LifeCanvas Technologies
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Introduction

This document outlines the full LifeCanvas Technologies protocol, from beginning to end using SmartClear II Pro and SmartLabel. The general protocol is as follows:

1. SHIELD preservation
2. Delipidation with SmartClear II Pro
3. Immunolabeling with SmartLabel
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. 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.**

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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.

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

- SmartClear II Pro
- Delipidation Buffer – stored at 4°C
- Conduction Buffer – stored at 4°C
- SmartClear Membranes

Protocol:

1. Incubate the samples in Delipidation Buffer overnight at RT. Only 400 mL of solution will be used in the device, so 100 mL can be removed from the bottle to be used for this purpose.
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. 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 to 1000 or 1200 mA.
7. Turn on Electrophoresis power and clear the samples. **The samples will not appear transparent at this stage, and they will almost appear unchanged.** The clearing time is sample dependent. Adult mouse brains will clear in 24 hours at 42°C. Rat brains will clear in roughly 7 days at 42°C. Smaller samples will clear faster.

8. When the samples are done delipidating, remove the mesh bag from the device and transfer the sample to PBS with 0.02% sodium azide. The samples can be stored in this solution at 4°C until you are ready for the next steps.
9. Consult the Appendices for Shutdown Procedures and Maintenance Information.

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Immunolabeling with SmartLabel

Reagents / Equipment Required

- SmartLabel
- SmartLabel Reagents – check bottles for storage conditions. **This protocol is intended for use with Buffers containing lot numbers listed as PS#, PD#, SS#, and SD#. If your reagents have different lot number formats please reach out for the correct protocol.**
- 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.

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 – 16 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 – 8 hours
7. Actively wash out excess secondary – 4 hours

Prepare the Samples for Labeling (Immediately after delipidation)

In this step we will incubate the samples in the buffers to prepare them for labeling.

1. On the day before you are ready to start labeling (or on a Friday if labeling on Monday), transfer the sample to ~20 mL of Primary Sample Buffer and incubate at RT with light shaking overnight. If you are not ready to start labeling, you should store the samples in PBSN (PBS with 0.02% sodium azide) at 4°C until this step.
2. 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 sample cups from their 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. Remove the mesh bag and optional mesh strips from the storage solution.
6. Rinse them with tap water, then distilled water and add them to the water with the cups.

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. Setup the device as shown in the SmartLabel QuickGuide.
2. Wash the device:
    a. Turn off the pumps and drain any liquid from the reservoirs.
    b. Add ~500 mL of distilled water to each reservoir being washed.
    c. Turn on the pump and run it for at least a minute.
    d. Repeat steps a-c three more times or until the reservoirs are not bubbly.
3. Turn off the pumps and drain any liquid from the reservoirs.
4. Pour one bottle of Primary Device Buffer into each reservoir that will be in use. Make sure to screw down the reservoir lid to prevent bubbles from escaping. Note that voltage is applied to both chambers even if you are only running 1 experiment. The best practice is to only run the pump in the chamber which is actively staining something.
5. Turn on the pumps and change the settings to the following:

Setting	Proper Value
Voltage	90 V
Current Limit	1 Chamber = 500 mA, 2 Chambers = 1000 mA
Temperature	25 °C
Rotation Speed	0.01 rpm
Timer	16 hours

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Prepare the Primary Antibody Cocktail (Just before staining)

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.

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.

Finally, if you wish to do multiple rounds of staining by stripping antibodies after staining we recommend using Sequential staining with whole IgG secondaries.

1. Pipette 1 mL of Primary Sample Buffer into a 1.5 mL conical tube and keep it on ice or in a cold block.
2. Add antibodies / dyes to the tube, 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 cocktail, 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.
3. Shake or vortex the tube and briefly cover it from light until the next step.

Start Primary Labeling

In this step the sample cup will be prepared, added to the device, and labeling will begin.

1. Remove the sample cup from the distilled water and dump out the water.
2. Fold a kimwipe in half and roll it into a tube. Carefully insert it into the cup to soak up remaining water.
3. Carefully pour Primary Sample Buffer into the cup, filling it nearly to the top.
4. Dump and discard the liquid in the cup.
5. Repeat step 2 to soak up the liquid.
6. Carefully pour Primary Sample Buffer into the cup again. This time, the liquid level should be about 5 mm below the lower plastic rim of the cup, or about 2/3 full. This leaves space for the antibody cocktail.
7. Remove the mesh bag and strips from the distilled water and dry them with kimwipes.
8. Open the mesh bag into a cylinder (folding the bottom can help keep it open) and insert it into the cup.
9. Use a lab spoon or spatula to lower the sample into the mesh bag inside the cup. For mouse brain tissue it is best to do this with the cerebellum down.
10. It is best for the tissue to be standing up vertically. If the sample falls over, fold a mesh strip in half and use blunt tweezers to insert it into the mesh bag beside the sample to hold it in place.
11. Use a pipette to add the Antibody Cocktail to the cup. Once added, gently mix with the pipette.
12. If your sample is particularly tall, add some more Primary Sample Buffer to cover it up.
13. (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.
14. Take the sample cup and place it into the Labeling chamber. Line up the hex on the bottom of the cup with the hex in the chamber. You can turn off the pump if it makes it easier, just make sure to turn it back on after.
15. Turn on Stirring and look at the sample in the cup. The sample should not be moving. If it is, briefly remove it from the chamber and add additional mesh strips to prevent movement.
16. Turn on Sample Rotation and ensure the timer is set to 16 hours.
17. Place the magnetic lid over the chamber.

18. Turn on Electrophoresis Power and the timer and close the main device lid. The timer will start to count up. After a few seconds the voltage will click up to 90V and the current will start at roughly 300 mA per chamber. Over the course of the experiment the current will increase to the 500 mA limit and the voltage will decrease accordingly.
19. The timer will automatically turn off electrophoresis and rotation. It is okay for this step to end overnight or even over the weekend – simply leave the device as it is.

Finish Primary Labeling (Next morning after staining)

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

1. With the pump running, drain a small volume of buffer (roughly 30 mL) out of the reservoir into a small beaker.
2. Measure the pH of the solution with a pH meter or pH strip. The pH should be below 8 and ideally around 7.50.
3. (Optional) If the pH is still above 8.0, you will need to extend the duration of the experiment to complete 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 hours. Then turn on Sample Rotation, Electrophoresis Power and the timer. Repeat until the pH is below 8.0.
4. Open the device, remove the magnetic lid, and remove the sample cup from the device.
5. Transfer the sample to a fresh conical tube filled with PBS. The best way to do this is to remove the mesh bag from the sample cup using blunt tweezer (be careful not to poke the membrane!). Then dip the mesh bag into the PBS upside down. The sample will fall out of the bag and sink.
6. Wash the sample in the PBS over the course of the day at RT with light shaking. Protect the sample from light.
7. Wash the Sample Cups and mesh:
    a. 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.
    b. Use a gentle stream of distilled water to finish rinsing the cup and transfer it back to its storage solution.

c. Rinse the mesh bag and strips with water and transfer them to the storage solution as well.

8. Wash the device:
    a. Turn off the pumps and drain liquid from the reservoirs being washed. Used buffer can be disposed down the drain as it is not hazardous.
    b. Add 500 mL distilled water to each reservoir being washed.
    c. Turn on the pump and run it for at least a minute.
    d. Repeat steps a-c three more times or until the reservoirs are not bubbly.
    e. Once the device is washed you can turn off the main power.
9. 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.
10. 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. **This is required for many targets, including cFos and NeuN.** In preparation for this, refresh the PBS in the tube a few times during the day to facilitate washing. If you plan to do multiple rounds of staining, you cannot fix the antibodies because you will need to strip them out after imaging. In this case, transfer the sample to ~20 mL of Secondary Sample Buffer and wash overnight at RT with light shaking. Then skip the fixation step and go directly to "Prepare the Sample for Sequential Secondaries".

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).

1. Prepare a solution of 4% PFA in 1X PBS.
2. After washing the sample in PBS during the day, transfer it to PFA and incubate overnight at RT with shaking. Protect the sample from light.
3. Note: if you are not able to do this step in the same day, simply leave the sample in PBS for up to a few days before fixing.
4. 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 (PBS + 0.02% sodium azide).

5. If you need to apply sequential secondaries, continue to the next step!

Prepare the Sample for Sequential Secondaries (First thing in the 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. Transfer the sample to a fresh tube of ~20 mL of Secondary Sample Buffer at 37°C. Refresh the solution at least once after about 2 hours.

Prepare the 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 each of the reservoirs to be used.
    b. The current limit and timer should be adjusted. The settings should now read as follows:

Setting	Proper Value
Voltage	90 V
Current Limit	600 mA – 1 Chamber 1200 mA – 2 Chambers

Temperature	25 °C
Rotation Speed	0.01 rpm
Timer	4 hours

Wash the Sample 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. Fold a kimwipe in half, roll it up into a tube and insert it carefully into the cup to soak up remaining water inside.
3. Carefully pour Secondary Sample Buffer into the sample cup, filling it up nearly to the top.
4. Dump and discard the liquid in the cup.
5. Repeat step 2 to soak up remaining liquid.
6. Pour Secondary Sample Buffer into the cup and fill it nearly to the top.
7. Remove the mesh bag and strips from the distilled water and dry them with kimwipes.
8. Open the mesh bag into a cylinder and insert it into the sample cup.
9. Use a lab spoon or spatula to lower the sample into the mesh bag inside the cup. For mouse brain tissue it is best to do this with the cerebellum down.
10. It is best for the tissue to be standing up vertically. If the sample falls over, fold a mesh strip in half and use blunt tweezers to insert it into the mesh bag beside the sample to hold it in place.
11. Take the sample cup and place it into the Labeling chamber, lining up the hex on the bottom of the cup with the hex on the bottom of the chamber. The cup should sit flat in the chamber.
12. Turn on the Stirring power and look at the cup. The sample should not be moving. If it is, briefly remove it from the chamber and add additional mesh strips to prevent movement.
13. Cover the labeling chamber with the magnetic lid and close the main device lid.
14. Turn on Sample Rotation.

15. Turn on the Electrophoresis Power and the timer. The timer will begin to count up. After a few seconds the voltage will click up. The current will reach the limit value of 600 mA per chamber and the voltage will drop to around 50V.
16. After about 2 hours, refresh the buffer in the cup. This is not necessary but likely improves staining quality. Here is how to do that:
    a. Remove the cup from the device.
    b. Remove the mesh bag from the cup with some tweezers and place it on a kimwipe.
    c. Dump out the solution in the cup.
    d. Roll up a kimwipe and insert it carefully into the cup to soak up remaining liquid.
    e. Pour fresh Secondary Sample Buffer into the cup and put the mesh bag with the sample back into the cup.
    f. Put the cup back in the device and continue washing.

Prepare Secondary Antibody Cocktail (Just before secondary staining ~5pm)

In this step the Secondary Antibody Cocktail will be made.

1. Pipette 1 mL of Secondary Sample Buffer into a 1.5 mL conical tube and keep it on ice or in a cold block.
2. Add secondary antibodies to the tube. We recommend 2:1 molar ratio of Secondary:Primary, but this is antibody dependent and is a good starting point.
3. Shake or vortex the tube and briefly cover it from light until the next step.

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, remove the mesh bag from the cup with tweezers and place it on a kimwipe.
2. Dump out the liquid in the cup.
3. Fold a kimwipe in half, roll it up into a tube and insert it carefully into the cup to soak up remaining liquid.
4. Carefully pour Secondary Sample Buffer into the sample cup, filling it up nearly to the top.

5. Dump and discard the liquid in the cup.
6. Repeat step 3 to soak up remaining liquid.
7. Carefully pour Secondary Sample Buffer into the cup again. This time, the liquid level should be about 5 mm below the lower plastic rim of the cup, or about 2/3 full. This leaves space for the antibody cocktail.
8. Put the mesh bag with the sample back into the cup.
9. Use a pipette to add your Secondary Antibody Cocktail to the cup. Once added, mix it gently with the pipette.
10. If your sample is particularly tall, add some more Secondary Sample Buffer to cover it up.
11. (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.
12. Take the sample cup and place it into the Labeling chamber. Line up the hex on the bottom of the cup with the hex on the bottom of the chamber. You can turn off the pump if it makes it easier, just make sure to turn it back on after.
13. Turn on Stirring and look at the sample in the cup. The sample should not be moving. If it is, briefly remove it from the chamber and add additional mesh strips to prevent movement.
14. Place the magnetic lid over the chamber and close the main lid.
15. Turn on Sample Rotation.
16. **Change the timer to 8 hours.**
17. Turn on Electrophoresis Power and the timer and close the main device lid. The timer will start to count up. After a few seconds the voltage will click up and the current will hit the limit of 600 mA per chamber. The voltage will be around 50V.
18. The timer will automatically turn off electrophoresis and rotation. It is okay for this step to end overnight or even over the weekend – simply leave the device as it is.

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 to “Finish Secondary Labeling Experiment”.

1. After the Secondary Staining is finished, remove the sample cup from the device, remove the mesh bag from the cup and place it on a kimwipe.
2. Dump and discard the liquid in the cup.
3. Fold a kimwipe in half, roll it up into a cylinder and carefully put it into the sample cup to soak up remaining liquid.
4. Pour Secondary Sample Buffer into the cup nearly to the top, then dump it out and discard the liquid.
5. Repeat step 3 to soak up remaining liquid.
6. Pour fresh Secondary Sample Buffer into the cup.
7. Put the mesh bag with the sample back into the cup.
8. Put the cup back into the device and **change the timer to 4 hours**.
9. Put the magnetic lid back on the chamber and close the main lid.
10. Turn on Stirring, Sample Rotation, Electrophoresis Power and the timer.
11. After about 2 hours you can remove the cup and repeat steps 2-10 for optimal washing. (Note – you can do this without resetting the timer so it is effectively 2 x 2hr washes).

Finish Secondary Labeling Experiment (After optional electrophoretic washing)

In this step the sample will be removed from the device, and the device / cup will be washed.

1. Open the device, remove the magnetic lid, and remove the sample cup from the device.
2. Transfer the sample to a fresh conical tube filled with PBS. The best way to do this is to remove the mesh bag from the sample cup using blunt tweezer (be careful not to poke the membrane!). Then dip the mesh bag into the PBS upside down. The sample will fall out of the bag and sink.
3. Wash the sample in the PBS over the course of the day at RT with light shaking. Protect the sample from light.
4. Wash the Sample Cups and mesh:
    a. 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.**
    b. Use a gentle stream of distilled water to finish rinsing the cup and transfer it back to its storage solution.

   c. Rinse the mesh bag and strips with water and transfer them to the storage solution as well.

5. Wash the device:
   a. Turn off the pumps and drain liquid from the reservoirs being washed. Used buffer can be disposed down the drain as it is not hazardous.
   b. Add 500 mL distilled water to each reservoir being washed.
   c. Turn on the pump and run it for at least a minute.
   d. Repeat steps a-c three more times or until the reservoirs are not bubbly.
   e. Once the device is washed you can turn off the main power.
6. You are done labeling! You can now continue to Index Matching.

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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.

Note: If a sample does not contain any antibodies or only contains fixed antibodies, you can increase incubation temperature to 37°C to speed up RI matching.

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. After index matching your sample, use a pipette or degassing chamber to remove any bubbles from the sample. It is easier to also remove bubbles from ventricles using a P10 pipette or a 30 gauge syringe needle.
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 55-60°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 carefully 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 for about 10-15 minutes. Opening the tube will release some bubbles.
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 1.520.
2. Fill the imaging chamber with the immersion oil.
3. Index match and mount your sample as normal with EasyIndex.
4. 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.

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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.
15. Pour 400 mL of Delipidation Buffer into Reservoir A.
16. Pour the entire bottle of Conduction Buffer into Reservoir B.
17. Turn the pumps back on. You are now ready to clear!
18. 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 Bufffer 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:

Reagent	Vendor	Product Number	Final Concentration (mM)
Sodium dodecyl sulfate	Sigma-Aldrich	75746	300
Boric acid	Alfa Aesar	12680	10
Sodium sulfite	Sigma-Aldrich	S0505	100
Sodium hydroxide	Sigma-Aldrich	S5881	Titrate to pH 9

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:

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

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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:

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:

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