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Guideline

Subject: **Fixation of Tissues**
Approval Date: August 2016
Review Date: August 2020
Review By: Macroscopic Cut-Up Manual Committee
Number: 2/2016

Fixation

Fixation of tissues is essential for successful dissection, processing and microscopic examination of diagnostic histopathology specimens.[1,2] The fixative of choice for routine specimens [3] is 10% phosphate-buffered formalin.

Benefits of fixation

- Allows thin sectioning of tissue[3] by hardening tissue
- Prevents autolysis and inactivates infectious agents (except prion diseases)
- Improves cell avidity for special stains[3]

Drawbacks

- Alters protein structure affecting tissue antigenicity and prevents some forms of cytogenetic testing
- Shrinks and distorts tissue affecting measurements taken post-fixation
- Loss of some tissue constituents e.g. fat, carbohydrates, crystals (water soluble)

Tissue fixation guidelines

The following guidelines are provided for optimal tissue fixation.

Recording

The time to fixation, the fixative used and the total fixation time (including fixation before decalcification) should be recorded.[4-6]

- Ischaemic time (time until the submerging of tissue in fixative) should be minimised and recorded in relevant cases.[4]
- Fixation for less than the optimally specified time below should be recorded as inadequately fixed.

Pre-laboratory

Specimens should be transferred to a fixative within 1 hour of surgical excision.[4,5] If this cannot be achieved, keep the specimen at 4°C including during transfer to the laboratory.

Deterioration will commence very quickly with the loss of blood supply after surgery. [4,5] Results have shown that the quality of RNA and DNA is reliable for specimens stored at 4 °C overnight. [7]

Preparation

Large specimens should be sliced immediately after macroscopic evaluation to allow appropriate penetration of fixative.[8] Slices should be a minimum of 10mm; 5mm is ideal but may be difficult to

achieve e.g. in large fatty specimens.

- Sufficient time must be allowed for penetration of fixative; rates of penetration vary according to fixative type.
- Formalin penetrates tissues slowly (approx. 1mm per hour) 1,9 so specimens need to be opened, incised or sliced and left to fix for an adequate period of time prior to processing.
- Size of tissue blocks taken should be small enough to allow adequate permeation of fixative (and subsequent processing solutions) through the perforations in cassettes. For this reason, tissue sections should be no thicker than 2-3 mm. [10,11]
- Anatomical barriers to fixation must be removed or incised (e.g. fascia, bone, faeces, thick tissue) and large specimens must be sectioned or inflated with fixative (e.g. lung) or opened and cleaned (gastrointestinal tract) to allow penetration.
- Bony or densely calcified tissues should be completely fixed before decalcification.[10]
- Although decalcification almost always results in some loss of cytological detail and/or alters staining properties, decalcification of incompletely fixed tissues almost invariably leads to unacceptable results. [11]
- If bony/calcified tissue is likely to be needed for molecular testing, an appropriate sample should be kept aside for routine formalin fixation without decalcification as acid decalcification destroys nucleic acids. Alternatively a gentle decalcification using EDTA can be considered.[12,13] The specific decalcification solution used and duration of decalcification should be recorded.
- Fixatives diluted and/or contaminated by bodily fluids (e.g. bile, blood, faeces) will be reduced in concentration and must be replaced to ensure effectiveness.
- Pinning specimens to a corkboard or use of a paper or gauze “wick” in tubular structures can improve fixation and reduce tissue distortion.

Fixative

10% neutral buffered formalin is the most common fixative for routine histology [1,2,3,11] and is suitable for immunohistochemistry (IHC) and most molecular testing if appropriately applied.

Formalin for routine fixation

- The fixative of choice for routine histological specimens [3] is 10% phosphate-buffered formalin.
- Neutral buffered formalin (NBF) is 37% formaldehyde in water, therefore 10% formalin is approx. 4% formaldehyde. [11]
- Formalin should be replaced with fresh solution after 24 hours if the specimen requires longer fixation before processing, to reduce the effect of polymerisation and ensure a stable concentration. [14]
- A ratio of specimen to formalin less than 5:1 is considered inadequate.
- Formic acid develops over time producing an acidic solution requiring a buffer to remain neutral (pH 7.0).
- Colour pH indicator can be added to formalin to monitor acidity.
- Acidic formalin causes haematin pigment deposition in tissues particularly in haematogenous tissue after storage for extended periods of time.
- Methods are available to remove haematin [16,17] but it is better to prevent its deposition by maintaining a neutral pH.
- Formalin fixes by cross-linking proteins. [1] This can be reversed to varying degrees. [1,18]
- Antigen-retrieval techniques are required for successful immunohistochemistry. [3,17,18]

Insufficient fixation will result in unfixed tissues being damaged by dehydrating fixative effect of ethanol during processing [1,19] causing:

- Loss of immunohistochemical antigenicity
- Difficulties in microtomy

Other fixatives

Formalin-fixed, fresh, frozen, or alcohol-fixed specimens are suitable for polymerase chain reaction (PCR) and fluorescent in situ hybridisation (FISH) tests. Other tissue treatments (e.g. acidic or heavy metal fixatives, or decalcifying solutions) must not be used on specimens destined for molecular testing. [20]

Other solutions may be preferred in certain circumstances and are necessary for specialist studies and techniques. Ensure fresh tissue is not placed into formalin or other fixative until samples are taken for other requested procedures such as microbiology, immunofluorescence, cytogenetics, flow cytometry and electron microscopy. Consult the relevant laboratory for specific procedures required.

- Microbiology – do not fix, send fresh tissue to microbiology as soon as possible, refrigerate if there are any delays.
- Transport media for other techniques* include:
- Immunofluorescence – use fresh tissue and flash freeze in optimum cutting temperature (O.C.T.) compound. For transportation prior to freezing, place tissue on PBS soaked gauze at 4 °C or for longer periods ideally place in transport medium such as Michel's transport solution (citric acid, ammonium sulphate, n-ethylmaleimide, and magnesium sulphate). [21]
- Cytogenetics – Tissue culture media (e.g. RPMI), containing antibiotics[22,23] (Also refer to guidelines on the handling of samples for genetic testing. [24,25]
- Flow cytometry – RPMI 1640 or other tissue culture medium
- Electron microscopy – 1-4% glutaraldehyde in buffer e.g. 0.1M sodium cacodylate buffer pH 7.4. [26] Substitutes for formalin are available that may provide adequate fixation with reduced risk of occupational health and safety hazards and improvements to staining results. [27,28>

**Choice of fixatives and transport media for specimens requiring IHC and molecular testing will requires validation before use.*

- Tissues should be fixed in a sufficient volume of solution; optimally in a ratio of 10:1 but at least 5:1 fixative to specimen, for penetration to occur in the most efficient manner. [1,9] (Note 6)

Containers must be large enough to hold specimens without distortion and allow penetration from all sides. [29] If a specimen container of adequate size is not available at the time of surgery, the specimen should be promptly sent to pathology as if "fresh" to enable appropriate handling.

Duration

Specimens should be fixed for approximately 6 to 72 hours,[5] preferably for a minimum of 8 hours especially for larger specimens.

- "Overnight" fixation (i.e. 8-12 hours) is generally indicated for 10 mm thick slices of tissues. Fixation for 12-24 hours is considered optimal for most immunohistochemistry.
- Minimum fixation of 6 hours to a maximum of 72 hours for breast cancer specimens, including core biopsies, is required for demonstration of oestrogen, progesterone and HER2 biomarkers.[4,5,30]
- Prolonged fixation i.e. >72 hours in formalin or other aldehyde-based fixatives should be avoided because it may produce nonspecific background staining, be more difficult to reverse and also result in loss of immunohistochemical antigenicity. [8,19]
- If new blocks of the specimen are made after initial blocks were taken a comment regarding their time of fixation should be included e.g. in a supplementary report describing the blocks and any findings from those blocks.
- Microwave irradiation has been used to expedite tissue fixation and processing. [31,32] However, it is possible that microwave treatment may cause alteration to immunohistochemical staining characteristics.

- Adequate fixation should not be compromised in the interests of minimising turn-around times.

Temperature

Room temperature is adequate for most purposes but where molecular testing is required, cold-fixation at 4°C better preserves DNA and RNA, allowing penetration before cross-linking commences and may be considered under special circumstances.[1,7]

Warming the buffered formaldehyde prior to fixation can increase the rate of fixation, [11] but can also increase the rate of autolysis and has the potential to create a “rind” of fixed tissue which impedes formalin penetration to the centre of large specimens. Primary fixation in the cold can slow autolysis, but also slows the process of fixation, and premature cooling of a specimen in fixative can lead to inadequate fixation. [10]

Cytology

Cytology specimens processed into cell blocks should be fixed without delay and treated as a small biopsy.

The cell clot is best placed on a moistened piece of filter paper (by dipping the filter paper into NBF) and placed in the tissue cassette. A pipette may be used to gently remove the clot from the centrifuge tube and transfer it onto the filter paper in a tissue cassette.

Controls

IHC positive controls should be fixed and prepared in the same manner as the tissue samples.

Occupational Health and Safety

Procedures for appropriate handling, storage and disposal must be considered in the use of fixatives in the laboratory. Reference should be made to material safety data sheets (MSDS) for specific chemicals. [31,32]
In Australia, although 10% formalin is not classed as a dangerous good, it is regarded as a hazardous substance according to the National Occupational Health and Safety Commission (NOHSC).[33-35]
Concentrated formalin (37% formaldehyde) has greater potential as a hazard and has been upgraded in risk to a Category 2 carcinogen.[34,35] It is recommended that laboratories avoid the use of concentrated formalin solutions by purchasing commercially prepared 10% formalin.[1] Provision of the best possible ventilation and air quality monitoring is recommended for compliance with exposure standards. Consult guidelines in your jurisdiction.[30]
The following factors should be considered when using formalin: [1,33-37]

Safe handling of formalin

- Avoid contact to skin and eyes.
- Use personal protective equipment.
- Gloves.
- Safety glasses.
- Clean up spillages.
- Avoid inhalation of vapour.
- Keep containers closed.
- Ensure well-ventilated work spaces.
- Store in well-ventilated areas, out of direct sunlight below 30°C.
- Use of respirator masks may be considered if required.

Accidental release

- Use an absorbent such as sand, “kitty litter” or a commercial product to collect spills and contain spread.
- Collect and seal absorbed material into labelled containers for disposal.
- Prevent release into soil, drains and waterways.
- Wash surfaces with excess water after collection.

Fire-fighting

- Formalin is not flammable.
- Extinguishers with water, carbon dioxide, dry chemical powder or foam are all suitable depending on other products involved in a fire.
- Breathing apparatus may be required due to hazardous vapours released.

First aid

- Obtain medical advice (first aid officer, doctor, poisons information centre, ambulance) immediately if major exposure occurs.
- Eye wash station, safety shower and first aid kits should be standard facilities in a laboratory.
- Treat symptomatically.

Inhalation

- Remove from exposure into fresh air.
- If not breathing, apply artificial respiration.

Ingestion

- Rinse mouth thoroughly with water
- Do not induce vomiting
- Give water or milk to drink

Eye contact

- Immediately hold eyes open and flood with water
- Continue for at least 15 minutes.

Skin contact

- Wash affected area thoroughly with water and mild soap.
- Remove contaminated clothing –wash thoroughly or discard.

Disposal

- Collect and seal in appropriately labelled containers and arrange disposal with a commercial waste service.
- Depending on regulations in specific jurisdictions, incineration under controlled conditions may be used for disposal.
- Wash all empty containers thoroughly with water.
- Prevent release into soil, drains and waterways.

References

1. Suvarna KS, Layton C and Bancroft JD. *Bancroft's Theory and Practice of Histological Techniques.* Churchill Livingstone, 2012.
2. Bancroft JD and Gamble M . *Theory and practice of histological techniques.* Churchill Livingstone Elsevier, Philadelphia, PA, 2008 .
3. Grizzle WE. Special symposium: fixation and tissue processing models. *Biotech Histochem* 2009;84(5):185-193.
4. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J Clin Oncol*. 2010;28(16):2784-95.
5. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013;31(31):3997-4013.
6. Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors: Guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J Thorac Oncol. 2013;8(7):823-859.
7. Bussolati G, Annaratone L, Medico E, D'Armento G, Sapino A. Formalin fixation at low temperature better preserves nucleic acid integrity. PLoS ONE 2011;6(6):e21043.
8. Torlakovic EE, Riddell R, Banerjee D, El-Zimaity H, Pilavdzic D, Dawe P, et al. Canadian Association of Pathologists-Association canadienne des pathologistes National Standards Committee /Immunohistochemistry: best practice recommendations for standardization of immunohistochemistry tests. Am J Clin Pathol.2010;133(3):354-365.
9. Medawar PB . The rate of penetration of fixatives. Journal of the Royal Microscopical Society. 1941;61:46-57.
10. Washington School of Medicine, Department of Pathology & Immunology, Formalin Fixation. Available here.
11. Lester SC. Manual of Surgical Pathology. 3rd ed. Philadelphia: Saunders Elsevier, 2010.
12. Wickham CL, Sarsfield P, Joyner MV, Jones DB, Ellard S and Wilkins B. Formic acid decalcification of bone marrow trephines degrades DNA: alternative use of EDTA allows the amplification and sequencing of relatively long PCR products. Mol Pathol. 2000:53(6):336.
13. Korac P, Jones M, Dominis M, Kusec R, Mason DY, Banham AH and Ventura RA . Application of the FICTION technique for the simultaneous detection of immunophenotype and chromosomal abnormalities in routinely fixed, paraffin wax embedded bone marrow trephines. J Clin Pathol. 2005;58(12):1336-1338.
14. Cree IA, Deans Z, Ligtenberg MJ, Normanno N, Edsjo A, Rouleau E, et al . Guidance for laboratories performing molecular pathology for cancer patients. J Clin Pathol. 2014;67(11):923-931.
15. Pizzolato P. Formalin pigment (acid hematin) and related pigments.Am J Med Technol.1976; 42(11):436-440.
16. Waldrop FS, Puchtler H and Terry MS. Removal of acid hematin-type pigments from sections; efficacy of alcoholic picric acid solution. Stain Technol. 1969;44(6):279-281.
17. Henwood A. What is the Best Procedure to Remove Formalin Pigment from Formaldehyde-Acetic Acid-Alcohol Fixed Tissues? J Histotechnol. 2010;33(3):109-111.
18. Eltoum I, Fredenburgh J and Grizzle WE. Advanced Concepts in Fixation: 1. Effects of Fixation on Immunohistochemistry, Reversibility of Fixation and Recovery of Proteins, Nucleic Acids, and other Molecules from Fixed and Processed Tissues. 2. Developmental Methods of Fixation. J Histotechnol. 2001;24(3):201-210.
19. Leong AS and Gilham PN. The effects of progressive formaldehyde fixation on the preservation of tissue antigens. Pathology. 1989;21(4):266-268.
20. Sewart S, Barraclough R, Rudland PS, West CR and Barraclough DL. Molecular analysis of a collection of clinical specimens stored at 4 degrees C as an alternative to snap-freezing. Int J Oncol.2009;35(2):381-386.

21. Odell ID, Cook D Optimizing direct immunofluorescence. In: Day CE (editor). Histopathology: Methods and Protocols, Methods in Molecular Biology, vol. 1180, SpringerScience+Business Media New York, 2014.
22. Rooney DE. Human Cytogenetics: constitutional analysis. A Practical Approach. Oxford University Press, Oxford, 2001.
23. Rooney DE. Human Cytogenetics: malignancy and acquired abnormalities. A Practical Approach. Oxford University Press, Oxford, 2001.
24. National Pathology Accreditation Advisory Council (NPAAC).Requirements for Medical Testing of Human Nucleic Acids. Australian Government, Department of Health and Ageing, Canberra, 2013.
25. National Pathology Accreditation Advisory Council (NPAAC).Requirements for Cytogenetics Testing. Australian Government, Department of Health and Ageing, Canberra, 2013.
26. Bozzola J, Russell L. Chapter 2. Specimen preparation for transmission electron microscopy. Electron Microscopy Principles and Techniques for Biologists. Jones and Bartlett Publishers, Boston, USA, 1992.
27. Prento P and Lyon H. Commercial formalin substitutes for histopathology. Biotech Histochem. 1997;72(5):273-282.
28. Dapson RW. Fixation for the 1990's: a review of needs and accomplishments. Biotech Histochem. 1993;68(2):75-82.
29. Goldstein NS, Ferkowicz M, Odish E et al. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. Am J Clin Pathol. 2003;120(1):86-92.
30. Ellis IO. Tissue pathways for breast pathology. The Royal College of Pathologists, London, 2010.
31. Emerson LL, Tripp SR, Baird BC et al. A comparison of immunohistochemical stain quality in conventional and rapid microwave processed tissues. Am J of Clin Path. 2006;125:176-183.11.
32. Mayers CP. Histological fixation by microwave heating. J Clin Pathol.1970;23(3):273-275.
33. Perrigo Australia. Material Safety Data Sheet Buffered Formalin 10% Clear. Balcatta, WA ,2013.
34. PODC Scientific. Material Safety Data Sheet 10% Neutral Buffered Formalin. Artamon, NSW, 2012.
35. National Pathology Accreditation Advisory Council (NPAAC).Requirements for the Packaging and Transport of Pathology Specimens and Associated Materials. 3rd Ed. Australian Government, Department of Health and Ageing, Canberra, 2013.
36. National Industrial Chemicals Notification and Assessment Scheme.Formaldehyde. Priority Existing Chemical Assessment Report No. 28.Australian Government Department of Health and Ageing, Garran, ACT, 2006.
37. Royal College of Pathologists of Australasia. Formaldehyde Exposure in Laboratories. Surry Hills, NSW, 2014.