3D Tissue Clearing with Passive CLARITY

A handbook for HMRI/UoN researchers

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Abbreviations

3DISCO: *three-dimensional imaging of solvent cleared organs*
BABB: *benzyl alcohol and benzyl benzoate (1:2 ratio)*
CLARITY: *clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue hydrogel*
CUBIC: *clear unobstructed brain imaging cocktails and computational analysis*
COLM: *CLARITY-optimized light-sheet microscopy*
DBE: *dibenzylether*
DCM: *dichloromethane*
EtOH: *ethanol*
GFP: *green fluorescent protein*
iDISCO: *immunolabelling-enabled three dimensional imaging of solvent-cleared organs*
PEA-CLARITY: *plant enzyme assisted clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue hydrogel*
PACT: *passive CLARITY technique*
PARS: *perfusion assisted agent release in situ*
PFA: *paraformaldehyde*
RIMS: *refractive index matching solution*
SDS: *sodium dodecyl sulfate*
SeeDB: *see deep brain*
THF: *tetrahydrofurane*
YFP: *yellow fluorescent protein*

Glossary

Monomer: *A molecule that may bind chemically to other molecules to form a polymer.*

Polymer: *A large molecule (macromolecule) consisting of repeated monomer subunits.*

Refractive index: *the* ***refractive index*** *or* ***index of refraction*** *'n' of an optical medium is a dimensionless number that describes how light, or any other radiation, propagates through that medium.*

Background

Our interest in 3D tissue clearing began after hearing of the CLARITY technique in 2012 during a talk by Prof. Karl Deisseroth. He described the technique in mouse brain (Chung et al., 2013), but coming from a diverse background (Jamie is a medical researcher, while Will and Antony are plant biologists), we wanted to see if CLARITY could work in a diverse range of tissues, including plants. As a result, we developed PEA-CLARITY, which utilises enzymes to overcome penetration issues with the plant cell wall (Palmer et al., 2015). Afterward, our thoughts immediately turned to how this advance could be transferred back into mammalian tissues.

Seeing how well one field could borrow from another inspired us to start a dedicated 3D Tissue Clearing and Light Sheet Microscopy Facility at the Hunter Medical Research Institute, University of Newcastle, Australia. This way, standardised 3D clearing techniques can be applied to a wide range of different tissues and allow information to be easily shared between research groups.

This handbook was first written so we could compile the latest 3D tissue clearing references and rapidly update them so we could keep track of things. Given there has been a remarkable level of local and international collaboration surrounding the development and dissemination of these techniques, it's fitting that any compilation we have prepared is shared with as many groups as possible. We believe 3D tissue clearing protocols have enormous potential to shape how anatomy and histology is performed. So if you find this handbook useful, please offer us suggestions, corrections, criticisms or updates and we'll add them to the next edition.

Happy clearing!

1. Introduction

Achieving optical transparency in intact, three-dimensional (3D) biological tissue has a number of clear benefits for any biologist. Apart from eliminating the need for labour intensive cutting of thin '2D' sections, optical clearing allows the complete 3D structure of biological tissue to remain intact for analysis, providing the following advantages:

1) An immediate understanding of spatial relationships between structures or molecules of interest.
2) Accurate and complete cell counting[1]
3) Global visualisation of branching networks such as neural pathways, vasculature and airways.

Techniques to optically clear 3D tissue aim to homogenise the refractive index (RI) throughout the sample, allowing light to pass through without heterogeneous scatter. Unfortunately, early techniques that use harsh reagents such as ethanol, benzyl alcohol and benzyl benzoate (BABB) severely quench fluorescent probes such as GFP, limiting the ability to molecularly interrogate cleared samples (Hama et al., 2011; Becker et al., 2012). Within the last 5 years however, a range of new clearing techniques and reagents including Sca*l*e (Hama et al., 2011; Hama et al., 2015), ClearT (Kuwajima et al., 2013), 3DISCO/iDISCO (Erturk et al., 2012a; Erturk and Bradke, 2013; Renier et al., 2014), SeeDB (Ke et al., 2013; Ke and Imai, 2014), CUBIC (Susaki et al., 2014; Susaki et al., 2015), 'active' CLARITY (Chung et al., 2013; Kim et al., 2013; Lee et al., 2014; Tomer et al., 2014; Epp et al., 2015; Zheng and Rinaman, 2015) and 'passive' CLARITY (PACT/PARS; (Tomer et al., 2014; Yang et al., 2014; Palmer et al., 2015; Zheng and Rinaman, 2015) have been developed to circumvent this and many other issues (see Table 1 for comparison). The focus of this handbook is the application of 'passive' CLARITY, with useful insights from recent publications and our own experience.

In brief, CLARITY involves the fixation of molecules of interest (proteins and nucleic acids) within a solid polymer network using formaldehyde and hydrogel monomers (acrylamide/bisacrylamide). This is followed by removal of light scattering lipids with a strong ionic detergent (SDS). The tissue is then amenable to immunohistochemistry, chemical staining or *in situ* hybridisation. After molecular labelling, the sample is submerged in a refractive index (RI) matched fluid for imaging (Figure 1).

[1] Due to time and labour constraints, generally only a fraction of thin '2D' sections (e.g. 1 out of every 5) are analysed. 3D tissue clearing makes it feasible to collect and analyse 100% of data contained within each sample.

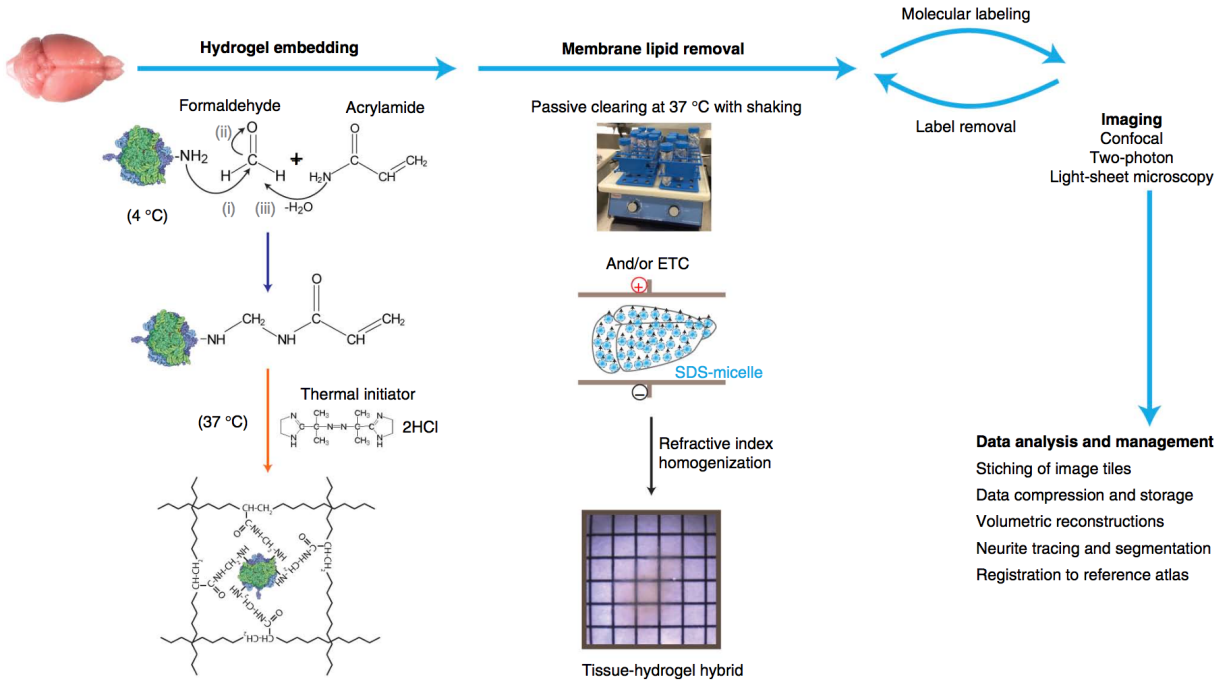

Figure 1. CLARITY pipeline overview. The tissue sample, e.g., an intact mouse brain, is perfused with a hydrogel solution that contains a cocktail of acrylamide, bisacrylamide, formaldehyde and a thermal initiator. Formaldehyde mediates cross-linking of biomolecules to acrylamide/bisacrylamide monomers via amine groups; presumptive chemistry of this process is shown. Hydrogel polymerization is initiated by incubating the perfused tissue at 37 °C, resulting in a meshwork of fibers that preserves biomolecules and structural integrity of the tissue. Lipid membranes are removed by passive clearing at 37 °C (or by electrophoretic tissue clearing). The resulting intact tissue-hydrogel hybrid can undergo multiple rounds of molecular and structural interrogation using immunohistochemistry and light microscopy. Adapted from Tomer et al. (2014).

CLARITY has the following useful attributes:

- Can be applied to any organ or tissue
- Does not quench endogenous fluorescent probes (e.g. GFP)
- Amenable to multiple rounds of immunolabelling or *in situ* hybridisation
- Can be applied to historical/previously fixed samples
- Once cleared, tissue can be kept for long periods of time (several months or more)

'Passive' CLARITY takes longer than 'active' CLARITY (Table 1), however, it does not require a complicated electrophoresis/filtration setup and strikes a balance between effective clearing and simplicity. Thus, it provides a good introduction to 3D tissue clearing techniques.

While the focus of this handbook is the passive CLARITY technique, it is important that researchers looking to employ a 3D tissue clearing protocol carefully consider the range of techniques available alongside the biological question (Table 1). For example, if you want to clear multiple tissue types, maintain endogenous fluorescence, attempt multiple rounds of

immunolabelling/*in situ* hybridisation and maintain good tissue integrity, then CLARITY is a good option. However, if you simply want to look at endogenous fluorescence in a tissue sample and are able to image within a day or two, then 3DISCO is much faster (hours/days compared to weeks). Recent evidence points to iDISCO as an excellent option for immunolabelling studies (Belle et al., 2014; Renier et al., 2014) and reports from our collaborators suggest that CUBIC is also an effective technique (particularly for dense organs rich in haemoglobin or extracellular matrices such as kidney and spleen). For further reading, a recently published review by Richardson and Lichtman provides an excellent description of current clearing techniques (Richardson and Lichtman, 2015).

The following procedure is an edited version of the protocols by Tomer et al. (2014); Yang et al. (2014). Alternative/optional steps are denoted by three asterisks (***) and are appropriately referenced. Updates and corrections to the protocol are welcome. Any changes will be included in the next edition of the handbook.

If you need any specific equipment, reagents or SOPs to get started, contact us using the email addresses found on the title page.

Table 1. Comparison of 3D Tissue Clearing Techniques

Technique and original references	Mechanism of action	Tissues validated	Preservation of endogenous fluorescence	Molecular phenotyping	Tissue integrity	Complexity	Time investment	Comments	References
3DISCO (EtOH/BABB) [1]	Dehydration with EtOH followed by RI homogenisation with BABB	Rodent CNS and embryos. *D. Melanogaster*	No	IHC confirmed. Must be performed before clearing	Tissue shrinkage.	Incubation in multiple solutions over variable periods of time	Several days for whole mouse brain or embryos. 1 day for small tissues.	Rapidly quenches endogenous fluorescence. Not effective in heavily myelinated tissue. Only minimal validation of IHC	[1,3,6,10]
3DISCO (THF/DBE) [2 and 7]	Dehydration with THF followed by RI homogenisation with DBE	Rodent CNS, embryos, kidney, heart, muscle, vasculature	Yes. However signal will quench within 1-2 days	IHC confirmed. Must be performed before clearing	Tissue shrinkage. Becomes solid	Incubation in multiple solutions over variable periods of time	Overnight for whole mouse brain or embryos. A few hours for small tissues	Less background fluorescence and faster/better clearing than EtOH/BABB. Extensive validation of IHC [4,5]	[2,3,4,5,6,7]
Sca*l*eA2/U2 [8]	RI homogenisation via aqueous urea and glycerol mixture. Results in lipid removal.	Mouse brain and embryos	Yes. However signal undergoes some quenching [9]	IHC confirmed in 30 um sections	Significant swelling and fragility. Clearing is reversible	Incubation in a single solution	Weeks to months	Not effective in heavily myelinated tissue	[5,8,9,10,12]
Sca*l*eS [20]	RI homogenisation via aqueous urea, sorbitol and glycerol mixture	Mouse and human brain	Yes	IHC, lipophilic dyes and chemical stains confirmed	Excellent. No significant change in size. Compatible with EM.	Incubation in multiple solutions over variable periods of time	Several days (or several hours using elevated temperature)	Major improvements over Sca*l*eA2/U2. Heavily myelinated regions still cause problems	[20]
Clear$^{T/T2}$ [10]	RI homogenisation using formamide (*Clear^{T}*) or formamide/PEG mixture (*Clear^{T2}*)	Rodent brain and embryos, intestine, muscle	Yes (*Clear^{T2}* only)	IHC confirmed with *Clear^{T2}* to a depth of 120 um	No significant change in size for *Clear^{T}*. Slight swelling in *Clear^{T2}*. Clearing is reversible	Incubation in multiple solutions over variable periods of time	Overnight for whole mouse brain or embryos. Minutes/hours for small tissues sections	Only brain sections up to 1mm thickness tested. Very high 1°Ab conc. used. Compatible with lipophilic dyes	[5,10]
SeeDB [9]	RI homogenisation with aqueous fructose solution	Rodent brain and embryos	Yes	IHC confirmed	No significant change in size. Clearing is reversible	Incubation in multiple solutions over variable periods of time.	Several days	Compatible with lipophilic dyes. Optical transparency is limited compared to other techniques	[5,9,12]
CUBIC [11 and 12]	RI homogenisation via urea and aminoalcohol-based chemical cocktails. Results in lipid removal. Unbinds heme, increasing transparency in tissue.	Mouse brain, lung, kidney, liver, spleen, heart, pancreas, muscle, skin, intestine	Yes	IHC confirmed. Performed during clearing process	No significant change in size	Incubation in multiple solutions over variable periods of time	Approx. 2 weeks for whole mouse brain	Whole-body clearing is possible via transcardial perfusion with CUBIC reagents. Elution of the endogenous chromophore, heme, results in excellent transparency.	[5,11,12, 22]
CLARITY (active) [13]	Formation of tissue-hydrogel hybrid followed by lipid removal via electrophoresis and SDS. RI matching in imaging medium	Mouse brain, spinal cord, spleen, pancreas, intestine, kidney, lung, testis, muscle	Yes	IHC and *in situ* hybridisation confirmed. Performed after clearing process	Some tissue swelling after clearing. Reduction in size when incubated in Focusclear	Polymerisation followed by complex electrophoresis step and mounting in imaging medium	Several days for small tissues. Approx. 2 weeks for whole mouse brain, or 3 days with stochastic electrotransport [21]	Requires complicated and expensive ETC set up. Variable results with standard ETC chamber. Marked improvements described with stochastic electrotransport [21]. Variability reported with IHC.	[5,13,15,16,17, 21]
CLARITY (passive) [14, 15 and 19] **aka PACT/PARS or PEA-CLARITY**	Formation of tissue-hydrogel hybrid followed by lipid removal with SDS. RI matching in imaging medium	Rodent brain, spinal cord, intestine, kidney, lung, liver, pancreas, human brain, zebrafish.	Yes	IHC and *in situ* hybridisation confirmed. Performed after clearing process	Some tissue swelling after clearing. Reduction in size when incubated in Focusclear	Polymerisation followed by clearing in a single solution and mounting in imaging medium.	Several days for small tissues. Several weeks for whole mouse brain.	No ETC chamber required. Simple 'low maintenance' procedure	[14,15,18,19, 23, 24, 25]

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2. Passive CLARITY Method

Step 1: Hydrogel embedding

CLARITY builds on chemical principles to grow hydrogel polymers from inside the tissue in order to provide a support framework for structural and biomolecular content. This is achieved by infusing cold (4°C) hydrogel solution containing acrylamide/bisacrylamide monomers and a thermally triggered initiator into formaldehyde fixed tissue. Formaldehyde serves the dual purpose of cross-linking amine-containing tissue components to each other *and* covalently binding the acrylamide/bisacrylamide monomers to these native biomolecules. These biomolecules include proteins, nucleic acids and other small molecules, but not the vast majority of cellular membrane phospholipids (Figure 2). Tissue can be embedded with hydrogel solution via a transcardial perfusion, or by simply 'drop fixing' (as described in the protocol in this handbook).

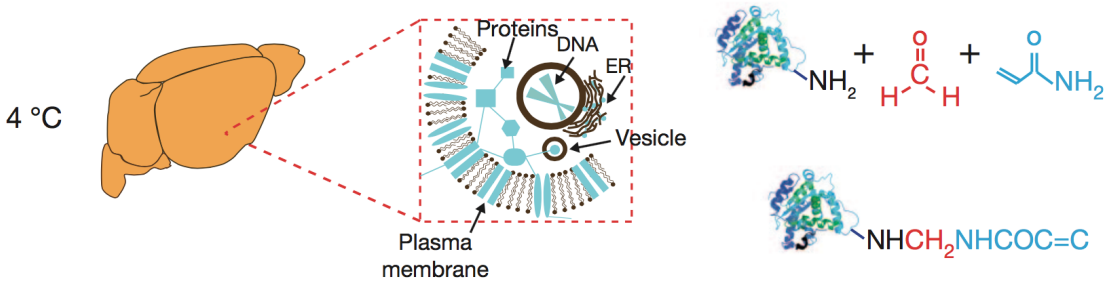

Figure 2. Hydrogel embedding. Biomolecules such as proteins and nucleic acids are cross-linked with formaldehyde (red) in the presence of hydrogel monomers (blue). This covalently links tissue elements to hydrogel monomers. Adapted from Chung et al. (2013).

Step 2: Hydrogel polymerisation

Once the tissue is thoroughly infused with hydrogel solution, polymerization of acrylamide/bisacrylamide monomers (which are now covalently bound to formaldehyde-fixed proteins and nucleic acids) is required to create a cross-linked network within the tissue. This polymer network maintains the 3D structure and position of native biomolecules throughout the clearing procedure (Figure 3).

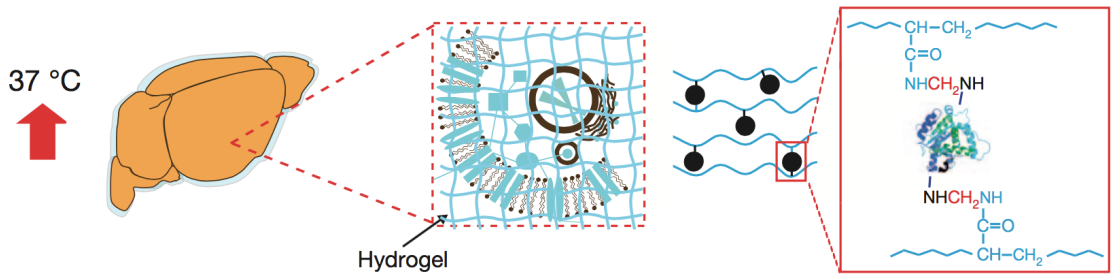

Figure 3. Hydrogel polymerization. Hydrogel monomers become cross-linked after polymerization is thermally initiated at 37°C. This forms a supportive framework throughout the clearing process. Adapted from Chung et al. (2013).

Step 3: Passive clearing (lipid removal)

After hydrogel polymerization, lipids (responsible for preventing access of both photons and molecular labels to deep structures) are removed. A strong ionic detergent-based clearing solution containing SDS is used to remove the vast majority of lipids, while the hydrogel polymers maintain the structure and position of molecules of interest such as proteins and nucleic acids (Figure 4).

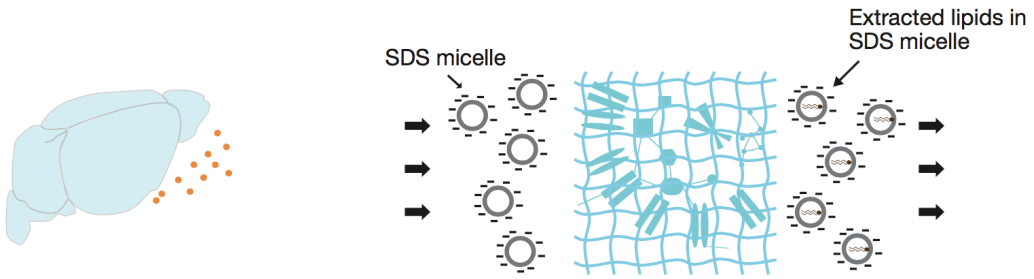

Figure 4. Clearing (lipid removal). SDS micelles diffuse into the tissue and remove lipids, leaving fine-structures and cross-linked biomolecules (proteins and nucleic acids) in place. Adapted from Chung et al. (2013).

Step 4: Molecular labelling (optional)

A tissue sample may have endogenous transgenic expression of fluorophores to label molecular and structural details in the sample (e.g. GFP), but immunohistochemistry or stains (e.g. DAPI) can also be used to label structures and molecules of interest. Importantly, clarified tissue can undergo multiple rounds of immunolabelling (Figure 5).

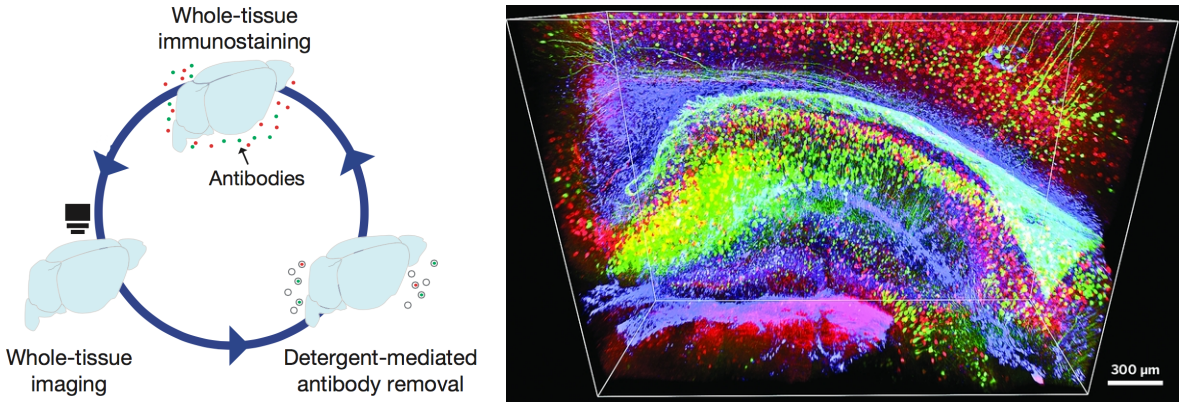

Figure 5. Molecular labeling. *Left*: Multiple round whole-mount immunolabelling procedure in clarified tissue. *Right*: Three-dimensional view of an immunolabelled hippocampus showing eYFP-expressing neurons (green), parvalbumin-positive neurons (red) and GFAP (blue). Adapted from Chung et al. (2013).

Step 5: Refractive index matching

Owing to inherent RI inhomogeneity of tissues, photons (both the excitation light and the emitted fluorescence signal) have inconsistent scatter when traveling through the sample,

limiting the quality of images that can be acquired, as well as the imaging depth that can be achieved. Therefore, a crucial final part of sample preparation is the homogenization of the microscopic environment inside the tissue using a chemical solution that closely matches the average RI of the tissue (Figures 6 and 8).

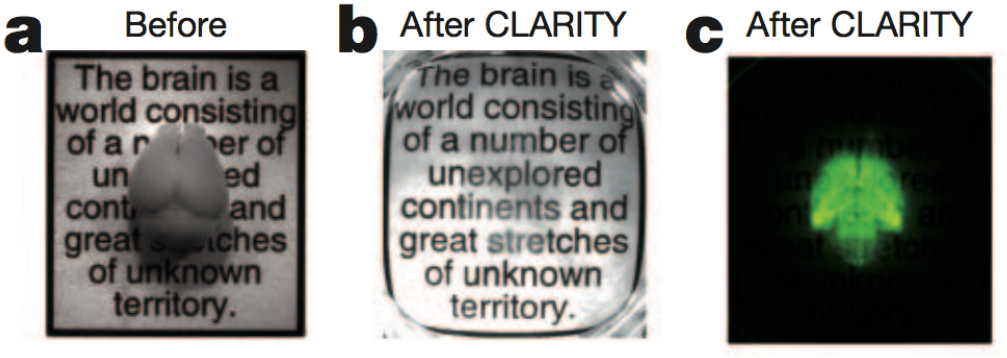

Figure 6. Refractive index matching. (a) Cajal quote under a Thy1–eYFP line-H mouse brain after hydrogel embedding. (b) Cajal quote after clearing and RI matching. (c) Fluorescence image of brain depicted in b. Adapted from Chung et al. (2013).

2.1 Materials

2.1.1 Hydrogel solution

• Acrylamide solution (40% (wt/vol); Bio-Rad, cat. no. 161-0140)
• Bisacrylamide solution (2% (wt/vol); Bio-Rad, cat. no. 161-0142)
• Polymerization thermal initiator VA044 (Novachem cat. no. 017-19362 or Wako, cat. no. VA-044)
• 10× PBS (University stores or Invitrogen/GIBCO cat. no. 70011-044)

2.1.2 Clearing solution

• SDS (Sigma-Aldrich, cat. no. L3371)
• Sodium hydroxide pellets (University stores or Sigma-Aldrich, cat. no. S5881)

2.1.3 Molecular labeling reagents

• 1× PBS with Triton X-100 (PBST; 0.1%, vol/vol; Sigma Aldrich cat. no. X100)
• Primary and secondary antibodies (concentrations ranging from 1:20 to 1:200 dilutions are recommended starting points)
• Tissue stains (e.g. DAPI, propidium iodide, Neurotrace etc; concentrations used for thin sections are generally sufficient)

****** Alternative step ***** *A 0.2M borate buffer with 0.1% Triton-X can be used instead of PBST to reduce background staining (Chung et al., 2013; Tomer et al., 2014).*

2.1.4 Available refractive index matching solutions

• FocusClear (CelExplorer Lab, cat. no. FC102)
• RIMS (88% Histodenz solution as per Yang et al. (2014); Treweek et al. (2015); Sigma Aldrich cat. no. D2158)
• sRIMS (70% sorbitol solution as per Yang et al. (2014); Sigma Aldrich cat. no. S1876)
• 2, 20 thiodiethanol (60% solution, vol/vol as per Zheng and Rinaman (2015); Sigma Aldrich cat. no. 166782)
• Glycerol (80 - 87% solution, vol/vol; University stores or Sigma Aldrich cat. no. G5561)

2.2 Equipment

2.2.1 Solution preparation

- Magnetic stirrer
- pH meter
- Balance
- Beakers
- Weigh boats
- Spatulas
- Transfer pipettes

2.2.2 Hydrogel embedding

- Eppendorf tubes
- Parafilm
- Ice

2.2.3 Hydrogel polymerization

- Temperature controlled waterbath
- Eppendorf tubes
- Parafilm
- Kimwipes

2.2.4 Passive clearing

- Temperature controlled shaker incubator
- 15 or 50 mL conical tubes
- Transfer pipette

2.2.5 Molecular labeling

- Temperature controlled shaker incubator
- Multiwell plate or any small, sealable containers
- Pipettes
- Spatula

2.2.6 Refractive index matching and sample mounting

- Eppendorf or conical tubes (depending on sample size)
- Spatula
- Transfer pipette
- Microscope slides
- Coverslips
- BluTak™
- Vaseline or silicone grease

2.3 Reagent Setup

2.3.1 Hydrogel solution

!CAUTION! Acrylamide, bisacrylamide and VA-044 are toxic. Perform all procedures in a fume hood and wear appropriate PPE including a lab coat, gloves, safety goggles, face mask and closed-toe shoes.

1) Ensure all reagents and mixing vessels/containers are chilled and kept on ice to stop premature polymerization of the hydrogel during preparation.

2) Prepare 200ml of hydrogel solution by mixing 20 ml of 40% (wt/vol) acrylamide (4% (wt/vol) final concentration), 2.5 ml of 2% (wt/vol) bisacrylamide (0.025% (wt/vol) final concentration), 20 ml of 10× PBS, 157.5 ml of distilled water and 0.5 g of VA-044 thermal initiator (0.25% (wt/vol) final concentration). Use serological pipettes to transfer solutions efficiently.

****** Alternative step ****** *The % of acrylamide, % or presence of bisacrylamide and % or presence paraformaldehyde can be varied increase the pore size of the polymerized hydrogel for faster clearing and immunohistochemistry (**see Table 2 in Appendix**). Our recipe is similar to A4P0 used by Yang et al., (2014) but with added bis-acrylamide. Note that tissue samples must be initially fixed with 4% paraformaldehyde if it is not included in the hydrogel solution (Yang et al., 2014).*

3) Divide hydrogel solution into 10-15 mL aliquots in 15 ml conical tubes and store them at 4°C if using within several days, or -20 °C for storage over several weeks.

4) Label containers with contents, maker, date, and safety warnings.

2.3.2 Clearing solution

!CAUTION! SDS is a toxic irritant. Perform all procedures in a fume hood and wear appropriate PPE including a lab coat, gloves, safety goggles, face mask and closed-toe shoes.

1) Use a large beaker and magnetic stirrer to prepare 5 L of clearing solution. Mix 400g of SDS (8% wt/vol) into 3 L dH_20. Make up to 5L with dH_20 and pH to 8.5 with NaOH solution (1-5M).

2) Use a large funnel to pour clearing solution into 1 or 2 L reagent bottles. Label containers with contents, maker, date, and safety warnings.

2.4 Procedure

2.4.1 Hydrogel embedding (via passive diffusion)

The following steps describe how to embed the hydrogel via passive diffusion. **Please note that tissue must be fixed with 4% paraformaldehyde or 10% formalin (as per your standard fixation protocol) prior to hydrogel embedding**. If preferred, the hydrogel solution can be infused via transcardial perfusion (see Chung et al. 2013). Transcardial perfusion of the hydrogel is recommended for large structures (e.g. an entire mouse brain).

!CAUTION! The hydrogel solution is toxic. Perform all procedures in a fume hood and wear appropriate PPE including a lab coat, gloves, safety goggles, face mask and closed-toe shoes.

!CAUTION! DO NOT PUT HYDROGEL SOLUTION DOWN THE SINK. The hydrogel will polymerise at room temperature and block drains. Collect paraformaldehyde and hydrogel in a designated waste container for proper disposal.

1) Place all solutions, containers, and fixed tissue on ice. This is to ensure the hydrogel does not polymerise due to high temperatures.

2) If hydrogel solution is frozen, thaw frozen vial on ice or in a refrigerator. *Gently* mix the thawed monomer solution by inverting. Make sure that there is no precipitation floating in the hydrogel solution; this is an indicator of spontaneous polymerization of monomers in the stored hydrogel solution.

3) Place fixed tissue(s) into the hydrogel solution. Ensure there is enough volume of hydrogel solution so that it does not become appreciably diluted. For small tissues (e.g. a mouse kidney), an Eppendorf tube is suitable.

4) Incubate the sample in hydrogel solution at 4°C for a minimum of 1 d. Protect the sample from light if fluorophores are present.

****** Alternative step ***** *To properly embed larger samples for 3D clearing (e.g. an entire mouse brain) the hydrogel solution should be transcardially perfused (see Chung et al., 2013)*

2.4.2 Hydrogel polymerization

!CAUTION! The hydrogel solution is toxic. Perform all procedures in a fume hood and wear appropriate PPE including a lab coat, gloves, safety goggles, face mask and closed-toe shoes. Dispose hydrogel in a designated waste container for proper disposal.

1) After incubation in hydrogel solution at 4°C (1-2 days), place sample into a small container (e.g. an Eppendorf tube) and completely fill it with hydrogel solution. There must be no air bubbles present in the container because oxygen will inhibit hydrogel polymerisation. Seal lid with parafilm to ensure no air can enter.

****** Alternative step 1 ***** *A 4- to 5-mm-thick layer of mineral oil can be gently laid over the top of the hydrogel solution before/during the previous 4°C incubation step. This will prevent oxygen contact with the hydrogel solution. With this method, the sample container can be taken directly from the fridge and transferred to the water bath in the next step (Zheng and Rinaman, 2015).*

Alternative step 2 ** *Originally, a 'de-gassing' procedure which involves the vacuum removal of air from the sample container and purging with nitrogen gas was used. This adds a significant layer of complexity to the process, but may lead to enhanced polymerization within the tissue (Chung et al., 2013; Tomer et al., 2014; Epp et al., 2015).*

2) Transfer the closed, air-tight container to a 37 °C room or to a water bath or incubator shaker for 3 h. Make sure that the hydrogel has fully solidified before proceeding to the next step. Note that lower acrylamide or bisacrylamide concentrations (see section 2.3.1) may not solidify fully upon polymerization. In these cases, the alternative 'de-gassing' step (see above) may be crucial to ensure absolutely no oxygen is present in for polymerization.

3) Carefully extract the sample from the solidified hydrogel by softly rubbing all the hydrogel from the surface. Kimwipes can be used to remove the residual gel from the tissue surface.

4) Thoroughly wash the tissue with at least 2 changes of clearing solution over 24 h at *room temperature* to dialyze the remaining paraformaldehyde, initiator and monomer. This is important to limit excessive fixation and polymerization within the tissue.

2.4.3 Passive clearing (lipid removal)

!CAUTION! The clearing solution is toxic (contains SDS and boric acid). Perform all procedures in a fume hood and wear appropriate PPE including a lab coat, gloves, safety goggles, face mask and closed-toe shoes. Dispose clearing solution in a designated waste container for proper disposal.

1) Place the sample in a 50 mL conical tube and fill with clearing solution.

2) Gently shake the sample in a shaker incubator at 37 °C. **Replace the solution every 2 to 3 days to maximize the diffusion gradient for lipids out of the sample**.

3) Continue until tissue is transparent (easy visualization of high-contrast signals such as a black-and-white grid or printed text through the tissue; Figure 7B) and clearing is homogenous (even distribution of transparency across the tissue). A typical sample such as a 1-2 mm brain section takes several days to a week. Each tissue type is different and will require consideration of clearing time and possible variations of the hydrogel and clearing solution (see 'Alternative step 1' below for references).

Note 1 ** *Sample will not become completely 'see through' in clearing solution. This is because the refractive index of the clearing solution is not the same as the tissue. Do not incubate sample in clearing solution for more than 4 months as tissue antigenicity becomes degraded after this time.*

4) After clearing, wash the sample for at least 48 hours in PBST (1X PBS with 0.1% Triton X). Change PBST 2 times daily. It is important to make sure all the SDS is washed out of the tissue, or it will precipitate after it goes into the imaging medium and form an opaque mass.

Note 2** *Sample will become less 'see through' when placed in PBST (Figure 7C). This is because the refractive index of PBST is quite different from the cleared tissue. It will only become completely 'see through' when incubated in a refractive*

index matched imaging solution.

****** Note 3 ****** *Always ensure the pH of the clearing solution is consistent and that no microbial growth occurs within the stock clearing solution or sample. 0.01% sodium azide can be added to inhibit microbial growth.*

****** Alternative step 1 ****** *The clearing process can be sped up by using lower concentrations of acrylamide/bisacrylamide in the hydrogel solution (see Table 2 in Appendix) or increasing the SDS concentration in the clearing solution (Yang et al., 2014).*

****** Alternative step 2 ****** *Higher temperatures, up to 60°C, can be used for faster clearing, however this may quench endogenous fluorescence. In our experience, yellowing of the sample can also occur at higher temperatures. This is a result of the Maillard reaction and can be inhibited by the addition of 0.5% α-thioglycerol to the clearing solution (Ke and Imai, 2014).*

****** Alternative step 3 ****** *'Stochastic electrotransport' (a modified version of ETC originally described for 'active' CLARITY) can be used to rapidly and consistently clear thick tissues without damage. However, this requires specialised equipment (Kim et al., 2015).*

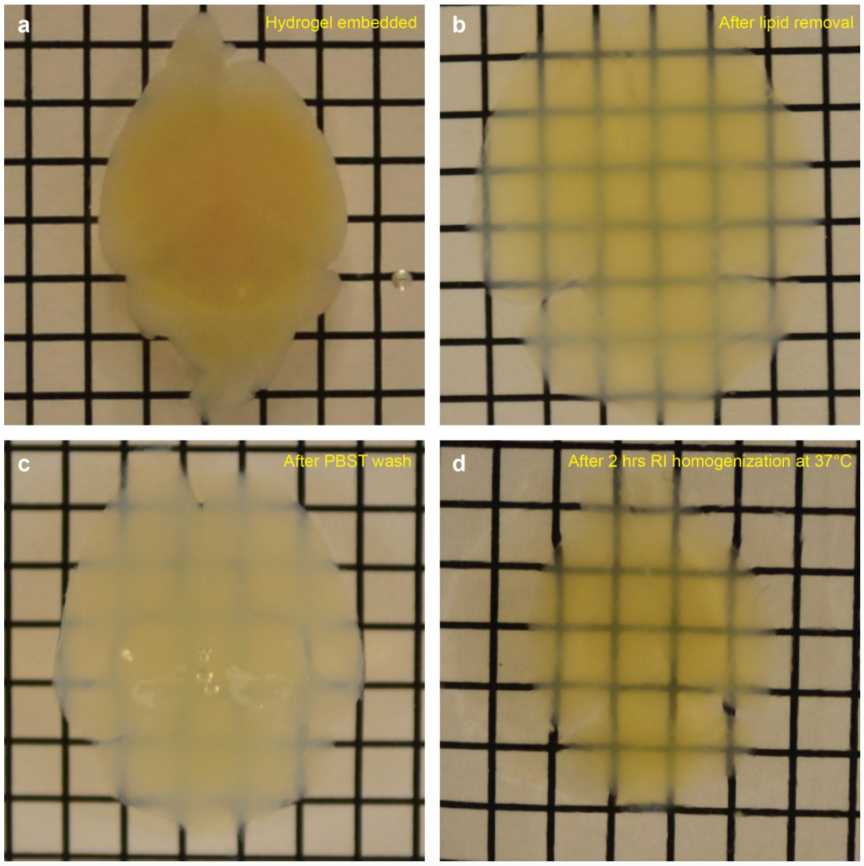

Figure 7. Sample transparency at key stages of the CLARITY protocol. (a) hydrogel embedded whole mouse brain, (b) after clearing/lipid removal, (c) after washing of the residual clearing solution with PBST, and (d) after 2 hours of RI homogenization with FocusClear. Adapted from (Yang et al., 2014).

2.4.4 Molecular labeling

Follow this section for immunostaining a tissue block or whole organ. If you do not wish to perform immunohistochemistry or tissue staining, proceed directly to section 2.4.5.

1) Using a sealable container, incubate sample in 1-2 mL of 1° antibody/PBST solution (beginning with a 1:20 or 1:50 dilution) for 2 days at 37°C for small tissue blocks. For whole organs such as an intact brain, incubate in 5 mL 1° antibody/PBST solution for up to a week. For whole organs, the larger volume of antibody solution should be supplemented periodically over the long incubation times. As with any staining procedure, it is important to systematically optimize staining conditions (detergent, temperature, concentrations and so on) for the particular antibody used.

****** Note 1 ***** *High antibody concentrations (1:20–1:100) are usually required for effective immunostaining to ensure deep penetration into tissue and to overcome the large aggregate number of antibody binding sites over the volume. 1:1,000 dilutions typically lead to inadequate tissue penetration.*

****** Note 2 ***** *A major factor for successful immunostaining is complete removal of lipids during the clearing step.*

****** Alternative step 1 ***** *Immunolabelling can be sped up via application of an intermittent vacuum to assist penetration of antibodies into the tissue sample (Palmer et al., 2015).*

****** Alternative step 2 ***** *'Stochastic electrotransport' can be used to rapidly and consistently label thick tissues with antibodies or dyes. However, this requires specialised equipment (Kim et al., 2015). A more simple method is described by (Li et al., 2015)*

****** Alternative step 3 ***** *0.2 M sodium borate buffer with 0.1% (vol/vol) Triton X-100 (pH 8.5), can be used in place of PBST for antibody incubations to reduce background staining (Chung et al., 2013; Tomer et al., 2014).*

2) Wash off the 1° antibodies with PBST solution at 37 °C for 1 d for tissue blocks and for 2–3 d for whole organs (refresh PBST every 4–6 h).

3) Incubate with the desired 2° antibody (1:50–1:100) in PBST for 2 d at 37 °C for tissue blocks, or for up to 1 week for whole organs. **Nuclear labeling dyes, such as DAPI (1 µg/ml), can also be added at this step.**

****** Note 1 ***** *Smaller secondary antibody formats such fragment antigen-binding (Fab): 55 kDa, Fab dimer (F(ab')2): 110 kDa, or camelid nanobodies: 15 kDa are suggested for faster diffusion throughout the hydrogel matrix (Treweek et al., 2015).*

4) Wash off the secondary antibodies with PBST at 37 °C for 1 d for tissue blocks and 2–3 d for whole organs.

****** Note 2 ***** *It is possible to elute (remove) prior labels by incubating in clearing solution at 37-60 °C overnight in preparation for* ***another round*** *of labeling after imaging has been performed. Once labels have been removed, repeat the immunolabelling procedure with the alternative antibodies.*

****** Note 3 ***** *Make sure that there is no microbial growth during long incubations at 37°C. 0.01% (wt/vol) sodium azide can be added to the primary and secondary*

antibody solutions to prevent this.

****** Note 4 ***** *To make immunolabelling more cost-effective, recycle 1° antibody solutions by adding 0.01% sodium azide and storing at 4°C.*

2.4.5 Refractive index matching and sample mounting for imaging

FocusClear, glycerol (80 - 87% vol/vol), RIMS (88% Histodenz wt/vol in 0.02M phosphate buffer), sRIMS (70% sorbitol wt/vol in 0.02M phosphate buffer) and 2,20 thiodiethanol (60% vol/vol) have been tested in several studies (Chung et al., 2013; Tomer et al., 2014; Yang et al., 2014; Epp et al., 2015; Liu et al., 2015; Zheng and Rinaman, 2015). We have achieved good results with Focusclear and RIMS, but are yet to test sRIMS or 2,20 thiodiethanol.

1) Transfer the PBST-washed tissue into RI matching medium (e.g. FocusClear). Periodically check the visual clarity of the sample over the next few hours. 1 or 2 mm thick tissue blocks will typically complete the RI homogenization process in a few hours, but can be left overnight. Entire organs should be left overnight.

****** Alternative step ***** *Treatment at 37°C accelerates this process several-fold.*

2) Place the sample in *fresh/unused* RI matching medium several hours before imaging. This is because PBST remaining in the tissue sample dilutes the RI medium during the first incubation, changing the RI.

3) Once the tissue is completely 'see-through' (Figure 8), take a clean glass slide and place it on a dust-free surface.

4) Take a small piece of BluTak™ and prepare a constant-diameter worm-shape using gloved hands. Make it around the same thickness as the sample.

5) Make a circular 'tissue well' in the middle of the slide using the BluTak™.

6) Using a small spatula, apply Vaseline or silicone grease around the base of the BluTak™ tissue well to ensure it is fluid-tight and will not leak.

7) Carefully take the sample with a spatula and place it within the BluTak™ tissue well.

8) Use a plastic transfer pipette to completely fill the tissue well with RI matching medium. Fill so that the fluid surface is convex over the BluTak™ walls.

9) *Carefully* place a glass coverslip over the tissue, starting at one side of the tissue well and gently laying it across to the other side. Ensure that there are no bubbles between the sample and coverslip (Figure 8).

10) The sample is now ready for imaging using confocal microscopy.

****** Note ***** *Before RI homogenization (or after), the tissue may be stored indefinitely in PBST (with 0.01% (wt/vol) sodium azide) at 4°C or room temperature. Extended incubation of tissue in FocusClear is not advised, as this can result in the formation of a white opaque precipitate that will hinder deep imaging.*

****** Alternative step ***** *Light sheet microscopy is a much faster alternative for imaging cleared 3D tissue (Richardson and Lichtman, 2015).*

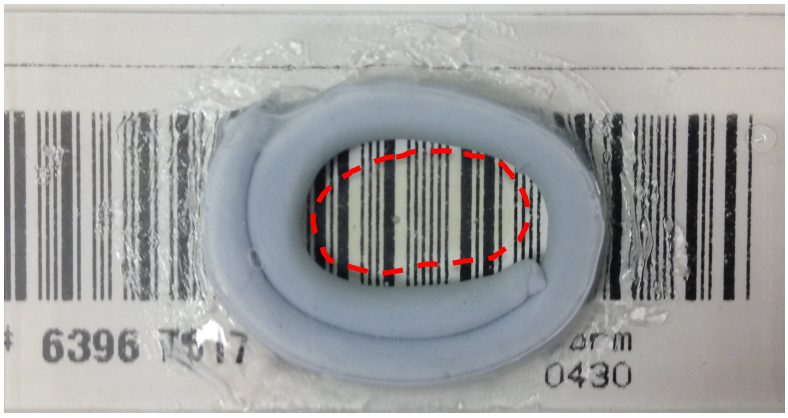

Figure 8. Passively cleared, RI homogenised, 1 mm thick coronal section of mouse brain (bordered by dashed, red line). Mounted with a standard microscope slide, BluTak™, Vaseline, FocusClear and a coverslip. Fine lines of barcode are clearly visible and undistorted.

1.5 Troubleshooting

Step	Problem	Possible Reason	Possible Solution
Hydrogel embedding	Transcardial perfusion is not possible (e.g. human tissue samples)		Passively infuse the hydrogel monomers into post-fixed tissue by incubating the sample in hydrogel solution at 4°C for overnight or several days (depending on the tissue size) with gentle shaking. 3–4 d of passive infusion is sufficient for an entire pre-fixed mouse brain.
	The hydrogel polymerizes prematurely	Hydrogel can polymerize at room temperature	Keep the tubing and needles on ice during perfusion to prevent clogging. It is also crucial to use ice-cold PBS to chill the tissues before perfusing them with the hydrogel monomer solution
	Poor hydrogel polymerization after 37 °C incubation	Bad reagents	Use fresh PFA for fixation; try preparing hydrogel solution immediately before use and store the thermoinitiator, acrylamide and bis-acrylamide stock solutions at 4 °C
	Fragile, easily damaged tissue	Inadequate infusion of hydrogel solution throughout tissue	Leave samples in hydrogel solution longer when passively infusing at 4°C
		Low levels of tissue fixation	Add/increase PFA content in hydrogel formulation for subsequent sample preparations. Extend the PFA postfixation step time
		Insufficient density of tissue cross-linking	Add/increase the concentration of PFA (1–4%) and/or include bisacrylamide (0.05%) in the hydrogel formulation
		Excessive oxygen present in container during polymerization step	Ensure no air bubbles are present in the container or around the sample during polymerization step
Passive clearing	Tissue turns yellow	Maillard reaction (reaction between amino acids and sugars that causes discoloration)	Reduce incubation temperature or add 0.5% α-thioglycerol to the clearing solution (Ke et al., 2013).
		Use of PFA-containing hydrogel solutions	Reduce or remove PFA from the hydrogel solution. If PFA is used in hydrogel solution, ensure it is freshly made. Slight tissue yellowing will not negatively affect imaging results—tissue generally becomes clear upon RIMS mounting. However, very occasionally, some samples become very yellow during the first half of the clearing process: these samples should be cleared for a longer length of time—until they are very transparent—or the yellowing will cause high background during imaging.
	Clearing rate appears to slow down before the tissue is clear	Clearing may slow down as the clearing buffer acidifies	Regularly exchange the clearing solution and ensure pH does not become acidic.
		Dense cross-linking	If PFA was used in the hydrogel solution, remove it in subsequent

			experiments; reduce the PFA postfixation incubation time by half
	Tissue does not become transparent	Blood was not completely removed during transcardial perfusion	Ensure blood is completely removed from vasculature during transcardial perfusion by checking perfused PBS is completely clear prior to injecting hydrogel solution or 4% paraformaldehyde.
		Acrylamide/bisacrylamide polymer network is too dense to allow efficient lipid removal	Lower acrylamide/bisacrylamide or paraformaldehyde content of hydrogel solution to limit polymer formation and cross-linking. See Table 2 and the following references for suggested concentrations: Tomer et al. (2014); Yang et al. (2014); Epp et al. (2015); Zheng and Rinaman (2015).
		SDS concentration of clearing solution is not high enough	Increase SDS concentration of clearing solution (e.g. 8%; Yang et al. (2014)).
		Dense, light scattering structures such as collagen or other connective tissues are present	Perform specific enzyme degradation step after clearing (Palmer et al., 2015).
	Tissue appears to degrade	Bacterial contamination	Regularly exchange the clearing solution (at least every 2 days); add 0.01–0.05% (wt/vol) sodium azide to clearing solution.
		Poor hybridization of tissue to hydrogel monomers	In subsequent experiments, prepare the hydrogel solution with fresh reagents, increase the PFA content by 1%, extend the tissue incubation in HM by 12–24 h and/or before polymerizing the tissue-hydrogel
	Hydrogel softening during clearing	Over-clearing and/or initial poor hydrogel polymerization	Consider doubling the time for the postfixation step or including PFA in the hydrogel solution formulation in subsequent experiments; consider underclearing tissue, as RIMS incubation will cause translucent tissues to become transparent for imaging
	Tissue becomes turbid; white precipitate appears in the tissue	Incomplete washing after clearing, causing SDS and/or salts to precipitate in tissue when it is moved from 37 °C to RT	Double the time for of all wash steps, making sure to perform several exchanges of 1× PBS each day; wash with PBST or BBT instead of 1× PBS
		Tissue becomes white and nearly opaque upon transfer to 4 °C	Salts and, in particular, residual SDS will precipitate in tissue if it is moved to 4 °C. However, the precipitate should disappear upon gradual warming of tissue to RT or 37 °C. Consider performing more extensive wash steps in future experiments, particularly after SDS clearing
Molecular labeling	Low staining reagent or label penetration	Insufficient lipid clearing	Return sample to clearing solution at 37°C for further lipid removal
		Poor antibody penetration	Up to 1% (vol/vol) Triton X-100 (more typically 0.2–0.5%) in PBS can be employed to facilitate further antibody penetration; increase the antibody concentration in the primary antibody cocktail or replenish the antibody halfway through extended incubations, by either adding additional antibody directly to the original antibody

		cocktail or by preparing a fresh antibody dilution; employ stochastic electrotransport as described in (Kim et al., 2015); use an intermittent vacuum procedure during staining as described in (Palmer et al., 2015)
	Insufficient incubating solution	Use large volumes of incubating solutions (e.g. 5 mL) for big tissue samples. Frequent refreshment of the buffer and antibody can lead to further improvement
	Incomplete delipidation, which obstructs labeling	Increase the clearing time
	Epitope loss or epitope masking (unlikely if adhering to protocol)	If tissue was damaged because of microbial contamination, consider adding 0.01–0.05% (wt/vol) sodium azide to all buffers and solutions that are used in long incubations; overfixation may lead to antigen masking, so postfixation steps should be decreased
	In FISH experiments, degradation of nucleic acid transcripts, or diffusion of transcripts out of sample during clearing	Ensure that all hydrogel, clearing and labeling reagents are RNase-free; embed samples in a hydrogel solution that contains PFA and/or bis-acrylamide.
	Poor quality of antibody or dye, which results in weak labeling	Only use high-quality antibodies that have been first veri- fied in standard thin-section immunolabeling; experiment with a different antibody supplier—different antibodies against the same target may vary greatly in their labeling abilities, such as in their binding affinity and in their capacity to access intracellular compartments for cell-fill- ing labeling versus only superficial or extracellular epitope binding. Finally, it can be helpful to simultaneously prepare a thin section (40–100 ⌈m) alongside a thick, cleared sec- tion while troubleshooting to ensure that the visualization of a strong signal is possible
	Dense, light scattering structures such as collagen or other connective tissues are present	Perform specific enzyme degradation step after clearing (Palmer et al., 2015).
Heavy background or weak signal	Long incubation in FocusClear can lead to fine precipitation (invisible to the naked eye) that generates heavy autofluorescence	FocusClear treatment should be kept to the minimum needed. Residual SDS must be thoroughly washed from the samples in PBST before FocusClear incubation; Try using RIMS (Yang et al., 2014; Treweek et al., 2015)
	Photobleaching	To prevent photobleaching, minimize any unnecessary light exposure to the sample before and during imaging (protect with foil at all times). Keep laser power to a minimum while setting up the imaging parameters for the tissue
	Tissue damage during processing	Review procedures carefully, and ensure that no reagents introduced bacterial contamination of sample; lengthen the wash steps to remove potential precipitate (SDS, donkey serum–antibody immunocomplexes)

		Sources of autofluorescence— part 1: fixative-induced autofluorescence, elastin, collagen	Many standard histological techniques for reducing autofluo- rescence, such as tissue bleaching, performing wash steps in PBST containing 100 mM glycine to quench aldehydes and treating tissue with histology stains that quench or mask autofluorescence, may be adapted to thick-sectioned cleared tissues—typically by performing longer wash steps after the appropriate countermeasure; photobleaching the tissue before IHC at wavelengths that exhibit the highest autofluo- rescence may also help.
		Sources of autofluorescence— part 2: heme chromophores, lipofuscins	Thoroughly remove all blood during initial cardiac perfusion; to elute heme, incubate hydrogel-embedded in aminoalcohol CUBIC reagent-1 (Susaki et al., 2015) for 12–24 h at 37°C with shaking, and then transfer the sections directly into 8% (wt/vol) SDS for clearing; lipofuscin autofluorescence is partially combatted by tissue clearing; however, thick tissue sections may be incubated in 0.2% to 1.0% (wt/vol) Sudan Black B for 1–3 hours immediately before hydrogel embedding in order to reduce high autofluorescent background—tissue clearing will allow Sudan Black B treated sections to become sufficiently transparent for imaging
	High background, but with high signal of correctly labeled epitopes	Nonspecific antibody binding	Extend the wash steps after both primary and secondary antibody incubations an additional day, by performing four or five buffer exchanges each day, and wash the samples in PBST instead of 1× PBS; in rodent tissue samples, avoid using antibodies that require anti-mouse secondary antibody labeling[23]; also some chicken antibodies show strong staining with high background and/or aggregation—these antibodies should be diluted to 1:400 to 1:1,000
Mounting/imaging	Poor image quality and/or poor imaging depth	Tissue is of insufficient transparency for light to penetrate	Extend the tissue incubation time in RI matched mounting medium (e.g. RIMS) to several days before imaging.
	Morphological distortion	Tissue size fluctuations	Immediately before incubation in RI matched imaging medium (e.g. FocusClear or RIMS), postfix cleared, immunolabeled tissue in 4% PFA for a few hours at RT. If using RIMS, incubate for several days to one week before imaging; consider including or increasing the amount of PFA in hydrogel solution and/or consider a longer postfixation step after before hydrogel embedding.
	Bubbles in mounted tissue	Air trapped in tissue or dissolved air in RI matched mounting solution;	Apply strong vacuum to RI matched mounting solution containing tissue sample to remove dissolved air. Ensure sample mounted with insufficient solution to avoid the introduction of air bubbles between the mounting medium meniscus and cover glass
	Sample appears turbid or white	Mounted sample was placed at 4 °C, causing salts, etc., to precipitate	The precipitate should disappear upon gradual warming of tissue to RT or 37 °C. Store RIMS-mounted tissue at RT, protected from light, or mount tissue in cRIMS for cold storage

Adapted from (Tomer et al., 2014; Treweek et al., 2015).

1.6 Appendix

Table 2. Modifications to hydrogel solution

	Standard (Chung et al., 2013)	**Standard + saponin** (Chung et al., 2013)	**Standard variations** (Tomer et al., 2014)	**A4P0** (Yang et al., 2014; Treweek et al., 2015)	**Standard variations** (Epp et al., 2015)	**Hydrogel 'A'** (Zheng and Rinaman, 2015)	**Hydrogel 'B'** (Zheng and Rinaman, 2015)
Acrylamide (% wt/vol)	4	4	0.5 - 4	4	3 - 4	4	4
Bisacrylamide (% wt/vol)	0.05	0.05	0.0125 - 0.05	×	0.025 - 0.05	×	×
Paraformaldehyde (% wt/vol)	4	4	4	×	3 - 4	4	2
VA-044	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Saponin	×	0.05	×	×	×	×	×
Acrolein (% vol/vol)	×	×	×	×	×	×	2
Notes:	Original hydrogel solution. We suggest this as as a good starting point.	Saponin enhances penetration of hydrogel into sample. May be useful for passive infusion of historical/pre-fixed samples.	Lower concentrations of monomers enhance speed of lipid clearing.	Significantly increases speed of lipid clearing. Leads to greater tissue swelling than 'standard' hydrogel. Requires tissue to be fixed prior to hydrogel infusion due to exclusion of paraformaldehyde.	Lower monomer and formaldehyde concentrations enhance speed of lipid clearing and tissue transparency. Lower concentrations also cause some tissue swelling	Same as standard solution but without bisacrylamide	Includes acrolein to enhance tissue rigidity and signal to noise ratio for immunolabelling. Acrolein imparts some tissue discoloration but does not impede imaging

All hydrogel variations are made up with 1X PBS.

Recommended Reading

1) Richardson DS, Lichtman JW. 2015. **Clarifying Tissue Clearing.** Cell 162(2):246-257.

**Excellent review covering the basic principals behind light scatter and how different tissue clearing techniques achieve transparency.*

2) Chung K, Wallace J, Kim SY, Kalyanasundaram S, Andalman AS, Davidson TJ, Mirzabekov JJ, Zalocusky KA, Mattis J, Denisin AK, Pak S, Bernstein H, Ramakrishnan C, Grosenick L, Gradinaru V, Deisseroth K. 2013. **Structural and molecular interrogation of intact biological systems.** Nature 497(7449):332-337.

**The original 2013 CLARITY paper.*

3) Tomer R, Ye L, Hsueh B, Deisseroth K. 2014. **Advanced CLARITY for rapid and high-resolution imaging of intact tissues.** Nat Protoc 9(7):1682-1697.

**Describes high-throughput modifications for clearing and imaging using both active or passive CLARITY.*

4) Yang B, Treweek JB, Kulkarni RP, Deverman BE, Chen CK, Lubeck E, Shah S, Cai L, Gradinaru V. 2014. **Single-cell phenotyping within transparent intact tissue through whole-body clearing.** Cell 158(4):945-958.

**The original PACT (passive CLARITY) paper.*

5) Treweek JB, Chan KY, Flytzanis NC, Yang B, Deverman BE, Greenbaum A, Lignell A, Xiao C, Cai L, Ladinsky MS, Bjorkman PJ, Fowlkes CC, Gradinaru V. 2015. **Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping.** Nat Protoc 10(11):1860-1896

**In depth description of the passive CLARITY protocol.*

6) Palmer WM, Martin AP, Flynn JR, Reed SL, White RG, Furbank RT, Grof CPL. 2015. **PEA-CLARITY: 3D molecular imaging of whole plant organs**. Scientific Reports 5.

**Application of passive CLARITY to difficult tissues such as plant tissue using specific enzymes. This technique could be applied to dense, hard to clear mammalian tissue.*

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Epp JR, Niibori Y, (Liz) Hsiang H-L, Mercaldo V, Deisseroth K, Josselyn SA, Frankland PW. 2015. Optimization of CLARITY for Clearing Whole-Brain and Other Intact Organs. eneuro 2(3).

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