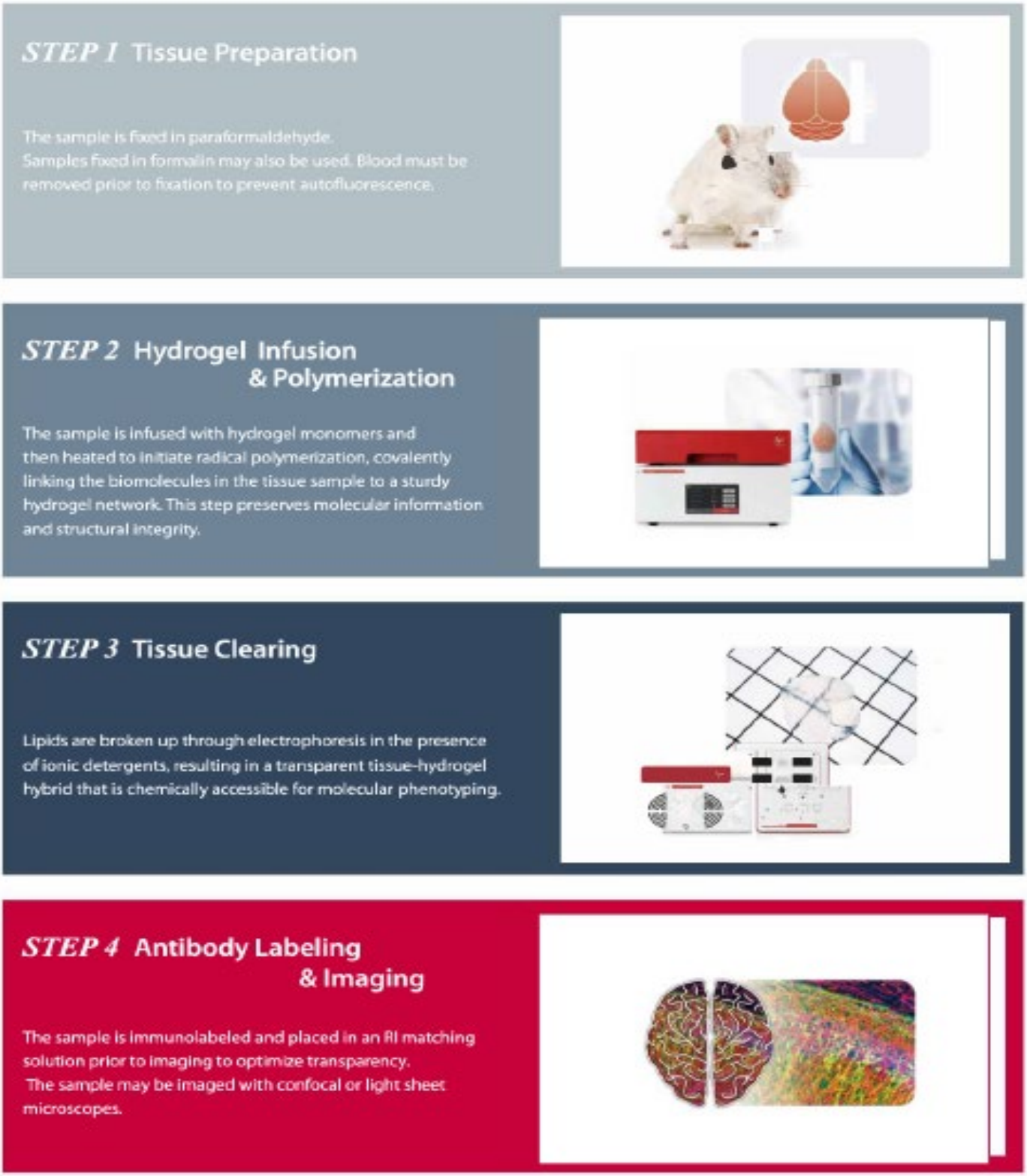
**OVERVIEW OF THE X-CLARITY TISSUE CLEARING PROCESS**



**Researcher Provided Steps**

Step 1

- See sample preparation recommendations below.
- Samples should be provided to the AMCF in PBS.

Step 4 (antibody labeling)

- Post-tissue clearing, samples will be returned to researchers (in PBS) for antibody labeling (if needed).
- See antibody labeling reference materials below.
- The AMCF may assist with antibody labeling. Discuss individual needs with the AMCF Director.

**AMCF Provided Steps**

Step 2

- Hydrogel infusion and polymerization will be performed following Logo's SOPs (tissue specific recommendations)

Step 3

- Tissue clearing will be performed following Logo's SOPs (tissue specific recommendations)
- If antibody labeling is needed, this should be performed by the researcher post-clearing. The AMCF may provide labeling of samples if researchers provide reagents and verify labeling strategy.
- Samples will be returned to researchers (in PBS) if performing their own labeling.

Step 4 (RIM, Imaging)

- The AMCF will provide researchers performing their own labeling with RIM media, if so desired.
- The AMCF will RIM incubate samples prior to imaging (>12h).
- Samples should not be maintained in RIM for long periods of time as this may reduce fluorescence. Samples may be returned to PBS but may appear cloudy afterwards. Cloudiness can be reduced by returning to RIM with incubation at 37° C for 12 – 24h. Cloudiness should not impact additional labeling or imaging (subsequent sectioning and re-imaging).
- Imaging is performed with assistance from AMCF staff. Researchers with high light sheet microscope usage needs may request training for independent use of this instrumentation. Discuss individual needs with the AMCF Director.

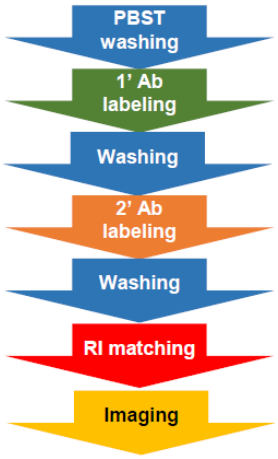
**RECOMMENDATIONS FOR TISSUE FIXATION/PREPARATION FOR TISSUE CLEARING (STEP 1)**

1) Fix samples in 4% PFA.
   - A) *Small/Thin Tissue Samples: Immerse in 4% PFA for 8 to 16 h.*
   - B) *Large Tissues (Organs): Perfusion followed by 4% PFA immersion (24 h).* Perfusion: Initial perfusion with 1X PBS followed by perfusion with 4% PFA.
   - C) *Freshly Frozen* Thaw sample in ice cold 4% PFA and keep immersed for 24 – 36 h.
   - D) *Fixed Samples in PFA or formalin*, e.g. FFPE samples (no need for additional fixation)
2) Wash Samples in 1X PBS several times.

**Notes:**
Perfusion with b-acrylamide not necessary.
Larger samples may be sliced at this point.
Recommended to prepare fresh 4% PFA each time.
After fixation, may store in 1X PBS (4ºC) for up to 3 mths.

X-CLARITY™

**RECOMMENDATIONS FOR TISSUE LABELING POST-TISSUE CLEARING (STEP 4)**




Note:
When post-labeling, best with ≤ 2 – 3 mm thick sections, for brain.
For denser tissues, recommended 1 – 2 mm thick sections.
1° Ab, work with 1:100 to 1:500 dilution from (1 mg $ml^{-1}$ stock).
2° Ab, 1:250 dilution from (1 mg $ml^{-1}$ stock).
Incubate at least ~ 48 h per 1 mm of tissue thickness per Ab. Whole brain may require ≥ 2 weeks. Lower concentrations (1:500) may require 3 – 7 days per mm.

***In a hydrogel-like matrix, the diffusion coefficient of intact lgG is reported to be 1.1 x 1 o·7cm2/s. Antibody incubation time will depend on various factors such as antibody size, concentration, quality, tissue type, and sample thickness.***

***Below is a general guideline.***

1. Incubate in a primary antibody solution (1:100 or higher in antibody dilution solution) at 37°C with gentle shaking.

2. Anti-collagen type IV is recommended as a positive control. Incubation time will depend on various factors and must be optimized.
   NOTE: For a 1 mm mouse brain slice, incubation will take at least two days with most antibodies. For a whole mouse brain, at least four weeks is required.

3. Rinse the sample several times with PBST at RT with gentle shaking. Replace with fresh PBST and wash for 2 hours/mm tissue thickness at RT with gentle shaking. Increase washing time if high background signal is observed.

4. Incubate the sample in a secondary antibody solution (1:100 or higher) for the same amount of time as the primary antibody in the dark at 37°C with gentle shaking.

5. Rinse the sample several times with PBST in the dark at RT with gentle shaking. Replace with fresh PBST and wash for 2 hours/mm tissue thickness in the dark at RT with gentle shaking.

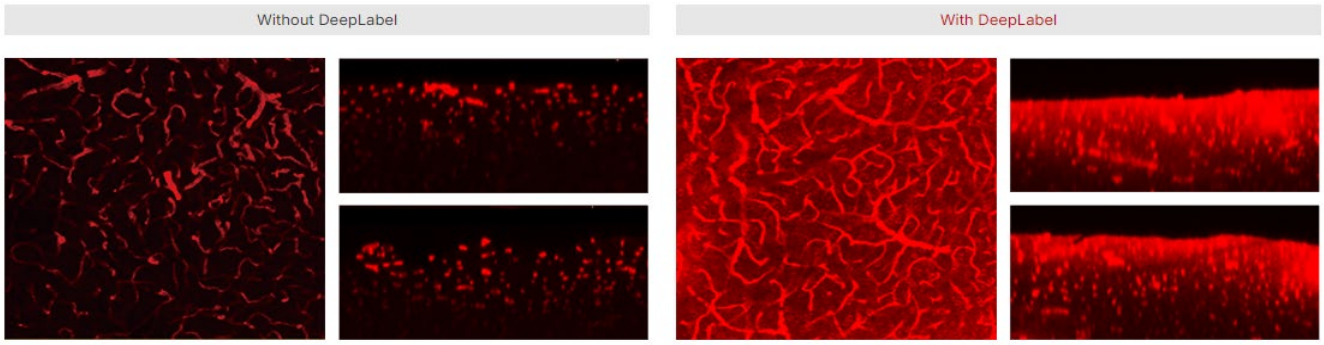
6. Wash the sample three times with distilled water for 5 minutes each in the dark at RT with gentle shaking.

7. Incubate the sample in X-CLARITY™ Mounting Solution (RIM) for 1 hour in the dark at RT with gentle shaking. Replace with fresh X-CLARITY™ Mounting Solution and incubate for an additional 1-2 hours in the dark at RT with gentle shaking.

NOTE: Incubating samples in RI matching media (e.g. X-CLARITY" Mounting Solution) reduces the RI variations within cleared tissue and increases the level of transparency. It also helps with the RI alignment of the tissue samples, objective immersion media, and objectives, which is crucial for high resolution subcellular imaging.

**Additional Notes:**

- Directly conjugated antibodies may reduce non-specific binding but may also reduce fluorescence requirements for sufficient signal amplification for downstream imaging and analysis goals.
- LOGOS/X-Clarity does offer a deep-label antibody staining kit that may facilitate labeling of volumetric samples.




Adult mouse brain tissue stained with anti-Collagen IV (red) and imaged with a confocal microscope.

**Antibodies Verified (LOGOS) to Label X-Clarity Cleared Tissues**

| Antigen | Species | Type/Clone | Antigen localization | Supplier | Cat # | Dilution |
|---|---|---|---|---|---|---|
| Acetylated tubulin | Mouse | Monoclonal | Cytoplasm | Sigma | T7451 | 1:500 |
| β-catenin | Mouse | Monoclonal | Cytoplasm | BD Biosciences | 610153 | 1:500 |
| β-Tubulin III (TUBB3) | Mouse | Monoclonal | Cytoplasm | BioLegend | 801201 | 1:350 |
| β-Tubulin III (TUBB3) | Mouse | Monoclonal | Cytoplasm | Sigma | T8660 | 1:300 |
| β-Tubulin III (TUBB3) | Rabbit | Polyclonal | Cytoplasm | Sigma | T2200 | 1:300 |
| Bcl-2 | Mouse | Monoclonal | Cytoplasm/Mitchondria | Santa Cruz | SC7382 | 1:250 |
| Calbindin D-28k | Mouse | Monoclonal | Cytoplasm | Swant | 300 | 1:500 |
| Calcitonin gene-related peptide (CGRP) | Goat | Polyclonal | Cytoplasm | Abcam | ab36001 | 1:300 |
| Calretinin | Rabbit | Polyclonal | Cytoplasm | Swant | 7697 | 1:500 |
| Caveolin | Rabbit | Polyclonal | Membrane | Abcam | ab18199 | 1:300 |
| CD133 | Rat | Monoclonal | Membrane | Millipore | MAB4310 | 1:500 |
| CD31 | Rat | Monoclonal | Membrane | BD Biosciences | 557355 | 1:500 |
| c-Fos | Rabbit | Polyclonal | Nuclei | Santa Cruz | SC253 | 1:500 |
| Choline acetyltransferase (ChAT) | Goat | Polyclonal | Cytoplasm | Millipore | AB144P | 1:100 |
| Cleaved caspase-3 | Rabbit | Polyclonal | Cytoplasm | Cell Signaling | 9661 | 1:500 |
| 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) | Mouse | Monoclonal | Membrane | Millipore | MAB326 | 1:300 |
| 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) | Mouse | Monoclonal | Membrane | Sigma | C5922 | 1:300 |
| Collagen type IV | Rabbit | Polyclonal | Extracellular matrix | Abcam | AB6586 | 1:300 |
| Collagen type III | Rabbit | Polyclonal | Extracellular matrix | Abcam | AB7778 | 1:300 |
| Ctip2 | Rat | Monoclonal | Nuclei | Abcam | AB18465 | 1:800 |
| Doublecortin (DCX) | Goat | Polyclonal | Cytoplasm | Santa Cruz | SC8066 | 1:500 |
| Epidermal growth factor receptor (EGFR) | Rabbit | Polyclonal | Membrane | Abcam | AB2430 | 1:500 |
| γ-Aminobutyric acid (GABA) | Rabbit | Polyclonal | Secretion | Sigma | A2052 | 1:500 |

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| Glial fibrillary acidic protein (GFAP) | Rabbit | Polyclonal | Cytoplasm | Abcam | AB7260 | 1:800 |
| Glial fibrillary acidic protein (GFAP) | Mouse | Monoclonal | Cytoplasm | Cell Signaling | 3670 | 1:500 |
| Glial fibrillary acidic protein (GFAP) | Rabbit | Polyclonal | Cytoplasm | Dako | Z0334 | 1:500 |
| Glial fibrillary acidic protein (GFAP) | Rat | Monoclonal | Cytoplasm | Thermo Fisher | 130330 | 1:500 |
| Glucose transporter 1 (GLUT1) | Rabbit | Polyclonal | Membrane | Abcam | AB15309 | 1:800 |
| Glutamic acid decarboxylase 67 (GAD67) | Mouse | Monoclonal | Cytoplasm | Millipore | MAB5406 | 1:500 |
| GFP | Chicken | Polyclonal | - | Abcam | AB13970 | 1:500 |
| GFP | Rabbit | Polyclonal | - | Abcam | AB290 | 1:300 |
| Growth associated protein 43 (GAP43) | Rabbit | Polyclonal | Cytoplasm | Abcam | AB16053 | 1:500 |
| Heme oxygenase 1 (HO-1) | Mouse | Monoclonal | Endoplasmic reticulum | Abcam | AB13248 | 1:250 |
| Inositol 1,4,5-trisphosphate 3-kinase A (IP3KA) | Goat | Polyclonal | Cyotoplasm | Santa Cruz | SC11206 | 1:500 |
| Ionized calcium binding adapter molecule 1 (Iba1) | Rabbit | Polyclonal | Cytoplasm | Wako Chemicals | 019-19741 | 1:500 |
| Laminin | Rabbit | Polyclonal | Extracellular matrix | Sigma | L9393 | 1:500 |
| Microtubule-associated protein 2 (MAP2) | Mouse | Monoclonal | Cytoplasm | Millipore | MAB3418 | 1:500 |
| Microtubule-associated protein 2B (MAP2B) | Rabbit | Polyclonal | Cytoplasm | BD Biosciences | 610460 | 1:500 |
| Myelin basic protein (MBP) | Rabbit | Polyclonal | Membrane | Abcam | AB40390 | 1:500 |
| Myelin basic protein (MBP) | Chicken | Polyclonal | Membrane | Aves Labs | MBP | 1:300 |
| Nestin | Mouse | Monoclonal | Cytoplasm | Millipore | MAB353 | 1:500 |
| Neural cell adhesion molecule (NCAM) | Rabbit | Monoclonal | Membrane | Millipore | AB5032 | 1:250 |
| Neurofilament H, non-phosphorylated (NFH) | Mouse | Monoclonal | Cytoplasm | BioLegend | 801701 | 1:500 |
| Neurofilament H (NFH) | Mouse | Monoclonal | Cytoplasm | Cell Signaling | 2836 | 1:300 |
| Neurofilament M (NFM) | Mouse | Monoclonal | Cytoplasm | Santa Cruz | SC51683 | 1:500 |
| Neuronal nuclear antigen (NeuN) | Mouse | Monoclonal | Nuclei | Millipore | MAB377 | 1:500 |
| Neuron-glial antigen 2 (NG2) | Rabbit | Polyclonal | Membrane | Millipore | AB5320 | 1:500 |
| Nitric oxide synthase 1 (NOS1) | Rabbit | Polyclonal | Membrane | Santa Cruz | SC648 | 1:250 |
| Olfactory marker protein (OMP) | Rabbit | Polyclonal | Cyotoplasm | Thermo Fisher | OSP00001W | 1:250 |
| Oligodendrocyte lineage transcription factor 2 (Olig2) | Rabbit | Polyclonal | Nuclei | IBL International | JP-18953 | 1:250 |
| Oligodendrocyte lineage transcription factor 2 (Olig2) | Rabbit | Polyclonal | Nuclei | Millipore | AB9610 | 1:300 |
| O4 | Mouse | Monoclonal | Membrane | Millipore | MAB345 | 1:250 |
| Parvalbumin | Mouse | Monoclonal | Cyotoplasm | Millipore | MAB1572 | 1:500 |
| Platelet-derived growth factor receptor (CD140a) | Rat | Monoclonal | Membrane | BD Biosciences | 558774 | 1:500 |
| Polysialic acid neural cell adhesion molecule (PSA-NCAM) | Mouse | Monoclonal | Membrane | Millipore | MAB5324 | 1:500 |
| Postsynaptic density protein 95 (PSD95) | Rabbit | Polyclonal | Membrane | Thermo Fisher | 51-6900 | 1:500 |
| Proliferating cell nuclear antigen (PCNA) | Mouse | Monoclonal | Nuclei | Santa Cruz | SC56 | 1:250 |
| Regulated in development and DNA damage responses 1 (REDD1) | Rabbit | Polyclonal | Membrane | Proteintech | 10638-1-AP | 1:250 |
| RFP | Rabbit | Polyclonal | - | Abcam | AB62341 | 1:300 |
| S100 | Mouse | Monoclonal | Membrane | Sigma | S2532 | 1:500 |
| Smooth muscle protein 22-alpha (SM22α) | Rabbit | Polyclonal | Membrane | Abcam | AB14106 | 1:300 |
| Special AT-rich sequence-binding protein 2 (SATB2) | Rabbit | Monoclonal | Nuclei | Abcam | AB34735 | 1:500 |

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| Trombosphondin 1 | Goat | Polyclonal | Cytoplasm | R&D Systems | AF3074 | 1:500 |
| Tropomyosin receptor kinase A (TrkA) | Goat | Polyclonal | Membrane | R&D Systems | AF1056 | 1:500 |
| Tyrosine hydroxylase (TH) | Rabbit | Polyclonal | Cytoplasm | Millipore | AB152 | 1:300 |
| Tyrosine hydroxylase (TH) | Mouse | Monoclonal | Cytoplasm | Santa Cruz | SC14007 | 1:250 |
| Vesicular glutamate transporter 1 (VGLUT1) | Mouse | Monoclonal | Membrane | Synaptic Systems | 135311 | 1:500 |
| Vimentin | Goat | Polyclonal | Cytoplasm | Millipore | AB1620 | 1:300 |