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Brain SeqStain Protocol

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ABSTRACT

Multiplex immunofluorescence imaging allows detection of multiple molecular and cellular markers in a single tissue section and their spatial organization in the tissue, thereby providing new knowledge via spatial relationship maps. However, such techniques have been challenging to use on brain tissues, given the delicate nature of these tissues and the difficulty antibodies face in permeating through their entire volume (Hickey et al. 2022). SeqStain is a novel multiplex immunofluorescence (IF) imaging technique that utilizes fluorescent DNA-tagged antibodies in combination with enzymatic-based removal of fluorescent signals to provide multiplexed spatialomic characterization of tissue specimens (Rajagopalan et al. 2021). This method allows for the profiling of a wide array of molecular entities and cellular constituents addressing the limitations of conventional immunohistochemical tissue staining and immunofluorescence techniques. However, applying SeqStain and other multiplex IF imaging methodologies to brain tissue sections, typically 40 µm thick sections, has been significantly limited due to some of the unique challenges inherent in brain tissues. Here, we present an optimized protocol for multiplexed IF staining and imaging of typical brain tissue sections that addresses many of the previous challenges and provides high-quality IF images. We have termed this protocol BRAIN SEQSTAIN. We expect these improvements to contribute to a more comprehensive understanding of the spatialomic organization in multiple cellular and molecular components of the brain in the future.

GUIDELINES

This protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 94630

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MATERIALS

	A	B	C
	Multi-well plates (6 well)	Corning	07-200-83
	Multi-well plates (24 well)	Corning	09-761-146
	WD-40	Home Depot	-
	Phosphate Buffer Saline	Gibco	10010-023
	SHIELD Kit	Life Canvas Technologies	SH-250
	Glycine	Sigma Aldrich	410225
	Antigen Unmasking Solution, Citric Acid Based	Vector Laboratories	H-3300
	Hydrogen Peroxidase	Sigma Aldrich	H1009
	β-Cyclodextrin	Sigma Aldrich	H107-5G
	Normal Donkey Serum	Jackson Immuno Research	017-000-121
	Glass Bottom Petri Dish	Electron Microscopy Sciences	70-674-02
	anti-MHCII	eBiosciences	56-5321-82
	anti-CD86	eBiosciences	56-0862-82
	anti-PGP9.5	Abcam	ab302664
	anti-Neurofilament-L	Cell Signaling Technology	8024
	anti-CD31	BioLegend	102520
	anti-Alpha synuclein	Abcam	ab311067
	anti-Tau	Cell signaling technology	27370
	anti-Myelin Basic Protein	BioLegend	836508
	Anti-alpha-synuclein	Cell signaling technology	33611
	anti-CD68	eBioscience	53-0681-82
	anti-NeuN	Sigma Aldrich	ABN78A4
	anti-Tyrosine Hydroxylase	BioLegend	818004
	anti-GFAP	eBioscience	53-9892-82
	anti-Parvalbumin	Abcam	ab313816
	anti-Alpha synuclein (MJFR1)	Abcam	ab195025
	Lithium Borohydride	Strem Chemicals	93-0397
	Tween 20	Sigma Aldrich	P2287

Instrumentation:

1. Microtome with a razor-sharp blade
2. Leveling platform with adjustable brass cutting platform
3. Microscope for blade inspection

4. Microscope for immunofluorescence imaging
5. Vortexer
6. Pipettes

BRAIN SEQSTAIN PROTOCOL

1 Schematic Representation of Brain SeqStain Protocol:

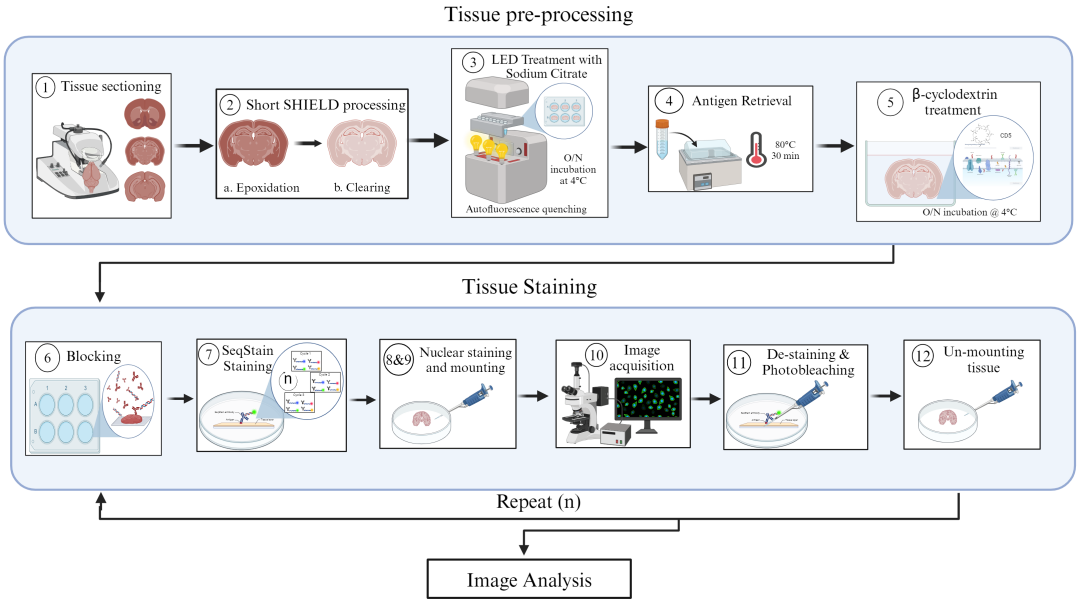

Figure 1: The diagram represents the steps and methods involved in Brain SeqStain Protocol

2 Steps of brain SeqStain protocol:

Step 1. Preparation of murine brain tissue sections

Step 2. Crosslinking step using an optimized shortened SHIELD protocol (Short SHIELD)

Step 3. LED treatment for quenching tissue autofluorescence
Step 4. Antigen retrieval using unmasking solution and peroxidase quenching
Step 5. Tissue permeabilization using cyclodextrin-based buffer
Step 6. Blocking
Step 7. Staining of unmounted brain tissues with antibodies
Step 7 (a). Staining of unmounted brain tissues with fluorescently labelled primary antibodies.
Step 7 (b). Staining of unmounted brain tissues with SeqStain antibodies
Step 8. Nuclear staining using DAPI Solution
Step 9. Mounting brain sections for imaging
Step 10. Confocal imaging
Step 11. De-staining tissue sections for removing fluorescent signal and re-imaging
Step 12. Unmounting of brain tissue sections for repeating next round of staining

Images ready for analysis.

Step 1. Preparation of (murine) brain tissue sections

3 **Preparation of instruments:**
This protocol is adapted from the published brain tissue sectioning protocol (Williams et. al, 2018).

3.1 Ensure the microtome blade is devoid of irregularities by inspecting it under a microscope and marking any uneven areas.

3.2 Apply a lubricant, such as WD-40, to the blade.

3.3 Securely affix the blade to the microtome, confirming the presence of a gap between the blade holder and the brass platform.

3.4 Utilize a leveler to confirm the evenness of the brass platform, adjusting as necessary via the lateral adjustment screw.

4 Sample Preparation and Sectioning

4.1 Insert a custom spacer into the platform to accommodate the brain specimen.

4.2 Crush dry ice into both powder and small crystal forms; distribute onto the brass platform to establish an optimal cryogenic environment.

4.3 Encase the isolated, whole, PFA-fixed murine brain tissue in a hydration square and position it centrally on the platform, allowing it to freeze.

4.4 Gently cover the tissue with powdered dry ice and, after a brief interval, brush away excess to expose the area of interest.

4.5 Lower the platform incrementally until the tissue contacts the blade, then section at a thickness of approximately 40µm.

5 Storage and post-processing

5.1 Carefully label wells for placing the tissue sections. For example, wells of a 24-well tissue culture plate.

5.2 Sequentially transfer each section into a well containing cyroprotectant.

5.3 Post-sectioning, cleanse the brass platform with water and properly dispose of residual tissue.

5.4 Securely cover the 24-well plate with parafilm and store it at -20 °C.

6 Tissue washing and preparation

6.1 Gently pick up the desired tissue section from the well of a 24-well plate using a synthetic paintbrush, transferring it to the cell strainer in the first well of the plate.

6.2 Fill 1 X PBS in a new 6 well plate and place a cell strainer within one well.

6.3 Wash the tissue in the well for 3 min at room temperature, with gentle agitation.

6.4 Next, move the tissue with the cell strainer to a new PBS-filled well in the 6-well plate to wash the tissue.

6.5 Repeat for a total of 12 times.

Step 2. Crosslinking step using an optimized shortened SHIELD protocol (…

7 This method is adapted from the published SHIELD protocol (Park et al. 2019) and has been optimized for use on floating brain tissue sections.
Note: While this protocol was in the process of submission, a similar optimized SHIELD protocol was recently published with many similarities (Porter et al., 2023).

8 Obtain necessary components such as ice, distilled water, SHIELD buffer, SHIELD epoxy, and conical falcon centrifuge tubes.

9 **Application of SHIELD OFF solution to tissue sections**

9.1 Prepare SHIELD OFF buffer by combining SHIELD buffer, SHIELD Epoxy and water of 1:2:1 ratio. For example, add 5 mL of SHIELD buffer and 10 mL of SHIELD Epoxy to 5 mL of distilled water in a conical tube.

9.2 Gently mix the prepared SHIELD OFF buffer by vortexing and leave it on ice to preserve reactivity, as published (Park et al. 2019).

9.3 Wash wells of a 6-well plate with 1X PBS.

9.4 Subsequently, fill the wells with 2mL of SHIELD OFF solution.

9.5 Transfer tissue sections to the SHIELD OFF containing well using a cell strainer.

9.6 Ensuring complete submersion of the sections, incubate at 4°C with agitation on an orbital shaker for 30 minutes.

10 Application of SHIELD ON solution to tissue sections

10.1 Prepare SHIELD ON solution in a 7:1 ratio by combining, for example, 7 mL of the base reagent with 1 mL of SHIELD Epoxy (Park et al. 2019).

10.2 Add SHIELD ON solution to a new well of a 6-well plate.

10.3 Transfer the tissue sections from the wells containing SHIELD OFF solution to the new wells containing the SHIELD ON solution along with a cell strainer.

10.4 Place the plate in a CO_2 incubator at 37°C with shaking for 30 min to induce polymerization of the tissue.

10.5 Subsequently, wash the tissue sections three times with 1X PBS for 10 minutes each at room temperature, utilizing the cell strainer for handling.

11 Quench reactive groups with 1M glycine solution

11.1 To neutralize excess reactive groups post-application of the SHIELD ON solution, prepare quenching solution by dissolving 3.75g of glycine in 35mL of Ambion nuclease-free water and adjust
the pH 7.5 with NaOH.

11.2 Add nuclease-free water to the quenching solution to get a total of 50mL then filter the solution before use.

11.3 Add 2mL of quenching solution to each well of the 6-well plate, containing different tissue sections. For example, well 1 wild type, well 2 LPS treatment, etc.

11.4 Carefully transfer tissue sections from previous steps to the quenching solution containing wells using a cell strainer and incubate the plate at 4°C with agitation for 15 minutes. Ideally, this step is conducted in a cold room to prevent any potential degradation or alteration of the tissue morphology.

11.5 Subsequently, wash the tissue sections with three successive washes with 1X PBS for 5 minutes at room temperature.

11.6 **Note:** The quenching step is pivotal in preventing non-specific binding during subsequent imaging and analysis, ensuring that only specific antigen-antibody interactions are visualized.

Step 3. LED Treatment for quenching tissue autofluorescence

12 To remove any background autofluorescence that is typically present in prepared tissue sections, the sections are treated with an optimized LED protocol, based on published literature.(Nolta et al. 2020; Duong and Han 2013)

13 Prepare sodium citrate buffer by diluting 15 ml of sodium citrate solution in 1600 ml of Millipore water; a scaled-down aliquot to280 µl in 30 ml of Millipore water and use for the experiment.

14 Transfer tissue sections to a newly labeled 6-well plate, with each well containing the sodium citrate buffer, ensure that no more than two sections were placed in each well.

15 After placement, seal the 6-well plate securely to prevent contamination and evaporation.

16 Position the plate on an elevated platform that places the 6-well plate approximately30 inches above the LED light source. Cover the setup to protect the people in the lab environment, leaving a small gap for heat dissipation. Place the entire setup in a cold room, to prevent any heat-damage to the tissue sections.

17 Leave the assembly undisturbed overnight to allow for adequate exposure to the LED light.

18 **Safety Note**: Activation of the LED light source should only be performed after the entire assembly is securely placed and covered to avoid direct exposure to the light and potential heat build-up.

Step 4. Antigen retrieval using unmasking solution and peroxidase quenc…

19 After the LED treatment, the tissue sections undergo a series of immunostaining steps to unmask antigens prior to primary antibody incubation.

20 Incubate the brain sections in a sodium citrate buffer (10 mM, pH 6) and place them within an 80°C water bath for 30 minutes.

21 Next, remove the brain sections from the water bath to room temperature (RT) for 40 minutes.

22 Transfer the brain sections into 15% H_2O_2 solution (dilute 30% H_2O_2 in PBS) for 15 minutes at RT on a shaker to quench endogenous peroxidase activity.

Step 5. Tissue permeabilization using cyclodextrin-based buffer

23 Note: This method is adapted from a recently published protocol (Mai et al., 2023).

24 Incubate the tissue sections overnight at 4°C in a 1% beta-cyclodextrin in PBS buffer.

25 Wash with 1X PBS wash for 3x5 mins at RT on shaker.

Step 6. Blocking

26 Block tissue sections in 10% Normal Donkey Serum in PBS for 1 hour at RT on the bench to prevent non-specific antibody binding.

27 **For SeqStain antibodies:** Block samples with a DNA-blocking solution (0.5M NaCl in 1X PBS with 200µg/mL salmon sperm DNA and 3nanomoles/mL ssDNA) for one hour at room temperature.

28 Discard the blocking solution.

Step 7. Staining of unmounted brain tissues with antibodies

29 **Step 7 (a). Staining of unmounted brain tissues with fluorescently labelled primary antibodies**

29.1 Prepare appropriate dilutions of pre-labelled primary antibodies in 0.1% PBS-Tx 100 and 1% β-cyclodextrin and 2% serum, e.g., GFAP at 1:300 and IBA1 at 1:500 dilutions, based on the manufacturer's recommendation.

29.2 Incubate tissue sections overnight with the diluted primary antibodies at RT, cover with aluminum foil to avoid exposure to light.

30 **Step 7 (b). Staining of unmounted brain tissues with Seqstain antibodies**

30.1 Prepare appropriate dilutions of Seqstain antibodies in DNA-blocking solution containing 0.1% PBS-Tx 100 and 1% β-cyclodextrin and 2% serum. e.g., anti-α-synuclein at 1:2000 dilution.

Seqstain antibodies are prepared according to our previously published protocol (Rajagopalan et al. 2021a).

30.2 Incubate tissue sections overnight with the diluted Seqstain antibodies at 4°C , cover with aluminum foil to avoid exposure to light.

Step 8. Nuclear Staining Using DAPI Solution

31 Wash tissue sections for three times with 0.1% PBS-Tween20 for 10 minutes each and add DAPI stain to counterstain the nuclei for 10 minutes at room temperature.

32 Wash the tissue sections three times with 0.1% PBS-Tween20 for 5 minutes each to remove excess DAPI stain.

33 Perform one final wash with 1X PBS was for 5 minutes.

Step 9. Mounting Brain Tissue Sections for Imaging

34 Mount tissue sections onto a glass bottom petri dish using 1XPBS.

35 Remove the 1XPBS carefully using a Kimwipe and add a ring of blue tack surrounding the tissue to create a well to hold liquid in.

36 Air dry petri dish until the tissue looks dry.

37 Add enough EasyIndex optical clearing solution to cover entire tissue section until it becomes translucent and seal it with a cover-glass by gently pressing it into the blue tack.

Step 10. Confocal Imaging

38 Capture Confocal images with either a Leica TCS-SP5 laser scanning confocal microscope or a Nikon Eclipse Ti-C2 confocal microscope.

39 Export images and process using image analysis software.

Step 11. De-staining tissues sections for removing fluorescent signal and …

40 **Preparation of Lithium Borohydride solution (1mg/mL)**

40.1 Add 0.005g of Lithium Borohydride to 5ml Millipore water and pipette gently to mix.

40.2 Leave solution uncapped in a biosafety hood for 10 mins at room temperature.

40.3 Filter solution with a syringe.

41 De-staining

41.1 Wash the tissue sections with 1X PBS thrice for three minutes each on the shaker to remove the EasyIndex.

41.2 Incubate tissue sections stained with **SeqStain antibodies** with DNase I enzyme (50 µL of DNase, 50 µL of DNA buffer in 400 µL of PBS) for 10 minutes and briefly wash with 1X PBS before imaging and again to confirm successful removal of fluorophores.

41.3 Incubate tissue sections stained with directly **fluorescently labeled antibodies** with lithium borohydride solution and photobleach each quadrant for two mins in Cy3/Cy5, AF488 channel at 60-70% lamp intensity.

41.4 Wash the tissue sections with 1X PBS and re-mount onto a fresh petri dish.

41.5 Subsequently, block the de-stained tissue section by following the above mentioned (Step F a/b) protocol before undergoing additional rounds of staining.

Step 12. Unmounting of Brain Tissue Sections for repeating next round of …

42 Add 1X PBS gently on the tissue sections until the tissue starts floating in the PBS.

43 Gently use the tissue brush to peel the tissue from the glass surface, if required.

44 Remove the tissue carefully from the petri dish and proceed for re-staining.

45 **Note:** Repeat Steps 6 through 11 as many times as needed in order to stain and image tissues with additional set(s) of fluorescent antibodies and other reagents to obtain a desired level of multiplexed data.

Image Analysis

46 The acquired images can subsequently assembled, processed and analyzed using a number of software tools, such as Nikon NiS elements, ImageJ, Adobe Photoshop and Illustrator etc.