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Light sheet Sample Processing - Mouse Brain

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ABSTRACT

An improved method for light-sheet imaging sample preparation. We use this protocol to process brain tissue (cerebellum) for light sheet imaging. First, we fix the tissue overnight at 4C, then we steam the tissue in Citric acid to retrieve epitopes after crosslinking and bleach the autofluorescence of tissue and blood vessels. This simple step saves time avoiding further bleaching steps in other staining protocols and improves the quality of antibody staining. We adapted *pre-staining clearing* delipidation using SDS in boric acid to reach optical transparency (McCreedy et al. 2021). For staining, we use conventional immunofluorescence. We proceed with *post-staining clearing*, based in concept on RTF method (Yu et al. 2018) with modifications. The final imaging solution of 80% glycerol should already have a refractive index of 1.45 matching that of Zeiss lightsheet Z1 X5 lens, and used to store the samples and fill the light-sheet microscope chamber for imaging and for sample storage.

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GUIDELINES

Whole mount staining is based on conventional Immunufluorescence staining method with the added steps of steaming in Citric acid to bleach the samples.
Tissue clearing process is based on PACT (McCreedy et al. 2021) and RTF (Yu et al. 2018) methods.

MATERIALS TEXT

- Phosphate Buffered Saline (PBS)
- Paraformaldehyde (PFA)
- Triton X-100 (Sigma, 9002-93-1)
- Citric Acid Anhydrous (Fisher Scientific, BP339)
- NDS: Heat-inactivated Donky Serum stored at -20 C
- Primary antibodies to epitope of interest. (up to three from different species)
- Species-specific fluorescent secondary antibodies. (non-overlapping excitation/emission ranges)
- Triethanolamine hydrochloride 99.5% (Sigma, T1502)
- Boric Acid
- NaOH
- SDS
- Formamide 99.0% (Sigma, F7503)
- Glycerol (Fisher Scientific, BP229-1)
- 2 ml round-bottom Eppindorf tubes
- Oster food steamer

SAFETY WARNINGS

Paraformaldehyde is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves.
Formamide is toxic. prepare solutions under the fume hood.
SDS causes respiratory tract irritation in solid powder form. Avoid contact with skin and eyes. Avoid formation of dust and aerosols.

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REAGENT SETUP

1 **PFA:** 4% Paraformaldehyde. Can be stored at **4 °C** to be used within 3-4 weeks.

The following solutions can be prepared in large volumes and stored in room temperature for months:

- 1X Phosphate Buffered Saline (PBS)
- 0.01 mM Citric Acid Buffer (**pH 6**)
- **TEA:** 0.1 M Triethanolamine in water
- 10% SDS
- **BA Buffer** (**pH 8.5**): Dissolve 61.83 g of Boric Acid and 12 g of sodium hydroxide pellets (NaOH) in 900 mL of ddH_2O. Stir until fully dissolved and clear. Add ddH_2O up to final volume of 1L.
- **BBT:** 800 mL ddH_20, 200 mL BA buffer, and 1 mL of Triton^{TM}X-100.

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Tissue Fixation

2 Dissect the mouse brain tissue and try to peel off the meninges. Rinse with PBS.

The cerebellum is our tissue of interest, however, this method can be applied to any other neuronal tissue (forebrain, cerebellum, brainstem, spinal cord,...).

3 Fix tissue with 4% PFA **4 °C** **Overnight**

Apply PFA under the fume hood. Wear gloves and avoid inhaling.

4 Wash with PBS for 10 min.

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5 Repeat Step 4 twice.

Delipidation 45m

6 Apply 2 ml of preheated Citric Acid to each sample and incubate samples in steamer for 45 min 45m **00:45:00**

95 °C

> **CRITICAL STEP:** This step replaces bleaching in other protocols, to eliminate blood vessels and tissue autofluorescence.

7 Let samples sit on bench for 5-10 min to cool down **Room temperature** . Meanwhile, prepare 12.5 mL/sample of SDS delipidation solution by combining 10 mL of 10% SDS with 2.5 mL of 1 M boric acid buffer.

> Do not store the SDS delipidation solution at RT for more than 1 day since SDS may precipitate in boric acid buffer.

8 Rinse the samples with PBS.

9 Transfer tissue from PBS to 12.5 mL of SDS delipidation solution in a 15 mL conical. Seal lid with parafilm and incubate at **37 °C** on a nutating rocker.

10 Replace SDS delipidation solution every other day until the tissue is optically transparent (5-7 days). 5d **120:00:00**

> Avoid leaving tissues in SDS clearing solution after becoming optically transparent, as this can lead to sample degradation.

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11 Transfer the sample to 14 mL of BBT and rotate at **Room temperature** for **00:30:00** min on tube revolver to wash out the residual SDS. 30m

12 Repeat step 11 twice

13 Leave the sample in BBT solution **Overnight** rotating at **Room temperature** 45m

14 Repeat steps 11-13

Staining 2d 0h 45m

15 Prepare the staining buffer: primary antibody + 1ml BBT + 2% (v/v) normal donkey serum (NDS)

Concentration of primary antibody varies.

16 Transfer the tissue into the staining buffer with primary antibodies in 2 mL tube. Protect from light and rotate for 2 days at RT on tube revolver. **48:00:00** **Room temperature** 2d

17 Wash tissue with BBT solution with 0.01% with 3 buffer changes within 1-2 h on a tube revolver at RT.

18 Prepare secondary antibodies 1/500 in 1ml BBT + 2% (v/v) normal donkey serum (NDS). **Overnight** **Room temperature** 45m

Filter staining buffer with secondary antibodies using syringe filter to remove any aggregates of secondary antibody that may affect IF labeling.

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19 Wash tissue with BBT solution with 0.01% with 3 buffer changes within 1-2 h on a tube revolver at RT.

Post-staining clearing 1h 45m

20 Prepare fresh 50%TEA/30%Formamide/20%Water mixture (12.5 ml per sample) and incubate the samples in mixture, rocking. **Room temperature** **Overnight**

21 Prepare fresh 70%TEA/15%Formamide/15%Water mixture (about 1.5 ml per sample) and incubate the samples in mixture, rocking **01:00:00** 1h

22 Meanwhile, prepare 50 ml of fresh **Imaging solution :** 40 ml Glycerol + 10 ml ddH2O.

23 Wash samples in 1.5 ml of (50% imaging solution + 50% Mixture in step 19) a gradual transition to glycerol with gentle mixing, and incubate, rocking **01:00:00** 1h

24 Change to 100% of imaging solution and incubate, rocking, for about 15 min

25 Store the samples at **4 °C** in fresh imaging solution

Imaging solution (80% Glycerol) is expected to have a refractive index of 1.45 (matching Zeiss lightsheet Z.1 X5 lens) and will also be used to fill the light sheet imaging chamber.

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