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Detailed Discussion of Tissue Prep and Formalin/Formaldehyde Fixation Recommendations:

1. **Time to fixation is critical**. The **quicker the better**, as stress responses are initiated almost immediately after the tissue is deprived of its blood supply. Ideally, the time between death and initiation of fixation should be **less than 20 minutes**, and this should be recorded. K**eeping tissues near (4°)** prior to fixation slows down biological processes induced by oxygen and nutrient deprivation. Prior to fixation, tissues should be maintained in a neutral, isotonic solution with physiologic Ca and Mg concentrations (e.g. PBS plus Ca & Mg) to avoid unnecessary disruption of adhesion systems and activation of signaling cascades.
2. **Type of fixative**: paraffin embedded tissues are most often fixed in either **10% (v/v) neutral buffered formalin (NBF) or fresh 4% (w/v) formaldehyde solution ("PFA") made from paraformaldehyde power**. It is important to realize that **the concentrations of formaldehyde in both 10% (v/v) NBF and 4% (w/v) PFA are almost identical**. Confusion arises because the concentration of formaldehyde in NBF is given as a percent *volume* per final volume (v/v) whereas the concentration of formaldehyde in PFA is given as a percent *weight* per final volume (w/v). The working solution of commercially available **NBF is a 1:10 dilution of a 37% (w/v) formalin stock.** This results in a 10% *volume/volume*, which is almost equivalent to 3.7% *weight per volume* formaldehyde concentration – similar to the 4% (*weight*/volume) PFA made from powder paraformaldehyde. There are, however, **differences between these two related fixative solutions**, which can impact on the ultimate choice. (See discussion under Fixative Options).
3. **Modes of formaldehyde fixation (immersion and cardiac perfusion)**. Immersion fixation is the easiest and most common mode of fixation. Human samples are exclusively immersion fixed. For animal tissues, cardiac perfusion is advantageous in some cases because fixation initiates more rapidly than immersion perfusion, but only if performed by a skilled individual who knows how to evaluate whether or not the procedure is effective. Perfusion is more effective because the fixative is delivered directly to tissue capillary beds, and since all cells are within a few cell diameters of a capillary, they are awash in fixative within seconds to minutes. To ensure completeness, most perfused tissues are also subjected to immersion fixation for some period. **PERFUSION IS STRONGLY RECOMMENDED FOR ADEQUATE FIXATION OF RODENT BRAINS,** unless the brain is sliced into small pieces (bread-loafed) immediately after dissection. Perfusion is unnecessary and impractical in many other instances.
4. V**olume of fixative should be 10-20 times** the **volume of the tissues** for immersion fixation. Inadequate volume is a common mistake and leads to poor and variable fixation.
5. **Size of tissues**: **samples must be less than 0.5 cm (5 mm) in one dimension** for adequate immersion fixation. Some recommend no more than 2-3 mm, especially for dense tissues. Formadehyde penetrates tissues at a rate of 1 mm/hr, at best. If tissue samples are thicker than 4 mm, then fixation in the center will not initiate until few hours after dissection. By this time, the central cells will be quite hypoxic and possibly necrotic. The recommended overall dimensions: 1.5x1.5x0.4 cm.
6. **Time of fixation**: **6-18 hrs for biopsy specimens and 24-72 hrs for standard samples.** Both under and over fixation alter the quality of molecular analytes. A recent report recommends that tissue not be fixed for more than 36hrs to avoid overfixation and excessive cross-linking. We have also found that extended fixation can cause excessive brittleness of paraffin-embedded mouse tissues, which are inherently "dryer" than human tissues. After fixation, tissues can be dehydrated first in 50% EtOH and then in 70% EtOH and subsequently stored for several days (at least) before processing into paraffin.
7. **Temperature of fixation:** is important, but controversial. Metabolic artifacts – induced by the abrupt cessation of oxygen and nutrient delivery as well as waste removal when the tissue is removed from its blood supply – can be minimized by slowing down cellular metabolic processes and/or accelerating the rate of fixation. To slow down metabolic processes prior to complete fixation, many researchers fix at **4°**. Clinical labs, on the other hand, typically fix at room temperature in part because of work-flow considerations and because of the increased rate of fixation. Some groups recommend using a special microwave process to simultaneously fix the tissue and inactivate endogenous phosphatases, proteases and nucleases. For tissues destined for paraffin processing, we currently

recommend fixation for 24-48 hrs at room temperature or 48-72 hrs – but this assumes the thickness of the tissue is 4mm or less and the volume of fixative is at 10X the volume of the tissue.

8. We recommend ***gentle* nutation or slow shaking** to replenish fresh fixative around the tissue. Exhaustion of surrounding fixative is particularly problematic when tissues are at the bottom of conical tubes. Avoid big bubbles, as shear forces at the air-surface interface will shred the tissue edges.

Types of fixatives:

There are **two basic classes of fixative: cross-linking fixatives and coagulant fixatives**. They both have advantages and disadvantages – and each produces its own set of artifacts.

Cross-linking fixatives: Formaldehyde-based fixatives are the most common type of cross-linking fixative used, and they work by covalently coupling molecules to each other and creating a stable meshwork within tissues. Two similar though not identical formaldehyde fixatives are neutral buffered formalin and freshly prepared paraformaldehyde. (See Freshly prepared paraformaldehyde vs neutral buffered formalin fixatives). **Glutaraldehyde** is another cross-linking fixative. The latter is used for EM, but rarely for standard paraffin-embedded tissues, as it makes the tissues very brittle and is more likely to produce autofluorescence if doing IF. The latter problem can be avoided if the free aldehydes are reduced using sodium borohydride or similar reagent. The major disadvantage of cross-linking fixatives is that they can mask epitopes recognized by certain antibodies – either by chemically modifying a critical amino acid or by blocking antibody access to the antigen because of the dense cross-linked meshwork "cage" that is generated. Many of the latter epitopes can be re-exposed after antigen retrieval, although this may cause unwanted secondary tissue damage. The latter is especially problematic for cryosections.

Coagulant fixatives: organic solvents are typical components of coagulant fixatives. They function by precipitating and often denaturing proteins in situ. Examples include ethanol, methanol, acetone or a combination. Organic solvent fixatives work well when immunostaining cytoskeletal proteins and other components of insoluble large macromolecular complexes. However, lipids and many lipid-modified proteins are extracted, and many aqueous-soluble cytoplasmic proteins are lost during subsequent wash steps. Organic solvents cause much greater tissue shrinkage than cross-linking fixatives and usually destroy the integrity of cell organelles. On the other hand, they penetrate tissues rapidly, initiating the fixation process faster than formaldehyde fixatives. Also, because they destroy cell membranes, they permeablize cells at the same time they fix the cells. If the antigens are not extracted, epitopes may be better preserved than with cross-linking fixatives.

Some complex fixatives contain multiple components, including an organic solvent, an acid and a cross-linking fixative (e.g. Bouin's fixative).

Formaldehyde based fixatives:

Neutral buffered (NB) formalin vs freshly prepared paraformaldehyde (PFA):

For paraffin embedded tissues, neutral buffered formalin (NBF) is the most commonly used fixative. (Note, it must be buffered to avoid precipitates and other artifacts). NBF is convenient to store (liquid at room temperature) and can be ordered as a working solution and therefore requires no prior preparation. In addition to its convenience, some prefer it

because the methanol additive penetrates the tissue faster than formaldehyde, this initiating fixation more quickly than with pure formaldehyde solutions freshly prepared from paraformaldehyde power.

"Paraformaldehyde" (PFA) is preferred for certain protocols because most commercial NB formalin solutions contain methanol as an additive in order to prevent formaldehyde polymerization over time. Since PFA is either freshly prepared from paraformaldehyde powder or freshly diluted from a frozen stock or stock stored under oxygen-free gas, this polymerization process is not an issue. (Note, although the solution is typically called "Paraformaldehyde", it is really a formaldehyde solution.) Moreover, NBF degrades with age, with the production of formic acid, and this issue is also avoided with freshly prepared PFA solutions. PFA is the formaldehyde fixative of choice for: 1) cardiac perfusions; 2) short fixation for cryo-embedded tissues where antigen retrieval needs to be avoided; 3) in situ hybridizations and 4) TEM, as an alternative to glutaraldehyde. Some researchers also prefer PFA for immersion fixation of tissues destined for paraffin embedding, if they are performing subsequent immunostaining. Because PFA lacks additives and is prepared freshly each time, officinados feel they can more rigorously standardize the extent of cross-linking, facilitating consistency of subsequent antigen retrieval protocols. Note, it has recently become possible to buy MeOH-free formaldehyde single-use ampule stocks, which is easier and safer to dilute than making up solutions from paraformaldehyde powder.

Recipes for preparing PFA-based fixatives:

20% (wt/vol) Paraformaldehyde (PFA) Stock in PBS (plus Ca++ and Mg++)

- Mix and heat to no greater than 60°C. It will take some time for the polymerized paraformaldehyde to break down to formaldehyde and go into solution.
- Check pH to make sure approx 7.4-7.6. Use pH paper NOT pH meter. Do NOT stick paper into the solution – put drop on paper. Adjust pH as necessary.
- Filter if particles remain.
- Store 10ml in 50ml conical tubes at -20°C. Note: if possible store in explosion-proof freezer.
- When needed, freshly thaw and resuspend in PBS plus Ca++ & Mg++ (50ml total). Final working solution: 4% w/v. Heat to 37°C if necessary to get all PFA into solution.
- Use for fixation after chilling on ice.
- Freshly prepared PFA is ideally used the same day. If necessary store at 4° and use within 24-48 hrs. Can also store longer and use as a final post-fix step, after immunohistochemistry or RNA in situ steps are complete.
- If PFA is to be used to fix tissues for any downstream RNA-dependent analyses (e.g. in situ hybridizations), then all above procedures should utilize solutions, chemicals and containers that are RNase free.

Alternative fixative with glutaraldehyde (good for subsequent β-gal histochemical staining)**:**

Alt Fixative:

Fixative:	Recipe (20 ml)
1% formaldehyde	0.540 ml 37% stock
0.2% glutaraldehyde	0.160 ml 25% stock
2mM MgCl2	0.040 ml 1M stock
5mM EGTA	0.200 ml 0.5M stock

0.02% NP-40	or equivalent	0.200 ml 2% stock
		Volume to 20 ml with PBS

Reagents:

From Sigma

Add fresh:

a. Glutaraldehyde, 0.2% v/v final concentration (1:800 dilution of a 25% stock—concentration in most commercial glutaraldehyde solutions).
b. Formalin, 1.5% v/v final concentration (1:25 dilution of a 37% stock – concentration of commercial formaldehyde solutions).

2. Fix tissue for 1-2 hrs (preferably on ice).
3. Wash 3x in PBS to remove fixative.

Notes on fixation and fixatives:

Note, if we (Loomis lab) want to keep our tissues for a long time after fixation, we will keep in PBS at 4° with a drop or two of 4% PFA to prevent bacterial overgrowth. This method is not published, but we have not observed problems with epitopes sensitive to cross-linking fixatives nor bacterial/fungal overgrowth.

Although excellent when staining for β-gal activity, glutaraldehyde-containing fixatives often result in autofluorescence due to the many residual free aldehydes so it should not be used when planning to do IF unless you first reduce with sodium borohydride or equivalent. Glutaraldehyde also makes tissue a little more brittle and distorts morphology.