


Clearing for Deep Tissue Imaging

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Biologic tissues are generally opaque due to optical properties that result in scattering and absorption of light. Preparation of tissues for optical microscopy often involves sectioning to a thickness of 50–100 μm , the practical limits of light penetration and recovery. A researcher who wishes to image a whole tissue must acquire potentially hundreds of individual sections before rendering them into a three-dimensional volume. Clearing removes strongly light-scattering and light-absorbing components of a tissue and equalizes the refractive index of the imaging medium to that of the tissue. After clearing, the maximum depth of imaging is often defined by the microscope optics rather than the tissue. Such visibility enables the interrogation of whole tissues and even animals without the need to section. Researchers can study a biological process in the context of its three-dimensional environment, identify rare events in large volumes of tissues, and trace cells and cell-cell interactions over large distances. This article describes four popular clearing protocols that are relevant to a wide variety of scenarios across biologic disciplines: CUBIC, CLARITY, 3DISCO, and SeeDB. © 2018 by John Wiley & Sons, Inc.

Keywords: clear • CLARITY • tissue clearing • three-dimensional imaging of solvent-cleared organs (3DISCO) • see deep brain (SeeDB) • unobstructed brain/body imaging cocktails (CUBIC)

How to cite this article:

Muntiferer, M., Castranova, D., Gibson, G. A., Meyer, E., Kofron, M., & Watson, A. M. (2018). Clearing for deep tissue imaging. *Current Protocols in Cytometry*, e38. doi: 10.1002/cpcy.38

INTRODUCTION

Traditionally, optical microscopy required physical sectioning of thin discrete regions of tissue for imaging by fluorescence microscopy. Such tissue preparation was a requirement because light cannot penetrate more than a few hundred microns through most tissues. However, imaging sections of tissue necessarily decontextualizes the section from the surrounding environment. With the prominence of volume imaging technologies like confocal, multiphoton, and light-sheet, researchers need to image deeper than physical sectioning generally allows. Deep imaging enables the characterization of interesting features in the context of a larger volume. When combined with fast confocal imaging systems, researchers can interrogate whole tissues and organisms. This enables the identification of relatively rare events that would have been missed through traditional thin sectioning and tracking of individual cells and cell-cell connections over long distances (e.g., neurons).

Enabling deep imaging involves clarifying the tissue so that light can penetrate further than would otherwise be possible. Choosing a tissue clearing approach is an application-specific decision that is most strongly influenced by the tissue type, the desired depth, the need to immunostain and/or visualize endogenous fluorescence, and the available resources. All tissue clearing approaches aim to reconcile the refractive index of the tissue to that of the medium in which the tissue is immersed. When done well, this reduces scattering of light and can lead to the transmission of visible wavelengths hundreds of times further than otherwise possible. Optimal clearing often involves the removal of unwanted components (generally lipids and chromophores) and preserves proteins of interest. This is because the biomolecular complexity of tissues (i.e., lipids, proteins, sugars, pigments, chromophores, etc.) results in refractive index mismatches within individual tissues and the absorption of light by pigments and chromophores. By removing undesirable components, **the refractive index can be more easily matched, and absorption can be reduced.** Altogether, light travels further in cleared tissue, enabling imaging at depth.

Tissue clearing methods vary widely in their approaches (Azaripour et al., 2016; Richardson & Lichtman, 2015), ranging from: (1) simple immersion in a refractive index-matching solution; (2) active and passive detergent-based removal of lipids and chromophores, and (3) the use of organic solvents for dehydration, lipid removal, and refractive index matching. This article describes four independent clearing approaches using CUBIC (see Basic Protocol 1; Susaki et al., 2014, 2015), Passive and Active CLARITY (see Basic Protocol 2 and Alternate Protocol 1; Chung & Deisseroth, 2013; Chung et al., 2013; Lee et al., 2016; Yang et al., 2014), 3DISCO (see Basic Protocol 3 and Alternate Protocol 2; Ertürk et al., 2012; Pan et al., 2016; Renier et al., 2014), and SeeDB (see Basic Protocol 4; Ke, Fujimoto, & Imai, 2013). These represent popular and well-established methods that can be used to clear most mammalian tissues and enable interrogation of both proteins and lipids within biologic tissues. These methods are compatible with endogenous fluorescence proteins and immunohistochemical labeling.

STRATEGIC PLANNING

When deciding which clearing protocol will work best, two defining variables should be considered: (1) what marker is being imaged (**endogenous fluorescence, immunostained antigens and/or lipids**) and (2) what tissue type is being imaged. As guidance, a flow chart is provided in Figure 1 that will narrow down which clearing protocols are compatible with the subject to be imaged. In addition, Table 1 provides examples of how well tissues of distinct types clear with each method. Note that Figure 1 is specific to the protocols presented in this publication, and readers may find variations of each method that will work in alternative scenarios. Table 1 is intended as a guide to lead the reader towards a protocol that will yield a high likelihood of success. The table is not based on a scientific survey, but is based on the authors' experience. Clearing protocols marked as 'nt' (not tested) do not indicate that a method will not be successful with a given tissue, but only that it has not been evaluated by the authors. Success with these methods is expected to vary because of the idiosyncratic nature of different researchers, resources, environments, experiments, and tissue types. When feasible, multiple clearing approaches should be tested to maximize success.

UNOBSTRUCTED BRAIN/BODY IMAGING COCKTAILS (CUBIC)

CUBIC (Susaki et al., 2014, 2015) is a tissue clearing method that removes both lipids and iron-based light-absorbing chromophores prior to refractive index matching. The method is compatible with a wide range of tissues, relatively inexpensive, technically simple, and useful for tissues that have endogenous fluorescence and/or require antibody staining. CUBIC is an excellent starting point for researchers exploring tissue clearing for the first time.

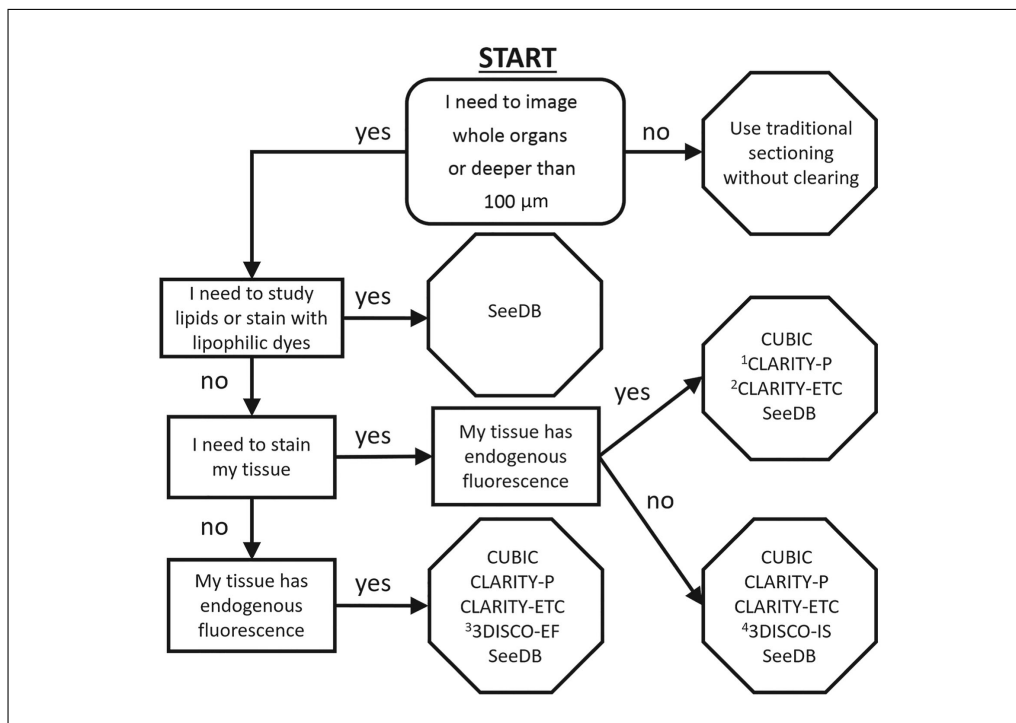


Figure 1 Decision tree for choosing a clearing protocol. Keep in mind the tissue to be cleared and the method of visualization (immunostaining, endogenous fluorescent proteins, lipids or lipophilic dyes). Begin at 'start' and follow the yes/no questions to narrow down which clearing protocol(s) are a proper fit for the experiment. Each branch of the tree terminates at an octagon suggesting clearing procedures that are compatible with the choices made. In most cases, multiple clearing protocols are suggested. Before deciding, consult Table 1 as a guide for protocols most likely to succeed with the specific tissue. ¹CLARITY-P: Passive; ²CLARITY-ETC: electrophoretic tissue clearing; ³DISCO-EF: endogenous fluorescence; ⁴DISCO-IS: immunostaining.

Materials

PBS with and without 0.1% (w/v) sodium azide
 4% (w/v) paraformaldehyde (see recipe)
 Hydrogel (*optional*; see recipe)
 Nitrogen gas or vacuum chamber (*optional*)
 Distilled water
 CUBIC R1 solution (see recipe)
 IHC buffer (*optional*; see recipe)
 Antibodies for tissue staining (*optional*)
 CUBIC R2 (*optional*; see recipe) with or without TEA

37°C water bath or incubator
 Nutating mixer (Fisher Scientific, cat. no. 88-861-041)

NOTE: The use of 50-ml plastic conical tubes is recommended for all steps in this protocol, as they provide sufficient room for most tissue sizes and enable ample movement of fluids during incubation steps on a nutating mixer. In general, polypropylene tubes are recommended for use in all of the protocols described herein, as their overall durability and resistance to organic solvents make them compatible with all of the clearing procedures. However, any type of tube will work for this protocol.

Fix and harvest tissue

1. *Optional:* Perfuse animal transcardially with PBS and then an equal volume of 4% PFA.

Table 1 Tissue Compatibility of Various Clearing Protocols^a

| Tissue | CUBIC | CLARITY | CLARITY-ETC | 3DISCO | SeeDB |
|--------------------|-------|---------|-------------|--------|-------|
| Artery | ** | nt | nt | ***** | nt |
| Bone | X | X | X | **** | X |
| Bone marrow | * | ** | ** | X | nt |
| Brain | ***** | *** | **** | ***** | *** |
| Brain tumor | * | nt | **** | ***** | nt |
| Eye | ** | nt | nt | **** | ** |
| Heart | ** | * | **** | **** | nt |
| Intestine | ***** | nt | nt | ***** | nt |
| Liver | ** | * | ***** | X | nt |
| Lung | ***** | nt | nt | ***** | nt |
| Lymph node | ***** | nt | nt | *** | nt |
| Mouse placenta | nt | *** | **** | nt | nt |
| Ovary | ***** | nt | nt | ***** | nt |
| Skeletal muscle | *** | nt | nt | ***** | nt |
| Skin | *** | nt | nt | ***** | nt |
| Spinal cord | *** | *** | **** | ***** | *** |
| Spleen | ** | ** | **** | * | nt |
| Whole adult mouse | *** | * | nt | ***** | nt |
| Whole mouse embryo | *** | nt | nt | ***** | nt |
| Whole zebrafish | ***** | nt | nt | nt | *** |

^a Asterisks, relative success of method for the indicated tissue; X, method does not work for the indicated tissue; nt, not tested.

The volumes used will depend on the size of the animal.

2. Remove tissue from the animal and then immerse in 3 volumes of 4% PFA or at least enough to cover the tissue. Fix overnight to 24 hr at 4°C.

Embed in hydrogel (optional)

Embedding in hydrogel is not necessary when performing CUBIC. It provides support and rigidity to fragile tissues and provides a means to make thick sections (> 500 µm) without distorting the tissue. It does not affect the efficiency of clearing, however, and can make mounting the sample more difficult and increase the time required for immunostaining.

3. Immerse tissue in 3 volumes of hydrogel overnight to 24 hr at 4°C.
4. *Optional*: Remove excess oxygen by degassing for 10 min.

To degas with nitrogen, gently bubble the gas into the hydrogel solution or place the hydrogel solution, open-capped, in a chamber in which oxygen has been purged with nitrogen.

To degas under vacuum, place the hydrogel solution, open-capped, in a vacuum chamber and apply vacuum. Bubbles will become visible in the hydrogel solution and may form around the sides of the container. After the vacuum is gently released, the remaining bubbles can be released from the sides by gently tapping the tube.

VA-044 acts as a thermal initiator of hydrogel polymerization. VA-044 will preferentially react with oxygen as opposed to the hydrogel. This step can minimize the amount of oxygen

diffused in the sample and in the head space above in the tube. Although recommended, it is not required, as the gel will still polymerize without degassing.

5. Polymerize hydrogel by moving to a 37°C water bath or incubator for 4 or 6 hr, respectively.

Polymerization will proceed more slowly in an incubator, requiring additional incubation time. When necessary, polymerization can be allowed to proceed overnight. Caution: Although clearing is unlikely to be affected by an increased incubation time, immunostaining may proceed more slowly.

6. Remove tissue from the polymerized hydrogel.

The polymerized gel should be viscous and thick, not firm but not runny.

7. *Optional:* At this stage, the tissue can be stored for up to 1 month. Wash tissues three times for 10 min in PBS with 0.1% (w/v) sodium azide (with same volume used in step 2) and store up to 1 month at 4°C in PBS with 0.1% sodium azide.

Clear tissue

8. Place tissue in 3 volumes of CUBIC R1 diluted 1:1 with distilled water. Incubate 24 hr at 37°C on a nutating mixer.
9. Change with fresh undiluted CUBIC R1 solution. Incubate up to 2 days at 37°C on a nutating mixer.

The tissue and solution may take on a green-brown appearance. This color is evidence that iron-based chromophores are being dissolved from the tissue.

10. Replace solution with fresh undiluted CUBIC R1 every 2 days or when it takes on a greenish-brown color. Move to the next step when the tissue appears clear and the green-brown color is washed out of the tissue.

This may take up to 2 weeks for very large tissues. For some tissues, the color may never fully dissipate.

If the tissue appears clear after CUBIC R1 incubation, it can be mounted in CUBIC R1 and imaged for endogenous fluorescence. Most tissues will benefit from a solution with a higher refractive index. In this case, proceed to step 19. If immunohistochemistry is required, proceed to step 11.

Stain tissue (optional)

NOTE: For additional information, see Critical Parameters discussion of tissue staining.

11. Wash tissue three times for 2 hr each in IHC buffer at 37°C on a nutating mixer.

The tissue will become opaque and may shrink to a more physiologic size.

12. Wash one additional time in IHC buffer overnight at 37°C on a nutating mixer.

This step ensures that all the CUBIC R1 reagents are removed prior to staining. The ingredients in CUBIC R1 can interfere with antibody binding.

13. Add stain to the tissue, diluted in IHC buffer, and incubate at 37°C on a nutating mixer.

The concentration of the stain will be specific to the reagent being used and should be determined by the researcher. The length of incubation needs to be empirically determined based on the tissue type and size. For example, when staining with antibodies, a whole mouse brain requires 1 week, whereas mouse ovaries and lungs require only 24 hr. Hydrogel-embedded tissue may require longer incubations.

14. Wash as in step 10. If desired, perform an additional overnight wash as in step 12.

The additional overnight wash may help reduce background.

15. *Optional:* Repeat steps 11–12 if a secondary stain is required.

Store tissue (optional)

Cleared tissues should be imaged immediately after clearing by continuing with the mounting steps. Tissue should always be imaged as quickly as possible after processing. Storage can result in degradation of the tissue and fluorescent signal, particularly from endogenous proteins. When necessary, however, tissues can be stored for up to a few months at 4°C and imaged later.

16. Wash tissue three times for 2 hr at 37°C on a nutating mixer. For stained tissue, wash with IHC buffer; for unstained tissue, wash with PBS containing 0.1% sodium azide.
17. *Optional:* For stained samples, fix in 3 volumes of 4% PFA overnight at 4°C. Wash out PFA by repeating step 16.

Fixing samples can make stains permanent through crosslinking. This is helpful when storing antibody-stained samples for an extended period.

18. Immerse tissue in PBS with 0.1% sodium azide and store up to several months at 4°C.

When the tissue is ready to be imaged, continue with the mounting steps.

Mount tissue

19. Wash tissue three times for 2 hr at 37°C on a nutating mixer. For stained tissue, wash with IHC buffer; for unstained tissue, wash with PBS containing 0.1% sodium azide.
20. For stained tissue, wash an additional time in IHC buffer overnight at 37°C on a nutating mixer.
21. Add CUBIC R2 solution to the tissue and incubate for 1 hr at 37°C on a nutating mixer.

The TEA in CUBIC R2 can negatively impact fluorescence in some stains. This is best determined by testing with a spare tissue. To avoid this, CUBIC R2 can be substituted with CUBIC R2 without TEA. The refractive index of CUBIC R2 without TEA is slightly lower than that of CUBIC R2, which may impact clearing of the tissue. Other refractive index-matching solutions (e.g., RIMS; see recipe) may be used in place of CUBIC R2.

22. Replace with fresh CUBIC R2 solution and incubate overnight to 24 hr.

The tissue will shrink.

23. Replace with fresh CUBIC R2 every 24 hr until the tissue appears to have maximally cleared.

The time required to re-clear will vary based on the size and type of tissue. Prolonged incubation (>1 week) will cause the tissue to swell again. Many weeks of incubation in CUBIC R2 can cause the tissue to swell to many times its original volume. To image after prolonged storage, simply repeat the mounting protocol, which will cause the tissue to shrink.

24. Mount cleared tissue in CUBIC R2 and image.

After imaging, the tissue can be stored for longer than 1 week by following steps 16–18.

CLARITY-PASSIVE

The passive CLARITY technique (PACT; Yang et al., 2014) is a method of delipidation and refractive index matching based on mounting tissue in a hydrogel monomer, then removing the lipids in a strong detergent. Any detergent that micelles at a reasonable concentration or pH may be used, but the most common is sodium dodecyl sulfate (SDS). After delipidation, the tissue is mounted in a refractive index–matching solution (RIMS) and imaged. The procedure is simple and relatively gentle to tissue. It is suitable for tissue with endogenous fluorescence as well as tissue that will be antibody stained post-clearing. It is also possible to lectin-stain tissue prior to performing the protocol.

Materials

PBS with and without 0.1% (w/v) sodium azide
4% (w/v) paraformaldehyde (see recipe)
Hydrogel (see recipe)
Nitrogen gas or vacuum chamber (*optional*)
8% (w/v) SDS solution, pH 7.5
IHC buffer (*optional*; see recipe)
Antibodies for tissue staining (*optional*)
RIMS (see recipe)

37°C water bath or incubator
Nutating mixer (Fisher Scientific, cat. no. 88-861-041)

NOTE: The use of 50-ml plastic conical tubes is recommended for all steps in this protocol, as they provide sufficient room for most tissue sizes and enable ample movement of fluids during incubation steps on a nutating mixer. In general, polypropylene tubes are recommended for use in all of the protocols described herein, as their overall durability and resistance to organic solvents make them compatible with all of the clearing procedures. However, any type of tube will work for this protocol.

Fix and harvest tissue

1. *Optional:* Perfuse animal transcardially with PBS and then an equal volume of 4% PFA.

The volumes used will depend on the size of the animal.

2. Remove tissue from the animal and then immerse in 3 volumes of 4% PFA or at least enough to cover the tissue. Fix overnight to 24 hr at 4°C.

A gentler fixation using 2% PFA or a shorter fixation period can improve clearing results; however, protein loss during clearing will be increased, reducing the available antigen and quenching endogenous fluorescence. A cross-linking fixative is necessary for the tissue to survive the process.

Embed in hydrogel

3. Immerse tissue in 3 volumes of hydrogel overnight to 24 hr at 4°C. Embedding in hydrogel is necessary to maintain tissue integrity during the clearing process.
4. *Optional:* Remove excess oxygen by degassing for 10 min.

To degas with nitrogen, gently bubble the gas into the hydrogel solution or place the hydrogel solution, open-capped, in a chamber in which oxygen has been purged with nitrogen.

To degas under vacuum, place the hydrogel solution, open-capped, in a vacuum chamber and apply vacuum. Bubbles will become visible in the hydrogel solution and may form around the sides of the container. After the

vacuum is gently released, the remaining bubbles can be released from the sides by gently tapping the tube.

VA-044 acts as a thermal initiator of hydrogel polymerization. VA-044 will preferentially react with oxygen as opposed to the hydrogel. This step can minimize the amount of oxygen diffused in the sample and in the head space above in the tube. Although recommended, it is not required, as the gel will still polymerize without degassing.

5. Polymerize hydrogel by moving to a 37°C water bath or incubator for 4 or 6 hr, respectively.

Polymerization will proceed more slowly in an incubator, requiring additional incubation time. When necessary, polymerization can be allowed to proceed overnight. Caution: Although clearing is unlikely to be affected by an increased incubation time, immunostaining may proceed more slowly.

6. Remove tissue from the polymerized hydrogel.

The polymerized gel should be viscous and thick, not firm but not runny.

7. *Optional:* At this stage, the tissue can be stored for up to 1 month. Wash tissues three times for 10 min in PBS with 0.1% (w/v) sodium azide (with same volume used in step 2) and store up to 1 month at 4°C in PBS with 0.1% sodium azide.

Clear tissue

8. Place tissue in ~3 volumes of 8% SDS solution or at least enough to cover the tissue. Incubate on a nutating mixer at 37°C until the tissue is mostly transparent. Change the solution if it becomes cloudy.

The SDS concentration can be altered depending on tissue fragility and lipid content. Lower concentrations of SDS will clear more slowly and be a bit gentler on the tissue, whereas higher concentrations will clear faster and be harsher. Clearing can take anywhere from hours to weeks, depending on tissue size and lipid content.

9. Once clear, remove tissue from SDS and wash repeatedly with 37°C PBS containing 0.1% (w/v) sodium azide until foaming from the SDS is no longer observed.

It is imperative that all of the detergent be washed out, as excess SDS will impact immunohistochemistry and refractive index matching. A tissue the size of a mouse brain generally requires five washes for 30 min at 37°C. Wash numbers and times are expected to vary according to tissue type and size and SDS concentration. At the completion of each wash, gently shake the tube to observe foaming. During the wash process, the tissue may turn white. This is expected, as the refractive index of fixed cleared tissue is different from that of water.

Stain tissue (optional)

NOTE: For additional information, see Critical Parameters discussion of tissue staining.

10. Wash tissue three times for 2 hr each in IHC buffer at 37°C on a nutating mixer.
11. Add stain to the tissue, diluted in IHC buffer, and incubate at 37°C on a nutating mixer.

The concentration of the stain will be specific to the reagent being used and should be determined by the researcher. The length of incubation needs to be empirically determined based on the tissue type and size. For example, when staining with antibodies, a whole mouse brain requires 1 week, whereas mouse ovaries and lungs require only 24 hr.

12. Wash as in step 10. If desired, perform an additional overnight wash at 37°C on a nutating mixer.

The additional overnight wash may help reduce background.

13. *Optional*: Repeat steps 11–12 if a secondary stain is required.

Store tissue (optional)

14. Store tissue as described in Basic Protocol 1, steps 16–18.

Optimize refractive index

15. Incubate tissue in RIMS until transparent.

Multiple changes may be required.

16. Mount in fresh RIMS and image.

Alternative solutions designed for refractive index optimization can be used in place of RIMS, including CUBIC R2 with or without TEA.

CLARITY-ETC

Electrophoretic tissue clearing (ETC; Chung & Deisseroth, 2013; Chung et al., 2013; Lee et al., 2016) is an active form of CLARITY that applies an electric field across the tissue to draw out the lipid micelles. Because SDS is an anionic surfactant, it carries a net negative charge and can thus be moved by a difference in potential between two electrodes. Several commercial applications exist (X-CLARITY, Logos biosystems; SmartClear II Pro, Life Canvas Technologies) or a system can be built in-house (Chung & Deisseroth, 2013; Chung et al., 2013; Lee et al., 2016; also see Internet Resources for “CLARITY wiki”). Electrophoretic tissue clearing greatly reduces the amount of time required to clear tissue, allowing the entire protocol to be completed in less than 2 days. Because the commercial systems have propriety solution and protocol requirements, the following protocol describes CLARITY-ETC under the assumption that the reader is using a custom-built clearing chamber.

Additional Materials (also see Basic Protocol 2)

CLARITY-ETC running buffer (see recipe)

Electrophoretic tissue clearing chamber (see Internet Resources or Chung & Deisseroth, 2013; Chung et al., 2013; Lee et al., 2016)

1. Fix, harvest, and embed tissue as described (see Basic Protocol 2, steps 1–7).
2. Place tissue into the ETC chamber, fill with ETC running buffer, and energize the device. Start with 1.5 A and no more than 60 V (see Critical Parameters for an extended discussion of current and voltage).

See CLARITY-ETC under the critical parameters section. The time required for clearing will vary depending widely on a variety of factors including: tissue type, tissue size, tissue thickness, tissue protein/lipid content, temperature, design of the ETC chamber, and the voltage applied.

3. Once clear, remove tissue from the buffer and wash repeatedly with room-temperature PBS containing 0.1% (w/v) sodium azide until foaming from the SDS in the buffer is no longer present.

It is imperative that all of the detergent be washed out, as excess SDS will impact immunohistochemistry and refractive index matching. A tissue the size of a mouse brain generally requires five washes for 30 min at 37°C. Wash numbers and times are expected to vary according to tissue type and size and SDS concentration. At the completion of each wash, gently shake the tube to observe foaming. During the wash process, the tissue may turn white. This is expected, as the refractive index of fixed cleared tissue is different from that of water.

ALTERNATE PROTOCOL 1

4. Stain tissue and optimize refractive index as described (see Basic Protocol 2, steps 10–17).

BASIC PROTOCOL 3

THREE-DIMENSIONAL IMAGING OF SOLVENT-CLEARED ORGANS (3DISCO) FOR ENDOGENOUS FLUORESCENCE

Organic solvent-based clearing methods generally offer the best clearing results over a wide range of tissues. This is because organic solvents can yield refractive indices that are characteristically higher than water-based solutions, and thus more accurately match that of the protein meshwork. These methods are particularly advantageous for tissues that are dense in connective tissues, for which water-based methods clear poorly. Numerous solvent-based methods have been developed that are broadly based on the benzyl alcohol/benzyl benzoate (BABB) and 3DISCO methods. Popular variations on these themes include immunolabeling-enabled DISCO (iDISCO) and ultimate DISCO (uDISCO). Each has improved upon 3DISCO with the goal of enabling immunohistochemistry or enhancing visualization of endogenous fluorescence.

Dealing with organic solvents offers challenges for both laboratory practices and imaging. Solvents require the use of safety practices that include working in fume hoods and using glass or specialized plastics that will not dissolve. Additionally, care must be taken when mounting cleared tissues, because the solvents can dissolve some mounting materials as well as the seals on some immersion microscope objectives. In addition, preparation of the tissue requires dehydration, which can cause shrinkage of up to 8-fold in volume. Shrinkage effectively decreases the resolution that can be achieved and increases autofluorescence of the surrounding tissue. Finally, fluorescence from endogenous proteins is often very bright initially, but wanes quickly, requiring samples to be imaged within a few days to maintain optimal signal.

This protocol, although referred to as 3DISCO, describes how to stain and clear tissues using a variation of 3DISCO, iDISCO, and uDISCO. It is recommended that researchers explore how the original BABB (Dodt et al., 2007), 3DISCO (Ertürk et al., 2012), iDISCO (Renier et al., 2014), and uDISCO (Pan et al., 2016) protocols may benefit their research. However, this protocol is an excellent starting point for exploring solvent-based clearing methods. The steps below are specific for imaging endogenous fluorescence. The method for immunostaining (see Alternate Protocol 2) includes a methanol treatment that will quench endogenous fluorescent proteins such as GFP and thus should not be used.

Materials

- PBS with and without 0.1% (w/v) sodium azide
- 4% (w/v) paraformaldehyde (see recipe)
- tert*-Butanol (Sigma, cat. no. 360538)
- Distilled water
- Dichloromethane (DCM; *optional*; Sigma, cat. no. 270997)
- Dibenzyl ether (DBE; Sigma, cat no. 108014)

- Nutating mixer (Fisher Scientific, cat. no. 88-861-041)
- 37°C incubator

NOTE: The use of 50-ml plastic conical tubes is recommended for all steps in this protocol, as they provide sufficient room for most tissue sizes and enable ample movement of fluids during incubation steps on a nutating mixer. Polypropylene tubes are recommended here due to their overall durability and resistance to the organic solvents used in this protocol. Alternatively, glass tubes can be used, but this is discouraged for reasons of safety.

Fix and harvest tissue

1. *Optional:* Perfuse animal transcardially with PBS and then an equal volume of 4% PFA.

The volumes used will depend on the size of the animal.

2. Remove tissue from the animal and then immerse in 3 volumes of 4% PFA or at least enough to cover the tissue. Fix overnight to 24 hr at 4°C.

Dehydrate and clear

3. Dehydrate tissue by immersing in ~3 tissue volumes of 30% *tert*-butanol diluted in dH₂O for 1 hr at 37°C on a nutating mixer and repeating with 50%, 70%, 80%, 90%, 100%, and 100% *tert*-butanol.

The volume of tert-butanol should be at least enough cover the tissue.

NOTE: Because 100% tert-butanol will freeze at room temperature, it should be stored at 37°C or melted at 37°C prior to use.

4. *Optional:* Incubate in the same volume of DCM for 1 hr at room temperature.

DCM acts as a strong delipidating agent. It is often unnecessary for small tissues, but is recommended for large tissues (e.g., whole brain) because it will significantly improve tissue clarity.

CAUTION: Care should be taken when handling DCM. Skin contact or inhalation will cause irritation.

5. Move tissue to 3 volumes of DBE and incubate for 1 hr at 37°C on a nutating mixer.

The tissue should noticeably begin to clear within 30 min of adding DBE.

6. Replace with fresh DBE and incubate at 37°C on a nutating mixer until the tissue appears completely clear.

Additional changes of DBE may be required if the tissue is very large.

Tissues may take on an amber color, but are generally completely transparent. For example, words on a page can often be read with uniform clarity and no obvious distortion through cleared tissues.

If the interior regions of the tissue appear opaque after multiple DBE changes, this usually indicates poor dehydration, often due to incubating for too short of a time or not providing movement during dehydration. DBE is hydrophobic and will not penetrate regions containing moisture. To correct for this: rehydrate and then dehydrate the tissue using a tert-butanol series of 100%, 100%, 90%, 80%, 80%, 90%, 90%, 100%, 100%. Clear with DBE as in steps 5–6.

7. Mount sample in DBE for imaging.

Care must be taken when mounting samples, as DBE will soften or melt many plastics and may dissolve the seals on immersion objectives.

Samples can be stored indefinitely in DBE; however, most of the endogenous fluorescence will fade after 1–2 weeks.

THREE-DIMENSIONAL IMAGING OF SOLVENT-CLEARED ORGANS (3DISCO) FOR IMMUNOSTAINING

This protocol uses methanol to remove lipids and permeabilize the tissue to facilitate the diffusion of antibodies and other stains through the tissue. Some antibodies will not bind their antigens after methanol treatment. Compatibility of individual antibodies with methanol treatment should be verified prior to continuing with this protocol. Testing

**ALTERNATE
PROTOCOL 2**

Muntifering et al.

11 of 21

can be done on frozen sections that are incubated in 100% methanol for 3 hr and then rehydrated in PBS prior to immunostaining. The sections should be evaluated for retention of antibody specificity and signal-to-noise ratio.

Additional Materials (also see Basic Protocol 3)

Methanol (Fisher, cat. no. A412SK-4)
Hydrogen peroxide (H₂O₂; *optional*; Sigma, cat. no. 216763)
PTx.2 solution (see recipe)
3DISCO permeabilization solution (see recipe)
3DISCO blocking solution (see recipe)
3DISCO staining solution (see recipe)
Antibodies of interest
PTwH solution (see recipe)

Fix and harvest tissue

1. *Optional*: Perfuse animal transcardially with PBS and then an equal volume of 4% PFA.

The volumes used will depend on the size of the animal.

2. Remove tissue from the animal and then immerse in 3 volumes of 4% PFA or at least enough to cover the tissue. Fix overnight to 24 hr at 4°C.

Pretreat tissue

3. Dehydrate tissue by immersing in 3 volumes of 30% methanol for 1 hr at room temperature with gentle movement and repeating with 50%, 70%, 90%, 100%, and 100% methanol.

A nutator, rocker, or orbital shaker is effective for providing gentle movement.

4. Incubate in 3:1 (v/v) DCM/methanol overnight at room temperature with gentle movement.

CAUTION: Care should be taken when handling DCM. Skin contact or inhalation will cause irritation.

5. Wash tissue two times with 100% methanol for 1 hr at room temperature with gentle movement.

6. *Optional*: Bleach tissue with 5% H₂O₂ in methanol overnight at 4°C.

Bleaching will provide enhanced clearing results, but may interfere with some staining reactions.

7. Rehydrate with 90%, 70%, 50%, and 30% methanol for 1 hr with gentle movement.
8. Wash three times in 3 volumes of PBS with 0.1% (w/v) sodium azide for 1 hr each with gentle movement.

Stain tissue

NOTE: For additional information, see Critical Parameters discussion of tissue staining.

9. Wash tissue twice in 3 volumes of PTx.2 for 1 hr at 37°C with gentle movement.
10. Permeabilize with 3DISCO permeabilization solution at 37°C with gentle movement for up to 2 days.

11. Block with 3DISCO blocking solution at 37°C with gentle movement for up to 2 days.

Blocking solution contains BSA; however, this can be substituted by serum from a species of interest (e.g., mouse, donkey, goat).

12. Stain with primary antibodies in 3DISCO staining solution at 37°C with gentle movement for up to 2 weeks.

The concentration will be specific to the antibody being used and should be determined by the researcher. The length of incubation needs to be empirically determined based on the tissue type and size.

13. Wash four times in PTwH solution for at least 1 hr and then again overnight at 37°C with gentle movement.

14. *Optional:* Stain with secondary antibodies in 3DISCO staining solution at 37°C with gentle movement for up to 2 weeks. After the secondary stain, wash again as in step 13.

Incubation times should be the same as for the primary stain.

Dehydrate and clear

15. Dehydrate and clear as described for endogenous fluorescence (see Basic Protocol 3, steps 3–7).

Immunostained samples can be stored indefinitely in DBE. Fluorescent tags generally maintain their fluorescence indefinitely after processing.

SEE DEEP BRAIN (SeeDB)

See Deep Brain (SeeDB) is a clearing technique that uses fructose to clear tissues and α -thioglycerol to prevent browning (Maillard reaction) and prevent the development of autofluorescence (Ke et al., 2013). Practical advantages of SeeDB include the short duration and simplicity of the protocol. Technical advantages include the preservation of fluorescent proteins and lipophilic tracers, and minimal morphologic distortion compared to some other clearing methods. Whole-mount immunostaining can be done on samples prior to SeeDB clearing, though antibody penetration is limited to 100–250 μ m (Ke et al., 2013), making other clearing methods described here more suitable for whole-mount immunostaining. SeeDB is reversible and samples can be sectioned and immunostained after SeeDB clearing is reversed. This clearing method has been applied to mouse embryos and brains, as well as eyes of several species (Hohberger, Baumgart, & Bergua, 2017), and zebrafish. The high viscosity of SeeDB limits its penetration into larger tissues.

Modifications can increase the penetration and clearing capabilities of SeeDB, as in FRUIT (Hou et al., 2015). The FRUIT protocol adds urea, which decreases viscosity and allows clearing of adult rabbit brains. The SeeDB clearing protocol is described here and will be appropriate for most applications. However, researchers are encouraged to consider FRUIT as an alternative.

Materials

PBS
4% (w/v) paraformaldehyde (see recipe)
IHC buffer (*optional*; see recipe)
Antibodies for tissue staining (*optional*)
SeeDB solutions (see recipe)

BASIC PROTOCOL 4

Muntifering et al.

Solutions 1–5: 20%, 40%, 60%, 80%, and 100% (w/v) fructose
Solution 6: 80.2% (w/w) fructose
SeeDB37: 86.7% (w/w) fructose

Nutating mixer (Fisher Scientific, cat. no. 88-861-041)
37°C incubator
50°C water bath (*optional*)

NOTE: The use of 50-ml plastic conical tubes is recommended for all steps in this protocol, as they provide sufficient room for most tissue sizes and enable ample movement of fluids during incubation steps on a nutating mixer. In general, polypropylene tubes are recommended for use in all of the protocols described herein, as their overall durability and resistance to organic solvents make them compatible with all of the clearing procedures. However, any type of tube will work for this protocol.

Fix and harvest tissue

1. *Optional:* Perfuse animal transcardially with PBS and then an equal volume of 4% PFA.

The volumes used will depend on the size of the animal.

2. Remove tissue from the animal and then immerse in 3 volumes of 4% PFA or at least enough to cover the tissue. Fix overnight to 24 hr at 4°C.

A gentler fixation using 2% PFA or a shorter fixation period can improve clearing results; however, protein loss during clearing may be increased, quenching endogenous fluorescence. A cross-linking fixative is necessary for the tissue to survive the process.

Stain tissue (optional)

Although SeeDB is an excellent protocol for imaging endogenous fluorescent proteins, a key advantage to SeeDB is that it retains lipids. This enables the use of lipophilic molecular probes. In addition, immunostaining can be successfully applied to SeeDB. However, unlike molecular probes, relatively bulky antibodies do not efficiently penetrate large whole-mount tissues. Penetration is less of an issue in protocols like CUBIC, CLARITY, and 3DISCO, where stringent detergents are used to remove lipids and permeabilize the tissue. Antibody stains prior to SeeDB clearing can be expected to penetrate no more than 200–500 μm . For additional information, see Critical Parameters discussion of tissue staining.

3. Wash tissue 3 times for 2 hr each in IHC buffer at 37°C on a nutating mixer.

The Triton X-100 in IHC buffer permeabilizes the tissue without significant loss of lipids.

4. Add stain to the tissue, diluted in IHC buffer, and incubate at 37°C on a nutating mixer.

The concentration of the stain will be specific to reagent being used and should be determined by the researcher. The length of incubation needs to be empirically determined based on the tissue type and size.

5. Wash as in step 3. If desired, perform an additional overnight wash in IHC buffer at 37°C on a nutating mixer.

The overnight wash may help reduce background.

6. *Optional:* Repeat steps 4–5 if a secondary stain is required.

Clear tissue

7. Wash tissue three times for 10 min in PBS.

8. Incubate tissue in increasing concentrations of fructose on a nutating mixer at room temperature as follows:

4–8 hr in SeeDB solution 1
4–8 hr in SeeDB solution 2
4–8 hr in SeeDB solution 3
12 hr in SeeDB solution 4
12 hr in SeeDB solution 5
24 hr in SeeDB solution 6.

9. *Optional*: Place tissue in SeeDB37 for 48 hr at 37°C

SeeDB37 increases the speed and penetration of clearing as well as the refractive index of the final solution. This may be beneficial for larger tissues and tissues with higher refractive indices.

Tissues can be stored for a week in SeeDB solution 6 at room temperature or in SeeDB37 at 37°C. Samples should be kept in SeeDB solution 6 or SeeDB37 during imaging.

Final clearing time and temperature is highly sample dependent. Larger tissues may require longer incubations and higher temperatures. Higher temperatures may cause quenching of fluorescent proteins and slight tissue expansion, so care should be taken to calculate the best final incubation parameters for each sample type.

Protocol modifications

For SeeDBp

SeeDBp prevents tissue expansion, which is seen in some tissues such as neonatal brain samples. Follow the protocol above, but substitute 0.1× PBS for distilled water when making SeeDB solutions 1–4.

For SeeDB37ht

SeeDB27ht increases the speed and penetration of clearing. Follow the protocol above, but change incubation times and temperatures as follows:

3 hr each in SeeDB solutions 1–4 at 50°C
12 hr in SeeDB solution 5 at 50°C
24 hr in SeeDB solution 6 at 50°C
24 hr in SeeDB37 at 50°C.

Store and image samples at 37°C.

REAGENTS AND SOLUTIONS

CLARITY-ETC running buffer

Add 12.37 g (200 mM) boric acid to dH₂O
Add 40 g SDS
Bring to 1 liter
Adjust pH to 8.5 with NaOH
Store up to 1 month at room temperature

CUBIC R1 solution

Mix 125 g urea (Sigma-Aldrich, cat. no. U5378) and 175 ml dH₂O in a glass beaker. Stir on a hot plate over low heat or place in a water bath, up to 56°C, until the urea dissolves. Add 123 g (or 124 ml) Quadrol (*N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine; Sigma-Aldrich, cat. no. 122262) and stir over low

heat until the Quadrol dissolves. Add 70 ml Triton X-100 (Fisher, cat. no. BP151-500). Remove from heat and stir until dissolved. Store up to 1 month in a sealed container at room temperature.

Allowing the mixture to reach a temperature of up to 56°C after addition of urea will facilitate other components going into solution, but is not necessary.

Quadrol is very viscous and should therefore be weighed directly into the urea solution. If the reagent must be measured by volume, heat it to 56°C in a water bath prior to pouring.

If the solution takes on a strong ammonia smell, it has expired. If the temperature is too high when the solution is made, the ammonia smell will be immediately present, and the solution should be discarded.

CUBIC R2 solution

Mix 125 g urea (Sigma-Aldrich, cat. no. U5378) and 75 ml dH₂O in a glass beaker. Stir on a hot plate over low heat or place in a water bath, up to 56°C, until the urea dissolves. Slowly add 250 g sucrose (Sigma-Aldrich, cat. no. S9378) with stirring over low heat and continue stirring until the sucrose dissolves. Turn off heat and add 44.5 ml triethanolamine (TEA) with stirring. Add 380 µl Triton X-100 (Fisher, cat. no. BP151-500) and stir until well mixed. Store up to 1 month in a sealed container at room temperature.

When preparing solution without TEA, increase the volume of water to 119.5 ml.

Allowing the mixture to reach a temperature of up to 56°C after addition of urea will facilitate other components going into solution, but is not necessary. The container should remain loosely capped to limit evaporation.

When the sucrose has dissolved, the solution will be extremely viscous.

If the solution takes on a strong ammonia smell, it has expired. If the temperature is too high when the solution is made, the ammonia smell will be immediately present, and the solution should be discarded.

3DISCO blocking solution

Add 5 ml DMSO to 44.5 ml PTx.2 solution (see recipe)

Add 0.5 ml of 10% (w/v) sodium azide

Add 2.5 g BSA

Stir until dissolved

Store indefinitely at room temperature

3DISCO permeabilization solution

Add 11.5 g glycine to 400 ml PTx.2 solution (see recipe)

Stir until dissolved

Add 0.5 ml of 10% (w/v) sodium azide

Add 99.5 ml DMSO

Store indefinitely at room temperature

3DISCO staining solution

Add 2.5 g BSA to 49.5 ml PTwH solution (see recipe)

Stir until dissolved

Add 0.5 ml of 10% (w/v) sodium azide

Store indefinitely at room temperature

Hydrogel

5 ml 10× PBS
38.75 ml dH₂O
5 ml 40% acrylamide (Bio-Rad, cat. no. 161-0140)
1.25 ml 2% bis-acrylamide (Bio-Rad, cat. no. 161-0142)
0.175 g VA-044 (Wako Chemicals, Fisher Scientific, cat. no. NC0632395)

Combine ingredients in order in a 50-ml conical tube and mix gently until dissolved. Keep at 4°C for all steps prior to polymerization. Prepare fresh when possible. If necessary, store up to 1 week at 4°C or up to 1 month at –20°C.

Immunohistochemistry (IHC) buffer

500 ml PBS
0.5 ml Triton X-100
2.5 g bovine serum albumin
0.5 ml 10% (w/v) sodium azide
Store up to 1 month at room temperature

Paraformaldehyde, 8% and 4% (w/v)

For 8% stock solution, add 8 g paraformaldehyde resin (Fisher Scientific, cat. no. O4042-500) to 70 ml dH₂O. Heat to 60°C (no higher) with stirring. Add 1 N NaOH until the solution clears (usually a couple of drops). Cool to room temperature. Add 9 ml of 10× PBS and adjust volume to 100 ml with dH₂O. For 4% solution, dilute 1:1 with dH₂O. Store up to 2 weeks at 4°C.

Phosphate buffer

Add 3.1 g NaH₂PO₄ (monohydrate) and 10.9 g Na₂HPO₄ (anhydrous) to 500 ml dH₂O
Adjust pH to 7.4
Bring to 1 liter with dH₂O
Filter sterilize
Store indefinitely at 4°C

PBS with 0.1% (w/v) sodium azide

445 ml dH₂O
50 ml 10× PBS
5 ml 10% (w/v) sodium azide
Store at room temperature

PTwH solution

Add 50 ml of 10× PBS to 447.5 ml dH₂O
Add 1 ml Tween 20
Stir until dissolved
Add 1 ml of 10 mg/ml heparin
Stir until dissolved
Add 0.5 ml of 10% (w/v) sodium azide
Store indefinitely at room temperature

PTx.2 solution

Add 50 ml of 10× PBS to 448.5 ml dH₂O
Add 1 ml Triton X-100
Stir until dissolved

Add 0.5 ml of 10% (w/v) sodium azide
Store indefinitely at room temperature

Refractive index–matching solution (RIMS)

40 g iohexol (Histodenz, Nycodenz)
30 ml of 0.02 M phosphate buffer (see recipe)
0.01% sodium azide
0.1% Tween 20
1 g 1,4-diazabicyclo[2.2.2]octane (DABCO)

Dissolve iohexol in phosphate buffer. Add remaining ingredients, then adjust pH to 7.5 with NaOH. Store up to 1 month at room temperature.

SeeDB solutions

For solutions 1–5: Add 2, 4, 6, 8, or 10 g fructose (Sigma, cat. no. 1286504) to 10 ml dH₂O in a 15-ml conical tube and place on a nutating mixer until dissolved. Once the fructose is dissolved, add 50 µl α-thioglycerol (Sigma, cat. no. M1753). Store up to 1 week at 4°C (solutions 1-2) or room temperature (solutions 3-5).

For SeeDB solution 6: Add 40.1 g fructose to 9.9 ml water in a 50-ml conical tube and place in a 65°C water bath until dissolved or up to 5 hr. If fructose is not dissolved after 5 hr, remove from the bath to prevent caramelization and place on a nutating mixer until the fructose is completely dissolved. Once the fructose is dissolved, add 50 µl α-thioglycerol. Store at up to 1 week at room temperature.

For SeeDB37: Prepare as described for solution 6, using 43.4 g fructose and 6.7 ml water in a 50-ml conical tube. Once the fructose is dissolved, add 50 µl α-thioglycerol. Store up to 1 week at 37°C.

Final concentrations are 20%, 40%, 60%, 80% and 100% (w/v) fructose for solutions 1–5, respectively; 80.2% (w/w) fructose for solution 6; and 86.7% (w/w) fructose for SeeDB37. All solutions contain 0.5% α-thioglycerol.

SeeDB37 must be stored at 37°C to prevent the fructose from precipitating.

COMMENTARY

Background Information

The value of imaging deep into tissues has been appreciated since the early 1900s, when Werner Spalteholz developed his formula for clearing tissues using benzyl benzoate, methyl salicylate, and wintergreen oil (Spalteholz, 1914). Until this time, looking into tissues could only be done using tools outside the visible light spectrum, mainly X-rays. Clearing led to advancements in the understanding of gross anatomy. However, the earliest methods could not be directly applied to modern optical imaging technology because they damaged tissue and quenched endogenous fluorescent proteins.

Currently, tissue clearing is enjoying a renaissance, corroborated by the flood of high-impact papers reporting the development of novel and hybrid techniques. Clearing methods are now applicable to a wide variety of organs, tissue types, and whole animals.

This is driven by the availability of high-quality reagents and instrumentation. Fluorescent proteins, fluorescent molecules, antibodies, and molecular probes are applicable to nearly all biological disciplines. In addition, modern confocal, light sheet, multiphoton, and ultra microscopes can build volumes through optical sectioning and, when paired with modern computing technologies, researchers can reconstruct whole tissues or whole organisms without the need for physical sectioning. As researchers discover the utility of these approaches, tissue clearing techniques are being implemented across disciplines. Clearing protocols now exist for most applications. Although methods vary widely in their approach, they are generally designed to preserve fluorescent proteins and lipid components and enable immunostaining (Azaripour et al., 2016; Richardson & Lichtman, 2015).

Critical Parameters

Paraformaldehyde fixation

Each of the protocols presented herein indicates that samples should be fixed in 4% PFA for an extended period (overnight to 24 hr) prior to clearing. Long fixation steps at high concentrations of PFA are often discouraged when planning imaging experiments, as increases in autofluorescence can occur along with masking of some epitopes. However, when clearing tissue, it is critical to adhere to these fixation conditions, since extensive cross-linking of proteins is required to maintain the integrity of the tissue during extended incubations in warm temperatures and when lipids are removed. Although exceptions can be made for all protocols, it is suggested that they be carefully tested prior to designing an experiment. Methods like SeeDB or CLARITY will be more forgiving of a lighter fixation due to support of the tissue from retained lipids or the hydrogel matrix, respectively. However, in the case of CLARITY, more protein will be lost during processing, which will adversely affect fluorescent signal and immunostaining.

Tissue staining

Staining of large tissues requires special considerations to allow staining reagents to diffuse throughout the large volume, primarily extended incubation times. Success can vary widely based on the tissue type, antibodies/antigens, and tissue size. Generally, staining should be avoided when a suitable endogenous marker can be substituted. If staining must be used, measures that reduce incubation time and increase the probability of even staining throughout the tissue should be considered. 'Small' alternatives to traditional antibodies that will diffuse quickly and evenly can be used, such as fluorescent molecular probes, FAB fragments, and nanobodies. Finally, conjugated primary antibodies can be used instead of traditional primary-secondary combinations to avoid lengthy secondary stains and a potential increase in background.

CLARITY-ETC

When designing or operating the ETC device, it is recommended to maintain a constant current and a variable voltage. The limits of each should be no greater than 1.5 A and 60 V. As the lipid-detergent micelle concentration and temperature increase in the running buffer, the resistance of the buffer changes. With a constant-current DC power supply, the system will adjust the voltage to maintain a

constant current. As current generates heat, it is best to maintain a constant current to ensure that heat is dissipated at a predictable rate (either passively or via a Peltier-based system). Starting with a high voltage will also begin the clearing with a high current (due to the low initial resistance of the clearing buffer) and generate excess heat, thus possibly denaturing proteins of interest. If a higher average voltage is desired, to decrease the clearing time or due to ETC device-specific constraints, the concentration of the boric acid electrolyte in the running buffer can be reduced. The relationship between the electrolyte concentration and the amperes required to maintain a voltage is roughly linear. Thus, halving the concentration of boric acid will result in a two-fold increase in voltage with no change to the current. Careful testing should be undertaken before applying changes to the running buffer in an experiment, as variations in osmolarity of the running buffer and voltage can result in tissue damage and over-clearing. Over-clearing will damage fluorescent proteins and reduce antigen.

It is imperative to not over-fix the tissue when performing CLARITY-ETC, as over-fixed tissue will not adequately clear. The opposite holds true, as well. It is important to ensure that any fixatives are fresh and are at the proper pH. Failure to clear or failure to maintain tissue integrity is almost always caused by a problem with the initial fixation. Additionally, it is important to not over-clear the tissue. In ETC, scorching is a potential problem with over-clearing. In passive CLARITY and CLARITY-ETC, fluorescent protein loss and epitope loss can occur with extended clearing times. It is highly recommended to test each tissue under a variety of conditions prior to designing an experiment.

Clearing buffer may also be reused between ETC runs, but close attention must be paid to the color of the buffer between uses. When fresh, it should be clear. As lipids are extracted from the tissue, the buffer will take on a pale, straw-yellow hue. When the buffer is noticeably yellow, it should be discarded. Twenty-four hours of run time is generally a good lifetime for a liter of running buffer.

Troubleshooting

The success of a clearing approach should be evaluated empirically according to specific experimental goals. The ability to detect markers of interest and collect imagery at the required tissue depths are examples of

important parameters. Hence, a tissue that remains partially opaque is not necessarily an indicator of failure if the outcomes of the experiments can be achieved. When those outcomes are not satisfactory, the method should be modified or an alternative clearing method should be chosen. The clearing protocols presented herein are diverse, with numerous stages where alterations may be required to maximize success. Alterations and points of caution are addressed within each protocol where appropriate. Additionally, some of these points have been addressed in more detail in the Critical Parameters section. Generally, it is suggested that one become familiar with multiple clearing protocols. Often, unsatisfactory clearing results can be ameliorated by using a different clearing approach, as evident when examining Table 1.

Anticipated Results

All the protocols described herein are intended to optically clear mammalian tissues to enable deeper imaging on optical microscopes. Some tissues, for example brain, will clear well with all described protocols, whereas others, for example liver, will only clear well with CLARITY-ETC. See Table 1 for examples.

Time Considerations

The time required to complete clearing will depend on many factors, including the clearing protocol, the size and composition of the tissue, and whether staining is required. Times can be expected to vary from 2 days to 1 month.

In general, it is recommended to use tissues as quickly as possible after harvesting from an animal. However, for all protocols, tissues can be stored at 4°C in PBS with 0.01% sodium azide for an undefined period of time. The authors have had excellent results clearing tissues that had been stored for many months in this way. Archived tissues frozen in sucrose can also be used for clearing, however, this is not recommended because fragility of the tissue increases, making it more likely to dissociate under clearing conditions. Nonetheless, the authors do not discourage the use of archived material, since various tissues and experimental conditions may yield excellent results.

Acknowledgements

The authors would like to thank members of the Center for Biologic Imaging at the University of Pittsburgh for their support. D.C. was supported by the intramural program of the Eunice Kennedy Shriver National Insti-

tute of Child Health and Human Development, National Institutes of Health (ZIA-HD008808 and ZIA-HD001011, awarded to Brant M. Weinstein). G.A.G. and A.M.W. were supported by the National Institute on Aging (5P01AG043376-05).

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Internet Resources

<https://wiki.claritytechniques.org/>

An excellent resource for all aspects of the CLARITY and CLARITY-ETC protocols. Information on everything from preparing solutions to making an ETC chamber can be found here.

<https://cubic.riken.jp/>

This website links to information on the CUBIC protocol.

<https://idisco.info/>

This website links to information on the iDISCO protocol and maintains updates to the originally published protocol.