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

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Whole Mouse Brain Delipidation, Immunolabeling, and Expansion Microscopy

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Naveen Ouellette

ABSTRACT

The mammalian brain contains approximately 1,000 brain areas and each brain area contains multiple (up to 100) cell types. Neurons in one brain region can send projections to dozens of target regions, and distinct neuron types could project to different combinations of target regions. The enumeration and description of the brain's cell types, and their brain-wide connectivity, is foundational for understanding how neural activity is routed across brain-wide neural circuits during normal behavior and how these processes are dysregulated in mental disorders.

Obtaining brain-wide single neuron reconstructions requires high-resolution, high-contrast imaging of entire brains – neuronal axons travel many centimeters (in the mouse) while individual axon collaterals could be finer than 100nm. Here, we present an integrated protocol for labeling and isotropic expansion of whole mouse brains that results in optically clear specimens ideally suited for high-resolution selective plane illumination microscopy (SPIM) imaging. Pipeline steps are modular, and the protocol is extensible to other large volume clearing and expansion applications.

We have imaged expanded whole mouse brains generated using this protocol on our custom built ExA-SPIM microscope without need for any tissue slicing. These whole brain data sets are being used for tracing complete axonal morphology of individual neurons.

MATERIALS

Dichloromethane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #320269**

Keywords: Expansion Microscopy, Whole Brain, Tissue Clearing, Delipidation, Hydrogel, Immunolabeling, Light Sheet, Clearing, Antibody, SPIM

Tetrahydrofuran **Merck MilliporeSigma (Sigma-Aldrich) Catalog #186562**

Sodium Dodecyl Sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #74225**

Sodium phosphate dibasic **Merck MilliporeSigma (Sigma-Aldrich) Catalog #7558 79-4**

Sodium phosphate monobasic monohydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9638**

2-methyl-2-butanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #152463**

2-propanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #278475**

Glycine **Fisher Scientific Catalog #BP381-500**

PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalo #AM9625**

Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787-50ML**

Tween 20 **Contributed by users Catalog #P1379**

5% Sodium Azide **Fisher Scientific Catalog #71448-16**

MES or 2-(N-Morpholino)ethanesulfonic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3671**

5M Sodium Chloride, 1000ml **Promega Catalog #V4221**

10N NaOH **Merck MilliporeSigma (Sigma-Aldrich) Catalog #SX0607N-6**

Acryloyl-X, SE **Thermo Fisher Scientific Catalog #A20770**

DMSO anhydrous **Thermo Fisher Scientific Catalog #D12345**

Acrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9099**

NN Methylene-Bis-acrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M7279**

Sodium Acrylate (purity note:*) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #408220**

Acrylic Acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #147230**

Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #P8107S**

1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #15568025**

EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #AM9260G**

Phosphate Buffer Solution 1.0 M pH 7.4 (25 °C) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3619**

SSC (20X) RNase-free **Invitrogen - Thermo Fisher Catalog #AM9765**

EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #AM9260G**

Tris-HCl (1M pH 8) **Thermo Fisher Scientific Catalog #AM9856**

Agarose for ≧1kbp fragment **Nacalai Tesque Inc. Catalog #01163-76**

	Materials	Product Number
	1 gallon Slider plastic storage bags	Amazon, COMINHKG109462
	Cover glass, 24X55MM	Epredia, 12455S
	Diamond Scriber	Ted Pella, 62107-ST
	Glass Serological Pipet	Fisher Scientific, PYREX™708710
	Glass Slides, 1"x3"	EMS, 71867-01
	Heavy Duty Carbon Steel Razor	EMS, 71965
	Instrument Soaking Tray	Sklar, 10-3052
	Large Cover Glass, #2	Brain Research Laboratories, 4342-2
	Pelco Single edge uncoated carbon steel	Ted Pella, 121-95
	S/S or S/A Press to Seal Gasket 32X19mm(D), various depths: 0.5, 1.0, 2.0, 2.5 mm	EMS, 70337
	WHEATON® Shorty Vials clear with PTFE faced rubber lined cap	DWK, DWK986546
	WHEATON® Liquid Scintillation Vials, Caps Attached to Vials, Glass, Polyethylene Cone, 22-400, 20 mL	DWK, DWKW224607

Equipment

Hefty Slider Freezer Storage Bags, Gallon Size, 56 Count

NAME

Hefty

BRAND

COMINHKG109462

SKU

LINK

https://www.amazon.com/Hefty-Slider-Freezer-Bags-Gallon/dp/B01JLPJM7G/ref=sr_1_1_sspa?keywords=ziploc+bags&qid=1685583549&rdc=1&sr=8-1-spons&psc=1&spLa=ZW5jcnlwdGVkUXVhbGlmaWVyPUEyTUVIRDZBWFI3Wk5DJmVuY3J5cHRlZElkPUEwMjI2NTA0MVA3NEtVWVJOWFQ3NCZlbmNyeXB0ZWR

SPECIFICATIONS

1 gallon

Equipment

Cover glass NAME

Epredia BRAND

12-455-S SKU

https://www.fishersci.com/shop/products/12455S/12455S LINK

24x55 mm SPECIFICATIONS

Equipment

Deluxe Diamond Scribing Pen NAME

EMS BRAND

54468 SKU

https://www.tedpella.com/tools_html/54410.aspx LINK

Equipment

PYREX® Reusable Serological Pipettes, Glass, Corning, 10 mL NAME

pipet TYPE

PYREX®, Corning BRAND

7085-10 SKU

https://us.vwr.com/store/product/4760135/pyrex-reusable-serological-pipets-glass-corning LINK

Equipment

Microscope Slides NAME

slides TYPE

EMS BRAND

71867-01 SKU

https://www.emsdiasum.com/1mmt-superfrosted-slide-1grpk LINK

25x75mm, thickness: 1mm, Frosted End SPECIFICATIONS

Equipment

Single Edge Carbon Steel Razor NAME

blade TYPE

EMS BRAND

71960 SKU

https://www.emsdiasum.com/c-single-edge-carbon-steel LINK

Equipment

Instrument Soaking Tray NAME

Sklar BRAND

10-3052 SKU

https://mms.mckesson.com/product/947692/Sklar-10-3052 LINK

Equipment

Large Cover Glass no.2 thickness (0.19 – 0.25mm) NAME

cover glass TYPE

Brain Research Laboratories BRAND

4342-2 SKU

https://brainresearchlab.com/product/large-cover-glass/ LINK

3-3/4" x 4-1/2" (95mm x 114mm) no.2 thickness (50pc/pack) SPECIFICATIONS

Equipment

Single edge uncoated carbon steel blade NAME

blade TYPE

Pelco BRAND

121-95 SKU

https://www.tedpella.com/dissect_html/blades.aspx#_121_95 LINK

118mm long x 19mm wide x 0.229mm thick. (4.65 x 0.75 x 0.009") SPECIFICATIONS

Equipment

S/S Press to Seal Gasket 32X19mm(D) NAME

EMS BRAND

70337 SKU

https://www.emsdiasum.com/press-to-seal-silicone-isolators LINK

0.5, 1.0, 2.0, 2.5 mm depths. S/S or S/A SPECIFICATIONS

Equipment

WHEATON® Liquid Scintillation Vials, Caps Attached to Vials, Glass, Polyethylene Cone, 22-400, 20 mL

NAME

Vial

TYPE

Wheaton

BRAND

DWK986546

SKU

https://www.dwk.com/na/wheaton-liquid-scintillation-vials-caps-attached-to-vials-glass-polyethylene-cone-22-400-20-ml-986546

LINK

20 mL Glass Vial with Polyethylene cone Caps

SPECIFICATIONS

Equipment

WHEATON® Shorty Vials clear with PTFE faced rubber lined cap

NAME

vial

TYPE

WHEATON

BRAND

DWKW224607

SKU

https://www.sigmaaldrich.com/US/en/product/aldrich/dwkw224607

LINK

Equipment

Nutating Mixer

NAME

Mixer

TYPE

Fisherbrand

BRAND

88-861-043

SKU

https://www.fishersci.com/shop/products/nutating-mixers-variable-speed/88861043

LINK

16.3 x 11.5 x 10.7 in.(415 x 293 x 273 mm)

SPECIFICATIONS

Equipment

Fisherbrand™ Multi-Purpose Tube Rotator

NAME

Carousel

TYPE

FisherBrand

BRAND

88-861-049

SKU

https://www.fishersci.com/shop/products/multi-purpose-tube-rotators/88861049

LINK

RECIPES

10 mM Phosphate Buffer pH 8.3

Combine the following reagents, adjust pH to 8.3.

	Reagent	Amount	Final Concentration
	1M Phosphate Buffer	5 mL	10 mM

	Reagent	Amount	Final Concentration
	Milli-Q water	495 mL	

SBiP Solution: 0.08% SDS, 16% 2-methyl-2-butanol, 8% 2-propanol, in H_2O

Combine the following reagents on ice. Use a fume hood when adding 2-methyl-2-butanol and 2-propanol. Mix On ice until solution is uniform and clear. Store immediately at 4 °C until ready for use. Use each batch within a month for best effect.

	Reagent	Amount	Final Concentration
	4% SDS (in H2O, pH 7.4)	10 mL	0.08%
	2-Methyl-2-butanol	80 mL	16%
	2-Propanol	40 mL	8%
	50mM Na2HPO4	2 mL	
	Milli-Q water (ice cold)	350 mL	
	Total	482 mL	

B1n Buffer: 0.1% Triton X-100, 2% Glycine, 0.02% NaN_3 in H_2O

Combine the following reagents and stir at Room temperature until fully dissolved. Store for a few months at Room temperature.

	Reagent	Amount	Final Concentration
	Triton X-100	500 uL	0.1%
	Glycine	10 g	2%
	5% Sodium azide	2 mL	0.02%
	10N NaOH	50 uL	
	Milli-Q Water	up to 500 mL	
	Total	500 mL	

PTxw: 0.05% Tween 20, 0.1% Triton X-100, 0.04% NaN_3 in PBS

Combine the following reagents and stir at Room temperature until fully

dissolved, store at 4 °C.

	Reagent	Amount	Final Concentration
	10X PBS	50 mL	1X
	Triton X-100	500 uL	0.1%
	Tween 20	250 uL	0.05%
	5% Sodium azide	4 mL	0.04%
	Milli-Q Water	up to 500 mL	
	Total	500 mL	

MBS solution: 100mM MES Buffered Saline, 150mM NaCl, pH 6.0

Combine the following reagents at Room temperature until fully dissolved. Adjust to pH 6. Store for a few months at Room temperature.

	Reagent	Volume	Final Concentration
	MES	1 g	100 mM
	5M NaCl	1.5 mL	150 mM
	10N NaOH	200 uL	
	Milli-Q Water	48 mL	
	Total	50 mL	

10 mg/mL Acryloyl-X (AcX) in DMSO:

To make 10 mg/mL AcX, add DMSO directly to the bottle of AcX. Vortex to dissolve. Aliquot and store at -20 °C in a desiccated environment. Aliquots should be used within one month.

	Reagent	Volume	Final Concentration
	Acryloyl X	5 mg	10 mg/mL
	Dimethyl Sulfoxide (DMSO)	500 uL	

10% (w/v) VA-044 (polymerization initiator):

Combine the following reagents and stir On ice until dissolved. Store at -20 °C up to one month.

	Reagents	Volume	Final Concentration
	VA-044	1 g	10%
	Milli-Q water	10 mL	

Stock X (Monomer Solution) Preparation:

To make the Stock X solution, the following stock solutions must be prepared in advance:

- 50% (w/v) Acrylamide
- 2% (w/v) N,N Methylene-bis-acrylamide
- 4.04M Sodium acrylate (2 options: made from acrylic acid or powder form)

50% (w/v) Acrylamide:

Combine the following reagents and stir until dissolved. Store at -20 °C up to one month.

	Reagents	Volume	Final Concentration
	Acrylamide	5 g	50%
	Milli-Q water	10 mL	

2% (w/v) N,N Methylene-Bis-Acrylamide

Combine the following reagents and stir until dissolved. Store at -20 °C up to one month.

	Reagents	Amount	Final Concentration
	N,N Methylene-bis-acrylamide	0.2 g	2%
	Milli-Q water	10 mL	

4.04M Sodium Acrylate

Add 22.5 mL milli-Q water into a 250 mL glass bottle and cool the solution down to 0 °C on ice. Slowly add in 27.5 mL acrylic acid with stirring until fully mixed. Cover the bottle to the neck with ice and then, with stirring, add 36 mL 10N NaOH over the course of 00:10:00. Make sure to keep the solution at 0 °C.

Insert a pH meter and begin adding 1N NaOH in 1 mL increments until pH 7.6 – 8.0.

Keep track of the volume needed to reach this range.

Once desired pH is reached, let the solution warm to Room temperature and check the pH to make sure it is still in correct range. Add water to a final volume of 100mL and store at -20 °C.

	Reagents	Amount
	14.6M Acrylic acid	27.5 mL
	Milli-Q water	22.5 mL + extra to reach 100mL
	10N NaOH	36 mL
	1N NaOH	~5-10 mL

4.04M Sodium Acrylate (from powder form)

Combine the following reagents and stir until dissolved. Store at -20 °C up to one month.

	Reagents	Amount	Final Concentration
	Sodium acrylate	18.99 g	4.04 M or 38%
	Milli-Q water	50 mL	

Note

Powder Sodium Acrylate can be used. However, a yellow solution indicates low purity. If this is observed, discard and use a different batch.

Stock X (Monomer Solution)

Combine the following On ice. Aliquot and store at -20 °C for up to one month.

	Reagents	Amount	Final Concentration
	4.04M Sodium acrylate	4.554 mL	9.2%
	50% Acrylamide	1 mL	2.7%
	2% N,N Methylene-bis-acrylamide	1.5 mL	0.16%
	5M NaCl	8 mL	12%

	Reagents	Amount	Final Concentration
	10X PBS	2 mL	1X
	Milli-Q water	1.745 mL	
	Total	18.799 mL	

Proteinase K Digestion Buffer: 50mM Tris-HCl pH 8, 1mM EDTA, 0.5% TritonX, 50mM NaCl, 0.3%SDS

Combine the following reagents. Aliquot and store at -20 °C for several months.

	Reagent	Amount	Final Concentration
	1M Tris-HCl pH 8	2 mL	50 mM
	10% Triton X-100	2.5 mL	0.5%
	5M NaCl	500 uL	50 mM
	0.5M EDTA	100 uL	1 mM
	10% SDS	1.5 mL	0.3%
	Milli-Q Water	42.9 mL	
	Total	50 mL	

SAFETY WARNINGS

Tetrahydrofuran (THF) and dichloromethane (DCM) are toxic and carcinogenic. THF is flammable. When exposed to air, THF may form explosive peroxides if concentrated by distillation or evaporation. Test for peroxide formation or discard THF after 1 year. Perform the steps that involve these reagents under the fume hood. Dispose of THF and DCM in a hazardous waste stream. Wear lab coat, safety goggles or glasses, and chemical resistant gloves (7.8 MIL). If these solvents contact your gloves, remove immediately and don new gloves.

2-methyl-2-butanol and 2-propanol are corrosive and flammable. Perform the steps that involve these reagents under the fume hood. Dispose of 2-methyl-2-butanol and 2-propanol in a hazardous waste stream. Wear a lab coat, safety goggles or glasses, and gloves.

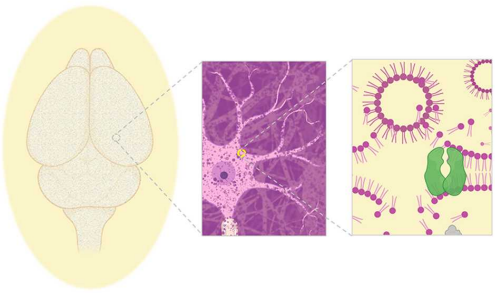
Sodium azide may be harmful if inhaled. It may cause respiratory tract, skin, and eye irritation and may be fatal if absorbed through skin or swallowed. Sodium azide can react with metal spatulas and metal lab equipment to form shock sensitive salts. Sodium azide reacts with lead, copper, silver, gold and metal halides to form heavy metal azides which are shock sensitive and explosive. Additionally, contact with acids liberates toxic gas. Dispose of sodium azide in a hazardous waste stream. Wear a lab coat, safety goggles or glasses, and gloves.

Acrylamide powders and solutions are toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, teratogen and a carcinogen. Dispose of acrylamide and any contaminated consumables in a hazardous waste stream. Wear a lab coat, safety goggles or glasses, and gloves.

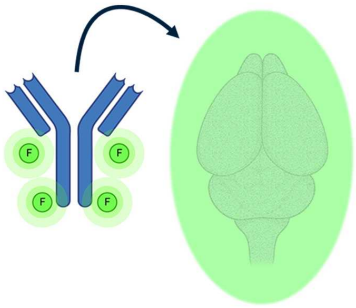
Protocol Overview

1 This protocol prepares a whole mouse brain for expansion microscopy (ExM). Methods of tissue processing include organic and aqueous delipidation, immunolabeling, ExM (gel embedding and expansion), and mounting the sample in the imaging chamber.

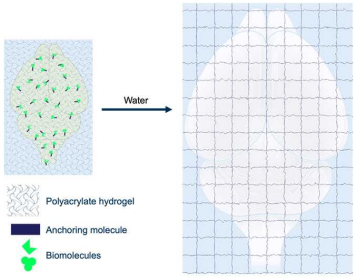
Days 1-14: Organic Solvent and Aqueous Delipidation
Reduce light scattering and improve antibody penetration



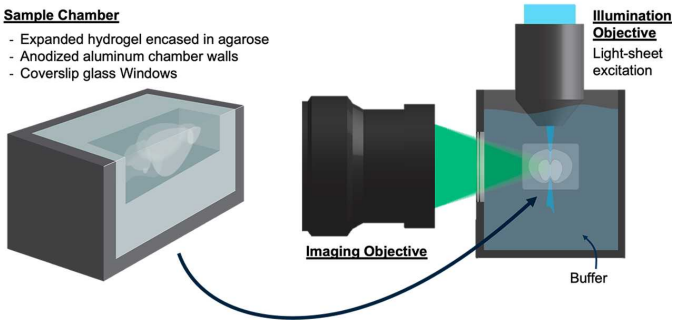
Days 15-45: Immunolabeling
Label target neurons with custom fluorescent tags to amplify signal



Days 46-70: ExM
Anchor tissue to an expandable hydrogel; break peptide bonds and expand tissue isotropically (3-4X)



Day 71: Sample mounting
Equilibrate hydrogel and secure in the chamber for imaging



Tetrahydrofuran / Dichloromethane Delipidation

1w 1d

2 Reference Tetrahydrofuran and Dichloromethane Delipidation of a Whole Mouse Brain protocol.

Protocol

NAME

Tetrahydrofuran and Dichloromethane Delipidation of a Whole Mouse Brain

CREATED BY

Naveen Ouellette

PREVIEW

SBiP Delipidation

1w

3 Reference Aqueous (SBiP) Delipidation for a Whole Mouse Brain protocol.

Protocol

NAME

Aqueous (SBiP) Delipidation of a Whole Mouse Brain

CREATED BY

Naveen Ouellette

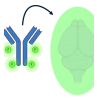
PREVIEW

Immunolabeling

4w 3d

4 Reference Immunolabeling of a Whole Mouse Brain protocol.

Protocol



NAME

Immunolabeling of a Whole Mouse Brain

CREATED BY

Naveen Ouellette

PREVIEW

Gelation and Digestion

4w 2d 6h 23m

5 **Day 1 – MBS equilibration**

5.1 For all ExM steps, use small-volume glass tubes or vials to contain the brain. This way, we use a smaller amount of valuable reagents for gelation. We typically use a 4 mL glass vial that has a wide enough opening to fit an adult mouse brain.

5.2 Wash sample in MBS at Room temperature, filling vial to the top (typically 4 mL). 2h

Replace solution for each step:

- MBS for 01:00:00 +
- MBS for 01:00:00 +

5.3 Replace MBS and store On ice at 4 °C Overnight 16h

6 Day 2-5 – Acryloyl X (AcX) treatment 4d

6.1 Prepare a new tube or vial with 3 mL MBS for each brain. Place On ice.

6.2 Mix AcX with MBS (2500 µg per brain or 250 µL of 10 mg/mL AcX with 3 mL MBS) for each whole brain.

6.3 Replace MBS solution from previous step with AcX solution for each whole brain and fill remaining space in vial with MBS to minimize air.

6.4 Incubate On ice at 4 °C, mix by inverting tubes once per day for 4 days. 4d

7 Day 6-7 – PBS washes 2d

7.1 Replace solution with cold 1X PBS, mix by inverting, keep on On ice at 4 °C, Overnight. 16h

7.2 Replace solution with cold 1X PBS, mix by inverting, keep on On ice at 4 °C, Overnight. 16h

8 Day 8-11 – Stock X equilibration 4d

8.1 Prepare Stock X on On ice.

Safety information

Acrylamide powders and solutions are toxic if swallowed, inhaled, or absorbed through the skin. It is a mutagen, teratogen and a carcinogen.
Dispose of acrylamide and any contaminated consumables in a hazardous waste stream.

8.2 To activate Stock X, add VA-044. The amount of VA-044 required is equal to 1.2% of the total volume of Stock X used. (Typically we add 234 µL of 10% (w/v) VA-044 in ~ 20 mL Stock X).

8.3 Fill each whole brain tube with activated Stock X solution to minimize air.

8.4 Save any extra activated Stock X solution on On ice.

8.5 Incubate on On ice at 4 °C, mix by inverting tubes once per day for 4 days. 4d

9 Day 12 – Gelation and Proteinase K

9.1 Bring brain vials and activated Stock X solution to Room temperature on the bench. Swirl Stock X conical tube carefully to avoid bubbles.

> Note
>
> Typically a 5 mL preparation of activated Stock X is more than sufficient for gelling one adult mouse brain. If more activated Stock X is required, a fresh preparation may be added to the Stock X left over from the previous step.

9.2 De-gas activated Stock X solution in a vacuum chamber ~ 00:20:00. This reduces the appearance of bubbles during polymerization. 20m

9.3 Prepare polymerization chamber by stacking silicone isolator gaskets of various thicknesses to an appropriate height on an uncharged 1"x3" microscope slide. The gaskets should be stacked high enough so the entire mouse brain will be embedded within the hydrogel. Typically, we stack the gaskets to allow about 2 mm of gel to polymerize around the brain. Inspect chamber for dust and debris before beginning embedding process.

9.4 Add activated Stock X to partly fill polymerization chamber.

9.5 Avoid and eliminate all bubbles.

9.6 Transfer brain to chamber and fill with Stock X just to the top.

9.7 Seal chamber with a clean, long coverslip, taking care to avoid bubbles.

9.8 Place in petri dish, and seal in 2 sequential zip lock bags, each thoroughly purged with nitrogen gas.

9.9 Incubate at 37 °C for 04:00:00 + 4h

9.10 Remove coverslip and gasket; gel should be firm without extra liquid dripping out.

9.11 Cut gel with a razor blade, making a rectangular cuboid shape. Leave about a 2 mm border of gel on all sides of the brain. If needed, the gel may be trimmed down further after expansion.

9.12 Wash in 50 mL 1X PBS at Room temperature in a 50 mL conical tube for about 00:03:00 with swirling. 3m

9.13 Replace solution with 40 mL Proteinase K (ProK) buffer spiked with 100U ProK (~ 126 µL); incubate at Room temperature with gentle swirling or rocking on a nutator for 5 days. 5d

Note

Store Proteinase K at -20 °C and keep On ice until added to the ProK Buffer.

10 Day 18 – Proteinase K digest (continued) 5d

10.1 Swirl whole brain conical tube carefully and thoroughly.

10.2 Add 126 µL (100U) Proteinase K to the tube.

10.3 Move to 37 °C and incubate 5 days+. Cortex should look transparent and the white matter should look mostly clear. Digestion time may be extended if needed.

11 Day 23 – Final washes (after judging that digest is complete) 3d

11.1 Wash brain in ~50 mL 1X PBS briefly at Room temperature, then replace with enough 1X PBS to fill the 50 mL conical tube; wash at Room temperature with gentle swirling or rocking for 2 days. 2d

11.2 Replace 1X PBS solution for a few hours and/or Overnight. 16h

11.3 Change final wash with 1X PBS Azide 0.05% and store at 4 °C until ready for expansion.

Expansion of Hydrogel Embedded Brain

12 Submerge the hydrogel in 0.05X SSC.

- Replace 0.05X SSC once per day for at least 3 days at Room temperature.

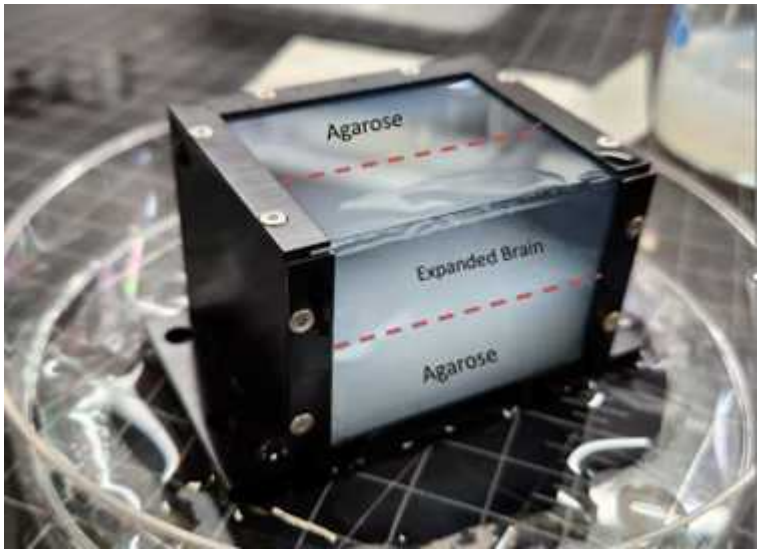
The hydrogel should expand to about 3 times its original size before digestion. If the gel does not seem to be expanded fully, change the SSC buffer again.

As it expands, the hydrogel becomes more fragile. When handling the hydrogel, use a gloved hand to carefully transfer it to another container if needed. To exchange 0.05X SSC, we use a 2 L Instrument Soaking Tray which has a strainer insert. The gel and strainer are lifted out of the solution, the solution is refreshed, and the strainer and gel are gently placed back in.

Mounting of Hydrogel in ExA-SPIM Chamber

13 **Mounting of Expanded Hydrogel Embedded Brain in ExA-SPIM Chamber**

The expanded hydrogel is embedded in a chamber that will hold it in place during imaging on the ExA-SPIM. The hydrogel is placed against the upper corner of the chamber and held in place with agarose. Once the agarose is solidified, the solid top and front panels that the sample is resting against are replaced with glass. The sample will be securely held in place while allowing access on two sides for imaging.



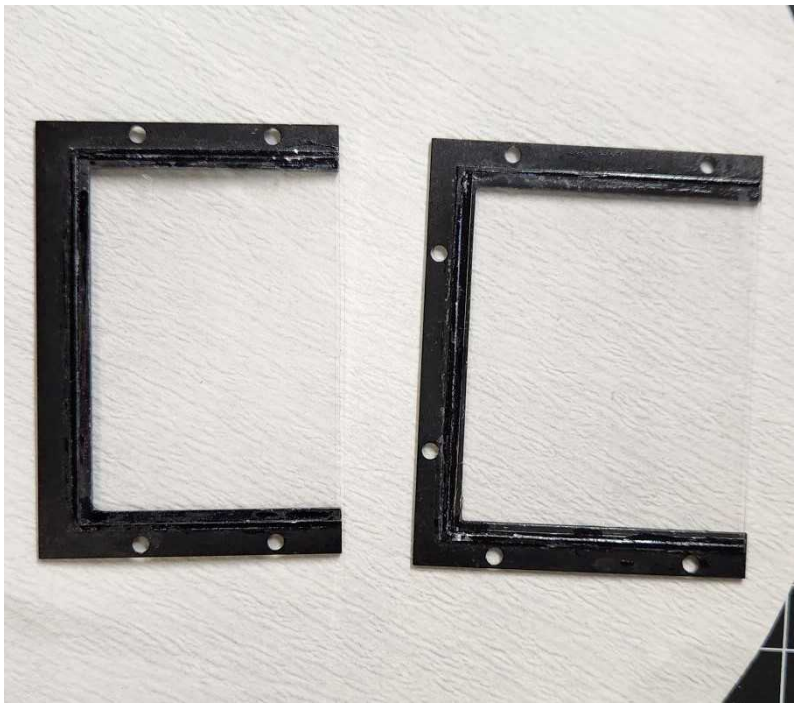
Assembled ExA-SPIM sample chamber with expanded brain sample and agarose support.

13.1 Assemble the glass window panels

Using a glass cutting pen and ruler, cut #2 glass panels to fit the top and front window frames. Along the longest edge, trim the glass about 1 mm shorter than measured. When the panels are assembled on the chamber, this will leave a gap at the corner where the two glass edges meet along the top-front corner. This opening allows fluid to move between the chamber and surrounding imaging buffer, helping keep the sample equilibrated.

Use Krazy glue to apply the glass to the metal frame. Make sure the glass is clean and free of debris or glue.

Optional: a glass window may be cut for the back panel viewing window, but this is not crucial. The back solid panel may remain in place during imaging.

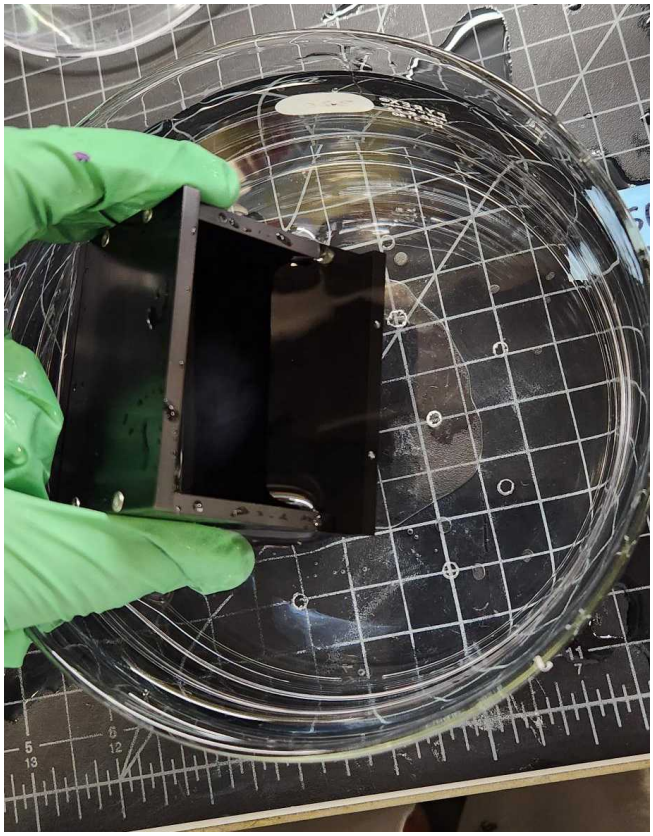


Top and Front glass panels assembled.

13.2 Assemble the chamber to prepare for agarose embedding

The chamber is first assembled with solid sides so that the hydrogel can be placed inside and embedded in 2% Agarose without leakage. Leave one side open (Back or Bottom side) for inserting the hydrogel. Fill chamber with water to test for any leaks. If leaks are observed, ensure that all screws are tight. If there is a very slow leak, this is usually OK, agarose will not

leak as easily as water.



The chamber is assembled with bottom panel left open for hydrogel insertion.

13.3 Trim the expanded hydrogel

The lateral side of the embedded brain hydrogel will be mounted against the front glass panel. This side of the gel should have a smooth surface. If the cut sides of the hydrogel are uneven, it will be difficult to mount without agarose leaking around the uneven side and obscuring the brain.

Use a long, thin blade to smoothly trim the sides along the lateral, anterior and posterior sides of the hydrogel. It is best to leave about 6 mm buffer of empty hydrogel surrounding the brain.

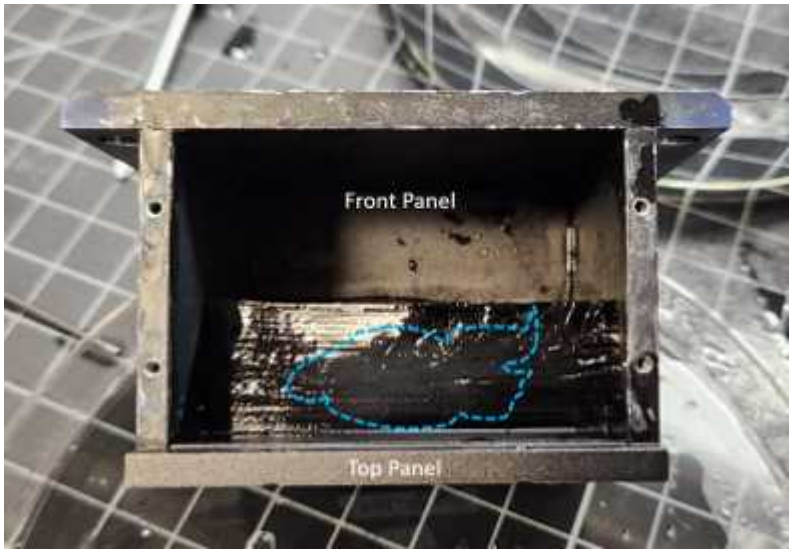
Trimming is not necessary unless the the gel is too large or front facing side is very uneven.

Trimmed hydrogel, smooth cuts on lateral sides.

13.4 Position the hydrogel in the imaging chamber

Submerge the hydrogel and assembled chamber in a wide dish filled with the same 0.05X SSC that the brain was soaking in. Position the hydrogel so the dorsoventral axis of the brain intersects with the top and bottom of the chamber (Z-axis of the ExA-SPIM). The dorsal surface should be facing the top panel.

While submerged, carefully use your hand to slide the hydrogel through the open panel into the chamber. Position the chamber so the open panel faces up. The hydrogel should rest against the corner of the Front and Top sides. Carefully drain any 0.05X SSC that is left in the chamber. A transfer pipet may be used to remove any remaining 0.05X SSC.



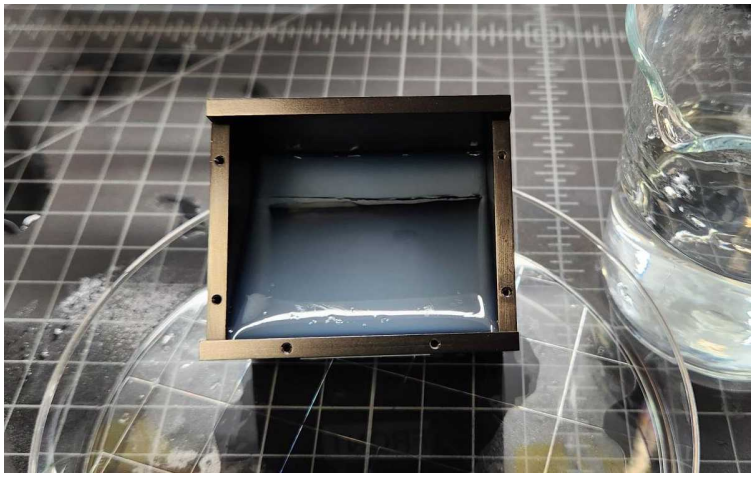
ExA-SPIM chamber positioned with hydrogel resting in top-right corner.

13.5 Prepare 150 mL of 2% agarose. Add 3 g of agarose to 150 mL 0.05X SSC. Stir with a spatula and heat about 2 minutes in the microwave until boiling and solution is clear.

> **Note**
>
> IMPORTANT NOTE: The agarose must be made from the very same batch of 0.05X SSC that the hydrogel was expanding in. Any difference in salt concentration in the agarose can alter the size of the hydrogel after it has been embedded.

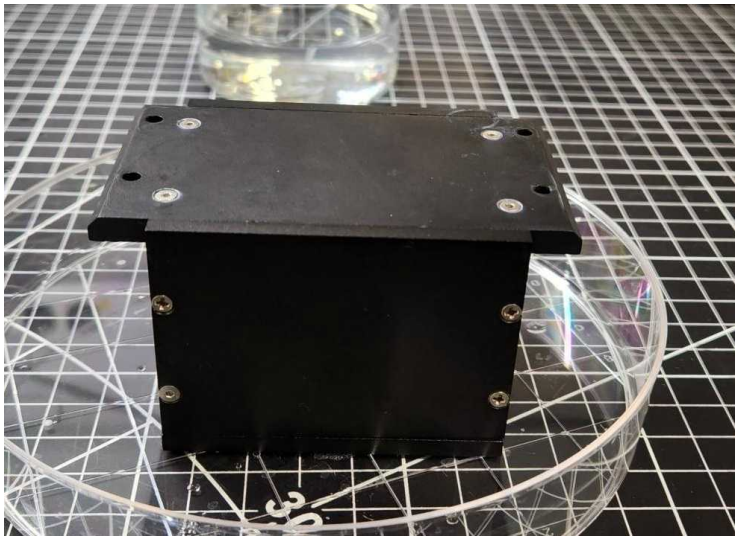
13.6 Wait until the agarose has cooled to about 55 °C.

To prevent the agarose from leaking underneath the hydrogel, prop the chamber at an angle (against the side of a petri dish) so gravity is gently pulling the hydrogel against the Front-Top corner of the chamber. Gently pour the agarose in so it just reaches the lowest edge of the open panel. Wait for it to solidify. Keep remaining agarose on a hot plate at ~ 55 °C.



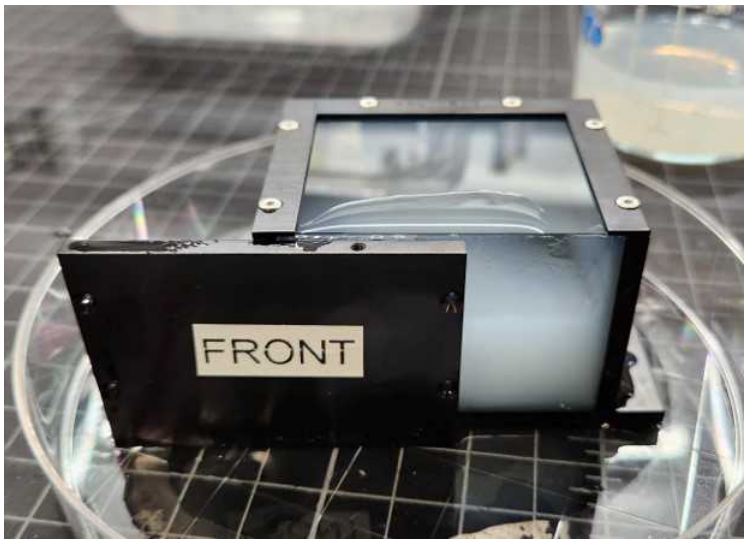
Molten agarose is poured into the chamber.

13.7 Once the first pour is solid, place the chamber on a level surface and fill the remaining space with agarose. Insert the last panel and wait for it to solidify. The bottom panel has 4 screw holes that are used to attach it to the imaging chamber. It must placed so the the holes are positioned farther away from the sample.

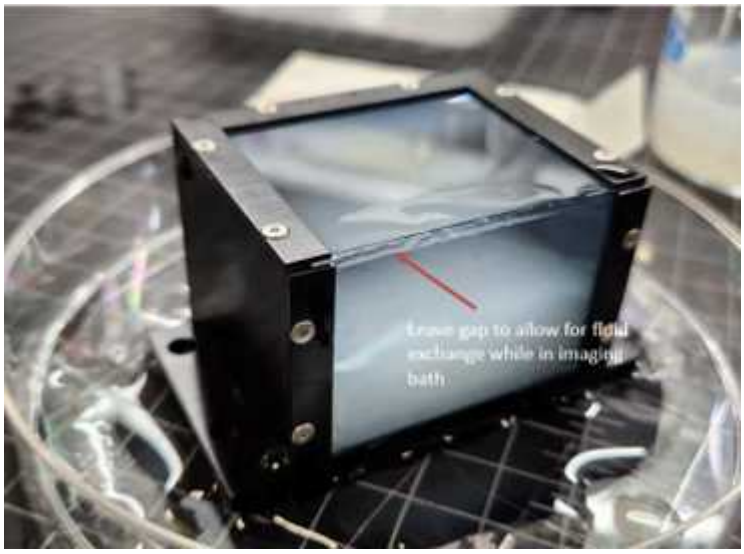


13.8 The front and top panels must be replaced with glass panels to conduct imaging. Carefully remove these panels and replace with the glass panels that were prepared earlier. Avoid trapping bubbles.

It helps to wet the surfaces of the exposed agarose and hydrogel with 0.05X SSC when the new panels are applied. There should be a small gap visible at the top-front corner where the glass panels meet. This opening helps keep the sample equilibrated with the surrounding imaging buffer.



Front panel is removed.



Front and Top panels have been replaced with glass. A small gap remains in the corner to allow for fluid exchange.

13.9 Submerge embedded sample back in to the 0.05X SSC it was previously soaking in. Protect container from light and leave the embedded hydrogel to soak overnight.

Mounted sample is submerged in 0.05x SSC before imaging.

14 The sample is now ready to be imaged on the ExA-SPIM.