Special Topic: Advanced Basics of Immunostaining and Antigen Retrieval

W. Gray (Jay) Jerome
Vanderbilt University Medical Center
jay.jerome@vanderbilt.edu

The ability to immunologically link microscopic tags to specific proteins has produced major advances in all forms of microscopy. Initially researchers needed to make and label their own antibodies. This required a good working knowledge of immunology. Today's labeled antibodies are readily available from a variety of vendors. Although this has greatly facilitated the expansion of immunolabeling techniques, it has also led to their use by researchers with little or no background in immunology. While this is not a problem for simple, straightforward situations, the quality of data produced in more specialized experiments can suffer. Being an expert in immunology is not necessary for the average user of immunohistochemistry techniques, but some basic understanding of what antibodies are, how they are formed, and how best to use them as a cellular probe can greatly enhance the results of "non-routine" staining procedures. In this regard it is worth reviewing some basic immunology principles for the naive user and sharing some insights with the more experienced user. This is the general goal of a session that will be presented at Microscopy and Microanalysis 2003. Specifically, the session will cover what antibodies are, the variables in a "standard" immunostaining protocol (fixation, washing, choice of primary and secondary antibodies), and will cover some more specialized techniques such as antigen retrieval. This text is taken from the outline of that session.

Immunoglobulins, particularly immunoglobulin G (IgG), are the basic tool of immunohisto/cyto-chemistry. An antibody is an immunoglobulin molecule. It is produced by cells of the immune system in response to a foreign challenge (antigen). A good antibody binds to its antigen specifically and with high affinity. This binding is exploited to tether a microscopically visible marker to the antigen. In this way, the molecule can be specifically localized within cells or tissue. To produce superior localization without artifact, a good antibody is required. This point cannot be overemphasized. A little time spent obtaining and testing antibodies to insure they are useful in immunostaining will save the investigator a lot of time and trouble later.

There are a variety of paradigms for immunostaining. The most basic procedure is to label the antibody directly with a microscopic marker. This simplifies the staining procedures, but interactions between the label and the immunoglobulin often lessens the usefulness of the antibody as a histochemical reagent. The most common method is the two-step or indirect method. In this method, the antibody is reacted with the sample and allowed to bind to the antigen. Then, the preparation is incubated with a second antibody. This antibody contains the label and has been carefully selected because its properties are not adversely affected by the tag and because of its specificity for binding to the first antibody. For instance, to localize human acid phosphatase enzyme an IgG antibody can be raised in a rabbit that recognizes human acid phosphatase. After tethering the anti-acid phosphatase to the acid phosphatase molecules in the cell, the anti-acid phosphatase can be localized by tethering a labeled goat IgG that specifically binds all rabbit IgGs but does not bind human IgG. These general labeled secondary antibodies are readily available commercially. Although this two-step method takes a little more time, it saves the researcher the time of making multiple primary antibodies, labeling each antibody, and testing each for specificity after labeling in order to find one that is useful. Variations on this theme include exploiting the ease of biotinylating antibodies and the strong affinity of biotin for avidin as a means of localizing an antigen. Since each avidin will bind four biotins, modifications of the technique can be used to amplify the labeling of an antigen that occurs in low abundance.

Among the more mystical of variables in deciding on a specific immunohistochemistry technique are those classified as "antigen retrieval" procedures. The goal is to unmask "hidden" epitopes and thus increase immunostaining or allow immunostaining with antibodies that were previously not useful for *in situ* staining. Antigen retrieval procedures have been most useful with paraffin embedded sections, but are now receiving some notoriety for their success with whole mount preparations (Figure 1) and with specimens for electron microscopy. Basically, the idea is to open up sites that were hidden during processing or are occult in the native protein. The method by which these procedures work is not known, but it is thought that most function by denaturing the protein, "renaturing" the protein to its more native state, or removing the crosslinking effect of fixative within an active site. The procedures can be divided into two basic techniques. The first employ chemical means such as detergents or proteases and the second physical means such as heat and/or pressure. Heat/pressure are the more popular and appear to be more the universally useful. However, it should be realized that what works for some antibodies may not work for others. In fact, in some cases antibody staining can be reduced. Temperature, time, and pH, in that order, appear to be the most critical variables in heat-activated antigen retrieval protocols. Because of the variable of each unique antigen-antibody pair, Shi and colleagues have suggested the use of a "test battery" of techniques to determine the optimum conditions for each antigen. Although somewhat time consuming, this approach provides a high degree of certainty that your results

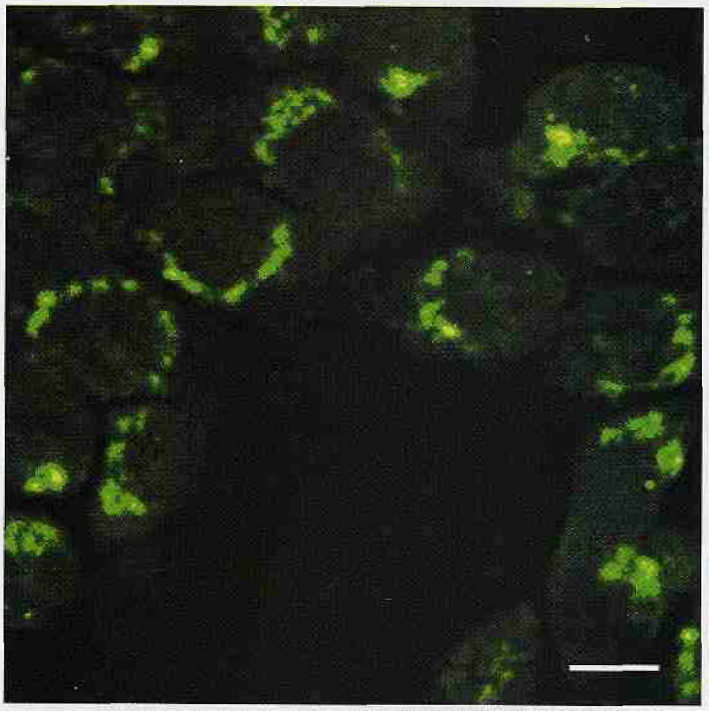
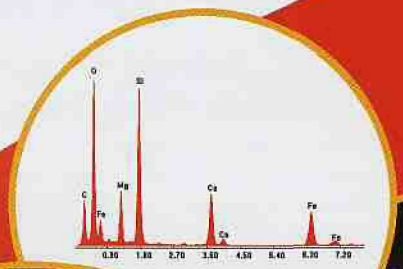
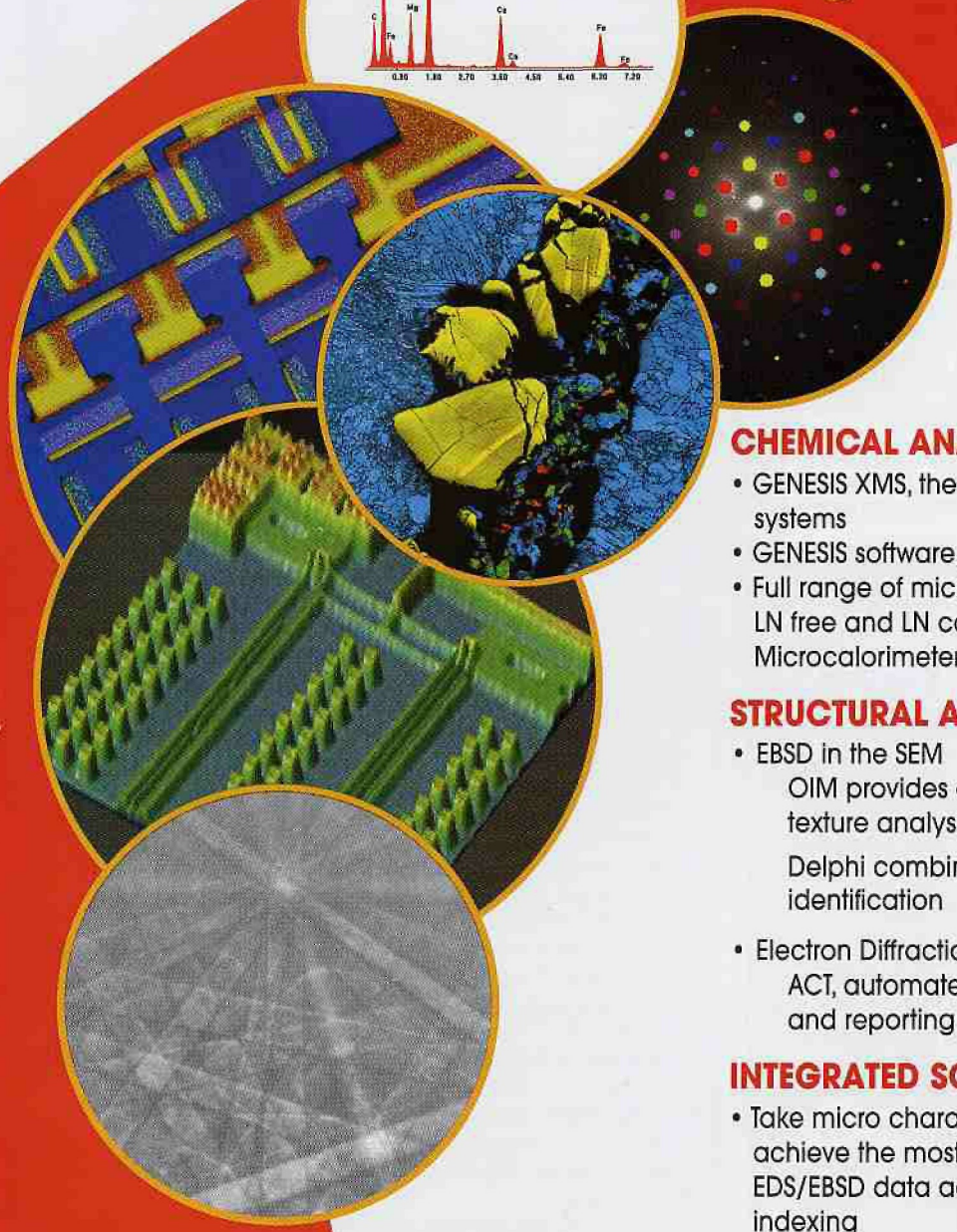


Figure 1. Microwave heating used to retrieve low abundance antigen. Anti-NPC-1 was used to localize the NPC-1 protein to hypertrophied areas of the Golgi, TGN, and associated vesicles in macrphages. Without antigen retrieval, antibody was only moderately detected by fluorescence microscopy although Western blotting indicated its presence in these membranes.

EDAX Micro Characterization Tools

Right tool for the right job ...Only from EDAX





CHEMICAL ANALYSIS FOR THE SEM & TEM

- GENESIS XMS, the next generation of microanalysis systems
- GENESIS software, easy to use with full functionality
- Full range of microanalysis detectors including Si(Li) LN free and LN cooled, WDS, Silicon Drift and Microcalorimeter

STRUCTURAL ANALYSIS IN THE SEM & TEM

- EBSD in the SEM
  OIM provides crystal orientation mapping and texture analysis
  Delphi combines EBSD and EDS data for phase identification
- Electron Diffraction in the TEM
  ACT, automated tools for the acquisition, analysis and reporting of TEM diffraction data

INTEGRATED SOLUTIONS

- Take micro characterization to the next level and achieve the most accurate results with simultaneous EDS/EBSD data acquisition and Chl-Scan chemical indexing

PHASE-CONTRAST IMAGING

- Reveal the internal microstructure of a sample with resolutions below 200nm

Americas 201-529-4880
Japan +81-3-3740-5172
Europe, Africa and Middle East
+31-13-536-4000
S.E. Asia +852-2698-7373
Email:
edax.info-americas@ametek.com or
edax.info-international@ametek.com
Visit our website at
www.edax.com

EDAX® ...advancing materials characterization TSL™

EDAX Inc. is a unit of AMETEK Inc.
Process & Analytical Instruments Division

are valid. If semi-quantization of immunostaining or comparison with other staining approaches is undertaken, the test battery or a similar approach is imperative.

Several critical factors go into the decision of the exact protocol to be used in an immuno-localization. These include how cell integrity is to be maintained, how the antigen is going to be made accessible to the antibody, what type of tag will be used, and how the localization data will be acquired and analyzed. For instance, are the cells to be fixed and, if so, what fixative should be employed? Do the cells need to be permeabilized to allow the antibody access to the antigen or is the antigen readily accessible? Do you need to visualize multiple antigens at the same time? Do you need the resolution of an electron microscope or is light microscopy sufficient? All of these are considerations in choosing a methodology. The use of immunostaining methods has become so widespread that many great articles and books have been written on the subject. Listed below are only a few examples that the author has found useful.

Some Useful References

Polak JM, Van Noorden S. 1997. Introduction to Immunocytochemistry. Second Edition. New York, Springer-Verlag.- *A nice basic, easy to read, introduction with useful protocols.*

Javois LC (editor). 1999. Immunocytochemical Methods and Protocols. Second Edition. Totowa NJ, Humana Press- *Detailed description of many important aspects of Immunocytochemistry with detailed protocols.*

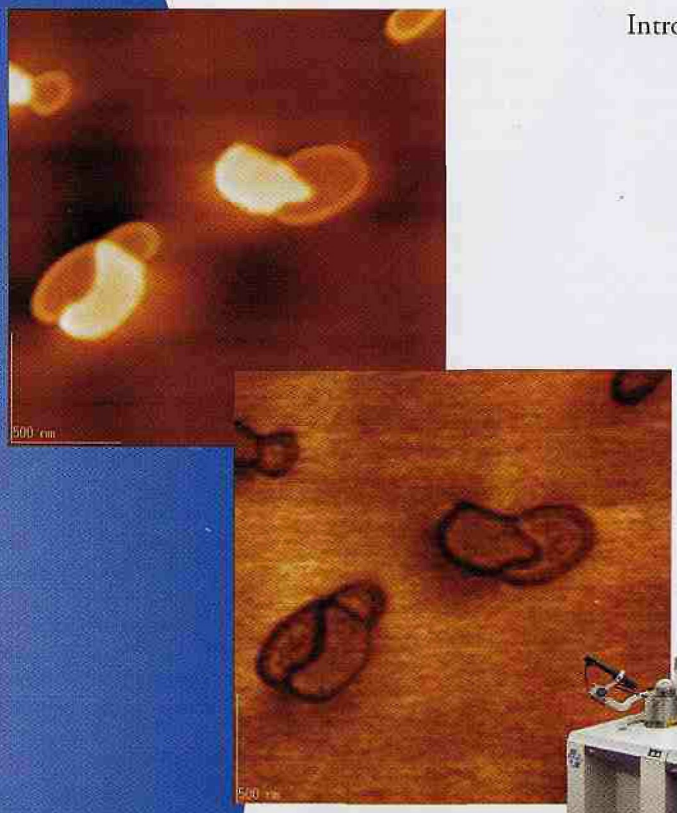
Shi S-R, Gu J, Taylor CR (Eds). 2000. Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology. Westborough MA, BioTechniques Press. - *Historical review and authoritative protocols.*

Shi S-R, Cote RJ, Taylor CR. 2001. Antigen retrieval techniques: current perspectives. J Histochem Cytochem 49:931-937. - *Brief review of key variables.*

Hayat MA. 2002. Microscopy, Immunohistochemistry, and Antigen Retrieval Methods for Light and Electron Microscopy. New York, Kluwer Academic / Plenum Publishers. - *Good review, particularly concerning electron microscopy antigen retrieval.*

Go Native!

Take total control of your SPM imaging environment



Scanning Kelvin Probe Microscopy (SKPM) and Topography Images of Sn Alloy

Introducing the JSPM-5200 from JEOL, the newest scanning probe microscope to let you explore the structure of surfaces in their native environments. Analyze samples in fluid at body temperature, investigate optical coatings, and observe magnetic and electrical phenomena.

You control the imaging environment– fluid, vacuum, ambient air, or controlled atmosphere and the temperature (130K-800K) of your samples.

Powerful image processing and analysis functions– plus more than 20 data collection modes – will let you see things you've never seen before. Easy to operate and easy to upgrade, the JSPM-5200 is the most versatile SPM/AFM instrument for your research needs.

Contact the imaging experts at JEOL or visit www.jeol.com/jspm-5200.html to see things as they really are.

- **Fluid, vacuum, ambient air or controlled atmosphere**
- **Cold and hot stages: 130K to 800K**
- **More than 20 data collection modes**
- **Easy upgrade path**

Another Extreme Imaging Solution from

JEOL

978-535-5900 eod@jeol.com