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v5.05

Introduction

This document outlines the full LifeCanvas Technologies protocol, from beginning to end using SmartBatch+. The general protocol is as follows:

1. SHIELD preservation
2. Delipidation with SmartBatch+
3. Immunolabeling with SmartBatch+
4. Index Matching – **RI = 1.52 EasyIndex Required**
5. Imaging

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. Also, either steps 2 or 3 can be replaced with passive methods (see the Passive Pipeline Protocol) or with SmartBatch+.

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

Dae Hee Yun, Young-Gyun Park, Jae Hun Cho, Lee Kamentsky, Nicholas B. Evans, Alex Albanese, Katherine Xie, Justin Swaney, Chang Ho Sohn, Yuxuan Tian, Qiangge Zhang, Gabi Drummond, Webster Guan, Nicholas DiNapoli, Heejin Choi, Hae-Yoon Jung, Luzdary Ruelas, Guoping Feng, and Kwanghun Chung. Ultrafast immunostaining of organ-scale tissues for scalable proteomic phenotyping, ***bioRxiv***, 2019 June 05, DOI: 10.1101/660373. Preprint.

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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. We have also found a SHIELD post-fix of PFA fixed samples to give the most reproducible results as it is less dependent on good perfusions. The post-fix also uses less reagent but is slightly longer. It is still possible to perfuse with SHIELD if you wish, and that protocol is listed below.

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

If you have some unique samples or are unsure what protocol to use, please contact us at: science@lifecanvastech.com.

The protocol below starts with a PFA fixation and subsequent drop-fix. If you are unable to perfuse the animal, start at **Step 3.**

1. Before proceeding, please check the Expiration Date on the SHIELD-Epoxy bottle. If the solution is used after the expiration date the mechanical stability of the sample can be compromised.
2. Transcardially perfuse the animal with ice-cold PBS. For mice, use about 20 mL and a 5 mL/min flow rate. For rats, use 200 mL and a 60 mL/min flow rate. We recommend using heparinized PBS to remove as much blood as possible (20 U/mL concentration). Make sure the fluid is running completely clear before next perfusing with ice-cold 4% PFA in PBS. Use the same amounts and flow rates as before. 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 the needle in the heart.
3. Dissect out the brain / organ of interest.
4. Incubate the sample in 4% PFA in PBS overnight to 24 hours at 4°C with shaking.
5. Prepare **fresh** SHIELD OFF Solution. 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.

Reagent	For 1 Mouse Brain or smaller (mL)	For 1 Rat Brain (mL)
DI Water	5	12.5
SHIELD-Buffer Solution	5	12.5
SHIELD-Epoxy Solution	10	25

6. Incubate the sample in SHIELD OFF Solution at 4°C with shaking for the duration shown in the table below:

Step 6	SHIELD OFF Solution Volume (mL)	Incubation Time (days)
Mouse Brain	20	3
Rat Brain	50	6

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.

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

Step 7	SHIELD ON BUFFER Volume (mL)	Incubation Time (hr)
Mouse Brain	20	24
Rat Brain	40	24
Smaller Samples	> 10X Sample Volume	24

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.

8. You may now proceed to the tissue clearing section of the protocol.

Clearing (Delipidation)

Reagents / Equipment Required

- SmartBatch+
- Delipidation Buffer – stored at RT
- Conduction Buffer – stored at 4°C
- SmartBatch+ Clearing Cup

Protocol:

1. Setup the device.
2. Fill an **Incubation Jar** with ~250 mL of Delipidation Buffer. This buffer can be set aside and re-used for incubations until you run out of the rest of the buffer for the device.
3. Insert SHIELD fixed samples into **Mesh Bags**, taking note of which sample is in which bag. When inserting mouse brains into the bags put the cerebellum facing down to avoid damage to olfactory bulbs.
4. Place the **Sample Ring** on the **Ring Stand.**
5. Hang the **Mesh Bags** on the **Sample Ring** and secure them with the **Lock Ring.**
6. Place the **Ring Stand** in the **Incubation Jar** and close the lid.
7. Incubate the samples in Delipidation Buffer overnight at RT with light shaking.
8. Wash the device and turn off **Auxiliary Power**.
9. Drain out any liquid from the device and ensure the **Drainage Valve** is closed. Soak up any remaining liquid from the **Chamber** using a paper towel.
10. Pour a whole bottle of Conduction Buffer into the **Chamber**.
11. Remove the **Clearing Cup** from its storage container and wash carefully with a gentle stream of water.
12. Place the **Clearing Cup** into a beaker of distilled water for several minutes to finish washing the cup.
13. Remove the **Clearing Cup** from the liquid and dump out any water. Carefully use a kimwipe to soak up any remaining water inside the cup.
14. Fill the cup with 32mL of Delipidation Buffer.
15. Place the **Sample Ring** on top of the **Clearing Cup** with the samples inside. Add more Delipidation Buffer if the samples are not fully submerged.

16. Insert the cup in the **Chamber**, lining up the pegs on the bottom with those in the **Chamber Hex Piece.** The cup will fit in snugly.
17. Turn on the **Auxiliary Power.**
18. Close the **Chamber Lid** and secure it with the thumbscrew.
19. Close the **Case Lid**.
20. Press **"Preset"** until it indicates **Clearing Mode.** Change any settings or apply the timer if desired. The preset settings are the recommended settings for mouse brains.
21. Turn on the **Electrophoresis Power**.
22. Wait for your samples to clear. For mouse brains, run for 24 hours. For rat brains, run for 6 days, and refresh the Delipidation Buffer every 3 days.
23. Once fully delipidated, turn off **Electrophoresis Power** and **Auxiliary Power**.
24. Open the **Case Lid** and **Chamber Lid** and remove the **Clearing Cup** from the **Chamber.**
25. Remove **Sample Ring** from the **Clearing Cup** and place on the **Ring Stand.** Carefully rinse the **Clearing Cup** with distilled water and store it in its storage solution. It is important to keep the membrane hydrated at all times. We recommend refreshing the storage solution every few months. More can be made here.
26. When done clearing samples, or every 10 days (whichever comes first) wash the system and replace the buffer in the device. We recommend refreshing the Delipidation Buffer when adding new samples, or every ~3 days, whichever comes first.
27. Wash the device before turning it off to prevent detergent buildup.
28. If your samples contain endogenous FPs and do not require immunolabeling, you can proceed to Index Matching. If you are not yet ready to start index matching, you can move samples to PBSN (PBS with 0.02% sodium azide) for long term storage at 4°C.
29. If you plan to immunolabel your samples, proceed to Immunolabeling. **If you plan to batch label the samples, you can leave them in the mesh bags and on the Sample Ring for all processing.** If you will not start labeling immediately, you can store samples in the **Incubation Jar** with PBSN (PBS with 0.02% sodium azide) until the day before labeling as described in the Immunolabeling section.
30. Consult the Appendices for Shutdown Procedures and Maintenance Information.

Immunolabeling with SmartBatch+

Reagents / Equipment Required

- SmartBatch+
- SmartBatch+ Batch Single Sample Staining Kit or Batch Staining Kit
- Labeling Reagents, such as primary and secondary antibodies or fluorescent nuclear dyes. Please consult Validated Antibody List for more information.
- Batch Staining Cup (Or Single Sample Staining Cup)

Outline

Here is a brief description of the protocol:

1. Wash samples and incubate in Primary Sample Buffer overnight.
2. Deliver primary antibodies, dyes, and simultaneous Fab secondaries – 18 hours
3. Wash samples passively in PBS – 8 hours
4. Fix antibodies / dyes with 4% PFA – overnight
5. Wash out PFA with Secondary Sample Buffer passively (4 hours) and actively (4 hours)
6. Deliver secondary antibodies – 12 hours
7. Actively wash out excess secondary – 6 hours

Prepare the Samples for Labeling (After delipidation)

In this step we will incubate the samples in the buffers to prepare them for labeling. If you aren't ready to start labeling just yet, store samples in PBSN at 4°C.

1. On the day before you are ready to start labeling, wash and fill an **Incubation Jar** with ~225 mL of Primary Sample Buffer.
2. Transfer the samples on the **Sample Ring** and **Ring Stand** to the **Incubation Jar** and incubate overnight at RT with light shaking.
3. **Note:** if you will be staining a single sample or smaller samples that can fit in the **Single Sample Staining Cup**, do the incubations in a conical tube with 20 mL of solution.
4. On the morning of the day that you will start labeling, replace the solution with fresh Primary Sample Buffer.

Prepare the Sample Cups (Morning of day of staining)

In this step the cups and mesh are rinsed and soaked in distilled water to ensure the SDS in the storage solution is properly washed out.

1. Remove the **Staining Sample Cup** from its storage solution.
2. Use a very gentle stream of tap water to carefully rinse the cup, holding the cup close to the tap. **The cups are fragile so do not subject them to high water pressure.**
3. Once rinsed out, carefully rinse the cup with distilled water.
4. Place the cup in a volume of distilled water until the later steps.
5. **If you are using the Single Sample Staining Cup**, get a **Mesh Bag** from the storage solution, wash it well with water and add it to the distilled water with the cup.

Prepare the Device (Just before staining)

In this step the device is washed, buffer is added and settings are prepared for the primary staining.

1. Wash the device.
2. Turn off **Auxiliary Power** and drain any liquid from the reservoirs and make sure the **Drainage Valve** is closed.
3. Soak up any remaining liquid from the **Chamber** using a paper towel.
4. Pour one bottle of Primary Device Buffer into the **Chamber.**
5. Press **"Preset"** until it indicates **Labeling 1 Mode.** The preset settings are the recommended settings for primary antibody staining.
6. Change the timer to 18 hours (18:00).

Start Primary Labeling

Before starting this step it is vital to plan out what antibodies will be used and with what method of secondary. The protocol can accommodate staining with dye-conjugated antibodies, simultaneous delivery of primary along with monovalent Fab fragment secondaries, and sequential delivery of primary and secondary (can be Fab or IgG). Please consult the Validated Antibody List for more information as we have found that some antibodies require specific secondary delivery schemes. If you anticipate a signal being dim, we recommend sequential delivery of secondary using whole IgG secondaries in the 647 nm channel. If you are planning to use an antibody for the first time, we recommend first following the Antibody Validation Protocol. If you wish to skip that step, we recommend using a sequential delivery of primary and secondary for the first test since simultaneous Fab fragments can change the binding affinity of the primary antibody. It is also recommended to try new antibodies with single samples first, as batch experiments can require large amounts of antibody.

You can deliver as many antibodies or dyes as you want at once without any host or wavelength conflicts so plan ahead for that. If you are delivering a nuclear dye or vasculature stain, please add them during this primary step. In general, we recommend using higher wavelengths (red or far red) for antibodies and saving lower wavelengths for brighter targets or nuclear dyes.

1. Remove the sample cup from the distilled water and dump out the water.
2. Use a kimwipe to carefully soak up any remaining liquid from inside the cup.
3. Carefully pour a small amount of Primary Sample Buffer into the cup and swirl it around to coat the membrane.
4. Dump and discard the liquid in the cup and soak it up again with a kimwipe.
5. Fill the **Batch Staining Cup** with 40 mL of Primary Sample Buffer. If you are using the **Single Sample Staining Cup**, fill it with 9 mL of Primary Sample Buffer.
6. Remove the **Sample Ring** from the **Incubation Jar** and place it on top of the **Sample Cup** with the samples in their **Mesh Bags** in the cup. If you are using the **Single Sample Staining Cup**, remove the **Mesh Bag** from the distilled water, dry it with kimwipes, add the sample to the bag, and put the bag into the **Staining Cup.**
7. Add antibodies / dyes to the **Sample Cup**, following the considerations listed above. Consult the Validated Antibody List for recommended amounts of antibodies to use. **If you are going to use whole IgG secondaries, they cannot be added during this step and must be delivered sequentially.** If you are going to use simultaneous Fab fragment secondaries, you should add them to the cup, generally in a 2:1 molar ratio. Please note that IgG antibodies have a MW of 150 kDa, while monovalent Fab fragments have a MW of 50 kDa.
8. If your samples are particularly tall, add some more Primary Sample Buffer to cover it up.
9. (Optional) Add Normal Donkey Serum or Normal Goat Serum (matching your secondary antibody host) to the cup. For mouse brains we recommend 100 µL per hemisphere.
10. Take the sample cup and place it into the **Chamber**. Line up the hex on the bottom of the cup with the hex in the **Chamber**.
11. Turn on the **Auxiliary Power** while the lid is open and ensure that the sample cup rotates and is mixing well.

12. Close the **Chamber Lid** and **Case Lid** and turn on **Electrophoresis.**
13. Turn on **Timed Shutdown**.
14. The timer will automatically turn off electrophoresis at the end of the experiment.

Finish Primary Labeling (Next morning after staining)

In this step, the samples will be moved to PBS and the device / sample cup will be washed.

1. Use a pipette to remove ~150 µL from the cup and pipette it onto a pH strip.
2. (Optional) If the pH is still above ~8.0, you will need to extend the duration of the experiment to complete antibody binding. For every 0.1 above 8.0, the experiment should run for an additional hour. So if you measure a pH of 8.2, change the timer to 2:00 (2 hours) and turn on **Electrophoresis Power** and **Timed Shutdown**.
3. When the pH has dropped below 8 and labeling is complete, open the device and remove the **Sample Cup**.
4. For batch labeling, wash out an **Incubation Jar** and fill it with ~250 mL of PBSN. Then remove the **Sample Ring** from the **Sample Cup** and place it on a **Ring Stand**. Place the **Ring Stand** in the **Incubation Jar** and close the lid. Wash the samples until the end of the day in PBSN at RT with light shaking, refreshing the solution at least once.
5. For single sample experiments, prepare a conical tube with ~40 mL of PBSN. Then remove the **Mesh Bag** from the **Sample Cup** and place it in the tube. Wash the sample until the end of the day in PBSN at RT with light shaking, refreshing the solution at least once.
6. Carefully rinse the **Sample Cup** with distilled water and store it in its storage solution. It is important to keep the membrane hydrated at all times. We recommend refreshing the storage solution every few months. More can be made here.
7. Wash the device before shutting it down.
8. If you do not need to add any sequential secondaries, the staining is done! We recommend that you fix the antibodies and dyes in place using PFA to prevent dissociation during index matching.
9. If you do need to add sequential secondaries, it is important to fix the primaries in place. It is possible they could dissociate later and form aggregates during the secondary step.

Fix the Antibodies (End of day after staining)

In this step we will fix the antibodies and dyes delivered during the Primary step to prevent them from dissociating during index matching (and possible secondary staining steps). **Note: if you are not able to do this step in the same day, simply leave the sample in PBSN for up to a few days before fixing.**

1. Prepare a solution of 4% PFA in 1X PBS. For batch experiments you will need ~250 mL. For single samples you will need 20 mL.
2. For batch staining, remove the **Ring Stand** from the **Incubation Jar** and wash out the **Jar**. Then fill it with the 4% PFA and insert the **Ring Stand**.
3. For single samples, move the sample from PBSN to a fresh tube of 4% PFA.
4. Incubate samples overnight at RT with light shaking protected from light.
5. If you do not have any sequential secondaries to add, wash out PFA with a PBS incubation at RT with shaking (protected from light). You can now continue to Index Matching when you are ready. You can store the sample long term at 4°C in PBSN.
6. If you need to apply sequential secondaries, continue to the next step!

Prepare Samples for Sequential Secondaries (Morning after PFA fixation)

In this step we will begin to wash out PFA from the sample and equilibrate it to Secondary Sample Buffer.

1. If you are ready to complete secondary staining in this day, you can immediately continue to Step 2. Otherwise, move the sample to PBSN (PBS + 0.02% sodium azide) and store it at 4°C protected from light until you are ready.
2. For batch staining, wash out the **Incubation Jar** and replace the liquid with ~250 mL of Secondary Sample Buffer. For single samples, move the sample to a fresh tube of ~20 mL of Secondary Sample Buffer.
3. Incubate samples for 4 hours at 37°C with light shaking, refreshing the solution at least once.

Prepare Sample Cups for Secondary Staining (Morning on the day of secondary staining)

In this step the cups and mesh will be washed and prepped for staining.

1. Prepare the cups in the same manner as they were for Primary Staining.

Prepare the Device for Secondary Staining (Just before secondary staining)

In this step the device will be prepared for staining.

1. Prepare the device in the same manner as the Primary Staining **with these exceptions**:
    a. After washing the device, pour one bottle of Secondary Device Buffer into the **Chamber**
    b. Change the timer to 4:00 (4 hours).
2. Press **"Preset"** until it indicates **Labeling 2 Mode.** The preset settings are the recommended settings for secondary antibody staining.

Wash Samples Before Adding Secondaries (Afternoon of secondary staining ~1pm)

In this step the sample will be washed electrophoretically to remove any nonfixed primary antibodies and any remaining aldehydes.

1. Remove the **Sample Cup** from the distilled water and dump out the water.
2. Use a kimwipe to carefully soak up any remaining liquid from inside the cup.
3. Carefully pour a small amount of Secondary Sample Buffer into the cup and swirl it around to coat the membrane.
4. Dump and discard the liquid in the cup and soak it up again with a kimwipe.
5. Fill the **Batch Staining Cup** with 40 mL of Secondary Sample Buffer, or the **Single Sample Staining Cup** with 9 mL of Secondary Sample Buffer.
6. Insert the samples into the cup as before.
7. Take the sample cup and place it into the **Chamber**. Line up the hex on the bottom of the cup with the hex in the **Chamber**.
8. Turn on the **Auxiliary Power** while the lid is open and ensure that the sample cup rotates and is mixing well.
9. Close the **Chamber Lid** and **Case Lid** and turn on **Electrophoresis.**
10. Turn on **Timed Shutdown**.

11. The timer will automatically turn off electrophoresis at the end of 4 hours. We do recommend refreshing the solution inside the cup at least once however. To do so:
    a. Turn off **Electrophoresis Power**. The other settings can remain **ON**.
    b. Remove the **Sample Cup** from the device.
    c. Remove the samples from the **Cup** and repeat steps 2-6 above.
    d. Put the **Sample Cup** back into the **Chamber**, close the lids and turn **Electrophoresis Power** back on.

Start Secondary Labeling

In this step the cup will be removed from the device, solutions refreshed, and secondary antibodies added.

1. Remove the **Sample Cup** from the device and transfer samples to the **Ring Stand** or on a petri dish if a single sample.
2. Dump out the liquid in the cup and use a kimwipe to carefully soak up any remaining liquid from inside the cup.
3. Carefully pour a small amount of Secondary Sample Buffer into the cup and swirl it around to coat the membrane.
4. Dump and discard the liquid in the cup and soak it up again with a kimwipe.
5. Fill the **Batch Staining Cup** with 40 mL of Secondary Sample Buffer, or the **Single Sample Staining Cup** with 9 mL of Secondary Sample Buffer.
6. Add secondary antibodies directly to the **Sample Cup**. We recommend 2:1 molar ratio of Secondary:Primary, but this is antibody dependent and is a good starting point. Consult the Validated Antibody list for more details on specific antibodies.
7. (Optional) Add Normal Donkey Serum or Normal Goat Serum (matching your secondary antibody host) to the cup. For mouse brains we recommend 100 µL per hemisphere.
8. Insert the samples into the cup as before.
9. Take the sample cup and place it into the **Chamber**. Line up the hex on the bottom of the cup with the hex in the **Chamber**.
10. Turn on the **Auxiliary Power** while the lid is open and ensure that the sample cup rotates and is mixing well.
11. Change the timer to 12:00 (12 hours).

12. Close the **Chamber Lid** and **Case Lid,** turn on **Electrophoresis**, and turn on **Timed Shutdown.**

Wash the Sample (Optional – Morning after secondaries)

In this step, the device will be used to actively wash out excess secondary antibodies. We do highly recommend this step, but if you cannot do this step, you can skip ahead. You can also passively wash samples in PBSN for several days before fixing as an alternative.

1. Remove the **Sample Cup** from the device and transfer samples to the **Ring Stand** or on a petri dish if a single sample.
2. Dump out the liquid in the cup and use a kimwipe to carefully soak up any remaining liquid from inside the cup.
3. Carefully pour a small amount of Secondary Sample Buffer into the cup and swirl it around to coat the membrane.
4. Dump and discard the liquid in the cup and soak it up again with a kimwipe.
5. Fill the **Batch Staining Cup** with 40 mL of Secondary Sample Buffer, or the **Single Sample Staining Cup** with 9 mL of Secondary Sample Buffer.
6. Insert the samples into the cup as before.
7. Take the sample cup and place it into the **Chamber**. Line up the hex on the bottom of the cup with the hex in the **Chamber**.
8. Turn on the **Auxiliary Power** while the lid is open and ensure that the sample cup rotates and is mixing well.
9. Change the timer to 6:00 (6 hours) and turn on **Electrophoresis Power** and **Timed Shutdown.**
10. It is recommended to refresh the solution in the cup one time, roughly around the 3 hour mark using the same procedure. (You can do this without turning off **Timed Shutdown** so that it continues counting down).

Finish Secondary Labeling Experiment and Fix Secondaries (After electrophoretic washing)

In this step the sample will be removed from the device, and the device / cup will be washed. The samples will then be PFA fixed to ensure that secondary antibodies do not dissociate. This fixation is recommended but not entirely necessary.

1. Open the device and remove the **Sample Cup**.

2. For batch labeling, wash out an **Incubation Jar** and fill it with ~250 mL of PBSN. Then remove the **Sample Ring** from the **Sample Cup** and place it on a **Ring Stand**. Place the **Ring Stand** in the **Incubation Jar** and close the lid. Wash the samples until the end of the day in PBSN at RT with light shaking, refreshing the solution at least once.
3. For single sample experiments, prepare a conical tube with ~40 mL of PBSN. Then remove the **Mesh Bag** from the **Sample Cup** and place it in the tube. Wash the sample until the end of the day in PBSN at RT with light shaking, refreshing the solution at least once.
4. Carefully rinse the **Sample Cup** with distilled water and store it in its storage solution. It is important to keep the membrane hydrated at all times. We recommend refreshing the storage solution every few months. More can be made here.
5. Wash the device before shutting it down.
6. At the end of the day prepare a solution of 4% PFA in 1X PBS. For batch experiments you will need ~250 mL. For single samples you will need 20 mL.
7. For batch staining, remove the **Ring Stand** from the **Incubation Jar** and wash out the **Jar**. Then fill it with the 4% PFA and insert the **Ring Stand**.
8. For single samples, move the sample from PBSN to a fresh tube of 4% PFA.
9. Incubate samples overnight at RT with light shaking protected from light.
10. Transfer samples to PBSN in the morning to wash out PFA.
11. You are done labeling! You can now continue to Index Matching.

Index Matching

Now that you are ready to image your samples, you need to index match them so they are optically transparent. For this protocol, it is required to use the 1.52 RI version of EasyIndex. If a sample will not be imaged until a later date, store the sample in PBS with 0.02% sodium azide.

Reagents Required

EasyIndex – RI = 1.52 – stored at RT in sealed container

Protocol:

Note: If a sample does not contain any antibodies or only contains fixed antibodies, we recommend index matching at 37°C. Otherwise you should incubate at RT.

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 50% EasyIndex + 50% distilled water 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:

Sample	EasyIndex Volume (mL)	Incubation Time
Mouse Brain or Hemisphere	20	1 day
Rat Brain Hemisphere	50	1 day
100 µm slice	0.5	10 minutes
1 mm thick slice	2	3-6 hours

3. Incubate the tissue in 100% EasyIndex at RT or 37°C for the same duration or until transparent.

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 RT with gentle shaking overnight and stored appropriately. You can also store samples in EasyIndex at RT, but be aware that they can take on a more yellow color over time that does not effect imaging.

Sample Mounting and Imaging Tips

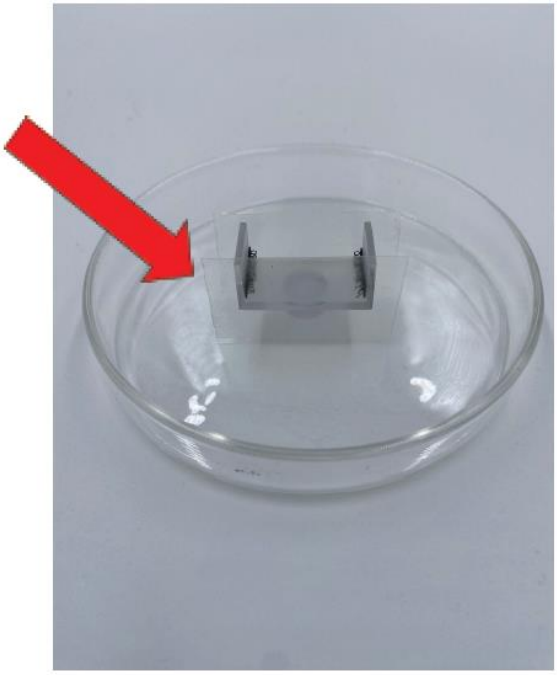
Since every imaging system is different, it is difficult to devise a singular mounting protocol for every setup. However, the following requirements always apply:

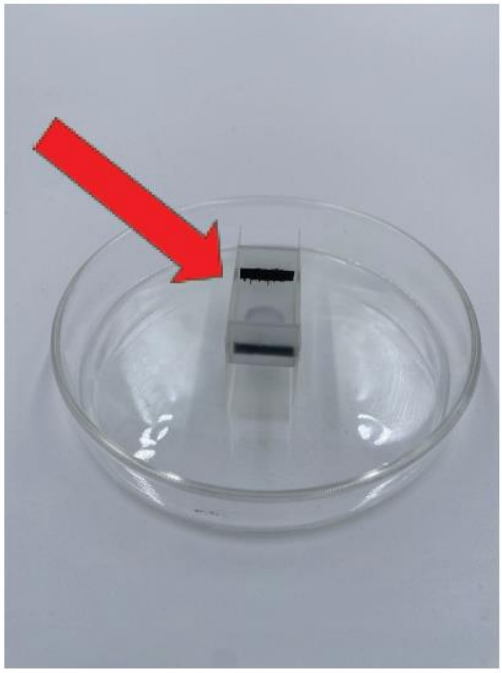
1. The sample must have the same RI as the imaging medium.
2. The sample must be immobilized.
3. EasyIndex, like many RI matching solutions, is water based. To avoid local changes in RI due to evaporation, the imaging chamber should be sealed from the air. This can be achieved with a sealed imaging chamber 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 gel made of EasyIndex. Here is the protocol:

1. Add ultra low melting point agarose (Sigma A5030) to EasyIndex so it is 2% agarose (0.6 g in 30 mL for example). It is best to mix in larger volumes (~20 mL) for more accurate percentages.
2. Wait a few hours for the agarose particles to be fully hydrated. If the particles are not hydrated the sides of the falcon tube will have visible chunks of agarose. This can be stored in the fridge for later use.
3. Mix the solution well and pour it into a smaller centrifuge tube. You will need about 5 mL per sample.
4. Place the vial in a water bath at 90°C for about 30 minutes.

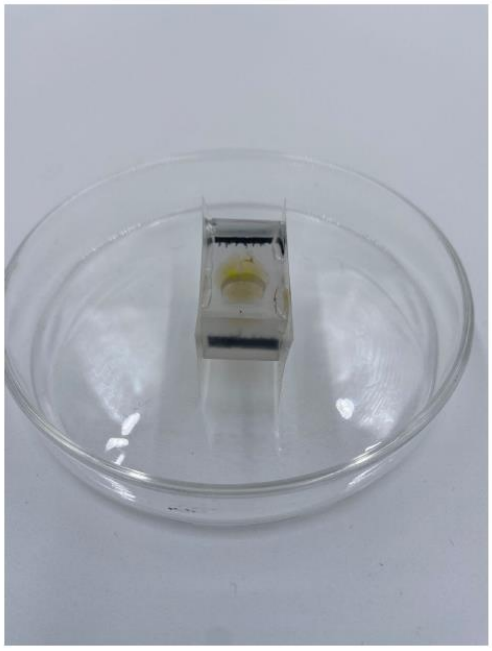
5. Once the gel has started to melt, mix it well to ensure homogenous melting of the agarose particles. Don't worry about introducing any bubbles. The tube and gel will be very hot so handle with care and use tweezers if needed.
6. Once the gel is well mixed, return the tube to the water bath and leave it there for at least another 30 minutes.
7. When the gel is ready it should be fully clear. A good way to check is by looking at something through the gel. It should not distort it or cause any streaky lines. If your gel still has bubbles at this point, you can leave it in the bath for a bit longer or centrifuge it for a few minutes.
8. Reduce the temperature of the bath to 70°C so it can be handled.
9. Prepare your sample holder. In the case of SmartSPIM, add thermal seal strips, sticky well plate covers, or blu-tack to the sides to form walls. See the image below:

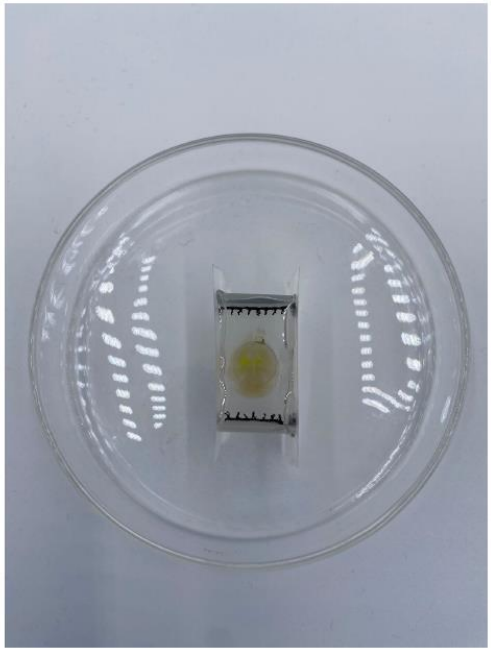




10. Prepare the sample by placing it in a glass petri dish and pipetting out the excess EasyIndex. Examine the sample under a light to remove any internal or external bubbles with a P10 pipette. If there are any internal bubbles that cannot be accessed via ventricles, use a small gage needle to aspirate the bubbles.
11. Pour the prepared gel slowly into the sample holder.

12. Examine the gel and pipette out any bubbles that might arise after pouring.
13. Slowly slide the sample into the gel using a spatula or spoon.
14. Examine the sample under a direct light and pipette out any bubbles in the gel.
15. Place the holder at 4°C for at least 30 minutes.
16. Once the gel has set, carefully remove the sides from the holder and place the mounted sample back into EasyIndex.
17. For best imaging quality, we recommend floating the sample overnight in the EasyIndex you will use to image to rematch the gel.





SHIELD Appendices

SHIELD Perfusion Protocol

1. Prepare SHIELD Perfusion Solution **fresh** on ice. 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.

Reagent	For 1 Mouse (40 mL total)	For 1 Rat (250mL total)
DI Water	5	31.25
SHIELD-Buffer Solution	10	62.5
32% Paraformaldehyde Solution	5	31.25
SHIELD-Epoxy Solution	20 **(add in 10 mL increments)**	125 **(add in 25 mL increments)**

2. 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.

Step 2	PBS (mL)	SHIELD Perfusion Solution (mL)	Flow Rate (mL/min)
Mouse	20	20	5
Rat	200	200	60

- 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.

3. Dissect out the brain / organ of interest.
4. Incubate the sample in the remaining SHIELD Perfusion Solution at 4°C with shaking.

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:

Step 4	SHIELD Perfusion Solution Volume (mL)	Incubation Time (day)
Mouse Brain	20	2
Rat Brain	50	2
Smaller Samples	> 10X Sample Volume	2

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.

5. Prepare **fresh** SHIELD OFF Solution. 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.

Reagent	For 1 Mouse Brain or smaller (mL)	For 1 Rat Brain (mL)
DI Water	5	12.5
SHIELD-Buffer Solution	5	12.5
SHIELD-Epoxy Solution	10	25

6. Incubate the sample in the SHIELD OFF Solution at 4°C with shaking. Use the following volumes and incubation times:

Step 6	SHIELD OFF Solution Volume (mL)	Incubation Time (days)
Mouse Brain	20	1
Rat Brain	50	3
Smaller Samples	> 10X Sample Volume	1

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.

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

Step 7	SHIELD ON BUFFER Volume (mL)	Incubation Time (hr)
Mouse Brain	20	24
Rat Brain	40	24
Smaller Samples	> 10X Sample Volume	24

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.

8. You may now proceed to the tissue clearing section of the protocol.

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 7** 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.

SmartBatch+ Appendices

SmartBatch+ Setup

Please see the SmartBatch+ User's Manual for more information and pictures.

1. Place the device on a flat surface in a dry environment.
2. Plug the Power Cord into the back of the device and turn on the power switch above the plug entry.
3. Open the Case Lid and Chamber Lid and remove any paper towels from the chamber used during shipping.
4. Always wash the device before and after use.

SmartBatch+ Device Washing

Follow these steps to wash the device.

During these steps it is important to not run the **Auxiliary Power** without any liquid in the system. It could potentially damage the pump.

1. Turn off **Auxiliary Power**.
2. Drain any liquid out of the system with the **Drainage Valve** and close the valve.
3. Pour 400 mL distilled water into the **Chamber**.
4. Turn on **Auxiliary Power** and run the pump for several minutes.
5. Turn off **Auxiliary Power** and drain the liquid.
6. Repeat steps 3-5 at least 3 more times, or until the liquid in the **Chamber** is no longer bubbly.

SmartBatch+ Shutdown Procedure

If the device is being used for clearing and the buffer has not yet been used for its full 10 day lifetime, turn off **Auxiliary Power** and drain the Conduction Buffer back into its bottle. It can be re-used later. After draining out buffer (either for clearing or labeling), wash the device. Then simply power the device down.

SmartBatch+ Maintenance

We recommend re-calibrating the temperature sensors every 6 months to maintain proper system function and to ensure the best results. For the best results, use a well calibrated digital thermometer to collect temperature measurements.

1. Fill the device with 400 mL distilled water and turn on the **Auxiliary Power.**
2. Open the **Case Lid** and **Chamber Lid.**
3. Put the thermometer into the **Chamber** at an angle and close the **Chamber Lid** such that the thermometer is still in the liquid, and the sensor on top of the **Chamber Lid** points down into the liquid.
4. Wait ~30 seconds for the temperature to stabilize.
5. Press the **"Mode"** button so the indicator shows **"Cal".** This means that the device is in calibration mode.
6. Type the measured temperature into the keypad.
7. Press the **"Set"** button in the **Temperature** panel. This will set the current temperature to the value you are measuring.
8. Press the **"Mode"** button again to re-enter calibration mode.
9. Press the **"Cal Stats"** button.
10. Press the **"Save"** button.
11. Press **"Return"** to go back to the main screen.
12. The device temperature is now calibrated!
13. If there are any errors with temperature calibration, you can reset to the Factory Calibration from the **"Cal Stats"** page in calibration mode.

Leaky Sample Cups

The sample cups are fragile and can be damaged. Leaks can occur if the membrane is punctured, high pressure water is used for washing, or if the membrane is left out to dry. We recommend testing your Sample Cups every few experiments to ensure they are still intact. Here is how to do so:

1. Remove the cup from its storage solution.
2. Carefully rinse both the inside and outside with a gentle stream of distilled water.
3. Place the cup onto a kimwipe on its side and roll it up in the kimwipe to dry off the outside of the cup. Repeat this another time to dry the outside.
4. Dry off the bottom of the cup with a kimwipe and place the cup down on a fresh kimwipe.
5. Carefully fill the sample cup to the top with distilled water, making sure not to spill any on the lid of the cup or the sides.

6. Wait for ~10 minutes and return to confirm that the liquid remained in the cup and did not leak out onto the kimwipe below.

If your cups are leaky, please reach out to science@lifecanvastech.com.

Sample Cup Storage Solution

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

Reagent	Vendor	Product Number	Final Concentration
Sodium dodecyl sulfate	Sigma-Aldrich	75746	5%
Boric acid	Alfa Aesar	12680	1%
Sodium hydroxide	Sigma-Aldrich	S5881	Titrate to pH 9

Protocol last updated: 06/02/2022

v5.05: Added missing instructions to turn on Electrophoresis Power during secondary wash steps.