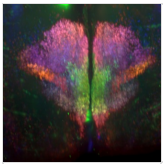

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LightsheetSampleProcessing-EmbryonicBrain

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1 Works for me dx.doi.org/10.17504/protocols.io.bi8jkhun

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ABSTRACT

An imropved method for Lightsheet imaging sample preparation. We use this protocol for embryonic brain tissue (the embryonic cerebellum in particular), it could also be optimized for other types of tissues with few modifications to incubation periods. First, we fix the tissue for two hours on ice, then we pereamibilize using a mixture of Triton and Tween. **An added ingenious step** is steaming the tissue in Citric acid to retrieve epitopes after crosslinking and bleach the autofluorescence of tissue and blood vessels. That simple step saves a lot of time avoiding H_2O_2 bleaching steps in other staining protocols and improves the quality of antibody staining. After staining, the tissue clearing is based in concept on RTF method (Yu et al. 2018) with modifications and added incubation steps in increasing glycerol concentrations. The final imaging solution of 80% glycerol should already have a refractive index of 1.45 matching that of Zeiss lightsheet Z1 X5 lens, thus, it can be used to fill the lightsheet chamber for imaging and for sample storage.

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GUIDELINES

Whole mount staining is based on cryosection Immunufluorescence staining method with the added steps of Triton/Tween permeabilisation and steaming in Citric acid to bleach the samples.
Tissue clearing process is based on RTF method (Yu et al. 2018).

MATERIALS

NAME	CATALOG #	VENDOR

NAME	CATALOG #	VENDOR
Triethanolamine hydrochloride	T1502	Sigma – Aldrich

MATERIALS TEXT

- Phosphate Buffered Saline (PBS)
- Paraformaldehyde (PFA)
- Triton X-100 (Sigma, 9002-93-1)
- Tween-20 (Sigma, P5927)
- 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)
- 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.

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)
- TP1: 0.1% Tween-20 in PBS
- TP2: 0.4% Triton in PBS
- **TTP:** 50% TP1+ 50% TP2 (Final concentrations: 0.05% Tween-20 and 0.2%Triton)
- 0.01 mM Citric Acid Buffer pH **6.0**
- **TEA:** 0.1M triethanolamine in water

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Tissue Fixation 2h 40m

2 Dissect the embryonic brain tissue and rinse with PBS. 10m

3 Fix brain tissue with 4% PFA for 2h **02:00:00** **On ice** 2h

Perform this step under the fume hood. Wear gloves and avoid inhaling.

4 Wash with PBS for 10 min. 10m

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5 Repeat Step 4 twice. 20m

Whole Mount Staining 1d 3h

6 Remove PBS and apply 1.5 ml of **TTP** for each sample in 2ml round-bottom Eppendorf tube. Place in the incubator, rocking, at **37 °C** for 45 min **00:45:00** 45m

- Triton and Tween are detergents used to permeabilize the tissue and allow antibody penetration.
- About 30 min into the incubation period, preheat Citric acid solution in steamer **95 °C** to use in the next step.

7 Discard TTP and rinse with PBS.

8 Apply 1.5 ml of preheated Citric Acid to each sample and incubate samples in steamer for 30 min **00:30:00** **95 °C** 30m

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

9 Let samples sit on bench for 5-10 min to cool down **Room temperature** . Meanwhile, prepare Blocking solution: 10% NDS in TTP , and keep on ice. 5m

- Total volume of blocking solution depends on sample size and number of samples. Account for blocking, primary and secondary antibody solutions.
for example: ~200 µl per sample, per step, is largely enough for dissected early embryonic cerebellum.

10 Discard the Citric acid and rinse with PBS.

11 Apply Blocking solution and incubate, rocking in the cold room for at least 1h **4 °C** . Meanwhile prepare the proper concentrations of primary antibodies in blocking solution. 1h

Concentrations of primary antibodies depend on their quality and specificity. In general, for the embryonic cerebellum, double the concentration used in cryosections IF staining is a good starting point to test the antibody in wholemount.

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12 Discard Blocking and apply Primary antibody solution. Incubate in the cold room, rocking, overnight **Overnight** **4 °C** 12h

Primary antibody can be recycled and reused within 3-4 weeks, stored at **4 °C** .
For larger tissues and better staining, incubation in primary antibody can be extended to 2-3 days.

13 PBS wash, rocking, for 10min **4 °C** 10m

14 Repeat Step 13 twice. 20m

15 Apply secondary antibodies 1/400 in Blocking solution at **4 °C** for 3h **03:00:00** (leave overnight for best results and large samples) 3h

The samples are **photosensitive** beyond this step. Keep in the dark or wrap in foil through all the following steps.

16 PBS wash, rocking, for 10 min **4 °C** 10m

17 PBS wash 10min twice. (You can stop at this point and keep the samples in PBS at 4°C overnight, to continues with clearing the next day). 20m

Clearing 7h 30m

18 Prepare fresh 30%TEA/40%Formamide/30%Water mixture (1.5 ml per sample) and incubate the samples in mixture, rocking, in the cold room for 1h. 1h
01:00:00 **4 °C**

Prepare solutions containing Formamide under the fume hood. Wear gloves and avoid inhaling.

19 Prepare fresh 60%TEA/25%Formamide/15%Water mixture (1.5 ml per sample) and incubate the samples in mixture, rocking, in the cold room for 4h. **04:00:00** **4 °C** 4h

Can be extended to overnight for larger tissues.

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20 Prepare fresh 70%TEA/15%Formamide/15%Water mixture (about 1.5 ml per sample) and incubate the samples in mixture, rocking, in the cold room for 2h. **02:00:00** **4 °C** 2h

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

21 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, for about 15 min **4 °C** 15m

22 Change to 100% of imaging solution and incubate, rocking, for about 15 min . **4 °C** 15m

23 Store the samples in fresh imaging solution, which is expected to have a refractive index of 1.45 (matching Zeiss lightsheet Z1 X5 lens) and will also be used to fill the light sheet imaging chamber, and storing the samples **4 °C**

Keep the samples in the imaging solution, rocking in the cold room, for at least 24h before imaging (few days for larger samples).

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