Handout for the NSH One-day Immunohistochemistry forum.
Tuesday, September 16, 2008

'ROUTINE' TISSUE PREPARATION AND IHC: FIXATION/PROCESSING RE-VISITED.

Bryan R. Hewlett, ART, MLT
Quality Management Program-Laboratory Services,
Ontario Medical Association,
Toronto, Ontario, Canada

ABSTRACT

Diagnostic Histopathology, based largely upon histomorphological criteria, is a subjective "art" which relies heavily on the skills and experience of the pathologist. The histomorphological criteria are influenced by the preparative techniques which are chosen for use.

Increasingly, pathologists are being asked to provide additional diagnostic, prognostic or predictive information, particularly in the area of tumor classification. This information is critical for proper therapeutic management of the patient. Much of this information is gathered by application of immunohistochemistry (IHC) to the tissue sample.

IHC has evolved to become an established routine staining technique in many hospital laboratories. IHC has the capacity to identify, and even quantify, specific substances which cannot be characterized by conventional tinctorial staining techniques. The sensitivity with which IHC can detect a specific substance can vary dramatically with only slight changes in the protocol used. Consequently, it is very difficult to compare the results obtained by different laboratories, since no standard protocol exists. Even within a single laboratory, using a standardized IHC protocol, day-to-day results may differ widely due to variation in the histological techniques utilized prior to IHC.

Over the past fifty years, advances in tissue-processing technology and other preparative techniques have dramatically reduced the time necessary to produce a tissue section with little compromise to the histomorphology. Unfortunately, acceptable histomorphology does not always indicate acceptable preservation of the specific substance to be identified by IHC. In fact, the majority of IHC sensitivity problems — particularly in the area of prognostic and/or predictive markers such as ER, HER2, EGFR and CD117 — may be attributed to sub-optimal tissue preparation.

FIXATION/PROCESSING of TISSUES FOR IHC.

Background

The microscopical analysis of body tissue requires that tissue be sufficiently thin to be transparent and possess sufficient optical contrast to permit resolution of structural detail. Most tissues do not meet these requirements; it therefore becomes necessary to manipulate them. However, it is not sufficient for the histologist that a specimen meet solely these requirements. In addition, the cells and extracellular materials must be preserved in such a way that there is as little alteration as possible to the structure and chemical composition of the living tissue. Without being spatially displaced, the structural proteins and other constituents of the tissue must be rendered insoluble in all of the reagents to which they will be subsequently exposed.

The single most important manipulation technique is stabilization of the tissue.

Two principle techniques are in use today.

1) Stabilization may be achieved by rapid freezing of the tissue to a very low temperature, followed by a low-temperature sectioning process in order to obtain thin slices. This is followed by a chemical stabilization of the sections in an organic solvent in order to perform IHC. Today this technique is principally utilized only for research purposes.

2) Fresh tissue is stabilized immediately in one of several chemical solutions or 'fixatives'. Subsequently, the stabilized tissue is either frozen for sectioning or, much more commonly, further processed through a variety of solvents to render the tissue miscible with a medium (paraffin wax) which will provide support during the sectioning process. This is the technique of choice for clinical histopathology laboratories worldwide.

The most commonly used fixative for clinical histopathology is an aqueous solution of formaldehyde gas commonly referred to as 'formalin'. There are very good reasons for this choice. Formalin's mild cross-linking action with proteins provides tissue stabilization with minimum structural change, a loss of less than 1% of protein (Ostrowski, 1961), and the least amount of loss of biological activity and change in chemical reactivity (Horobin, 1982).

Formaldehyde fixation actions are largely reversible, making it possible to subsequently restore much of the structural change and the biological and chemical reactivity (Baker, 1958).

Tissues **optimally** stabilized in aqueous formaldehyde fixatives will provide excellent morphology and allow the broadest range of histochemical and immunohistochemical procedures. Unfortunately, there are many factors which effect optimum formaldehyde fixation. The actions of formaldehyde are greatly modified by concentration (4-6%), buffer type, pH and osmolality of the buffer solution (phosphate, pH 7.2-7.4, physiological), other added salts (avoid) and the temperature of fixation (22C-37C). Tissue thickness (3 -5 mm), the volume ratio between fixative and tissue (20:1), and the duration of fixation (minimum 24-48 hours) are all important factors that are relatively easy to control.

Tissues which are sub-optimally stabilized in aqueous formaldehyde fixatives will show the deleterious effects of subsequent reagents to which they have been exposed.

Penetration Rates of formaldehyde (Hewlett, 2002)

Formaldehyde is one of the most rapidly penetrating fixatives currently in use. Unfortunately, it is also one of the slowest to complete the fixation process. This paradox is explained in (Burnett, 1982), and an excellent description of the properties of formaldehyde is available (Kiernan, 1999). It is a commonly held belief that in practical terms aqueous formaldehyde fixatives penetrate at the rate of approximately 1.0mm per hour. In reality, the penetration rate of aqueous formaldehyde fixatives in mm/hr is variable, depending upon how the data is obtained. In particular, the penetration rate obtained will depend upon the tissue thickness. It may also vary slightly depending on tissue type. The penetration rate of formaldehyde fixatives has been extensively studied, often with conflicting results. One should conclude that the penetration of non-coagulating fixatives such as formaldehyde is difficult to physically measure accurately.

The original experiments of Medawar (1941) utilized plasma clots with an indicator to mark depth of penetration. Medawar showed that fixatives obey the diffusion laws, that is, the depth penetrated is proportional to the square root of time. He also determined a coefficient of diffusibility for each fixative, the Medawar constant *K*.

Using the equation $d = K\sqrt{t}$, where *d* is distance penetrated in mm, *t* is time in hours and *K* the Medawar constant for the fixative in question, it is possible to determine the penetration rate. Medawar determined that $K = 5.5$ for aqueous formaldehyde. Using this value, neutral buffered formaldehyde (NBF) would penetrate 27.5 mm in 25 hours. Plasma clots are easier to penetrate than solid tissues, so for the latter the rate is probably less.
Baker (1958) chose a gelatine-albumen gel to more closely mimic solid tissue and determined $K = 3.6$ for aqueous formaldehyde, or a penetration of 18 mm in 25 hours. Baker also pointed out that the actual penetration into tissue would probably be less, possibly due to the resistance of lipid containing cell membranes. He quotes the data of Tellyesnicsky (1926) who, mainly using large liver tissue samples, indicated a more conservative $K = 0.78$ for aqueous formaldehyde. That would translate to 3.9 mm in 25 hours.

From the equation $d = K\sqrt{t}$, it follows that fixatives penetrate more quickly into small samples of tissue compared to large ones. The initial rate of penetration into tissue is extremely rapid. The first layer of cells (20 - 40 μm) takes less than a second (>300 mm/hr).
Using Baker's $K = 3.6$, the following examples will illustrate further;

1 second	d = 0.06 mm (>300 mm/hr)
1 minute	d = 0.465 mm (27.88 mm/hr)
4 minutes	d = 0.93 mm (13.94 mm/hr)
16 minutes	d = 1.86 mm (6.97 mm/hr)
1 hour	d = 3.6 mm (3.6 mm/hr)
4 hours	d = 7.2 mm (averages to 1.8mm/hr),
8 hours	d = 10.18 mm (averages to 1.27mm/hr),
16 hours	d = 14.4 mm (averages to 0.9mm/hr),
24 hours	d = 17.6 mm (averages to 0.73mm/hr),
96 hours	d = 35.3 mm (averages to 0.36mm/hr).

From these examples, it can be seen that the penetration rate slows dramatically over time. To double the depth of penetration takes four times as long. This has practical implications for the fixation of large (thick) specimens. Although the surface cells of the tissue are rapidly penetrated by fixative, deeper portions of the tissue may not receive any fixative for several hours. Consequently, ischemic changes such as agonal imbibition, diffusion of cellular components, and autolysis will continue to occur at these deeper locations. The very best way to prevent these effects is to apply the fixative by means of vascular perfusion. Using this technique, all tissue components are exposed to fixative within minutes. Unfortunately, this procedure is seldom practical for clinical specimens.

A more practical technique to minimize deep tissue autolysis in large specimens is to immediately cut the fresh specimen into 5 mm thick slices for optimal fixation. As penetration of the tissue by the fixative occurs from both sides of the slice, this ensures that the deepest portions of the tissue slice will receive fixative within 30 -90 minutes of the surface cells (***see worst case penetration rates below***). Subsequently these slices can be trimmed to 3-4 mm thick blocks for processing. If the common practice of linked serial slices (bread-loafing) of thick large tissues prior to fixation is used, the individual slices should NOT exceed 1.0 cm in thickness (a surface cell - deep cell fixation time variance of between 1.5 - 4 hours respectively). This is an important practical application of the formaldehyde penetration rate. However, the more important issue is the formaldehyde fixation rate, i.e. penetration rate plus binding time.

Fixation rate of formaldehyde (Hewlett, 2002).

The time required for the covalent binding of formaldehyde, to form addition compounds with tissue proteins, has proven more difficult to study. Baker (1958) quotes experiments that, after 8 hours at the elevated temperature of 70C, indicate that only 50% of the eventual binding of formaldehyde was complete and that only 90% even after 24 hours at the same elevated temperature. On the basis of this finding, he suggested that the only complete fixation by formaldehyde occurred when the tissue was fixed for some time (5-7 days) and remained stored in the formaldehyde solution.
Pearce (1980) states that "It is clear that in using 10% formalin at room temperature, and even more so at 4C, we are making little use of its capacity to form addition compounds and bridges". ***Note: it is a common research practice to fix at 4C!***

Fox et al. (1985) used ^{14}C labelled formaldehyde to study the covalent binding time for rat kidney tissues. At a temperature of 25°C, the amount of formaldehyde bound to tissue increased with time until equilibrium (maximal binding) was achieved at 24 hours. At 37°C the reaction was slightly faster and equilibrium was reached at 18 hours. A later study by Helander(1994) also used ^{14}C labelled formaldehyde to study binding time for the fixation of rabbit liver. At 25°C. equilibrium was achieved at 25 hours.

The correlation of results between these two studies is impressive, particularly in view of the fact that Fox used **16 µm** thick sections of fresh rat kidney whereas Helander used **4 mm** cubes of fresh rabbit liver. The one-hour difference in equilibrium times achieved by each study, despite a wide difference in tissue thickness, indicates that penetration time is **NOT** a

significant factor in the kinetics of the reaction. Despite the fact that thin slices of tissue will be penetrated faster than much thicker tissue cubes, it would seem that the equilibrium binding time is the limiting factor for the formation of addition compounds, and hence for tissue stabilization.

Through a comparison of the tissue thickness used in the Fox [(1985)] and Helander [(1994)] studies, we can also infer that *K* must be at least =2.0. (*The use of shorter time intervals between 24 and 25 hours would have allowed a more accurate determination of K*).
Therefore using K = 2.0, as a worst case, the following tissue penetration examples would apply;

1 second	d = 0.033 mm (124 mm/hr)
1 minute	d = 0.26 mm (15.5 mm/hr)
4 minutes	d = 0.52 mm (7.8 mm/hr)
16 minutes	d = 1.04 mm (3.9 mm/hr)
1 hour	d = 2.0 mm (2.0 mm/hr)
4 hours	d = 4.0 mm (averages to 1.0mm/hr),
8 hours	d = 5.66 mm (averages to 0.7mm/hr),
16 hours	d = 8.0 mm (averages to 0.5mm/hr),
24 hours	d = 9.8 mm (averages to 0.41mm/hr),
96 hours	d = 19.6 mm (averages to 0.2mm/hr).

In a further study by Helander [(1999)] using rat brain and kidney, equilibrium was not achieved until 50 hours. However, the tissue in Helander's later study was twice the thickness (8mm) of that used in the original study. This factor must be considered when comparing the data.

Failure to recognize the importance of formaldehyde binding time is the leading cause of the tremendous intra and inter- laboratory variability in immunohistochemical (IHC) performance. A clinical laboratory's so called 'routine formaldehyde fixation', actually consists of allowing the tissues to fix for widely variable periods of time after specimen receipt. The end of the fixation time periods is then commonly determined by the scheduled daily start-up time for the tissue processing instrument!

Formaldehyde fixes not by coagulation, but by addition, as it reacts at random with available basic amino acids (primarily lysine, cysteine and arginine) to form several adducts. These reactions are readily reversible by water and alcohol. These adducts have free hydroxymethyl groups which, provided that they are closely spaced, are capable of further reaction to form more stable methylene bridges between proteins (see Kiernan [1999] for more information). This type of mild cross-linking is responsible for the stabilization of proteins by formaldehyde that we term fixation.

Despite the common belief that smaller (thinner) tissues will fix more rapidly than thicker tissues, as demonstrated by both Fox and Helander, the reality is that while a single layer of cells is penetrated by formalin extremely rapidly the chemical reaction requires 24 hours to complete: a 4mm thick slice of tissue is fully penetrated within an hour and requires 25 hours for the chemical reaction to complete. Therefore, the formaldehyde chemical reaction time and hence the minimum stabilization time is 24-25 hours at ambient temperatures for both 1 mm thick needle core biopsies and 5 mm thick tissue slices. In addition, at a 1.0cm slice

thickness, the tissue will be fully penetrated within approximately 4 hours but the chemical reaction will require 50 hours for completion.
Unfortunately, the minimum stabilization time does not, denote complete fixation time. The initial cross-links are still relatively weak and easily reversible; stronger cross-linking continues to occur over time. Complete fixation is thought to take at least 5 - 7 days. Even after this time, cross-links continue to form slowly.

Werner [2000], quoting the two papers above, considers cross-linking complete in 24 - 48 hours, but also expresses concern about 'over-fixation' (***sic. See below***) due to excessive cross-linking, which may occur if fixation is allowed to exceed 24-48 hours. I agree with Werner, in that further additional cross-linking may mask some epitopes, but in my experience this does not occur with the vast majority of antibodies in use until after 5 - 7 days of fixation. After even longer periods of fixation, provided that the IHC technique has been optimized, the majority of clinically relevant antibodies will still give reliable results and fixation times of up to 90 days are acceptable. Considering that the majority of clinical laboratories will dispose of most surgical specimens after the diagnosis is reported (14-30 days), it is unlikely that such lengthy fixation times as 90 days will be relevant in practice. The major strength of formaldehyde, as a fixative for IHC, lies in the fact that the mild cross-linking is 90% reversible [Baker, 1958, Helander, 1994, Shi, 2000]. This reversibility allows the successful use of various 'antigen retrieval' techniques. Far more serious is the problem of short (<24 hour) inadequate fixation.

Inadequate formaldehyde fixation.

For fixation times below 18 -24 hours, reproducibility of IHC results becomes a major problem. This is because inadequate exposure to the fixative leads to the formation of fewer and more randomly spaced addition compounds, weaker and more unpredictable cross-links, and the ready reversibility of formaldehyde fixation. The shorter the initial exposure times the more rapid and unpredictable the degree of reversal is. In fact, for times less than approximately 8 - 12 hours (at 37C - 22C respectively), the weak cross-links are rapidly reversed and the tissue is largely re-fixed by the next reagent in the tissue processing cycle. This is usually ethanol, a protein denaturing solvent which has marked effects on the secondary and tertiary structure of proteins [Baker, 1958. Horobin, 1982]. Ethanol fixation can result in the loss of up to 40% of tissue protein [Ostrowski, 1961], is known to disrupt cellular membranes, and may even strip cytoplasmic membranes completely. This can have disastrous consequences if the target antigen is one of the proteins lost, or if it is located in or on such membranes. In addition, these effects of ethanol fixation are not reversible, thus rendering 'antigen retrieval' techniques largely ineffective or even deleterious to the tissue.

In aqueous solution, formaldehyde rapidly becomes hydrated to form methylene hydrate (methylene glycol). The equilibrium of the reaction lies so far in favour of the hydrated form, that little (<1 part in 100,000) true free formaldehyde is present. The reactivity of aqueous solutions of formaldehyde is known to physical chemists as an example of a "clock" reaction. The conversion of methylene glycol to formaldehyde by removal of the little free formaldehyde present can be used as a "real-time" clock, measured in hours [Burnett, 1982].

Formaldehyde fixation begins at the periphery of the tissue. The initial layers of cells rapidly bind all of the available free formaldehyde and start the 'clock' reaction. Methylene glycol continues to rapidly penetrate the tissue, in the process halting cellular activity, poisoning enzymes and thus preventing further autolysis. However, this is cannot be considered fixation in the accepted sense.

Over hours, more free formaldehyde is generated from methylene glycol. As this free formaldehyde is generated, it randomly binds with the available reactive side chains on proteins, forming hydroxylmethyl adducts, which in turn re-starts and perpetuates the 'clock'.

In tissue slices of less than 5 mm thickness, 24-25 hours are required at room temperature for the maximal binding of formaldehyde to occur and for all of the adducts to form, and double that time in tissue of twice that thickness. If this process is interrupted before completion, the formation of addition compounds will be incomplete, easily reversed and full stabilization by mild cross-linking will not occur. Depending upon the time of interruption, the periphery may show adequate cross-linking whereas the remainder of the tissue is fixed by coagulant alcohol during processing. This type of zonal fixation can have disastrous effects upon IHC staining. The zonal effect will occur whether the tissue is a small biopsy or a 4 mm slice. This variable admixture, of additive (formaldehyde) and coagulative (alcohol) fixation, is a major factor in the huge variability of both intra- and inter-laboratory IHC results.

"Over-fixation".

Over-fixation was a term used by early histologists to describe the unexpected and excessive hardening and shrinkage of tissues which occurred following lengthy exposure to certain fixatives. It gave rise to the concept of 'tolerant' and 'intolerant' fixatives. Heidenhain's SUSA and Zenker's fluid are examples of 'intolerant' fixatives. Textbooks suggest that for these types of fixative, times should not exceed 24 hours to avoid 'over-fixation' effects. On the other hand, an aqueous solution of formaldehyde is the classic example of a 'tolerant' fixative. Tissue may be left in it for extended periods of time with no excessive hardening or shrinkage.

So-called 'over-fixation', as applied to fixation of tissues in aqueous solutions of formaldehyde for IHC, is a complete misnomer. Simply put, it never occurs in any clinically relevant time frame (many months).

USEFUL FIXATION REFERENCES FOR IHC

Arber DA. Effect of prolonged formalin fixation on the immunohistochemical reactivity of breast markers. *Appl Immunohistochem Mol Morphol.*2002; 10(2):183-186

Baker JR. (1958).*Principles of Biological Microtechnique*, Methuen & Co. Ltd, pp 37-46.

Burnett MG. The mechanism of the formaldehyde clock reaction: Methylene glycol dehydration. *J Chem. educ.* 1982; **59,** 160

Fox CH., et.al. Formaldehyde fixation. *J Histochem. Cytochem.* 1985; **33,** 845 -853

Goldstein NS, Ferkowicz M, Odish E, Mani A and Hastah F. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. *Am J Clin Pathol.* 2003; **120(1)**, 86-92.

Gown AM Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am J Clin Pathol.*2004; **121**, 172-174.

Helander, KG. Kinetic studies of formaldehyde binding in tissue. *Biotechnique and Histochemistry.* 1994; **69,** 177 -179

Helander, K.G. Formaldehyde binding in Brain and Kidney: A kinetic study of fixation. *The Journal of Histotechnology.* 1999; **22(4),** 317-318.

Hewlett BR. Penetration rates of Formaldehyde. *Microscopy Today. 2002;10(6): 30*

Hewlett BR. *Optimizing IHC technique: A laboratory handbook.* 2003. IHC publications.

Horobin RW. (1982). *Histochemistry*, Butterworths. ISBN 3-437-10700-3. p19 -55.

Hopwood D. Fixatives and fixation: A review. *Histochemical journal.*1969; **1,** 323 -360.

Kiernan J.A., *Histological and Histochemical Methods: Theory and Practice*, 3rd Edition, 1999. Oxford: Butterworth-Heinemann. ISBN # 0-7506-3106-6.

Larsson L-I. Section pre-treatment, epitope demasking, and methods for dealing with unwanted staining. In: *Immunohistochemistry: theory and practice.* Boca Raton: CRC Press,1988:147-170.

Mason J.T., and O'Leary T.J. Effects of formaldehyde fixation on protein secondary structure: A calorimetric and infrared spectroscopic investigation.
J. Histochem. Cytochem. 1991; **39**: 225-229.

Medawar PB. The rate of penetration of fixatives. *J R Microsc Soc.* 1941; **61,** 46

NCCLS. *Quality Assurance for Immunocytochemistry; Approved guideline.* NCCLS document MM4-A (ISBN 1-56238-396-5) 1999.

O'Leary T.J. Standardization in Immunohistochemistry. *Applied Immunohistochemistry and Molecular Morphology*. 2001; **9(1)**: 3-8.

Ostrowski K, Komender J and Kwarecki K. Quantitative investigations on the solubility of proteins extracted from tissues fixed by different chemical and physical methods. *Annls. Histochim.* 1961; **6,** 501-506.

Pearse AGE. *Histochemistry: Theoretical and Applied.* 4th Edition, 1980. Churchill Livingstone. ISBN 0-443-01998-3. Volume 1, p 101.

Petsko GA and Ringe D. *Protein structure and function.* 2004. Sinauer Associates Inc. ISBN 0-87893-663-7

Shi S.R., Gu J. and Taylor C.R. (Editors) *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology*. 2000. Eaton Publishing. ISBN 1-881299-43-0

Sompuram SR, Vani K, Messana E and Bogen SA. A molecular mechanism of formalin fixation and antigen retrieval. *Am J Clin Pathol* 2004;121: 190-199

Taylor C.R. A Total Test Approach to Standardization of Immunohistochemistry. *Arch. Pathol. Lab. Med.* 2000; **124**: 945-951.

Tellyesnicsky, K., (1926). Article on 'Fixation' in R. Krause's *Enzyklopädie der mikroskopischen Technik*, vol.2. Berlin (Urban & Schwarzenberg).

Werner M., et.al. Effect of formalin fixation and processing on immunohistochemistry. *Am J Surg Pathol.* 2000; 24(7), 1016 -1019

Williams JH, Mepham BL, Wright DH. Tissue preparation for immunohistochemistry. *J Clin Pathol.* 1997; **50**: 422-4