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WHOLISTIC ExM: Whole-Body Expansion Microscopy with Fluorescence Insitu Hybridization (WB-ExM FISH)

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Abstract

Whole-Body Expansion Microscopy with Fluorescence In-situ Hybridization for young zebrafish.

The interpretation of WHOLISTIC 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.

Protocol materials

- PBS, pH 7.4 **Thermo Fisher** **Catalog #**10010001
- Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**X100-5ML
- 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
- Acryloyl-X, SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester) **Thermo Fisher Scientific** **Catalog #**A20770
- Melphalan **Cayman Chemical Company** **Catalog #**16665
- 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
- Corning® 25×25 mm Square #2 Cover Glass **Corning** **Catalog #**2855-25
- Melphalan **Cayman Chemical Company** **Catalog #**16665
- MOPS (Fine White Crystals/Molecular Biology), Fisher BioReagents™ **Thermo Fisher Scientific** **Catalog #**BP308100
- RNase-Free DNase Set **Qiagen** **Catalog #**79254
- Poly-L-lysine hydrobromide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**Poly-L-lysine hydrobromide
- Photo Flo 200 Solution **Electron Microscopy Sciences** **Catalog #**74257
- SSC (20X), RNase-free **Thermo Scientific** **Catalog #**AM9763
- NaCl (5 M), RNase-free **Thermo Fisher Scientific** **Catalog #**AM9760G
- Proteinase K, Molecular Biology Grade **New England Biolabs** **Catalog #**P8107S
- EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Scientific** **Catalog #**AM9260G
- UltraPure™ SDS Solution, 10% **Thermo Fisher Scientific** **Catalog #**15553027
- 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
- Ammonium persulfate (APS) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A3678-100G
- N,N,N′,N′-Tetramethylethylenediamine (TEMED) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7024-25ML
- Sodium Hydroxide Solution (10N/Certified) **Fisher Scientific** **Catalog #**SS255-1

- 4-Hydroxy-TEMPO (4HT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4-Hydroxy-TEMPO
- 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
- CS-8R Coverslips, 0.15 mm (0.006 in), 8 mm diameter, pkg of 100 **Multi Channel Systems MCS GmbH Catalog #**640701

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

2 **PBST-0.5**

Can be prepared in advance.
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

3 **MOPS Buffer**

Stock concentration: 200 mM (10x)
Dissolve 1046.5 mg in 25 ml in nuclease-free water, pH to 7.7 with **10N** NaOH.

Store at -20ºC.

MOPS (Fine White Crystals/Molecular Biology), Fisher BioReagents™ **Thermo Fisher Scientific Catalog #**BP308100

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

4 **Melphalan stock**
Stock concentration: 2.5 mg/ml
Dissolve Melphalan (2.5 mg per ml) in anhydrous DMSO. To dissolve, heat to 37ºC and vortex vigorously and place on a shaker. This may take an hour to dissolve. Aliquot in 800 µl batches. Store in a desiccated environment at -20ºC.

Melphalan **Cayman Chemical Company Catalog #**16665

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

5 **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

Reagents

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

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

6 **Melphalan-X Solution**
Stock concentration: 2 mg/ml
Combine an equal concentration of **Acryloyl-X** (10 mg/ml) and **Melphalan** (2.5 mg/ml) (1-part AcX to 4-parts **Melphalan** (i.e., 200 µl: 800 µl).
Incubate overnight at RT with shaking.
Store in 50 µl aliquots in a desiccated environment at -20ºC.
Use at 1 mg/ml by 1:1 dilution in 20 mM **MOPS Buffer**.

Melphalan **Cayman Chemical Company Catalog #**16665

MOPS (Fine White Crystals/Molecular Biology), Fisher BioReagents™ **Thermo Fisher Scientific Catalog #**BP308100

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

	A	B	C	D	E	F
					to make 1ml	to make 10ml
	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
					to make 1ml
	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 #**147230-5G

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

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

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

8 **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

	A	B	C	D	E
	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
- 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
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

9 **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

10 **SSCT**

0.5x SSC, 0.1% Tween

SSC (20X), RNase-free **Thermo Scientific Catalog #**AM9763

11 **Disruption Buffer #1:**
500 mM NaCl, 0.3% SDS, 50 mM Tris-HCL pH8 .0, 1 mM EDTA with Proteinase K diluted 1:50 from 800 U/ml stock

Disruption Buffer #2:
50 mM NaCl, 1% SDS, 50 mM Tris-HCL pH 8.0, 1 mM EDTA with Proteinase K diluted 1:50 from 800 U/ml stock

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

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

EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Scientific Catalog #**AM9260G

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

12 **Gelation Chambers**
Silicone gaskets (Invitrogen P24743)
Glass slides (SuperFrost 12550123)
Scotch tape
Poly-l-lysine
Shaker (nutator - 75rpm)

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

Fixation

2h

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

14 Prepare fresh 4% PFA.

15 Place samples in 1 ml of 4% PFA.
Room temperature

16 Keep overnight in 4% PFA at 4ºC on a shaker. 1h
Overnight
4 °C

17 Rinse samples in 4 × 15 min 1 ml 1x PBS at 4ºC. 1h
This should be done in a cold room in order to minimize RNA degradation.
01:00:00

RNA Anchoring 50m

18 Place each fish in separate PCR tube and add 150 µl of 20 mM **MOPS Buffer**.

19 Incubate samples 1× 30mins. 30m
00:30:00

20 Thaw **Melphalan-X** and **Acryloyl-X** solutions during incubation.

21 Dilute **Melphalan-X** 1:1 with **MOPS Buffer**.
Add **Acryloyl-X** 1:100 to **Melphalan-X/MOPS Buffer**.
(100 µl needed per sample)

22 Remove as much of **MOPS Buffer** from the PCR tube as possible.
Add 100 µl **Melphalan-X/MOPS Buffer/Acryloyl-X** solution to each PCR tube.

23 Incubate overnight at 37ºC.
37 °C
Overnight

24 Transfer fish to 24-well plate, one per well. 10m
Wash samples 2× 5 min of 1x PBS (600 µl/sample).
00:10:00

Permeabilization 1h

25 Permeabilize for 1 h in **PBST-0.5** at RT in 500 ul.

01:00:00 1h

Preparation of Gelation Chamber #1

26 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**.

Incubation with Gelation Solution 1 45m

27 Rinse samples with 1x PBS (3× 5 min). 15m
00:15:00

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

29 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 ~1 ml of **Gelation Solution #1**.

30 Remove as much PBS from each sample well.

31 Incubate samples in **Gelation Solution #1** on ice (3× 10 min at 4ºC) with 400 µl of gelation solution on a shaker in a 24-well plate. 30m
00:30:00

Gelation 1 2h

32 With spatula, transfer fish into the **Gelation Chamber #1**.

33 Gently place cover slip over the fish lying on the walls of **Gelation Chamber #1** (made of scotch tape).

34 Slowly pipette ~200 µl of **Gelation Solution #1** into **Gelation Chamber #1** until full. Avoid any air bubbles.
Solution will hold by water tension.

35 Once **Gelation Chamber #1** is filled, place the chambers at 37ºC for 2 h to induce polymerization. 2h
Ensure incubator is humidified.
37 °C
02:00:00

Disruption 4h 5m

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

37 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.

38 Transfer each gel to a 2 ml Eppendorf tube and add 750 µl of **Disruption Buffer #1**.

Disruption Buffer #1:
500 mM NaCl, 0.3% SDS, 50 mM Tris-HCL pH8 .0, 1 mM EDTA with Proteinase K diluted 1:50 from 800 U/mL stock

39 Incubate samples (50ºC for 4 hr). 4h
50 °C
04:00:00

40 Remove liquid from Eppendorf tube.
Add 1ml of **Disruption Buffer #2** to Eppendorf tube**.**

Disruption Buffer #2:
50 mM NaCl, 1% SDS, 50 mM Tris-HCL pH 8.0, 1 mM EDTA with Proteinase K diluted 1:50 from 800 U/mL stock

41 Incubate each samples at 50ºC overnight.
50 °C
Overnight

Chamber 2 preparation

42 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

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

Room temperature

01:00:00

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

45 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.

46 Incubate samples in **Gelation Solution #2** on ice (3× 10 mins @ 4ºC) with 2ml of **Gelation Solution #2** on a shaker in 12-well plate. 30m

4 °C

00:30:00

Gelation 2

2h 5m

47 With spatula, transfer gel block containing fish into a **Gelation Chamber #2**.
Make sure there are no bubbles.
Add small drop of gelation chamber.
Carefully place coverslip on top (Corning #2855-25)

Reagents

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

48 Slowly pipette **Gelation Solution #2** into **Gelation Chamber #2** until full. Avoid any air bubbles.
Solution will hold by water tension.
Cover.

49 Once **Gelation Chamber #2** is filled, place the chambers at 37ºC for 2 hr to induce polymerization. 2h
Ensure incubator is humidified.
37 °C
02:00:00

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, 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.

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

Second Disruption 4h

53 Transfer each gel to a 2 ml Eppendorf tube and add 750 µl of **Disruption Buffer #1**.

Disruption Buffer #1:
500 mM NaCl, 0.3% SDS, 50 mM Tris-HCL pH8 .0, 1 mM EDTA with Proteinase K diluted 1:50 from 800 U/mL stock

54 Incubate samples (50ºC for 4 hr). 4h
50 °C
04:00:00

55 Remove liquid from Eppendorf tube.
Add 1ml of **Disruption Buffer #2** to Eppendorf tube**.**

Disruption Buffer #2:

50 mM NaCl, 1% SDS, 50 mM Tris-HCL pH 8.0, 1 mM EDTA with Proteinase K diluted 1:50 from 800 U/mL stock

56 Incubate each samples at 50ºC overnight.
50 °C
Overnight

Hybridization with Probes

30m

57 Wash samples 4× 15 min with 1 ml 1x PBS.

58 Thaw and mix **Hybridization Buffer** and **Probe Wash Buffer**.

59 Incubate each gel in 600 µl **Hybridization Buffer** for 30 min at 37ºC.
30m
37 °C
00:30:00

60 Dilute **HCR probes** 6 µl in 600 µl **Hybridization Buffer** per gel (10 nM final concentration). Vortex.

61 Incubate gel with **HCR probes** overnight at 37ºC. No shaking is necessary.
37 °C
Overnight

62 Place **Probe Wash Buffer** and 1x PBS at 37ºC in preparation for washing the next day.

Probe Wash

1h 30m

63 Wash gels in pre-warmed (37ºC) **Probe Wash Buffer** for 3× 30min.
1h 30m
Keep in 37ºC incubator.
01:30:00

64 Wash gels in pre-warmed (37ºC) 1x PBS for 3× 30 min, then 1x PBS for 3× 60 min.
Keep in 37ºC incubator.

Wash gels in 1x PBS overnight at RT.
Overnight

Hybridization Chain Reaction

5h 20m

65 Incubate each gel in 1 ml **Amplification Buffer** for at least 30 min at RT.

66 Snap cool hairpins with PCR machine at 95ºC for 90 sec and cool to 25ºC for 30 min.

67 For each fluor mix, add hairpins h1 and h2 at 1:50 in 400 µl **Amplification Buffer** (1x per gel).
Vortex.

68 Incubate each gel with hairpins for 4 hr at RT in the dark.
04:00:00

4h

69 Wash gels 2 × 20 min in 1 ml 5x SSCT at RT.
00:40:00

40m

SSCT: 5x SSC, 0.1% Tween

SSC (20X), RNase-free **Thermo Scientific Catalog #**AM9763

70 Wash gels 2 × 40 min in 1 ml 0.5x SSCT at RT.
00:40:00

40m

71 Place in 1x PBS and leave to equilibrate at least 1 h before imaging.

Mounting

72 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

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

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

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

74 Mount gel, sample side up.

Imaging

75 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.

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

DNAse treatment for Stripping Hairpins

77 Incubate gel for 30 min in 1 ml of **DNase1 Buffer** at 37ºC

RNase-Free DNase Set **Qiagen Catalog #**79254

78 Add 450 µl of **DNase Buffer** to 50 ml DNase1. Mix. (increase to 750 µl if gel is not fully covered).

RNase-Free DNase Set **Qiagen Catalog #**79254

79 Incubate gel in DNase1 for 2 hr at 37ºC.

RNase-Free DNase Set **Qiagen Catalog #**79254

80 Wash 4 × 15 min with 1 ml 1x PBS.

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