LMD
Paraffin / Frozen tissue protocol

1- Making the slides RNase free:

The foil slides can be treated to remove RNases by dipping them into a bath of pure RNase Zap (Ambion Corp) for 15 seconds.
Follow this with two rinses in DEPC water to remove all of the RNase Zap.
The slides then should be completely dried by placing them in a heater at 37°C for as long as is required to dry.
Then one may follow with UV treatment as described below.
Note: Heating in an autoclave may not always ensure that all RNases are destroyed. If this method is performed the heating must be at 180°C for up to 4 hours.

2- Preparing the slide with UV Irradiation:

Prior to placing specimens on the membrane slides, it is advisable to prepare the membrane by placing the blank slides to be used, into a UV Cross-linker device. The slides should be irradiated at 220nm to 260nm at full power for 30 minutes. This will usually destroy RNases, improve the laser cutting of the foil (reducing static), and help make the foil more hydrophilic to improve adherence of the specimen.

If you don't have a UV Cross-linker, please place the slide/ membrane facing up in a hood with UV light for 30 minutes.

3- Coating treatment:

For poorly adhering tissues, the slides may be treated with a microlayer of Poly-L-Lysine, Fibronectin or Laminin.

Paraffin Block Sectioning:

- Clean the paraffin microtome before sectioning to avoid contamination
- Mount the tissue block onto the microtome
- Trim the sample to obtain a plane surface and an approach to the tissue
- Cut the tissue into 5–15 um sections
- Place the paraffin ribbons on the surface of the water in a waterbath at 37°C– 40°C; in this way, the floating ribbons are easier to stretch and subsequently can be placed without folds on the slides. Please note that the water must be pure for PCR experiments or RNase-free, if RNA analysis is intended (Antigen retrieval might be required).
- Mount the sections using a brush on the pretreated membrane slides.
- Incubate the membrane slides at 60°C for 2 hours in a dry oven to further improve tissue adhesion to the membrane.

The paraffin must be removed prior to staining the paraffin-embedded sections. This is achieved by washing the slides with xylene followed by a series of descending concentrations of ethanol as follows:

- Xylene 20 seconds 3x (three separate containers)
- 100% ethanol 30 seconds 2x (two separate containers)
- 95% ethanol 30 seconds 2x (prepared with RNase-free water)

- 70% ethanol 30 seconds 2x (prepared with RNase-free water)
- Store slides at –80°C or go to your 'Staining Dehydration' step

Frozen tissue Sectioning:

- Cryostat must be cleaned prior to use. All surfaces must be wiped down with 95-100% ethanol and RNAzap, especially knife holder and anti-roll plate
- Transport the cryo block on dry ice from -80°C freezer
- Mount the cryo block onto the specimen clamp and wait at least 20 minutes for the block to equilibrate to the cutting temperature (about -20°C).
- Trim the block to remove as much as OCT before cutting and trim the sample to get a plane surface and an approach to the tissue
- Cut the block into 8um-10um sections and immediately place them on RNAse-free UV-C-treated coated membrane slides for LMD (some people go up to 30um).
- Mount sections directly onto room temperature slides. Do not allow slides to come to room temperature prior to initiating staining process. Hold slides in slide box on dry ice or within cryostat
- Store slides at –80°C or go to 'Staining Dehydration' step

Staining and Dehydration

- Frame Slides sections or glass slide sections could be fixed with one of the below suggestion straight from -80°C or the cryostat

1- 100% ice-cold acetone — 2 minutes (or 30 sec for RNAse) (better for non-lipid tissue such as heart, kidney, liver, lung)

2- 70%-75% or 100% Ethanol — 2 minutes (**RNAse-free for 20 seconds to 30sec** [thick sample] better for Brain, spinal cord, intestine, etc)

3- mixture of ethanol : acetic acid (19:1) to increase the adhesion of the tissue to the PPS-, PEN-, PET-, POL- or FLUO-membranes (also 20 sec) *(This latest fixation is also use for FISH techniques but at a much lower concentration (3:1). Keep in mind it also increase autofluorescence in the background if you are planning to do immunofluorescence)*

- Dip rinse with RNAse free water at RT, let dry 3- 5 minutes before cutting with the LMD or proceed immediately with your staining procedure.

Staining or Immunofluorescence

Staining

Quick Toluidine Blue Staining (LMD Protocol Guide p. 23)

From ice-cold 70%-75% ethanol

1-	Distilled H_2O	8 dips
2-	Toluidine blue solution	3 minutes
3-	Distilled H_2O	15 seconds
4-	Distilled H_2O	15 seconds
5-	75% ethanol	3 minutes
6-	Air-dry	5-10 minutes

Light H&E (Hematoxylin and Eosin) – (LMD Protocol Guide p. 24)
From ice-cold Acetone

1-	Distilled H_2O	30 seconds
2-	Hematoxylin	15-30 seconds (up to 1 minute)
3-	Distilled H_2O	30 seconds
4-	Blueing reagent	30 seconds
5-	Eosin solution	10 seconds
6-	70% ethanol	30 seconds
7-	95% ethanol	30 seconds
8-	100% ethanol	30 seconds
9-	Air-dry at room temperature (3, 5 to 10 minutes)	

Result:

Nuclei - blue-black	Fibrin - deep pink
Cytoplasm - varying shades of pink	Red blood cells - orange/red
Muscle fibers - magenta	

Modified Cresyl Violet Staining for RNA-Research (LMD Protocol Guide p. 26)
From ice-cold 75% ethanol

1-	Distilled H_2O	8 dips
2-	Crezyl violet solution	1 minutes
3-	75% ethanol	5 seconds
4-	90% ethanol	5 seconds
5-	100% ethanol	5 seconds
6-	100% ethanol	1 minutes
7-	Air-dry	5-10 minutes

Quick Thionin Staining (LMD Protocol Guide p. 27)
From ice-cold 70%-75% ethanol

1-	Distilled H_2O	8 dips
2-	Thionin solution	20 seconds (up to 1 minute)
3-	Distilled H_2O	15 seconds
4-	Distilled H_2O	15 seconds
5-	75% ethanol	1 minute
6-	95% ethanol	30 seconds,
7-	100% ethanol	30 seconds
8-	Air-dry	5-10 minutes

Congo Red for Amyloid plaque-associated brain tissue

From: Sethi, S., Theis, J., et al. ***Medullary amyloidosis associated with apolipoprotein A-IV deposition****. Kidney International (2012) Vol 81-2 pages 201-6*

(0.2% Congo Re in 0.9% NaCl in 80% ethanol using RNase-free water)

From ice-cold 75% ethanol

1-	Distilled H2O	8 dips
2-	Congo Red	10 minutes
3-	Distilled H2O	8-10 dips
4-	80% ethanol	8-10 dips
5-	Air-dry	5-10 minutes

IHC Antigen retrieval

Most formalin-fixed tissues require an antigen retrieval step before immunohistochemical staining can proceed.

1- Buffer solutions for heat-induced epitope retrieval

The following solutions are three of the more popular buffers for HIER. In the absence of datasheet information or advice from other researchers for a particular antibody, choice of retrieval buffer is best determined by experimentation.

1- **Sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0)**

Tri-sodium citrate (dihydrate) 2.94 g

Distilled water 1000 ml.

Mix to dissolve. Adjust pH to 6.0 with 1N HCl.

Add 0.5 ml of Tween 20 and mix well. Store at room temperature for 3 months or at 4°C for longer storage.

Pre-mixed, concentrated sodium citrate buffers for HIER

2- **1 mM EDTA, adjusted to pH 8.0**

EDTA 0.37 g.

Distilled water 1000 ml.

Adjusted to pH 8.0 with NaOH.

Store at room temperature for 3 months.

3- **Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0)**

Tris 1.21 g.

EDTA 0.37 g.

Distilled water 1000 ml (100 ml to make 10x, 50 ml to make 20x).

Mix dissolve. Adjust pH to 9.0.

Add 0.5ml of Tween 20 and mix well. Store at room temperature for 3 months or at 4°C for longer storage.

Pre-mixed, concentrated Tris-EDTA buffers for HIER

2- Heat-Induced epitope retrieval Methods

1- Pressure Cooker

2- Microwave

- Scientific or domestic microwave (850W)
- Microwaveable vessel with slide rack to hold approximately 400-500 ml or Coplin jar
- Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, sodium citrate pH 6.0, etc)

Methods:

After deparaffinization and rehydration the sections.

1- Add the appropriate antigen retrieval buffer to the microwaveable vessel (See note **i**).

2- Remove the slides from the tap water and place them in the microwaveable vessel. Place the vessel inside the microwave. If using a domestic microwave, set to full power and wait until the solution comes to a boil. Boil for 20 min from this point. If using a scientific microwave, program so that antigens are retrieved for 20 min once the temperature has reached 98°C. (See note **ii**).

3- When 20 min has elapsed, remove the vessel and run cold tap water into it for 10 min. **Use care with hot solution.** (See notes **iii** and **iv**).

4- Continue with the immunohistochemical staining protocol.

Notes

i. Use a sufficient volume of antigen retrieval solution in order to cover the slides by at least a few cm.

ii. Use a non-sealed vessel to allow for evaporation during the boil. Be sure to monitor for evaporation and watch out for boiling over during the procedure and do not allow the slides to dry out!

iii. 20 min is only a suggested antigen retrieval time. Less than 20 min may leave the antigens under-retrieved, leading to weak staining. More than 20 min may leave them over-retrieved, leading to non-specific background staining and also increasing the chances of sections dissociating from the slides. A control experiment is recommended beforehand, where slides of the same tissue section are retrieved for 5, 10, 15, 20, 25 and 30 min before being immunohistochemically stained to evaluate optimum antigen retrieval time for the particular antibody being used.

iv. This allows the slides to cool enough so they may be handled and allows the antigenic site to re-form after being exposed to high temperature

Immunofluorescence

- Rapid Immunofluorescence staining

  After fixation,

  1. Dip rinse in RNAse free 1X PBS at RT – 2x 10 seconds
  2. Block with 10% Normal Goat Serum in 1x RNAse-free TBS or RNAse-free PBS at RT - 5 minutes
     If you select TBS, keep TBS until step 6.
  3. Ist Antibody/ies incubation in 5% NGS in 1xTBSTor PBST (tween-20 used) at RT – 10 to 15 minutes
  4. Dip rinse in RNAse free 1X TBS or RNAse-free PBS at RT – 2x 10 seconds
     The following steps should be done in the dark
  5. 2nd Antibody/ies incubation in 5% NGS in 1x TBST at RT – 10 to 15 minutes
  6. Dip rinse in RNAse free 1X PBS at RT – 2x 10 seconds
  7. Rinse one more time with RNAse free water at RT
  8. Let dry 5 minutes before cutting with the LMD.

- Cells immunofluorescence

  1- Cells grow on # 1.5 coverslip, 18 mm^2 coverslip, #1.5 in 6 wellplate, or 8 mm^2 coverslip, # 1.5 in 24 wellplate, until 50 to 70% cells confluence.
  2- Cell fixation in ice cold methanol, 7 min, at -20 ºC.
  3- Rinse 3x in PBS, 5 min each
  4- Block with 10% Normal Goat Serum (NGS), 0.2% Triton X-100, in PBS, pH 7.4. 15 min, at RT
  5- Incubate in primary antibodies in blocking buffer 1 hr, RT
  6- Rinse 3x in PBS, 5 min each
  7- Block with 10% Normal Goat Serum (NGS), 0.2% Triton X-100, in PBS, pH 7.4. or 15 min.
     The following steps should be done in the dark
  8- Incubate in secondary antibodies, in *blocking buffer* for 1 hr at RT
  9- Rinse 3x in PBS at RT
  10- Rince once in tap water

- NeuroTrace. Fluorescent Nissl Stains

  1- Prepare the cryosections using standard protocols and place on a slide.
  2- Rehydrate the sections for at least 40 minutes in 0.1 M phosphate-buffered saline (PBS), pH 7.2.
  3- Wash the sections for 10 minutes in PBS plus 0.1% Triton® X-100.
     This step permeabilizes the tissue and is required for optimal staining.
  4- Wash the sections two times for 5 minutes each in PBS.

  The following steps should be done in the dark

  5- Dilute the NeuroTrace stain (1:20) in PBS.
  6- Apply approximately 80 µL of the diluted stain to the slide, so that the section is covered, and incubate for 20 minutes.
  7- Remove the stain and wash the sections for 10 min in PBS plus 0.1% Triton X–100.
  8- Wash the sections two times for 5 minutes each in PBS.
  9- Wash the sections for 2 hours at room temperature
  10- Rinse the sections in distilled H_2O 1x 10 seconds