

Step by Step instruction for frozen sample preparation for histology assay in non-histopathology lab environment

Note: Before planning a frozen section project, please note that:

- ❖ Frozen tissue work always needs an appointment in advance. Before you bring your samples to the core facility, please contact us to make an appointment first.
- ❖ Both fixed and un-fixed tissue samples can be frozen sectioned. If tissue is not being preserved by a fixative, it is imperative that the tissue should be frozen as quickly as possible after the tissue is devascularized.
- ❖ The submitted samples can be accepted either as fresh tissue on ice, formaldehyde fixed tissues in 30% sucrose, snap-frozen tissues, or OCT embedded frozen blocks.
- ❖ If the sample has been fixed with formaldehyde containing fixatives, it must be cryoprotected with 30% sucrose in 1×PBS (See procedure below: 3. Fixation). Submit the tissue in 30% sucrose in 1×PBS or frozen embed your tissue samples in OCT compound and submit the tissue to the core facility as your earliest convenience.
- ❖ On the day of your frozen section appointment, put the samples on dry-ice or in liquid nitrogen and bring it to our Core Facility.
- ❖ Never freeze and thaw the frozen tissue, or ice crystal artefact will occur. Any leftover frozen tissues can be paraffin embedded through a special processing procedure.

This instruction is specifically designed for those who work in non-histopathology lab environment to prepare frozen sample for histology assay.

Please always be aware that it is important to keep fresh tissues on ice and process them within 5-10 minutes of devascularization to well preserve the nuclear acid and protein in the fresh tissue samples.

1. Grossing:

Samples should be grossed the same way as described in the “Instruction for sample preparation for paraffin processing”. Avoid crushing artefacts by gently but firmly securing the specimen while cutting.

2. Size of the sample:

Smaller size is preferred as it can be frozen faster than a larger one. But while you prepare fresh sample for frozen section, the thickness is not as critical as paraffin processing sample, it could be 1 cm or a little bit more.

3. Fixation (optional):

The sample can be fixed before or after the grossing. Again, if the sample has been fixed with formaldehyde fixatives, it should be rinsed with 1×PBS for 10 minutes, 3 times, transferred into 15% sucrose in 1×PBS at 4°C until tissue sinks, then transferred into 30% sucrose in 1×PBS at 4°C until tissue sinks. You might need to perform antigen retrieval procedure to expose the

epitopes due to the cross-link effect of aldehyde fixatives when perform immunostaining on these samples.

4. Rinse and dry:

Remove excess liquid surround the tissue by absorption with Kim-wipe, gauze or paper towel prior to freezing. Otherwise, these liquid will form ice crystal on the surface of tissue and prevent tissue attach to frozen embedding media (e.g. OCT compound) when the tissue is frozen embedded and cause a lot of difficulties during sectioning. This procedure only takes a few more seconds, but it will make a dramatic difference for the sectioning process later on.

5. Snap-Frozen (optional):

Samples could be snap-frozen on dry-ice or in liquid nitrogen when:

- They are collected over a period of time and cannot be processed simultaneously.
- There are too many samples to process at once.
- Samples are collected in the field, or the mechanical processing of fresh samples is insufficient for thorough disruption.

While freezing allows the researcher to postpone DNA/RNA/protein isolation, it is time consuming and makes subsequent RNA isolation a more laborious process. Ideally, specimens should be snap-frozen or placed in appropriate reagent within 5 minutes from loss of vascularization to prevent the DNA, RNA and protein from degradation and keep the best morphology.

Transfer the frozen sample into a cold, labeled cryo-vial, with a screw-top lid. It is not recommended to put the fresh tissue inside the cryo-vial first and snap-freezing the tissue in the vial. It will become very difficult to take the tissue out of the tube later. Powder Dry-ice/Cold isopentane in Granular Dry-ice / Liquid nitrogen + cryo-vial method provides excellent specimen integrity and a wide array of options for tissue analysis (See Cold source below). The vials can be stored in a liquid nitrogen storage tank or -80°C freezer for long-term storage.

6. Cold Source:

An ideal cold source should be able to freeze down the tissue relative quickly and evenly. Because the faster you freeze down the samples, the less chance of ice crystal artefact will occur. But on the other hand, freezing tissue too quickly, such as submersing the tissue directly into the liquid nitrogen will cause the tissues or blocks crack, which will make them very difficult or impossible to section. This happens because the outside tissue begins to freeze much more quickly than internal portion.

The following cold sources are recommended for those who want to embed their samples by themselves in a research laboratory without special frozen apparatus:

- 1) One simple method is to use dry ice (-78.5 °C) in block form placed in a Styrofoam container. Place the filled cryomold on the block to freeze it. This method has the advantages of simplicity and safety, but does not freeze the tissue as rapidly as immersion in a freezing medium.

Note: Freezing tissue on granular/pellet dry-ice or in the freezer is not recommended. These cold sources neither provide an even freezing nor freeze the tissue quickly enough. This will cause freezing artefacts, and desiccation of the tissue.

- 2) The 2nd method we recommend is to use dry ice in pellet form. Place a small stainless steel bowl (or Pyrex or polypropylene beaker) in the bottom of a Styrofoam container and fill the space around the bowl with dry ice pellets. Place some pellets in the bowl and slowly add isopentane/2-methylbutane. Work in a fume hood, of course, as these are flammable. When the pellets stop bubbling vigorously, the "slurry" is ready. Once you've filled the mold and oriented the tissue, immerse it in the liquid to freeze it. Isopentane/2-methylbutane also can be chilled in liquid nitrogen (-176°C). With the liquid nitrogen in a Styrofoam container or Dewar flask, use a tongs to lower a stainless steel, Pyrex, or polypropylene container of isopentane into the liquid nitrogen. The isopentane will start to become opaque as it nears freezing. Take the isopentane out of the liquid nitrogen and freeze the specimen as described above. Chill the isopentane again as necessary for subsequent tissues. This method has the advantage of very rapid freezing.

Note: DO NOT use Methanol, Ethanol or Acetone instead of isopentane/2-methylbutane. Once OCT contacts with Methanol, Ethanol or Acetone, you will not be able to freeze it down at -20°C.

- 3) Vapor phase of liquid nitrogen in a proper container with a relative wide opening which allow you put the embedding mold in the vapor phase of the liquid nitrogen very easily.

Note: DO NOT freeze the tissue by submersing into the liquid nitrogen, it will cause the OCT blocks to crack which makes them very difficult or impossible to section. This happens because the outside tissue begins to freeze much more quickly than internal portion.

- 4) If you have a metal block with a flat surface, you can combine this with dry-ice/Liquid Nitrogen as an alternative cold source. Using this method, you have to pre-chill down the metal block in -80°C freezer/Dryice before you use the cold surface as cold source to frozen embedding your sample on it. It is recommended to have another piece of metal block which you can put it on the top of the frozen sample to accelerate the freezing process.

7. Frozen embedding unfrozen tissue in OCT compound

- 1) Label the sample ID on the surface of the Cryomold with a Permanent Maker.

- 2) Place few drops of OCT (depends on the size of the tissue to be embedded) onto the center of the bottom of cryomold. Be careful to select the proper size embedding mold according to the size of the tissues to be embedded.
- 3) Place the unfrozen tissue sample in the drops and oriented. Make sure that the side touching the bottom of the cryomold is the side you want sectioned first. Gently push the tissue with a forceps to ensure that the bottom surface of the tissue is placed properly, level with the container, touching the face of the bottom and the tissue is located in the center of the mold. *Be very careful to orient the sample because it is important for the demonstration of proper morphology.*
- 4) Carefully drop more OCT onto the specimen until it is completely covered. None of the tissue should remain exposed.
- 5) Try to avoid the formation of air bubbles. Remove any bubbles inside the OCT. This is important because the air bubbles will create problems when cutting sections.
- 6) Let it settle for 15-30 seconds to allow the OCT to completely wet the surface of the tissue.
- 7) Place cryomold with OCT covered sample in it on the surface of the cold isopentane/2-methylbutane or in the vapor phase right next to the liquid nitrogen with the flat side down using a long forceps.
- 8) After hardening of the OCT compound (it will happen in 0.5-1 minute), wrap the OCT embedded block in foil and placed in a labeled zip bag.

8. Frozen embedding frozen tissue in OCT compound:

Embedding frozen tissue is similar as embedding unfrozen tissue, except that you must freeze down the frozen tissue in OCT compound as soon as possible without any delay whenever two of them touch each other. Otherwise, a bigger portion of the frozen tissue will experience a freeze-thaw-freeze cycle and ice crystal will form inside of it and result in a very bad morphology due to the ice crystal arteffect.

9. Storage:

The frozen blocks can be temporary stored in dry ice. Transfer the blocks to a liquid nitrogen storage tank (Years) or -80°C freezer (Months).

From this point over, the sample should never be thaw unless there is specific requirement.