Introduction

This document outlines the full LifeCanvas Technologies protocol, from beginning to end. With some variability depending on sample size and type, the timeline for this protocol for each sample looks like this:

<table>
<thead>
<tr>
<th>Days</th>
<th>SHIELD</th>
<th>Clearing</th>
<th>Labeling</th>
<th>RI Matching</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>5</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
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<td>8</td>
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<tr>
<td>9</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

You will always follow these steps in the order shown, with the exception that Immunolabeling can be skipped if your signal of interest is endogenous and you don’t need to exogenously label anything.

The technology in our pipeline is based on the following original publications:

Young-Gyun Park, Chang Ho Sohn, Ritchie Chen, Margaret McCue, Dae Hee Yun, Gabrielle T Drummond, Taeyun Ku, Nicholas B Evans, Hayeon Caitlyn Oak, Wendy Trieu, Heejin Choi, Xin Jin, Varoth Lilascharoen, Ji Wang, Matthias C Truttmann, Helena W Qi, Hidde L Ploegh, Todd R Golub, Shih-Chi Chen, Matthew P Frosch, Heather J Kulik, Byung Kook Lim & Kwanghun Chung. Protection of tissue physicochemical properties using polyfunctional crosslinkers, Nature Biotechnology, 2018 Dec 17, DOI: 10.1038/nbt.4281

Sung-Yon Kim, Jae Hun Cho, Evan Murray, Naveed Bakh, Heejin Choi, Kimberly Ohn, Luzdary Ruelas, Austin Hubbert, Meg McCue, Sara L. Vassallo, Phillipp J. Keller, and Kwanghun Chung. Stochastic electrotransport selectively enhances the transport of highly electromobile molecules, PNAS, 2015 Nov 17, DOI: 10.1073/pnas.1510133112

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SHIELD

Introduction

Before removing lipids from samples, it is important to properly fix them. If you skip this step and proceed with a sample only fixed with PFA, it will fall apart during delipidation. It is possible to replace SHIELD with acrylamide fixation as in CLARITY (Nature, 2013), or glutaraldehyde fixation as in SWITCH (Cell, 2015), although SHIELD provides superior preservation with a more repeatable, simpler protocol. It is important to note that the polyepoxy works in conjunction with PFA to preserve biomolecules, so PFA is required in some form.

Reagents Required

- **SHIELD-Epoxy Solution (SH-ES)** - Store at 4°C upon delivery.
- **SHIELD-Buffer Solution (SH-BS)** - Store at RT
- **32% Paraformaldehyde Solution (15714-S Electron Microscopy Sciences)**
- **SHIELD-ON Buffer (SH-ON)** - Store at 4°C upon delivery.

Standard Protocol

In most samples, the general protocol below will work well. However, there are some modifications to the protocol for the following sample types:

- **PFA-fixed human brain slices (1 mm thick)**
- **Thin PFA-fixed slices (<~200 µm thick)**

Different sample types have different starting points, but you always need to complete the protocol through Step 6. If your experiment allows it, the best preservation technique involves perfusion of the live animal. If you can perfuse the animal, please start at Step 1. If you cannot perfuse an animal, but you do have fresh tissue or an embryo, please start at Step 3. If the sample is fresh frozen, please thaw the sample first, and then start at Step 3. If your sample is already 4% PFA-fixed, please start at Step 4. If you are unsure where to start, please contact us at: science@lifecanvastech.com.
1. Prepare SHIELD Perfusion Solution fresh on ice (see Reagent Setup), and transcardially perfuse the animal with ice-cold PBS followed by ice-cold SHIELD Perfusion Solution in the following volumes and flow rates. Keep the remaining SHIELD Perfusion Solution on ice for use in Step 3.

<table>
<thead>
<tr>
<th>Step 1</th>
<th>PBS (mL)</th>
<th>SHIELD Perfusion Solution (mL)</th>
<th>Flow Rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>20</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Rat</td>
<td>200</td>
<td>200</td>
<td>60</td>
</tr>
</tbody>
</table>

- We recommend using heparinized PBS to remove as much blood as possible. (20 U/mL concentration)
- Perfuse with PBS until the fluid is running completely clear before perfusing with SHIELD Perfusion Solution.
- Be careful not to introduce air bubbles inside tubing. When the fluid comes out of the mouth or a lung swells, adjust the position of needle in the heart.

2. Dissect out the brain / organ of interest.

3. Incubate the sample in fresh SHIELD Perfusion Solution at 4°C with shaking (See Reagent Setup if you are starting here). The volumes shown are optimized for brain samples, but similar volumes should be used for samples of similar size. For smaller samples, use a volume that is 10X the volume of the sample itself. Make sure the sample is immersed in the solution during shaking. Use the following volumes and incubation times:

<table>
<thead>
<tr>
<th>Step 3</th>
<th>SHIELD Perfusion Solution Volume (mL)</th>
<th>Incubation Time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Brain</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Rat Brain</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>Smaller Samples</td>
<td>&gt; 10X Sample Volume</td>
<td>2</td>
</tr>
</tbody>
</table>

We recommend cutting the brain into hemispheres with a razor blade after this step. If your study requires an intact whole-brain, you do not need to cut it.
4. Prepare fresh **SHIELD OFF Solution** (see [Reagent Setup](#)) and incubate at 4°C with shaking. Use the following volumes and incubation times, and please note the different incubation times for post-fixing PFA-fixed samples.

<table>
<thead>
<tr>
<th>Step 4</th>
<th>SHIELD OFF Solution Volume (mL)</th>
<th>Incubation Time (day) For Perfusion / Fresh</th>
<th>Incubation Time (day) For PFA-fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Brain</td>
<td>20</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Rat Brain</td>
<td>50</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Smaller Samples</td>
<td>&gt; 10X Sample Volume</td>
<td>1</td>
<td>Sample Dependent</td>
</tr>
</tbody>
</table>

If your sample’s smallest dimension is 1.5 mm or smaller, please stop here after incubation and continue to the **Small Sample SHIELD-ON** protocol.

5. Transfer the sample to **SHIELD ON Buffer** (RT) and incubate at 37°C with shaking:

<table>
<thead>
<tr>
<th>Step 5</th>
<th>SHIELD ON BUFFER Volume (mL)</th>
<th>Incubation Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Brain</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Rat Brain</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>Smaller Samples</td>
<td>&gt; 10X Sample Volume</td>
<td>24</td>
</tr>
</tbody>
</table>

SHIELD preservation is now complete. The sample can be stored in 1X PBS with 0.02% sodium azide at 4°C for several months without significant loss of fluorescence signal and structural integrity.

6. You may now proceed to the [tissue clearing](#) section of the protocol.
Reagent Setup

**SHIELD Perfusion Solution** (prepare fresh before perfusion)

Mix the following in the order shown in the table and keep on ice. After adding each reagent, please vortex or mix well to prevent precipitate formation. This is the total volume required for the entire Standard Protocol (starting at Step 1). If you cannot perfuse your sample, but have a fresh sample, please prepare 20 mL for samples the size of mouse brains, and 50 mL for samples the size of rat brains. For smaller samples, use the same ratio of chemicals with a total volume at least 10X the volume of the sample.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>For 1 Mouse (40 mL total)</th>
<th>For 1 Rat (250mL total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI Water</td>
<td>5</td>
<td>31.25</td>
</tr>
<tr>
<td>SHIELD-Buffer Solution</td>
<td>10</td>
<td>62.5</td>
</tr>
<tr>
<td>32% Paraformaldehyde Solution</td>
<td>5</td>
<td>31.25</td>
</tr>
<tr>
<td>SHIELD-Epoxy Solution</td>
<td>20 (add in 10 mL increments)</td>
<td>125 (add in 25 mL increments)</td>
</tr>
</tbody>
</table>

**SHIELD-OFF Solution** (prepare fresh for Step 4 of the Standard Protocol or Step 1 of the Post-Fixing Human Samples protocol.)

Mix the following in the order shown in the table and keep on ice. After adding each reagent, please vortex or mix well to prevent precipitate formation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>For 1 Mouse (mL)*</th>
<th>For 1 Rat (mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI Water</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>SHIELD-Buffer Solution</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>SHIELD-Epoxy Solution</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>

SHIELD works with a wide variety of tissues, but the total volume of solution required is dependent on tissue size. For smaller samples, use the same ratio of chemicals with a total volume at least 10X the volume of the sample.
Clearing (Delipidation)

Reagents / Equipment Required

- SmartClear II Pro
- SmartClear Clearing Buffer A and B (1 bottle each) – stored at RT
- SDS Clearing Solution (or extra Buffer A) – stored at RT
- SmartClear Membranes

Protocol:

1. Incubate the sample in SDS Clearing Solution (or Clearing Buffer A) overnight.
2. Install membranes and buffer in the SmartClear II Pro. See Buffer and Membrane Installation for details.
3. Insert the sample into an appropriately sized mesh bag. Always choose a bag that is larger than the sample to allow for expansion during clearing. It is best to align the sample with the longest dimension vertically for best clearing speed (cerebellum down is best). Use the notches at the top of the mesh bags to identify samples.
4. Insert the mesh bag into the cylindrical sample holder and insert that in the clearing chamber. It is best to use the dividers to split the holder into quarters.
5. Tighten the knob on the clearing chamber and close the lid of the device.
6. Adjust temperature accordingly. Generally, if your samples contain endogenous fluorescence, it is best to clear at 42°C for Buffer A. This is equivalent to the ‘Gentle’ setting in beginner mode. If your sample doesn’t have endogenous signal, you can increase the temperature of Buffer A to 50°C, or ‘Fast’ setting for faster clearing speed. Note: If you want further control of temperature, operate the device in Expert Mode. For preservation of RNA for future FISH studies, clearing should be performed at 37°C. To operate at this temperature, you may need to reduce the current from 1500 mA.
7. Turn on Electrophoresis power and clear the samples until they are homogeneously translucent. The samples will not appear transparent at this stage. An opaque center means the samples are not done clearing. The clearing time is highly sample dependent but SHIELD preserved hemispheres usually clear in 3-4 days with the ‘Gentle’ setting. Please check out this blog post for more information and images.
8. When the samples are clear, remove the mesh bag from the device, and wash out SDS with an overnight PBS wash. After washing out SDS, the samples can be stored at 4°C in PBS with 0.02% sodium azide until you are ready for index matching or immunolabeling.

[Note] Do not put the sample into 4°C environments without first washing out SDS. The SDS will precipitate inside the sample. To dissolve SDS, warm the sample up to 37°C and refresh the solution.

9. Consult the Appendices for Shutdown Procedures and Maintenance Information.
Immunolabeling with SmartLabel

Reagents / Equipment Required

- SmartLabel
- Labeling Buffer (one bottle) – stored at 4°C
- Sample Buffer (~50 mL total) – stored at 4°C
- Labeling Reagents, such as primary and secondary antibodies or fluorescent nuclear dyes. Please consult Validated Antibody List for more information.
- SmartLabel Sample cup
- Mesh bag insert and strips
- SmartLabel Sample cup storage solution. More can be made fresh here.

Protocol:

1. Setup the device (see SmartLabel Setup for more details and SmartLabel Maintenance for more information).
2. Before labeling, samples must be equilibrated in Sample Buffer (~20 mL per wash) in a conical tube for overnight or until sinking (some sample types may not sink). Incubate the sample in Sample Buffer overnight at RT with light shaking.
3. Refresh the Sample Buffer and continue incubation for 3-4 additional hours at RT with shaking.
4. Prepare an antibody cocktail in 1 mL Sample Buffer. See Antibody Cocktail Preparation for more details. Store this cocktail at 4°C while prepping the device and sample.
5. Rinse the SmartLabel thoroughly. To do so, pour 500 mL distilled water into the reservoirs and run the pumps for ~30 seconds before turning the pump off and draining the water. Repeat a minimum of 3 times.
6. Remove the mesh bag insert and mesh strips from the Sample cup storage solution and rinse thoroughly in distilled water.
7. Pour a small amount of Sample Buffer in a petri dish and wet the mesh bag insert and strips with Sample Buffer.
8. Remove the Sample Cup from the storage solution and rinse it gently with distilled water. **[WARNING: it is important to execute the following steps quickly to prevent the membrane from drying out]:**
   a. Pipette 4 mL fresh Sample Buffer into the Sample cup.
   b. Discard this Sample Buffer.
   c. Pipette 3 mL of fresh Sample Buffer into the Sample cup. Be careful not to introduce any bubbles. (Pipette 8 mL if using the Large Sample cup).

9. Using tweezers, open the mesh bag insert and slide it into the Sample cup, making sure to push it all the way to the bottom with the top open.

10. Carefully lower the sample into the mesh bag insert inside the Sample cup such that it stands vertically (Figure 1, 2). The sample must be located near the bottom of the cup. Cerebellum down is best to prevent damage.

11. Add the antibody cocktail to the Sample Buffer in the Sample cup. Avoid introducing bubbles if possible. Gently mix the buffer by trituration with the pipette.

12. Using tweezers, lower the mesh strips into the mesh bag insert alongside the sample to prevent it from rotating inside the bag during the experiment (Figure 3). This step is not required if your sample remains stationary without the mesh strip. *Be careful not to puncture the membrane with the tweezers.*

13. Ensure that the entire sample is covered with Sample Buffer. If your sample is particularly tall, add additional Sample Buffer 100 µL at a time until the sample is fully submerged.

14. Insert the Sample cup into the hex piece on the bottom of the Labeling Chamber (Figure 4). Make sure the Sample cup sits flat and that it is not forced into place (Figure 5).

15. Turn on the stir bar by pressing the corresponding button on the SmartBox under the ‘Stirrer’ section. Check the Sample cup to ensure that the sample does not spin inside the cup. If it does, simply add more mesh strips to prevent spinning.
16. Dispense the entire volume of Labeling Buffer from one bottle into the SmartLabel reservoir and screw down the lid.

17. Ensure Labeling Chamber dams are in place (Figure 6).

18. Turn on the Pump to circulate Labeling Buffer into the Labeling Chamber.

19. Change the Rotation Speed Settings by pressing the ‘Rotation’ Button on the SmartBox. Then drag the bar on the right to ~2 rpm and press ‘Save’. Then exit the screen and turn on the Sample Rotation by pressing the gear. Look into the chamber to ensure that the Sample cup is rotating.

20. Decrease the Rotation Speed back to 0.01 rpm.

21. **[IMPORTANT]** Check the settings on the SmartBox and change them if needed. The settings should be as follows:

<table>
<thead>
<tr>
<th>Setting</th>
<th>Proper Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage</td>
<td>90 V</td>
</tr>
<tr>
<td>Current Limit</td>
<td>500 mA – 1 Chamber 1000 mA – 2 Chambers</td>
</tr>
<tr>
<td>Temperature</td>
<td>25 °C</td>
</tr>
<tr>
<td>Rotation Speed</td>
<td>0.01 rpm</td>
</tr>
<tr>
<td>Timer</td>
<td>21 hours</td>
</tr>
</tbody>
</table>

22. Place the magnetic lid on the chamber and close the lid of the device.

23. Turn on electrophoresis and the timer. **Do not run the experiment for less than 21 hours.**

24. After 21 hours, the timer will automatically turn off the electrophoresis power.

25. To complete binding of antibodies, the final pH of the main buffer must be between 7-8. Use pH paper to measure the pH of the Labeling Buffer in the chamber (Not in the Sample cup). **[IMPORTANT]** If the Labeling Buffer pH is above 8, set the timer to 3 hours and restart electrophoresis and sample rotation.

26. Remove the Sample cup from the Labeling Chamber and turn off the pump.
27. Transfer the sample to PBS to wash out any unbound probes. The easiest way to do this is to remove the bag from the cup and tip the bag upside down into a conical tube filled to the top with PBS using tweezers. The sample will sink out of the bag into the solution.

28. Cover the conical tube with the sample in aluminum foil or keep in the dark. The sample should be protected from light from here on until it is imaged to prevent bleaching.

29. Immediately discard the remaining Sample Buffer in the Sample cup and rinse the cup with distilled water. Do this quickly to keep the cup wet. Then place the Sample cup and mesh in the storage solution. They can be reused for many months. It is important to wash the cups well. When using nuclear dyes especially it is possible to contaminate a future experiment. If you are seeing this, we suggest purchasing sufficient cups to dedicate a cup to nuclear dye staining.

30. Drain out the buffer from the SmartLabel and wash the system with distilled water a minimum of 3 times to prevent detergent buildup. Due to the eFLASH mechanism, buffers cannot be reused due to changes in pH and deoxycholate.

31. Some antibodies can dissociate from their antigens in many immersion liquids over time. To prevent this dissociation, it is best to fix the probes in place*. Here is how to do so:
   a. Incubate the sample in PBS at RT with shaking for at least 6 hours, refreshing the solution at least 3 times to wash out any unbound probes.
   b. Incubate the sample in 4% PFA in PBS overnight at RT with shaking.
   c. Incubate the sample in PBS at RT with shaking for at least 6 hours, refreshing the solution at least 3 times to wash out any PFA.

32. The samples can now be stored in the dark at 4°C with 0.02% sodium azide until you are ready to index match and image.

*If you wish to do multiple rounds of labeling and imaging, you cannot PFA fix the samples after labeling. For multiple round labeling, see the Appendix.
Index Matching

Now that you are ready to image your samples, you need to index match them so they are optically transparent. Samples should not be stored in EasyIndex for more than 2-3 days. If a sample will not be imaged until a later date, store the sample in PBS with 0.02% sodium azide.

Reagents Required

EasyIndex – stored at RT in sealed container

Protocol:

1. Shake the bottle of EasyIndex well to homogenize the solution. Let the bottle sit for ~30 minutes to allow the bubbles to settle.

2. Incubate the tissue in EasyIndex with shaking at RT or 37°C. It is important to incubate in a sealed container to prevent evaporation. Perform in the dark or cover any tubes with aluminum foil to protect from light. Use the following volumes and recommended incubation times:

<table>
<thead>
<tr>
<th>Sample</th>
<th>EasyIndex Volume (mL)</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Brain Hemisphere</td>
<td>20</td>
<td>Overnight</td>
</tr>
<tr>
<td>Rat Brain Hemisphere</td>
<td>50</td>
<td>1 day</td>
</tr>
<tr>
<td>100 µm slice</td>
<td>0.5</td>
<td>10 minutes</td>
</tr>
<tr>
<td>1 mm thick slice</td>
<td>2</td>
<td>3-6 hours</td>
</tr>
</tbody>
</table>

After index matching, the sample should be clear enough to easily see through while submerged in EasyIndex. If the solution surrounding the sample seems inhomogeneous, it suggests that the sample has not yet been fully equilibrated with the solution and should be incubated further, or that the sample is not fully delipidated. Please consult this article for more information and images. If it is not fully delipidated, simply wash out EasyIndex and clear it further.

[Note] We strongly advise against reusing EasyIndex as its Refractive Index (RI) changes after the first usage.

[Note] If a sample has been index matched and needs to be recovered and saved, the sample should be washed in PBS at 37°C with gentle shaking overnight and stored appropriately.
Sample Mounting and Imaging Tips

Since every imaging system is different, it is difficult to devise a singular mounting protocol for every setup. However, the following requirements always apply:

1. The sample must have the same RI as the imaging medium.
2. The sample must be immobilized.
3. EasyIndex, like many RI matching solutions, is water based. To avoid local changes in RI due to evaporation, the imaging chamber should be sealed from the air. This can be achieved with a sealed imaging chamber or by covering the surface of the liquid with a layer of mineral oil.

It is possible to glue the sample to a holder to immobilize it. However, the glue will interfere with imaging in those planes and can damage the sample for future imaging. To avoid this, it is possible to embed the sample in agarose made of EasyIndex. Here is the protocol:

1. After index matching your sample, use a pipette or degassing chamber to remove any bubbles from the sample.
2. Mix low melting point agarose (Agarose, Type I, low EEO, CAS Number: 9012-36-6) and EasyIndex (0.9% wt/vol). It is best to mix in larger volumes (~20 mL) for more accurate percentages.
3. Vortex the mixture until the agarose powder is evenly distributed in the solution. Close the cap, wrap it in parafilm, and let it sit at RT for ~30 minutes to hydrate the powder.
4. When the agarose has become hydrated and is hardly visible, vortex the solution well and aliquot. These solutions can be refrigerated for later use.
5. Prepare a water bath / heat block for 56-60°C.
6. Put the solution in a 15 mL conical tube and secure the cap.
7. Microwave the tube until the solution boils. The exact boiling time will differ based on the microwave. Generally, we microwave the tube for 8 seconds – 5 seconds – 5 seconds with a 4-5 second interval between to open the cap and release pressure.
8. Once any agarose lumps are no longer visible, sonicate the tube for ~10 seconds to remove bubbles from the gel solution.
9. Place the tube in the water bath / heat block and wait for any remaining bubbles to dissipate.
10. Get something to hold the gel and sample while it sets. We use custom holders, but a small well or dish can work.
11. Carefully pipette the solution into the holder and use a P10 pipettor to remove any bubbles that form. Pipette enough to form a layer at least the thickness of the sample.

12. Being mindful of the orientation of the sample, use a thin spatula to gently deposit the sample onto the surface of the gel in the holder. The sample may float on the gel, and it is okay if the top sticks out slightly.

13. If you need to reposition the sample, work quickly since the gel will start to set soon (sometimes within ~1 minute).

14. Use a P10 pipette to remove any bubbles that may form. A stereoscope may help locate any small bubbles.

15. Move the holder with the sample to a -20°C freezer for ~5 minutes or 4°C for ~30 minutes until set.

16. Remove the sample from the holder and cut any excess gel. If using a lightsheet with sided illumination, it is best to cut the gel so it is flat facing the illumination.

17. Put the gel embedded sample in a fresh volume of EasyIndex, and index match it for at least an hour at 37°C.

18. You can now mount the gel embedded sample how you wish depending on your imaging setup.

19. To remove the sample from the gel, incubate it in 20% DMSO for ~30 minutes to an hour at RT. The sample will begin to pop out of the gel and you can carefully cut or peel the gel away from the sample.

To prevent evaporation of water from the imaging chamber, you can cover the surface of the liquid with mineral oil and dip the objective through the oil into the EasyIndex to image the sample. When doing this it is important to remove any bubbles or oil from the objective. We do this by swiping the objective carefully with the tip of a bent plastic zip tie. Due to the mechanics of the chamber, this is particularly difficult with the LaVision Ultramicroscope. To get around this problem, you can try this workaround:

1. Purchase a custom refractive index immersion oil from Cargille with a refractive index of 1.465.
2. Fill the imaging chamber with the immersion oil.
3. Index match and mount your sample as normal with EasyIndex.
4. Quickly dip the sample into the chamber and image in the oil.
**SHIELD Appendices**

**Small Sample SHIELD-ON**

This SHIELD-ON protocol should be used for any sample with its smallest dimension 1.5 mm or smaller. This should be used as a replacement for Step 5 of the Standard Protocol.

It can be used for drop-fixation of mouse spinal cords.

1. In a 50 mL conical tube, mix SHIELD-ON Buffer and SHIELD-Epoxy Solution in a ratio of 7:1. The total volume needed will be about 20 mL. Incubate the sample at 37°C with shaking for 3-6 hours. This time is dependent on tissue size.
2. Transfer the sample to a new conical tube with the same volume of fresh SHIELD-ON Buffer (containing NO SHIELD-Epoxy Solution) and incubate at 37°C with shaking overnight.
3. SHIELD post-fixation is now complete. The sample can be stored in 1X PBS with 0.02% sodium azide at 4°C for several months without significant loss of fluorescence signal and structural integrity.
4. You may now proceed to the tissue clearing section of the protocol.

**Post-Fixing PFA-fixed Human Brain Samples**

1. Prepare SHIELD-OFF Solution according to the Reagent Setup and incubate the sample in it at 4°C with shaking for 2 days for 1 mm thick slices. Thicker slices may require longer incubation.
2. In a 50 mL conical tube, mix SHIELD-ON Buffer and SHIELD-Epoxy Solution in a ratio of 1:1. Use the same volume of solution as Step 1. Incubate the sample at 20°C with shaking for 1 day.
3. SHIELD post-fixation is now complete. The sample can be stored in 1X PBS with 0.02% sodium azide at 4°C for several months without significant loss of fluorescence signal and structural integrity.
4. You may now proceed to the tissue clearing section of the protocol.
**Post-Fixing PFA-fixed Thin Slices**

This protocol can be used to quickly SHIELD fix thin sections.

1. In a small sealed tube, mix **SHIELD-ON Buffer** and **SHIELD-Epoxy Solution** in a ratio of 7:1.
2. Incubate the slice in this solution at 4°C for 6 hours with shaking.
3. Move the sample to RT and incubate in the same solution with shaking for 24 hours.
4. SHIELD post-fixation is now complete. The sample can be stored in 1X PBS with 0.02% sodium azide at 4°C for several months without significant loss of fluorescence signal and structural integrity.
5. You may now proceed to the tissue clearing section of the protocol.
SmartClear II Pro Appendices

SmartClear Setup

Please see the SmartClear II Pro QuickGuide for pictures and more details.

1. Place the device on a flat surface in a dry environment.
2. Place the SmartBox next to the device with at least 6” of space to allow for airflow.
3. Insert the ‘Alternating’ and ‘Direct’ cables from the back of the SmartBox into the respective connectors on the SmartClear. Push them in securely and fasten the threaded locks.
4. Plug the power cable into the SmartBox and turn the switch in the back to power the device on and off.
5. Upon startup, the device will allow 3 options: Install Buffers, Beginner Mode, or Expert Mode. If Buffers and membranes are not yet installed, please choose that option. Otherwise, you can directly enter your operating mode of choice.

Buffer and Membrane Installation

Buffer and membrane installation can be performed in a guided manner using the Buffer Installation menu at startup, or by pressing the Change Buffer button in Beginner Mode. The procedure can be replicated manually in Expert Mode with the following procedure. For pictures and more details, consult the SmartClear QuickGuide.

*Note – Never run the pumps without any liquid in the reservoir. This can damage the pumps. Please turn off the pumps or the device before draining liquid from the system. Also, never run the pumps without membranes installed. This can cause leaks.

1. Open the lid of the SmartClear, and locate Reservoir A and B. Please remove any paper towels from the reservoir (to prevent spills during shipping).
2. Locate the drainage tubes in the front compartment and ensure that the valves are closed (valve handles pointing to the side).
3. Pour 500 mL distilled water into each reservoir.
4. Unscrew the lid to the clearing chamber and locate the electrodes. They are platinum wire assemblies on either side of the chamber.
5. Open a new package of Membranes and locate the black rubber gasket. These gaskets will cover the electrodes.
6. Lower one membrane into the chamber with the rubber facing the electrodes and place it against one side. Repeat with the other side.

7. Locate the Membrane spacer with the small hole at the bottom. With the membranes covering the electrodes, push the spacer down into the chamber at the back of the chamber with the corresponding hole. This will sandwich the membranes in place.

8. Locate the other Membrane spacer with the hole at the top and push it down on the front side of the membranes.

9. Power on the SmartBox and enter Expert Mode.

10. Turn on Pump B with the button in the bottom right. Look into the clearing chamber. You should not see any liquid escaping from the membranes indicating no leaks.

11. Turn on Pump A and open the reservoirs. Check the water level.

12. Screw down the clearing chamber lid and leave the device with pumps powered on for ~1 hour and check the water level when returning. The levels should remain the same. If you see the level of Reservoir A is extremely high (near the top of the lid), but B is low, this indicates a leaky membrane.

13. Turn off the pumps.

14. Drain the water out of the system and pour Buffer into their respective reservoirs.

15. Turn the pumps back on. You are now ready to clear!

16. The lifetime of the buffer and membranes is 10 days (electrophoresis power on only).

*Note – the volume of each buffer can vary once the electrophoresis power is turned on. Some buffer will travel through the membrane from Buffer B to Buffer A due to electroosmotic flow. This is expected and normal. Buffer A can reach ~600 mL and Buffer B can drop to ~350 mL due to the flow to A and evaporation.

If you suspect a membrane break or leak, consult the Membrane Breaks section.
SmartClear Shutdown Procedure

The lifetime of the buffer and membranes is 10 days of active clearing. **This 10-day lifetime only counts when the electrophoresis power is turned on.** This means that membranes and buffers can be saved for later use if the 10 days is not up. If you intend to begin clearing another sample soon (within 3-4 days), you can just leave the buffer and membranes in the system in Beginner Mode or in Expert Mode with the pumps powered on. This will keep the membranes hydrated. If you don’t intend to clear again for a longer time period, follow this shutdown procedure:

1. Enter Expert Mode and turn off the pumps. **[IMPORTANT] – turn off the pumps before draining liquid to prevent pump damage.**
2. Drain out each reservoir into a sealed container.
3. Pour 500 mL distilled water into each reservoir and turn the pumps back on for a few minutes to wash the system.
4. Turn the pumps back off and drain out the water.
5. Power down the device with the switch on the SmartBox.
6. Open the clearing chamber and remove the Membrane Spacers.
7. Remove the membranes and store them in a sealed container of distilled water or PBS until you are ready to reinstall them.

SmartClear Maintenance

We recommend thoroughly washing the system every 3-4 buffer changes. To wash the system, follow this protocol before changing to fresh buffer:

1. With buffers and membranes installed, enter Expert Mode.
2. Turn off the pumps and drain out the buffer. **[IMPORTANT] – turn off the pumps before draining liquid to prevent pump damage.**
3. Pour 500 mL distilled water into each reservoir and turn the pumps back on.
4. Run the system for ~5 minutes and turn the pumps back off.
5. Drain the water from the system and repeat 2 more times with fresh water.
6. When finished, turn off the power with the switch on the SmartBox and remove the membranes.

We also recommend calibrating the temperature sensors every 3-4 months. To do this, please consult the SmartClear II Pro Temperature Calibration document.
Membrane Breaks

On very rare occasions, it is possible for one of the nanoporous membranes to break during operation. This is not common, but it is important to be on the lookout for it. When a membrane break or leak occurs, here is what to look for:

1. The current will be lower and erratic, jumping between 600 mA and 1200 mA. This is the easiest way to check for a leak. Note, when the system first starts up with cool buffers, the current will not immediately reach 1500 mA, but will slowly increase as buffers heat up. This is normal behavior. Lower currents happening after startup indicate a leak.

2. In addition to number 1, the buffer will travel through the membrane and transfer from Reservoir B to Reservoir A. This will result in extremely high levels of Buffer A. To check, open the reservoir lid. The buffer will be almost to the top (about 15 mm from the top). Please note that as explained earlier it is normal for there to be some increase in Buffer A volume. This high level is not normal however.

After confirming a break or leak, here is what to do:

1. Send an email to info@lifecanvastech.com explaining the issue. Pictures always help.
2. Go to Expert Mode and turn off the pumps. Always turn off pumps before removing liquid from the system.
3. Remove any samples from the system.
4. Drain out the buffers and discard them.
5. Remove membrane spacers and membranes.
6. Install new membranes and insert membrane spacers.
7. Pour 500 mL distilled water into each reservoir and turn on the pumps for ~5 minutes.
8. Turn the pumps off and drain out the water.
9. Repeat steps 7 and 8 twice more to completely wash the system.
10. After draining out water, you can dispense new Buffer A and B into the appropriate reservoirs and continue clearing.
**SDS Clearing Solution**

This buffer can be used for passive clearing, incubating samples in solution before *active clearing*, and for stripping antibodies for *multi-round labeling*. **This solution will not work in the SmartClear II Pro and could damage the system.** Prepare the following solution in distilled water. These are just suggested vendors, you can use any vendor you would like:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vendor</th>
<th>Product Number</th>
<th>Final Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>Sigma-Aldrich</td>
<td>75746</td>
<td>300</td>
</tr>
<tr>
<td>Boric acid</td>
<td>Alfa Aesar</td>
<td>12680</td>
<td>10</td>
</tr>
<tr>
<td>Sodium sulfite</td>
<td>Sigma-Aldrich</td>
<td>S0505</td>
<td>100</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Sigma-Aldrich</td>
<td>S5881</td>
<td>Titrate to pH 9</td>
</tr>
</tbody>
</table>
SmartLabel Appendices

SmartLabel Setup

Please see the SmartLabel QuickGuide for pictures and more details.

1. Place the device on a flat surface in a dry environment.

2. Place the SmartBox next to the device with at least 6” of space to allow for airflow, with the small SmartBox+ under the larger SmartBox.

3. Insert the ‘Cooling’, ‘Alternating’ and ‘Direct’ cables from the back of the SmartBox into the respective connectors on the SmartLabel. Push them in securely and fasten the threaded locks.

4. Plug the power cable into the SmartBox and turn the switch in the back to power the device on and off.

5. Locate the plastic dams from the accessory box and push them into the slots in the Labeling Chambers. These control the buffer level.

6. Locate the buffer reservoirs and remove any paper towels from shipping.

7. Locate the drain tubes in the front compartment of the device. Ensure that they are closed, and then pour 500 mL distilled water into each reservoir.

8. Start both pumps and run for several minutes to wash the system.

9. You are now ready to start labeling!

*Note – Never run the pumps without liquid in the system. This can damage the pumps. Please turn off the pumps before draining any liquid from the system.

SmartLabel Maintenance

We recommend thoroughly washing the system before and after every experiment. This is outlined in the Labeling Protocol.

We also recommend calibrating the temperature sensors every 3-4 months. To do this, please consult the SmartLabel Temperature Calibration document.

The Sample cups must be stored in the storage solution to keep the membrane hydrated and to wash out any unbound probes. It is best practice to also keep the mesh bag inserts and strips in this storage solution to wash out probes. Please refresh this solution regularly to keep it clean.
SmartLabel Sample Cup Storage Solution

This solution is used to wash and store SmartLabel Sample cups and mesh. Prepare the following solution in distilled water. These are just suggested vendors, you can use any vendor you would like:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vendor</th>
<th>Product Number</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>Sigma-Aldrich</td>
<td>75746</td>
<td>5%</td>
</tr>
<tr>
<td>Boric acid</td>
<td>Alfa Aesar</td>
<td>12680</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Sigma-Aldrich</td>
<td>S5881</td>
<td>Titrate to pH 9</td>
</tr>
</tbody>
</table>

Antibody Cocktail Preparation

Due to the electrophoretic mechanism of the SmartLabel (eFLASH), the absolute amount of antibody used (i.e., in µg) should be of primary consideration vs. antibody concentration or dilution.

1. In a 1.5 mL tube, add 1 mL of Sample Buffer and keep on ice or in a cold block.
2. Add antibodies and dyes to this tube.
3. Mix well by inverting the tube several times and keep at 4°C until it is added to the Sample cup.

Here are some notes regarding antibody amounts:

1. We highly recommend sticking to the list of Validated Antibodies. If you are interested in labeling something that isn’t on the list, please reach out to science@lifecanvastech.com for more information. There is also an Antibody Validation Protocol you can do to test the antibody before a SmartLabel experiment.
2. Antibody amounts should be determined empirically but can initially be estimated by considering the abundance of antigenic sites in the tissue as a function of the tissue’s size/mass. For example, 5 µg of anti-parvalbumin antibody or 3 µg of anti-TH antibody is enough for a mouse brain hemisphere. The Validated Antibody list has recommended amounts for samples like mouse brain hemispheres (double amounts for whole brains).
3. Both primaries and secondaries can be included in the same experiment. However, only monovalent F(ab) fragment secondaries can be used to prevent aggregate formation.
Either **Fc-specific F(ab) fragments** (best) or **generic F(ab) fragments** with your fluorophore of choice can be used. If you have no choice but to use a whole IgG secondary, please consult the Appendix.

4. Aim for a secondary-to-primary molar ratio of ~2:1. An embedded Excel Calculator is below, and can also be found in the User Resources Page on our website.

\[
\text{MW} = \text{molecular weight (primary, } 1° = 150 \text{ kDa; secondary, } 2° = 50 \text{ kDa)}
\]

\[
C = \text{antibody concentration (if available, i.e. from manufacturer)}
\]

\[
V = \text{volume of antibody solution, calculated from } C \text{ above and the target weight (in } \mu g \text{) of } 1° \text{ desired for the experiment, based on the mass of the tissue being labeled and your estimation of the number of antigenic sites present in the tissue (as described above)}
\]

\[
molar \text{ ratio } \frac{1°}{2°} = \frac{1}{2} \quad \frac{V_{1°} \cdot C_{1°}}{MW_{1°}} \frac{V_{2°} \cdot C_{2°}}{MW_{2°}}
\]

\[
V_{2°} = \frac{V_{1°} \cdot \frac{C_{1°}}{C_{2°}} \cdot \frac{MW_{2°}}{MW_{1°}} \cdot \frac{2}{1}}
\]

a. Example, for a fully intact adult mouse brain hemisphere:

Label #1 (e.g., PV, a marker of inhibitory interneurons):

- \( C_{1°} = 1 \mu g / \mu L \) (primary antibody concentration, from supplier)
- \( C_{2°} = 1.6 \mu g / \mu L \) (secondary antibody concentration, from supplier)
- \( V_{1°} = 5 \mu L \) (volume of primary antibody chosen to use)
- \( V_{2°} = 2.08 \mu L \) (volume of secondary antibody calculated to use)

Label #2 (e.g., TH, a marker of catecholaminergic neurons):

- \( C_{1°} = 1.0 \mu g / \mu L \)
- \( C_{2°} = 1.6 \mu g / \mu L \)
- \( V_{1°} = 3 \mu L \)
- \( V_{2°} = 1.25 \mu L \)

Below is an Excel calculator whose default values show a third example. If the table itself is not editable (i.e. embedded), you can either (1) use the Excel file provided by LifeCanvas or (2) copy the table into Excel and enter the formula below the table into the last cell (to the right of V2), which will make the calculator functional.
Excel formula to solve for \( V_2 \) is

\[
=\frac{C_2 \times C_3 \times C_6 \times C_7}{C_4 \times C_5 \times B_7}
\]

**Multi-Round Labeling**

It is possible to perform multiple rounds of labeling if necessary. Keep in mind however that it is very difficult to register multiple rounds of imaging to each other in very large samples.

1. Perform the normal SHIELD, clearing, and labeling steps without PFA fixing probes after labeling.
2. Index match and image round 1.
3. Wash out EasyIndex with PBS.
4. Incubate the sample in SDS clearing buffer for 2 hours at 70°C with shaking to strip away antibodies.
5. Perform the next round of immunolabeling with SmartLabel.
6. Repeat as desired.

**Whole IgG Secondary**

If you absolutely must use a whole IgG secondary along with your primary antibodies, you must run 2 separate SmartLabel experiments. Note: if you are staining a sample which already has an antigen (vascularly delivered primary antibody which is fixed in place for example), you can use a whole IgG without requiring a second labeling experiment.

**Protocol:**

1. Run a SmartLabel experiment using only the desired primaries.
2. At the end of the experiment, the primaries must be fixed in place with the 4% PFA fixation.
3. Wash out PFA with PBS several times over several hours and begin a second SmartLabel experiment using the secondaries only.
Digital Protocol and Other Documentation

These protocols and others referenced here can be accessed through our User Resources Portal: https://lifecanvastech.com/lifecanvas-user-resources/

Here is a QR Code for that website:

![QR Code for User Resources Portal]

The SmartLabel specific documentation as well as a Curriculum for first time users can be found at the SmartLabel User Resources Portal: https://lifecanvastech.com/smartlabel-user-resources/

Here is a QR code for that website:

![QR Code for SmartLabel User Resources Portal]