Dewan/Loomis-Protocol: Revised 12-16-2016

Freezing tissues for histology:

Tissues should be frozen as rapidly as possible to avoid ice crystals and to promote the formation of amorphous (vitreous) ice. Freezing tissues slowly allows the water molecules to line up during the transition and form crystals, which results in volume expansion with destruction of cell membranes and holes in loose connective tissue.

Liquid nitrogen (-190°C) is frequently used for rapid (flash) freezing. However, liquid nitrogen (LN_2) has a low specific heat constant and **when it contacts warm tissues or OCT it boils and forms a vapor barrier, which acts as an insulator. As a result, the inner core of the tissue sample freezes slowly and unevenly, often causing the block to crack. This is especially problematic if the tissue sample is large – such as a whole rodent brain**. **Isopentane (2-methylbutane), which has a high thermal conductivity, does not form a vapor halo. Therefore, isopentane chilled with LN2 freezes tissues more effectively and evenly than putting the tissues (especially large tissues) directly into LN_2.**

Note, isopentane chilled with dry ice will only reach a temp of -78.5^{o}C and therefore large samples will freeze more slowly than when isopentane is chilled with LN_2 and therefore the risk of ice crystal formation is significantly higher for larger samples and samples not cryopreserved in sucrose or other cryoprotectant. If isopentane is chilled with dry ice, the dry ice should be in a fine power and/or ethanol slurry to ensure a temp of -78.5^{o}C.

NEVER ALLOW FROZEN TISSUE TO THAW AND REFREEZE!!!!! The accumulation of moisture around the thawed, but cold tissue, will cause destructive ice crystal formation as the tissue re-freezes.

Embedding protocol for Frozen Samples:

1. Set up equipment for freezing the tissues:
    a. Put liquid nitrogen or a dry ice slurry with 100% EtOH in a styrofoam box.
    b. Pour isopentane (also called 2-methylbutane) (Fisher Scientific, Cat#O3551-4) in a metal canister to a height of 2” (or 1/3 to ½ full).
    c. Put the metal canister containing isopentane into the liquid nitrogen or dry ice EtOH slurry. Cover with lid and allow to equilibrate for 3 to 10 mins. If the isopentane boils when a piece of dry ice is dropped in, then it is not yet cold enough for freezing the tissue.

2. Fill wells in the labeled plastic molds (Fisher Scientific, Small: 15x15x5mm, Cat#22-363-553; Medium: 24x24x5mm, Cat#23-363-554 and Large: 22x22x20mm, Cat#18646A) with OCT (Optical Cutting Temperature) (Fisher Scientific, Cat#4583) or NEG50™ (Richard-Allan Scientific, Cat#6502), minimizing bubbles.
3. Wick off excess surrounding liquid from the tissue before dipping tissue in an unlabeled OCT-filled well. This OCT dip (or "wash") removes remainder carry-over fluid and allows the tissue to equilibrate. Do not allow the tissue to sit more than 10 mins at room temp in the OCT, as the tissue may become shriveled.
4. Transfer the sample to the appropriately labeled, OCT-filled mold. Again take care to avoid bubbles in the OCT, especially around the tissue itself (check under stereoscope if possible).
5. Using long (12") forceps, grasp the molds with the tissue and hold them in the isopentane chilled by either liquid-nitrogen or dry ice slurry. If the tissue has been well-fixed and cryopreserved with sucrose, glycerol or polyethylene glycol (which helps prevent ice crystal formation), then the tissue can be frozen at -80°C in isopentane chilled with an EtOH/dry ice slurry. However, if the tissue is unfixed (fresh) it MUST be frozen more rapidly at a colder temperature (closer to -160°) to prevent ice crystal formation; therefore the isopentane must be chilled by liquid nitrogen.
    a. The base of the molds should be in the liquid, but not deep enough to allow the isopentane to flow over the top of the mold and OCT.
    b. Do not to let the sample touch the metal sides of the canister.
    c. If using liquid nitrogen to cool the isopentane, remove isopentane canister from the liquid nitrogen bath, as needed, to keep isopentane from freezing solid (freezing temperature of isopentane is -160°C). A small amount of freezing at the bottom of the canister is fine and indicates the isopentane is chilled, but there should be at least an inch of liquid isopentane visible.
6. Check the progress of the tissue block freezing. It is critical that freezing occurs rapidly and evenly. One should be able to watch the OCT turn white, progressing from the edges/bottom to the center over 1-2 minutes.
7. Remove the blocks and temporarily put them on dry ice.

8. When all the blocks are frozen, store at -80°C. For long-term storage, best to wrap with foil or put in sealable plastic bag.
9. Let the isopentane warm up and save it for re-use in a glass bottle with a secure cap. Do NOT tighten the lid on the bottle until the isopentane has reached room temp. If you tighten while the isopentane is still cold, the resulting vaporization & expansion can cause a dramatic explosion.

NOTE: isopentane is highly flammable and its boiling temperature is just above room temp, so store appropriately.

Trouble shooting and notes:

Swiss chees holes in tissue sections: The tissue was either: 1) frozen too slowly with no cryoprotectants; 2) allowed to partially defrost (such as being left in a cryostat overnight during the defrost cycle); 3) stored at -20° for more than a few hours; 3) stored too long in the -80; 4). Ice crystals will slowly form even when tissues are frozen, but they form much more slowly at -80 than at -20.

Tissue blocks crack: tissues were frozen unevenly – the outer rim of tissue froze quickly whereas the inner core froze slowly forming large ice crystals, which expanded the tissue causing cracking. This is typically observed when larger tissue samples are placed in liquid nitrogen directly – not chilled isopentane.

Notes: For lung and intestine, it is advantageous to infuse them with a solution of 50% O.C.T. and 50% PBS prior to embedding.

Useful refs & links:

http://www.bdbiosciences.com/us/resources/s/frozentissue

http://www.thelabrat.com/protocols/prepfrozensections.shtml

http://www.leicabiosystems.com/pathologyleaders/freezing-biological-samples