Published in final edited form as:

Curr Protoc Cytom. 2018 October ; 86(1): e38. doi:10.1002/cpcy.38.

Clearing for Deep Tissue Imaging

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Abstract

Tissue clearing offers an excellent alternative to physical thin-sectioning that is often required with optical imaging. Generally, sections are thin, 50–100 μ m, to allow for the delivery and recovery of light. Tissue clearing reduces the limitations of light delivery and recovery, first by removing light-absorbing and light-scattering molecules, and then by matching the refractive index of the tissue with a solution in which the tissue is imaged. When clearing is done well, long-working distance optics can peer many millimeters deep. 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. Here, we describe four popular clearing protocols that will be relevant to a wide variety of scenarios across biologic disciplines: CUBIC, CLARITY, 3DISCO, and SeeDB.

Keywords

Tissue clearing; Clear; Unobstructed Brain/Body Imaging Cocktails (CUBIC); CLARITY; three-dimensional imaging of solvent-cleared organs (3DISCO); See Deep Brain (SeeDB)

INTRODUCTION

Traditionally, optical microscopy required physically sectioning thin discrete regions of tissue for imaging by fluorescence microscopy. Such tissue preparation was a requirement

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INTERNET RESOURCES (optional)

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

An excellent resource for all aspects of the CLARITY and CLARITY-ETC protocols. Information on everything from solutions to how to make ETC chambers can be found here.

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

This website links to information on the CUBIC protocol.

<https://idisco.info/>

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

since 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 the 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 that you wish to clear, the depth that you wish to image, whether you need to immunostain and/or visualize endogenous fluorescence and the resources that are available to you. 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, it 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 the proteins that we wish to study. This is because the bio-molecular 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 and 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 to dehydrate, remove lipids, and refractive index match. In this protocol, we describe four independent clearing approaches, CUBIC (Basic Protocol 1) (Susaki et al., 2014, 2015), Passive and Active CLARITY (Basic Protocol 2 and Alternative Protocol 1) (Yang et al., 2014; Lee et al., 2016; Chung et al., 2013; Chung and Deisseroth, 2013), 3DISCO (Basic Protocol 3) (Ertürk et al., 2012; Renier et al., 2014; Pan et al., 2016) and SeeDB (Basic Protocol 4) (Ke et al., 2013), that represent popular well-established methods that can be used to clear most mammalian tissues and enable the interrogation of both proteins and lipids within biologic tissues. These methods are compatible with endogenous fluorescence proteins and immunohistochemical labeling.

STRATEGIC PLANNING

When deciding what clearing protocol will work best, two defining variables should be considered. 1) What is being imaged (Endogenous fluorescence, immunostained antigens and/or lipids) and 2) what tissue type will be imaged. As guidance, a flow chart is provided in Figure 1 that will narrow down what 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. Instead, it 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 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, the authors suggest trying more than one clearing approach to maximize success.

Basic Protocol 1: Unobstructed Brain/Body Imaging Cocktails (CUBIC)

CUBIC (Susaki et al., 2015, 2014) 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 with endogenous fluorescence and requiring antibody staining. CUBIC is an excellent starting point for researchers exploring tissue clearing for the first time.

Materials

4% Paraformaldehyde (see recipe)
 Hydrogel (optional, see recipe)
 Nitrogen or vacuum chamber (optional)
 CUBIC R1 (see recipe)
 CUBIC IHC (optional)
 CUBIC R2 (see recipe)
 CUBIC R2 w/o TEA (optional)
 Distilled water
 PBS with 0.1% Sodium Azide (w/v) (see recipe)
 50 mL plastic conical tube (Fisher Scientific, cat. no. 14-959-49A)
 Nutating mixer (Fisher Scientific, cat. no. 88-861-041)
 37°C Incubator
 37°C Water bath

Fix and Harvest Tissue

- 1 (optional) Perfuse the 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 the tissue from the animal and then immerse in three volumes of 4% PFA, or at least enough to cover the tissue. Store at 4°C for overnight to 24 hours.

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

- 3 Immerse in the tissue in 3 volumes of hydrogel (see recipe) overnight – 24 hours at 4°C.

- 4 (Optional) Degas hydrogel with nitrogen or under vacuum for 10 minutes.

VA-044 will preferentially react with oxygen as opposed to the hydrogel, and 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.

When using nitrogen, the gas can be gently bubbled into the hydrogel solution or the hydrogel solution can be placed in a chamber, open capped, where oxygen has been purged with nitrogen.

When using a 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.

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

Note: Polymerization will proceed slower in an incubator, requiring the 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 slower.

- 6 Remove the tissue from the polymerized hydrogel (it should be a viscous, thick gel, not firm, but not runny).

- 7 (optional) Wash tissues three times for 10 minutes in PBS with 0.1% Sodium Azide (w/v) (with a volume used in step 2) and store for up to 1 month at 4°C in PBS with 0.1% Sodium Azide (w/v).

Clear Tissue

- 8 Place the tissue in 3 volumes of CUBIC R1 diluted 1:1 with distilled water. Incubate at 37°C on a nutating mixer for 24 hours.
- 9 Change with fresh CUBIC R1 solution. Incubate for up to two 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 the solution with fresh CUBIC R1 (undiluted) every two days or when the solution takes on a greenish-brown color.

Move to the next step when the tissue appears clear and when the green-brown color is washed out of the tissue. This may take up to two weeks for very large tissues. For some tissues, the color may never fully dissipate.

- 11 (optional) If the tissue appears clear after CUBIC R1 incubation, it can now be mounted in CUBIC R1 and imaged for endogenous fluorescence.

Most tissues will benefit from a solution with a higher refractive index. Thus, in most situations, continue to “Mount Tissue.” If immunohistochemistry is required continue to “Stain Tissue.”

Stain Tissue (optional): Note: See ‘tissue staining’ in the Critical Parameters section.

- 12 Wash the tissue 3 times for 2 hours each in CUBIC IHC buffer, at 37°C on a nutating mixer.

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

- 13 Wash an additional time in CUBIC IHC buffer overnight, at 37°C on a nutating mixer.

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

- 14 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. For example, when staining with antibodies, a whole mouse brain requires 1 week whereas mouse ovaries and lungs require only 24 hours.

Note: Hydrogel embedded tissue may require a longer incubation step.

- 15 Wash the tissue 3 times for 2 hours each in CUBIC IHC buffer, at 37°C on a nutating mixer.

- 16 (optional) wash an additional time in CUBIC IHC buffer overnight, at 37°C on a nutating mixer.

This step may help reduce background, but it is not necessary.

- 17 (optional) Repeat steps 14–16 if a secondary stain is required.

Store Tissue (optional): Cleared tissues should be imaged immediately after clearing by continuing with the “Mount Tissue” protocol. When necessary, tissues can be stored for up to a few months at 4°C and imaged later.

Note: Tissue should always be imaged as quickly as possible after processing. Storage can result in the degradation of the tissue and fluorescent signal, particularly from endogenous proteins.

- 18 Wash the tissue 3 times for 2 hours at 37°C on a nutating mixer.
 - a. If the tissue was stained using the optional “Stain Tissue” protocol: wash with CUBIC IHC.
 - b. If the tissue was NOT stained using the optional “Stain Tissue” protocol: wash PBS with 0.1% Sodium Azide (w/v).
- 19 (optional) For samples that were stained with the optional “Stain Tissue” protocol, fix in three volumes of 4% PFA overnight at 4°C. Wash out the PFA by repeating the previous step.

Fixing samples can make stains permanent through crosslinking. This is helpful when storing antibody-stained samples for an extended period.
- 20 Immerse the tissue in PBS with 0.1% Sodium Azide (w/v) and store at 4°C.

When the tissue is ready to be imaged, continue with the “Mount Tissue” protocol.

Mount Tissue

- 21 Wash the tissue 3 times for 2 hours at 37°C on a nutating mixer.
 - a. If the tissue was stained using the optional “Stain Tissue” protocol: wash with CUBIC IHC.
 - b. If the tissue was NOT stained using the optional “Stain Tissue” protocol: wash PBS with 0.1% Sodium Azide (w/v). The wash steps can also be abbreviated to 1 hour each
- 22 (optional) If the tissue was stained using the optional “Stain Tissue” protocol: wash an additional time in CUBIC IHC buffer overnight, at 37°C on a nutating mixer.
- 23 Add CUBIC R2 to the tissue and incubate at 37°C on a nutating mixer for 1 hour.

Note: 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 w/o TEA. The refractive index of CUBIC R2 w/o TEA is slightly lower than CUBIC R2, which may impact clearing of the tissue. Other refractive

index matching solutions may be used in place of CUBIC R2, like RIMS (see recipe).

- 24 Replace with fresh CUBIC R2 and incubate overnight – 24 hours. The tissue will shrink.
- 25 Replace with fresh CUBIC R2 every 24 hours until the tissue appears to have maximally cleared.

The time required to re-clear will vary based on the size and the type of tissue. Prolonged incubation (> 1 week) in CUBIC R2 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 “Mount Tissue” protocol which will cause the tissue to shrink.

- 26 Mount the cleared tissue in CUBIC R2 and image.
- 27 (optional) After imaging, the tissue can be stored for longer than 1 week by following the “Store Tissue” protocol.

Basic Protocol 2: 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

- 4% paraformaldehyde (see recipe)
- Hydrogel (see recipe)
- Nitrogen or vacuum chamber (optional)
- 8% SDS solution
- Refractive Index Matching Solution (RIMS, see recipe)
- 37°C water bath
- 37°C incubator
- Nutating mixer

Fix and Harvest Tissue

- 1 (optional) Perfuse the 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 the tissue from the animal and then immerse in three volumes of 4% PFA, or at least enough to cover the tissue. Store at 4°C for overnight to 24 hours.

A gentler fixation at 2% PFA or for a shorter 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.

- 3 After fixation, wash the tissue in room-temperature PBS

Embed in Hydrogel

- 4 Immerse the tissue in hydrogel overnight – 24 hours at 4°C.
- 5 (Optional) Degas hydrogel with nitrogen or under vacuum for 10 minutes.

VA-044 will preferentially react with oxygen as opposed to the hydrogel, and this step can minimize the amount of oxygen diffused in the sample and in the head space above in the tube. It is not required, as the gel will still polymerize without degassing.

When using nitrogen, the gas can be gently bubbled into the hydrogel solution or the hydrogel solution can be placed in a chamber, open capped, where oxygen has been purged with nitrogen.

When using a 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.

- 6 Polymerize the hydrogel by moving to a water bath or an incubator at 37°C for 4 or 6 hours, respectively.

Note: Polymerization will proceed slower in the incubator, requiring the additional incubation time. When necessary, polymerization can be allowed to proceed overnight. Caution: clearing efficiency may be reduced by increased polymerization time. Additionally, immunostaining may proceed slower.

- 7 Remove the tissue from the polymerized hydrogel (it should be a viscous, thick gel, not firm, but not runny).
- 8 (optional) Wash tissues three times for 10 minutes (with a volume used in step 2) in PBS with 0.1% Sodium Azide (w/v) and store for up to 1 month at 4°C in PBS with 0.1% Sodium Azide (w/v).

Clear Tissue

- 9 Place the tissue in approximately 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 concentration of the SDS solution is 8%, but the concentration can be altered depending on tissue fragility and lipid content. Lower concentrations of SDS will clear slower and be a bit gentler on the tissue, while higher concentrations of SDS will clear faster and be harsher. Clearing can take anywhere from hours to weeks, depending on tissue size and lipid content.

- 10 Once clear, remove the tissue from the SDS and wash with room-temperature PBS with 0.1% Sodium Azide (w/v) repeatedly until foaming from the SDS is no longer present.

It is imperative that all of the detergent is 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 minutes at 37°C. Wash numbers and times are expected to vary according to tissue, size and concentration of SDS. 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 than that of water.

Stain Tissue (optional)—Note: See tissue staining in the Critical Parameters section

- 11 Wash the tissue 3 times for 2 hours each in IHC buffer, at 37°C on a nutating mixer.
- 12 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 and temperature 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 hours.

- 13 Wash the tissue 3 times for 2 hours each in IHC buffer, at 37°C on a nutating mixer.
- 14 (optional) wash an additional time in IHC buffer overnight, at 37°C on a nutating mixer.

This step may help reduce background, but it is not necessary.

- 15 (optional) Repeat steps 12–14 when a secondary stain is required.

Optimize Refractive Index

- 16 Incubate tissue in RIMS until transparent. Multiple changes may be required.
- 17 Mount in fresh RIMS and image.

Alternative solutions designed for refractive index optimization can be used in place of RIMS including CUBIC R2 and CUBIC R2 w/o TEA.

Alternative Protocol 1: CLARITY - Electrophoretic Tissue Clearing (ETC)

Electrophoretic tissue clearing (Chung et al., 2013; Chung and Deisseroth, 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. Since 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 you may build your own system (Lee et al., 2016; Chung et al., 2013; Chung and Deisseroth, 2013) (also see “CLARITY wiki” under internet resources). Electrophoretic tissue clearing greatly reduces the amount of time required to clear tissue, allowing the entire protocol to be completed in less than two days. Since the commercial solutions 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.

Materials

- CLARITY-ETC Running Buffer (see recipe)
- Hydrogel (see recipe)
- Nitrogen or vacuum chamber (optional)
- Refractive Index Matching Solution (RIMS, see recipe)
- Electrophoretic Tissue Clearing Chamber (see internet resources or (Lee et al., 2016; Chung et al., 2013; Chung and Deisseroth, 2013))

Fix and Harvest Tissue

- 1 (optional) Perfuse the 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 the tissue from the animal and then immerse in three volumes of 4% PFA, or at least enough to cover the tissue. Store at 4°C for overnight to 24 hours.

A gentler fixation at 2% PFA or for a shorter 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.
- 3 After fixation, wash the tissue in room-temperature PBS

Embed in Hydrogel

- 4 Immerse in the tissue in hydrogel (see recipe) overnight – 24 hours at 4°C.
- 5 (Optional) Degas hydrogel with nitrogen or under vacuum for 10 minutes.

VA-044 will preferentially react with oxygen as opposed to the hydrogel, and this step can minimize the amount of oxygen diffused in the sample and in the head space above in the tube. It is not required, as the gel will still polymerize without degassing.

When using nitrogen, the gas can be gently bubbled into the hydrogel solution or the hydrogel solution can be placed in a chamber, open capped, where oxygen has been purged with nitrogen.

When using a 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.
- 6 Polymerize the hydrogel by moving to a water bath or incubator at 37°C for 4 or 6 hours, respectively.

Note: Polymerization will proceed slower in the incubator, requiring the additional incubation time. When necessary, polymerization can be allowed to proceed overnight. Caution: clearing efficiency may be reduced by increased polymerization time. Additionally, immunostaining may proceed slower.
- 7 Remove the tissue from the polymerized hydrogel (it should be a viscous, thick gel, not firm, but not runny).
- 8 (optional) Wash tissues three times for 10 minutes (with a volume used in step 2) in PBS with 0.1% Sodium Azide (w/v) and store for up to 1 month at 4°C in PBS with 0.1% Sodium Azide (w/v).

Clear Tissue

- 9 Place the tissue into the ETC clearing chamber, fill with ETC running buffer, and energize the device (start with 1.5A and no more than 60V, 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.
- 10 Once clear, remove the tissue from the SDS and wash with room-temperature PBS with 0.1% Sodium Azide (w/v) repeatedly until foaming from the SDS is no longer present.

It is imperative that all of the detergent is 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 minutes at 37°C. Wash numbers and times are expected to vary according to tissue, size and concentration of SDS. 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 than that of water.

Stain Tissue (optional)—Note: See tissue staining in the Critical Parameters section

- 11** Wash the tissue 3 times for 2 hours each in IHC buffer, at 37°C on a nutating mixer.
- 12** 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 and temperature 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 hours.

- 13** Wash the tissue 3 times for 2 hours each in IHC buffer, at 37°C on a nutating mixer.
- 14** (optional) wash an additional time in IHC buffer overnight, at 37°C on a nutating mixer.

This step may help reduce background, but it is not necessary.

- 15** (optional) Repeat steps 12–14 for secondary stains.

Optimize Refractive Index

- 16** Incubate tissue in RIMS until transparent. Multiple changes may be required.
- 17** Mount in fresh RIMS and image.

Alternative solutions designed for refractive index optimization can be used in place of RIMS including CUBIC R2 and CUBIC R2 w/o TEA.

Basic Protocol 3: Three-Dimensional Imaging of Solvent-Cleared Organs (3DISCO)

Organic solvent-based clearing methods generally offer the best clearing results over a wide range of tissues. This is because organic-solvents can obtain 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 poorly clear. Numerous solvent-based methods have arisen 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 have 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 since the clearing solvents can dissolve some mounting materials as well as the seals on some microscope immersion objectives. In addition, preparation of the tissue requires dehydration which can cause shrinkage 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 quickly wanes, requiring that samples are 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. The authors suggest 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.

Note: Two protocols are described below which are specific for either imaging either endogenous fluorescence or immunostaining. The protocol for immunostaining with 3DISCO requires methanol treatment which will quench endogenous fluorescent proteins like GFP.

Part 1: 3DISCO: Endogenous Fluorescence

Materials

4% Paraformaldehyde (see recipe)

Distilled water

tert-butanol (Sigma, cat. no. 360538)

Dibenzyl Ether (DBE; Sigma, cat no. 108014)

Dichloromethane (DCM; optional; Sigma, cat. no. 270997)

PBS with 0.1% Sodium Azide (w/v) (see recipe)

50 mL polypropylene plastic conical tube (Fisher Scientific, cat. no. 14-959-49A)

Note: Polypropylene plastic tubes (alternatively glass tubes) should be used when working with the organic solvents in this protocol.

Nutating mixer (Fisher Scientific, cat. no. 88-861-041)

37°C Incubator

Fix and Harvest Tissue

- 1 (optional) Perfuse the 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 the tissue from the animal and then immerse in three volumes of 4% PFA, or at least enough to cover the tissue. Store at 4°C for overnight to 24 hours.

Dehydrate and Clear

- 3 Immerse tissue in 30% tert-butanol diluted in dH₂O for 1 hour at 37°C on a nutating mixer and repeat for 50%, 70%, 80%, 90%, 100% and 100% dilutions of tert-butanol.

The volume of tert-butanol should be approximately three times the tissue volume or at least cover the tissue.

Note: 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 hour at room temperature.

DCM acts as a strong delipidating agent. For small tissues it is often unnecessary. However, DCM is recommended for large tissues like whole brains because it will significantly improve tissue clarity. Care should be taken when handling DCM. Skin contact or inhalation will cause irritation.

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

The tissue should noticeably begin to clear within 30 minutes 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.

Note: 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.

Note: If the interior regions of the tissue appear opaque after multiple DBE changes, this usually indicates poor dehydration of the tissue, often due to incubating the tissues for too short of a time or not providing movement during the dehydration phases. 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 the sample in DBE for imaging.

Reminder: Care must be taken when mounting samples since DBE will soften or melt many plastics and may dissolve the seals on microscope immersion objectives.

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

Part 2: 3DISCO: Immunostaining

Materials

4% Paraformaldehyde (see recipe)
 Distilled water
 Methanol (Fisher, cat. no. A412SK-4)
 tert-butanol (Sigma, cat. no. 360538)
 PTx.2 (see recipe)
 3DISCO permeabilization solution (see recipe)
 3DISCO blocking solution (see recipe)
 3DISCO staining solution (see recipe)
 PTwH solution (see recipe)
 Dibenzyl Ether (DBE; Sigma, cat no. 108014)
 Dichloromethane (DCM; optional; Sigma, cat. no. 270997)
 Hydrogen Peroxide (optional; Sigma, cat. No. 216763)
 PBS with 0.1% Sodium Azide (w/v) (see recipe)
 50 mL plastic conical tube (Fisher Scientific, cat. no. 14-959-49A)
 Nutating mixer (Fisher Scientific, cat. no. 88-861-041)
 37°C Incubator

Fix and Harvest Tissue

- 1 (optional) Perfuse the 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 the tissue from the animal and then immerse in three volumes of 4% PFA, or at least enough to cover the tissue. Store at 4°C for overnight to 24 hours.

Tissue Immunostaining Pretreatment: Note: 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 can be done on frozen sections that are incubated in

100% methanol for 3 hours and then rehydrated in PBS prior to immunostaining. The sections should be evaluated for retention of antibody specificity and signal to noise.

- 3 Dehydrate the tissue in 3 volumes of 30% methanol for 1 hour at room temperature with gentle movement, and repeat for 50%, 70%, 90%, 100%, and 100% dilutions of methanol.

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

- 4 Incubate in 3:1 DCM/methanol, overnight at room temperature with gentle movement.

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

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

- 6 (optional) bleach the tissue with 5% H₂O₂ in methanol, overnight at 4°C.

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

- 7 Rehydrate with 90%, 70%, 50% and 30% methanol for 1 hour with gentle movement.

- 8 Wash in PBS with 0.1% Sodium Azide (w/v) for 1 hour each with gentle movement.

Immunostaining: Note: See tissue staining in the Critical Parameters section

- 9 Wash twice in PTx.2 for 1 hour 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 for serum from a species of interest (mouse, donkey, goat, etc.).

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

- 13 Wash 4 times in PTwH Solution for at least 1 hour and then again overnight at 37°C with gentle movement.

- 14 (optional) Stain with primary antibodies in 3DISCO Staining Solution at 37°C with gentle movement for up to 2 weeks.

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

- 15** (optional) If a secondary stain was used: Wash 4 times in PTwH Solution for at least 1 hour and then again overnight at 37°C with gentle movement.

Dehydrate and Clear

- 16** Immerse tissue in 30% tert-butanol for 1 hour at 37°C on a nutating mixer and repeat for 50%, 70%, 80%, 90%, 100% and 100% dilutions of tert-butanol.

The volume of tert-butanol should be approximately three times the tissue volume or at least cover the tissue.

Note: 100% tert-butanol will freeze at room temperature. It should be stored at 37°C or melted at 37°C prior to use.

- 17** (optional) Incubate in the same volume of DMC for 1 hour at room temperature.

DCM acts as a strong delipidating agent. For small tissues it is often unnecessary. However, DCM is recommended for large tissues like whole brains because it will significantly improve tissue clarity.

- 18** Move the tissue to 3 volumes of DBE and incubate for 1 hour at 37°C on a nutating mixer.

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

- 19** *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.

Note: 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.

Note: If the interior regions of the tissue appear opaque after multiple DBE changes, this usually indicates poor dehydration of the tissue, often due to incubating the tissues for too short of a time or not providing movement during the dehydration phases. 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 11–12.

- 20** Mount the sample in DBE for imaging.

Reminder: Care must be taken when mounting samples since DBE will soften or melt many plastics and may dissolve the seals on microscope immersion objectives.

Note: Samples can be stored in DBE indefinitely. Fluorescent tags generally maintain their fluorescence indefinitely after processing.

Basic Protocol 4: 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 like those seen in 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 et al., 2017), and zebrafish. The high viscosity of SeeDB limits its penetration into larger tissues. Modifications can increase the penetration and clearing capabilities of SeeDB, like FRUIT (Hou et al., 2015). The FRUIT protocol adds urea which decreases viscosity and allows for the clearing 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% Paraformaldehyde (See Recipe)
 SeeDB Solutions 1–6 (See Recipe)
 SeeDB37 (optional, See Recipe)
 Nutating Mixer (Fisher Scientific, cat. no. 88-861-041)
 50 mL plastic conical tube (Fisher Scientific, cat. no. 14-959-49A)
 15 mL plastic conical tube (Fisher Scientific, cat. no. S50712)
 65°C water bath
 37°C Incubator (for protocol modification)

Fix and Harvest Tissue

- 1 (optional) Perfuse the 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 the tissue from the animal and then immerse in three volumes of 4% PFA, or at least enough to cover the tissue. Store at 4°C for overnight to 24 hours.

A gentler fixation at 2% PFA or for a shorter 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): Note: 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 200–500µm. Additionally, see tissue staining in the Critical Parameters section.

- 3 Wash the tissue 3 times for 2 hours each in IHC buffer, at 37°C on a nutating mixer.

The TritonX-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 the tissue 3 times for 2 hours each in CUBIC IHC buffer, at 37°C on a nutating mixer.

- 6 (optional) wash an additional time in CUBIC IHC buffer overnight, at 37°C on a nutating mixer.

This step may help reduce background, but it is not necessary.

- 7 (optional) Repeat steps 4–6 for secondary stains.

Clear Tissue

- 8 Wash tissues three times for 10 minutes in PBS.
- 9 Place the tissue in SeeDB Solution 1 (20% wt/vol fructose) at room temperature on a nutating mixer for 4–8 hours.
- 10 Place the tissue in SeeDB Solution 2 (40% wt/vol fructose) at room temperature on a nutating mixer for 4–8 hours.
- 11 Place the tissue in SeeDB Solution 3 (60% wt/vol fructose) at room temperature on a nutating mixer for 4–8 hours.
- 12 Place the tissue in SeeDB Solution 4 (80% wt/vol fructose) at room temperature on a nutating mixer for 12 hours.
- 13 Place the tissue in SeeDB Solution 5 (100% wt/vol fructose) Solution at room temperature on a nutating mixer for 12 hours.
- 14 Place the tissue in SeeDB Solution 6 (80.2% wt/wt fructose) at room temperature on a nutating mixer for 24 hours.

- 15** (optional) Place the tissue in SeeDB37 for 48 hours 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.

Store tissues in SeeDB solution 6 (SeeDB) at room temperature, or in SeeDB37 at 37°C for a week. Samples should be kept in solution 6 (SeeDB) or SeeDB37 during imaging.

Note: 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: In addition to the standard protocol shown above, several variations have been created to address specific sample types:

SeeDBp – prevents tissue expansion which is seen in some tissues like neonatal brain samples: Follow the standard protocol above with the substitution of 0.1x PBS for distilled water when making SeeDB solutions 1–4.

SeeDB37ht – increases the speed and penetration of clearing: Follow the standard protocol above but change incubation times and temperatures as follows: incubate in SeeDB solutions 1–4 at 50°C for 3 hours each, followed by a 12 hour incubation in SeeDB solution 5 at 50°C. Next, incubate in SeeDB Solution 6 at 50°C for 24 hours followed by an incubation in SeeDB37 for 24 hours at 50°C. Store and image samples at 37°C.

REAGENTS AND SOLUTIONS

Note: Use deionized, distilled water in all recipes and protocol steps.

Common Solutions

Sodium Azide, 10% (w/v) – 50mL

Add 5g of sodium azide (Sigma-Aldrich, cat. no. S2002) to 50mL of dH₂O.

Store at room temperature

PBS with 0.1% Sodium Azide (w/v) - 500mL

Mix 50mL of 10x PBS, 445mL of dH₂O and 5mL of Sodium Azide, 10% (w/v).

Store at room temperature

Paraformaldehyde, 8% and 4% - 100mL

Prepare an 8% paraformaldehyde stock

1. Add 8g of paraformaldehyde resin (Fisher Scientific, cat. no. O4042-500) to 70 ml dH₂O.
2. Heat to 60°C (no higher) with stirring.

3. Add 1 N NaOH until the solution clears, usually a couple of drops.
4. Cool to room temperature.
5. Add 9 ml of 10x PBS and adjust the volume to 100 ml with dH₂O.
6. (optional) filter sterilize.

Prepare 4% paraformaldehyde

1. Dilute 50ml of the 8% paraformaldehyde stock with 50 ml of dH₂O.
2. (optional) filter sterilize.

Store solutions at 4°C

IHC Buffer – 500mL

1. To 500mL of PBS, Add 500µL of TritonX-100
2. Add 2.5g of bovine serum albumin
3. Add 500µL of 10% sodium azide stock solution

Store at room temperature

Hydrogel - 50mL

1. Mix 5mL of 10x PBS to 38.75 mL of H₂O in a 50mL conical tube.
2. Add 5mL of 40% Acrylamide (Bio-Rad, cat. No. 161-0140)
3. Add 1.25mL of 2% Bis-Acrylamide (Bio-Rad, cat no. 161-0142)
4. Add 0.175g of VA-044 (Wako Chemicals, fisher scientific cat. no. NC0632395), mix gently until dissolved.
5. Keep at 4°C for all steps prior to polymerization.
6. (optional) Remove excess oxygen by degassing under vacuum or with nitrogen gas for 10 minutes

When using nitrogen, the gas can be gently bubbled into the hydrogel solution or the hydrogel solution can be placed in a chamber, open capped, where oxygen has been purged with nitrogen.

When using a 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.

7. Shift to 37°C in a water bath or incubator for 4 hours to overnight to polymerize.

Note: This solution should be made fresh when possible. However, it can be stored at 4°C for 1 week and –20°C for up to 1 month prior to use.

CUBIC Solutions

CUBIC R1 - 500g; ~420mL

1. Mix 125g of Urea (Sigma-Aldrich, cat. no. U5378) and 175mL of H₂O in a glass beaker.
2. Stir on a hot plate over low heat or place in a water bath, up to 56 degrees Celsius, until the urea dissolves.

Allowing the mixture to reach a temperature of up to 56 degrees, will facilitate other components going into solution, but this step is not necessary.

3. Add 123g (or 124mL) of Quadrol (*N,N,N',N'*-Tetrakis(2-Hydroxypropyl)ethylenediamine, Sigma-Aldrich, cat. no. 122262).

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

4. Stir over low heat until the Quadrol dissolves.
5. Add 70mL of TritonX-100 (Fisher, cat. no. BP151-500).
6. Remove from heat and stir until dissolved.

Store the solution sealed at room-temperature. The shelf-life is approximately 1 month. When the solution takes on a strong ammonia smell, it has expired. If the temperature is too high when making the solution, the ammonia smell will be immediately present, and the solution should be discarded.

CUBIC R2 - 500g; ~380mL

1. Mix 125g of Urea (Sigma-Aldrich, cat. no. U5378) and 75mL of H₂O in a glass beaker.
2. Stir on a hot plate over low heat or place in a water bath, up to 56 degrees Celsius, until the urea dissolves.

Allowing the mixture to reach a temperature of up to 56 degrees, will facilitate other components going into solution, but this step is not necessary. The container should remain loosely capped to limit evaporation.

3. Slowly add 250g of sucrose (Sigma-Aldrich, cat. no. S9378) with stirring over low heat.
4. Stir until dissolved using low heat. When dissolved, the solution will be extremely viscous.
5. Turn off heat and add 44.5mL of Triethanolamine (TEA) with stirring.
6. Add 380μL of TritonX-100 until well mixed.

Store the solution sealed at room-temperature. The shelf-life is approximately 1 month. When the solution develops a strong ammonia smell, it has expired. If the temperature is too high when making the solution, the ammonia smell will be immediately present, and the solution should be discarded.

CUBIC R2 w/o TEA - 495g; ~380mL

1. Mix 125g of Urea (Sigma-Aldrich, cat. no. U5378) and 119.5mL of H₂O in a glass beaker.
2. Stir on a hot plate over low heat or place in a water bath, up to 56 degrees Celsius, until the urea dissolves.

Allowing the mixture to reach a temperature of up to 56 degrees, will facilitate other components going into solution, but this step is not necessary. The container should remain loosely capped to limit evaporation.

3. Slowly add 250g of sucrose (Sigma-Aldrich, cat. no. S9378) with stirring over low heat.
4. Stir until dissolved using low heat. When dissolved, the solution will be extremely viscous.
5. Turn off heat and add 380μL of TritonX-100 until well mixed.

Store the solution sealed at room-temperature. The shelf-life is approximately 1 month. When the solution develops a strong ammonia smell, it has expired. If the temperature is too high when making the solution, the ammonia smell will be immediately present, and the solution should be discarded. The container should remain loosely capped to limit evaporation.

CLARITY Solutions

Phosphate Buffer – 1L

1. Add 3.1g NaH₂PO₄ (monohydrate) and 10.9g Na₂HPO₄ (anhydrous) to 500mL H₂O
2. Adjust the pH to 7.4
3. Bring to a total volume of 1L with H₂O
4. Sterile filter

Store indefinitely at 4°C.

Refractive Index Matching Solution (RIMS) - ~50mL

1. Dissolve 40g of iohexol (Histodenz, Nycodenz) in 30mL 0.02M phosphate buffer
2. Add 0.01% sodium azide

3. Add 0.1% Tween20
4. Add 1g DABCO (1,4-diazabicyclo[2.2.2]octane)
5. Adjust the pH to 7.5 with NaOH and store at room temperature

Store at room temperature for up to 1 month.

8% SDS Solution – 1L

Add 80g sodium dodecyl sulfate to H₂O and bring to a total volume of 1L, pH to 7.5.

Store indefinitely at room temperature.

CLARITY-ETC Running Buffer – 1L

1. Add 12.37g (200mM) Boric Acid to H₂O
2. Add 40g of SDS
3. Bring to a final volume of 1L, pH to 8.5 with NaOH.

Store at room temperature

3DISCO Solutions

PTx.2 - 500mL

1. Add 50mL of 10X PBS to 448.5mL of dH₂O
2. Add 1mL of TritonX-100, stir until dissolved
3. Add 0.5mL of Sodium Azide, 10% (w/v)

Store indefinitely at room temperature

PTwH Solution - 500mL

1. Add 50mL of 10X PBS to 447.5mL dH₂O
2. Add 1 mL of Tween-20, stir until dissolved
3. Add 1mL of 10mg/mL Heparin, stir until dissolved
4. Add 0.5mL of Sodium Azide, 10% (w/v)

Store indefinitely at room temperature

3DISCO Permeabilization Solution - 500mL

1. Add 11.5g of Glycine to 400mL of PTx.2, stir until dissolved
2. Add 0.5mL of Sodium Azide, 10% (w/v)
3. Add 99.5mL of DMSO

Store indefinitely at room temperature

3DISCO Blocking Solution - 50mL

1. Add 5mL of DMSO to 44.5mL of PTx.2

2. Add 0.5mL of Sodium Azide, 10% (w/v)
 3. Add 2.5g of BSA, stir until dissolved
- Store indefinitely at room temperature

3DISCO Staining Solution - 50mL

1. Add 2.5g of BSA to 49.5mL of PTwH, stir until dissolved
 2. Add 0.5mL of Sodium Azide, 10% (w/v)
- Store indefinitely at room temperature

SeeDB Solutions

SeeDB Solution 1: 20% (wt/vol) Fructose Solution with 0.5% α -thioglycerol

1. Add 2g of Fructose (Sigma, cat. no. 1286504) to 10 ml of distilled water, mix until dissolved
 2. Add 50 μ l of α -thioglycerol (Sigma, cat. no. M1753).
- Store at 4°C for up to 1 week.

SeeDB Solution 2: 40% (wt/vol) Fructose Solution with 0.5% α -thioglycerol

1. Add 4g of Fructose to 10 ml of distilled water, mix until dissolved
 2. Add 50 μ l of α -thioglycerol.
- Store at 4°C for up to 1 week.

SeeDB Solution 3: 60% (wt/vol) Fructose Solution with 0.5% α -thioglycerol

1. Add 6g of Fructose to 10 ml of distilled water, mix until dissolved
 2. Add 50 μ l of α -thioglycerol.
- Store at room temperature for up to 1 week.

SeeDB Solution 4: 80% (wt/vol) Fructose Solution with 0.5% α -thioglycerol

1. Add 8g of Fructose to 10 ml of distilled water, mix until dissolved
 2. Add 50 μ l of α -thioglycerol.
- Store at room temperature for up to 1 week.

SeeDB Solution 5: 100% (wt/vol) Fructose Solution with 0.5% α -thioglycerol

1. Add 10g of Fructose to 10 ml of distilled water, mix until dissolved
 2. Add 50 μ l of α -thioglycerol.
- Store at room temperature for up to 1 week.

SeeDB solutions 1–5 are easily prepared by combining fructose and water in a 15 ml conical tube and placing them on a nutating mixer until dissolved. Once the fructose is dissolved, add the α -thioglycerol.

SeeDB Solution 6: (SeeDB Final Solution): 80.2% (wt/wt) Fructose Solution with 0.5% α -thioglycerol

1. Add 40.1g of Fructose to 9.9 ml distilled water, mix until dissolved
2. Add 50 μ l of α -thioglycerol.

Store at room temperature for up to 1 week.

To prepare SeeDB Solution 6, combine fructose and water in a 50 ml conical tube and place in a 65° C water bath until dissolved or up to 5 hours. If fructose isn't dissolved after the 5 hour 65° C water bath incubation 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 the α -thioglycerol. Store at room temperature.

***SeeDB37: 86.7% (wt/wt) Fructose Solution with 0.5% α -thioglycerol**

1. Add 43.4g of Fructose to 6.7 ml distilled water, mix until dissolved
2. Add 50 μ l of α -thioglycerol.

Store at 37°C for up to 1 week.

To prepare SeeDB37 combine fructose and water in a 50 ml conical tube and place in a 65°C water bath until dissolved or up to 5 hours. If fructose isn't dissolved after the 5 hour 65°C water bath incubation, remove from the bath to prevent caramelization and place on a nutating mixer at 37°C. Once the fructose is dissolved, add the 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 1900's when Werner Spalteholz developed his formula for clearing tissues which used 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 since 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 biologic discipline. In addition, modern confocal, light sheet, multiphoton and ultramicroscopes can build

volumes through optical sectioning, and when paired with modern computing technologies, researchers can reconstruct whole tissues or whole organisms without the need 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 the methods vary widely in their approach, they are generally designed to preserve fluorescent proteins, lipid components or enable immunostaining (and all the above) (Azaripour et al., 2016; Richardson and 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-24 hours, prior to clearing. Long fixation steps at high concentrations of PFA are often discouraged when planning imaging experiments since 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. Exceptions can be made for all protocols; however, it is suggested that they are 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 example 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 a large volume, primarily extended incubation times. Success can vary widely based on the tissue type, antibodies/antigens, and the size of the tissue. Generally, staining should be avoided when a suitable endogenous marker can be substituted. If staining must be used, consider measures to reduce incubation time and increase the probability of even staining throughout the tissue. Use ‘small’ alternatives to traditional antibodies that will diffuse quickly and evenly like fluorescent molecular probes, FAB fragments and nanobodies. Finally, consider using primary conjugated antibodies as opposed to the 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 Amperes and 60 Volts. As the lipid-detergent micelle concentration and temperature increases in the running buffer, the resistance of the buffer changes. With a constant current DC power supply, the system will adjust the voltage accordingly to maintain a constant current. As current generates heat, it is best to maintain a constant current to ensure 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. 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 present themselves with extended clearing times. It is highly suggested to test each tissue in a variety of conditions prior to designing an experiment.

Clearing buffer may also be reused between ETC runs, but pay close attention 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, discard. 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 depths within the tissue 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 authors' suggest turning to an alternative clearing method or an alteration of the existing method. The clearing protocols presented herein are diverse with numerous stages where alterations may be required for maximizing 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 is 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 the described protocols whereas other tissues, 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.

Acknowledgments

(mandatory for NIH, optional for all others)

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 Institute 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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Significance Statement

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 μ m, 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 optical density 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. By eliminating the need to physically section, researchers can image whole intact tissues and even organisms.

Figure 1. Decision tree to assist with choosing a clearing protocol

Keeping in mind the tissue that you wish to clear and whether you must immunostain, detect endogenous fluorescent proteins, study lipids or use lipophilic dyes. Begin at 'start' and follow the yes/no questions to narrow down which clearing protocol(s) presented herein are a proper fit for your experiment. Each branch of the tree terminates at an octagon which suggests clearing procedures that are compatible with the choices that you have made. In most cases, multiple clearing protocols are suggested. Before deciding, consult Table 1 as a guide for which clearing protocol is most likely to succeed for your specific tissue.

¹CLARITY: Passive, ²CLARITY: Electrophoretic Tissue Clearing, ³DISCO: Endogenous Fluorescence, ⁴DISCO: Immunostaining.

Table 1

Tissue Compatibility with Various Clearing Protocols

	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
Lungs	*****	nt	nt	*****	nt
Lymph Nodes	*****	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	***

*, **, ***, ****, ***** = the relative success of a clearing method for the indicated tissue, X = the clearing method does not work for the indicated tissue, nt = not tested.