LifeCanvas Technologies
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Introduction

This document outlines the full LifeCanvas Technologies protocol, from beginning to end using passive methods only requiring no specialized equipment. It is possible to mix and match passive methods with active methods if you have the equipment (ie. passive clearing but active labeling). The general protocol is as follows:

1. SHIELD preservation
2. Clearing (delipidation)
3. Immunolabeling
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.

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

Evan Murray, Jae Hun Cho, Daniel Goodwin, Taeyun Ku, Justin Swaney, Sung-Yon Kim, Heejin Choi, Young-Gyun Park, Jeong-Yoon Park, Austin Hubbert, Margaret McCue, Sara Vassallo, Naveed Bakh, Matthew P. Frosch, Van J. Wedeen, H. Sebastian Seung, Kwanghun Chung. Simple, scalable proteomic imaging for high-dimensional profiling of intact systems, **Cell**, 2015 Dec 03, DOI: 10.1016/j.cell.2015.11.025

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v4.07

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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)

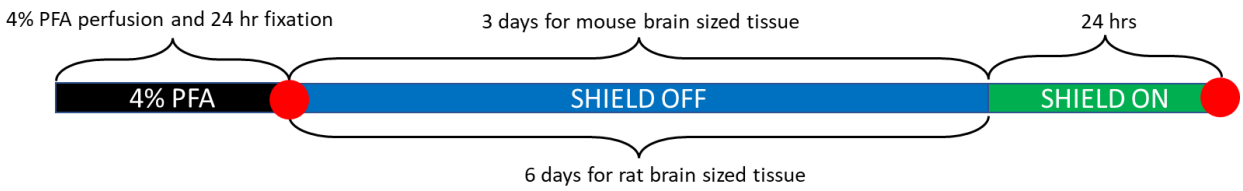
SHIELD-ON Buffer (SH-ON) - Store at 4°C upon delivery.

Standard Protocol

In most samples, the general protocol below will work well. It starts with a PFA fixation and subsequent drop-fix. If you are unable to perfuse the animal, start at **Step 3.** Please see the links below for specific sample types. It is still possible to perfuse with SHIELD if you wish, and that protocol is listed below. However, the Standard Protocol is recommended.

- 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.



Stopping Points: The protocol can only be stopped at the red circle points in the timeline above, so plan ahead!

1. 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.
2. Dissect out the brain / organ of interest. Careful dissection is essential to preserve the sample's structural integrity.
3. Incubate the sample in 4% PFA in PBS for 24 hours at 4°C with shaking. If you are not ready to continue to the next step, The sample can be stored in 1X PBS with 0.02% sodium azide at 4°C until you are ready to continue.
4. 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.
5. Prepare **fresh** SHIELD OFF Solution. Mix the following **in the order shown in the table** and keep on ice or move to 4°C after mixing. 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 3 days for mouse brains or similar sized samples. Rat brains should be incubated for 6 days. 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. Pre-warm SHIELD ON Buffer to 37°C. For mouse brains or similar sized samples use 20 mL. Rat brains require 40 mL.
8. Transfer the sample to SHIELD ON Buffer (RT) and incubate at 37°C with shaking for 24 hours.

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

Clearing (Delipidation)

Reagents / Equipment Required

- LifeCanvas Delipidation Buffer – stored at RT
- Temperature controlled shaking incubator (like EasyClear)

Protocol:

1. Incubate the sample in ~20 mL Delipidation at 37-45°C with shaking. We recommend clearing whole mouse brains for 1 week at 45°C, and hemispheres for 5 days. There is no need to refresh the solution. The samples will look the same as before clearing and should not have changed size. **You will not be able to tell that the sample is done clearing until the index matching stage.** Larger tissues will take longer to clear, while smaller tissues will take less time. For any questions about clearing time, please email: science@lifecanvastech.com for suggestions.
2. If you want to stain your sample with SYTO16 or Propidium Iodide, you can do this passively while the sample is being cleared. To do so, simply add dye to the tube in a 1:1000 dilution. This may not work with other dyes, but these have been tested.
3. When done clearing, incubate the sample in PBS (with 0.02% sodium azide) overnight at 37°C to wash out remaining buffer.
4. After washing, the samples can be stored at 4°C in PBS with 0.02% sodium azide until you are ready for index matching or immunolabeling.

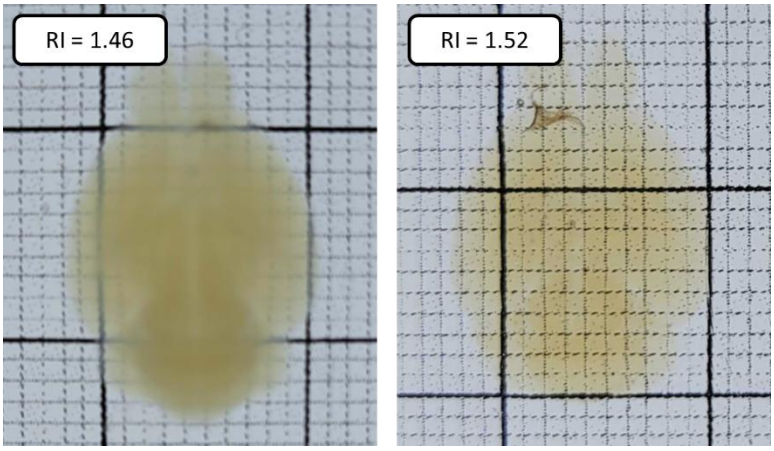
Immunolabeling

To increase penetration of antibodies into large, intact tissues, we recommend using active staining SmartLabel or SmartBatch+. You can proceed with normal IHC protocols but with extended staining durations and increased antibody amounts as needed. This optimization is highly empirical and variable from antibody to antibody. In some cases, it may not be possible to achieve uniform staining passively. If your samples are small (50-200 µm slices), normal IHC protocols as recommended by antibody manufacturers can be followed.

[Note] If you are interested in FISH, please consult the original SHIELD manuscript or contact the authors for more information.

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.** Here are images of 2 representative mouse brains index matched with 1.46 and 1.52 RI versions respectively which highlights the need for 1.52 RI.



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. If you are not 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 affect imaging.

Sample Mounting and Imaging Tips

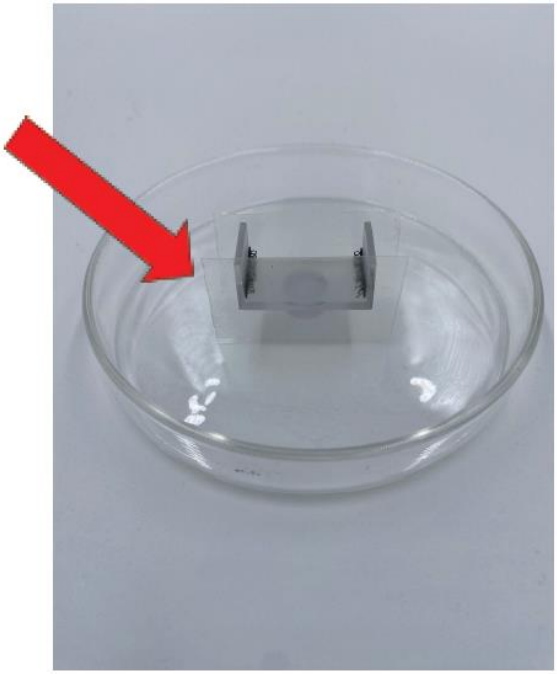
Since every imaging system is different, it is difficult to devise a singular mounting protocol for every setup. However, the following requirements always apply:

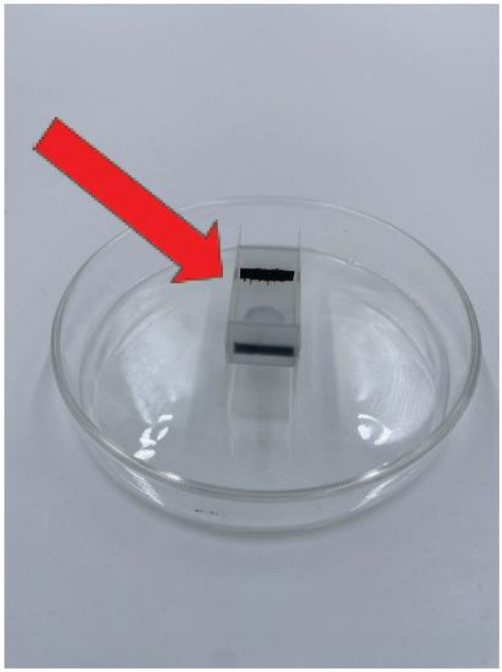
1. The sample must have the same RI as the imaging medium.
2. The sample must be immobilized.
3. EasyIndex, like many RI matching solutions, is water based. To avoid local changes in RI due to evaporation, the imaging chamber should be sealed from the air. This can be achieved with a sealed imaging chamber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

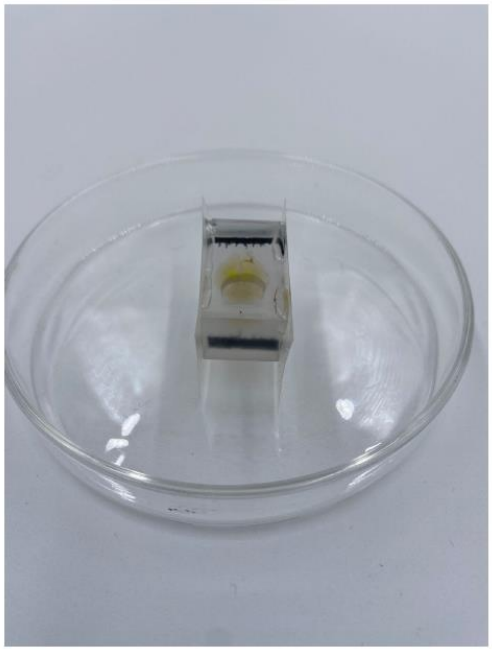
9. Prepare your sample holder. In the case of SmartSPIM, add thermal seal strips, sticky well plate covers, or blu-tack to the sides to form walls. See the image below:

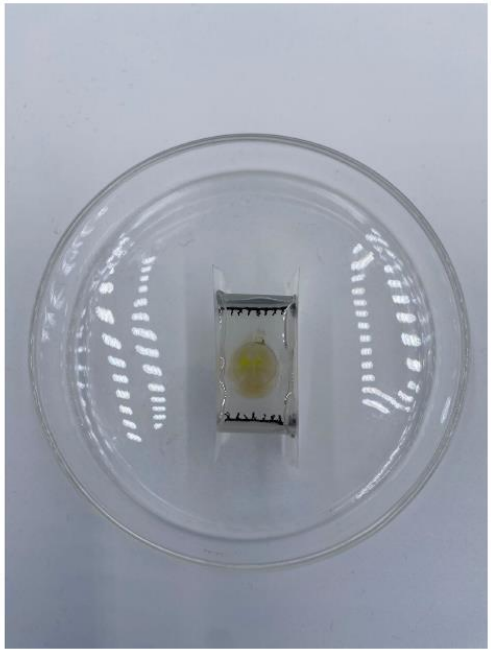




10. Prepare the sample by placing it in a glass petri dish and pipetting out the excess EasyIndex. Examine the sample under a light to remove any internal or external bubbles with a P10 pipette. If there are any internal bubbles that cannot be accessed via ventricles, use a small gage needle to aspirate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

Changelog

V4.07:

- Added timeline for SHIELD.
- Added image comparing 1.46 vs. 1.52 RI EasyIndex.