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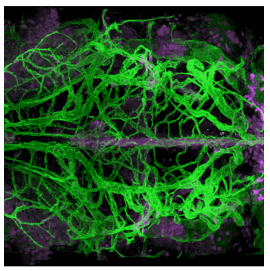
WHOLISTIC ExM: Whole-Body Expansion Microscopy with Immunofluorescence and Histological Stains V.5



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Abstract

The interpretation of Whole Body Imaging (WBI) data necessitates comprehensive anatomical knowledge to accurately determine cell-type identity; however, resources pertaining to the internal anatomy of larval zebrafish are limited. To mitigate this gap, we established an advanced Whole Body Expansion-Microscopy (WB-ExM) protocol, facilitating the acquisition of molecular and cell-type information at subcellular resolution throughout the entire organism. While high-quality resources are available for the larval zebrafish brain[1,2,3] there remains a deficiency in materials concerning its visceral anatomy. In order to supplement existing histological, X-ray, and Expansion Microscopy methods aimed at mapping body-wide anatomy[4,5,6,7] and to develop a technique that yields high-fidelity molecular and anatomical data with enhanced processing speeds and compatibility with older samples, we devised an enzyme-free, rapid, and robust whole-body expansion microscopy method[8,9], effective on larvae up to at least 14 days post-fertilization. This method involves the use of high-temperature (100°C) chemical hydrolysis to uniformly soften tissues, embedding within a medium-density gel with reduced protein-gel anchoring, and repeated embedding post-digestion with moderate ~1.5x expansion factors in each cycle, culminating in a robust and uniform expansion even of challenging structures such as cartilage embedded in soft tissue. This protocol results in excellent optical clearing, retains high levels of antibody signals.

A demo dataset can be viewed **here**. Dorsal view of a double transgenic zebrafish animal labeling the ventricular and vascular systems (*Tg(foxj1a:eGFP)* x *Tg(flk1:dsRed-CAAX)*), stained against eGFP (magenta) and dsRed (green) (10 days post-fertilization, expanded ~2×).

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3. Tabor, K. M. *et al.* Brain-wide cellular resolution imaging of Cre transgenic zebrafish lines for functional circuit-mapping.
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5. Ding, Y. *et al.* Computational 3D histological phenotyping of whole zebrafish by X-ray histotomography. *eLife* 8, e44898 (2019).
6. Steib, E. *et al.* TissUExM enables quantitative ultrastructural analysis in whole vertebrate embryos by expansion microscopy. *Cell Reports Methods* 2, 100311 (2022).
7. Sim, J. *et al.* Nanoscale resolution imaging of the whole mouse embryos and larval zebrafish using expansion microscopy. Preprint at https://doi.org/10.1101/2021.05.18.443629 (2021).
8. Chen, F., Tillberg, P. W. & Boyden, E. S. Expansion microscopy.
9. Wang, Y. *et al.* EASI-FISH for thick tissue defines lateral hypothalamus spatio-molecular organization. *Cell* 184, 6361-6377.e24 (2021).

Image Attribution

Virginia M. S. Ruetten

Protocol materials

- Corning® 25×25 mm Square #2 Cover Glass **Corning** **Catalog #**2855-25
- CS-8R Coverslips, 0.15 mm (0.006 in), 8 mm diameter, pkg of 100 **Multi Channel Systems MCS GmbH** **Catalog #**640701
- PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free **Thermo Fisher Scientific** **Catalog #**AM9625
- Pierce™ 16% Formaldehyde (w/v), Methanol-free **Thermo Scientific** **Catalog #**28906
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific** **Catalog #**10977023
- Normal Goat Serum **Jackson ImmunoResearch Laboratories, Inc.** **Catalog #**005-000-121
- PBS, pH 7.4 **Thermo Fisher** **Catalog #**10010001
- Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**X100-5ML
- Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2002-100G
- Agarose, low gelling temperature **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A9414-100G
- Fisherbrand™ Superfrost™ Disposable Microscope Slides **Fisher Scientific** **Catalog #**Catalog No.12-550-123
- Press-to-Seal™ Silicone Isolator with Adhesive, eight wells, 9 mm diameter, 0.5 mm deep **Thermo Fisher Scientific** **Catalog #**P24743
- Poly-L-lysine hydrobromide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**Poly-L-lysine hydrobromide
- Photo Flo 200 Solution **Electron Microscopy Sciences** **Catalog #**74257
- Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester) **Thermo Fisher Scientific** **Catalog #**A20000
- ATTO 647N maleimide **AAT Bioquest** **Catalog #**2857
- DMSO, Anhydrous **Thermo Fisher** **Catalog #**D12345
- anti RFP antibody **Synaptic Systems** **Catalog #**409 006
- Anti-Rabbit-IgG - Atto 647N **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**40839-1ML-F
- Donkey anti-Chicken IgY (H L) Highly Cross Adsorbed Secondary Antibody, Alexa Fluor™ 568 **Thermo Fisher Scientific** **Catalog #**A78950
- GFP Polyclonal Antibody **Invitrogen - Thermo Fisher** **Catalog #**A-11122
- Acryloyl-X, SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester) **Thermo Fisher Scientific** **Catalog #**A20770
- Hydrogen peroxide solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H1009-500ML
- NaCl (5 M), RNase-free **Thermo Fisher Scientific** **Catalog #**AM9760G
- UltraPure™ SDS Solution, 10% **Thermo Fisher Scientific** **Catalog #**15553027
- Acrylic acid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**147230-5G
- Sodium Hydroxide Solution (10N/Certified) **Fisher Scientific** **Catalog #**SS255-1

- N,N,N′,N′-Tetramethylethylenediamine (TEMED) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T7024-25ML
- 2% bis-acrylamide solution **Bio-Rad Laboratories Catalog #**1610142
- Ammonium persulfate (APS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3678-100G
- 40% Acrylamide Solution **Bio-Rad Laboratories Catalog #**1610140
- Acrylic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**147230-500G
- 4-Hydroxy-TEMPO (4HT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4-Hydroxy-TEMPO

Before start

Make sure you have all the reagents at hand.

Reagents

1 **4% PFA**

Cannot be prepared in advance.
Take a new ampule of 16% PFA (10ml). Aliquot it into 1 ml aliquots. Store at -80ºC. On the day take one aliquot out and thaw.
To make a total volume of 4 ml of 4% PFA: mix 1ml of 16% PFA, 0.4 ml of 10x PBS and 2.6 ml of distilled H20. Use within the next two days and store in a 4ºC fridge.

Note

PFA deteriorates even if stored at -80ºC. Avoid reusing aliquoted samples.
When PFA is stored in sealed ampules, it is protected from atmospheric oxygen and moisture. This isolation prevents oxidative degradation

Reagents

- PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free **Thermo Fisher Scientific Catalog #**AM9625
- Pierce™ 16% Formaldehyde (w/v), Methanol-free **Thermo Scientific Catalog #**28906
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

1 **Blocking Buffer**

Cannot be prepared in advance.
5% goat serum, 0.5% Triton-100 in 1x PBS, 0.1% Na Azide.
Keep in fridge at 4ºC

4 °C

To make 7.5 ml of Blocking Buffer
- 0.375 ml of goat serum
- 0.75 ml of PBS-T-5-NaAz-1 [PBS + Triton (5%) + NaAzide (1%)]
- 6.375 ml of 1x PBS

Note

Notes on Goat Serum:
This comes in a sealed vial. Do not open the seal but use a syringe to extract medium. The medium is nutrient rich and so easily can become contaminated. Do not use for more than 3 months. Reconstitute with distilled water.

Reagents

- Normal Goat Serum **Jackson ImmunoResearch Laboratories, Inc. Catalog #**005-000-121
- PBS, pH 7.4 **Thermo Fisher Catalog #**10010001
- Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**X100-5ML
- Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2002-100G

2 **Antibodies**

Primary antibodies (polyclonal)
rabbit anti-eGFP
chicken anti-RFP
anti-mRuby

Secondary antibodies
goat anti-rabbit Atto647N
donkey anti-chicken Alexa568

- GFP Polyclonal Antibody **Invitrogen - Thermo Fisher Catalog #**A-11122
- anti RFP antibody **Synaptic Systems Catalog #**409 006
- Anti-Rabbit-IgG - Atto 647N **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**40839-1ML-F
- Donkey anti-Chicken IgY (H L) Highly Cross Adsorbed Secondary Antibody, Alexa Fluor™ 568 **Thermo Fisher Scientific Catalog #**A78950

3 **PBST-0.5**
Can be prepared in advance and stored at room temperature.
1x PBS with 0.5% or 0.1% Triton
PBST-0.5 and PBST-0.1 respectively

Triton dissolves terribly. It hardens upon making contact with water and takes time to dissolve fully. Prepare a 10% stock solution in 1x PBS and use that for subsequent

rounds.
Keep at RT.

Room temperature

Reagents

PBS, pH 7.4 **Thermo Fisher Catalog #**10010001

Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**X100-5ML

4 **Bleaching Solution**

Cannot be prepared in advance.

Combine 3% H_2O_2, 60mM KOH and 100mM (or more) NaN_3 in PBS.

- Prepare 300mM NaN_3: add 195mg of NaN_3 to 10ml of PBS.

To make 5 ml of bleaching solution:
- 0.3 ml of 50% H_2O_2 solution
- 0.1 ml of 1M KOH solution (takes one hour with little bubbling) [tested]
- fill the rest of 100mM NaN_3 (i.e.: 4.25ml, 85mM NaN_3)

Note

Many biological tissues contain catalase, which rapidly breaks down hydrogen peroxide into water and oxygen. This leads to a large number of bubbles, which can rupture the tissue. The tissue should first be passivated. NaN_3 is a catalase inhibitor. Pre-incubate fish with NaN_3 to block catalase. Then add bleach solution containing NaN_3. The dissociation of H_2O_2 occurs faster at high pH, thus higher KOH should've used. The concentration of NaN_3 is in excess to saturate the reaction - can be lowered.

Hydrogen peroxide solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H1009-500ML

5 **1% low-melting temperature agarose**

Can be prepared in advance.

Dissolve 1 g of low-melting point agarose in 100 ml of 1x PBS.
Add 1 g of power to 100 ml 1x PBS at RT. Stir with magnetic stirrer.
Bring to a boil using a microwave. Stir with magnetic stirrer until full dissolved.

Repeat boil and stirring until solution is clear.

Reagents

- Agarose, low gelling temperature **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9414-100G
- PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free **Thermo Fisher Scientific Catalog #**AM9625

6 **Acryloyl-X Solution (AcX)**

Cannot be prepared in advance.
Stock concentration: 10 mg/ml
Dissolve to 10 mg/ml in anhydrous DMSO. Aliquot in 20 µl batches.
Store in a desiccated environment at -20ºC. Don't re-use AcX after thawing.

Working solution will be: 20 µg/ml, dilution 1:500 in 1x PBS

Note

Using anhydrous DMSO is important because DMSO is remarkably hygroscopic, readily absorbing water vapor from ambient air. DMSO-water mixtures have different solvation properties than pure DMSO, which can cause precipitation of hydrophobic compounds. The presence of water also enables hydrolysis of susceptible functional groups, degrading stock solutions over time.

Reagents

- Acryloyl-X, SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester) **Thermo Fisher Scientific Catalog #**A20770
- DMSO, Anhydrous **Thermo Fisher Catalog #**D12345

7 **Na Acrylate Solution**

Fill a 500 ml beaker with ~300 ml of water for a water bath.
Place an open 50 ml Eppendorf tube into the bath and bring the setup to a fume hood.
The purpose of the water bath is to provide a highly conductive medium to cool the solution.

Add 9.0 ml of molecular grade water to the Eppendorf tube.
Add 11 ml of pure acrylic acid to the Eppendorf tube. (Note that this is a flammable and reactive compound).

Add 14.4 ml of 10 M NaOH to the Eppendorf tube. This should be done dropwise to prevent excessive heating and boiling.

Leave it to cool.
The solution should be clear. If a yellow precipitate is observed this means the chemicals need to be changed.

Calibrate a pH meter.

Remove the acrylate-filled tube from the fume hood.
By now, most of the acrylic acid will have been converted to non-volatile sodium acrylate.

Measure the pH of the solution.
Add NaOH to adjust the pH gradually to between 7.5-8 using a pH meter. Do NOT use pH test strips.
We recommend starting by adding 10 M NaOH solution, and when the pH gets close to the desired pH adjusting via the use of 1 M NaOH solution.

(As a general guidance: Add about 500 µl 10 M NaOH, 250 µl 10 M NaOH, 120 µL 10 M NaOH, then some 1 M NaOH)
Add water up to a final volume of 40 ml.

(Note: Acrylic acid has a pKa of 4.76 at pH 7.75 - this solution has about 4 mM remaining buffering capacity)

Acrylic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**147230-5G

Sodium Hydroxide Solution (10N/Certified) **Fisher Scientific Catalog #**SS255-1

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

8 **Monomer and Gelation Solutions #1 (Medium Density Gel with high Bis)**

	A	B	C	D	E	F
	name	units	stock conc.	final conc.	vol (ul)	vol (ml)
	Acrylamide	%	40	10	250	2.5
	Na Acrylate	M	4	0.5	125	1.25

	A	B	C	D	E	F
	Bis	%	1	0.1	100	1
	10x PBS	x	10	1	100	1
	Water				365	3.65

Monomer Solution #1

Monomer Solution #1 can be stored safely at -20ºC for 1-3 months.

	A	B	C	D	E
	name (units)	units	stock conc.	final conc.	vol (ul)
	Monomer Solution #1				940
	APS	%	10	0.2	20
	TEMED	%	10	0.2	20
	4HT	%	0.5	0.01	20

Gelation Solution #1

Reagents

- 40% Acrylamide Solution **Bio-Rad Laboratories Catalog #**1610140
- 2% bis-acrylamide solution **Bio-Rad Laboratories Catalog #**1610142
- Acrylic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**147230-500G
- Sodium Hydroxide Solution (10N/Certified) **Fisher Scientific Catalog #**SS255-1
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- Ammonium persulfate (APS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3678-100G
- 4-Hydroxy-TEMPO (4HT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4-Hydroxy-TEMPO
- N,N,N′,N′-Tetramethylethylenediamine (TEMED) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T7024-25ML

9 **Monomer and Gelation Solutions #2 (Medium Density Gel with low Bis)**

	A	B	C	D	E	F
					to make 1ml	to make 10ml
	component	units	stock conc.	final conc.	vol (ul)	vol (ml)
	Acrylamide	%	40	10	250	2.5
	Na Acrylate	M	4	0.5	125	1.25
	Bis	%	1	0.02	20	0.2
	10x PBS	x	10	1	100	1
	Water				445	4.45

Monomer Solution #2

Monomer Solution #2 can be stored safely at -20ºC for 1-3 months.

	A	B	C	D	E
					to make 1ml
	component	units	stock conc.	final conc.	vol (ul)
	Monomer Solution #2				940
	APS	%	10	0.2	20
	TEMED	%	10	0.2	20
	4HT	%	0.5	0.01	20

Gelation Solution #2

Reagents

- 40% Acrylamide Solution **Bio-Rad Laboratories Catalog #**1610140
- 2% bis-acrylamide solution **Bio-Rad Laboratories Catalog #**1610142
- Acrylic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**147230-5G
- Sodium Hydroxide Solution (10N/Certified) **Fisher Scientific Catalog #**SS255-1

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

Ammonium persulfate (APS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3678-100G

4-Hydroxy-TEMPO (4HT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4-Hydroxy-TEMPO

N,N,N′,N′-Tetramethylethylenediamine (TEMED) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T7024-25ML

10 **Disruption Buffer**

	A	B	C	D	E	F
					to make 1ml	to make 100ml
	component	units	stock conc.	final conc.	vol (ul)	vol
	SDS	%	10	5	0.5	50
	Tris pH 7.5	mM	1000	50	0.05	5
	NaCl	M	5	0.2	0.04	4
	MilliQ water				0.41	41

Disruption Buffer

Stock **Disruption Buffer** can be prepared in advanced and stored at RT for a few months.

NaCl (5 M), RNase-free **Thermo Fisher Scientific Catalog #**AM9760G

UltraPure™ SDS Solution, 10% **Thermo Fisher Scientific Catalog #**15553027

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

11 **Total Protein Stains**

Prepare stock: 10 mg/ml stocks in anhydrous DMSO.
Aliquot in 5-10ul.
Store in a desiccated environment at -20ºC.

Reagents

Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester) **Thermo Fisher Scientific Catalog #**A20000

ATTO 647N maleimide **AAT Bioquest Catalog #**2857

DMSO, Anhydrous **Thermo Fisher Catalog #**D12345

12 **Gelation Chambers**

Silicone gaskets (Invitrogen P24743)
Glass slides (SuperFrost 12550123)
Scotch tape
Poly-l-lysine
Shaker (nutator - 75 RPM)

Reagents

Press-to-Seal™ Silicone Isolator with Adhesive, eight wells, 9 mm diameter, 0.5 mm deep **Thermo Fisher Scientific Catalog #**P24743

Fisherbrand™ Superfrost™ Disposable Microscope Slides **Fisher Scientific Catalog #**Catalog No.12-550-123

Poly-L-lysine hydrobromide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**Poly-L-lysine hydrobromide

Photo Flo 200 Solution **Electron Microscopy Sciences Catalog #**74257

13 **General note for sample handling**

Soak plastic transfer pipettes in PBST-0.5 before manipulating samples - otherwise, the fish will inevitably get stuck.
Never use forceps to handle or move the fish as this inevitably damages the sample.

Fixation and Permeabilization

10h

14 Euthanize samples with an overdose of MS-222 (a.k.a. tricaine) (200-300 mg/L).

15 Prepare fresh 4% PFA.

16 Place samples in 1 ml of 4% PFA.

Room temperature

17 Keep overnight (~9h) in 4% PFA at 4ºC on a shaker.

09:00:00

9h

4 °C

Note

Fixation time can be adjusted by an hour or two as convenient. Over-fixation causes excessive cross-linking, which masks epitopes and makes tissue stiff. Under-fixation results in poorer protein retention.

18 Rinse samples in 4 × 15 min 1 ml 1x PBS. 1h

01:00:00

Room temperature

Bleaching 1h

19 Prepare fresh **Bleaching Solution**

Combine 3% H_2O_2, 0.5% KOH (~90mM) and 50mM (or more) NaN_3 in PBS.

Prepare 100mM NaN_3:
Add 65mg of NaN_3 to 10ml of PBS.
NaN_3 is toxic so use a fume hood.
Lower concentrations of NaN_3 would likely be acceptable too. We aimed at saturating the reaction.

To make 5 ml of Bleaching Solution:
- 0.3 ml of 50% H2O2 solution
- 0.45 ml of 1M KOH solution (0.3ml is still quite aggressive)
- fill the rest of 100mM NaN_3 (i.e.: 4.25ml) to get to 85mM of 0.5% KOH (~90mM) and 50mM (or more) NaN_3 in PBS.

Note

NB: Bleaching is not a required step, and users should decide whether to include it. If using Casper fish, for instance, bleaching will ensure the eyes are void of pigments, but have little other benefit. Bleaching comes with the risk of micro bubbles being retained in the tissue and so if it does needed we recommend avoiding it.

Hydrogen peroxide solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H1009-500ML

Hydrogen peroxide solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H1009-500ML

20 Preincubate the samples in 100mM NaN3 (~10mins). 50m

Incubate the samples in 500 µL of **Bleaching Solution**.
Some bubbling will occur, but less is better. Leave the fish in the solution until eye pigments are visibly reduced (light brown), which should take approximately 30 min at RT. If pigments clear faster, the step can be stopped early. While we have not noticed any detrimental effects of 40 min incubation on antigenicity, shorter incubations are preferable as H_2O_2 can damage epitopes.

Room temperature

00:50:00

21 Rinse the samples with 1x PBS (2× 5 min at RT). 10m

Room temperature

00:10:00

Immunofluorescence (Optional) 5d 17h 10m

22

Note

If immunohistochemistry is desired, we strongly recommend performing it prior to expansion. The protocol uses high-temperature hydrolytic disruption to homogenize the tissue, which denatures proteins and tends to destroy epitopes. Post-expansion immunohistochemistry will be supported in future versions of the protocol.

23 Prepare fresh **Blocking Buffer**.

24 Incubate the samples in **Blocking Buffer** (3 hr at RT) on a shaker. 3h

Room temperature

03:00:00

25 Incubate the samples in primary antibodies diluted 1:100 in **Blocking Buffer** in PCR tube (3 days at RT) on a shaker. Make sure Na Azide is included in the **Blocking Buffer** to prevent microbial growth. 3d

72:00:00

Room temperature

Note

Can I place multiple samples in the same PCR tube?
Yes, multiple samples can be placed in the same PCR tube (~10). The level of antibody is high enough that it should not get significantly depleted.

What antibodies can I use?
As we do IHC pre-expansion and digestion, any antibody that works well in fixed whole-mount preparation should work in expansion.

26 Wash sample in 1 ml **Blocking Buffer** (4 × 15 min at RT) on a shaker. 1h

Room temperature

01:00:00

27 Wash the samples in **Blocking Buffer** (3 × 2 hr or overnight at RT) on a shaker. 9h

Room temperature

09:00:00

28 Incubate the samples in secondary antibodies (1:100) in **Blocking Buffer** in PCR tube (2 days at RT) on a shaker. 2d

48:00:00

Room temperature

29 Wash the samples in **Blocking Buffer** (3× 2 hr, at RT). 4h

Room temperature

04:00:00

30 Wash the samples in 1x PBS (2× 5 min at RT) and transfer to fresh 1x PBS. 10m

Room temperature

00:10:00

31 Image one sample to confirm immunofluorescence staining was successful.

Permeabilization (if no IHC) 5h 10m

32 For specimens that have not been stained with antibodies, permeabilize in **PBST-0.5** at 4ºC overnight (or for at least 5 hr). 5h

05:00:00

4 °C

33 Wash samples (2× 5 min at RT) in 1x PBS. 10m

Room temperature

00:10:00

Agarose Embedding

34 Heat agarose in microwave until it liquifies.
Cool agarose to 50ºC (by placing in 50ºC incubator).

Note

NB: Agarose embedding is not a mandatory step. Agarose embedding facilitates mounting the samples in a consistent orientation, which can greatly ease downstream data analysis and so is recommended, but not necessary.

35 Adhere silicone gasket (Invitrogen P24743 or Invitrogen P24740) on a glass slide (Superfrost Microscope Slides #12550123) to form a **Mounting Chamber**.

Mounting Chamber

Reagents

- Press-to-Seal™ Silicone Isolator with Adhesive, eight wells, 9 mm diameter, 0.5 mm deep **Thermo Fisher Scientific Catalog #**P24743
- Fisherbrand™ Superfrost™ Disposable Microscope Slides **Fisher Scientific Catalog #**Catalog No.12-550-123

36 Place fish in **Mounting Chamber**, remove any access PBS, and cover with ~70-100 µl of 1% low-melting-point agarose. Orientate as desired (on side or dorsal side up) and leave to solidify.

Note

Ensure that the fish if fully covered in agarose (both above and below it). It can sometimes be easier to add an excess of agarose to make sure fish is fully immersed, and then remove the excess. The agarose should fill the chamber while not doming much.
If the sample isn't fully immersed, this can lead to the fish sticking to the slide.

Note

Use a sharp and clean utensil to nudge the fish into the desired orientation.
If more time is needed, the mounting chamber can be placed on a heating block to ensure that the agarose doesn't solidify pre-emptively.
Always begin by nudging the fish to the very bottom of the agarose drop to avoid the sample being held by surface tension to the top of the drop. Make sure that the fish is fully submerged in agarose.

37 With a razor blade, cut out a rectangle around the agarose-embedded sample and delicately lift the pad of agarose and transfer it to 12 well-plate with 1 ml of 1x PBS/well. Cut as close to the fish as possible with some safety margin.

Note

To facilitate lifting of the agarose-embedded agarose, immersing the slide in 1x PBS can help. Ensuring that the sample is fully immersed in agarose also facilitates this step (see comments above).

Protein Anchoring

1h

38 Prepare **Acryloyl-X Solution** (stock solution 10 mg/ml, working solution: 20 µg/ml, dilution 1:500 in 1x PBS).
1 ml per sample is needed.
Do not prepare in advance.

39 Incubate each sample in 1 ml of **Acryloyl-X Solution** (1 hr at RT) shaking in 12-well plate. 1h

Room temperature

01:00:00

Preparation of Gelation Chamber #1

40 Prepare chambers for gelation, one for each sample.
Layer 11 pieces of Scotch tape together to create ~0.6 mm thick spacers.
Cut two strips of spacer material, about 2 cm long and 0.5 cm wide.
Stick two strips to a glass slide ~15 mm apart to form the side walls of the **Gelation Chamber #1**.

Gelation Chamber #1

Incubation with Gelation Solution 1

45m

41 Rinse samples with 1x PBS (3× 5 min) at RT.

15m

00:15:00

Room temperature

42 Thaw **Monomer Solution #1**, as well as 4HT, TEMED and APS. Vortex well and keep on ice.
Monomer Solution #1 and **#2** can be stored safely at -20ºC for a few months.

43 Mix **Monomer Solution #1** and 4HT, TEMED and APS at a ratio of 94:2:2:2 to produce **Gelation Solution #1**.
Vortex.
For reference: Each sample needs ~3 ml of **Gelation Solution #1 (**~1 ml per incubation round).

44 Incubate samples in **Gelation Solution #1** on ice (3× 10 min at 4ºC) with 1 ml of **Gelation Solution #1** on a shaker in a 12-well plate. 30m
00:30:00
4 °C

Gelation 1 2h

45 With spatula, transfer agarose block containing fish into the **Gelation Chamber #1**.

46 Gently place cover slip over the agarose block lying on the walls of **Gelation Chamber #1** (made of scotch tape).
The cover slip should lay flat on the agarose block.
Gently press to seal.

47 Slowly pipette **Gelation Solution #1** into **Gelation Chamber #1** until full. Avoid any air bubbles.
Solution will hold by water tension.

One can use **Gelation Solution #1** that was used during the previous incubation (no need to prepare fresh one).

Note

Avoiding bubbles is critical. If bubbles appear, the procedure should be repeated (cover slip removed and repositioned) until no bubbles are present. Bubbles contain oxygen, which inhibits polymerization by scavenging the free radicals that drive the chain reaction. Moreover, bubbles can become trapped within the gel and cause distortions and artifacts during imaging. Bubbles further than ~1mm away from the sample are acceptable though should still be minimized.

48 Once **Gelation Chamber #1** is filled, place the chambers at 37ºC for 2 hr to induce polymerization. 2h
Ensure incubator is humidified to prevent gel from drying out. This can be achieved by placing a beaker of water in the incubator.
37 °C
02:00:00

Note

Once polymerized, acrylamide forms non-toxic polyacrylamide, though gels may initially contain trace residual monomer so washing them thoroughly is recommended.

Disruption 9h 5m

49 Turn on the 100ºC heat block (for later disruption step).
Prepare **Disruption Buffer** (5 ml/sample).

Stock **Disruption Buffer** can be prepared in advanced and stored at RT for a few months (e.g.: 50-100 ml stock). The solution should be clear. If precipitates form in the solution, this is a sign that it should have been re-made.

50 Place gels at RT, and allow them to cool on the bench for > 5 min. 5m
Room temperature
00:05:00

51 Take off coverslip lid with a razor blade.
Under a stereomicroscope, trim the gels into a rectangle with a ~1-3 mm border on either side of the sample.
Add a nick on the top right corner to be able to track orientation of the samples.
The gel should be about 9 mm x 4 mm.

52 Transfer each gel to a 2 ml Eppendorf tube and add 1.8 ml of **Disruption Buffer** and leave for 10 min to dilute any unpolymerized residues.
Remove the **Disruption Buffer** and fill with fresh **Disruption Buffer**.

53 Incubate each samples in **Disruption Buffer** (overnight at 100ºC). 9h
Make sure that lid is closed tightly to avoid evaporation.
100 °C

09:00:00

After disruption, the gel should be about 12.5 mm x 6 mm.

Chamber 2 preparation

54 Prepare **Gelation Chamber #2**, one for each sample.

Layer 20 pieces of Scotch tape together to create ~1.2 mm thick spacers.
Cut two strips of spacer material, about 2 cm long and 0.5 cm wide.
Stick two strips to a glass slide ~15 mm apart to form the side walls of the **Gelation Chamber #2**.

Incubation with Gelation Solution 2

1h 30m

55 Wash each samples in 1x PBS at RT (3× 20 min).

1h

Room temperature

01:00:00

56 Thaw **Monomer Solution #2,** as well as 4HT, TEMED and APS. Vortex well and keep on ice.
Monomer Solution #1 and **#2** can be stored safely at -20ºC for a few months.

57 Mix **Monomer Solution #2** and 4HT, TEMED and APS at a ratio of 94:2:2:2 to produce **Gelation Solution #2**.
Vortex.
For reference: Each sample needs ~6 ml of **Gelation Solution #2 (**~2 ml per incubation round)

58 Incubate samples in **Gelation Solution #2** on ice (3× 10 mins @ 4ºC) with 2ml of **Gelation Solution #2** on a shaker in a 12-well plate.

30m

4 °C

00:30:00

Gelation 2

2h 5m

59 With spatula, transfer gel block containing fish into a **Gelation Chamber #2**.
Make sure there are no bubbles at the interface between chamber and gel.
If needed, add a small drop of gelation solution below the gel if air bubbles remain.

Add a small drop of gelation solution on top of the gel to minimize chances of air bubbles forming.
Carefully place coverslip on top (Corning #2855-25)

Reagents

Corning® 25×25 mm Square #2 Cover Glass **Corning Catalog #**2855-25

60 Slowly pipette fresh **Gelation Solution #2** into **Gelation Chamber #2** until full. Avoid any air bubbles on either side.
Solution will hold by water tension.
Wipe off any excess solution with a wipe.
The excess solution will prevent oxygen from reaching the gel too quickly, and avoid the inhibition of polymerization.

Note

Avoiding bubbles is critical. If bubbles appear, the procedure should be repeated (cover slip removed and repositioned) until no bubbles are present. Bubbles contain oxygen, which inhibits polymerization by scavenging the free radicals that drive the chain reaction. Moreover, bubbles can become trapped within the gel and cause distortions and artifacts during imaging. Bubbles further than ~1mm away from the sample are acceptable though should still be minimized.

61 Once **Gelation Chamber #2** is filled, place the chambers at 37 ºC for 2 hr to induce polymerization. 2h
Ensure incubator is humidified.

37 °C

02:00:00

62 Place gels at RT, and allow them to cool on the bench for > 5 min. 5m

Room temperature

00:05:00

63 Take off coverslip lid with a razor blade.
Under a stereomicroscope, remove the scotch side walls and trim the gels into a rectangle, close to the first gel.
Removing any second gel material that formed outside of the first gel.

64 Wash in 1x PBS (2× 10 min).

Staining

4h

65 Incubate gels in Alexa488-NHS and/or Atto647N-maleimide dye at 1:1000 each in 1x PBS (1 hr at RT) in 12-well plate on gentle shaker. DAPI can also be added to label nuclei.

1h

Room temperature

01:00:00

Both Alexa488-NHS and/or Atto647N-maleimide give great contrast and highlight complimentary features of the ultrastructure. Alexa488 is more hydrophilic than Atto647N-maleimide. Total protein dyes give a lot of context to interpret immunohistochemistry results - we recommend starting with Alexa488-NHS.
If staining is not needed, this step can be fully skipped.

Note

Be careful when storing these dyes. A desiccant should be added to container storing the stocks to avoid hydrolysis of the compounds.

Reagents

Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester) **Thermo Fisher Scientific Catalog #**A20000

ATTO 647N maleimide **AAT Bioquest Catalog #**2857

66 Wash in 1x PBS (3× 1 hr) on shaker at RT. Washing thoroughly is critical as unbound dye will increase background. Run the washes with a large excess volume. This is key, as long washes in smaller volumes won't result in

3h

Room temperature

03:00:00

Mounting

67 Attach a 8mm circular coverslips (CS-8R, Warner Instruments, 64-0701) to a Z1 sample holder using superglue.

Reagents

CS-8R Coverslips, 0.15 mm (0.006 in), 8 mm diameter, pkg of 100 **Multi Channel Systems MCS GmbH Catalog** #640701

LIGHTSHEETHOLDER V13 Short.ipt LIGHTSHEETHOLDER V13 Short.stl

68 Coat coverslip with poly-lysine and let it dry.

69 Mount gel, sample side up.

Imaging

70 Image using a Zeiss Z1 Light sheet Microscope. Allow 45 min (with gel in place) for system to equilibrate before acquiring multi-tile acquisitions.
Image with 20× 1.0 NA water immersion objective.
Exposure time: 100-150 ms.

71 After imaging, gels can be teased off holder with a paint brush.

72 For long term storage, keep gels in 1x PBS at 4ºC.

Protocol references

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