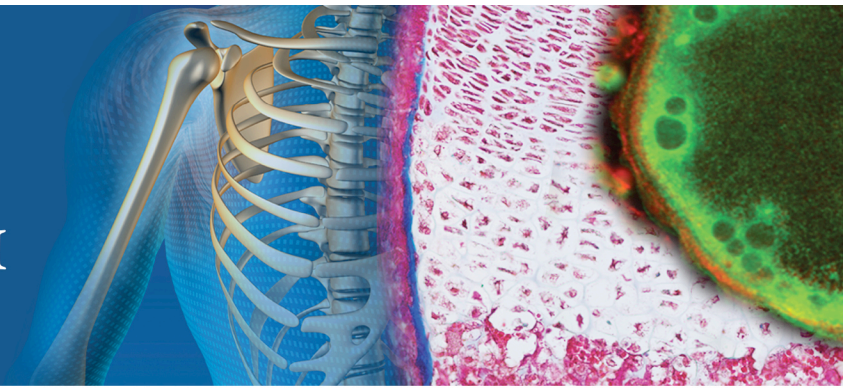
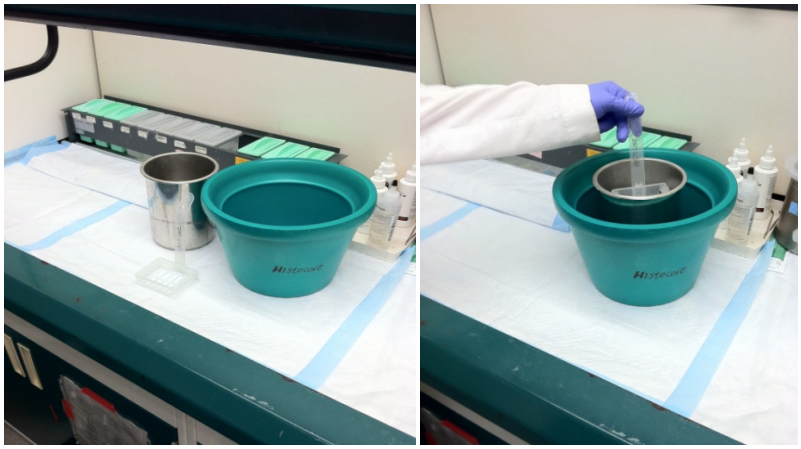


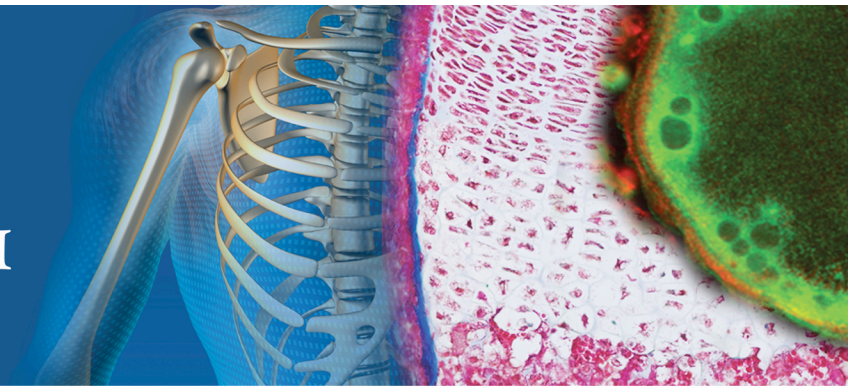
Preparation of Frozen specimens

In the HBMI Core, we use the method of snap freezing our tissue. Snap freezing refers to the ultra-low temperature freezing method used to prepare high-quality cryosections. Ice-crystals that form during a slow freezing process cause distortion in tissue morphology and can lead to more difficult sectioning. If the specimen is cooled rapidly, the crystals that form are much smaller and fewer which leads to much better morphology. Dry ice (-80^{o}C) can cool a standard sized specimen submersed in O.C.T. within 3 minutes typically, but is still not cold enough to eliminate crystal formation. The method below, uses a super-cooled bath of 2-methyl butane (-150^{o}C) that can freeze a standard specimen within 1 minute, thus greatly reducing crystal formation.

Materials:

- Ice bucket
- Stainless steel bucket
- Liquid nitrogen
- 2-methyl butane (Isopentane) (CAS# 78-78-4)
- Plastic staining rack with long handle
- O.C.T. Compound (Tissue Tek, cat# 4583)
- Standard Cryomolds (Tissue Tek, cat# 4557)
- A marking pen to identify specimen on Cryomold.
- Timer
- Aluminum foil





Procedure:

1. Specimen must already have been fixed in PFA or 10%NBF, decalcified. Times will vary depending on the results you are looking for. Please inquire with the histocore. Specimens are then run through 10%, 20%, and 30% sucrose gradients each for 24 hours.
2. Rinse samples in PBS and infiltrate in O.C.T. for 30 minutes prior to snap freezing.
3. When snap freezing, all steps should be done under a fume hood!
4. Place each specimen into cryomolds and fully cover with O.C.T. Specimens may be oriented once submersed in O.C.T. and will usually stay in place due to the viscosity of the medium.
5. Fill a cryobucket with liquid nitrogen, approximately 3cm deep. *****Take serious care using liquid nitrogen and wear proper PPE!! Transport bucket and liquid with a cover and on a cart for extra stability.*****
6. Fill stainless steel bucket with 2-methyl butane so that a thin layer of liquid covers the bottom of the container. The level of the liquid should just reach the top of the cryomold so that the blocks are never fully submersed during the freezing process, as the 2-methyl butane should never touch the O.C.T. Ideal depth should be approximately 1cm across the bottom, or ~150ml in a 1.5L container.
7. Carefully place steel bucket into the liquid nitrogen. Vapors forms at first as the 2-methyl butane rapidly cools. After 3 minutes, solid white ice is visible at the bottom of the steel bucket as the 2-methyl butane freezes. This is indication that liquid has reached its freezing point of -150^{o}C.
8. One specimen at a time, carefully dip block into 2-methyl butane with long-handled staining rack. Complete freeze is achieved at ~60 seconds (90 seconds maximum for larger specimens).
9. Remove block from 2-methyl butane and examine for characteristic bump that forms on the top center of the block. Wrap specimen in aluminum foil immediately and store in dry ice or -80^{o}C freezer. This should be done within a minute of snap-freeze so as to prevent any thawing.
10. Liquid nitrogen quickly boils away at room temperature and may need to be replaced if preparing many samples. Indication that the 2-methyl butane is warming up again is when the white layer of ice begins to disappear.
11. Remove steel bucket from liquid nitrogen and allow all materials to warm to room temperature under fume hood. Dispose of 2-methyl butane in hazardous waste container and label properly. All liquid nitrogen will boil and evaporate away.

Always ask the histocore if you have any questions. Please return any materials you have borrowed from the histocore.