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Why 4F:1G Fixative Works for Me

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Introduction

McDowell and Trump [1] published a seminal paper examining the efficacy of several fixative formulas. They were attempting to determine the best solution for producing samples that could be put in fixative during surgical procedures or in the morgue and then processed for both electron and light microscopy evaluation without significantly compromising either examination method.

They tried the formalin fixative recipe of Carson et al. [2] designed for electron microscopy samples and stated that they found ultrastructural preservation adequate for electron microscopy, though not producing excellent fixation. They pointed out that conventional wisdom of that time indicated that samples destined for ultrastructural evaluation should be diced into small pieces and fixed on ice with glutaraldehyde or mixtures of glutaraldehyde and formaldehyde, the latter freshly formulated from paraformaldehyde powder. It was pointed out that preparing fresh formaldehyde was inconvenient and that it did not have much shelf life because it begins re-polymerizing into paraformaldehyde shortly after it is made. These features of the contemporary fixation techniques in 1976 made them poorly suited to typical surgical or autopsy procedures.

McDowell and Trump tested their fixative mixtures on a variety of human and rat tissues and concluded that the 4 percent formaldehyde: 1 percent glutaraldehyde fixative (designated by them as 4CF–1G) in Millonig's or Sorenson's phosphate buffer at an osmolality of 176 mOsm was most successful for their purposes.

McDowell and Trump were aware that most electron microscopists of the time did not believe in using jug-grade formaldehyde (37–40 percent) solutions containing methanol as a preservative to prevent polymerization of the formaldehyde. They compared tissues fixed in formaldehyde freshly prepared from paraformaldehyde powder versus those fixed in jug-grade formaldehyde and saw no structural difference. This removed a time-consuming and somewhat tedious step from fixative preparations needing some formaldehyde, to the relief of those of us running service laboratories fixing thousands of samples for electron and light microscopy.

We have been using their formula, which we designate 4F:1G for over 30 years. Although they only tested their methods on human and rat tissues, we have applied their formula to organisms from all kingdoms of life. We have also shown long-term storage of samples in 4F:1G far beyond the 1-year period suggested by McDowell and Trump.

Materials and Methods

The recipe we use to prepare McDowell and Trump 4F:1G fixative (4F:1G) is as follows.

Add, in the order listed:
86 ml distilled water
10 ml Fisher P-79 37-40 percent formaldehyde
4 ml 25 percent glutaraldehyde (biological grade)
1.16 g $NaH_2PO_4 \cdot H_2O$
0.27 g NaOH
Stir while adding components.
Check the pH when done. Some 0.1 N NaOH can be added to raise the pH if it is below 7.2-7.4.

Once prepared, 4F:1G can be stored at 4°C for three months. Tissues fixed in 4F:1G can be stored up to 23 years (so far) at 4°C.

Results and Discussion

An electron microscopy service laboratory situated in a College of Veterinary Medicine has been an excellent setting in which to test 4F:1G for a number of applications beyond what was originally envisioned by McDowell and Trump. We have had the opportunity to use 4F:1G on viruses, prokaryotes, free-living protozoans, parasitic protozoans, fungi, arthropods, nematodes, plants, reptiles, amphibians, birds, and a wide variety of mammalian tissues from numerous species. There have been extremely rare instances where a sample did not fix well, but in these cases, it also would not fix well with 2 percent glutaraldehyde or 4 percent formaldehyde (some yeast cells are in this group, as well as some obscure protozoans such as *Guttulinopsis vulgaris*).

Histology laboratories historically are not fond of dealing with tissues fixed for electron microscopy in glutaraldehyde solutions with a concentration of 2 percent or higher or with mixtures of formaldehyde and glutaraldehyde containing over 2 percent glutaraldehyde, such as Karnovsky's fixative [3]. After paraffin embedding, these tissues tend to be brittle, producing poor sections.

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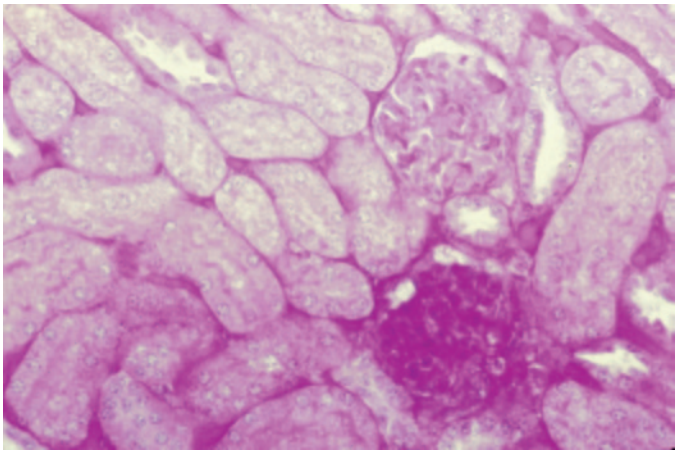

Figure 1: Paraffin section of pig kidney fixed with 2 percent glutaraldehyde and osmium and then stained with PAS. All elements in the section are stained pink due to Schiff's reactivity with the unbound hydroxyl groups of glutaraldehyde molecules in tissue. Original magnification 180×, image width = 360 µm.

In addition, some stains routinely used for histological preparations do not work as well as they do for samples fixed in the buffered neutral formalin (4 percent) typically used in histology laboratories. In particular, as explained by McDowell and Trump, periodic acid/Schiff's reagent (PAS) applied to samples fixed in 2 percent or higher concentrations of glutaraldehyde turn uniformly pink due to the interaction of Schiff's reagent with the vicinyl hydroxyl groups at either end of the glutaraldehyde molecule. Because 4F:1G has only 1 percent glutaraldehyde, the majority of the glutaraldehyde hydroxyl groups are bound to proteins in the tissue, and a PAS reaction still gives the normal specificity seen in formaldehyde-fixed histological samples. Figure 1 shows a pig kidney fixed with 2 percent glutaraldehyde and osmicated, followed by paraffin embedment, sectioning, and PAS staining of the sections. Non-specific pink staining is seen throughout the section. Figure 2 shows a pig kidney fixed with 4F:1G, osmicated

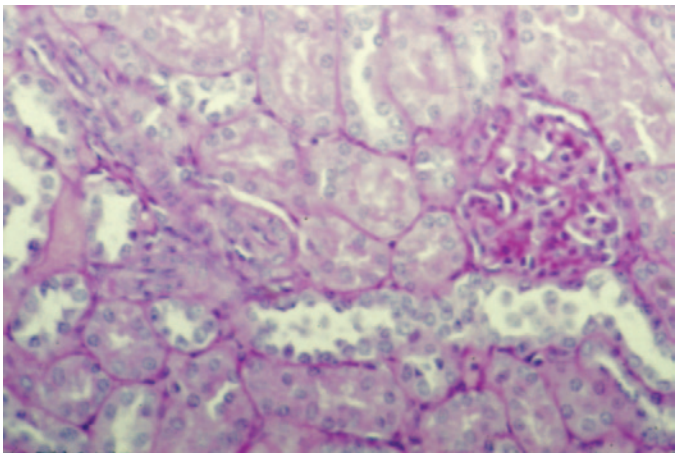

Figure 2: Paraffin section of pig kidney fixed with 4F:1G and osmium and then stained with PAS. Specific PAS reactivity is seen only in brush borders and the basement membranes because the majority of the hydroxyl groups of the glutaraldehyde are bound to tissue components. Original magnification 180×, image width = 360 µm.

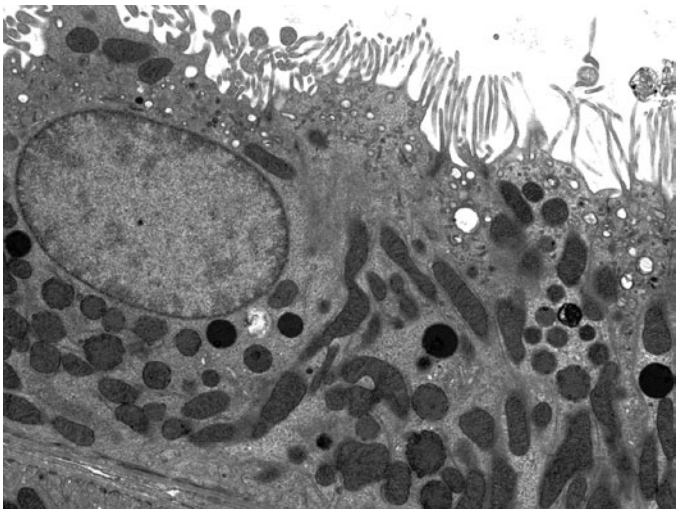

Figure 3: Rat kidney proximal convoluted tubule fixed in 4F:1G and osmium and processed to Spurr resin on the same day in 1986. Original magnification 5,000×, image width = 20 µm.

and embedded in paraffin. Following microtomy, the sections were stained with PAS. Specific pink staining of basement membrane and brush borders can be seen.

Storage of samples fixed for electron microscopy is frequently convenient, particularly if histological preparations of the same sample can be evaluated first by light microscopy to determine if electron microscopy is needed for analysis of finer detail. Some laboratories recommend storing fixed tissues in buffer solutions, often with added sucrose to retard extraction of cellular materials as suggested by Hayat [4]. This will often result in the development of bacterial or fungal growth associated with the samples that produce no host reaction, making it clear that they are a post-fixation artifact. We recommend strongly that samples be left in the primary fixative (4F:1G)

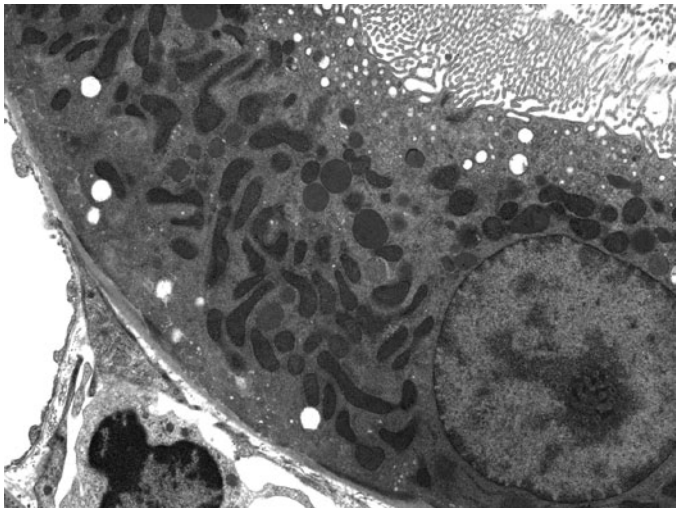

Figure 4: Rat kidney proximal convoluted tubule. A piece of the same rat kidney shown in Figure 3, fixed in 4F:1G in 1986 and stored in the fixative at 4°C until osmication and processing to Spurr resin in 2009. Original magnification 5,000×, image width = 20 µm.

at 4°C for long-term storage. We have no evidence that any serious degradation of ultrastructural details occurs in samples so stored. Figure 3 shows a proximal convoluted tubule (PCT) from a rat kidney that was fixed in 4F:1G in 1986 and post-fixed, dehydrated, and embedded in Spurr resin on the same day. Figure 4 shows a PCT from the same rat kidney fixed in 1986 and stored in the 4F:1G solution until 2009. In August 2009, a piece of the stored kidney was removed from the fixative and processed through the same series used in 1986 to Spurr resin, and the photograph was taken. Note that in both Figures 3 and 4 the nuclear envelopes and the rough endoplasmic reticulum consist of parallel membranes with no evidence of swelling. In addition, the mitochondria have dense matrices and no evidence of distension of the inner or outer membranes. There are no notable membrane discontinuities, as are often seen with formaldehyde fixation. Finally, both the nucleoplasmic and cytoplasmic ground substance seems satisfactorily dense, with no evidence of extraction of background material, as is so often seen with formaldehyde fixation.

Overall, 4F:1G has a lot to recommend it as a starting point for sample fixation. It is not superior to 2 percent glutaraldhyde for ultrastructural preservation, but it is equal. The fixative is easy to formulate from inexpensive chemicals. A sample can be put in the fixative solution and stored for years without having to be processed immediately, which offers more flexibility for an investigator doing field work, such as collecting samples from turkey flocks or collecting leaves from plant crops in the field because the samples can be processed at the leisure of the investigator. In addition, because the fixative does not compromise preparations for histological paraffin sectioning and staining with special stains, it allows the investigator to take one sample and do excellent light microscopy and excellent electron microscopy on pieces taken from a single fixed sample. Finally, 4F:1G has proved itself a superb fixative for perfusions of animals, particularly mammalian kidneys as well as an excellent immersion fixative for cells and tissues from all the kingdoms of life as illustrated in Dykstra and Reuss [5].

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