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Dako

Connection

SPECIAL ISSUE:

IHC STANDARDIZATION

Procedures in Immunohistochemistry

In this issue

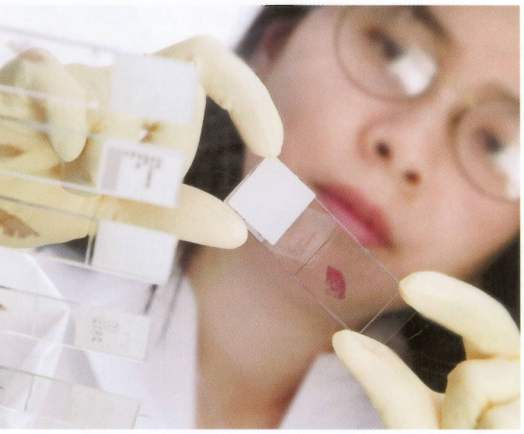
Q & A with the Pathologists on Immunohistochemistry Standardization

Profiles, interviews, opinion and articles from leading researchers

Q&A

Ask the Experts:
On Immunohistochemistry Standardization

The Interviews are based on the Review Article; Recommendations for Improved Standardization of Immunohistochemistry. Applied Immunohistochemistry & Molecular Morphology. 15(2):124-133, June 2007. Goldstein, Neal S. MD; Hewitt, Stephen M. MD, PhD; Taylor, Clive R. MD, DPhil; Yaziji, Hadi MD; Hicks, David G. MD; Members of the Ad-Hoc Committee On Immunohistochemistry Standardization.



Identification of a Slide for analysis

Connection: ***In your first recommendation, you say that non-formalin fixatives are strongly discouraged. However, Formalin's primary ingredient, formaldehyde, has been declared a possible human carcinogen by the Environmental Protection Agency and by the Occupational Health and Safety Administration. Studies have also found that formaldehyde impairs the sense of smell and the respiratory system, irritates the eyes and skin, causes migraine headaches and causes cancer in laboratory animals. Would you still recommend Formaldehyde as the fixative of choice and why?***

Dr. C. Taylor: Yes, formalin has these well known drawbacks, and should be used carefully. That being said – it is the 'best' fixative for regular morphology in that it is forgiving in time of exposure, produces morphologic features that pathologists are trained to interpret, and is cheap.

Times vary in fixation. The closest I have seen was in 70% ethanol and it has limitations. Then you also have to consider cost - both to purchase the fixative and to dispose of the fixative. Is it flammable? How long is it stable on the shelf? If it is, your costs escalate. With formalin, you need good ventilation, utilization and disposal, these risks are minimal.

Dr. H. Yaziji: Until a viable alternative is available, formaldehyde should continue to be the first and only fixative for clinical specimens.The above precautions are real; however, with proper ventilation, utilization and disposal, these risks are minimal.

Dr. S. Hewitt: Yes. Do not think I love formalin. It stinks and burns, but compared to the alternatives it offers the best trade-offs in economics and efficiency. You have to look at the big picture. There is some diagnosis that requires formalin for the cytologic artifacts. One example is the characteristic nuclei of papillary carcinoma of the thyroid. Step back and look at how a lab runs, you need a fixative that is flexible enough to deal with specimens that range from biopsies to organs.

> "Until a viable alternative is available, formaldehyde should continue to be the first and only fixative for clinical specimens."

Dr. S. Badve: Even water can cause death if one drinks too much of it. It is important to remember that nearly all the solutions that we use in the lab are toxic in some form or the other. It is imperative to use safe practices at work irrespective of the kind of substances one handles in the lab.

The main question is whether the toxicity is manageable or not. Good ventilation and not working with very strong solutions is the key to dealing with formalin. From the administrative and cost stand-point it is very cheap and easy to dispose off.

Far more importantly than the points above is the fact that we have used formalin for years and years. We know it is not an ideal fixative, however, over the years we have got used to the artifacts it creates and learned to interpret the morphology in a more or less consistent manner. Additionally, all the immunohistochemistry data related to reactivity of antibodies is based on standard formalin fixation. This becomes relevant not only while diagnosing relatively rare lesions but understanding the uncommon reactivity of common antibodies such as keratins in malignant melanoma.

> "The more fixatives you add to a laboratory, the more complicated you make it for everyone"

As we go marching ahead into the era of predictive and prognostic markers and associated targeted therapies, it is going to be even more important to have uniform fixation and processing standards. As highlighted by the poster child of targeted therapy, detection of HER2 expression is significantly influenced by fixation. For this reason it is mandated by the FDA that HER2 testing (by IHC or FISH) should be performed on formalin fixed tissues. More recently, the ASCO-CAP (American Society of Clinical Oncology- College of American Pathologists) guidelines have reiterated this view.

Lastly, we know from our experience with various antigen retrieval solutions that the exact constituents of the solutions matter. Not all low pH citrate-based antigen retrieval solutions perform the same. For a given antibody (and lab) one brand can work better than the other. The same might be true for buffered formalin; at the current time, effect(s) of additives to formalin has not been adequately studied.

Connection: ***Is it true that a particular histology feature may be better demonstrated by other fixatives, such as Glyo-Fixx for nuclear features and lymphocyte appearance and Omnifix for cytoplasmic detail (Arch Pathol Lab Med. 2005; 129: 502-506). Would you recommend these fixatives in the future?***

Dr. C. Taylor: NO

Dr. H. Yaziji: No. There is sufficient evidence to discourage the use of glyoxal-based fixatives because of detrimental effects on FISH (and to a lesser degree) IHC testing.

Dr. S. Hewitt: The claims are narrow. If I really wanted to make claims about cytologic features, I would be using B5 for lymph nodes and 70% ethanol for prostate. But that is not a reasonable approach. You need a broad diagnostic fixative. Specialty fixatives are just that. B5 is gone because of the mercury. 70% ethanol is not flammable and actually is easy to integrate, but makes lymph nodes look horrible.

The more fixatives you add to a laboratory, the more complicated you make it for everyone - histotechnicians, pathologist, immunohistochemist.

Dr. S. Badve: It is true that morphology with several fixatives is better than neutral buffered formalin. The hematopathologists have known it for years and were using formalin containing metals such as zinc or mercury to enhance the morphology. However, it was soon realized that these fixatives gave rise to problems when performing IHC analyses and most places have stopped using these fixatives. The simple truth, to put it rather bluntly is, "formalin is the devil you know"

Connection: ***Can you comment of the molecular mechanism of Formalin Fixation***

Dr. C. Taylor: Not really well understood, certainly by me, but also by pathologists in general. The general working concept involves 'cross linking' of protein groups by the aldehyde derivatives, and while I have published in this area (Immunomicroscopy - A Diagnostic Tool for Surgical Pathologists, Taylor CR , Cote RJ, Saunders 2006, and in Antigen retrieval techniques, Shi SR Gu J Taylor CR. Eaton Press 2000), I make no pretence to understand the chemistry.

Dr. H. Yaziji: Can you clarify the question a bit more?

Connection: ***How do Formaldehyde molecules act on tissues/cells to make it a good fixative? In other words, what makes this chemical a special fixative in your opinion?***

Dr. H. Yaziji: Formaldehyde is not a very good fixative. It infiltrates the tissue very fast, but it has a very slow fixation rate, and it does not really fit the rapid turnaround time needs of the modern world of pathology.

That said, there is no proven better alternative yet, and what makes it a standard fixative is the huge body of literature on predictive markers of thousands of formalin-fixed specimens. That is what gives formalin the advantage over other fixatives, none of which has been documented and/or tested to the extent that formalin has.

Dr. S. Hewitt: Not as well as the papers can. What I can say is that I do not think we actually have the chemistry completely understood, and users underappreciated the impact of different buffers in their formalin, or the impact of old, salted-out formalin.

Dr. S. Badve: The exact mechanism and pathophysiology of fixation is poorly understood, and results on a given protein are unpredictable. Formalin is a solution containing formaldehyde which dissociates at a slow rate. Upon immersion of tissue in formalin, it interacts with formaldehyde resulting in a gradual "fixation". The speed of this process is notoriously slow, partly due to the poor penetration of water soluble formaldehyde through cellular lipids. Hence the common observation, that fatty tissues (such as breast) are difficult to fix and require longer exposure to the fixative.

Connection: ***In your comment regarding recommendation two, you say that tissue processing is not a black box process and that "changes in the processing system have the capacity to alter the chemical makeup of the tissue specimen". Could you elaborate on the changes in the "chemical makeup" (or molecular alterations) of the tissue?***

Dr. C. Taylor: Again, in simple terms, the fact that chemical cross-linking occurs almost certainly affects the folding of the protein molecule, the tertiary structure, if you will. These structural changes in turn affect the recognition and binding ability of an antibody on formalin fixed tissue, because the antibody binds to its antigen through a 3-D charge/structure relationship. The idea is that AR may reverse, in part, these changes.

Sectioning with a microtome

Dr. H. Yaziji: I don't know the exact answer to this question.

Dr. S. Hewitt: "Tissue Processing" is three steps - dehydration, clearing and impregnation, and the latter steps are dependent on the former. If you look carefully, you can define differences in the biomolecules recovered from paraffin embedded tissue based on the process. This is easiest at the RNA level, but we can see it at the protein level. However you can not divorce this from the steps of preparing the tissue and fixing the tissue. They are all related.

Dr. S. Badve: Standard processing protocols entail the use of alcohols after the tissue is fixed in formalin. Alcohol is also a fixative with relatively rapid penetration. So if unfixed tissues are "loaded' onto a processor, they are more likely to undergo significant "alcoholic" fixation which can lead to altered IHC and false positive results.

Connection: ***In recommendation three (Antigen Retrieval) you recommend one AR protocol. Which protocol would be your number one recommendation? How would you standardize this method with so many variations?***

> "So far no one has enforced any rule about fixation; and no one has really tried"

Dr. C. Taylor: In general citrate pH 6.0, 20 minutes, 100 ^{0}C; but some antigens do better under different conditions. We like to do a "test battery'" of a few protocols to find the best one for each antibody/antigen pair.

Dr. H. Yaziji: The most popular AR that works on the overwhelming majority of antibodies worldwide is heating the tissue in citrate pH 6.

Dr. S. Hewitt: There are better people to ask this one than me.

Dr. S. Badve: There is no one protocol that works for all antibodies. Most labs, (including those that use black box technology i.e. fully automated systems where you load the unstained slides and program the machine to do all the staining without any additional input from humans) use more than one protocol. A useful method when introducing a new antibody is to run the positive and negative control slides with all the AR protocols available in the lab and select the one that works the best for your local conditions (with the least concentration of a given antibody).

Connection: ***Despite the popularity of antigen-retrieval (AR) techniques, the precise molecular mechanism underlying the process remains enigmatic. What is the most understood AR technique?***

Dr. C. Taylor: Don't think any one is most understood.

Dr. H. Yaziji: Like you said, they are mostly speculations without solid evidence. The cross-linking hypothesis is the most popular one, but again without solid proof.

Dr. S. Hewitt: AR is certainly shrouded in mystery. I view it as simple heat time and pressure. Go back to physical chemistry.

Dr. S. Badve: Akin to fixation, the process(es) associated with AR are poorly understood. Although the changes seem to be different for various proteins, the overall process seems to be relatively specific for a given protein.

Connection: ***How would you like to enforce recommendation four through ten? In other words, who will oversee these recommendations and enforce the rules (Food and Drug Administration (FDA), American Society for Clinical Pathology, College of American Pathologists (CAP).***

Dr. C. Taylor: Well so far no one has enforced any rule about fixation; and no one has really tried. CAP and others have recently made a limited set of recommendations, limited in turn to Herceptin; and CAP will try to enforce it starting January 1st. The most likely outcome will be to stop a lot of labs from doing the test; and nothing that is done prospectively will address all the "test data" currently out there.

Dr. H. Yaziji: I hope the pathology societies (CAP, perhaps) will enforce them without interference from the federal government. If we don't self-enforce them, they will be enforced by outside agencies such as the FDA, Clinical Laboratory Improvement Amendments/ Centers for Medicare & Medicaid Services.

Dr. S. Hewitt: No comment. I work for the government.

Dr. S. Badve: The recommendations are based on good practices and like morality cannot be legislated. This is not for the want of trying. There are several problems that one will encounter if one were to attempt to legislate. Let's take a Keratin antibody for instance, several vendors make these antibodies. Who is going to be the "decider" who proclaims one clone to be better than the rest and mandates its use to the exclusion of all others. This is not possible in a free society. It also assumes that we know and have the means to judge a product as good or bad i.e. we have a GOLD standard. The sad truth is that gold standards are rare in clinical practice. To go back to the classic example of HER2, where response to therapy can be possibly used as a parameter to develop a gold standard, we still do not have a consensus as to which technique (IHC or FISH) is better in predicting likelihood of response.

Connection: *Recommendation 13: How would you envision the development of a universal IHC scoring system?*

Dr. C. Taylor: Can only see that happening by automation; and then one antigen at a time.

Dr. H. Yaziji: A simple and practical one is semi-quantitative (focally positive, variably positive, uniformly positive). For predictive factors, this is different and should be decided on a marker-by-marker basis. But for non-predictive markers, the above system should suffice.

Observation under a bright field microscope

Dr. S. Hewitt: I do not. I envision objective scoring systems based on function of the assay.

Dr. S. Badve: Universal IHC scoring system is a good theoretical concept; however, it is often not practical. Data from the quality assurance studies conducted by the CAP and by United Kingdom National External Quality Assessment Service (NEQAS) have shown significant variation in the results obtained in different labs on more or less identical material. Depending on the lab the results can vary from negative to weak positive to strong positive even for relatively common antibodies such as estrogen receptor. The reasons for this variation include the pre-analytical variables which generate a need for major differences in IHC protocols.

Connection: *Recommendation 14: Would a template/procedure/scoring system for reporting IHC standardization assays be beneficial. Do you recommend it?*

Dr. C. Taylor: See above – specifics will have to be 'specific' for each test; some general approaches may have merit as recommendations.

Dr. H. Yaziji: Yes, and yes.

Dr. S. Hewitt: Yes

Dr. S. Badve: Any kind of standard format is better than not having one. It might be easier for predictive and prognostic factors ,but most of the other reagents defining true positive is difficult. When using IHC for classifying a tumor of uncertain histogenesis, the definition of positive varies significantly with the context. Many pathologists will call a tumor spindle cell skin tumor keratin positive even if only a few cells express keratins and particularly, if the tumor lacks positivity for S100 or HMB-45.

> "As more predictive assays come about, there will be greater pressure to standardize tissue handling"

Connection: *When do you expect these recommendations to become effective?*

Dr. C. Taylor: I couldn't guess; but not soon, except on a test-by-test basis.

Dr. H. Yaziji: When they trickle down to regulatory agencies (such as Clinical and Laboratory Standards Institute (CLSI), FDA, CAP).

Dr. S. Hewitt: Yes, I think this will happen.

Dr. S. Badve: Any kind of standard format is better than not having one. It might be easier for predictive and prognostic factors, but most of the other reagents defining true positive is difficult. When using IHC for classifying a tumor of uncertain histogenesis, the definition of positive varies significantly with the context.

Many pathologists will call a tumor spindle cell skin tumor keratin positive even if only a few cells express keratins and particularly, if the tumor lacks positivity for S100 or HMB-45.

Connection: *What are the emerging issues that might affect IHC Standardization (Automation, Robotics, Outsourcing)?*

Dr. C. Taylor: You named the three most important. In my view, automation of the "total test" is vital, plus image analysis for scoring. As it gets to be difficult and/ or expensive to meet the elevated standards, more and more tests will be sent to reference labs just as occurred in clinical pathology.

Dr. H. Yaziji: Can you be more specific? Connection: In a few years Robots/ Instruments may replace people to do the routine fixation, staining etc., (like the automobile industry). Also, some of the routine procedures such as mounting may be outsourced to India or China. Who will enforce the rules in these countries?

Dr. H. Yaziji: It's possible that some private equity firm will fund a major quest-like mega lab in India. I am not sure, if this is allowed/ allowable by law. Assuming it's legally feasible to outsource entire patient samples for pathology and laboratory processing, it becomes a logistical nightmare for the CAP (and other regulatory agencies) to ensure that these off-shore laboratories are doing what they are supposed to be doing. In my opinion, the module of outsourcing manufacturing jobs to China and India, if we can draw any valid conclusions from that module, does not carry good news for standardization of tissue processing, IHC and molecular procedures.

Dr. S. Hewitt: Robotics and automation are already here. Outsourcing, in the correct environment offers the capacity to improve immunohistochemistry. Volume can drive quality. Certainly when volume is too low, validation of assays is challenging. I believe "pre-analytic" issues remain the big issue. As more predictive assays come about, there will be greater pressure to standardize tissue handling. This is clearly counter to the push to see more diversity of fixatives and processors. We need objective data on quality. Histology is not an objective measure of quality. If we are going to have personalized medicine, then quality will be measured in the capacity to measure the biomolecules in tissue. This must be objective. IHC is certainly the first platform in clinical labs that enables this.

> "Who will pay for this new level of test performances-because it will cost more."

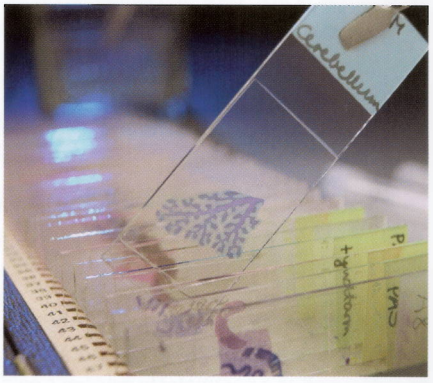
Dr. S. Badve: Automation and robotics is a double-edged sword. Although it may enable/promote standardization of practices, it might at the same time introduce novel variables. These might be in the form of alternative fixatives or use propriety fluids which contain additives; the effects of these may not be obvious for a period of time.

Connection: *If it is not asking too much - could you tell us how you would address the concerns of those who believe that these rules are difficult to implement?*

Dr. C. Taylor: Can't really answer this; I think that the 'rules' will be introduced piece meal – and will evolve and broaden as labs either adapt or give up and send out the assays. So it will be difficult; but if the payers only pay for properly performed tests, then that will solve the problem quickly!

In my opinion, the biggest and most important unanswered question (because it has not been raised at the highest levels) is who will pay for this new level of test performance? – because it will cost more.

Dr. H. Yaziji: Anatomic pathology is like baseball. All the rules and enforcements have been subjective. It is time for the pathology community to treat anatomic pathology the same way they treat clinical pathology before it's too late.



A stained tissue on a glass slide

Dr. S. Hewitt: I am listening, but I am also listening to the demands of clinicians and patients. I think the biggest challenge is economic. They want more and do not have plans for paying more. Well that is not going to work.

If they want more, and I do believe more information is warranted, then reimbursements have to rise to cover the costs- this is across the board. You do not know what specimens will need molecular testing, and it is not feasible only to pay additional money for those that do.

The model needs to be - Pathology - providing more information for better patient care. In the end it makes better sense. Invest in pathology and personalized medicine, and you will not have to give trials of ineffective, expensive drugs. A penny saved is a pound earned.

Dr. S. Badve: These recommendations have been made by practicing surgical pathologists. They range from the mundane to things we all agree are great goals to have, but will be difficult to implement. However, it is important to remember these recommendations are made by an ad hoc committee with no regulatory powers. They should be seen as a map of where things are and where we need to be in order to provide optimal quality and consistency of lab results; something every lab director strives to achieve.

Connection: ***How would you rate European, Asia-Pacific and Latin American IHC Standards? Are your recommendations followed in these countries? Who sets the standards in these countries?***

Dr. C. Taylor: UK NEQAS, in my view, is ahead of the field in most areas, but not all. They still don't really tackle fixation; and they don't have a good enforcement – police force in place.

Dr. H. Yaziji: The best QC methods in standardizing IHC are across the Atlantic and north of the border. There's ample data to suggest that the CAP proficiency testing doesn't come close to the European and Canadian systems.

Dr. S. Hewitt: Yes and no. Some medicine is international, but there are local variations. Overseas, you do not see the use of vacuum processors as commonly, and the paraffins are at higher melting temperatures commonly. I would anticipate some of them would be adopted.

Dr. S. Badve: The most well known quality assurance program is the UK NEQAS. Similar programs have been set up by many countries with variable results. Traditionally these have been set up by pathologists or pathology societies and the participation has been voluntary with little "regulatory activity". With the advent of targeted therapy, other bodies such as ASCO are also getting involved in ensuring quality assurance and quality control. One of the major issues that frequently arise is the availability of adequate control material for use in proficiency testing.

> "I feel that I have a responsibility to share the accumulative knowledge that I've learned over the years..."

Connection: ***As a leader in the industry, how have you kept up with the changes?***

Dr. C. Taylor: I talk to people, read the literature, attend selected meetings that appear key to me, and talk to some more people. No one individual, or one group or organization, can make significant change here; this requires a co-ordinated and global response. It is starting; but will take time; it has taken more than 20 years to date, and may take 20 more. Hope I can still read.

Dr. H. Yaziji: Yes, it is always challenging for anyone to balance between their family duties and their academic career. I can't claim that I am very successful in keeping this balance under control. On the professional side, I feel that I have a responsibility to share the accumulative knowledge that I've learned over the years with the community that needs it most.

What's worse, is that the more knowledge we acquire, the heavier the burden gets. I, like every academician, feel that it is my duty to contribute to the community what I think is useful. My consulting company is doing just that, and I hope that our work speaks for itself. Certainly, we are having fun doing that and I am truly fortunate that I'm part of a very interesting activity revolving around cutting-edge issues related to ancillary testing in pathology.

Dr. S. Hewitt: It is a chronic challenge. You will actually see me in the booths a meetings kicking the tires on instruments, and trying reagents for the fun of it, when I can afford to. We borrow and collaborate with other groups. My interest is very bio-specimen driven and less disease specific, so we tend to do lots of collaborations.

Dr. S. Badve: There are major developments going on in the field and it is impossible to keep up with all aspects of scientific and technological developments. I have learnt a lot during the discussion we had at this committee meeting. As in anything else, getting involved is an important part of the solution.

Connection: ***Thank you for taking the time to give us your opinions.***