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SHIELD Protocol with LifeCanvas Devices

ORIGINAL ARTICLE:

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REAGENTS:

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.

In most situations, the **STANDARD PROTOCOL** will work well. If you are post-fixing PFA-fixed human brain slices (~1 mm thick), please proceed to the **POST-FIXING PFA-FIXED HUMAN SAMPLES** protocol.

STANDARD PROTOCOL

Different samples have different starting points, but you always need to complete the protocol through Step 6. If your experiment allows it, the best preservation technique involves perfusion of the live animal. If you can perfuse the animal, please start at **Step 1.** If you cannot perfuse an animal, but you do have fresh tissue or an embryo, please start at **Step 3.** If the sample is fresh frozen, please thaw the sample first, and then start at **Step 3.** If your sample is already PFA-fixed, please start at **Step 4.**

PROCEDURE:

1. Prepare SHIELD Perfusion Solution **fresh** on ice (see **REAGENT SETUP**), and transcardially perfuse the animal with ice-cold PBS followed by ice-cold SHIELD Perfusion Solution in the following volumes and flow rates. Keep the remaining SHIELD Perfusion Solution on ice for use in Step 3.

Step 1	PBS (mL)	SHIELD Perfusion Solution (mL)	Flow Rate (mL/min)
Mouse	20	20	5
Rat	200	200	60

   - 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.

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

Step 3	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.

4. Prepare fresh SHIELD OFF Solution (see **REAGENT SETUP**) and incubate at 4°C with shaking. Use the following volumes and incubation times, and please note the different incubation times for post-fixing PFA-fixed samples.

Step 4	SHIELD OFF Solution Volume (mL)	Incubation Time (day) For Perfusion / Fresh	Incubation Time (day) For PFA-fixed
Mouse Brain	20	1	4
Rat Brain	50	3	6
Smaller Samples	> 10X Sample Volume	1	Sample Dependent

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

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

Step 5	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.

6. You may now proceed to the **TISSUE CLEARING** section of the protocol.

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REAGENT SETUP

REAGENT SETUP:

SHIELD Perfusion Solution (prepare fresh before perfusion)

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

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)**

SHIELD-OFF Solution (prepare fresh for Step 4 of the **STANDARD PROTOCOL** or Step 1 of **POST-FIXING PFA-FIXED HUMAN SAMPLES** protocol.)

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

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

* If you are post-fixing a human sample slice, the mouse brain volume will work. SHIELD works with a wide variety of tissues, but the total volume of solution required is dependent on tissue size. For smaller samples, use the same ratio of chemicals with a total volume at least 10X the volume of the sample.

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POST-FIXING PFA-FIXED HUMAN SAMPLES

PROCEDURE:

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.

SMALL SAMPLE SHIELD-ON

This SHIELD-ON protocol should be used for any sample with its smallest dimension 1.5 mm or smaller. This should be used as a replacement for **Step 5** of the **STANDARD PROTOCOL**.
It can be used for drop-fixation of mouse spinal cords.

PROCEDURE:

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.

TISSUE CLEARING

SHIELD-processed tissues can be cleared both passively with EasyClear (or temperature controlled shaking water bath) or actively with SmartClear II Pro. Tissues should be cleared prior to immunolabeling.

ACTIVE CLEARING (SMARTCLEAR II PRO)

PROCEDURE:

Install buffers and membranes in the SmartClear II Pro system. Wash the sample in SDS Clearing Solution (or a small volume of Buffer A) overnight, and carefully place the tissue in the sample holder and put it in the SmartClear II Pro system. For optimal clearing, align the long axis of your sample vertically in the holder. Buffer A temperatures of 40-45°C can clear a mouse brain hemisphere preserved with perfusion in 3-4 days, yielding better retention of fluorescence signal than passive clearing. Post-fixed samples may take longer to clear. For FISH experiments, it is best to clear at 37°C for 7-8 days. Higher temperature will lead to significant loss of RNAs. The sample will not appear transparent, but rather translucent. Samples will become transparent after **INDEX MATCHING (EASYINDEX).** Samples that are not fully cleared will show an opaque center. If your samples do not require immunolabeling, wash out clearing buffer with PBS at 37°C with shaking (overnight for small tissues, 1-2 days for a whole mouse brain) and proceed to **INDEX MATCHING (EASYINDEX)**. If your samples need to be immunolabeled, wash out clearing buffer with PBST at 37°C with shaking (overnight for small samples, 1-2 days for a whole mouse brain) before labeling. Please contact LifeCanvas at science@lifecanvastech.com for help with labeling.

PASSIVE CLEARING

REAGENT SETUP:

SDS Clearing Solution:

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

PROCEDURE:

Incubate the sample at 37-60°C with shaking. Higher temperatures will clear tissue faster but result in smaller retention of fluorescence signal. It will take 8-14 days to clear a mouse brain hemisphere at 45°C. For FISH experiments, it is best to clear at 37°C. The sample will not appear transparent, but rather translucent. Samples will become transparent after **INDEX MATCHING (EASYINDEX).** Samples that are not fully cleared will show an opaque center. If

your samples do not require immunolabeling, wash out clearing buffer with PBS at 37°C with shaking (overnight for small tissues, 1-2 days for a whole mouse brain) and proceed to **INDEX MATCHING (EASYINDEX)**. If your samples need to be immunolabeled, wash out clearing buffer with PBST at 37°C with shaking (overnight for small samples, 1-2 days for a whole mouse brain) before labeling. Please contact LifeCanvas at science@lifecanvastech.com for help with labeling.

INDEX MATCHING (EASYINDEX)

PROCEDURE:

1. Shake the bottle of EasyIndex well to homogenize the solution. Let the bottle sit for ~30 minutes to allow the bubbles to settle.
2. Incubate the tissue in EasyIndex with shaking at RT. Use the following volumes and recommended incubation times:

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

After index matching, the sample should be clear enough to easily see through. If the solution surrounding the sample seems imhomogeneous, it suggests that the sample has not yet been fully equilibrated with the solution and should be incubated further.

*We strongly advise against reusing EasyIndex as its Refractive Index (RI) changes after the first usage.
*Samples should not be stored in EasyIndex for more than 2-3 days. If a sample will not be imaged until a later date, store the sample in PBS with 0.02% sodium azide.
*If a sample has been index matched and needs to be recovered and saved, the sample should be incubated in PBS at 37°C with gentle shaking overnight then stored appropriately.