ANATECH LTD INNOVATOR

We can fix it!

We can fix it! That almost sounds like an ad for a home improvement store, designed to assist you in making home repairs. In histology "fix it" means preserving the specimen. In a sense Anatech Ltd can help you incorporate the "home improvement" and tissue preservation definitions at the same time.

When we think of preserving a tissue we typically think of formaldehyde fixation. The use of formaldehyde as a preservative dates back to 1893, when Ferdinand Blum published a paper showing that a 4% solution of formaldehyde had antiseptic properties. Blum's research was well received, since there were few bactericidal agents available in that era. During his research Blum made another interesting observation; he noticed that his fingers, which had been exposed to the 4% formaldehyde, became hardened. The degree of hardness matched that of tissues preserved in alcohol, a very common histological fixative at that time. To verify his findings, Blum fixed anthrax infected mouse tissue in 4% formaldehyde instead of alcohol. He subsequently processed and stained the tissues. Not only did he achieve excellent staining results, but he also observed less shrinkage than that seen in alcohol fixed specimens. He published his results of formaldehyde as a preservative in 1894. And as we say, "the rest is history!"

The chemical formula of formaldehyde gas is HCHO (Figure 1). It is the simplest of the aldehyde molecules. Aldehydes consist of a terminal carbonyl group (carbon atom double bonded to oxygen), which is responsible for the reactivity of the formaldehyde molecule with other chemicals. Formaldehyde's natural state is a gas but it's very soluble in water. To manufacture the liquid solution, formaldehyde gas is bubbled through water until no more gas can be dissolved, which happens at 37% (w/w). So formaldehyde is sold as a 37% aqueous solution where formaldehyde exists as a hydrate called methylene glycol, $CH_2(OH)_2$ (Figure 2). If eight or more methylene glycol molecules polymerize, a white, insoluble precipitate (paraformaldehyde, Figure 3) is formed. Paraformaldehyde can form over time or in solutions exposed to cold temperatures (e.g., 32˚F). The presence of paraformaldehyde reduces the formaldehyde in solution.

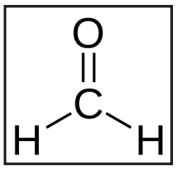

Figure 1.

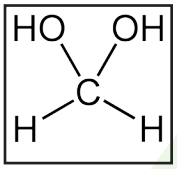

Figure 2.

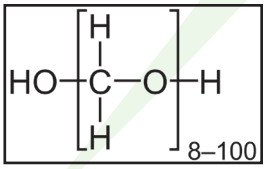

Figure 3.

To prevent paraformaldehyde precipitation, manufacturers add 7–13% methanol as an inhibitor.

Blum's research used one-part formaldehyde (identified by the manufacturer as 40% (w/v) formaldehyde) with nine-parts water to yield a solution of 4% formaldehyde. Today's commercial solution is sold as 37% (w/w) formaldehyde and 11% methanol and is considered in histology as "*100% formalin*." Therefore, 10% formalin fixatives contain 3.7% formaldehyde and 1.1% methanol.

Formaldehyde also has a tendency to react with oxygen and produce formic acid, creating an acidic formaldehyde solution with a pH as low as 2. In tissue fixation, acidic formalin can react with the hemoglobin of the red blood cells and produce hematin (formalin pigment), a black/brown granular pigment (Figure 4). To prevent this reaction, several buffered formalin fixatives have been developed. The most widely used today is 10% neutral phosphate buffered formalin (NBF). Sodium phosphate monobasic and dibasic salts are added to the 10% formalin to bring and maintain the solution at pH 7. Other buffer salts can be used to achieve the same end result, which is to keep the formalin solution outside the acid range where formalin pigment forms. While the buffers may vary, the active ingredient for fixation (3.7% formaldehyde) is the same.

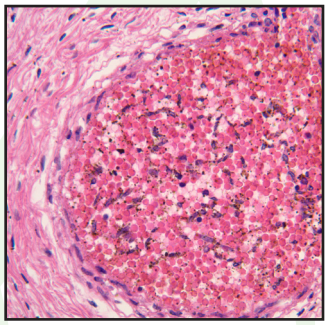
Figure 4. Formalin pigment (black granular material) caused by fixation in acidic formalin.

It's interesting to note that Blum's bactericidal research indicated formaldehyde as an effective but slow acting chemical. Formaldehyde's penetration rate versus fixation properties are still a consideration today. Formaldehyde is a small molecule that can penetrate the tissue quickly. However the formaldehyde must still go through a chemical reaction to actually fix the tissue, and this process is slow. First, the methylene glycol (specifically the aldehyde group) combines with the tissue. This addition product further reacts with tissue protein groups to form methylene bridges. These methylene bridges within tissue molecules provide the stable chemical structure that allows the tissue to subsequently handle the harsh world of tissue processing (alcohols, clearants, heated paraffin).

Histology textbooks have historically recognized formaldehyde's slow reaction time by stating that specimens should fix for at least 12–24 hours. These recommended times resulted in optimal cellular morphology showing diverse chromatin patterns and well defined cytoplasmic structures. However, in current times demanding fast turn around, specimens are lucky if they are fixed in formalin for 4–6 hours. In this short time the formalin may have penetrated the tissue but has not reacted with the cell structures to fix them. Now tissues have more exposure to alcohols than formalin. The multiple dehydration alcohols that the specimen passes through during processing will alcohol fix the tissue. But the characteristics of formalin fixation, especially nuclear detail, are lost. This can result in the artifacts commonly known as soapsuds artifact (Figure 5), blue halo (Figure 6) and generally unreadable slides.

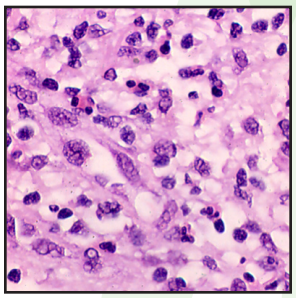

Figure 5. Soapsuds.

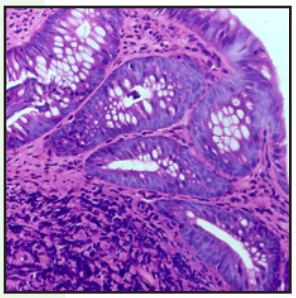

Figure 6. Blue halo.

Achieving optimal formalin characteristics requires more time than is currently being allocated in clinical laboratories. A comparison of uterus fixed in NBF for 4–6 hours (Figure 7) and 24 hours (Figure 8) show the importance of allowing adequate time for the formalin to react with the tissue.

Since the days of leaving tissues to fix in NBF for 12–24 hours are over, how can optimal morphology be obtained? Obviously, the fixative needs to work faster by increasing penetration rate, reaction time or both. Cell membranes, comprised of protein and lipid bilayers, present a natural barrier. NBF's aqueous nature and the cell membrane's lipid component have dissimilar solubilities, thus hindering penetration. Alcoholic fixatives were introduced to help the formalin penetrate faster through the lipid protein membranes, allowing more available reaction time.

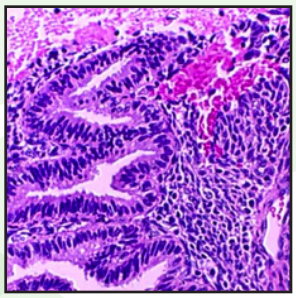

Figure 7. 4-6 hour fixation.

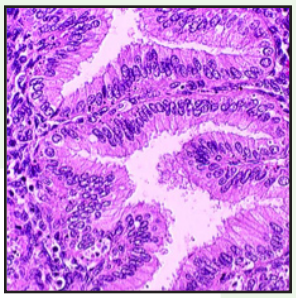

Figure 8. 24 hour fixation.

The drawback to most alcoholic formalins is their acidity, which can encourage the production of formalin pigment. The standard buffer salts used in aqueous formalin (e.g., sodium phosphates) are poorly soluble in alcohol and are not suitable for alcoholic formalin.

Anatech Ltd has formulated a buffered alcoholic formalin, CBA Formalin, with unique buffer salts that are alcohol soluble and provide a pH that prevents formalin pigment. CBA Formalin's rapid penetration rate assists with the difficult task of getting fixative through fatty, thickly grossed, or late arriving specimens. But its low alcohol concentration precludes any alcohol drying action on the tissue. To keep costs down, Anatech Ltd supplies CBA Formalin in a concentrated form. Proper dilution with either ethanol, reagent alcohol or isopropyl alcohol results in a 10% buffered formalin in 70% alcohol. Diluted CBA Formalin can be used in the first two stations of the processor or in station two after an initial station of NBF. The processor rotation schedule can be extended since there will be less water carryover from the 70% alcohol than from the aqueous NBF. Purchasing a concentrate eliminates the hazard surcharge for a flammable product, hence saving money on shipping costs.

A major improvement in formalin fixatives came about in the early 1980's when Jones published an abstract showing the use of zinc sulfate with formaldehyde to improve nuclear morphology and to enhance immunohistochemistry (IHC) results. A few years later Herman reported the use of zinc formalin on automated processors and showed that specimen immunoreactivity was well preserved. These were important findings for anatomical pathology.

While necessary for complete fixation, the methylene bridges formed with NBF blocked the antigenic sites in IHC procedures. The methods required to retrieve the antigenic sites (e.g., digestion, HIER) often produced artifacts of their own (tissue detachment, loss of morphology). Zinc formalin's ability to preserve antigenic sites often made antigen recovery unnecessary.

However, the published zinc formalin solutions had another inherent problem—they precipitated on the processor and produced crunchy specimens due to zinc sulfate's interaction with the dehydrants.

Anatech Ltd's Zinc Formalin and Z-Fix overcame those issues. First, Anatech reformulated a zinc formalin solution using zinc sulfate. While Zinc Formalin is unbuffered, this formulation all but eliminated precipitation and difficult-to-cut specimens. However, due to the occasional reports of formalin pigment when used on extremely bloody specimens, Anatech developed Z-Fix, a buffered zinc formalin that prevents formalin pigment and still provides superior morphology (Figure 9) and immunohistochemistry preservation. The chemistry of zinc formalin fixation is similar to NBF. The first step is the same with the addition of methylene glycol to the tissue. However, the zinc ion is also attaching to the tissue. Dapson believes that the zinc ions "staple" the molecule into a natural shape. Therefore, as methylene bridges form with the formaldehyde, the attached zinc maintains the tissue's general shape. The attachment of the zinc ions to the tissues also provides fewer sites for the formaldehyde to crosslink. With fewer methylene bridges, Anatech's Zinc Formalin and/or Z-Fix diminishes the need for antigen recovery steps in immunohistochemistry. Anatech provides Z-Fix in both ready to use and concentrated (just add water) formats. An Alcoholic Z-Fix Concentrate is also available when fixation time is short or when fatty tissues are encountered. Simply dilute with ethanol, reagent alcohol, or isopropyl alcohol and water to yield a buffered zinc formalin in 70% alcohol.

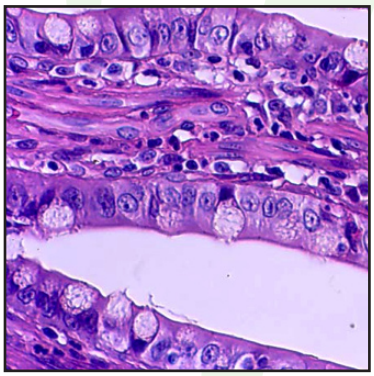

Figure 9. Intestine, Z-Fix.

Mercury-based fixatives like B-5 have been used in histology to provide delicate and superb nuclear detail. However, B-5 fixative cannot be used without releasing mercury into the sewer system, a result of contaminated reagents. Anatech Ltd realized these hazards and developed Z-5 as a replacement for mercury fixatives. It produces results indistinguishable from B-5, without the drawbacks. There are no crystals to remove from sections. Immunoreactivity is preserved with most antigens, reducing the need for antigen recovery. Z-5 is offered as a concentrate, just add anhydrous ethanol or reagent alcohol to dilute.

While zinc fixatives offer a great improvement, formaldehyde is the still the active ingredient. In 1987 formaldehyde was classified as a carcinogen. Occupational Safety and Health Administration (OSHA) enacted the Formaldehyde Standard to protect workers exposed to formaldehyde. Training, exposure monitoring, special labeling and warning signs were now part of the histology routine because of formaldehyde fixatives. This gave birth to the next major development in tissue fixation: the formaldehyde-free fixative.

Developing a formaldehyde-free fixative had major requirements. Pathologists were accustomed to making a diagnosis based on the gross morphology and microscopic appearance of formaldehyde fixation. Therefore any replacement had to mimic formaldehyde fixation as much as possible. The penetration rate would need to be equivalent, if not faster, than NBF. Also H&E, special stains and immunohistochemistry procedures should remain the same. Ideally, the replacement would not impair immunoreactivity with methylene bridges. Obviously, a formaldehyde-free fixative needs to have less health hazards than formaldehyde to justify a switch.

Anatech's formaldehyde-free fixative, Prefer, met the requirements. The active ingredient is glyoxal, a two-carbon, di-aldehyde that provides the aldehyde appearance of formaldehyde. Glyoxal is able to form crosslinkages, but does not impair immunoreactivity because the glyoxal crosslink is longer than formaldehyde crosslinks. Besides glyoxal, Prefer contains a very small amount of ethanol, to assist with penetration rate, and a non-toxic buffer. The alcohol content is very low and will not produce alcohol-type fixation morphology. Prefer is available as a ready to use solution and as a concentrate, which can be diluted traditionally or into a more alcoholic formulation to increase penetration. Prefer is a safer, and technically superior (Figure 10) alternative for formaldehyde.

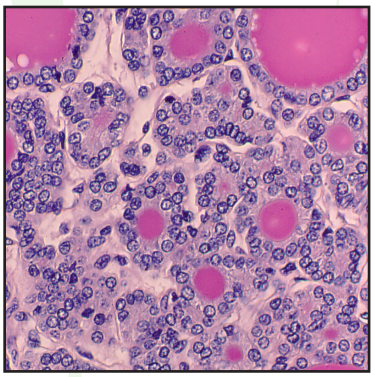

Figure 10. Thyroid, Prefer.

References

- 29 CFR 1910.1048 Formaldehyde Standard
- Dapson, R.W. (1993). Fixation for the 1990's: A Review of Needs and Accomplishments. *Biotechnic & Histochemistry*. **68**:75-82.
- Federal Register, Volume 52, No. 233, Friday, December 4, 1987.
- Fox C.H., Johnson F.B., Whiting J., et al. (1995). Formaldehyde Fixation. *J Histochemistry Cytochemistry*, **33**:845.
- Herman, G.E., Chipala, E., Bochenski, G., et al. (1988). Zinc Formalin Fixative for Automated Tissue Processing. *J Histotechnology*, **11**:85-89.
- Jones, M.J., Banks, P.M., Caron, B.L. (1981). Transition Metal Salts as Adjuncts to Formalin for Tissue Fixation (abstract). *Lab Invest*, **44**:32A.
- Kiernan, J.A. (2000). Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. *Microscopy Today*, **00-1**:8-12.
- Lillie, R.D., Fullmer, H.M. (1976). *Histopathologic Technic and Practical Histochemistry*. New York, NY: McGraw-Hill Book Company.
- http://en.wikipedia.org/wiki/File:Methanediol-2D.png
- http://en.wikipedia.org/wiki/File:Formaldehyde-2D.svg
- http://upload.wikimedia.org/wikipedia/commons/thumb/f/f4/Paraformaldehyd.svg/2000px-Paraformaldehyd.svg.png

Need more technical tips?

Get Anatech Ltd iWonder's delivered monthly to your inbox! Opt in by sending an email to info@anatechltdusa.com.

1020 Harts Lake Road

Battle Creek MI 49037

1.800.262.8324

www.anatechltdusa.com

ANATECH LTD

Have fixation problems?

We Can Fix It!

Choose the Anatech Ltd fixative that best meets your needs.

Traditional NBF, with several options available:

Cat# 111…CB Formalin
Save on shipping costs and storage. Just add water to make NBF!

Cat# 121…CBA Formalin
Just add water and alcohol for a convenient buffered alcoholic formalin.

Cat# 135…NBF
Ready to use neutral phosphate buffered formalin.

Improved morphology and antigenic preservation:

Cat# 144…Zinc Formalin
Ready to use, unbuffered.

Cat# 612, 616, 619…Zinc Formalin prefills

Cat# 174…Z-Fix
Ready to use, buffered.

Cat# 171…Z-Fix Concentrate
Just add water to make buffered zinc formalin.

Cat# 161…Alcoholic Z-Fix Concentrate
Add water and alcohol to make buffered alcoholic zinc formalin.

Cat# 622, 626, 629…Z-Fix prefills

Superior fixation that avoids the health risks and compliance costs of carcinogenic formaldehyde:

Cat# 414…Prefer
Glyoxal based formalin-free fixative.

Cat# 632, 636, 639…Prefer prefills

Cat# 411…Prefer Concentrate
Can be diluted to make a standard or alcoholic version; just add water and alcohol.