

TISSUE FREEZING METHODS FOR **CRYOSTAT SECTIONING**

Basic Tissue Freezing Methods

Preparing Tissue for Freezing

Then a quick overview of MHPL Cryostat sectioning Techniques:
Using The Brush;
Using The Anti-Roll Plate;

Speaker: Donna J. Emge, HT-ASCP, MHPL Manager



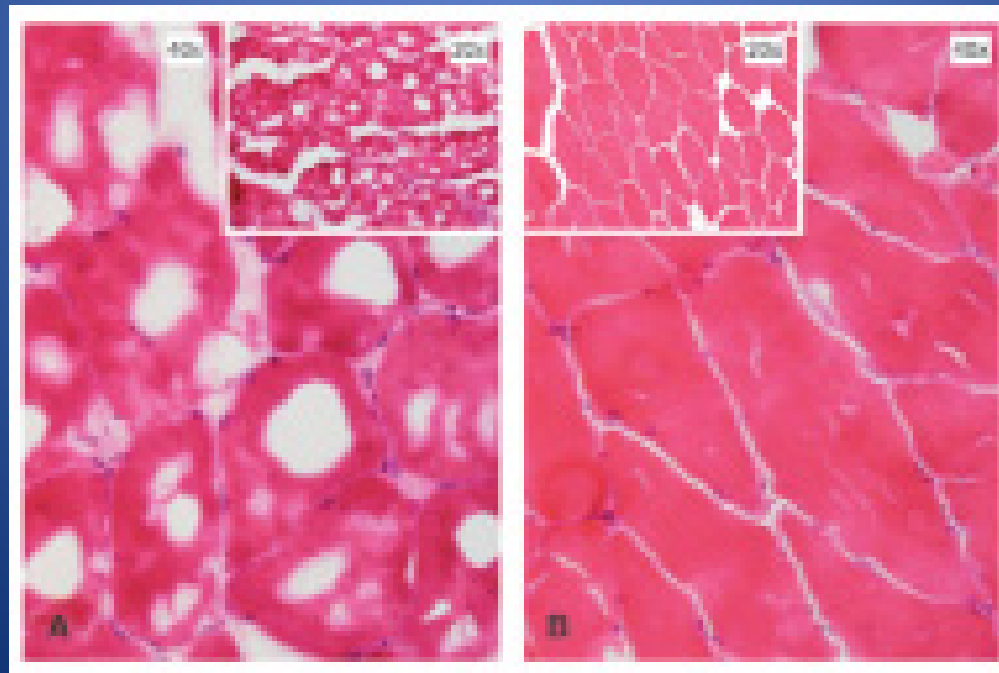
Preparing Tissue For Freezing

Tissue for freezing should be frozen or fixed as promptly as possible after cessation of circulation to avoid morphological distortions and damage due to:

- Tissue drying artifact.
- Autolysis - The destruction of tissues or cells by the action of substances, such as enzymes, that are produced within the organism. Also called *self-digestion*.
- Putrefaction - Decomposition by microorganisms.

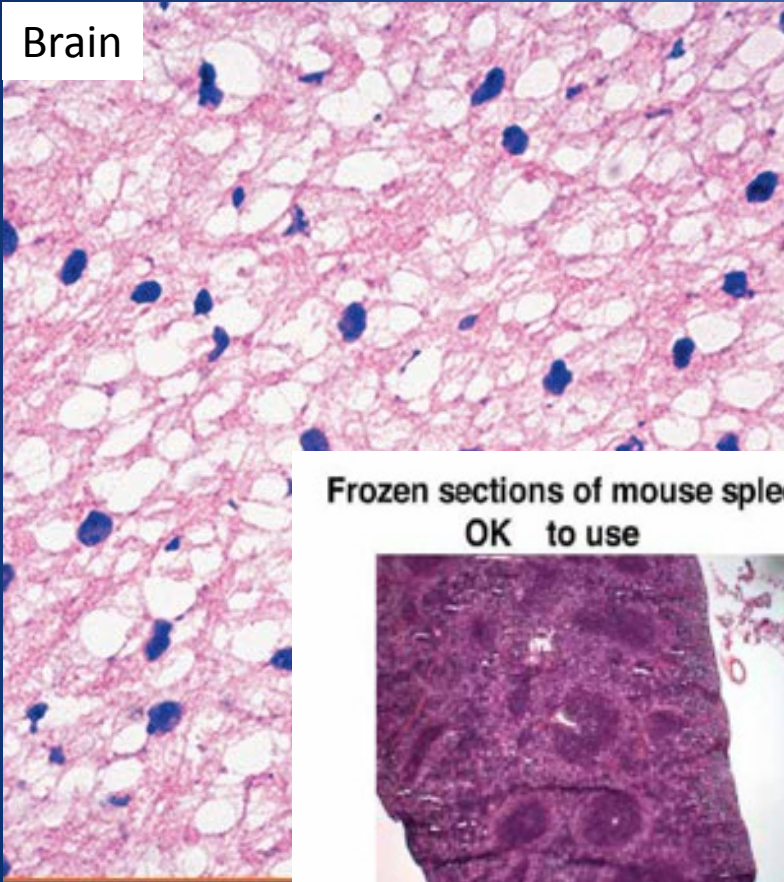
WHY SNAP FREEZE OR FIX AND CRYOPROTECT FOR FREEZING?

Slow freezing can cause distortion of tissue due to ice crystal formation that replaces the architecture with a “Swiss Cheese” effect.

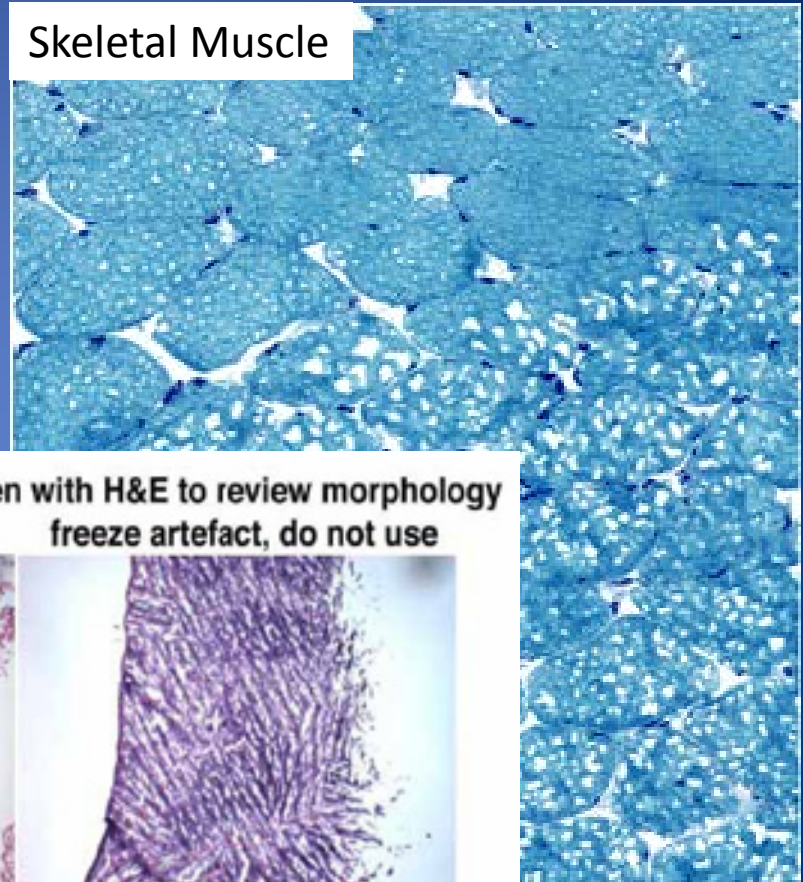


The object is to freeze so rapidly that water does not have time to form crystals and remains in a vitreous form that does not expand when solidified.

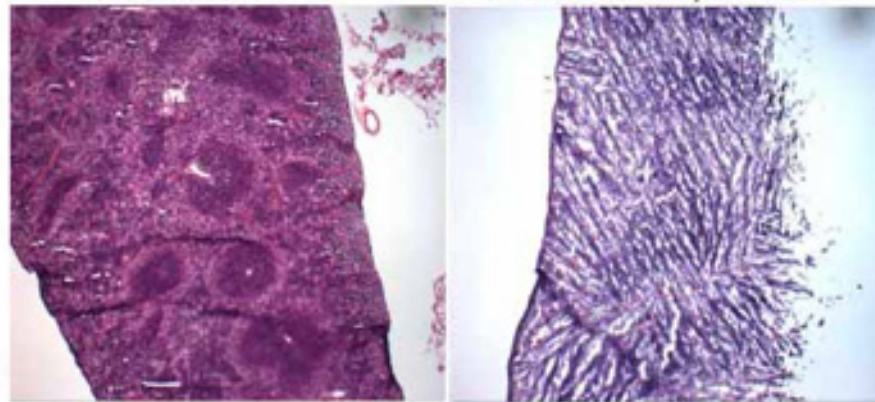
Brain



Skeletal Muscle



Frozen sections of mouse spleen with H&E to review morphology
OK to use freeze artefact, do not use



Freezing artifact in sections of Brain, Muscle, and Spleen

SHORT ARTICLE ON THE SUBJECT OF WATER CRYSTAL FORMATION:

“FREEZING BIOLOGICAL SAMPLES”

Charles W. Scouten & Miles Cunningham

<http://www.myneurolab.com/global/Manuals/Tips%20and%20Techniques%20Freezing%20Artifact.pdf>

METHODS OF TISSUE FREEZING

- 1. Fresh tissue freezing** – Tissue is in OCT and flash frozen fresh.
- 2. 4% PFA fixed, sucrose cryoprotected tissue freezing** – Tissue is in OCT and may be frozen using dry ice or the flash frozen method.
- 3. Enzyme study tissue freezing** – Often used for fresh muscle tissue. A fresh frozen method with no OCT matrix. Tissue protrudes from a Tragacanth or other support medium.

MHPL Protocols for these methods are included in your workshop folder.

WHY NOT JUST USE LIQUID NITROGEN?

AFTER ALL: “Liquid nitrogen is one of the coldest liquids routinely available and it does not mix with tissue.”

- **It Boils** – this creates a vapor barrier that causes freezing in a slower, unpredictable pattern.
- **Tissue and OCT often cracks** – due to this unpredictable freezing pattern.

Review the article:

“FREEZING BIOLOGICAL SAMPLES”

Charles W. Scouten & Miles Cunningham

<http://www.myneurolab.com/global/Manuals/Tips%20and%20Techniques%20Freezing%20Artifact.pdf>

GETTING STARTED:



Before you dissect the animal - organize and set-up:

Before you dissect the animal - organization and set-up:

- **Choose appropriate freezing method** - and depending on the method prepare liquid nitrogen, isopentane, dry ice.
- **Label ahead of time** - cryo molds, aluminum foil, specimen bags while at room temperature.
- **Covered Foam cooler with crushed dry ice** – to temporarily hold frozen samples as you work.
- **Tools & other supplies** - OCT, or Tragacanth, forceps, small labeled weigh boats or small labeled petri dishes.



FRESH TISSUE FREEZING

Pros

- Fastest of all methods.
- Excellent for IHC, IF, ISH. No antigen retrieval required since no cross-linking fixative.
- Often easiest to section – depending upon tissue.

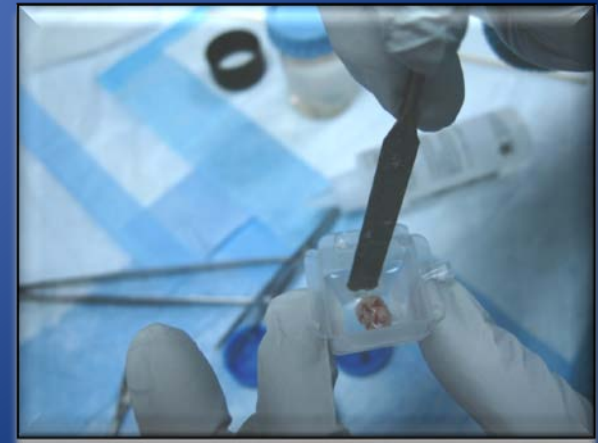
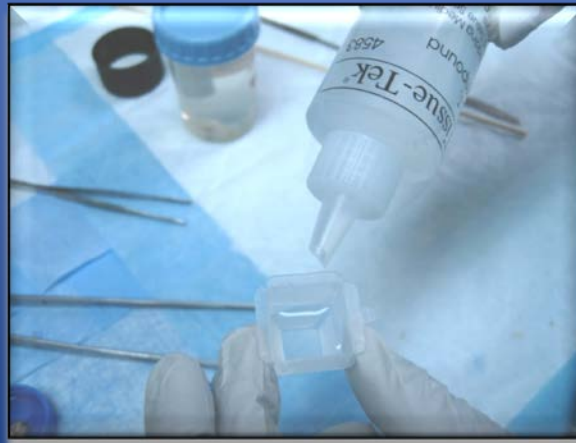
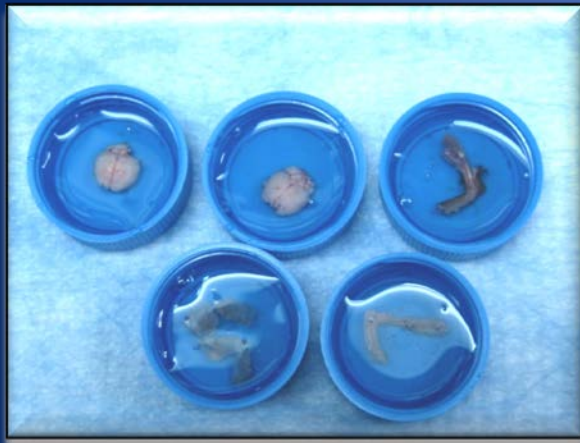
Cons

- Poorest morphology.
- Prone to freezing artifact – must be snap frozen.
- ISH integrity – extreme clean techniques required or RNA will be rapidly and easily degraded.

PREPARING FRESH TISSUE FOR FREEZING

(Not for enzyme study method)

- **Acclimate tissue to OCT** - cover freshly dissected tissue for a few minutes in OCT in a labeled small petri dish or small weigh boat.
- **Transfer and orientate in fresh OCT** in a labeled Cryomold – with just enough OCT to cover the tissue.
- **Avoid bubbles in the OCT** – especially near the tissue.
- **Sectioning surface** - is the bottom of the Cryomold.
- **Begin freezing.**



Fresh Tissue Freezing Procedure:

- **A metal beaker** is filled 2/3 with Isopentane and placed in a Dewar of Liquid Nitrogen enough to come up to about 1/3 of the metal beaker. Prepare at least 10 minutes before freezing sample.
- **With 12 inch forceps freeze** the cryo mold prepared sample in the clear portion of isopentane – **do not fully submerge**.
- **Avoid block cracking** - when there is still a small drop size of unfrozen OCT transfer sample to covered foam cooler of dry ice while continuing on to other samples.



Temporarily store frozen samples in a covered foam cooler of dry ice while continuing to freeze other samples



Wrap individual samples in labeled foil, seal in a plastic bag, place in a freezer box . Store at -80° C.



4% PFA FIXED, SUCROSE CRYOPROTECTED TISSUE FREEZING

Pros

- Excellent morphology compared to other methods.
- May use a slower freeze in **crushed powder dry ice** alone, **slush of dry ice and 100% alcohol**, or in a **beaker of isopentane surrounded by dry ice** - without incurring freezing artifact or block cracking.
- Any of the freezing methods discussed can be used.
- Good for most IHC, IF and ISH.

Cons

- Time consuming
- Most IHC will require antigen retrieval.
- Although the fixative cross-linking is protective for ISH techniques there is some RNA degradation.

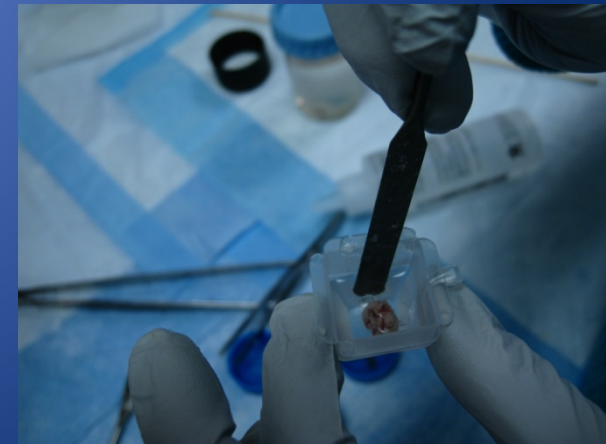
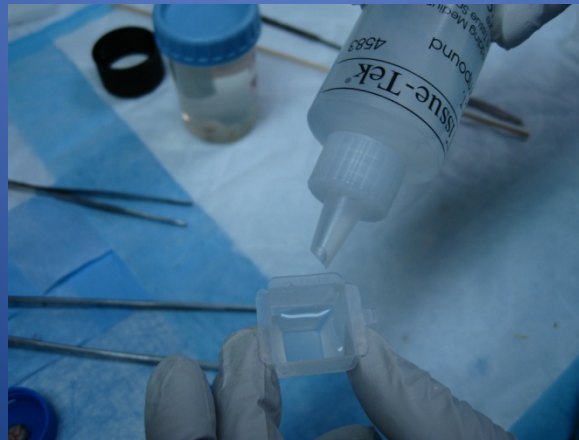
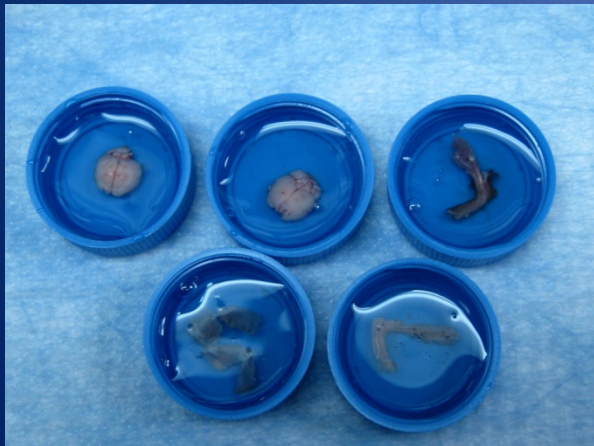
PREPARING FIXED, SUCROSE CRYOPROTECTED TISSUE

- 4% PFA transcardial perfuse animal.
- Drop fix in 4% PFA for a few hours to O/N.
- 15% sucrose in 1XPBS **until tissue sinks.**
- 30% sucrose in 1XPBS **until tissue sinks.**

SUCROSE CRYOPROTECTED TISSUE FREEZING

(Not for enzyme study method)

- **Acclimate tissue to OCT** - cover freshly dissected tissue for a few minutes in OCT in a labeled small petri dish or small weigh boat.
- **Transfer and orientate in fresh OCT** in a labeled Cryomold – with just enough OCT to cover the tissue.
- **Avoid bubbles in the OCT** – especially near the tissue.
- **Sectioning surface** - is the bottom of the Cryomold.
- **Begin freezing.**



FIXED, SUCROSE CRYOPROTECTED TISSUE FREEZING

- **A metal beaker** is ½ filled with isopentane, placed in a foam cooler or laboratory ice bucket and surround with crushed dry ice. Add a few pieces of dry ice to the isopentane and wait until boiling stops. Add more isopentane if necessary.
- **With 12 inch forceps freeze** the cryo mold prepared sample in the isopentane – do not fully submerge.
- **Alternatively:** Freeze cryomold prepared sample by surrounding it in finely powder crushed dry ice alone, or in a dry ice methanol or 100% Ethanol slurry.
- **Transfer frozen sample to a covered foam cooler of dry ice** while continuing on to other samples.
- **Wrap all samples in labeled foil**, place in a bag sealed bag, in a freezer box.
- **Store at -80**



ENZYME STUDY TISSUE FREEZING

Pros

- Excellent for Enzyme histochemistry and Immunohistochemistry studies.
- Best method for muscle tissue.

Cons

- Advanced skill needed for sectioning – no supportive OCT matrix. Anti-roll plate better than brush technique.
- Time and technique skill to prepare.
- Extremely susceptible to any freeze thaw – leading to loss of morphologic detail in muscle or brain tissue.

ENZYME STUDY METHOD

TISSUE PREP FOR FREEZING

Organize and set up:



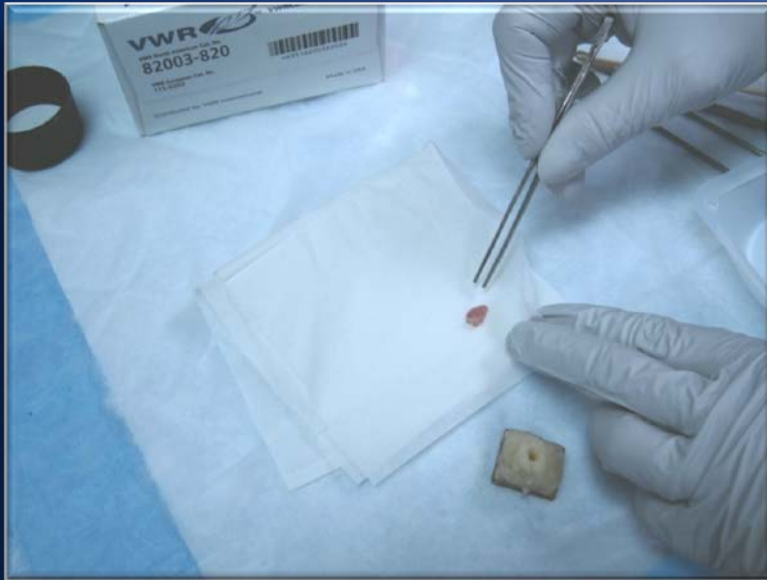
ENZYME STUDY METHOD PREP FOR TISSUE FREEZING



- Prepare a small pyramid of thick Tragacanth paste on a small piece of cork.
- Make a small hole in the Tragacanth paste pyramid.



ENZYME STUDY METHOD PREP FOR TISSUE FREEZING



- Gently remove any surface moisture from tissue with fresh tissue wipe.

- Place $1/8$ to $1/4$ of the tissue in a hole at top of pyramid, leaving the rest stick out above the traganth.
- Seal edges of traganth to the tissue.



ENZYME STUDY TISSUE FREEZING

Procedure:

- A metal beaker is filled 2/3 with Isopentane and placed in a Dewar of Liquid Nitrogen enough to come up to about 1/3 to 1/2 of the metal beaker. Prepare at least 10 minutes before freezing sample.
- **Hold the cork/tragacanth/sample with 12" forceps and submerge sample side down completely into the isopentane for 10 to 20 seconds.**



ENZYME STUDY TISSUE FREEZING

- Transfer sample to covered foam cooler of crushed dry ice or immediately to a -80 freezer.
- Rapidly wrap all samples in pre-cooled, labeled foil, and place in a pre-cooled plastic bag, in a freezer box.
- Store at -80°C.



CRYOSECTIONING PREP

- Remove the frozen block from the -70°C freezer and allow it to equilibrate in the cryostat chamber temperature for approximately 30 minutes.
- The optimal temperature for cryostat sectioning depends on the nature of the tissue and on whether the tissues have been freshly frozen or pre-fixed with subsequent cryoprotection.
- Note the reference chart for temperature setting guidelines for tissue types in your folder.

MHPL CRYOSTAT SECTIONING TECHNIQUES

- Using The Brush
- Using The Anti-Roll Plate



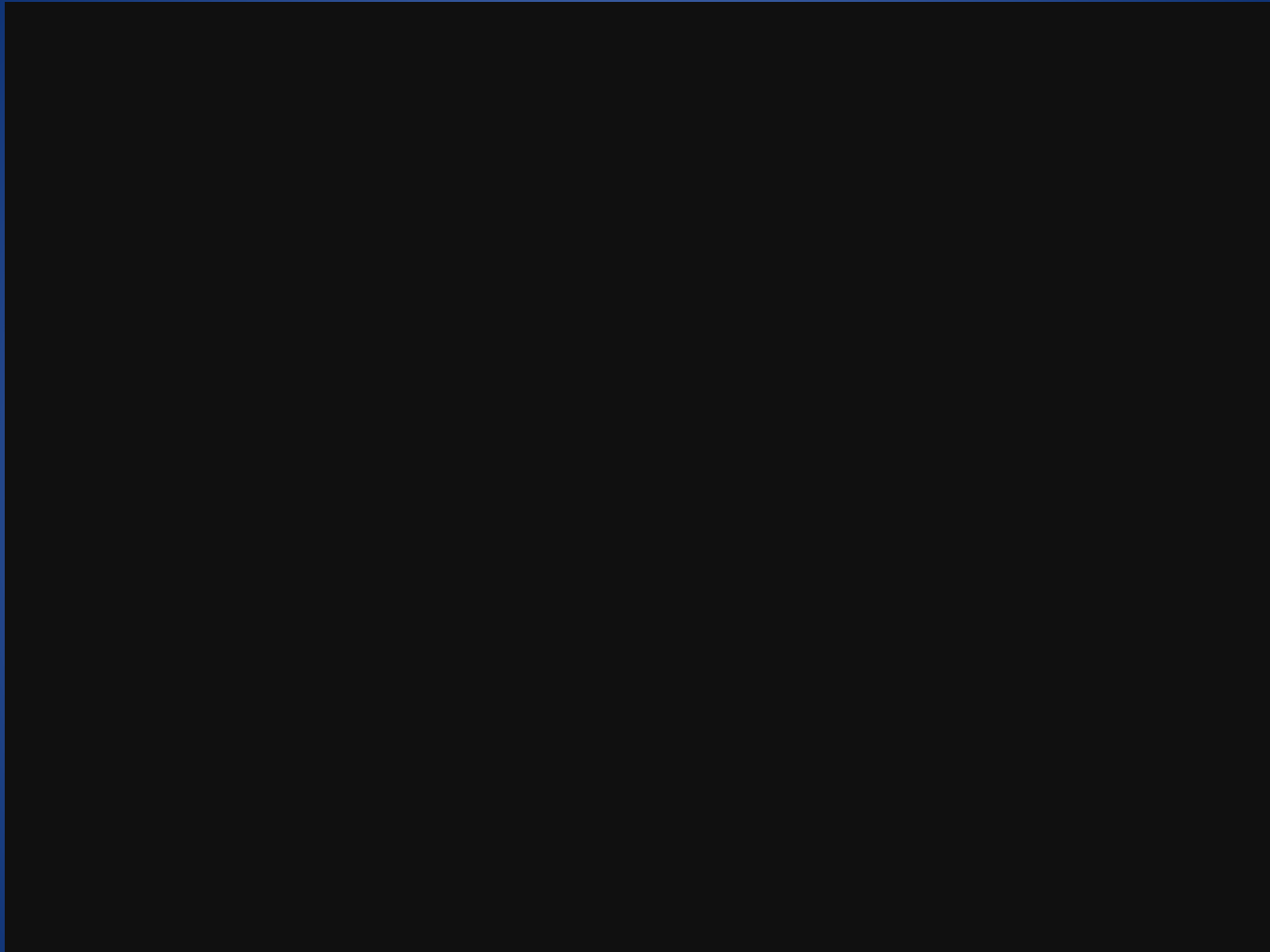
CRYOSTAT SECTIONING BRUSH TECHNIQUE

The purpose of the brush is to grab and maneuver the section across the stage.



You can buy a 1/4 inch, #2 flat, or bright brushes from an art supply store for about \$3 and cut them at an angle. With this angled tip, the brush meets the tissue flat like a broom because the brush is held at an angle.

CRYOSTAT SECTIONING BRUSH TECHNIQUE



Stephen R Peters M.D.

Pathology Innovations, LLC

http://www.pathologyinnovations.com/frozen_section_technique.htm

CRYOSTAT SECTIONING BRUSH TECHNIQUE



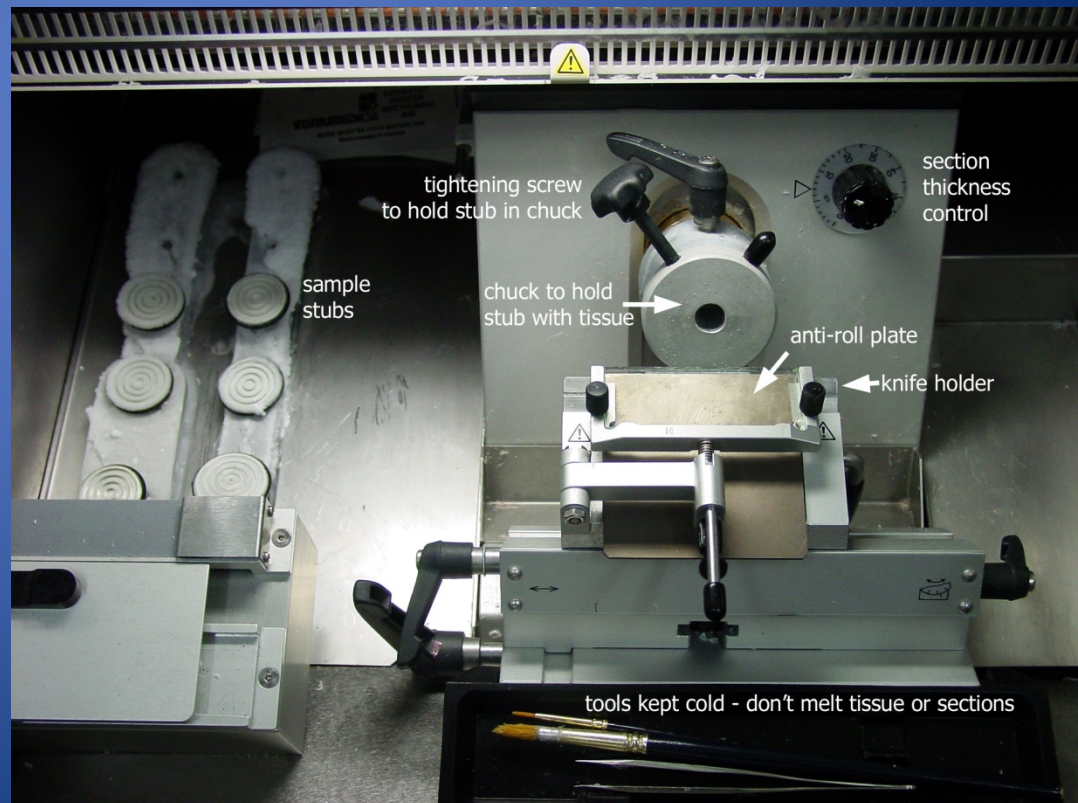
CRYOSTAT SECTIONING ANTI-ROLL PLATE

Used to prevent frozen sections from curling upwards, after sectioning.

The device is made of coated glass or plastic and is aligned parallel to the knife edge, a little above it.

The following points are to be considered:

- a. Correct height to knife edge
- b. Correct angle to knife
- c. Top edge not damaged
- d. At cabinet temperature



CRYOSTAT SECTIONING ANTI-ROLL PLATE



CRYOSTAT SECTIONING ANTI-ROLL PLATE TRAGACANTH SUSPENDED TISSUE



CARE AND HANDLING OF FROZEN SECTION SLIDES

EXCELLENT PAPER:

Evaluation of the Value of Frozen Tissue Section Used as “Gold Standard” for Immunohistochemistry

Shan-Rong Shi, MD, Cheng Liu, Llana Pootrakul, PhD, Laurie Tang, MS, Andrew Young, Ryan
Chen, Richard J. Cote, MD, and Clive R. Taylor, MD, PhD

Am J Clin Pathol 2008;129:358-366

DOI: 10.1309/7CXUYXT23E5AL8KQ

<http://ajcp.ascpjournals.org/content/129/3/358.full.pdf>

EXAMINES:

- *The use of acetone- or ethanol-fixed frozen tissue sections as the “gold standard” for immunohistochemical analysis*
- *Frozen sections fixed by 6 protocols: acetone, ethanol, NBF (2 durations), and NBF + calcium chloride (2 durations). With and without AR.*

Acknowledgements

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