Kevin Vaughn

Immunocytochemistry of Plant Cells

Springer

Immunocytochemistry of Plant Cells

Kevin Vaughn

Immunocytochemistry of Plant Cells

Springer

Kevin Vaughn
Salem, OR
USA

ISBN 978-94-007-6060-8 ISBN 978-94-007-6061-5 (eBook)
DOI 10.1007/978-94-007-6061-5
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2012956166

Printed on acid-free paper

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

Preface

Immunocytochemistry of plant cells has come a long way from the first review on this subject by Bruce Knox in the early 1980s. In that early review, our only tools were fluorescein-labeled antibodies for light microscopy and ferritin-labeled antibodies for electron microscopic observation. Frankly, in many of these early localizations the resolution of the tissue or the specificity of the labeling left much to be desired. Many of my traditional plant biochemist/physiologist colleagues said things like "I don't believe those immunocytochemical techniques Kevin". One can understand this level of skepticism when organelles were not readily discernable and the label was hard to determine from background. Embedding and sectioning plant tissue embedded in Lowicryl resin was very difficult and the tissue appeared extracted after prolonged embedding. Only certain unique tissues such as germinating seeds were preserved sufficiently to allow for good resolution of structures. However, things started to improve dramatically for plant immunocytochemistry with the introduction of the London resins. These resins infiltrated plant tissues easily and could be polymerized with standard electron microscopy techniques used for epoxy-based resins. The other breakthrough was the development of gold-labeled secondary antibodies. Unlike ferritin, these antibody-probes could be prepared in a variety of sizes and the preparation of the particles themselves was not difficult and they became available from numerous commercial sources as well. In addition, gold probes could be used at both the light and electron microscopic levels so that a single specimen block could be used to localize at the tissue level with the light microscope and at the organelle and sub-organelle level with the transmission electron microscope.

My goal when I entered this area was to produce micrographs that had a high level of structural preservation and a convincing immunolocalization as well. When these papers started to appear in the early 1980s, I had a steady stream of visitors to the lab to learn the protocols and my laboratory phone was dubbed "the immunogold hotline" by my post docs in the lab! "Why don't my localizations look like yours?" was the most frequent question. Luckily, this is not rocket science and most of my visitors and telephone correspondents after a bit of coaching were able to localize their protein of interest. A 1988 McKnight training

class at U. Georgia even resulted in a whole class full of students doing a successful electron microscopic localization even though most of the students had never performed electron microscopic studies previously.

Science is not done in a vacuum and certainly the development of techniques in my laboratory was heavily influenced by other plant and animal immunocytochemists. Prominent among those people that were influential in these projects are Dick Trelease, J. Paul Knox, John Harper, Roberto Ligrone, Andrew Staehelin, Karen Renzaglia, Tobias Baskin, and my former post-docs Andrew Bowling, John Hoffman, Timothy Sherman, Martin Vaughan, and Larry Lehnen. Each of these contributed a bit of knowledge or technique that helped these experiments progress and the protocols become more refined. I am also most grateful to my mentors, Martha Powell and Kenneth Stewart, in my initial training in microscopy while a graduate student at Miami University. I entered graduate school planning to be a geneticist but ended up a cell biologist thanks to Martha and Ken. Rex Paul, who maintained the microscopes at the Stoneville location for many years, kept the microscopes in impeccable shape and allowed a high productivity from my now retired Zeiss EM 10CR microscope with almost 38,000 micrographs produced. I am also indebted to several NRI funded proposals that allowed me to hire some of the above named post docs and to develop the techniques described in this volume. My retirement from the USDA in August 2010 has allowed me the time to focus more on the writing of this book, while the memories of the experiments and the many modifications we made over the years is still fresh in my mind. I thank my friends Paul Knox, Andy Bowling, Dave Collings, John Harper, Roberto Ligrone, Tobias Baskin, Lacey Samuels, and Bo Kwang for supplying me with a lovely set of micrographs to help illustrate this book.

Immunocytochemistry, like its predecessor cytochemistry, arose out of my frustration with trying to either use a very small amount of tissue (such as that occurring in variegated chimera plants) or to determine specific reactions in a subset of that tissue using biochemical methods. For example, the presence of RuBisCo in guard cell chloroplasts was the subject of much debate but immunocytochemical techniques allowed for unequivocal localizations. The development of immunogold-silver and immunofluorescence on semi-thin sections for light microscopy was similarly fruitful in answering some long-standing anatomical questions. Just in our lab, we have answered questions on the nature of gelatinous fibers in trees, the role of gelatinous fibers in vines, mechanisms for ballistic seed dispersal and leaf abscission. As more traditional anatomists embrace these techniques, I am sure that a number of other recalcitrant questions will be answered.

This book is organized essentially into two sections. The first chapter gives what we consider general protocols that work well on a variety of tissues and organelles, but also a number of variations that one might try in order to obtain a successful localization. Most of these were developed when the more standard protocols failed. The second portion of the book reviews by organelle of those techniques that may work better with that particular organelle, what unique immunocytochemical techniques can be used, and a review of some of the more

important studies on that organelle. Some of the chapters also address the questions that are still outstanding and which could benefit from immunocytochemical studies.

My hope with the protocols outlined in this book and the description of other studies that more people will attempt these techniques and that they become more widely adopted by the plant science community.

Salem, Oregon Kevin Vaughn

Contents

Chapter 1
Immunocytochemical Techniques

Introduction

Immunocytochemistry is the branch of microscopy in which antibodies are utilized to detect molecules at either the light or electron microscopic level. In some ways, immunocytochemistry is a further refinement of the older and well-established protocols of histochemistry (Gahan 1984) and enzyme cytochemistry (Vaughn 1987a; Sexton and Hall 1991), in which reactive molecules or enzyme activities are detected as a colored or electron-opaque reaction products. In some cases, these older, more classical techniques may be combined with immunocytochemistry. For example, the activity of glycolate oxidase could be detected by the cerium precipitation technique and the localization of the protein by immunocytochemistry (Vaughn 1989). Similarly, histochemical detection of vic-OH groups in cell wall polysaccharides by the PATAg technique (Roland and Vian 1991) can supplement the information from antibody labeling of polysaccharides (Vaughn 2002; Ligrone et al. 2011). Thus, immunocytochemistry is but a portion of the larger arsenal of techniques that a microscopist has available in order to characterize a sample beyond structure.

Plant scientists were surprisingly slow to adopt these technologies, partly because immunology was not part of the basic curriculum for plant science students. Although Coon developed the immunofluorescence protocol in the 1940s, it was 30 years later before the first convincing plant immunolocalization studies were published (reviewed in Knox 1982). In many ways, plants tissues are less amenable to direct adoption of the protocols developed in mammalian cells on plant tissues as the cell walls were effective barriers to the movement of the antibody into plant cells. However, a series of studies in the 1970s and 1980s on pollen allergens (reviewed in Knox 1982) and seed storage proteins (Craig and Miller 1984) convinced even ardent skeptics of this technology of its usefulness in higher plant studies. The next several decades resulted in many more immunocytochemical localization of proteins, nucleic acids, small molecules, and polysaccharides in plant cells and it was clear that immunocytochemical protocols

K. Vaughn, *Immunocytochemistry of Plant Cells*,
DOI: 10.1007/978-94-007-6061-5_1, © Springer Science+Business Media Dordrecht 2013

were now a standard part of the repertoire of techniques for plant scientists to utilize. Important amongst these more recent advances have been the detection of the hormones ABA and IAA, phytochrome (McCurdy and Pratt 1986), the distribution (Allred and Staehelin 1985) and development (Pettigrew and Vaughn 1988) of photosystem proteins in the thylakoids of the chloroplast, peroxisomal proteins (Doman and Trelease 1985; Vaughn 1989), and cell wall polysaccharides (Knox 1997; Bowling and Vaughn 2008). However, especially compared to the extensive literature on mammalian cell immmunolocalizations, localizations in plants lag far behind in numbers. Indeed, if the localization of cytoskeletal proteins (and many of these using antisera or monoclonals raised to fungal or mammalian proteins) and cell wall components were eliminated from this list, the list of plant epitopes that have been localized is quite small.

Many of the protocols that are used for excellent preservation of tissues of plant cells (e.g. Bozzola and Russell 1992; Vaughn and Wilson 1981) and utilized by microscopic laboratories around the country are mostly not useful for localizing plant proteins. Fixation with glutaraldehyde and osmium with subsequent dehydration and embedding in epoxy resins that are polymerized under high heat conditions are generally not amenable to immunolocalizations (for an exception see Vaughn and Turley 1999). However, most of the protocol changes required for successful immunolocalizations represent no dramatic change from these standard protocols, but rather, more subtle changes in the reagents and techniques. Those changes are summarized below in each of the techniques sections. Also presented is a sort of "standard" protocol, which is a good place to start when attempting an immunolocalization protocol. This is the protocol that has been used with success in my laboratory for a number of studies on a variety of different proteins or macromolecules. However, each antibody/antiserum and epitope to be recognized presents their own sets of problems. Suggested variations to obtain a successful immunolocalization are incorporated both in this chapter and, if a technique is especially useful for a given organelle, reference to those special techniques is included in this chapter.

Fixation

Fixation is a requirement of most immunocytochemical procedures in order to ensure that the tissues retain their structural integrity and that components are not lost or rearranged during the dehydration and embedding protocols. Fixatives for plant immunocytochemistry include glutaraldehyde, formaldehyde, acrolein and osmium, although most of what will be discussed involves the first two. Other than acrolein, all of these are commonly used fixatives in almost any electron microscopic laboratory.

My philosophy of plant fixation for even standard microscopic fixation is a bit different than many botanical microscopists in that I utilize a relatively high percentage (6 %, v/v) glutaraldehyde in my initial fixation. This regime was based

upon the studies of Salema and Brandao (1973) who hypothesized that a failure to adequately fix plant cells was from the relatively high water content (compartmentalized largely within the vacuole) so that the effective concentration of the fixative within the plant cell is much lower, leading to inadequate fixation. When I adopted this higher percentage of glutaraldehyde and the PIPES buffer, there was some skepticism from reviewers about the use of this high percentage of glutaraldehyde. However, the improvement in my micrographs was immediate and dramatic. The cells were fixed thoroughly and throughout even larger tissue pieces. Thus, for my immunocytochemical experiments I often start with an initial fixation of 1–3 % glutaraldehyde and sometimes have utilized 6 % on tissues that were not easily fixed. In my laboratory, we routinely utilized highly purified glutaraldehyde at a concentration of 70 % in sealed ampoules under nitrogen. The ampule is opened just before the fixation was to take place and mixed thoroughly with the fixation buffer. Any cloudiness in the fixative, indicative of glutaric acid being present in the glutaraldehyde, should result in the disposal of the solution. Gloves are worn at any times in which there is a potential exposure to glutaraldehyde. Work in a hood or a well ventilated facility if possible.

Glutaraldehyde fixes proteins by cross-linking amino groups with the aldehyde function (Hayat 1981) and because it is a di-aldehyde, it can cross-link one protein to another and also internally within the protein. This fixation causes a change in the configuration of the protein molecule such that an antibody raised to the protein may or may not recognize the fixed protein. Even the highly abundant and localized peroxisomal protein catalase was not successfully localized until antibodies were raised to catalase that had been fixed prior to immunization. See further discussion of this in the peroxisome chapter.

Besides a thorough fixation, the delivery of the fixative to all tissues within the sample also should be rapid. Many samples can be cut into small pieces (>1 mm^2 in size) in a drop of fixative on dental wax. These pieces are transferred to vials containing the same fixative using a transfer pipet or with fine forceps, taking all precautions not to wound the tissue. We have found that foil lined 20 ml scintillation vials especially useful for processing samples for microscopy as they are very sturdy and do not react substantially with any of the solutions standardly used for microscopy. Generally fixation for immunocytochemistry is at 4 °C, although microtubules and other cytoskeletal elements are not stable at this temperature and room temperature fixation is used instead. If the samples do not sink directly in the fixative, a gentle pressure may be applied in a bell jar. Alternately, a low concentration (up to 1 %) of Tween 20 or Silwet may be added to the fixatives as a wetting agent. We have had excellent luck in fixing tissues that have been treated briefly with a Silwet solution prior to immersion in the glutaraldehyde solution. These samples sink directly in the fixative and the quality of preservation of these samples is excellent. For tissues that are waxy (heavy cuticle), this addition is especially useful. The concentration of detergent should be kept low so as to prevent leaching of the cellular contents and removal of proteins and membranes. We normally kept a diluted solution of Tween 20 in PIPES buffer and added the

solution dropwise with swirling. As soon as the tissues sank uniformly, the additions of detergent are ceased.

Piperazine ethane sulphonic acid (PIPES) buffer is the buffer system that seems to work best for the fixation of most plant tissues (Salema and Brandao 1973). We generally use a 0.05 M PIPES solution in the range of pH 6.8–7.4. Unfortunately there is no logic to the optimum pH for any given tissue so it is a trial and error process. However, pH 7.2 works well for many plant tissues used in our laboratory and is a good starting point for buffer pH. When PIPES is added to water the solution is not clear. Start with a volume of water ~80 % of the final so as to adjust for the addition of base. Gradually add 1 N KOH or NaOH dropwise under constant clearing until the solution begins to clear. At ~pH 6.8 the solution will be nearly clear and the addition of base should proceed slowly under constant monitoring. We have stored PIPES at 4 °C up to a month with no problems. Some batches of PIPES seem to have some insoluble material and these batches should be avoided.

Formaldehyde is the other fixative often used in immunocytochemical experiments, especially in those where only light microscopic localizations are required or where a bigger tissue piece is required to maintain the orientation of the tissue. Formaldehyde as a fixative penetrates more rapidly than glutaraldehyde (Hayat 1981). Traditionally, formaldehyde is prepared by adding paraformaldehyde powder and heating in 60–70 °C water under constant stirring. A few drops of 1 N NaOH will generally clarify the solution. This preparation should be performed in a hood so as to minimize exposure to the fixative. Generally prepare a much more concentrated solution (10–16 %) and then dilute the fixative into double strength buffers. Alternatively, solutions of paraformaldehyde in ampoules under nitrogen may be used with success. These have the advantages of lessening the exposure to the fumes of the formaldehyde solution and having fixative only requiring mixing with buffer. Formaldehyde may be mixed with glutaraldehyde in the Karnovsky fixative. For standard microscopy, this fixative is of great use for plant material although it seems to offer little advantage for immunocytochemistry over the single fixatives in our trials. However, other laboratories utilize it as a standard mixture (Chen and Baldwin 2007).

Osmium tetroxide is a strong fixative that reacts with double bonds, such as those abundant in the lipid components of membranes. Osmium is slow to penetrate tissues on its own so it is used generally in a two step fixation with glutaraldehyde as a primary fixative and osmium as a post fixative. Although generally the samples for immunocytochemistry are not post-fixed in osmium tetroxide, tissues that are fixed with osmium may be restored to antigenicity with a sodium m-periodate treatment on grid (see below). The addition of osmium greatly improves the preservation of membranes so that if membranes need to be detected, inclusion of the osmium step is a necessary one. Osmium is not compatible with PIPES buffer so if osmium fixation is intended, the samples are either fixed either in cacodylate buffer throughout the fixation and wash regimes or the samples are fixed with glutaraldehyde in PIPES and then washed in two exchanges of cacodylate buffer so as to remove the PIPES buffer before the osmium fixation step.