HYBRiD: Hydrogel-Based Reinforcement of DISCO
Supplementary Protocol

Protocol Contents

1. **Reagents**
2. **Buffers**
3. **Sample Collection**
4. **Decalcification and Decoloring**
5. **Organic Clearing**
6. **Aqueous Clearing**
7. **Refractive Index Matching**
8. **Sample Mounting and Imaging**

---

1. Reagents

Reagent	Use	Supplier Information
Water	Buffers	MilliQ water
4% Paraformaldehyde solution	Fixation	Electron Microscopy Sciences 1224SK
EDTA (Ethylenediaminetetraacetic acid)	Decalcification	Sigma-Aldrich EDS
Imidazole	Decalcification	Sigma-Aldrich I202
10X PBS	Buffers	ApexBio Genesee Scientific 25-507
N,N,N',N'-Tetrakis(2-hydroxypropyl) ethylenediamine (Quadrol)	Decoloring, pH	Alfa Aesar L16280-AP
Tetrahydrofuran	Organic Clearing	Sigma-Aldrich 186562
Dichloromethane	Organic Clearing	Sigma-Aldrich 270997
Triton-X100	Buffers	Sigma-Aldrich X100
Sodium azide (NaN_3)	Buffers	Sigma-Aldrich S2002
16% Paraformaldehyde (or 32%)	Hydrogel	Electron Microscopy Sciences 15710-S
40% Acrylamide	Hydrogel	Bio-Rad 1610140
2% Bis-Acrylamide	Hydrogel	Bio-Rad 1610142
VA-044 (2,2'-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride)	Hydrogel	Wako Chemicals 01119365
20% Sodium dodecyl sulfate (SDS)	Aqueous Clearing	RPI Corp L2310020000.0
Lithium hydroxide monohydrate or Lithium hydroxide	Aqueous Clearing	Sigma-Aldrich 402974
Boric acid	Aqueous Clearing	Sigma-Aldrich B6768
EasyIndex	RI Matching	LifeCanvas Technologies EI-Z1001
Agarose	Sample Mounting	Sigma-Aldrich A6013

2. Buffers

Decalcification Buffer – E/I (1 L)
Mix at RT till fully dissolved, **kept at RT**.

Ingredient	Amount	Final Conc.
EDTA	100 g	10%
Imidazole	150 g	15%
MilliQ water	To 1 L	

Decoloring Buffer – Quadrol (1 L)
Stored at RT, protect from light for long term storage.

Ingredient	Amount	Final Conc.
N,N,N′,N′-Tetrakis(2-Hydroxypropyl)ethylenediamine (Quadrol)	250 g	25%
10X PBS	100 mL	1x
MilliQ water	To 1 L	

THF Gradients (100 mL)
Prepare fresh using glass bottles in a fume hood, **cool down to 4°C before use.**

Ingredient	50%	70%	80%	95%
Tetrahydrofuran	50 mL	70 mL	80 mL	95 mL
Decoloring buffer (25% Quadrol in 1X PBS)	50 mL	30 mL	20 mL	5 mL

1% Acrylamide Hydrogel – A1P4 (200 mL)
Prepare fresh, dissolve and add VA-044 last, **kept at 4°C.**

Ingredient	Amount	Final Conc.
40% Acrylamide	5 mL	1%
2% Bis-acrylamide	1.25 mL	0.0125%
VA-044 Initiator	0.5 g (in 5 ml of water)	
16% Paraformaldehyde	50 mL	4%
10X PBS	20 mL	1x
Pure water	To 200ml (~120ml)	

1M LiOH-Boric buffer (2 L, pH = 9)
Dissolve $LiOH \cdot H_2O$ in 1.5 L water, adjust pH to 9 with boric acid, then bring volume to 2 L with water, **kept at RT**.

Ingredient	Amount	Final Conc.
Lithium hydroxide monohydrate	84 g	1M
Boric acid		
MilliQ water	To 2 L	

LiOH/Borate/SDS buffer (2 L)
Mix at RT till fully dissolved, **kept at RT**.

Ingredient	Amount	Final Conc.
1M LiOH-Boric buffer, pH=9	40 mL	20mM
20% SDS	600 mL	6%
MilliQ water	To 2L	

0.2% PBST buffer (2 L)
Mix at RT till fully dissolved, **kept at RT**.

Ingredient	Amount	Final Conc.
10X PBS	200 mL	1x
Triton X-100	4 mL	0.2%
MilliQ water	To 2L	

3. Sample Collection

i) Anesthetize animals by the preferred method.
ii) Perfuse with ice-cold PBS (20 mL/2 min), making sure the liver turns a pale tan color to show that the vasculature has been thoroughly flushed of blood. Follow with cold 4% PFA (20 mL/2 min) until the sample is stiff and fixed. This trans-cardiac step provides better blood removal but can be skipped if necessary.
iii) Collect tissues of interest (if clearing whole-body samples remove hair and skin before continuing) and post-fix overnight at 4°C with shaking.
iv) After post-fixation, wash excess PFA off sample at RT with 1X PBS, for a total of 3 washes.
v) (Stopping point) Fixed samples may be stored at 4°C in PBS with 0.02% NaN_3 for a few weeks.

	Duration	*Buffer*	*Temp*
Day 1	overnight	4% Paraformaldehyde	4°C
Day 2	Up to1h	1X PBS	RT
	Up to1h	1X PBS	RT
	Up to1h	1X PBS	RT

4. Decalcification and Decoloring

i) Samples must be pretreated to obtain the best clearing results. Not all samples require the same steps, choose which preparation is required according to the characteristics of the sample (if both treatments are needed, decalcification should be completed before decoloring).
(1) Decalcification recommended: samples containing any bone
(2) Decoloring recommended: samples containing any skeletal muscle, spleen, kidney, liver, and/or heart

ii) Follow the recommended sample treatment outlined below. Samples can be put on a shaker with gentle agitation or a rotator to facilitate buffer exchange.
iii) (Stopping point) Samples may remain in 1X PBS with 0.02% NaN_3 at 4°C before delipidation for a few weeks.

	Duration	*Buffer*	*Temp*
Day 2	4 days	Decalcification buffer ^ Refresh daily	4°C
Day 6	1 hour	1X PBS	RT
	1 hour	1X PBS	RT
	1 hour	1X PBS	RT
Day 6	2 days	Decoloring buffer * Refresh daily	37°C
Day 8	overnight	1X PBS	RT
Day 9	1 hour	1X PBS	RT
	1 hour	1X PBS	RT
	1 hour	1X PBS	RT

^: For large samples containing bones, the decalcification buffer step can be extended to up to 7 days to facilitate decalcification.

*: For large and heavily colored samples, the decoloring buffer would turn light yellow or greenish. If the decoloring buffer remains light yellow or greenish after 2 days of decoloring, this step can be extended to up to 4 days till the buffer becomes colorless.

5. Organic Clearing

i) Prepare samples for organic clearing by placing each in a glass vial or bottle. **Do not use plastic containers.** Samples can be put on a shaker with gentle agitation to facilitate buffer exchange. Sample wash duration for each step differs with different sample size (see guideline below) and need to be determined by the users. Only ½ of the recommended duration is required in each of the reversed THF gradient steps (based on the iDSICO protocol).

Sample	*Wash Duration*
1mm Brain sections	30 min
Whole Mount Limbs	45 min
Newborn Mouse (P0-P3)	90 min
Adult Mouse Chest	90 min
Juvenile Whole Body (2wk-3wk)	120 - 180 min

(1) Prepare the 25% Quadrol in 1X PBS solution in advance to allow full mixing of the viscous liquid into a homogenous solution. This buffer should be stored protected from light, if the solution has become tinted yellow, it should be discarded and replaced.
(2) All dilutions of THF should be prepared in glass bottles inside a fume hood, fresh on the day of the experiment then cooled to 4°C. Wash duration for 1-mm brain sections shown here, please adjust accordingly for different samples.
(3) (Stopping point) Samples may remain in 1X PBS with 0.02% NaN_3 at 4°C for a few weeks before hydrogel embedding.

	Duration	*Buffer*	*Temp*
Day 10	30 min	50% THF	4°C
	30 min	70% THF	4°C
	30 min	80% THF	4°C
	30 min	95% THF	4°C
	30 min ^	95% THF	4°C
	30 min	100% DCM *	4°C
	30 min	100% DCM	4°C
	15 min ^	95% THF	4°C
	15 min	95% THF	4°C
	15 min	80% THF	4°C
	15 min	70% THF	4°C
	15 min	50% THF	4°C
	15 min	1X PBS	4°C
	overnight	1X PBS	4°C
Day 11	1 hr	1X PBS	4°C
	1 hr	1X PBS	RT
	1 hr	1X PBS	RT

^: If the sample needs to be stored overnight during organic clearing, stop at these steps.

*: If the samples contain a lot of lipids (e.g., adipose tissues), the DCM delipidation step can be repeated up to three times to facilitate lipid removal.

6. Aqueous Clearing

i) Prepare fresh 1% Acrylamide Hydrogel (1% acrylamide/4% paraformaldehyde (A1P4)) on ice in a fume hood and keep protected from light.
ii) Move the sample from PBS to hydrogel, fill the tube as close to the top as possible to remove room for air, and incubate at 4°C with gentle shaking for 72 hours.
iii) Degas sample with nitrogen and vacuum for 15 min at RT, then flush with nitrogen before tightly sealing tube to prevent entrance of oxygen.
iv) Place sample on a 37°C shaker to polymerize for 4 hours. Tubes should be upright to prevent the formation of bubbles during the polymerization process.

v) Drain excess hydrogel and wash sample overnight with LiOH/Borate/SDS buffer at 37°C.
vi) Refresh the LiOH/Borate/SDS buffer in a fume hood, ensuring proper waste practices for the drained buffer which may contain excess acrylamide monomers.
vii) Passively wash sample with LiOH/Borate/SDS buffer on a shaker at 37°C until sample is sufficiently cleared. Refresh buffer every 3-4 days.
viii) The LiOH/Borate/SDS buffer step can take 5 weeks before samples appear translucent depending on sample sizes.

Duration		*Buffer*	*Temp*
Day 11	72 hours	1% Acrylamide Gel (A1P4)	4°C
Day 14	overnight	LiOH/Borate/SDS buffer	37°C
Day 15	varies	LiOH/Borate/SDS buffer Refresh every 3-4 days.	37°C

7. Refractive Index (RI) Matching

i) Once the sample appears translucent (it will not be fully transparent in SDS buffer), move into a new tube with excess 0.2% PBST and wash on a 37°C shaker for 1 hour.
ii) Refresh the PBST and wash the sample on the 37°C shaker overnight to remove excess SDS from the tissue.
iii) Repeat refreshes of the PBST throughout the following day (wash duration and frequency are flexible, but at least three 1-hour washes should be complete at 37°C with shaking to ensure thorough SDS removal.
   (1) It is critical to remove all SDS from the sample to prevent the formation of damaging crystal structures in the high osmolarity RI Matching media.
   (2) Cold storage of samples should only take place after thorough removal of SDS, as low temperatures can also result in the formation of SDS crystals in the sample.
   (3) <u>(Stopping point)</u> Samples may be stored in PBST at 4°C before RI matching.
iv) Move the sample into a new tube containing EasyIndex. Samples should be carefully dried by Kimwipe to ensure the minimal amount of PBST is carried over to RI matching media.
v) Place sample on the 37°C shaker with tube horizontal to allow thorough mixing of the solution.
vi) Once the sample appears translucent, move to RT shaker positioned upright to stop bubble formation and allow to cool. Samples can be put in a vacuum to remove bubbles.
vii) <u>(Stopping point)</u> Samples can be stored in EasyIndex at RT for up to 1 month, for long-term storage, samples can be washed in PBST thoroughly and stored in PBST at 4°C, and repeat RI matching if samples need to be imaged again.

Duration	*Buffer*	*Temp*
1 hour	0.2% PBST in a new tube	37°C
overnight	0.2% PBST	37°C
2 hours ^	0.2% PBST	37°C
2 hours	0.2% PBST	37°C
2 hours	0.2% PBST	37°C
*overnight **	EasyIndex	37°C
4 hours		RT

^: For large samples, PBST wash can be repeated daily, and the total duration can be extended for up to 1 week to ensure thorough SDS removal.

*: For large samples, EasyIndex can be refreshed daily to facilitate refractive index matching. RI matching step should take no more than 3 days.

8. Sample Mounting and Imaging

i) Samples can be directly imaged in EasyIndex using a confocal microscope. For lightsheet microscope imaging, samples need to be mounted in EasyIndex/Agarose to be fixed on the sample holder.
ii) Prepare 1.0% (wt/vol) low melting agarose in EasyIndex. Shake well, vortex thoroughly to break up any clumps of agarose. Seal the tube with parafilm.
iii) Let the tube sit vertically. Allow agarose to hydrate in solution for 30 min, keeping the tube protected from light.
iv) Once the agarose particle is evenly suspended, microwave the tube in short 5-10 second increments to boil the solution and fully dissolve the agarose.
    (1) Keep tubes capped and carefully watch for boiling, the buildup of pressure left unattended may explode in the microwave.
    (2) Carefully vent the cap between rounds of microwaving to release pressure.
v) Vortex tube for 15s, then sonicate the melted 1% agarose mounting media for ~10-15 seconds to remove small bubbles.
vi) The EasyIndex/agarose can be used immediately when cooler than 60°C or being placed in a 60°C water bath to use later.
vii) Prepare sample holders for mounting and secure to a plastic petri dish for easy handling.
    (1) Blu-Tack putty may be used to create an enclosed chamber for mounting, then peel the putty off the sample holder once the gel is set to allow imaging.
viii) Pour a small amount of melted mounting gel into the prepared sample holder and quickly move the sample into the gel, taking care to avoid formation of bubbles on and around the sample between the imaging planes.
ix) Keep the samples from light, cool at RT to ensure agarose is hardened.

x) Retrieve samples and remove putty from the sample holders, careful to avoid breaking the smooth surface of the gel.
xi) Move sample holders into the lightsheet microscope imaging chamber and allow to equilibrate before imaging.
xii) (Stopping point) Mounted samples may be placed back into the container with EasyIndex if not being imaged immediately.