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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. Active clearing but passive labeling and vice versa). The general protocol is as follows:

1. SHIELD preservation
2. Clearing (delipidation)
3. Immunolabeling
4. Index Matching
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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Table of Contents

Introduction 1

Table of Contents 2

SHIELD 3

- Introduction 3
- Reagents Required 3
- Standard Protocol 3

Clearing (Delipidation) 6

- Reagents / Equipment Required 6
- Protocol: 6

Immunolabeling 7

- Reagents / Equipment Required 7
- Protocol: 7

Index Matching 9

- Reagents Required 9
- Protocol: 9
- Sample Mounting and Imaging Tips 10

SHIELD Appendices 12

- SHIELD Perfusion Protocol 12
- Small Sample SHIELD-ON 14
- Post-Fixing PFA-fixed Human Brain Samples 15
- Post-Fixing PFA-fixed Thin Slices 15

Digital Protocol and Other Documentation 16

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	4
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.

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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

- LifeCanvas Delipidation Buffer
- 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, you can do this passively while the sample is being cleared. To do so, simply add SYTO16 to the tube in a 1:1000 dilution.
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, it is best to use SWITCH-mediated immunolabeling as described below. If your samples are small (50-200 µm slices), normal IHC protocols as recommended by antibody manufacturers can be followed. You can also proceed with normal IHC protocols but with extended staining durations and adjust antibody amount / incubation times as needed.

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

Reagents / Equipment Required

- Sodium dodecyl sulfate (Sigma 75746)
- Triton X-100 (Sigma X-100)
- Temperature controlled shaking incubator (like EasyClear)

Protocol:

1. Prepare Antibody OFF Solution by adding Sodium dodecyl sulfate (SDS) to PBS to a final concentration of 0.5 mM. This is most easily accomplished by diluting a stock SDS solution.
2. Equilibrate the sample in Antibody Off Solution overnight to distribute SDS molecules in the sample.
3. Place the sample in a fresh volume of Antibody Off Solution just large enough to cover the sample.
4. Add primary antibodies to the solution in the desired proportions. The amount of antibody should be determined empirically depending on the antibody concentration, size of the tissue, and the antigen. If the antibodies or dyes are fluorescent, protect the sample from light from this point on.
5. Incubate the sample at 37°C with gentle shaking for 12 hours to 7 days depending on the size of the sample. (1 mm-thick section to whole mouse brain).
6. Prepare Antibody On Solution by adding Triton X-100 to PBS to a final concentration of 0.1% (v/v).
7. Incubate the sample in Antibody On Solution at 37°C with gentle shaking for 12 hours to 2 days to initiate antibody binding and wash out unbound probes.

8. If secondary antibody labeling is required after primary staining, incubate the sample in fresh Antibody On Solution containing secondary antibodies at 37°C for 12 hours to 7 days. It is important to use enough secondary antibodies to saturate all primary antibodies in the sample. From this point on the sample must be protected from light.
9. After labeling is complete, incubate the sample in fresh Antibody On Solution to wash out unbound probes.
10. Over time, antibodies can dissociate from their targets. If you intend to keep the samples around for an extended period of time for imaging at later dates we recommend fixing the antibodies in place. To do so, fix the samples overnight in 4% PFA in PBS at RT with light shaking. Then wash out PFA with PBS.
11. The sample can now be stored in PBS with 0.02% sodium azide until it is ready for index matching.

Index Matching

Now that you are ready to image your samples, you need to index match them so they are optically transparent. 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 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. 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. It is usually easier to remove bubbles from ventricles using a P10 pipette or 30 gauge needle syringe.
2. Mix low melting point agarose (Agarose, Type I, low EEO, CAS Number: 9012-36-6) and EasyIndex (2% 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 1.52.
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

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.

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:

Protocol last updated: 5/26/2021