

Nov 29, 2021

Version 2

Targeted ExSeq -- Tissue Preparation V.2

 In 2 collections

DOI

dx.doi.org/10.17504/protocols.io.b2emqbc6

Anubhav Sinha^{1,2,3,4}, Yi Cui⁵, Shahar Alon^{1,2,6,4}, Asmamaw T. Wassie^{1,2,7,4}, Fei Chen^{1,8,4}, Ed Boyden^{9,2,7,10,11,12,4}

¹Equal Contribution; ²McGovern Institute, MIT; ³Harvard-MIT Program in Health Sciences and Technology;

⁴Human Tumor Atlas Pilot Project; ⁵Massachusetts Institute of Technology;

⁶Faculty of Engineering, Gonda Brain Research Center and Institute of Nanotechnology, Bar-Ilan University;

⁷Department of Biological Engineering, MIT; ⁸Broad Institute of MIT and Harvard;

⁹Department of Media Arts and Sciences, MIT; ¹⁰Koch Institute for Integrative Cancer Research, MIT;

¹¹Howard Hughes Medical Institute; ¹²Department of Brain and Cognitive Sciences

NCIHTAN



Anubhav Sinha

MIT

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.b2emqbc6>



External link: <https://pubmed.ncbi.nlm.nih.gov/33509999/>

Protocol Citation: Anubhav Sinha, Yi Cui, Shahar Alon, Asmamaw T. Wassie, Fei Chen, Ed Boyden 2021. Targeted ExSeq -- Tissue Preparation. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.b2emqbc6> Version created by **Anubhav Sinha**

Manuscript citation:

Alon S*, Goodwin DR*, Sinha A*, Wassie AT*, Chen F*, Daugharthy ER**, Bando Y, Kajita A, Xue AG, Marrett K, Prior R, Cui Y, Payne AC, Yao CC, Suk HJ, Wang R, Yu CJ, Tillberg P, Reginato P, Pak N, Liu S, Punthambaker S, Iyer EPR, Kohman RE, Miller JA, Lein ES, Lako A, Cullen N, Rodig S, Helvie K, Abravanel DL, Wagle N, Johnson BE, Klughammer J, Slyper M, Waldman J, Jané-Valbuena J, Rozenblatt-Rosen O, Regev A; IMAXT Consortium, Church GM****, Marblestone AH****, Boyden ES****+ (2021) Expansion Sequencing: Spatially Precise In Situ Transcriptomics in Intact Biological Systems, Science 371(6528):eaax2656. (* equal contribution, ** key contributions to early stages of project, *** equal contribution, +co-corresponding authors)

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

Created: November 29, 2021

Last Modified: November 29, 2021

Protocol Integer ID: 55469

Keywords: expansion microscopy, in situ sequencing, expansion sequencing, targeted ExSeq, ExSeq, spatial transcriptomics, spatial omics, spatially resolved transcriptomics, tissue preparation for targeted exseq, possible tissue preparation route, specific tissue collection protocol, targeted exseq, tissue preparation, transcatheter pfa perfusion path, freezing fresh tissue, tissue from mice, exseq paper, harvesting tissue, harvesting tissue from model organism, tissue section, harvested tissue, tissue for this work, cryoprotection, fixed tissue section, mounted tissue section, exm gel, fresh tissue, exseq, tissue, tumor biopsy, cryosectioning, mouse brain section, human tumor biopsy sample, perfused with pfa, such as tumor biopsy, treatment with labelx, tissue type, robust digestion option in step

Disclaimer

This protocol is shared under the **HTAN Internal Data and Materials Sharing Agreement** and is provided as is. See section 14 of full agreement for full details.

This protocol is shared as an Open Access protocol (under HTAN's definitions). This protocol is Subject to IP Restrictions.

Abstract

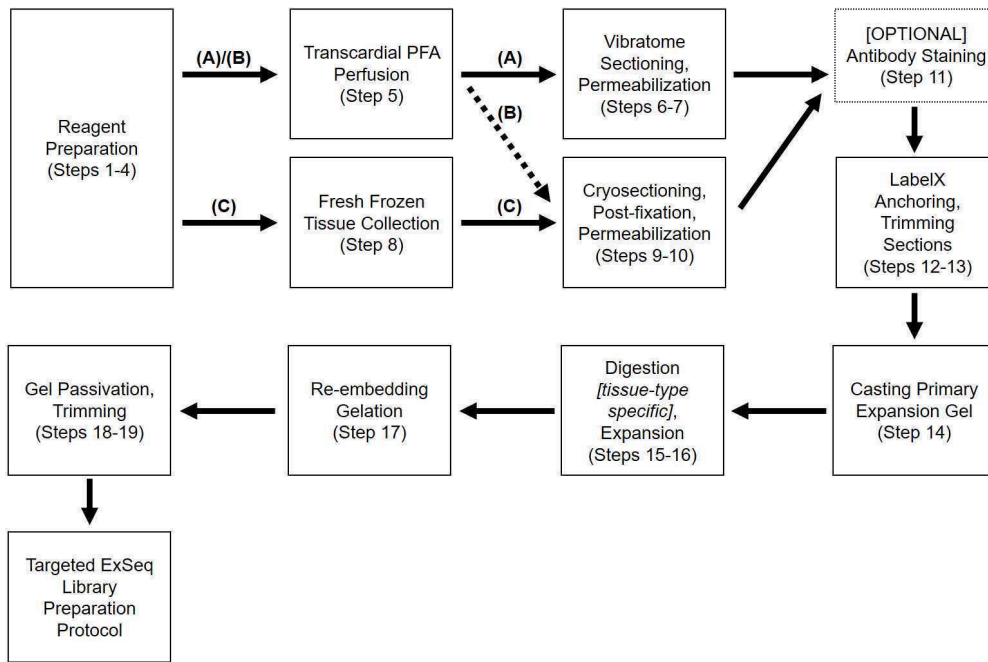


Figure 1. Flowchart of targeted ExSeq tissue preparation workflow.

This protocol accompanies **Expansion Sequencing (ExSeq)**, and describes the tissue preparation for Targeted ExSeq. The steps described here are a generalization of the protocols used for figures 4-6 of the paper, and represent our recommendations for future users of the technology. **Fig. 1** shows the structure of the protocol schematically.

There are three possible tissue preparation routes described in this protocol that are applicable to different experimental systems. Option (A): harvesting tissue from model organisms that can be transcardially perfused with PFA, followed by sectioning using a vibratome. We typically use this workflow for work on mouse brain sections (see figures 4-5 of ExSeq paper). Option (B): transcardially perfusing with PFA, followed by cryoprotection and cryosectioning. We occasionally use this protocol for work on mouse brain sections. Option (C): snap-freezing fresh tissue (i.e., human tumor biopsy samples, or freshly harvested tissue from mice), followed by cryoprotection and cryosectioning (see figures 2 and 6 of ExSeq paper).

The final result of options (A), (B), and (C) is the preparation of fixed tissue sections (either on a glass slide or free-floating). The protocols then briefly converge for optional antibody staining, treatment with LabelX, a chemical that enables anchoring of RNA to the expansion microscopy (ExM) hydrogel, followed by casting of the the ExM gel. There are minor differences in these steps between free-floating and slide-mounted tissue sections, which are noted in the individual steps. The next step, digestion, is tissue-type dependent and may require some optimization for your tissue type. We provide two potential options here: (1) a gentle digestion for tissues such as mouse brain, and (2) a harsh digestion for non-brain tissues such as tumor biopsies. The protocols then converge again for the rest of the process.

After digestion, the gels are expanded and re-embedded within a second non-expanding hydrogel to lock in the sample size. The carboxylates within the expansion gel are then chemically passivated, enabling enzymatic reactions to be performed within the gel. The samples are now ready for library preparation.

In more detail:

Steps 1-4 describe the preparation of reagents for downstream steps. The protocol begins either along options (A)/(B), the Transcardial PFA perfusion path (Step 5, continuing to vibratome sectioning in Steps 6-7 for option (A), or cryotome sectioning in Steps 9-10 for option (B)), or along option (C), the Fresh Frozen path (Step 8, continuing to cryotome sectioning in Steps 9-10).

The protocols then converge for optional antibody staining (Step 11), followed by LabelX anchoring (Step 12), optional sample trimming (Step 13), and formation of the expansion microscopy gel (Step 14). The details of the digestion step are tissue-type dependent (Step 15). The protocol then concludes with expansion (Step 16), re-embedding (Step 17), passivation, and optional trimming (Steps 18-19).

This protocol was used to profile human metastatic breast cancer biopsies as a part of the Human Tumor Atlas Pilot Project (HTAPP). The tissue for this work was collected (see HTAPP-specific tissue collection protocol). The tissue sections were then frozen, cryosectioned, post-fixed, and permeabilized (following steps 9-10). No antibody staining was performed (skipping optional step 11). The sections were then treated with LabelX and gelled (steps 12-14). The gels were then digested using the robust digestion option in steps 15-16. The samples were then re-embedded, passivated, and trimmed (following steps 17-19).

Image Attribution

All images (c) Massachusetts Institute of Technology.

Guidelines

This protocol significantly builds upon the tissue preparation from Expansion FISH ([Chen*, Wassie*, et al. \(2016\)](#)). A highly detailed Expansion FISH protocol (along with other expansion microscopy protocols) with detailed debugging advice has been published ([Asano*, Gao*, Wassie* et al. \(2018\)](#)). **Prospective users are very strongly encouraged to review that protocol, as it overlaps significantly with steps 5-16 of this protocol.**

All reagents used should be RNase-free. We have provided specific product numbers in the materials section. Of particular note, "water" always refers to UltraPure Water (nuclease-free).

Because the volumes used for each of the steps will vary dramatically on the size of the containers used, the majority of the volumes for solutions have been standardized to 1 mL for ease of scaling. Similarly, wash volumes will also vary significantly depending on the size of the container, and are not listed explicitly. In general, wash volumes should be significantly larger than the size of the sample being washed. As a few examples: for 1-2 free floating sections in an Eppendorf tube, the wash volume would be 500 to 1000 μ L; for sections on a slide in a well (as described in the protocol), the wash volume would be \sim 200 μ L; for gels in a 4-well plate, the wash volume would be 5 mL.



Materials

MATERIALS

- ☒ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**
- ☒ Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787-50ML**
- ☒ Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #P8107S**
- ☒ Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences Catalog #15710**
- ☒ Acrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9099**
- ☒ N,N'-Methylenebisacrylamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M7279**
- ☒ N,N,N',N'-Tetramethylethylenediamine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T7024**
- ☒ 4-Hydroxy-TEMPO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #176141**
- ☒ Tissue-prep Buffered 10% Formalin **Electron Microscopy Sciences Catalog #15742-10**
- ☒ Glycine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #50046**
- ☒ Label-IT® Amine **Mirus Bio Catalog #MIR 3900**
- ☒ Tissue-Tek® O.C.T. Compound, Sakura® Finetek **VWR International (Avantor) Catalog #25608-930**
- ☒ Ultra pure BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #cat# AM2616**
- ☒ 200 Proof Ethanol pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E7023**
- ☒ NaCl (5 M) RNase-free **Thermo Fisher Scientific Catalog #AM9759**
- ☒ DAPI **Thermo Fisher Scientific Catalog #62248**
- ☒ Ammonium persulfate (APS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A3678**
- ☒ UltraPure™ 1M Tris-HCl pH 8.0 **Thermo Fisher Scientific Catalog #15568025**
- ☒ NHS (N-hydroxysuccinimide) **Thermo Fisher Catalog #24500**
- ☒ Acryloyl-X, SE, 6-((acryloyl)amino)hexanoic Acid, Succinimidyl Ester **Thermo Fisher Catalog #A20770**
- ☒ Acrylamide/Bis 19:1, 40% (w/v) solution **Thermo Fisher Catalog #AM9022**
- ☒ DMSO, Anhydrous **Thermo Fisher Catalog #D12345**
- ☒ UltraPure™ 0.5 M EDTA pH 8.0 **Thermo Fisher Scientific Catalog #15575020**
- ☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermofisher Catalog #10977023**
- ☒ Hydrophobic Barrier PAP Pen ImmEdge **Vector Laboratories Catalog #H-4000**
- ☒ Frame-Seal in situ PCR and Hybridization Slide Chambers 15 × 15 mm **Bio-Rad Laboratories Catalog #SLF0601**
- ☒ MOPS **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3183-25G**
- ☒ Ethanolamine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E6133-100G**



- ⊗ MES 0.5M buffer soln. pH 6.5 **Alfa Aesar Catalog #J63778**
- ⊗ Sodium borate 0.5M buffer soln. pH 8.5 **Alfa Aesar Catalog #J62902**
- ⊗ Sucrose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #84097-250G**
- ⊗ Cell Culture Plate 24-well **greiner bio-one Catalog #662160**
- ⊗ 2-Methylbutane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3263**
- ⊗ VWR® Superfrost® Plus Micro Slide Premium **VWR International (Avantor) Catalog #48311-703**
- ⊗ 22 × 22 mm Thickness #0 Coverslips **Electron Microscopy Sciences Catalog #72198-10**
- ⊗ 24 × 50 mm Thickness #2 Coverslips **VWR International (Avantor) Catalog #48382-136**
- ⊗ Tris Base **Merck MilliporeSigma (Sigma-Aldrich) Catalog #648310**
- ⊗ 22 × 22 mm Thickness #1.5 Coverslips **VWR International (Avantor) Catalog #48366-227**
- ⊗ N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC HCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #03450-5G**
- ⊗ Cell Culture Plate 6-well **greiner bio-one Catalog #657160**
- ⊗ Sodium Hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5881**
- ⊗ Drierite **VWR International (Avantor) Catalog #EM-DX2515-1**
- ⊗ Sodium Acrylate **Santa Cruz Biotechnology Catalog #sc-236893**
- ⊗ 8M Guanidine-HCl Solution **Thermo Fisher Catalog #24115**

Previously tested for antibody staining (optional):

- ⊗ Invitrogen GFP Polyclonal Antibody **Thermo Fisher Scientific Catalog #A-11122**
- ⊗ Invitrogen Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody Biotin-XX **Thermo Fisher Scientific Catalog #B-2770**

Troubleshooting

Safety warnings

- ⚠ Please carefully read all safety datasheets for all reagents used in the protocol, and perform all steps in accordance with relevant guidelines.

For steps involving animal work or human subjects, follow all institutional requirements from the relevant IACUC or IRB.

Before start

Before starting, prepare stock solutions/reagents as described in the section Preparation of Stock Solutions (Steps 1-4).


Preparation of Stock Solutions

5m

1 Common Laboratory Stocks

5m

All solutions prepared using nuclease-free reagents, and stored at

 Room temperature .

1. 1X PBS
2. PBST (1X PBS with 0.1% Triton X-100 (v/v))
3. 70% Ethanol (v/v) in water

2 Preparation of Reagents for LabelX RNA Anchoring

1d

LabelX reacts with guanines of nucleic acids to install gel-anchorable moieties that can be co-polymerized with the expansion microscopy (ExM) gel. For further technical details, see [Chen*, Wassie* et al., 2016](#) (see Fig. 1A).

Here, we prepare the MOPS buffer (2.1) (used in the anchoring step), AcX (2.2) and LabelIT Amine (2.3) for the synthesis of LabelX, and describe the synthesis itself.

Note that the Mirus LabelIT Amine kit should be stored at -20C. The spin columns included with the kit are not used.

2.1 200 mM MOPS, pH 7.7

5m

10X Buffer A from the Mirus LabelIT Amine kit is 200 mM MOPS, pH 7.7.

Note: only prepare additional MOPS buffer if there is not enough in the kit.

To prepare 200 mM MOPS, pH 7.7, start by dissolving the appropriate amount of MOPS powder in water.

Add small amount of 5 M NaOH solution slowly until the pH reaches pH 7.7 (periodically sampling solution by pipetting a small amount onto narrow-range pH paper strips or using a pH meter).

Buffer can be stored at  Room temperature .

If preparing MOPS from powder, the suggested volume of MOPS buffer to prepare is ~10 mL.



2.2 10 mg/mL Acryloyl-X (AcX) Stock

5m

Upon receipt, 5 mg AcX powder stock should be stored in a parafilm-sealed 50 mL Falcon tube containing Drierite at -20 °C .

To prepare 10 mg/mL AcX stock, remove from freezer and allow to come to room temperature to avoid condensation. Add 500 μ L anhydrous DMSO and vortex to dissolve AcX.

Aliquot into PCR strips (~20–25 μ L/tube) and seal.

Store PCR strips inside a 50 mL Falcon tube containing Drierite. Seal tube with parafilm and store at -20 °C .

Aliquots should be used within 1 month of preparation.

2.3 Synthesis of LabelX

1d

Resuspend 100 μ g of LabelIT Amine (1 tube) in 100 μ L of Reconstitution Buffer (part of LabelIT kit), forming 1 mg/mL LabelIT Amine stock solution.

Add 10 μ L 10 mg/mL AcX stock to tube, and shake vigorously Overnight at Room temperature .

Seal with parafilm and store tube at -20 °C .

3 Preparation of Stock Solutions for Primary (Expansion) Gel

These steps describe the preparation of solutions needed for casting the ExM gel. A brief overview of the gelation process is provided in step 14.

There are four gelling reagents prepared here: (1) the monomer mix, referred to as StockX; (2) 0.5% 4-Hydroxy-TEMPO (4HT), a free radical scavenger; (3) 10% tetramethylethylenediamine (TEMED), the free radical polymerization accelerator; (4) 10% ammonium persulfate (APS), the free radical polymerization initiator.

The preparation of the 10X digestion buffer used for Proteinase-K digestion is also described here.

3.1 StockX: Gelation Monomer Solution

1h


Prepare the following three stock solutions:

1. 38% (w/v) Sodium Acrylate
2. 50% (w/v) Acrylamide
3. 2% (w/v) N,N'-Methylenebisacrylamide ("Bis")

Stock solutions should be sealed with parafilm and stored at  -20 °C .

Mix stock solutions as in table below to prepare StockX.

A	B	C	D
	Stock solution concentration	Amount	Final concentration
Sodium acrylate	38 g/100 mL (equivalent to 33% (w/w))	2.25 mL	8.6 g/100 mL
Acrylamide	50 g/100 mL	0.5 mL	2.5 g/100 mL
N,N'-methylenebisacrylamide	2 g/100 mL	0.75 mL	0.15 g/100 mL
Sodium chloride	5 M	4 mL	11.7 g/100 mL
PBS	10X	1 mL	1X
Water		0.9 mL	
Total		9.4 mL	


Divide StockX into 0.5 mL aliquots and store at  -20 °C for up to two months.

NOTE: 2% (w/v) Bis can be difficult to solubilize. A 1% Bis stock can be used if you double the volume of the Bis solution added and appropriately decrease the amount of water added.

NOTE: low-purity sodium acrylate may appear slightly yellow when dissolved in water. If this is the case, discard the solution and switch to a new bottle of sodium acrylate. Note that a number of sodium acrylate vendors, including MilliporeSigma, have had quality control issues since May 2019, leading to the inability to form gels in our hands. We currently recommend sodium acrylate from Santa Cruz Biotechnology.

3.2 0.5% 4HT

10m

Prepare a 5% 4HT (w/v) stock. Store at  -20 °C for up to two months.




Prepare a working stock of 0.5% 4HT (w/v) from the 5% 4HT stock by diluting in water. The working stock can be stored at  -20 °C for up to a month.

To limit freeze-thaw cycles for 4HT (as well as for TEMED and APS), the suggested amount of working stock is ~200 µL.

3.3 10% TEMED

5m

Prepare a 10% TEMED (w/v) solution in water. Store at  -20 °C for up to one month.


The suggested amount of 10% TEMED to prepare is ~200 µL.

Note: TEMED is toxic and corrosive. 100% stock should only be handled in the fume hood.

Note: we have found that a 10% TEMED (v/v) solution in water works essentially the same as 10% TEMED (w/v), and this is what we typically use.

3.4 10% APS

5m

Prepare a 10% APS (w/v) solution in water. Store at  -20 °C for up to two weeks.

The suggested amount of 10% APS to prepare is ~200 µL.

Note: APS is toxic and reactive. The powder should only be handled in the fume hood.

Note: a stock 10% APS (w/v) solution can be used for up to two weeks for the primary (expansion) gelation, but for the re-embedding gels, 10% APS (w/v) must be freshly prepared.

3.5 10X Digestion Buffer

5m

Prepare 10X Digestion Buffer by combining the following solutions:

	A	B	C	D
	Reagent	Stock Solution Concentration	Amount	Final Concentration
	Tris pH 8.0	1 M	5 mL	500 mM



	A	B	C	D
	EDTA	0.5 M	0.2 mL	10 mM
	Triton X-100	100%	0.5 mL	5%
	Water	n/a	Add up to total volume of 10 mL	
	Total		10 mL	

Note that this does not include NaCl or Guanidine HCl; the appropriate salt is added at the time of preparation of the 1X buffer.

The 10X Digestion Buffer can be stored for up to two months at  Room temperature .

4 Preparation of Stock Solutions for Passivation


Passivation is the chemical process performed after re-embedding in which the carboxylic acid functional groups in the expansion gel are converted to amides. A more detailed description is provided in step 18.

Three reagents are prepared here: (1) ethanolamine hydrochloride, the primary amine used in the amide-bond formation; (2) MES buffer, the acidic buffer used for EDC-NHS activation of the carboxylic acid; and (3) sodium borate buffer.

4.1 4 M Ethanolamine-HCl

5m

Prepare 4 M Ethanolamine-HCl. This can be stored for up to one month at

 Room temperature .

The suggested volume to prepare is ~20 mL, which should be sufficient for 3-4 samples (i.e. whole-brain sections).

4.2 200 mM MES Buffer, pH 6.5

5m

Prepare 200 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer at pH 6.5 from 0.5 M stock.

The suggested volume to prepare is ~20 mL, which should be sufficient for 7-8 samples.

This can be stored for several months at  Room temperature .



4.3 125 mM Sodium Borate Buffer, pH 8.5

5m

Prepare 125 mM sodium borate buffer at pH 8.5 from 0.5 M stock.

The suggested volume to prepare is ~20 mL, which should be sufficient for 7-8 samples.

This can be stored for several months at Room temperature .

Tissue Fixation and Sectioning -- Option (A)/(B): PFA Perfusion

2d

5 Transcardial PFA Perfusion and Fixation [Option (A)/(B)]

1d

Options (A) and (B) begin with transcardial perfusion with PFA. Option (A) goes on to section the tissue using a vibratome, while option (B) sections the tissue using a cryotome. The alternative to transcardial PFA perfusion is flash freezing (option (C)), described in steps 8-10.

Step 5 describes the transcardial perfusion and fixation. Step 6 describes tissue sectioning using a vibratome, permeabilization in ethanol, and storage. Step 7 describes rehydration for downstream processing.

For cryosectioning of PFA-perfused tissue, proceed from Step 5.5 (after sinking in sucrose) to Step 8.1 for OCT embedding/freezing and subsequent sectioning.

Here, we describe the general process of PFA perfusion and fixation for mouse, optimized for harvesting mouse brain. If you have a protocol that works well in your hands for harvesting tissue for single-molecule FISH, we recommend that you use that protocol.

5.1 Preparation for Fixation

1h

Store the following at 4 °C (or on ice) for at least 01:00:00 before starting:

1 ampule of 16% (w/v) paraformaldehyde;

~50 mL of nuclease-free water .

Freshly prepare 40 mL of 4% (w/v) paraformaldehyde in 1X PBS.

Reagent	Stock Concentration	Amount	Final Concentration
---------	---------------------	--------	---------------------



	Paraformaldehyde	16% (w/v)	10 mL (1 ampule)	4%
	PBS	10X	4 mL	1X
	Water	n/a	26 mL	n/a
	Total		40 mL	

Additionally, aliquot/prepare 40 mL of 1X PBS (or 1X DPBS).

Store both prepared solutions (4% (w/v) paraformaldehyde in 1X PBS, and 1X PBS) at

4 °C (or on ice) until starting procedure.

5.2 Transcardial PFA Perfusion

20m

Follow a standard transcardial PFA perfusion protocol for in situ hybridization work. We typically perfuse an anesthetized mouse with ice-cold 1X PBS (5 to 10 mL, until perfused solution runs clear; 1X DPBS can also be used), then change to ice-cold 4% PFA in 1X PBS (10 to 20 mL).

5.3 Post-Fixation

1d

Dissect out the tissue of interest and post-fix.

For mouse brain, we post-fix in 20-30 mL of 4% (w/v) paraformaldehyde in 1X PBS at

4 °C Overnight . We avoid post-fixing for longer than one night to avoid additional autofluorescence.

5.4 [OPTIONAL] Quenching Fixation

1d

After fixation, quench unreacted aldehydes in 20-30 mL of 1X PBS with 100 mM glycine

at 4 °C Overnight .

5.5 Washing with PBS

30m

Wash the tissue with 40 mL of 1X PBS for 00:30:00 at 4 °C .

For vibratome sectioning (option (A)), continue to Step 6.

For cryosectioning (option (B)), we cryoprotect mouse brains by sinking in 30% sucrose in 1X PBS at 4 °C , before freezing (described in Steps 8-10). Other protocols may work better for different tissues. Because the tissue has already been perfused with PFA



and post-fixed, the post-sectioning post-fixation (Step 9.2) is not necessary. Proceed to Step 8.

--- Pause Point ---

If needed, tissue can be washed again with 1X PBS, and stored for several days at

4 °C .

6 **Vibratome Sectioning and Storage [Option (A)]**

1d

Vibratome sectioning and permeabilization/storage conditions for tissue slices are described here.

6.1 **Preparation**

10m

Fill the wells of a 24-well plastic plate approximately half-full with 70% ethanol.

6.2 **Sectioning on Vibratome**

1h

Cut slices of desired thickness on a vibratome. Use 1X PBS or 1X DPBS to fill the sectioning chamber.

Transfer sections to wells of the 24-well plate, capturing 3-4 sections/well, and keeping adjacent sections in the same well.

For mouse brain, we typically cut 50 µm thick slices.

6.3 **Tissue Storage and Permeabilization**

1d

Seal the plate with Parafilm and store at 4 °C .

Slices are permeabilized after at least Overnight storage in 70% ethanol.

If slices are to be used immediately, they can be permeabilized in 70% ethanol for

01:00:00 at Room temperature .

We store slices in 70% ethanol at 4 °C for extended periods of time (up to months).

7 **Rehydration After Storage [Option (A)]**

30m

Slices require rehydration with 1X PBS after permeabilization.



Pre-fill Eppendorf tubes or the wells of an appropriate plate (typically 24-well or 48-well) with 1X PBS.

Transfer sections for further processing to the chosen wells/tubes. Multiple sections per well/tube is fine.

Wash the slices with 1X PBS for 00:15:00 × 2 times at Room temperature .

We rehydrate slices immediately before use.

Continue to Step 11.

Tissue Fixation and Sectioning -- Option (B)/(C): Frozen Tissue Blocks

3h 25m

8 Preparation of Frozen Tissue Blocks [Option (B)/(C)]

25m

There are two ways to prepare tissue for freezing: PFA perfusion and sucrose sinking (Option (B), continued from Step 5.5), or flash freezing (Option (C)). After freezing, the tissue is sectioned, post-fixed (for Option (C) only), permeabilized, and stored.

Step 8 describes the tissue collection (for fresh tissue) and flash freezing steps. Step 9 describes the cryosectioning, post-fixation, and permeabilization/storage steps. Step 10 describes two options for creating a chamber around the sample used for subsequent steps, as well as the rehydration washes. For option (C), follow all steps; for option (B), follow steps for freezing cryoprotected tissue

The section here is described without transcardial perfusion. One potential variation on this protocol would be to perfuse with 1X PBS to flush out blood before dissecting out the tissue of interest.

8.1 Preparation for OCT Embedding

15m

Prepare a dry ice/isopentane bath.

Cover the bottom of a cryomold with OCT.

8.2 [Option (C) ONLY] Fresh Tissue Collection

10m

Collect tissue of interest (i.e. harvest from anesthetized mouse, collect tumor biopsy, etc).

8.3 Freezing Tissue

Place tissue into prepared cryomold.



Add additional OCT around and on top of tissue, keeping note of the orientation of the tissue with respect to the cryomold.

[OPTIONAL] Allow cryomold to sit for ~5 minutes before freezing. For some tissues, this allows for better tissue integration with the OCT block during sectioning.

Lower cryomold into the dry ice/isopentane bath until completely frozen.

Store blocks at -80 °C until cryosectioning. Frozen OCT blocks are stable for months.

9 Cryosectioning and Post-Fixation [Option (B)/(C)]

1h 50m

Cryosectioning, formalin post-fixation, permeabilization in ethanol, and storage are described here.

9.1 Cryosectioning

20m

Section the tissue into slices of desired thickness on a cryotome, collecting sections on SuperFrost Plus slides.

Collect slides with sections in 4-well plates, keeping them cold at bottom of cryostat until post-fixation.

9.2 [Option (C) ONLY] Formalin Post-Fixation

15m

Fix slices with ice-cold 10% Buffered Formalin for 00:15:00 (for 15-20 µm sections; longer for thicker sections) at Room temperature (in fume hood).

Approximately 4-5 mL of formalin is needed to cover each slide in the 4-well plates.

Post-fixation is ONLY required for fresh frozen tissue, not PFA-perfused, cryoprotected tissue.

9.3 PBS Washes

15m



Wash the samples with 1X PBS for 00:05:00 × 3 times at Room temperature (in fume hood).



9.4 Tissue Storage and Permeabilization

1h


Briefly rinse slides with 70% ethanol and pipette away excess.

Then, cover slides with 70% ethanol for permeabilization.

If slides are needed immediately, permeabilization can be performed for  01:00:00 at  Room temperature .

For permeabilization and long term storage, store slides at  4 °C for at least  Overnight , ideally in a parafilm-sealed screw-top container (i.e. 50 mL Falcon tube or Coplin (slide staining) jar). If using a 4-well plate for storage, keep plate in sealed, humidified container to minimize evaporation.

--- Pause Point ---

Slides can be stored in 70% Ethanol at  4 °C (for up to months).

10 Chamber Construction and Rehydration After Storage [Option (B)/(C)]

1h 10m

Chambers for subsequent washes/incubations are formed directly on top of the slide-attached sections to reduce volumes for subsequent steps. There are two approaches to form the chamber -- using a hydrophobic (PAP) pen, or using a sticker to form a well.

10.1 Option 1: Constructing Chamber Around Tissue Using Hydrophobic Pen

40m

Using the hydrophobic pen, draw a box around the tissue section, and go over the box several times to form a chamber that will stand up to fluid exchanges. The box should have a margin of a few millimeters around the edges of the tissue.

Allow the hydrophobic pen to dry completely before proceeding, typically 20 - 40 minutes.

Suggested volume for subsequent steps is 100-200 µL.

10.2 Option 2: Constructing Chamber Around Tissue Using Stickers

1m

Place a chamber sticker (Bio-Rad Frameseal) around tissue section and seal.

Suggested volume for subsequent steps is 100-200 µL.

10.3 Rehydration Washes

30m



Rehydrate tissue by washing with 1X PBS for 00:15:00 × 2 times at

Room temperature .

For subsequent steps, the container holding slides in a 4-well plate should remain humidified. As sealing a 4-well plate with parafilm is difficult, a tupperware container with moist KimWipes should be used to hold the plate.

[OPTIONAL] Antibody Staining for Single Target

2d

11 Antibody Staining for Single Target

2d

Because the passivation chemistry quenches many fluorophores, we perform antibody staining as a split process, decoupling staining from visualization. We perform antibody staining using a biotinylated secondary antibody. The antibody signal is subsequently recovered and imaged at the end of the *in situ* sequencing process by staining with fluorophore-labeled streptavidin (described in subsequent protocol).

The following steps assume that no blocking with BSA is performed, and so PBST is used in all steps. If blocking is needed, 1 - 2.5% BSA (w/v) in PBST can be prepared and used for the solutions for antibody staining. Note that the BSA should be RNase-free.

11.1 PBST Wash

15m

Wash with PBST for 00:15:00 at Room temperature .

11.2 Primary Antibody Staining

1d

Stain with primary antibody in PBST Overnight at 4 °C .

Optimal concentration should be determined and used for each antibody.

11.3 Primary Antibody Washes

1h

Wash with PBST 00:20:00 × 3 times at Room temperature . Washes may also need to be optimized for each antibody.

11.4 Secondary Biotinylated Antibody Staining

1d


Stain with secondary antibody in PBST Overnight at 4 °C .

Optimal concentration should be determined and used.



11.5 Secondary Antibody Washes

1h

Wash with PBST  00:20:00 × 3 times at  Room temperature . Washes may also need to be optimized for each antibody.

LabelX Treatment of Slices

45m

12

LabelX Treatment of Slices

1d

LabelX is a bifunctional molecule, with a guanine-reactive moiety on one end (derived from LabelIT Amine), and an acryloyl group on the other (derived from AcX), enabling co-polymerization during the formation of the ExM gel.

LabelX treatment enables nucleic acids to be anchored to the expansion gel. For further details on the LabelX chemistry, see [Chen*, Wassie* et al. \(2016\)](#). LabelX also has some non-specific reactivity towards proteins, enabling antibodies from the antibody staining described in Step 11 to be anchored to the gel as well.

12.1 PBS Wash

20m

Wash the sections with 1X PBS for  00:20:00 at  Room temperature .

12.2 MOPS Buffer Pre-incubation



20m

Wash the sections with 1X Buffer A (Mirus Kit), or 20 mM MOPS pH 7.7 buffer for

 00:20:00 at  Room temperature .

12.3 LabelX Treatment

1d

Incubate the sections with 0.05 - 0.1 mg/mL LabelX (1:20 to 1:10 dilution) in 1X Buffer A (or MOPS)  Overnight (or for at least 6 hours) at  37 °C . For free floating sections in an Eppendorf tube, ~200 uL is typically enough to submerge 1-2 sections. For sections on slides, ~100 uL is typically enough to fill one well.

We have tested 0.1 mg/mL LabelX in mouse brain and human tumor tissue extensively. We have also found that 0.05 mg/mL LabelX performs similarly in thin tumor tissue sections, which can help save on reagent cost.

12.4 PBS Washes

40m



Wash the sections with 1X PBS for 00:20:00 × 2 times at Room temperature .

[OPTIONAL] Trimming Intact Sections

5m

13 Trimming Sections to Desired Size

5m

To prevent gels from becoming difficult to handle after gelation and expansion (due to size), tissue sections can be trimmed down to regions of interest.

For free-floating sections, a scalpel with a curved blade is ideal, though a flat-edged razor blade will work.

For a section on a slide, a flat-edged razor blade can be used to cut and scrape off excess tissue.

Casting Expansion Gel

2h 45m

14 Preparation and Casting of Expansion Gel

2h 45m

The expansion gel is formed by free radical polymerization throughout the entire specimen volume. The sample is first pre-incubated with gelling solution, containing gel monomers (StockX), a free radical scavenger (4HT), a free radical polymerization accelerator (TEMED), and a free radical polymerization initiator (APS). The presence of 4HT and the pre-incubation temperature of 4 °C prevents premature gelation, allowing the monomers to diffuse into the tissue. When the temperature is raised, gelation occurs. Note that oxygen inhibits gelation, so avoiding air bubbles and backfilling the gelation chamber to maximally separate the tissue from any air is important.

14.1 Preparation of Gelling Solution

5m

Thaw aliquots of StockX, 0.5% 4HT (w/v), 10% TEMED (w/v), and 10% APS (w/v). Once thawed, keep solutions in a cold freezer block or on ice.

Mix StockX, 4HT, TEMED, and APS at a 47:1:1:1 ratio in an Eppendorf tube. Add 4HT, TEMED, and APS to StockX in that order, taking care to vortex between each addition, and keeping the solution in the cold freezer block.



Suggested volumes: if the samples are free-floating in an Eppendorf or 24/48-well plate, 400 µL total should be sufficient. If the samples are attached to a slide, 200 µL should be sufficient.





	Stock solution concentration (w/v)	Amount	Final concentration (w/v)
Stock X solution	n/a	188 μ l	n/a
4HT stock solution	0.5%	4 μ l	0.01%
TEMED stock solution	10%	4 μ l	0.2%
APS stock solution	10%	4 μ l	0.2%
Total		200uL	

14.2 Pre-incubation of Sections with Gelling Solution

30m

Transfer free-floating tissue sections from PBS to the gelling solution using a paintbrush. Transfer the Eppendorf tube/plate to the fridge for  00:30:00 at  4 °C , ideally in/on a pre-chilled cold block.

For sections on slides, pipette out as much PBS as possible from the well, tilting the slide to help pull PBS into droplets. Pipette 100-150 μ L gelling solution onto the tissue, forming a bulging well. Place the slides in a 4-well plate and transfer to the fridge for

 00:30:00 at  4 °C , ideally on top of a pre-chilled cold block.

14.3 Preparation of Gelation Chamber

5m

Place two spacers, separated by ~10-15 mm on top of a glass slide.

Note: For free-floating tissue sections, the spacers can be placed while the sections are pre-incubating.

Note: For sections on slides, this step is performed at the end of preincubation, on the glass slide holding the section. The majority of the pre-incubation gelation solution should be removed at this stage, but the tissue section should remain covered with gelation solution. If an adhesive sticker was used to form a chamber, peel it off the glass slide. If a hydrophobic pen was used to form the chamber, scrape away the hydrophobic pen residue using a razor blade, taking to care to scrape away from the tissue.

Note: Spacers can be, for example, thickness #0 coverslips (100 μ m), or a single pieces of Scotch tape (~60 μ m thick). For 50 μ m sections or thinner, either Scotch tape or thickness #0 coverslip is fine. For thicker sections, a coverslip of appropriate thickness should be used. For first time users, we recommend using thickness #0 coverslips, as the thicker gels are easier to handle.

Note: If coverslips are used, a 1 μ L droplet of water can be pipetted onto the slide before placing the coverslip to promote better adherence between the coverslip and the glass.

Note: If Scotch tape is used as a spacer, the excess tape should be cut so as to leave no excess overhang off the edge of the slide.

14.4 Construction of Gelation Chamber

5m

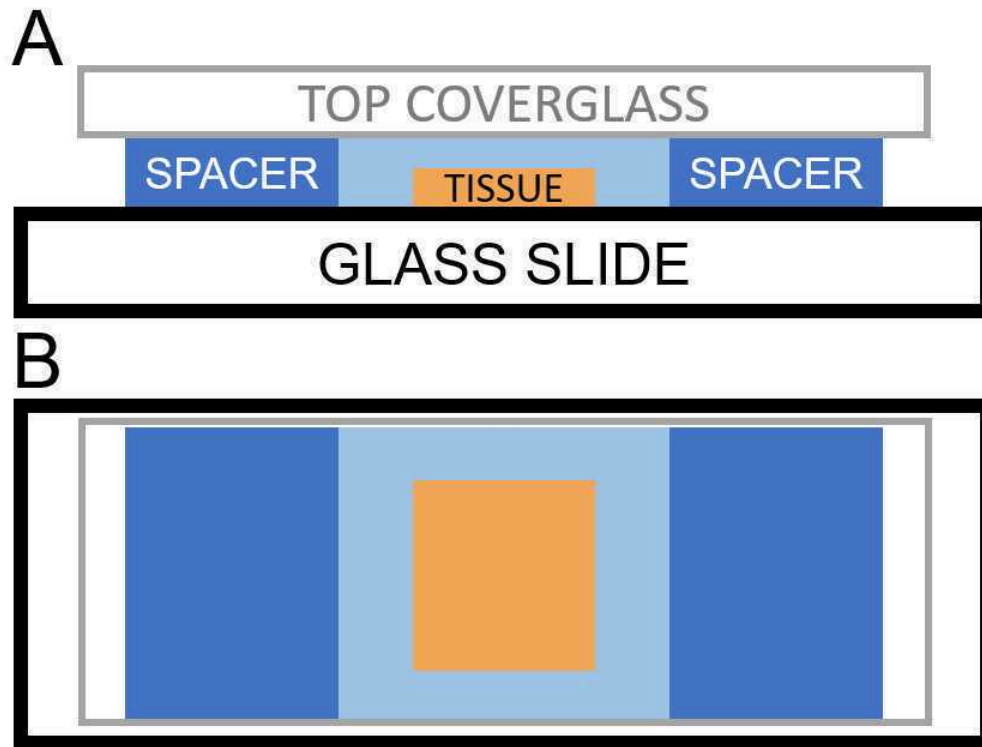


Figure 2. Sketch of gelling chamber for casting the expansion gel. (A) Longitudinal cross-section of gelling chamber. Tissue is orange, spacers are dark blue, gelling solution is light blue. (B) Top-down view of same gelling chamber from (A).

For free-floating tissue sections: When the pre-incubation is complete, pipette a droplet of gelling solution from the pre-incubation solution to the center of the chamber. Then, use a paintbrush to transfer the tissue section from the Eppendorf tube to the gelation chamber. If necessary, gently unroll/unfold the tissue with the paintbrush to flatten the tissue. Pipette an additional droplet of gelling solution on top of the gel.

For sections on slides: ensure that the tissue is covered with a droplet of gelling solution, but that there is not an excess of solution that may spill over when the lid of the gelling chamber is lowered.

Gently lower a coverslip or a slide on top of the gelling chamber, forming the lid. The lid should be lowered from one side to the other, i.e. so that the one edge of the lid is first lowered onto one of the spacers, and the second edge is slowly lowered onto the other spacer. The droplet on top of the sample will make contact with the lid and spread out over the tissue.

It is critical that bubbles near the tissue section be avoided during this process. If bubbles occur, the lid can be removed and the lowering can be attempted again.



After lowering the lid, the gelation chamber should be back-filled by pipetting additional gelation solution into the gap between the lid and the base slide. The gelling solution will spread to fill the chamber via capillary action. **Fig. 2** shows the final setup of the gelation chamber.

Take care not to overfill the chamber -- the lid should not be floating. The lid coverslip should be at least thickness #2 (200 μm) to avoid bending of the coverslip, leading to a non-flat gel. If there is spillage, the excess solution can be wiped away with a KimWipe.

These steps should be completed within 5 minutes to avoid premature gelation. Eppendorf tubes containing tissue/gelling solution should be kept on a cold-block until use to minimize warming.

14.5 Gelation

2h

Place the gelation chamber into a humidified container and incubate for  02:00:00 at  37 °C .

Digestion and Expansion

1d

15 Digestion

1d

To enable isotropic expansion, mechanical homogenization is performed by digesting proteins in the tissue-gel composite with Proteinase-K.

The recommended digestion protocol is highly tissue-type dependent. We provide two digestion protocols here -- one for tissues that require a mild digestion to expand (i.e. mouse brain), and one for tissues that require a robust digestion to expand (most non-brain tissues, including human tumor biopsies and mouse tumor models).

For the robust digestion, a chaotropic salt (guanidine hydrochloride) is included to denature proteins and increase their accessibility for Proteinase-K digestion. In addition, increased temperature and digestion duration are used to promote increased digestion.



15.1 Preparing Digestion Buffer

5m

Prepare the appropriate volume (described below) of either a Mild 1X Digestion Mix or a Robust 1X Digestion Mix, as is appropriate for the tissue.

Recipe for 1 mL of Mild 1X Digestion Mix (containing NaCl and Proteinase-K):

A	B	C	D
Solution	Stock Concentration	Volume	Final Concentration
10X Digestion Buffer	10X	100 μ L	1X
NaCl	5 M	100 μ L	500 mM
Proteinase-K	800 U/mL	10 μ L	8 U/mL
Water		790 μ L	
Total		1000 μ L	

Recipe for 1 mL of Robust 1X Digestion Mix (containing Guanidine HCl and Proteinase-K):

A	B	C	D
Solution	Stock Concentration	Volume	Final Concentration
10X Digestion Buffer	10X	100 μ L	1X
Guanidine HCl	8 M	100 μ L	800 mM
Proteinase-K	800 U/mL	10 μ L	8 U/mL
Water		790 μ L	
Total		1000 μ L	

Volume of digestion mix to prepare:

For free-floating sections: for 1-2 sections, digestion can be performed in 1 mL of 1X Digestion Mix in an Eppendorf tube. For more sections, a well of a 6-well plate is recommended, requiring 3-4 mL of 1X Digestion Mix. Additionally prepare an additional 0.5 mL of 1X Digestion Mix in a separate Eppendorf tube (Supplemental Digestion Mix). The supplemental Digestion Mix must have the appropriate salt (either NaCl or Guanidine HCl), but does not need Proteinase-K.

For sections on slides (in 4-well plates): prepare 5 mL per slide of 1X Digestion Mix.

The total volume of the Digestion Mix used should be >100 times the volume of the sample gel.

15.2 Opening Gelling Chamber

5m

Remove the humidified container from the incubator and remove the samples.

Pry the lid off of the gelation chamber by inserting a razor blade from the side between the lid and the spacers. Take care that the gel adheres to either the lid or the base slide. If it is partly stuck to the lid and the base slide, wet a paintbrush with Supplemental Digestion Mix and gently peel the the gel off of one of the surfaces.

Make cuts between the gel and the spacers with the razor blade, then lift off the spacers in a similar fashion to the lid. Using the razor blade, trim away excess gel. Leave a small margin (~1 mm) around the tissue.

For free-floating sections: pipette ~100 μ L of digestion buffer from the Supplemental Digestion Mix onto each gel. Also wet a paintbrush with Supplemental Digestion Mix. Wait 10-30 seconds for the buffer to wet the borders of the gel. Then, use the paintbrush to gently peel the gel off of the slide by slowly moving the paintbrush back and forth underneath the edge of the gel, gradually probing further underneath the gel until the entire gel lifts off. Transfer the gel to the container with 1X Digestion Mix (prepared in the previous sub-step) for digestion. If the digestion is being performed in a plate, add a few mL of water to another well to humidify the plate, then seal with parafilm.

For sections on slides: after opening the gelling chamber, place the slide (with the gel on top) into a well of a 4-well plate, and add the 1X Digestion Mix (prepared in the previous sub-step), ensuring that the gel is fully submerged in digestion mix. Add a few mL of water to another well, then place back into a humidified container for the next steps.

15.3 Option 1: Mild Digestion Conditions

1d

Mild digestion conditions (using 1X Mild Digestion Mix) were optimized around mouse brain slices. There are three options for mild digestion that perform comparably. The



lower temperature digestion conditions are preferred, as they avoid accelerating RNA degradation.

Option 1A: Digest  Overnight at  Room temperature .

Option 1B: Digest for  03:00:00 at  37 °C .



Option 1C: Digest for  01:00:00 at  60 °C .



The gel will expand ~1.5X during digestion. If the tissue was originally on a slide, the tissue-gel composite should lift off during digestion.



15.4 Option 2: Robust Digestion Conditions

2d 1h

Robust digestion conditions (using 1X Robust Digestion Mix) were optimized around tumor tissue sections on SuperFrost Plus slides.

Digest for  01:00:00 at  60 °C .

Then, prepare a fresh batch of 1X Robust Digestion Mix, replace the digestion mix, and then incubate  Overnight at  37 °C .



When 24 hours from the start of digestion have elapsed, prepare a fresh batch of 1X Robust Digestion Mix, replace the digestion mix, and then incubate  Overnight at  37 °C again.

Digestion is complete after  48:00:00 of total digestion.


For sections on slides, the tissue-gel composite should have lifted off from the slide.

15.5 PBS Washes

30m

Wash the gel with 1X PBS for  00:15:00 × 2 times at  Room temperature . The gel will be approximately 2X linearly expanded in 1X PBS.

--- Pause Point ---

The gel can be stored in 1X PBS at  4 °C for up to 4 weeks. Take care to seal the container holding the gel to avoid evaporation.


16 Expansion


1h

Place a glass slide at the bottom of the container that will be used for expansion. Samples will expand to 4.5X of the original size. The suggested container for expansion (and subsequent re-embedding/passivation) is a 4-well plate. A petri dish may also be used.

Transfer gel on top of the glass slide in the container.

Briefly wash the sample with water, and pipette away excess fluid.

Wash the samples with excess volumes of water for  00:20:00 × 3 times at

 Room temperature .

Note: gels become fragile during the expansion process.

Casting Re-embedding Gel

3h 20m

17 Gel Re-embedding

3h 20m

The ExM gel changes expansion factor based on salt concentration. To lock the gel into a fixed expansion factor, a non-expanding acrylamide gel is cast within the expanded gel. This second gel embedding, called re-embedding, results in a gel that is locked at 3.3X linear expansion factor, relative to the original tissue.

17.1 Preparation of Re-embedding Solution

5m

Prepare a fresh batch of 10% APS (as described in Step 1). Store on a cold block until use.

Prepare re-embedding solution. The volume prepared should be enough to cover the expanded gel. Suggested volume for one well of a 4-well plate is 5 mL.

Re-embedding solution:

	A	B	C	D
	Solution	Stock Concentration	Volume	Final Concentration
	Acrylamide/ Bis 19:1 solution	40% (w/v)	375 µL	~2.85% (w/v) acrylamide; 0.15% (w/v) N-N'- Methylenebisacryla mide
	Water		4525 µL	



	A	B	C	D
	Tris Base	1 M	25 μ L	5 mM
	10% TEMED	10% (w/v)	37.5 μ L	0.075%
	10% APS	10% (w/v)	37.5 μ L	0.075%
	Total		5000 μL	

Add reagents in the listed order. Vortex after adding TEMED and APS.

Note: acrylamide is toxic and the concentrated stock should be handled in a fume hood.



Note: re-embedding solution must be used immediately.

Note: we have also used higher percentage gels (up to 4% acrylamide final concentration) without issues. Increasing the acrylamide concentration can help if you are having issues with gels breaking.

17.2 Pre-Incubation of Gels with Re-embedding Solution

30m

Remove the final wash from the sample.

Add re-embedding solution, ensuring gels are submerged, and pre-incubate gels with very gentle shaking for  00:30:00 , at  Room temperature .

Note: gel will shrink slightly (~25%) during this pre-incubation, to 3.3X linear expansion factor.

17.3 Construction of Re-embedding Chambers

5m

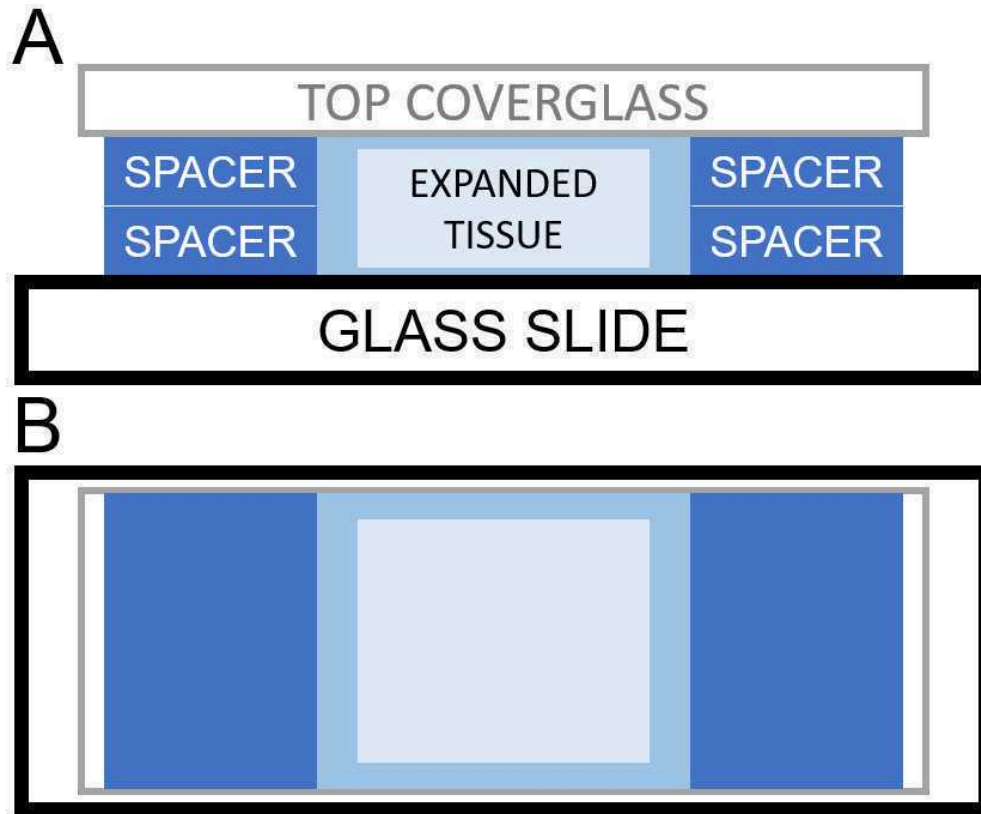


Figure 3. Sketch of gelling chamber for casting the re-embedding gel. (A) Longitudinal cross-section of re-embedding gelling chamber. Expanded tissue is pale blue, spacers are dark blue, gelling solution is light blue. Example spacers in figure are two stacked thickness #1.5 coverslips. (B) Top-down view of same gelling chamber from (A).

Remove samples from shaker, and remove excess re-embedding solution. Collect excess in a 50 mL Falcon tube.

Use paintbrush to gently nudge gel so that it is centered on top of the glass slide.

Place appropriate spacers on either side next to the gel. For expansion gels originally cast with scotch tape spacers, a thickness #2 (200 μm) coverslip should be sufficient. For gels originally cast with a thickness #0 coverslip spacer, two thickness #1.5 (170 μm each) coverslips stacked on top of each other should be sufficient.

To limit slippage of glass-glass contacts, small droplets ($\sim 1 \mu\text{L}$) of water can be placed between glass surfaces to promote better adhesion.

Pipette $\sim 20 \mu\text{L}$ of re-embedding solution on top of the gel. Then, lower the lid (a large thickness #2 coverslip, or a glass slide) on top of the chamber. As before, the lid should be lowered from one side of the gel to the other, and care should be taken to avoid trapping air bubbles.



After lowering the lid, the re-embedding chamber should be back-filled by pipetting additional re-embedding solution into the gap between the lid and the base slide. The gelling solution will spread to fill the chamber via capillary action. Take care not to overfill the chamber -- the lid should not be floating. **Fig. 3** shows the setup of the final gelation chamber.

These steps should be completed within 5 minutes to avoid premature gelation.

17.4 Nitrogen Purge and Re-embedding Gelation

1h 40m



Place re-embedding chambers into a 4-well plate. Place the 4-well plate into a humidified Tupperware container with two small holes in the lid, and close the container.

Purge Tupperware container with nitrogen gas. To do this, start a slow flow of nitrogen gas into a nitrogen line connected to tubing with a blunt needle at the end. The flow rate should be barely perceivable when pointed at skin.

Insert the needle through one of the holes in the lid of the container. Ensure that the other hole is open, allowing for airflow out of the container. Ensure that the container does not immediately bulge or pop-open, which would be indicative of too high of a flow rate.

Purge for  00:10:00 .

After purging, withdraw the needle, and seal both holes by covering with tape.



Cast re-embedding by incubating for  01:30:00 at  37 °C .



17.5 PBS Washes

1h

Remove re-embedding chambers from incubator.

As before, use a razor blade to pry open the lid of the re-embedding chamber. Make cuts between the gel and the spacers, and remove the spacers.

Wash with 1X PBS for  00:15:00 at  Room temperature .

If proceeding immediately to passivation, wash again with 1X PBS for  00:45:00 at  Room temperature .

--- Pause Point ---



The re-embedding gel can be stored in a fresh wash of 1X PBS for up to 1 month at

4 °C .

Passivation

3h 50m

18 Chemical Passivation of Carboxylic Acids

3h 50m

To enable enzymatic reactions to be performed, carboxylic acids/carboxylates within the original expansion gel need to be chemically passivated by transforming the carboxylic acid functional group into non-charged amides. This is performed by using carbodiimide crosslinker chemistry to form amides bonds between the carboxylic acids and ethanolamine. Unpassivated gels inhibit enzymatics, possibly through chelation of essential ions or interactions between the negative charges of carboxylates and enzymes.

Passivation is performed in two steps, an activation step, using EDC-NHS at pH 6.5 to activate carboxylic acids, and a coupling step at pH 8.5 to efficiently form amide bonds with ethanolamine.

Volumes here for slides in 4-well plate; can scale up/down accordingly for different containers.

18.1 Preparation of Passivation Solutions

10m

For each sample, prepare ~3 mL of the two following solutions. Amounts for 1 mL solution are listed below.

300 mM NHS in 4M Ethanolamine hydrochloride:

	A	B	C	D
	Reagent	Stock Concentration	Volume/Amount	Final Concentration
	NHS	powder	34.53 mg	300 mM
	Ethanolamine HCl solution	4 M	1 mL	~4 M
	Total		1 mL	

300 mM EDC in 200 mM MES buffer:



A	B	C	D
Reagent	Stock Concentration	Volume/Amount	Final Concentration
EDC	powder	57.51 mg	300 mM
MES Buffer	200 mM	1 mL	200 mM
Total		1 mL	

For both solutions, it is typically easier to mass out an approximate amount of NHS or EDC, then calculate the correct buffer volume to add to reach the correct concentration.

18.2 Passivation: Carboxylate Activation

2h

For each sample, combine 2.5 mL of the NHS in Ethanolamine with 2.5 mL of the EDC in MES, vortex, and add to sample in 4-well plate.

Carboxylate Activation Solution:

A	B	C
Solution	Volume	Final Concentration
300 mM NHS in 4 M Ethanolamine HCl	2.5 mL	150 mM NHS; 2 M Ethanolamine
300 mM EDC in 200 mM MES Buffer	2.5 mL	150 mM EDC; 100 mM MES
Total	5 mL	

Incubate gels in 5 mL carboxylate activation solution with gentle shaking for

02:00:00 at Room temperature .

18.3 Passivation: Coupling

40m

Prepare 5 mL of coupling solution.

A	B	C	D
Solution	Stock Concentration	Volume	Final Concentration



	A	B	C	D
	Ethanolamine HCl	4 M	2.5 mL	2 M
	Sodium Borate, pH 8.5	125 mM	2.5 mL	62.5 mM
	Total		5 mL	

Incubate gels in 5 mL coupling solution with gentle shaking for 00:40:00 at

Room temperature .

18.4 PBS Washes

1h

Wash gels with 1X PBS for 00:20:00 × 3 times at Room temperature .

--- Pause Point ---

After 1X PBS washes, gels can be stored in 1X PBS for up to one month at 4 °C . Be sure to store gels in a sealed container to avoid evaporation. 4- or 6-well plates, or petri dishes are suggested for long term storage.

[OPTIONAL] Trimming and Cutting

1h 20m

19 Further Trimming and Cutting for Long-Term Storage

1h 20m

It can be convenient to trim and store the gels as smaller fragments that are immediately ready for library preparation. This can be done in an image guided way by using DAPI staining to identify and extract regions of interest.

If image guidance is not necessary, the gels can be trimmed as in Step 19.2.

19.1 DAPI Staining

30m

Transfer gel to a suitable container for imaging, such as a 4-well or 6-well plate. The plate does not need to have a glass-bottom for this step.

Prepare ~3-5 mL of DAPI in 1X PBS. Recipe for 1 mL is below.

	Reagent	Stock Concentration	Volume	Final Concentration
	PBS	1X	999 µL	1X



	DAPI	1 mg/mL	1 μ L	1 mg/L
	Total		1000 μL	

Stain gel with DAPI solution for ⌚ 00:15:00 at 🌡 Room temperature .

Wash gel with 1X PBS for ⌚ 00:10:00 at 🌡 Room temperature .

19.2 Imaging and Trimming/Cutting

30m

Image gel on microscope using 4X objective in wide-field mode, using the imaging settings for DAPI.

Trim gel as appropriate using DAPI staining as a landmark. To cut the gel, use a thickness #2 (or #1.5) coverslip as a blade. After pushing the coverslip down (and holding in place), sweep a paintbrush alongside the coverslip to ensure the two sides are separated.

19.3 Additional Cutting and Storage

20m

After trimming the gel, the interior can be cut into smaller sections. This can either be done on the microscope using DAPI as a landmark (as above), or without imaging at the bench.

Smaller gel sections can be transferred to individual PCR tubes in 1X PBS, and stored for up to one month at 🌡 4 °C .