Introduction

Everything we do in our shared resource is affected by how a sample is prepared and handled before it is brought to our lab. If the sample is poorly fixed, it can be difficult to section, staining quality can be affected, and immunostaining results can be compromised. What follows are “best practices” based on years of experience.

Questions? – Email us at UACC-TACMASS@uacc.arizona.edu or call the lab at 520-626-7319.

Chemical fixation – formaldehyde

Chemical fixation of cells and tissues for histology is typically done with aqueous solutions containing formaldehyde. Formaldehyde causes the cross-linking of proteins in the sample, with binding between amino acids that have sulfhydryl groups. The cross-linking stops autolysis and preserves tissue and cellular structure.

The names used for the different forms of this chemical have a historical basis and can be confusing. Perhaps the most important issue is that formaldehyde in all its forms has been classified as a human carcinogen¹, meaning that it should be handled with care and disposed of properly (hazardous waste).

- **Paraformaldehyde** – is a white powder. It can be dissolved in water, but often must be heated to get it to dissolve. Heating the aqueous solution should always be done in a chemical fume hood, as the hot vapors are particularly toxic. Labs will often go this route to make “fresh” formaldehyde solutions with little or no methanol (a coagulating fixative that is usually considered undesirable for preserving samples that will be immunostained later).

- **Formaldehyde** – is a gas that can be dissolved in water to a maximum concentration of 37%. The 37% solution is often used as the stock for making routine histology fixatives. The stock solution does not store well, because over time the concentration of methanol and formic acid (byproducts of oxidation) increases in the stock bottle, often leading to a white precipitate.

- **Neutral buffered formalin (NBF), or 10% Formalin** – a 10% buffered histology fixative solution made from the stock 37% formaldehyde, meaning that the effective concentration of formaldehyde in the NBF fixative is 3.7%. Some people will refer to this as a 4% formaldehyde solution, which is a good approximation.

Given the safety hazards of working with paraformaldehyde and the problems related to aging stock solutions of 37% formaldehyde, we recommend:

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• Labs purchase 10% neutral buffered formalin in small quantities as needed.
• Documenting the date when the bottle is opened and discarding the bottle when it has been opened for more than 6 months or if there is evidence of precipitate before that time.
• Because suppliers may use different buffering solutions, for consistency’s sake we recommend sticking with a single supplier.
• If having a fixative that is methanol-free is important, see the appendix of this document for instructions on using glass ampules of 16% methanol free formaldehyde to make small batches of 4% formaldehyde fixative.

**Bouin’s fixative** is a variant of 10% NBF that includes picric and acetic acid, giving the fixative a yellowish color. It is sometimes used with gastrointestinal, embryonic, and other small and delicate tissues. Since concentrated picric acid requires very special handling, we recommend that labs purchase appropriate quantities of pre-made Bouin’s fixative and follow the advice above about keeping it for 6 months at most.

Warning: tissue fixed with Bouin’s can give very nice histologic staining, but if your lab is interested in harvesting nucleic acids or immunostaining, we do not recommend using Bouin’s fixative.

*NOTE: according to US government guidelines, all containers with a concentration of formaldehyde greater than 1% are required to have an appropriate warning sticker.*

**Chemical fixation – sample size and fixation time**

**Optimal tissue size:**

• Harvested tissues should be approximately 3mm thick x 15mm wide for optimal fixation. This is about the width of a US penny ($0.01) and the thickness of two penny’s stacked on top of each other. This is to ensure that the formaldehyde can penetrate into the tissue, cross-link the proteins, and stop autolysis as quickly as possible.

• The maximum size should be no larger than 5mm thick, and no larger than 20mm wide. This is about the width of a US nickel ($0.05) and the thickness of two nickel’s stacked on top of each other. This upper limit is because the tissue **must** fit in the space inside a histology cassette. If we have to trim down the size of your fixed tissue pieces to fit this space it is very likely that the center of the tissue was poorly fixed.

• TACMASR can provide users with tissue cassettes for their samples. Users must be label the cassettes clearly with a Tissue-Tek marking pencil #4160 (other pencils tend to rub off, leaving unmarked cassettes) before the tissue is placed inside and the cassette is placed in the fixative. There are also special histologic “markers” that are solvent resistant, but they must be purchased by the user’s lab. **NOTE:** the cassettes will go through many solvent changes in the tissue.
processor before the tissue is finally embedded in a paraffin block. Poorly marked cassettes are very difficult for us to read.

Fixation time:

- Immediately after collecting the tissue, place a correctly sized piece in 10% neutral buffered formalin for approximately 6-24 hours at room temperature. The shorter time period is only appropriate for pieces of tissue that are considerably smaller than the optimal size discussed above.
  
  - Do not delay fixation! Cellular morphology starts to change, genomic expression changes, and there is a loss of the integrity of biomarkers in the tissue that happens very rapidly after the circulation is cut off.
  
  - Fixation with formaldehyde is a chemical reaction. Diffusion into the tissue and the cross-linking of proteins happens more rapidly at room temperature. Do not store tissue that is being fixed in the refrigerator!
  
  - The tissue should be immersed in a volume of fixative that is approximately 20 times that of the volume of the tissue.
  
  - If the fixative solution becomes bloody, replace it with fresh fixative solution after one hour.
  
  - Larger pieces of tissue can be placed on a rotary shaker (room temperature) with gentle agitation to assist diffusion of the fixative. The fixative solution should be changed after a few hours.
  
  - Fixation time of longer than 24 hours in formaldehyde can “over fix” the tissue and should be avoided. Over-fixation can make immunostaining impossible and the FFPE block can be difficult to cut.
  
  - Fixation of much less than 24 hours (except perhaps for very small and thin samples) can lead to under-fixation. This means that inside the tissue there has been protein and nucleic acid degradation and some loss of morphology. IHC on under-fixed tissue will usually demonstrate non-specific antibody staining.

- After a maximum of 24 hours of formaldehyde fixation, transfer the tissue to 70% Ethanol and store at 4°C (refrigerator) for up to 7 days.
  
  - Do not store longer than 7 days in 70% ethanol, tissues can become very brittle and difficult to section after prolonged storage in ethanol.

**Chemical Fixation: over-fixation issues relevant to immunohistochemistry:**

Once tissue has been embedded in a paraffin block it can be stored and used for decades. Having your tissues processed and embedded by TACMASR is relatively inexpensive and gives your lab time to determine what to do next with the tissue without compromising the integrity of the sample. Please do not store wet tissue samples in your lab for long periods of time, you make it much more difficult to get good data at a later time.

- Overfixed tissues are difficult, though not impossible, to cut. While histological stains are not usually compromised with overfixation, immunohistochemistry is compromised.
• Long term preservation of epitope specificity is antigen and protein dependent. Immunohistochemistry stains will be compromised by overfixation.

• Do not store cut paraffin sections for long periods of time at ambient temperature. In our experience IHC can sometimes be compromised by incorrect storage. That said, optimal preservation of specific antigens is protein dependent, so incorrect storage does not preclude analysis.

• Long term antigen preservation in the FFPE block is safest, with plans to cut sections within a month or less before IHC staining. Cut sections should be stored at -80°C. Paraffin blocks should be stored at room temperature and kept away from heat sources.

**Research Animal Tissue (mouse) and EMSR:**

• Fresh organs and tissues harvested by the investigator can be snap frozen or fixed in formalin for downstream histological, immunostaining or molecular analysis techniques.

• Experimental mouse organs, tissues and xenographs generated by the UACC Experimental Mouse Shared Resource (EMSR) can be processed for histological techniques by TACMASR. If you are working with EMSR, please request that they hand off formalin fixed mouse tissues, bones and xenographs to TACMASR for processing/embedding and other services as indicated.

**Submitting fixed samples to TACMASR:**

• Submit fixed tissue samples in 70% ethanol to TACMASR as soon as possible after transferring to alcohol. TACMASR will process and embed the tissue into formalin-fixed, paraffin-embedded (FFPE) blocks.

• IMPORTANT: We generally batch process fixed tissues and embed weekly (Thursday at 1pm).

• Please talk to the TACMASR staff if you have specific or special instructions about how to orient and cut your specimens.

• If your samples include bony tissue, please let us know on the paperwork. We will need to decalcify the sample before we can embed it in paraffin. If you have decalcified the sample in your lab, please let us know that as well. Decalcification before embedding the tissue in paraffin softens the bones for subsequent sectioning. Histological and immunohistochemistry can be performed on decalcified bone tissue FFPE blocks. TACMASR decals bones in using Immunocal solution ([http://www.statlab.com/immunocal.html](http://www.statlab.com/immunocal.html)).

• All biological materials are potential biohazards, some more than others. As a courtesy, please include relevant information about potential biohazardous samples or conditions on the Request Form. Notify the lab staff if your samples present any undue biological hazard.

• TACMASR does not accept:
  • Fresh tissues without making prior arrangements with staff
  • Cell pellets on Fridays without prior arrangements
  • Any radiolabeled material
**Special instructions for the preparation of cultured cells:**
Cultured cell lines can also be preserved as formalin-fixed, paraffin-embedded (FFPE) blocks for histological staining and immunostaining. While treated vs. untreated cells can often be used as positive or negative controls for immunohistochemistry, in our experience there are times when the expected expression does not happen in cells.

**Cell Pellets:** Please arrange with TACMASR in advance, prior to harvesting cells, and plan to submit Monday through Wednesday so that we can fix them in formalin 6-8 hours and prepare the cells for blocking the next day.

Researchers grow their own cells and submit a pellet to TACMASR to make an FFPE block. Generally, \(1\times10^7\) cells from a \(T75\) flask will make a nice cell pellet FFPE block.

- Lift cultured cells in whatever manner you choose.
- Rinse in PBS in a 15ml conical tube, leaving a small amount of PBS on the pelleted cells.
- Transport cell pellet to the TACMASR lab on wet ice.
- TACMASR will fix cells in 10% neutral buffered formalin and prepare cells in a paraffin block, which can then be analyzed similar to an FFPE tissue block.
Recipe for 4% Formaldehyde (using methanol-free 16% formaldehyde ampoules)
(Courtesy of Claire M. Payne, Ph.D.)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Instructions</th>
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<tbody>
<tr>
<td>16% Formaldehyde (methanol free)</td>
<td>10ml ampoules (10 per package)</td>
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<td></td>
<td>• #28908 – Thermo-Fisher</td>
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<td><a href="https://www.thermofisher.com/order/catalog/product/28908">https://www.thermofisher.com/order/catalog/product/28908</a></td>
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<td>• #100503-916 – VWR</td>
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<tr>
<td>2x Dulbecco’s PBS (no calcium or magnesium)</td>
<td>• #21600-010 Thermo-Fisher/Life Technologies/Invitrogen</td>
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<td>(fixative diluent)</td>
<td><a href="https://www.thermofisher.com/order/catalog/product/21600010">https://www.thermofisher.com/order/catalog/product/21600010</a></td>
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<td>• Dissolve powder from a one liter packet in <strong>500ml</strong> distilled/ultra pure water</td>
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<td>• Do not pH</td>
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<tr>
<td>To make 4% Formaldehyde</td>
<td>• Diluting 16% formaldehyde with an equal volume of 2x D-PBS produces an 8% formaldehyde solution similar to the isotonicity of 1x D-PBS. Further dilution of the formaldehyde with an equal volume of 1x D-PBS maintains the isotonicity.</td>
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<tr>
<td></td>
<td>• Using one ampoule makes 40 ml of fixative.</td>
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<td>• Store in refrigerator for about 1 month.</td>
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<tr>
<td></td>
<td><strong>Caution</strong> – solutions of formaldehyde &gt;1% should have an appropriate hazard label.</td>
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