

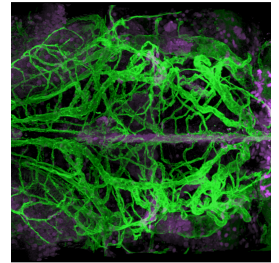
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Version 3

WHOLISTIC ExM: Whole-Body Expansion Microscopy with Immunofluorescence and Histological Stains V.3

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We use this protocol routinely.

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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(foxf1a:eGFP)* x *Tg(flk1:dsRed-CAAX)*), stained against eGFP (magenta) and dsRed (green) (10 days post-fertilization, expanded ~2×).

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Image Attribution

Virginia M. S. Ruetten



Protocol materials

- ✕ Corning® 25×25 mm Square #2 Cover Glass **Corning Catalog #2855-25**
- ✕ Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester) **Thermo Fisher Scientific Catalog #A20000**
- ✕ ATTO 647N maleimide **AAT Bioquest Catalog #2857**
- ✕ 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**
- ✕ Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-5ML**
- ✕ Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2002-100G**
- ✕ Normal Goat Serum **Jackson ImmunoResearch Laboratories, Inc. Catalog #005-000-121**
- ✕ PBS, pH 7.4 **Thermo Fisher Catalog #10010001**
- ✕ Agarose, low gelling temperature **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9414-100G**
- ✕ Acryloyl-X, SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester) **Thermo Fisher Scientific Catalog #A20770**
- ✕ DMSO, Anhydrous **Thermo Fisher Catalog #D12345**
- ✕ Ammonium persulfate (APS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A3678-100G**
- ✕ 4-Hydroxy-TEMPO (4HT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #4-Hydroxy-TEMPO**
- ✕ 2% bis-acrylamide solution **Bio-Rad Laboratories Catalog #1610142**
- ✕ Acrylic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #147230-5G**
- ✕ 40% Acrylamide Solution **Bio-Rad Laboratories Catalog #1610140**
- ✕ Sodium Hydroxide Solution (10N/Certified) **Fisher Scientific Catalog #SS255-1**
- ✕ N,N,N',N'-Tetramethylethylenediamine (TEMED) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T7024-25ML**
- ✕ 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**
- ✕ NaCl (5 M), RNase-free **Thermo Fisher Scientific Catalog #AM9760G**
- ✕ UltraPure™ SDS Solution, 10% **Thermo Fisher Scientific Catalog #15553027**



⊗ 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**

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

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 -80°C. 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 H₂O. Use within the next two days and store in a 4°C fridge.

Note

PFA deteriorates even if stored at -80°C. 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 4°C

🌡 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% H₂O₂, 60mM KOH and 100mM (or more) NaN₃ in PBS.

- Prepare 300mM NaN₃: add 195mg of NaN₃ to 10ml of PBS.

To make 5 ml of bleaching solution:

- 0.3 ml of 50% H₂O₂ solution
- 0.15 ml of 1M KOH solution (takes one hour with little bubbling)
- fill the rest of 100mM NaN₃ (i.e.: 4.25ml, 85mM NaN₃)

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. NaN₃ is a catalase inhibitor. Pre-incubate fish with NaN₃ to block catalase. Then add bleach solution containing NaN₃. The dissociation of H₂O₂ occurs faster at high pH, thus higher KOH should've used.

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 -20°C. Don't re-use AcX after thawing.

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

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 water to the Eppendorf tube.

Add 11 ml of 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.

A yellow precipitate should be observable. Leave it to cool.

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 μ l 10 M NaOH, 250 μ l 10 M NaOH, 120 μ L 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
	Bis	%	1	0.1	100	1
	10x PBS	x	10	1	100	1
	Water				365	3.65

Monomer Solution #1

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

	A	B	C	D	E
	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 #147230-5G**

⊗ 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**

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**

	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



	A	B	C	D	E	F
	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.

⊗ 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 -20°C.

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 in 4% PFA at 4°C on a shaker.



09:00:00



4 °C

9h

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



01:00:00



Room temperature

1h

Bleaching

19 Prepare fresh **Bleaching Solution**

Combine 3% H₂O₂, 0.5% KOH (~90mM) and 50mM (or more) NaN₃ in PBS.



Prepare 100mM NaN₃:

Add 65mg of NaN₃ to 10ml of PBS.

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 NaN₃ (i.e.: 4.25ml) to get to 85mM of NaN₃ 0.5% KOH (~90mM) and 50mM (or more) NaN₃ in PBS.



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).

Incubate the samples in 500 µL of **Bleaching Solution**. Very little bubbling should occur.



Room temperature



Room temperature

21

Rinse the samples twice with 1x PBS.



Room temperature

Immunofluorescence (Optional)

5d 17h 10m

22

Prepare fresh **Blocking Buffer**.

23

Incubate the samples in **Blocking Buffer** (3 hr at RT).



Room temperature



03:00:00

3h

24

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

3d



prevent microbial growth.

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.

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

Room temperature

01:00:00

1h

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

Room temperature

09:00:00

9h

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

48:00:00

Room temperature

2d

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

Room temperature

04:00:00

4h

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

10m



🌡 Room temperature

🕒 00:10:00

- 30 Image one sample to confirm immunofluorescence staining was successful.

Permeabilization (if no IHC)

5h 10m

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

🕒 05:00:00

5h

- 32 Wash samples (2× 5 min at RT) in 1x PBS.

🌡 Room temperature

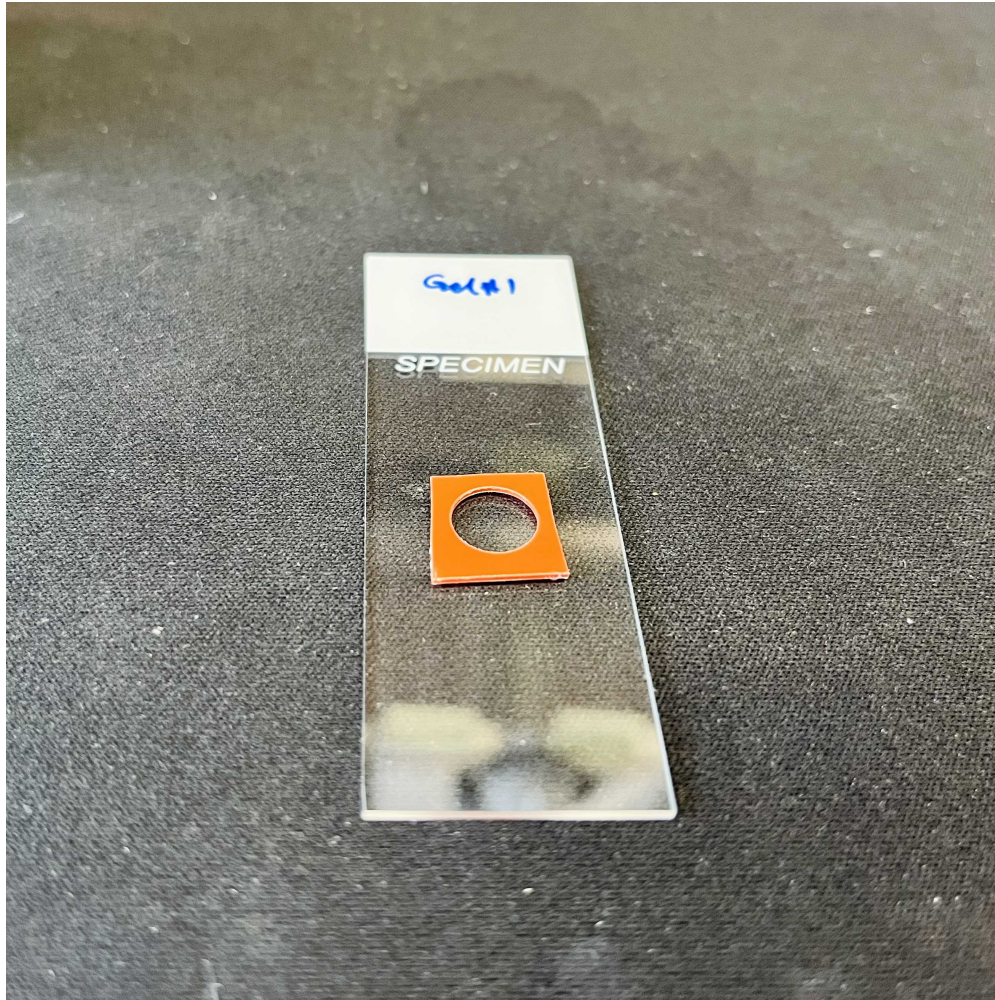
🕒 00:10:00

10m

Agarose Embedding

- 33 Heat agarose in microwave until it liquifies.
Cool agarose to 50°C (by placing in 50°C incubator).

- 34 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

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

Additional notes:

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.

- 36 With a razor blade, cut out a rectangle around the agarose-embedded sample and delicately lift the sample 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.

Protein Anchoring

1h

- 37 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.
- 38 Incubate each sample in 1 ml of **Acryloyl-X Solution** (1 hr at RT) shaking in 12-well plate.

🔥 Room temperature

🕒 01:00:00

1h

Preparation of Gelation Chamber #1

- 39 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

- 40 Rinse samples with 1x PBS (3× 5 min).

 00:15:00

15m

- 41 Thaw **Monomer Solution #1**, as well as 4HT, TEMED and APS. Vortex well and keep on ice.

- 42 Mix **Monomer Solution #1** and 4HT, TEMED and APS at a ratio of 94:2:2:2 to produce **Gelation Solution #1**.

Vortex.

Each sample needs ~3 ml of **Gelation Solution #1**.

~1 ml per incubation round.

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

 00:30:00

30m



Gelation 1

2h

- 44 With spatula, transfer agarose block containing fish into the **Gelation Chamber #1**.
- 45 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.
- 46 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).
- 47 Once **Gelation Chamber #1** is filled, place the chambers at 37°C for 2 hr to induce polymerization.
Ensure incubator is humidified.

37 °C

02:00:00

2h

Disruption

9h 5m

- 48 Turn on the 100°C heat block (for later disruption step).
Prepare **Disruption Buffer** (5 ml/sample).
- Stock **Disruption Buffer** can be prepared in advanced and stored at RT (e.g.: 50-100 ml stock).
- 49 Place gels at RT, and allow them to cool on the bench for > 5 min.
- Room temperature
- 00:05:00
- 50 Take off coverslip lid with a razor blade.
Under a stereomicroscope, trim the gels into a rectangle with a ~2-3 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.
- 51 Transfer each gel to a 2 ml Eppendorf tube and add 1.8 ml of **Disruption Buffer**.

5m



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

🔥 100 °C

🕒 09:00:00

9h

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

Chamber 2 preparation

- 53 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

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

🔥 Room temperature

🕒 01:00:00

1h

- 55 Thaw **Monomer Solution #2**, as well as 4HT, TEMED and APS. Vortex well and keep on ice.

- 56 Mix **Monomer Solution #2** and 4HT, TEMED and APS at a ratio of 94:2:2:2 to produce **Gelation Solution #2**.

Vortex.

Each sample needs ~6 ml of **Gelation Solution #2**.

~2 ml per incubation round.

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

🔥 4 °C

🕒 00:30:00

30m

Gelation 2

2h 5m

- 58 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**

- 59 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.

- 60 Once **Gelation Chamber #2** is filled, place the chambers at 37 °C for 2 hr to induce polymerization.
Ensure incubator is humidified.

 37 °C

 02:00:00

2h

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

 Room temperature

 00:05:00

5m

- 62 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.

- 63 Transfer gel to a 12-well plate with 1x PBS.

Transfer each gel to a 5 ml Eppendorf tube and fill with **Disruption Buffer**.
Incubate overnight at 95°C.
Make sure that lid is closed tightly to avoid evaporation.

 100 °C



09:00:00

Staining

2h 30m

- 64 Incubate gels in Alexa488-NHS and Atto647N-maleimide dye at 1:1000 each in 1x PBS (1 hr at RT) in 12-well plate on gentle shaker.

1h

Room temperature

01:00:00

Reagents

 Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester) **Thermo Fisher Scientific Catalog #A20000** ATTO 647N maleimide **AAT Bioquest Catalog #2857**

- 65 Wash in 1x PBS (3× 1 hr) on shaker at RT.

1h 30m

Room temperature

01:30:00

Mounting

- 66 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

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

- 68 Mount gel, sample side up.

Imaging

- 69 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.
- 70 After imaging, gels can be teased off holder with a paint brush.
- 71 For long term storage, keep gels in 1x PBS at 4°C.

Protocol references

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