

Protocol for Testing Antibody Compatibility with SHIELD & SmartLabel

- Q: *Why does antibody compatibility need to be validated for use with SHIELD & SmartLabel?*
- A: Like other methodologies that differ from the widely-used practice of perfusing & post-fixing tissue with 4% paraformaldehyde (PFA) (e.g., fresh frozen tissue, formalin-fixed paraffin-embedded tissue), antigen presentation and the ability of antibodies & other molecular probes to bind to these sites differs depending on the processing regime used. For example, PFA itself masks many sensitive epitopes such as those on certain neurotransmitter receptors, which are readily accessible for staining when using fresh frozen tissue. Antibody manufacturers list these distinct approaches on their product webpages and indicate whether a specific antibody or clone has been validated to work with a given approach.
- As was initially done when researchers started using these approaches that are now well-established, it is best practice to confirm the compatibility of individual antibodies with SHIELD & SmartLabel by performing a series of validation experiments involving different tissue preparation and buffer conditions.
 - The central purpose of this antibody validation procedure is to test whether satisfactory staining is retained throughout this series of conditions, each of which takes the sample increasingly further away from the base case of standard PFA fixation. If good staining is observed across all of the conditions, including the final one which approximates SmartLabel staining of a SHIELD-preserved, delipidated sample, then the antibody is a good candidate for trying actual SmartLabel active immunostaining.

Reagents, supplies, and equipment needed:

- Primary and secondary antibodies = LifeCanvas recommends either (1) fluorophore-conjugated primary antibodies or (2) primaries + [Fabulight Fc-specific monovalent F\(ab\) fragment secondaries](#)
 - For mouse host antibodies of unknown isotype, use pan-mouse F(ab) fragments such as [this one](#) (other conjugates available from drop-down menu)
 - When using goat host primary antibodies, use [non- FC-specific F\(ab\) fragments produced in donkey](#)
 - 3 conjugates that offer good spectral separation for multiplexing are: Alexa Fluor 488, Rhodamine Red-X (RRX), and Alexa Fluor 647
- Tissue samples = multiple conditions needed (see below for details)
- [SHIELD reagent kit](#)
- PBST (PBS with 1% Triton-X 100)
- Passive Clearing / delipidating Buffer = either purchased from LifeCanvas or prepared from an open-source recipe such as follows: 300 mM [SDS](#), 10 mM [boric acid](#), 100 mM [sodium sulfite](#), titrate to pH 9 with [sodium hydroxide](#)
- SmartLabel Labeling-ON buffer (= “SL buffer”) with pH titrated to ~8.45 using a small amount of phosphoric acid. (The pH of Labeling-ON buffer shipped to you in LifeCanvas buffer kits may be slightly higher than this, which is okay for SmartLabel experiments, but it needs to be titrated for passive staining experiments.)
- Microscope to visualize antibody staining results (a confocal is best) and related mounting materials

Notes on tissue samples needed and recommended preparation:

Depending on your exact research question, as long as the tissue sections contain the target proteins of interest it may be more or less important to look at the exact same region of tissue when comparing across the

conditions below. For example, if you are interested in a widely-expressed neuronal marker like NeuN or PV, you can likely safely get away with looking at any piece of cerebral cortex for a given condition. If this is the case, a parsimonious way to prepare your tissue would be to:

- a) Perfuse 2 animals, one with PFA and one using the SHIELD perfusion protocol. Hemi-sect both brains following initial post-fixation steps (i.e., after 24 hrs post-fixation in PFA-containing solution, before proceeding with the rest of the SHIELD perfusion protocol).
- b) In brief, you can then (1) vibratome section (~50-100 μm thick) individual PFA-only and SHIELD-preserved hemispheres and (2) clear/delipidate half of the SHIELD sections to (3) arrive at the following set of sections needed to test a given antibody:
 - PFA-only = 1 section
 - SHIELD-preserved non-cleared = 2 sections
 - SHIELD-preserved cleared = 2 sections
 - **Depending on your level of familiarity with clearing and with a given tissue type, it can also be beneficial to have an extra PFA-only and SHIELD-preserved section on-hand so that each can be visualized without any antibody stains, enabling observation of background autofluorescence and other native properties of the tissue.*
 - **If you are working jointly with LifeCanvas to optimize antibody staining for your application, please do include images from these sections in the panel that you send to us.**

In more detail, to arrive at the set of sections described above you can follow the steps and considerations outlined below:

- a. From one of the PFA brain hemispheres, use a vibratome to prepare tissue sections between ~50-100 μm thick. Your choice of thickness can depend on the following:
 - i. How much material you have available (e.g., if you need to validate antibodies specifically within a smaller brain region like the amygdala, you will want to work with thinner sections).
 1. There is no other use for the second PFA hemisphere, so use it if desired.
 - ii. How many antibodies you want to test using the tissue (using one antibody per tissue slice can help reduce complexity when troubleshooting, but it is not essential).
 - iii. What thickness of tissue you are most comfortable handling for mounting and imaging.
- b. Using one of the SHIELD-preserved brain hemispheres, also prepare ~50-100 μm thick sections. You will need 4 times as many SHIELD sections as you do PFA sections to test a given antibody.
 - i. **Prepare additional PFA and SHIELD sections as needed to look at background autofluorescence levels and for other purposes.*
- c. Clear/delipidate however many SHIELD sections are needed to test your set of antibodies of interest. Please refer to LifeCanvas protocols on SmartClear 2 Pro, EasyClear, and our Clearing Buffers for best practices. In general, SHIELD-perfused sections of ~50-100 μm thickness can be cleared by incubation in clearing buffer (either Buffer A or Passive Clearing Buffer) at 37-42° C on a shaking incubator.
 - i. Start with overnight incubation to be conservative and you can decrease clearing duration as you become more familiar with judging tissue clarity. You can always incubate one of the cleared sections (which are only translucent) in LifeCanvas's EasyIndex refractive index matching solution to turn them fully transparent and judge clearing accordingly. If parts of the tissue remain opaque especially outside of white matter regions, then the sections may require longer incubation in clearing buffer.
 - ii. Please see our *Quick Guide to Beginners in Tissue Clearing & Checking Clearing Progression with LifeCanvas Products* for more information. If you are relatively new to tissue clearing, please feel free to send photographs of your cleared samples to LifeCanvas

(science@lifecanvastech.com) for help judging tissue clarity before proceeding with staining.

Conditions to be tested:

As indicated in the table below, 3 different types of tissue are needed to validate antibodies as compatible with SHIELD & SmartLabel:

<i>Buffer condition</i>	<i>Tissue condition</i>		
	PFA	SHIELD	SHIELD + cleared
(Non-stained)	0-PFA	(not needed)	0-S+C
PBST	1	2	3
SL buffer	(not needed)	4	5

- PFA = tissue perfused with 4% PFA and post-fixed overnight
- SHIELD = tissue that has been through a complete SHIELD preservation protocol (perfusion or post-fix)
- SHIELD + cleared = tissue that has been SHIELD-preserved and then delipidated (either actively or passively)
- Additionally, as mentioned above: extra PFA-only (condition '0-PFA') and SHIELD + cleared (condition '0-S+C') sections to judge autofluorescence

The central purpose of this antibody compatibility check is to test whether satisfactory staining is retained throughout the following series of conditions, each of which takes the sample increasingly further away from the base case of standard PFA fixation and closer to conditions that approximate staining in SmartLabel:

- 0-PFA) **PFA fixed, non-stained:** to observe baseline tissue properties such as autofluorescence
- 0-S+C) **SHIELD preserved, cleared, non-stained:** to observe baseline tissue properties such as autofluorescence after clearing, as well as retention of any endogenous fluorescence reporters
- 1) **PFA fixed, PBST:** the base case, like standard IHC protocols
 - 2) **SHIELD preserved, (non-cleared), PBST:** to test the effects of SHIELD preservation on antigenicity, while still using standard IHC solutions
 - 3) **SHIELD preserved, cleared, PBST:** to test the effects of SHIELD preservation & delipidation on antigenicity, while still using standard IHC solutions
 - 4) **SHIELD preserved, (non-cleared), SL buffer:** to test the effects of SHIELD preservation combined with the reaction environment conferred by the SL buffer
 - 5) **SHIELD preserved, cleared, SL buffer:** to test the effects of SHIELD preservation & delipidation combined with the reaction environment conferred by the SL buffer

Validation steps:

With the above tissues (sections of PFA, SHIELD, and SHIELD + cleared) and reagents (PBST + SL buffer) on-hand you are now ready to begin the antibody validation protocol, which is simply a series of passive IHC experiments.

**Note: The antibody amounts and incubation volumes discussed here are generalized and are stated in reference to a tissue slice such as a coronal section of a mouse brain hemisphere, which is several mm by several mm in size. Depending on various factors such as (1) the size of your tissue slices, (2) the abundance of the target protein in the tissue, (3) the volume of solution you intend to use to incubate each slice, and (4) your past experience with antibody concentrations used to obtain satisfactory staining, you can use your best judgment to adjust the amount of antibody used in the steps below.*

- A. **Primary antibody incubation:** In reference to the table above, when preparing the base for your antibody incubation solution you can use one preparation tube for buffer/tissue conditions #1-3 (PBST) and another for conditions #4-5 (SL buffer).
- 1) For SmartLabel staining, the absolute amount of antibody (in μg) used in the experiment matters more than its relative concentration in the incubation solution, which is what is often referred to when designing free-floating labeling experiments that rely on passive diffusion.
 - i. For the purposes of these validation experiments, prepare your antibody incubation solutions with the goal of having 0.5-1 μg of primary antibody per tissue section. (*In practice, this amount can be adjusted based on the note above. For antibodies of unknown concentration, a dilution of $\sim 1:500$ can be a good starting place and you can refer to the manufacturer's suggestions.)
 - ii. *Note: If you currently have appropriate Fc-specific monovalent F(ab) fragment secondary antibodies, these can be included at this time along with the primary antibodies for one-step labeling. Calculate the amount of secondary to include using the formula described in the SmartLabel Labeling Protocol, which is as follows:*
 - Aim for a secondary-to-primary ratio of $\sim 2:1$, which should be calculated using the ratio of the antibody types' respective molecular weights (an embedded Excel calculator is provided below), where:
 - MW = molecular weight (primary, $1^\circ = 150 \text{ kDa}$; secondary, $2^\circ = 50 \text{ kDa}$)
 - C = antibody concentration (if available, i.e. from manufacturer)
 - V = volume of antibody solution, calculated from 'C' above and the *target weight (in μg) of 1° desired for the experiment, based on the mass of the tissue being labeled and your estimation of the number of antigenic sites present*

$$\text{molar ratio } \frac{1^\circ}{2^\circ} = \frac{1}{2} = \frac{\frac{V_{1^\circ} \cdot C_{1^\circ}}{MW_{1^\circ}}}{\frac{V_{2^\circ} \cdot C_{2^\circ}}{MW_{2^\circ}}}$$

$$V_{2^\circ} = V_{1^\circ} \cdot \frac{C_{1^\circ}}{C_{2^\circ}} \cdot \frac{MW_{2^\circ}}{MW_{1^\circ}} \cdot \frac{2}{1}$$

- a. Example, for a fully intact adult mouse brain hemisphere:
 - Label #1 (e.g., PV, a marker of inhibitory interneurons that accounts for $\sim 10\%$ of all neurons):
 - $C_{1^\circ} = 1 \mu\text{g} / \mu\text{L}$ (primary antibody concentration, from supplier)
 - $C_{2^\circ} = 1.6 \mu\text{g} / \mu\text{L}$ (secondary antibody concentration, from supplier)
 - $V_{1^\circ} = 25 \mu\text{L}$ (volume of primary antibody chosen to use)
 - $V_{2^\circ} = 10.42 \mu\text{L}$ (volume of secondary antibody calculated to use)
 - Label #2 (e.g., TH, a marker of catecholaminergic neurons, a sparser cell-type):
 - $C_{1^\circ} = 1.0 \mu\text{g} / \mu\text{L}$
 - $C_{2^\circ} = 1.6 \mu\text{g} / \mu\text{L}$
 - $V_{1^\circ} = 10 \mu\text{L}$
 - $V_{2^\circ} = 4.17 \mu\text{L}$

Below is an embedded Excel calculator whose default values show a third example. Depending on if/how you have both Microsoft Word & Excel installed on your computer it may or may not be editable here within Word by double-clicking it repeatedly. If it is not editable, you can either (1) use the Excel file provided by LifeCanvas or (2) copy the table into Excel and enter the formula below the table into the last cell (to the right of V2), which will make the calculator functional.

Secondary Antibody Volume Calculation formula		
Volume of Primary Antibody (in μL)	V1	10
Concentration of Primary Antibody (in mg/mL as indicated on the datasheet)	C1	1
Concentration of Secondary Antibody (in mg/mL as indicated on the datasheet)	C2	1.7
Molecular weight of Primary Antibody (in kDa)	MW1	150
Molecular weight of Secondary Antibody (in kDa)	MW2	50
Molar Ratio of Primary to Secondary	1	2
Volume of Secondary Antibody (in μL)	V2	3.921568627

Excel formula to solve for V2 is “=C2*C3*C6*C7/(C4*C5*B7)”

- 2) Incubate the tissue sections 1 per well/tube, numbered 1-5, for overnight to 24 hours on an RT shaker.
- B. **Wash sections --> Secondary antibody incubation:** Following completion of primary antibody incubation, wash the sections 3 times for ~20 minutes per wash in fresh either PBST or SL buffer, whichever was used during the prior step (but see *Note* below if one-step labeling was performed), on an RT shaker.
- 1) If you included F(ab) fragment secondaries along with the primaries, then labeling is complete and the samples can be slide-mounted for imaging. Proceed to step D.
 - i. *Note: If one-step labeling is complete, wash the SL buffer -incubated sections as follows (same as section C below): (1) 50% SL buffer + 50% PBST, (2) 100% PBST, and (3) 100% PBST.*
 - 2) If secondaries were not included, then prepare secondary antibody incubation solutions using fresh quantities of the same buffer used in the prior step (PBST or SL buffer) and add enough secondary antibody as indicated by the above formula.
 - 3) Incubate the tissue sections 1 per well/tube for overnight to 24 hours on an RT shaker.
- C. **Wash sections --> Mount & image:** Following completion of secondary antibody incubation, wash the sections 3 times for ~20 minutes per wash in fresh either PBST or SL buffer, whichever was used during the prior step, on an RT shaker. **For slices stained using SL buffer, the composition of the 3 washes should be as follows: (1) 50% SL buffer + 50% PBST, (2) 100% PBST, and (3) 100% PBST.**
- 1) Labeling is now complete and the samples can be slide-mounted for imaging.
- D. **Please prepare a panel of images** that highlight the staining observed across each of the different conditions, label the images #1-5 (along with conditions 0-PFA & 0-S+C, described above), and share the image series with LifeCanvas in a presentation file so that we can help judge antibody compatibility for use with SHIELD & SmartLabel. A template presentation file is available at this [link](#), which you can copy to use for yourself.
- 1) To properly assess staining intensity across conditions, it is recommended to keep the acquisition settings as consistent as possible between sections. Note that the intensity of some stains will appear brighter in cleared tissue vs. non-cleared tissue, so it is best practice to preview most or all of the sections before committing to acquisition settings to use for the whole set.