The Eye in Safety Studies
Preparation for Microscopic Assessment

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QUESTIONS BEFORE PROTOCOL

- Where is the study conducted?
  - Are the technicians comfortable with the method for preparing eyes?
  - What SOP/procedure is going to be used?
  - Pathologist should be involved

- What is the purpose of the study?
  - Are special procedures (e.g., IHC, TEM, measurement of the ONL)

QUESTIONS BEFORE NECROPSY

- What type of study? (systemic, topical, intravitreal injection, subTenon injection)
- Ophthalmic exam findings? (want microscopic correlates)

ENUCLEATION

- Be gentle during enucleation
  - prevents retinal detachment & artifacts in optic nerve
- Cut close to orbit to get optic nerve
  - need several mm’s to get cross section of optic nerve
  - Prevents artifacts in optic nerve
- Clean off extraocular tissue
  - includes hardarian gland in rodents
  - prevents retinal detachment
  - see landmarks & easier to trim globe
- Do not incise the globes prior to initial fixation
  - distorts structures
- Keep right and left globes separate
  - needed for clinical correlation
- May need to mark globe for orientation
  - tattoo ink, indelible dye, suture, extra tissue

Retinal detachment caused by leaving the Harderian gland attached
Tattoo ink used to mark superior aspect of albino rat globe
Tattoo ink used to mark white optic nerve of albino rat globe fixed in Davidson's fixative

optic nerve, rabbit Davidson's fixation
Spherical to irregularly shaped, hyaline deposits in the optic nerve with paraffin-embedding and H&E staining
- Mucocytes (Grynfeltt or Buscaino bodies)
- Artifact of myelin associated with handling
- Fix & Garman, 2000
- Inadequate decetration with retention of paraffin
- Thompson & Luna, 1978

**Fixation**
- Begin fixation as soon as possible after enucleation / death (minimizes autolysis)
- Intraocular masses, blood, large eyeballs (e.g., horse) needs longer fixation time (prevents autolysis)
- Fixation volume: 1 part globe: 10 (20) parts fixative
- Use gauze to submerge the eyeball (ensures 360º fixation)
- Systemic perfusion is not necessary (may give inconsistent results and spaces beneath the RPE)
- Keep right (OD) and left (OS) globes separate

**There is no ideal ocular fixative!**

**Ocular fixatives**
- Submersion technique
  - Davidson's solution
  - Bouin's solution
  - Zenker's solution & others
- Injection or window techniques
  - 2-6% glutaraldehyde
  - Post-fixed in 10% NBF
  - Mixed with paraformaldehyde (TEM)
  - 10% neutral buffered formalin (NBF)

**Injection technique**
- Use a small (25-27 ga) needle
- Locate the long posterior ciliary artery
- Insert the needle (with syringe attached) into the vitreous body through the sclera at a location along the plane of the long posterior ciliary artery (non-primate) or 90º superior or inferior to the long posterior ciliary artery (primate)
  - The point of entry should be posterior to the limbus and at the thickest part of the eyeball to avoid hitting the lens
- Holding the eyeball in one hand, slowly inject the fixative into the vitreous body. Stop when the eyeball feels turgid
  - Approximately 0.15 - 0.3 ml of fixative will be injected

For canine globes, inject along the posterior ciliary artery, just posterior to the lens
Window Technique

- Fix in 6% glutaraldehyde for 1-2 hours.
  - This initial fixation hardens the eyeball to allow a window to be made in each eyeball.
- Position the cornea down
- Note orientation of the long posterior ciliary artery
- Using a sharp tissue slicer blade make a parasagittal cut through the sclera to open a small (~5 mm) window at the nasal or temporal limbus (non-primate) or superior or inferior (primate) of each eye
- Place the eyeballs back into 6% glutaraldehyde for remainder of fixation period
**Glutaraldehyde Fixation**
- 6% buffered with 0.17% w/v sodium phosphate monobasic and 0.9% w/v sodium phosphate dibasic has proven to be an acceptable fixative if used with injection or window technique (non-rodents) and submersion for 2 days (Saby et al, 1991)
- Good fixative for cornea, lens, retina, but artifacts may be seen vacuoles in photoreceptors, corneal spaces and lenticular cracks (Saby et al, 1991)
- Less than ideal osmolarity with cause distortion of the globe

**Davidson’s Fixative**
- Composition (Ref: Latendresse et al, 2002)
  - 2% 37-40% formaldehyde
  - 35% ethanol
  - 10% glacial acetic acid
  - 53% distilled water
- Fixation time: 24 hours (small eyes), 48 hours (larger eyes), but do a pilot study
- Often used for rodent globes Not suitable for electron microscopy
- Often used for rodent globes Use to fix the lens for ICH

**DAVIDSON’S SOLUTION**
- Advantages from proper fixation
  - Maintains retinal attachment (exception noted in the rabbit) & good preservation of retina
  - May be used to fix fetal eyes within orbits
- Disadvantages from prolonged fixation
  - Opaque albino globes; can’t see landmarks
  - Clefts in corneal stroma, endothelial cell vacuolation & corneal epithelial pseudoedema
  - Shattering of lens and inconsistent staining
  - Indistinct appearance of rods & cones and retinal detachment
  - May cause excessive swelling of the lens
  - May cause "cataract-like" artifacts
Cornea, rat, prolonged fixation with Davidson's fixative (note the spaces)

Rat globe, Davidson’s fixative

Lens, primate, injected with modified Davidson’s fixative

Note the artifactual spaces and spherules

Fetal rat head, MDF 2 day, hold in NBF, decalcify 2 days, trim

Minimal artifacts

HARDENING

- Freezing (may cause freeze-thaw artifacts)
- Use of a series of alcohols (excess exposure hardens the lens)
- Post-fixation with 10% neutral buffered formalin used to hold and firm globes after initial fixation, especially injected globes or globes with windows
Trimming

- **Tissue slicer blade** or disposable microtome blade vs razor blade
  - Sawing motion = retinal detachment
- Standard cassette vs megacassette
  - Thin trimmed eyeball = more artifacts
  - Forcing ocular sections into standard (narrow) cassettes may cause retinal holes & folds
- **Keeping the vitreous** vs removing the vitreous
  - Removed to prevent retinal detachment (especially in primates), but may miss a diagnosis
- Get cross section of optic nerve
- Handle the globes gently

Using a deep megacassette, just trim off enough tissue so lid will close without smashing the tissue.

- Artifactual retinal folds from forcing trimmed eyeball into standard cassette
- Artifactual retinal tear from using forceps to force a trimmed eyeball into standard cassette

Evaluate routine sagittal section of retrobulbar optic nerve and cross section of retrobulbar optic nerve.

If optic nerve is a target, embed in plastic and evaluate cross section with paraphenylenediamine (PPD) stain.

Trimming: Planes of Section

Superior-inferior, vertical mid-sagittal
- dog, cat - tapetal & nontapetal retina
- pig, rabbit, rat - to be similar
- Optic disc serves as point of reference

Nasal-temporal, horizontal sagittal human & nonhuman primate
- macula & optic disc

Different or additional section to include ophthalmic findings (e.g., intravitreal injection site)

Trimming Non-primate Eyeballs

- Locate the long posterior ciliary artery extending from the optic nerve along a horizontal plane towards the temporal limbus and the medial limbus.
- Position the blade adjacent to the optic nerve and parallel to the long posterior ciliary artery and make one smooth downward and forward cut.
- The blade will meet some resistance at the lens. Put a hand on each side of the blade and push down (cutting through the lens and cornea).
- Pull back on the blade completing the cut.
- Note: The vitreous body stays as a gel that helps keep the lens and retina in place.
- Place the cut surface of the eyeball down in a megacassette.
- Avoid hitting the lens when using the blade to cut off the portion of the eyeball that is above the edge of the megacassette. This creates a window.
- Note: This minimizes artifacts in the lens and retina.

Location of blade and direction of movement

When at the lens, put even pressure on both sides of the globe and push downward

A smooth cut with a long, sharp blade should result in an even cut surface with no artifacts (e.g., retinal detachment)

The second cut should avoid the lens
Trimming Primate Eyeballs

- The primate eyeball needs to be trimmed in order to examine the macula which is located temporal and slightly superior to the optic disc.
  - The nasal long posterior ciliary artery is more prominent.
  - The optic nerve curves in a nasal and superior direction.
  - The superior oblique muscle inserts in a temporal and dorsal position to the optic nerve and is very tendinous in appearance.
  - The inferior oblique muscle inserts in a temporal and ventral position to the optic nerve and is very muscular in appearance.

- Note: Histologically, the retina, in the area of the macula, has a thick ganglion cell layer and a slight indentation (the fovea).
Ideal stained section of posterior segment of a primate eye

Macula with fovea

Window Technique

- After an additional fixation for at least 48 hours, trim the eyeball.
- Position eyeball with cornea down. Cover window in eyeball with a finger or the thumb to "seal" it.
- Using a sharp blade slicer blade, cut perpendicular to the long posterior ciliary artery and adjacent to optic nerve in one continuous smooth motion.
- Cover open part of eyeball with the thumb. Repeat a parallel cut on other side of optic nerve, continuing through eyeball in one smooth motion.
- Since a hole already exists in the eyeball, the eyeball only needs to be trimmed once. Use megacassettes.

Trimming: Rodent Eyeballs

- For globes of rats, make an off center longitudinal cut (prefer tissue slicer blade or disposable microtome blade) through the eyeball removing a calotte 1-2 mm thick, leaving the optic nerve, lens and most of the cornea on the larger section. An ideal cut would be perpendicular to the long posterior ciliary artery
- For globes of mice, do not trim, but may wish to mark eyeballs for orientation purposes.
Registry Nomenclature
Information System, ILSI

Trim as little lens as possible

- Make sure the lens is laying flat against the bottom of the embedding mold
- Second cut during trimming is for escape of air bubbles at embedding
- Positioning the globe at an angle may help reduce artifacts when sectioning
- Low melting point paraffins with plastic polymers may be helpful
Lens not pushed down when embedded, so portion is missing.

Glutaraldehyde fixation

**Blocks, Sectioning and Water Bath**

- Softening of lens may be necessary for sectioning
- Temperature of the water bath is important; avoid too much heat or too much time on the water bath
- Adherents (poly-L-lysine or gelatin) are added to the water bath to adhere sections to slides
- May need to use 2 water baths: one at room temperature to remove wrinkles and another at 2º above the melting point of the paraffin

Lens positioned at angle to get a section with minimal artifacts caused by the knife.

Ideal unstained section of posterior segment of a primate eye

- Macula with fovea
- Optic disc
- Optic nerve

Temperature of the water bath is important.
References

- Lewis, P. College of Veterinary Medicine, University of Florida, (1) 27th Annual Symposium/Convention, National Society of Histotechnology, 9/22-27/01,(2) NSH Teleconference, 3/20/02, (3) 28th Annual Symposium/Convention, NSH, Workshop #18, 9/28-10/3/02

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