Passive CLARITY for *N. furzeri* brains

Materials

- 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] Dihydrochloride (TCI 27776-21-2)
- ddH_2O
- Diatrizoic acid (Sigma-Aldrich 117-96-4)
- Lithium hydroxide monohydrate, 99.95% trace metals basis (Sigma-Aldrich 1310-66-3)
- N-methyl-D-glucamine (Sigma-Aldrich 6284-40-8)
- Boric Acid (Sigma-Aldrich 10043-35-3)
- SDS (GE Life Sciences 17-1313-01)
- UltraPure water (Thermo Fisher 10977015)
- 40% Acrylamide solution (Bio-RAD 1610140)
- OptiPrep Density Gradient Medium, 60% Iodixanol (Sigma-Aldrich 92339-11-2)
- PBS (Research Products International 7647-14-5)
- 8% PFA (Thermo Fisher Scientific 50-00-0)
- 32% PFA aqueous solution, methanol and RNase free, EM grade (Thermo Fisher Scientific 50-980-495)
- Tween 20 (Sigma-Aldrich 9005-64-5)

Recipes

4% PFA in PBST (20.2 mL)	**Reagent**	**Volume**	**Final**
	8% PFA	10 mL	4%
	10X PBS	2 mL	1X
	10% Tween 20	200 µL	0.1%
	ddH_2O	8 mL	

10% Azo-initiator (11 mL)	**Reagent**	**Volume**	**Final**
	UltraPure water	10 mL	10%
	2,2'-Azobis[2-(2-imidazolin-2-yl)propane] Dihydrochloride	1 g	

Hydrogel monomer solution (400 ml)	**Reagent**	**Volume**	**Final**
Extra solution can be stored at -20℃ indefinitely.	10% Azo-initiator	10 mL	0.25%
	40% Acrylamide	40 mL	4%
	10X PBS	40 mL	1X
	32% PFA	50 mL	4%
	UltraPure water	260 mL	

SDS clearing solution (60 mL)	**Reagent**	**Volume**	**Final**
Add boric acid to adjust the pH to 8.5.	$LiOH \cdot H_2O$	0.05 g	20 mM
	SDS	3.46 g	200 mM
	UltraPure water	56.49 mL or until 60 mL	

47% Iodixanol solution (12.75 mL)	**Reagent**	**Volume**	**Final**
	OptiPrep Density Gradient Medium	10 mL	47% Iodixanol
	UltraPure water	2.75 mL	

Optical clearing solution (17.63 mL)	**Reagent**	**Volume**	**Final**
	47% Iodixanol	10 mL	32.4%
	N-methyl-D-glucamine	3.39 g	23.5%
	Diatrizoic acid	4.24 g	29.4%

Fixation [Day 1 and 2]

1. Place samples into individually labeled eppendorf tubes.
2. Pipette 1 mL of 4% PFA into each tube. (Make sure to properly dilute solution)
3. Incubate the samples at 4℃ with agitation overnight.
4. Remove the 4% PFA and add 1 mL 10X PBS
5. Store samples at 4℃ with agitation overnight.
6. The sample may be frozen at -20℃ after removing PBS or proceed immediately to the next steps.

Hydrogel Monomer Solution Infusion and Hybridization [Day 3 and 4]

1. Remove PBS and pipette 1 mL of hydrogel monomer solution into each tube and incubate at 4℃ with agitation overnight.
2. Replace hydrogel monomer solution with 10X PBS. Fill the tube to the top with PBS so that air is minimized.
3. Incubate sample 37℃ for 4 hours with agitation.

Passive Clearing [Day 5 until fully cleared under light microscope]

1. Replace PBS with SDS clearing solution and incubate for 24 hours at 37℃.
2. Replace SDS with 10x PBS in 37℃ for 24 hours with agitation. It is okay to leave sample in PBS for over 24 hours, but do not go over 48 hours.
3. Replace 10x PBS with optical clearing solution and incubate in 37℃ for 14 to 16 days with agitation. Replace with 1 mL of fresh optical clearing solution after the first day and then again after 1 week.
4. To store the samples, wash them with 10X PBS in 37℃ for 24 hours with agitation after determining that they are clear under the microscope. The sample will look cloudy after washing.

5. Store samples in 10X PBS in 4℃.
6. When ready to use the samples, incubate each sample in 1 ml of optical clearing solution for approximately 2 days to clear the sample again.

Adapted from CLARITY Protocol and Tissue Clearing Guide | Abcam and Tissue Clearing Using CLARITY Method

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