A Commentary On Immunolabeling

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Is immunocytochemistry science or magic?

We have witnessed a slow but steady decline in electron microscopy (EM) skills in the biological sciences over the last decade. This decline is illustrated by the continued closures of EM facilities throughout the world. It is, however, difficult to rationalize this decline when the need for these skills is still under a constant demand. Certainly, the introduction of new microscopes has played a part in this. High-resolution confocal light microscopes, two photon imaging and other new technologies are providing impressive amounts of new information on biological systems. Many of these approaches require simple preparation protocols providing information that can be easily interpreted and are thus easily adapted to fast-moving research.

The application of EM techniques is also deceivingly simple. For this reason the field of immunocytochemistry is undergoing an increase in popularity. Laboratories with little or no previous experience in EM are discovering the impressive and convincing results achievable using these high-resolution methods. Antibodies that label well at the light microscopic (LM) level are taken to the EM level without a second thought. The result is that electron microscopists are under pressure to provide routine immunocytochemical services for experts and non-experts all looking for simple preparation protocols and easy answers. As many people are now discovering, the wide variability of specimen preparation protocols, reagents and biological systems under investigation make immunocytochemistry a more complex tool than was first expected. Often results from EM experiments do not match up with LM labeling patterns or biochemical information obtained during antibody preparation.

One response to the production of inconsistent results is to put the blame on the mystical nature of antibodies and then catalog them in arbitrary lists of numbers to be compared with other researchers. Numbers of antibodies that only work for light microscopy are compared with numbers that work both for light and EM and percent success rates are calculated. To do this undermines the scientific premise on which immunolabeling is based, that an antibody will bind to the antigen it has been designed to recognize.

If we accept that antibody labeling is as unpredictable as we are led to think, we relegate our work to the realms of black magic. As with any scientific system, immunolabeling is only unpredictable when we have insufficient information on which to base our experimental design and subsequent interpretation. Protocol modifications or a better understanding of the biology of the system can often solve the mysteries of unsuccessful EM immunolocalizations. Therefore, comparisons of antibody labeling properties must be accompanied by detailed descriptions of preparation and labeling protocols as well as details of antibody and antigen preparation. Only then can we make quantitative, comparative statements about our results. To compare antibodies only on their general success will provide no useful data for anyone working in the field and may only serve to put off new practitioners.

If immunolabeling is so easy how come antibodies are unpredictable?

A common problem for immunocytochemists is to translate biochemical data into a morphological result. An antibody is made

by injecting a purified protein (antigen) into a host animal. This animal generates an immune response to the foreign protein resulting in the generation of specific antibodies. Specific antibodies can be purified from serum obtained from the host animal and used to bind to the protein in different systems (in solution, on nitrocellulose paper, in whole cells or tissue slices, or on thin sections of chemically fixed samples). The antibody obtained is determined by the purity of the initial antigen, the response of the host animal to the antigen and to the purification procedure used to prepare the final product. Unskilled antibody preparation will result in the production of antibodies with unpredictable affinities. Performing localizations with poorly prepared or poorly characterized antibodies will produce uninformative results.

My antibodies have been carefully prepared but I still can't see a signal.

Antibody quality is only a part of the story for successful immunolocalization. To be able to see a signal either by light microscopy or in the electron microscope requires careful specimen preparation and immunolabeling techniques. Designing these protocols requires an understanding of how the original antigen and antibody were prepared to allow for special preparation requirements. For example, if the antigen is located in inaccessible parts of the molecule, or is surrounded by other inaccessible molecules, extreme permeabilization efforts may have to be made to make the antigen accessible for antibody binding.

Should I aim for good morphology?

Years of training and superb text book images have shown us what the inside of cells should look like in the transmission electron microscope (TEM). Images of cells consist of exquisite line diagrams showing dark membranes in a cytoplasm containing dark inclusions and lots of empty space. For many people this is what TEM images should look like and there still is a pressure to produce these aesthetically pleasing images. Of course, it is obvious that this image does not fit with what we currently know of cells, that they contain molecules of low atomic number suspended in an aqueous matrix. To get the high contrast images that have set the standard for fine structure morphology, cells and tissues have to undergo a harsh series of treatments aimed at denaturing and extracting proteins, replacing biological tissue with heavy metals, and replacing water with various resins. If our aim is to visualize specific individual molecules within this low contrast, wet environment using microscopes that demand dry, high contrast images, our expectations of what we expect to see must change.

Should I aim for retention of antigens?

If we are to examine the contents of cells to study the three dimensional distribution of an increasing number of subcellular molecules, then our preparation protocols should retain these molecules in their "normal" location within the cells. We should also expect that as many other molecules surrounding our molecule are retained thus giving us the option of studying molecular interactions. These associated molecules should also be preserved in a form as close as possible to their natural structure. The disadvantage of this approach is that we set limitations on the number and form of treatments we can expose our samples to, and because we are not extracting components of cells, we have to accept that our final images may have lower contrast than is usual for electron microscopy.

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My antigen is easily accessible, but I still have no signal.

Gaining access to allow antigen-antibody binding may be as simple as preparing sections. Large amounts of antigen are exposed and accessible on the section surface. If this is so and there is still no detectable label, then the lack of labeling may be traced to other causes. Sometimes the labeling protocol used to apply the antibodies may not be optimal. For labeling at the EM level it is important that the antibody concentration be optimal. It is often good practice to use primary antibodies at the highest concentration that does not produce background label. The time of exposure to antibody should be optimized to this dilution. Using antibodies at sub-optimal dilution or exposure time may result in variation in specific signal. Other reagents used in the labeling protocol will also effect the specific signal. For example, if serum is used as a blocking step for localizing antibodies to proteins that are present in serum, specific signal will be reduced or even eliminated. Liver sections labeled with antibodies to serum albumin (which should label the secretory pathway of hepatocytes) will show little or no signal if the antibodies are diluted in serum (which obviously will have serum albumin present!).

Why does my antibody work for light microscopy but not for electron microscopy?

If an antibody works for light microscopy then it should work for EM too. If this is not so, then the specimen preparation methods used for each localization experiment are so different that comparisons cannot be made. A typical preparation protocol for LM labeling is to label unfixed, air-dried, methanol treated cryostat sections. A cryostat section is at least 10 µm thick. Dry this down to a thin smudge on the slide, make it totally accessible to antibodies by exposure to methanol, and you basically have a two dimensional sample with antigen concentrated down onto the glass substrate which is totally accessible to antibody. Apply antibody, add a secondary fluorescent antibody (and maybe a bridging antibody to amplify the signal) and a very obvious result is obtained.

Now look at the typical EM protocol. The sample is chemically fixed to preserve morphology (maybe 4% formaldehyde and 0.1% glutaraldehyde), then it undergoes a series of dehydration steps and is finally embedding in (*e.g.*) LR White. An alternative approach is to prepare the sample by freeze substitution into Lowicryl resin after rapid freezing and use no chemical fixative. For either approach the morphology in the sections will generally be very good. But where is the signal?

One possibility is that the signal cannot be generated because the sample has been so well fixed and embedded that the antigen, or the gold probe, or both, cannot gain access. If the same protocol used for LM was also used for the EM preparation, there is a high probability that signal would be detected. The problem is that although it is perfectly acceptable to air-dry unfixed samples for LM immunocytochemistry, there is a great pressure to always aim for good morphology at the EM level. This is often the first prerequisite for any immunolabeling approach at the EM level. We have to ask why there is such importance attached to good morphology when we only require morphology to enable us to identify labeled structures. One reason is that electron microscopists are trained to produce aesthetically pleasing images with almost artistic quality.

A good practice that will save many hours of frustration is to test samples and immunoreagents to be used at the EM level by light microscopy first. Usually this involves preparing thicker resin or cryosections from the EM blocks and mounting these on a glass substrate. They can then be immunolabeled, with the primary antibody being visualized either with fluorescent-conjugates or with silver enhanced gold probes. This confirms immunoreactivity in the system and also gives a good indication of amounts and distribution of antigen.

Another reason why there may not be a signal detected at the EM level could be that antigen amounts are too low. The best immunocytochemical approaches give us information on the location of antigens and their relative number. If an antigen is only present in small number, then the signal should also be present in small number. Sometimes the amount of antigen present is so small that it requires a long and patient examination of many sections before a meaningful answer can be extracted. Even then, rigorous quantitation of signal may be the only way of presenting the data.

Remember that at the EM level, the amount of specimen that is sampled is a very small part of the whole. To be seen in the EM, the sections have to be hundreds of times thinner than cryostat sections (60 nm for EM but 10,000 nm for LM). To make things even worse, the immuno-reagents seldom penetrate the section. This leaves only antigens at the section surface available for binding. It is possible that the specific signal on any section may be one gold particle over one structure on the whole section.

It is easy to check if such a result will be expected. First find out how many antigens are present in the sample and work out how many should be exposed on the section surface. Labeling efficiency (the relationship between antigen number to observed signal) is usually between 5-20% (*i.e.*, if 100 antigens are available for binding only between 5 and 20 will label). From this it is possible to get an estimate of how many antibodies will bind to the section. It is often surprising that signal is ever detected.

Why can I get a good signal by LM but have too much background by EM?

One reason for this may be that the antibody dilutions or the exposure times are not optimal. This can be easily remedied by careful experimentation. A more difficult problem is the persistent background over biological tissue that should not be there. This may take the form of gold particles over the cell cytoplasm being observed with antibodies that should only label membranes. Or mitochondrial labeling being detected when using anti-biotin antibodies to visualize cell surface biotin. Or mitochondrial labeling occurring when using antibodies to detect lysosomal membranes inside the cell. These are annoying problems that often condemn the antibody to the trash heap when it comes to EM labeling.

A more careful examination of the labeling patterns might have rescued them and also produced interesting observations. The first step for any EM localization is to first examine the antibody labeling pattern by LM. This will deliver a good idea of the optimal dilution for labeling. It will also give a good idea of how plentiful the antigen is and its relative distribution. More importantly, it will also give information on the levels of background labeling. This will only be present if the LM labeling is honestly evaluated. Careful manipulation of LM images, either with the microscope or, more usually, at the computer may produce images of little use for interpreting our EM results. It is an easy task to re-

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move annoying background from fluorescent images. In the EM this same background will appear as gold particles that cannot easily be ignored or removed.

The examples given above are actual examples of background labeling resulting from the presence of specific antibodies in the primary antibody. The problem was that they were specific to targets other than the ones under study. Cell cytoplasm, as a contaminant of the membrane fractions and present in the injected antigen produced specific labeling of cytoplasmic proteins. Only a pre-incubation of the diluted antibody with purified cytoplasm removed this "background". Similarly, contaminating mitochondria in lysosomal antigen preparations produced specific label over mitochondria. In this case, this "background" was not detected at the LM level as the antibody produced labeling patterns expected for lysosomes. Finally, anti-biotin antibodies used on cell sections to detect surface biotin specifically labeled biotin-like molecules present in mitochondria. Interestingly, biotin-like molecules are also present in serum. This has the profound effect of removing specific anti-biotin activity of antibodies or streptavidin if serum is used as a blocking agent.

Why are anti-peptide antibodies so difficult to work with?

No antibody should be difficult to work with. Problems arise when we attempt to fit all our antibody reagents with the same labeling protocols. Antibodies and other affinity markers are not inert chemicals. They are biologically active molecules that usually have predictable binding properties. Once we understand how they bind and where the targets of this binding are located, then they become useful tools. This process of figuring out the best preparation protocol can often be tedious and may not be possible in busy laboratories that are only able to apply routine protocols.

If an antibody is made to a short peptide sequence, this is usually located on a small part of the target protein. Antibodies will only bind to this sequence. They will not bind to other parts of the molecule. If the sequence is embedded deep inside a folded protein, antibody binding may be difficult to achieve. Similarly, sequences embedded in transmembrane domains may also be relatively inaccessible to antibody binding.

It is also important to remember that titers and affinities will differ for different antibodies. A polyclonal antibody raised to a whole protein will contain immunoglobulins able to bind to all parts of the protein that elicited the immune response. Specific signal should be easily detected as the antibody will bind to all parts of the molecule that is exposed. Anti-peptide antibodies will only contain immunoglobulins that specifically bind to the target sequence. Only if this target sequence is exposed will labeling occur. We should expect lower signal levels for these antibodies.

Why do my IgM antibodies not work at the EM level?

Proper storage and handling of antibodies and other immunoreagents are critical parts of the immunolabeling process. Remember that immunoreagents are proteins that depend upon their structural integrity to retain their function. Freezing proteins for storage will produce damage to these molecules that may or may not be detectable. Certainly, larger molecules will be more susceptible to this freezing damage than smaller molecules. One round of a freeze-thaw cycle may be enough to break IgM's sufficiently to

make them unusable at the EM level.

The surprising thing is that for LM, even low concentrations of low affinity antibodies can sometimes be detected. Imagine that only about 10% of the IgM is structurally intact but all the rest is totally fragmented. This will have two effects. First, the antibody dilution will have to be increased for EM detection when compared with LM experiments. Second, the fragments may still bind to antigens on sections, but because they are fragments, the secondary antibodies may not recognize them. The fragments will compete for the specific binding sites stopping the whole molecules (which can be visualized) from binding. For LM, where there will be many more antigens present, and where amplification techniques can be used, the signal will still be detected.

At the EM level, reduction in specific binding is often a disaster. The small sample means that the amount of antigen present to bind the antibody is reduced to begin with. If the immunoreagents are reducing the available antigen to levels we cannot predict, we have no idea what is happening. Understand how storage is affecting the immunoreagents and know what the secondary antibody recognizes. These are important steps in the process of understanding why the antibodies do not work.

Can I really use LM preparation methods for EM?

If we are able to perform multiple labeling experiments, where we can use one antibody to identify specific organelles, then absolute morphology is not important. We can disrupt our cells so that they become bags of loosely packed organelles and our antibodies have total accessibility to antigens. We can even partially purify these organelles and work with them as a pellet or adsorbed onto specimen grids. We will loose spatial information if we do this, but we will get a signal.

Similarly, if the only way we can get a signal by LM is by air-drying and acetone fixing, then use the same protocol for EM. The protocol will be easy (pre-embedding label) but the morphology will be awful. Remember, this is what light microscopists accept as good morphology! Once the label system has been established to work by EM (*i.e.*, all the reagents work to give a result), then it will be possible to start modifying the protocol to improve morphology. Perhaps fixing the sample in 2% formaldehyde for 30 seconds, followed by a short homogenization (improving membrane preservation and controlling cellular disruption) is the answer. Maybe a fixation in 5% glutaraldehyde in a low osmolality buffer, so that the cells swell, is the answer.

The exact recipe will depend on what is known about the antigen. If it is a membrane protein, is the epitope deeply embedded inside the membrane? If so, will this require membrane disruption (freeze-thaw? detergent?) or will it be sufficient to just remove the cytoplasm? Each system has to be approached as a completely new project and each will have an answer.

Knowing as much as we can about the antigen is our first step to success. Allowing ourselves to work with samples that do not have "text-book" quality morphology is the next step. Being totally open about how we are willing to prepare samples and look at our results, completes the process.

Conclusions

This commentary originated from a discussion started on the MSA listserver* and was aimed at opening a discussion on the problems faced when attempting immunolabeling experiments. It also aimed at stimulating immunocytochemists to think a little

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about their systems and immunoreagents before giving up their experiments.

The choices open to us for immunocytochemistry are wide and it is often confusing to know where to start. However, I hope that I have provided some help when faced with the ubiquitous problems we have all had to face with immunocytochemistry. This is not meant to be a complete guide. For this I recommend the book by Gareth Griffiths which covers this subject in great detail, and which has been my invaluable reference book for many years (G. Griffiths, 1993).

Immunocytochemistry is a very special branch of EM that is almost impossible to provide as a service. However, this is what is being asked of EM labs all over the world and I know it this will be virtually impossible to change. For this reason, it is our responsibility to educate our colleagues and to allow them to become involved in the discovery process of how their antibodies work. I have no good solution to this, but do know that if someone is involved in their own specimen preparation and data collection, their work will progress much faster. If new EM users are taught why particular approaches should be applied instead of being given one protocol to apply exactly as written, pretty neat ideas originate from their work.

We must stop being the "black box" of science. ■

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LR White Flat Embedding of Cells Grown on Coverslips

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We do this two different ways, using polyethylene (PE) molds or aluminum weigh boats, depending on the sample. You can either heat- or UV-cure these molds – just change the type of "lid" on the embedding mold.

The PE molds are used when the coverslip can be made to fit in the mold. We use JB-4-type molds, available from your favorite microscopy supplier. The key to using these molds is that they have to be pre-treated…fill the inner cup of the mold with LR White, cover with an aluminum JB-4 chuck (try to have enough resin in the mold to come up around the base of the chuck), and polymerize in a 60° C oven for a day or two, until the resin is hard. The Al chuck can usually be removed by hand, but a flathead screwdriver used as a pry will help pop it off if you have trouble. Dispose of the resin block and wipe out the cup with KimWipes…the mold is ready to use for real samples. The Al chuck can be cleaned by soaking it for a day in methanol (in the hood). Any resin remaining stuck to the chuck can be scraped off with a spatula. Our guess is that there are micropores in the molds that have to be sealed; we've had problems with incomplete polymerization of blocks when fresh molds were used. The molds last until you get sick of them or until someone turns up the oven without checking to make sure it is empty and melts everything. Thermanox or other plastic coverslips can be cut to fit the molds (before the cells are plated!); glass coverslips can be broken to fit. Tissue pieces that are too large for embed-