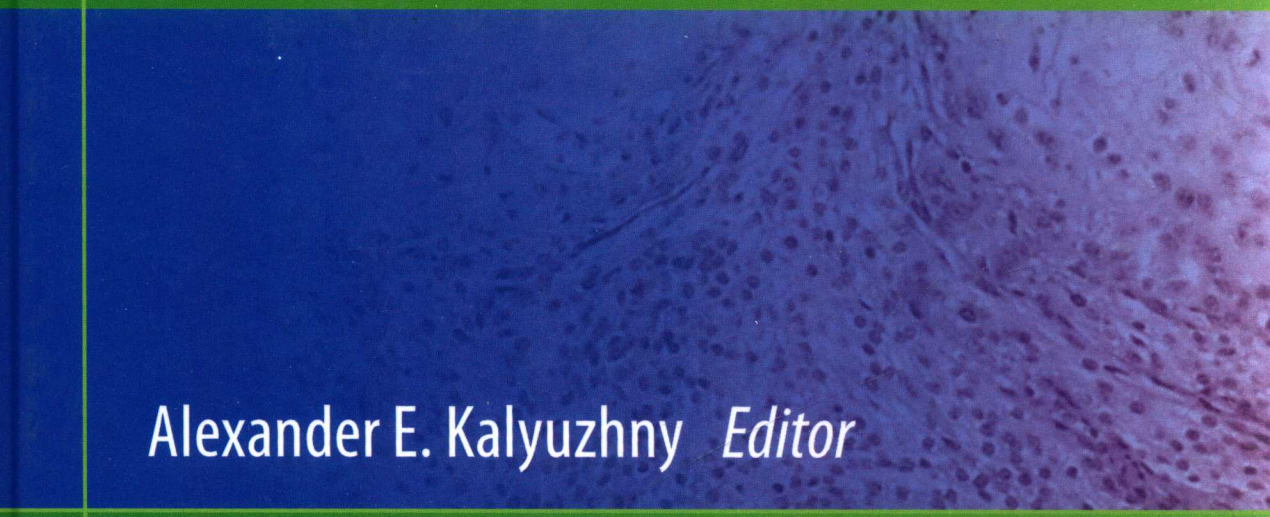
Methods in Molecular Biology 717

Springer Protocols



Alexander E. Kalyuzhny *Editor*

Signal Transduction Immunohistochemistry

Methods and Protocols

Humana Press

Signal Transduction Immunohistochemistry

Methods and Protocols

Edited by

Alexander E. Kalyuzhny

R&D Systems, Inc., Minneapolis, MN, USA

Humana Press

Editor
Alexander E. Kalyuzhny, Ph.D.
R & D Systems, Inc.
Minneapolis, MN
USA
Alex.Kalyuzhny@rndsystems.com

ISSN 1064-3745 e-ISSN 1940-6029
ISBN 978-1-61779-023-2 e-ISBN 978-1-61779-024-9
DOI 10.1007/978-1-61779-024-9
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011921260

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For other titles published in this series, go to
www.springer.com/series/7651

To my supporting family and encouraging friends

Preface

Immunohistochemistry (IHC) is one the most valuable research and diagnostic tools in biomedical research. Unlike detecting constitutively expressed targets, immunohistochemical detection of labile, low abundance, and short-lived signal transduction molecules appears to be a very challenging task. This book represents a set of detailed protocols written by IHC experts addressing the challenges of signal transduction immunohistochemistry (ST-IHC); because it would be fair to say that ST-IHC as a discipline is in its infancy and the chapters in the first part are of a more introductory nature which should help new investigators in their orientation in the field. The second part is dedicated to techniques used for the preservation of antigens and their unmasking. The third part presents protocols in digital imaging and image analysis of stained cells and tissues and high-throughput data collection and data analysis. The fourth part is focused on ST-IHC techniques used in neuroscience as well as cancer and stem cell research. And finally, the fifth part presents novel ST-IHC techniques that can be easily adopted for a wide variety of research tasks. This book can be used as a guide by novices and has a wealth of ideas that can be exploited by experienced researchers who are always on the lookout for new experimental tricks and hints. It can also serve as a troubleshooting guide for researchers in academia and in industry.

I wish to thank all the authors who, in addition to their own research projects, devoted a lot of time working on book chapters. In addition, I wish to thank R&D Systems, Inc., where I run the IHC department, for their support and for giving me the opportunity to gain invaluable IHC experience by validating thousands of antibodies over the years.

Minneapolis, MN *Alexander E. Kalyuzhny*

Contributors

Amy J. Archuleta • *PhosphoSolutions LLC, Aurora, CO, USA*
Jayant Avva • *Case Western Reserve University, Cleveland, OH, USA*
Christianne Bandeira-Melo • *Laboratório de Inflamação, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
Debabrata Banerjee • *Department of Medicine and Pharmacology, The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, UMDNJ, New Brunswick, NJ, USA*
Afsar Barlas • *Developmental Biology Program, Molecular Cytology Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
Juraj Bodo • *Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA*
Mats Borén • *Denator AB, Dag Hammarskjöldsv. 10A, Uppsala, Sweden*
Patricia T. Bozza • *Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil*
Michael D. Browning • *PhosphoSolutions LLC, Aurora, CO, USA*
Kathy Brumbaugh • *R&D Systems, Inc, Minneapolis, MN, USA*
Roberto Campos-Gonzalez • *R&D Systems, Inc, Minneapolis, MN, USA*
Charles Chavkin • *Department of Pharmacology and Program for Neurobiology and Behavior, University of Washington, Seattle, WA, USA*
Daniel Ciznadija • *Department of Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
Michael B. Cohen • *Department of Pathology, The University of Iowa, Iowa City, IA, USA*
Jeff Cooper • *R&D Systems, Inc, Minneapolis, MN, USA*
Skyler Dillon • *Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
Yaser Dorri • *Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
Anil Dsouza • *Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
Ning Fan • *Developmental Biology Program, Molecular Cytology Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
Niels T. Foged • *Visiopharm A/S, Hoersholm, Denmark*
Jillian Frisch • *R&D Systems, Inc, Minneapolis, MN, USA*

SHO FUJISAWA • *Developmental Biology Program, Molecular Cytology Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
MICHAEL GRAHEK • *R&D Systems, Inc, Minneapolis, MN, USA*
MICHAEL GRUNKIN • *Visiopharm A/S, Hoersholm, Denmark*
JODI HAGEN • *R&D Systems, Inc, Minneapolis, MN, USA*
YINGWEI HE • *Abgent, Inc, San Diego CA, USA*
J. P. HOUCHINS • *R&D Systems, Inc, Minneapolis, MN, USA*
ERIC D. HSI • *Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA*
JAMES W. JACOBBERGER • *Case Western Reserve University, Cleveland, OH, USA*
WADE JOHNSON • *R&D Systems, Inc, Minneapolis, MN, USA*
ALEXANDER E. KALYUZHNY • *R&D Systems, Inc, Minneapolis, MN, USA*
BIJI T. KURIEN • *Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
JULIA C. LEMOS • *Department of Pharmacology and Program for Neurobiology and Behavior, University of Washington, Seattle, WA, USA*
WEN-CHIEH LIAO • *R&D Systems, Inc, Minneapolis, MN, USA*
MONG-SHANG LIN • *R&D Systems, Inc, Minneapolis, MN, USA*
VERNON C. MAINO • *BD Biosciences Immunocytometry Systems, San Jose, CA, USA*
KATIA MANOVA • *Developmental Biology Program, Molecular Cytology Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
LEOPOLDO MENDOZA • *Solulink, Inc, San Diego, CA, USA*
PRAVIN J. MISHRA • *Department of Medicine and Pharmacology, The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, UMDNJ, New Brunswick, NJ, USA*
JENNIFER NGUYEN • *Solulink, Inc, San Diego, CA, USA*
KRISTIN M. NIXON • *PhosphoSolutions LLC, Aurora, CO, USA*
JAKOB RAUNDAHL • *Visiopharm A/S, Hoersholm, Denmark*
CLARISSE A. ROTH • *Department of Pharmacology, University of Washington, Seattle, WA, USA*
JORDAN SCHOEPHOERSTER • *R&D Systems, Inc, Minneapolis, MN, USA*
DAVID SCHWARTZ • *Solulink, Inc, San Diego, CA, USA*
R. HAL SCOFIELD • *Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; Department of Medicine, University of Oklahoma Health Sciences Center and Veterans Affairs Medical Center, Oklahoma City, OK, USA*
JERRY SEDGEWICK • *Sedgewick Initiatives, Saint Paul, MN, USA*
RADINA P. SOEBIYANTO • *Case Western Reserve University, Cleveland, OH, USA*
SREE N. SREENATH • *Case Western Reserve University, Cleveland, OH, USA*
STEVEN STOESZ • *R&D Systems, Inc, Minneapolis, MN, USA*
CRYSTAL A. STUTZKE • *PhosphoSolutions LLC, Aurora, CO, USA*
MARIA A. SUNI • *BD Biosciences Immunocytometry Systems, San Jose, CA, USA*
JOSEPH SWEET • *R&D Systems, Inc, Minneapolis, MN, USA*
SERGEI I. SYRBU • *Immunopathology Laboratory, Department of Pathology, The University of Iowa, Iowa City, IA, USA*

MESRUH TURKEKUL • *Developmental Biology Program, Molecular Cytology Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
WEI WANG • *Abgent, Inc, San Diego, CA, USA*
MICHAEL C. WEIS • *Case Western Reserve University, Cleveland, OH, USA*
PETER F. WELLER • *Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*
CHUN WU • *Abgent, Inc, San Diego, CA, USA*

Contents

Preface ..... *vii*
Contributors ..... *xi*

PART I ANTIBODIES AS A TOOL: FROM CONCEPT TO DESIGN AND APPLICATION

1 Overview of the Generation, Validation, and Application of Phosphosite-Specific Antibodies 3
Kathy Brumbaugh, Wade Johnson, Wen-Chieh Liao, Mong-Shang Lin, J.P. Houchins, Jeff Cooper, Steven Stoesz, and Roberto Campos-Gonzalez

2 Selection and Validation of Antibodies for Signal Transduction Immunohistochemistry 45
Juraj Bodo and Eric D. Hsi

3 An Overview of Western Blotting for Determining Antibody Specificities for Immunohistochemistry 55
Biji T. Kurien, Yaser Dorri, Skyler Dillon, Anil Dsouza, and R. Hal Scofield

4 Optimized Protocol to Make Phospho-Specific Antibodies that Work 69
Amy J. Archuleta, Crystal A. Stutzke, Kristin M. Nixon, and Michael D. Browning

PART II PRESERVATION AND UNMASKING OF TISSUE ANTIGENS

5 Methodology and Technology for Stabilization of Specific States of Signal Transduction Proteins 91
Mats Borén

6 An Enhanced Antigen-Retrieval Protocol for Immunohistochemical Staining of Formalin-Fixed, Paraffin-Embedded Tissues 101
Sergei I. Syrbu and Michael B. Cohen

PART III IMAGING TECHNIQUES AND HIGH-THROUGHPUT DATA ANALYSIS

7 Imaging Techniques in Signal Transduction IHC 113
Jerry Sedgewick

8 Practical Considerations of Image Analysis and Quantification of Signal Transduction IHC Staining 143
Michael Grunkin, Jakob Raundahl, and Niels T. Foged

9 Flow Cytometric Analysis of Cell Signaling Proteins 155
Maria A. Suni and Vernon C. Maino

10 CytoSys: A Tool for Extracting Cell-Cycle-Related Expression Dynamics from Static Data 171
Jayant Avva, Michael C. Weis, Radina P. Soebiyanto, James W. Jacobberger, and Sree N. Sreenath

PART IV NEUROSCIENCE, CANCER, AND STEM CELL RESEARCH

11 Signaling Events Initiated by Kappa Opioid Receptor Activation: Quantification and Immunocolocalization Using Phospho-Selective KOR, p38 MAPK, and K_{IR} 3.1 Antibodies 197
Julia C. Lemos, Clarisse A. Roth, and Charles Chavkin

12 Immunohistochemical Assessment of Signal Transduction and Cell-Cycle Networks in Neural Tumors 221
Daniel Ciznadija, Afsar Barlas, and Katia Manova

13 Novel Multicolor Immunofluorescence Technique Using Primary Antibodies Raised in the Same Host Species 233
Jillian Frisch, J.P. Houchins, Michael Grahek, Jordan Schoephoerster, Jodi Hagen, Joseph Sweet, Leopoldo Mendoza, David Schwartz, and Alexander E. Kalyuzhny

14 Activation and Differentiation of Mesenchymal Stem Cells 245
Pravin J. Mishra and Debabrata Banerjee

PART V NOVEL ASSAYS AND TECHNIQUES

15 Double *In Situ* Detection of Sonic Hedgehog mRNA and pMAPK Protein in Examining the Cell Proliferation Signaling Pathway in Mouse Embryo 257
Sho Fujisawa, Mesruh Turkekul, Afsar Barlas, Ning Fan, and Katia Manova

16 Identifying Intracellular Sites of Eicosanoid Lipid Mediator Synthesis with EicosaCell Assays 277
Christianne Bandeira-Melo, Peter F. Weller, and Patricia T. Bozza

17 Absorption Control in Immunohistochemistry Using Phospho-Peptides Immobilized on Magnetic Beads. 291
Jordan Schoephoerster, Jillian Frisch, Michael Grahek, Chun Wu, Yingwei He, Wei Wang, Jennifer Nguyen, David Schwartz, and Alexander E. Kalyuzhny

Index 301

Part I

Antibodies as a Tool: From Concept to Design and Application

Chapter 1

Overview of the Generation, Validation, and Application of Phosphosite-Specific Antibodies

Kathy Brumbaugh, Wade Johnson, Wen-Chieh Liao, Mong-Shang Lin, J.P. Houchins, Jeff Cooper, Steven Stoesz, and Roberto Campos-Gonzalez

Abstract

Protein phosphorylation is a universal key posttranslational modification that affects the activity and other properties of intracellular proteins. Phosphosite-specific antibodies can be produced as polyclonals or monoclonals in different animal species, and each approach offers its own benefits and disadvantages. The validation of phosphosite-specific antibodies requires multiple techniques and tactics to demonstrate their specificity. These antibodies can be used in arrays, flow cytometry, and imaging platforms. The specificity of phosphosite-specific antibodies is key for their use in proteomics and profiling of disease.

Key words: Antibody, Phosphosite-specific, Western blotting, ELISA, Multiplex, Flow cytometry, Immunocytochemistry

1. Introduction

Protein phosphorylation, like many other posttranslational modifications, introduces changes in mass and charge to an acceptor protein. This change alters the conformation of the acceptor protein, as well as its activity, binding properties, and subcellular distribution. Phosphorylation at key amino acids within a protein is considered a hallmark of the change in the protein's activity. Because of the rapid and reversible protein changes induced by phosphorylation, eukaryotic cells have preserved this modification and it has evolved as a tightly controlled regulator of key cellular processes, such as cell division, motility, neurotransmission, and metabolism. In eukaryotic cells, reversible protein

Alexander E. Kalyuzhny (ed.), *Signal Transduction Immunohistochemistry: Methods and Protocols*, Methods in Molecular Biology, vol. 717, DOI 10.1007/978-1-61779-024-9_1,

phosphorylation occurs primarily on serine, threonine, and tyrosine amino acids (1). In addition, dysregulated protein phosphorylation has been closely associated with several diseases, including cancer (2).

The phosphorylation status of a protein is due to the balanced activities between a protein kinase that transfers a phosphate from ATP to its target polypeptide, and a phosphatase that removes it from the polypeptide; thus, many phosphorylations are transient by nature (3). There are ~520 different protein kinases, the "kinome," in the human genome that are responsible for most cellular phosphorylations. Kinases have a degree of specificity and selectivity for their target proteins based on recognition and substrate-binding domains within their amino acid sequence (2). Some kinases, like MEK1, are very selective and may have only two protein substrates, ERK1 and ERK2, while other kinases, such as Akt1, are capable of recognizing and phosphorylating multiple protein substrates. On the other hand, there are approximately 150 phosphatases in the human genome (4). Thus, phosphatases appear not to be as selective as their kinase counterparts in choosing a protein substrate.

Approximately 30 years ago, the preferred and most widely used method to investigate protein phosphorylation was labeling cells and proteins with ^{32}P. Radioactive labeling of phosphoproteins was used to determine if a protein contained phosphate, to elucidate the type of phospho-amino acid, and to identify protein substrates and their corresponding kinases (5). This radioisotope as ^{32}P-ATP was used to label cells and proteins followed by lysis and immunoprecipitation, if required, electrophoretic separation and autoradiography of gels. Once the bands of interest were identified, they were excised and digested with enzymes like trypsin, followed by two-dimensional mapping and sequencing (6). While radio-labeling of proteins with ^{32}P is, without a doubt, one of the most sensitive ways to assess phosphorylation, these associated methods are remarkably cumbersome and stressful. The advent of the first successful antibodies to phospho-tyrosine (pTyr) and subsequent phosphosite-specific antibodies facilitated the study of phosphorylation and rapidly accelerated the study of this posttranslational modification in cellular events (7–10).

The initial pTyr antibodies were rapidly adopted by scientists and used to discover many phosphorylations that had not been seen before, e.g., after stimulation of cells with growth factors or oncogene activation. The same antibodies were used to further isolate and purify these novel phospho-proteins and to develop tools for their study. Among the proteins that were discovered by this immunopurification protocol are Insulin Receptor Substrate-1 (11), Caveolin (12), and pp120 Catenin (13). Because of the combination of the newly developed reagents and Western blotting, it became possible to generate a phospho-protein profile

from cells under many different conditions at a pace several-fold faster than with ^{32}P-labeling (14).

One of the major challenges is to obtain highly specific and sensitive antibodies capable of capturing intracellular phosphorylation events of low frequency or abundance, because of the rarity and transient nature of phosphorylations (15). Although with exceptions, on activation of a signaling pathway, only a small fraction (<10%) of a typical protein kinase target becomes phosphorylated with a defined kinetics of few minutes followed by de-phosphorylation by phosphatases or degradation terminating the signaling event (4, 16, 17). These phospho-protein's traits are useful when determining the specificity of an antibody but at the same time some of these properties pose serious limitations, therefore requiring antibodies of high affinity to fit in with methods of superior sensitivity.

According to Phosphosite (http://www.phosphosite.org), perhaps one of the most comprehensive database of phosphorylations today, there are more than 81,000 unique phosphorylations described in mammalian cells. Given a conservative average of three phosphorylations per protein, it is estimated that there are many more new phosphorylations in proteins yet to be discovered. Clearly, the scientific community still does not have phosphosite-specific antibodies to all of the already discovered sites to study and evaluate their individual importance and role in biomedicine.

Although currently there are several other very sensitive techniques, like mass spectrometry, to aid in the study of protein phosphorylation (18), antibodies will still provide a rapid, economical, and adaptable avenue to study these important modifications in years to come. As new phosphorylations are being discovered and validated by mass spectrometry or mutagenesis, phosphosite-specific antibodies will remain ideal for the everyday experiment to study the phosphorylation under a myriad of conditions, cells, and tissues.

There are two major issues in the world of antibodies determining their usefulness, one of them is specificity and the second is the applicability of the antibodies in different platforms or instruments. With this in mind, we have two main goals in writing this short review. First, to summarize the major techniques utilized in characterizing and validating phosphosite-specific antibodies. We believe that these techniques provide a minimum set of tools to determine these important reagents. The second goal is to illustrate some of the exciting uses for phosphosite-specific antibodies in biomedicine as powerful tools in elucidating the biology of normal and diseased cells. We should keep in mind that the strategies and methods described below can also be applied and used when characterizing antibodies to other posttranslational modifications such as acetylation and methylation among others.

2. Materials

2.1. Antibody Generation

There are two main routes to obtain antibody-based affinity reagents for biomedical research, one by the in vivo immunization of individual animals inducing B cells to secrete the antibodies, and the second by phage display of antibody libraries and screening for binders to the desired phospho-epitope. The former is the most widely used method and requires the active introduction of a foreign biological compound, either as a hapten-carrier conjugate or as a protein, to elicit an immune response in an animal, mostly mice, rats, chickens, and guinea pigs whose immune response is well understood. The latter method requires the availability of a library of arranged genes packed and individually displayed on a filamentous phage. The phage library carrying and expressing a large immunoglobulin gene repertoire is exposed to the antigen of interest, followed by cycles of enrichment and selection until a single "phage clone" is obtained. One of the main limitations to the phage display approach is the low affinity of the binders generated, limiting their use as everyday reagents. Since there are just a handful of published reports in generating antibodies to posttranslational modifications of the phage approach, it will not be covered in this review (19).

The serial immunization of animals takes advantage of the natural ability of the immune system to recognize an injected protein "immunogen," whether bacteria, cell debris, or a phosphopeptide, and to rapidly re-arrange the immunoglobulin genes to produce antibodies until the best fit for the immunogen is found. The immune system re-arranges the heavy and light chains of antibodies accordingly to provide the best antibody response to fight the foreign intruder. Scientists have been taking advantage of the immune system in animals such as rabbits, mice, and chickens, to generate superb antibodies for use in biomedical research, for decades.

Protocols for generation of phosphosite-specific antibodies, both polyclonal and monoclonal, adhere very closely to the ones used for a regular antibody and superbly summarized in the classic book by Harlow and Lane (20). Phosphosite-specific antibodies generated in vivo have been available for several years, and basically these reagents have been obtained by three main protocols: first by immunization with a phospho-peptide (8, 21, 22); second by using cells or mixed phospho-protein complexes (23); and third by fortuitous discovery and diligent characterization (24). In their pioneering publication, Sternberger and Sternberger superbly characterized a series of monoclonal antibodies to neurofilament proteins that reacted only when these proteins were phosphorylated (24). The Sternberger's antibodies have since become a standard for the study of neurodegenerative diseases.