17

Collagen 119, 120

Complement 120

Conjugation 7, 15, 16, 63

Glutaraldehyde 7, 16, 30, 64, 119

Connective tissue 113, 119, 139-142

Controls Chapters 4, 7, and 19; 59, 65, 88, 121, see also Chapter 21

Counterstain 17, 18, 106, also see Chapter 21

Cross-linking 7, 28, 30, 119, also see Chapter 21

Cytochrome 115

Cytology 81

Cell smears 17, 18, 31, 104, 119, 126, 141152

Cytoplasmic antigens 7

Dextran 7, 9, 58, 59,

DAB 17, 18, 59, 110, 114, 138, 145, 146

Diluent 109, 111, 112, 119, 120, 127, 137, 139, 149, 152

Disulfide bridges 1, 2, 3

Direct and indirect immunofluorescence 61

DNA 31, 60, Chapter 10, Chapter 11, Chapter 14, 103, 104, Chapter 18, 156

Double diffusion 127

Double staining 104, 105, 106, 116

EnVision™ procedure 58, 59, 60, 106

Elastin 119

Embedding Chapter 6

Enzyme(s) (See also specific **enzymes**) Chapter 3, Chapter 8, Chapter 21,

Epitope 2-6, 23, 26, 28, 29, 31, 51, 109, 111, 128, see also Chapter 21

Index

Epitope retrieval (See **Antigen retrieval**) 28, 51, 152, 153, 155, 156

EPOS™ technique 58, 59

Ethanol 31, 32, 67, 110,

Fading 99

Fast Garnet GBC 17

Fast Blue BB 17

Fast Red LB 17

Fast Red TR 17, 18

Fast Red Substrate 18

Fixation Chapter 1, 4, 5; page 36; Chapter 8; pages 58, 64; Chapter 11, Chapter 16, Chapter 17, Chapter 19 & 21

Fixatives Chapter 5, 68, 110, Chapter 21

Fluorescein isothiocyanate (FITC) 61-65, 68, 71, 77-79, 86, 90, 97-99

Fluorescence in situ hybridization (FISH) Chapters 11, 13 and 14

Fluorescyl-Tyramide Amplification 59

Fluorescence resonance energy transfer (FRET) 87

Formaldehyde 30

Formalin 6, Chapter 4, Chapter 5, 36, 38, 43, Chapter 8, Chapter 9, 65, 67, 68, 73, 99, 104, 105, 107, 109, 110, 115, 119, 125, Chapter 21

Freezing 9

Frozen sections 30, 31, 44, 58, 60, 65, 104, 115, 116, 119, 120, 131, 139, 141, 152,

FFPE Chapter 4, Chapter 11

Glutaraldehyde 7, 16, 30, 36, 64, 119

Horseradish peroxidase (See **Peroxidase**) 15, 16, 97, 104, 110, 115, 120.143, 144

Hybridoma 4, 5, 23

Hydrophobicity 5, 7, 8, 111, 119, 120, 140, 141, 143

Hyperimmunization 156

IgA 1

IgD 1

IgE 1

IgG (See **Immunoglobulin G**) 1

IgG1 1, 152,

IgM (See **Immunoglobulin M**) 1-3, 7, 8

Image analysis 27, 49, 107, 131, 156, see also Chapter 20,

Immune complex 5-7, 16, 119

Immunization 3-6, 23, 118

Immunocytochemistry 1.156

Immunodiffusion 118

Immunoelectrophoresis 118.127

Immunogen 2-5, 155, 156

  adjuvant 3

In situ hybridization Chapter 13, Chapter 14, Chapter 18

Iodonitrotetrazolium Violet (INT) 17

Labelled streptavidin-biotin method (LSAB) 16, 58, 59

Levamisole 16, 18, 110, 115, 116, 143, 147, 151

Limitations of IF techniques 63, also look in Chapter 10

Lipoproteins 9

Lymphocyte(s) 4, 5, 31, 124, 144

Lymphoid tissue 110, 116, 143, 145, 147

Macrophages 118, 144, 153

Mercuric chloride-based fixatives 110

Metaphase spread 124

Myeloma cells 4.5

Macrophages 118, 144, 153

Mercuric chloride-based fixatives 110

Naphthol 17, 18

Neutral buffered formalin 29-31, 67,

New Fuchsin 17, 18

Nitro blue tetrazolium (NBT) 17

Nordic Immunohistochemical Quality Control (NordiQC) 130

Nucleic Acid Probes 123, Chapter 14, Chapter 18

Osmium tetroxide 17, 31

Papain 1, 2

Penetration 7, 30, 32, 109, 120, 140, 141

Pepsin 1, 2, 54, 55, 68, 110

Peptide nucleic acid probes (PNA) Chapter 18, 67, 70-72

PharmacoDiagnostic 125, 133

Peroxidase (HRP) 12, Chapter 3, 31, 32, 57, Chapter 9, Chapter 14,page 97, 104, Chapter 16, Chapter 17, Chapter 21

Photobleaching 63, 64, 79, 85, 107

Pools of antisera 120

Post analytic Chapter 4, pages 24, 25, 26

Pre-analytic 5, Chapter 4, page 35, Chapter 6

Primary antibody Chapter 1, Chapter 2; Chapter 4, Chapter 8, Chapter 9, Chapter 10, Chapter 14, Chapter 15, Chapter 16, Chapter 19, Chapter 21

Prosthetic group 15

Pronase 110

Protease 68, 109, 110, 123

Proteinase K 54, 55, 68, 110

Proteolytic digestion 1, 2, 109, Chapter 16, Chapter 21

Quality control 24, 45, 46, 48, 61, 112, 121, 127, 129, 130,

Quantum dots 107

Retrieval solution Chapter 8, Chapter 17,

Signal-to-noise ratio 24, 70, 85, 89, 100, 125

Stability (antibody, light source) See Chapter 1, Chapter 13, page 11, 32, 63, Chapter 16,pages 119, 123, 124, 157

Streptavidin 12, 16, 30, Chapter 9, pages 104, 105, 107, 116, Chapter 21

Singlet oxygen scavengers 63

Tissue controls Chapter 19, 47, 127, 128, 129, 153

Tissue processing Chapter 6, pages 33, 38, 40, 73, 117, 128

TOP2A 67, 126, 127

Troubleshooting Chapter 21

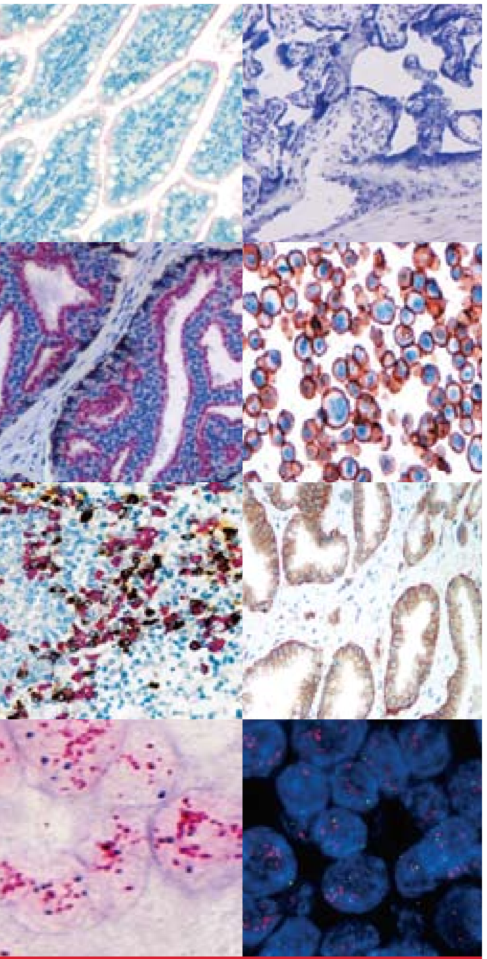
Trypsin 110

Virtual microscopy Chapter 20

Wash, Washing 5, 6, 31, 64, 72, 104, 112, 113, 117, 123, 131, 138, 139

Zenker's fixative 31

www.dako.com



dako.com

◀ *Detach bookmark*

SEND ME MORE...

The Illustrated Guide to Bone Marrow Diagnosis Second Edition

Immunohistochemical Staining Methods Fifth Edition

Special Stains

Demasking of Antigens Second Edition

USA: request Dako Education Guides via e-mail to:

LitReqUS@dako.com

Outside USA: please contact your local Dako office (see www.dako.com)

Corporate Headquarters
Denmark
+45 44 85 95 00

www.dako.com

Distributors in more
than 50 countries

Australia
+61 3 9357 0892

Austria
+43 1 408 4334 50

Belgium
+32 (0) 16 38 72 20

Canada
+1 905 858 8510

Denmark
+45 44 85 97 56

Finland
+358 9 348 73 950

France
+33 1 30 50 00 50

Germany
+49 40 69 69 470

Ireland
+353 1 479 0568

Italy
+39 02 58 078 1

Japan
+81 3 5802 7211

The Netherlands
+31 20 42 11 100

Norway
+47 23 14 05 40

Poland
+48 58 661 1879

Spain
+34 93 499 05 06

Sweden
+46 8 556 20 600

Switzerland
+41 41 760 11 66

United Kingdom
+44 (0)1 353 66 99 11

United States of America
+1 805 566 6655

08002 15OCT09