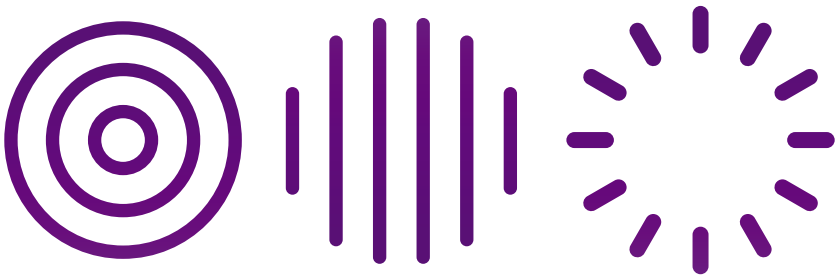
STOmics

Stereo-seq PERMEABILIZATION SET FOR CHIP-ON-A-SLIDE

USER MANUAL



Cat. No.: 211SP118 (8 RXNs)
Kit Version: V1.0
Manual Version: D

REVISION HISTORY

Manual Version: A
Kit Version: V1.0
Date: Jan. 2023

Description:

- Initial release

Manual Version: B
Kit Version: V1.0
Date: Apr. 2023

Description:

- Addition of Appendix B with instructions on performing antibody dilution prior to permeabilization testing.
- Upgraded Chip-on-a-slide layouts.

Manual Version: C
Kit Version: V1.0
Date: Aug. 2023

Description:

- Updated operational procedures to be compatible with PFA treated samples.
- Renamed 0.1X SSC (with 5% RI) as Wash Buffer.
- Extended pre-warming time of 1X Permeabilization Reagent Solution from 3 min to 10 min.
- Changed RT QC Mix addition volume from 90 uL/chip to 100 uL/chip.
- Updated tips for imaging by adding 1-2 μL of water for better Stereo-seq Chip Slide placment.
- Updated Tissue Removal time duration no later than 16hr.
- Minor format errata.

Manual Version:	D
Kit Version:	V1.0
Date:	Sep. 2023

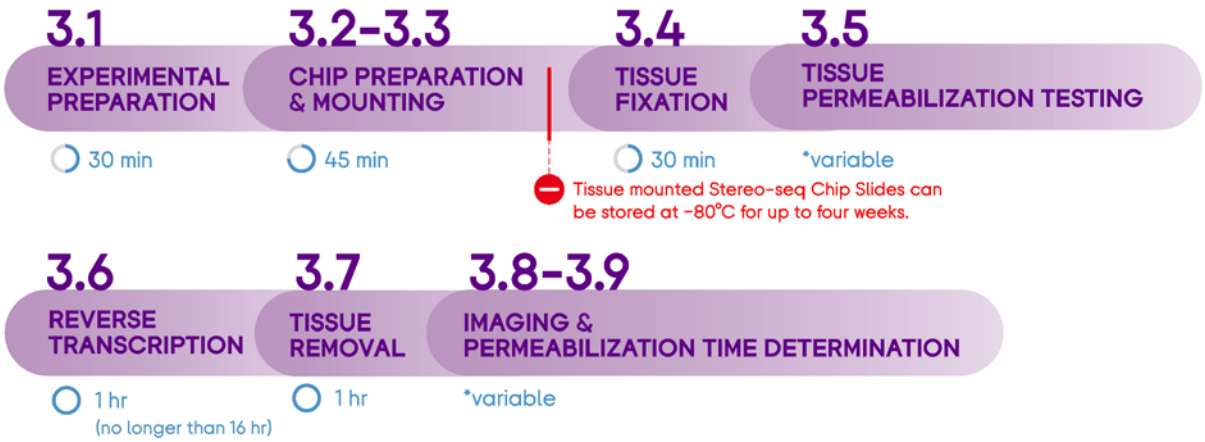
Description:

- Updated to be compatible with H&E application.
- Added steps to pre-warm Cassette and Gasket before tissue permeabilization testing.

Note: Please download the latest version of the manual and use it with the corresponding Stereo-seq Permeabilization Kit.

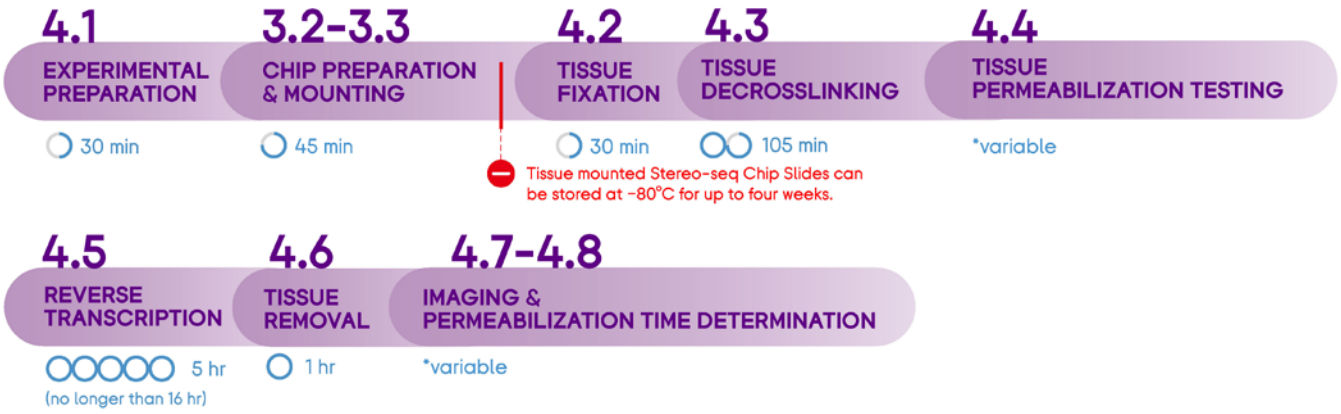
WORKFLOW

Fresh frozen sample



TOTAL TIME: ~5 HRS

PFA-fixed sample



TOTAL TIME: ~10 HRS

TABLE OF CONTENTS

CHAPTER 4: PFA FIXED SAMPLE, Stereo-seq PERMEABILIZATION SET FOR CHIP-ON-A-SLIDE STANDARD OPERATING PROCEDURE

NOTE: Additional operation tips and guidance.

CRITICAL STEPS: Pay extra attention for these steps to avoid experimental setbacks or problematic results.

QUALITY CHECK POINT

CAUTION: Proceed with extra care; improper handling or carelessness may cause experimental failure or accidents.

STOP POINT: Here you may pause your experiment and store your sample.

CHAPTER 1
INTRODUCTION

1.1. Intended Use

STOmics Stereo-seq Permeabilization Set for Chip-on-a-slide enables *in situ* capture of whole transcriptome information and is used for optimizing permeabilization conditions for a specific tissue of interest prior to STOmics Stereo-seq Transcriptomics Set for Chip-on-a-slide experiments. Featured with high resolution and large field of view, Stereo-seq Chip P Slides are patterned with capturing probes for capturing mRNA within tissues. Upon interacting with the tissue section, cDNA is synthesized *in situ* using fluorescently labeled nucleotides from captured mRNA. Through visualization using fluorescent microscopy, the optimal permeabilization time can be determined for a specific tissue of interest and will be required for further Stereo-seq Transcriptomics Set for Chip-on-a-slide experiments.

Stereo-seq workflow is also compatible with tissue H&E staining, which obtains better tissue morphological information, to assist with tissue type identification, to obtain gene expression profile of specific tissue region, and to conduct downstream differential analysis between selected regions of interests.

All reagents provided within this kit have passed stringent quality control and functional verification, ensuring performance stability and reproducibility.

1.2. List of Kit Components

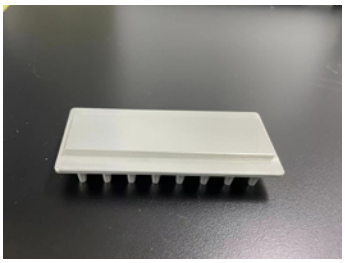
Each Stereo-seq Permeabilization Set for Chip-on-a-slide consists of:

- Stereo-seq Permeabilization Kit *1 (8 RXN)
- Stereo-seq Chip P Slide (1cm*1cm) *1 (8 EA)
- STOmics Stereo-seq Accessory Kit *2 (5 PCs)

Required for H&E applications:
H&E Mounting Medium (50μL/kit)

Compatible auxiliary but not included:

- Stereo-seq PCR Adaptor *1 (2EA)



Further information on catalog numbers, kit components, and specifications are listed below (next page).

Upon receiving the Stereo-seq Chip P Slide (1cm*1cm), please follow the instructions in Stereo-seq Chip P Slide Stereo-seq Chip T Slide Operation Guide For Receiving, Handling And Storing to properly store unused Stereo-seq Chip P Slides.

Performance of products may only be guaranteed before their expiration date. Proper performance is also subject to the products being transported, stored, and used in appropriate conditions.

Table 1-1

Stereo-seq Permeabilization Kit Cat. No.:111KP118			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
RI	1000028499		300 μL × 1
PR Enzyme	1000028500		10 mg × 1
RT QC Reagent	1000028501		748 μL × 1
RT Additive	1000028502	○ (transparent)	44 μL × 1
RT QC Enzyme	1000028503	○ (transparent)	44 μL × 1
TR Enzyme	1000028504		71 μL × 1
TR Buffer	1000028505		1725 μL × 2
Storage Temperature: -25°C~-18°C	Transported by cold chain	Expiration Date: refer to label	

Table 1-2

Stereo-seq Chip P Slide (1cm*1cm) Cat. No.: 210CP118	
Component	**Quantity (kit)**
Stereo-seq Chip P Slide (1cm * 1cm)	8 EA
Storage Temperature: -25°C~-18°C	Transported by cold chain	Expiration Date: refer to label

Table 1-3

STOmics Accessory Kit Cat. No.: 1000033700		
Component	**Reagent Cat. No.**	**Quantity (per kit)**
Cassette	10000033699	1 EA
Gasket	10000033698	4 EA
Sealing Tape	1000042970	6 EA
Storage Temperature: Room Temperature	Transported at room temperature	Expiration Date: refer to label

Table 1-4

Stereo-seq PCR Adaptor Cat. No.: 301AUX001		
Component	**Quantity (per kit)**	
Stereo-seq PCR Adaptor	2 EA	
Storage Temperature: Room Temperature	Transported at room temperature	Expiration Date: refer to label

Table 1-5 【Required for H&E applications】

H&E Mounting Medium Cat. No.: 1000041969		
Component	**Quantity (per kit)**	
H&E Mounting Medium	50 μL	
Storage Temperature: Room Temperature	Transported at room temperature	Expiration Date: refer to label

1.3. Additional Equipment and Materials

The table below (next page) lists equipment and materials needed for this protocol. Some common laboratory equipment not named in Table 1-5 are expected to be accessible by the user, for instance, an ice maker, biological safety cabinet, freezers, etc. For specific microscope requirements, please refer to **STOmics Microscope Assessment Guideline.**

Table 1-6

Equipment		
Brand	**Description**	**Catalog Number**
-	Cryostat	-
-	Benchtop centrifuge	-
-	Pipettes	-
	Metal heating block dry bath (optional)	-
-	Vortex mixer	-
Eppendorf	Microcentrifuge (for Stereo-seq mIF application, refrigeration function required)	5418 R
Bio-Rad*	T100 Thermocycler	1861096
ABI*	ProFlex 3 x 32-well PCR System	4484073

Choose either one of the listed brands (with * mark). Suitable PCR Adaptor will be needed.

Reagents		
Brand	**Description**	**Catalog Number**
Aladdin (or other brands)	2M NaOH - for PFA fixed tissue	S128511-1L
BBI (or other brands)	99% Sucrose - for PFA fixed tissue	A610498-0500
Gibco	PBS (10X), pH 7.4 - for PFA fixed tissue	70011044
BOSTER (or other brands)	4% Paraformaldehyde (with DEPC) - for PFA fixed tissue	AR1069
QIAGEN	RNeasy FFPE Kit - for PFA fixed tissue	73504
Ambion	Nuclease-free water	AM9937
	1X TE buffer, pH 8.0	AM9858
	20X SSC	AM9770
Sigma Aldrich	Hydrochloric acid, HCl (0.1N)	2104-50ML
	Methanol	34860-1L-R
	Triton X-100 Solution, 10% (for Stereo-seq mIF application)	93443-100ML
	Hematoxylin solution (for Stereo-seq H&E application)	51275
Sangon Biotech (or other brands)	Eosin Y, free Acid (for Stereo-seq H&E application)	A600190-0025
Agilent	Bluing Buffer, Dako (for Stereo-seq H&E application)	CS70230- 2

Thermo Fisher Scientific™	RiboLock RNase Inhibitor (40 U/μL) (for Stereo-seq mIF application)	EO0382
	Gibco™ Horse Serum (for Stereo-seq mIF application)	26050070
	Gibco™ Goat Serum (for Stereo-seq mIF application)	16210064
SAKURA	SAKURA Tissue-Tek® O.C.T. compound	4583

Consumables

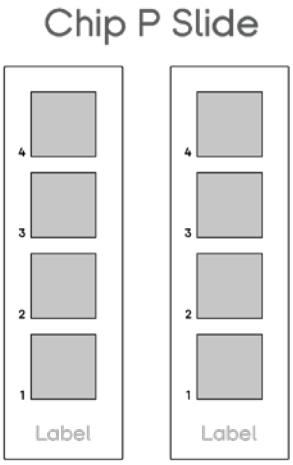
Brand	Description	Catalog Number
-	Stainless-steel base mold	-
-	Aluminum foil	-
-	Forceps	-
-	Slide Staining Rack	-
-	Sterilized Syringe (for Stereo-seq mIF application)	-
-	Microscope glass coverslip (area: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm)	-
Millipore	Millex Syringe Filter, Durapore PVDF, 0.22 μm pore size (for Stereo-seq mIF/H&E application)	SLGV033N
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
Kimtech	Kimwipes™ delicate task wipes	34155
MATIN	Power dust remover	M-6318
Axygen	1,000 μL filter tips	TF-1000-L-R-S
	200 μL filter tips	TF-200-L-R-S
	100 μL filter tips	TF-100-R-S
	10 μL filter tips	TXLF-10-L-R-S
Invitrogen	Qubit Assay Tubes	Q32856
BIOSHARP	Metal Block	-

1.4. Practice Tips

Stereo-seq Chip P Slide

Includes 2 Stereo-seq Chip P Slides, containing **four** Chip P (1cm*1cm) on each slide.

- Stereo-seq Chip P Slides and Stereo-seq Chip T Slides are differentiated by a laser engraved label at the end of the slide.

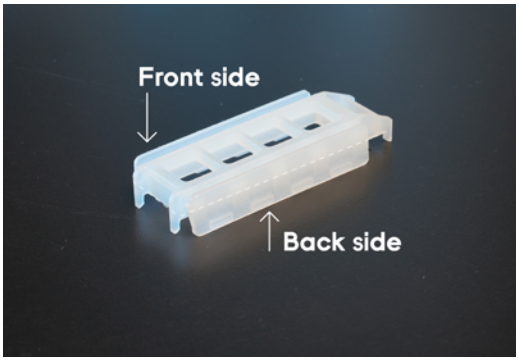


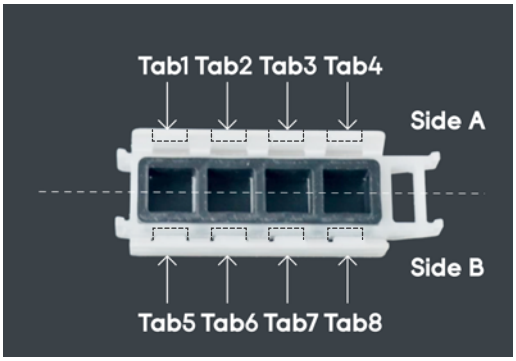
Stereo-seq Chip P Slide Storage

- Always store unused slides in their original slide container and then the aluminum bag at -25°C ~ 8°C. Keep sealed with tape or another re-sealable bag.
  Always KEEP the desiccant within the bag.

Stereo-seq Slide Cassette

STOmics Stereo-seq Accessory Kit contains a Stereo-seq Cassette and removable Gaskets which need to be assembled prior to use.

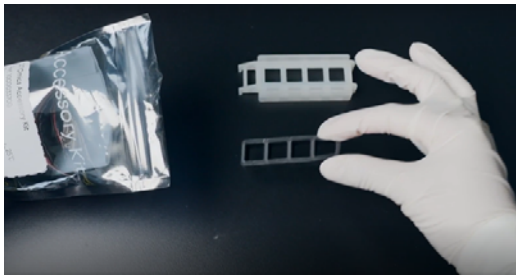




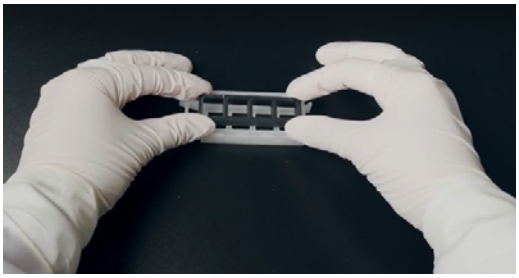
For a demonstration video of Stereo-seq Slide Cassette assembling and removal, please refer to the link or by scanning the QR code:
https://en.stomics.tech/resources/videos/list.html

Stereo-seq Slide Cassette Assembly

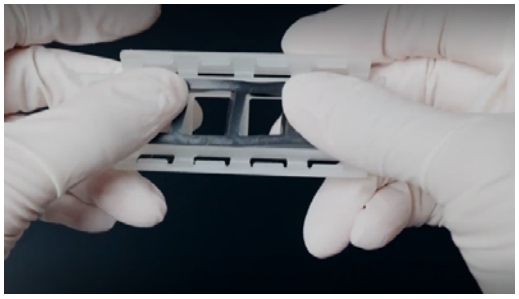
a. Take the Stereo-seq Slide Cassette and gasket out of the STOmics Stereo-seq Accessory Kit.



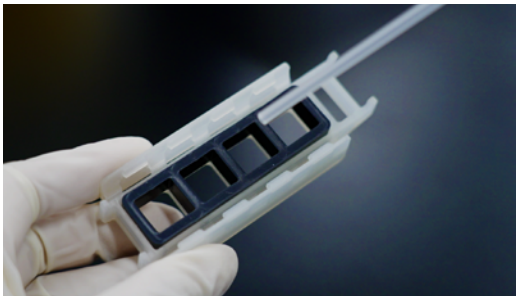
b. Pick up the Stereo-seq Slide Cassette and flip over. Insert the gasket into Stereo-seq Slide Cassette, ensuring the cutouts are aligned.



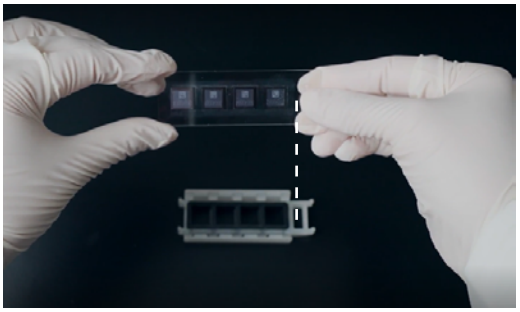
c. Press down the gasket to better fit the cassette.



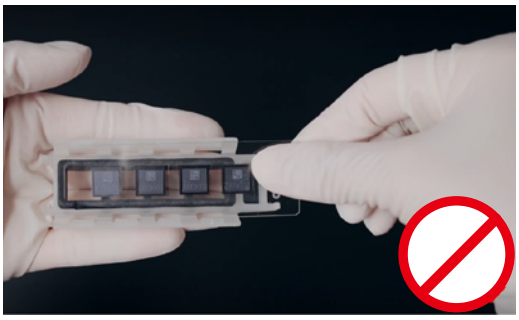
d. Use a power dust remover to blow off any debris on the gasket if necessary.



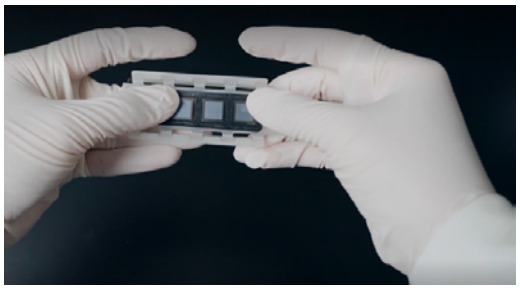
e. Pick up the Stereo-seq Chip Slide and flip over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.



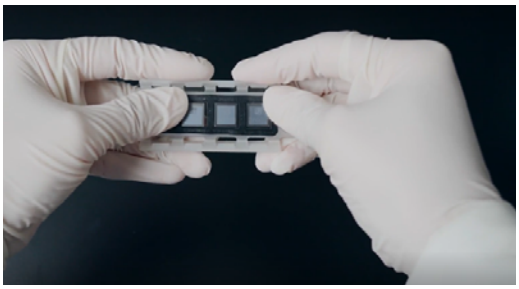
f. Make sure the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert Stereo-seq Chip Slide under the bottom 4 tabs.



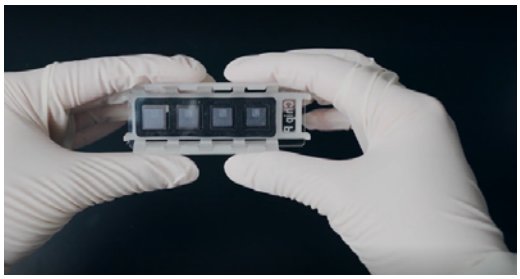
g. Support the back of the cassette with both middle fingers. Place left thumb between tab 1 and tab 2 while right thumb between tab 3 and tab 4.



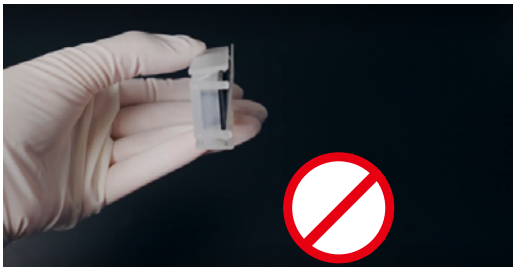
h. Press down evenly on the upper side (A side) of the slide (near the edge) and then simultaneously press down the top edge firmly with both index fingers to clip the slide in place until you hear a clicking sound.



i. Press along both edges of the Stereo-seq Slide Cassette to ensure the Stereo-seq Chip Slide is locked in place.



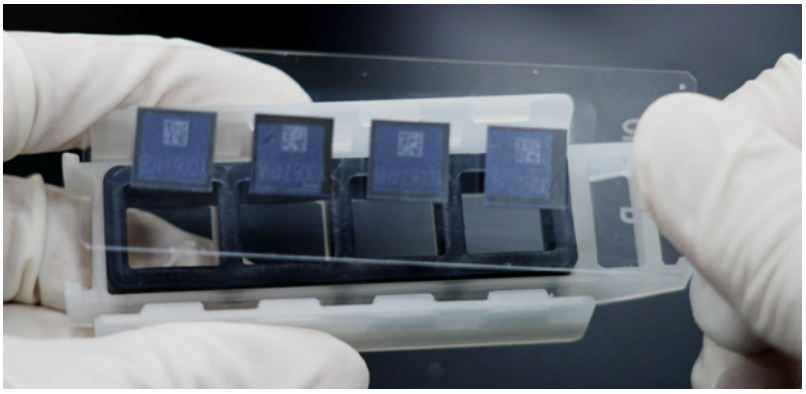
j. Take a final look at the Stereo-seq Slide Cassette to make sure the slide is clipped in place.



Stereo-seq Slide Cassette Removal

a. Flip the cassette over and firmly press down the upper side to release the slide from the tabs, while gently supporting the back of the Stereo-seq Chip Slide with both thumbs to prevent the Stereo-seq Chip Slide from falling off.

b. Lift the Stereo-seq Chip Slide from the side with the engraved label.



Stereo-seq Slide Cassette removal is not needed for the Stereo-seq Transcriptomics Set for Chip-on-a-slide.

1.5. Precautions and Warnings

- This product is intended for research use only, not for use in diagnostic procedures. Please read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, users are recommended to ensure that they are familiar with related instruments, and operate them according to manufacturer's instructions.
- Instructions provided in this manual are intended for general use only and optimization may be required for specific applications.
- Thaw reagents in the kits properly prior to use. For enzymes, centrifuge briefly and keep them on ice until further use. For other reagents, thaw them first at room temperature followed by inverting several times to mix them properly, and centrifuge them briefly before placing on ice for further use.
- mRNA capture will be compromised or absent for any scratched areas on the front-side surface of the chip.

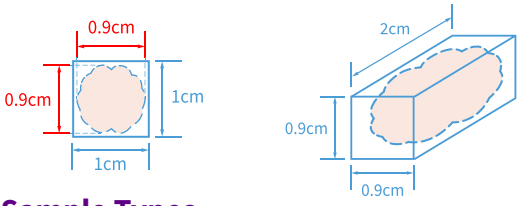
- To prevent cross-contamination, we recommend the use of filtered pipette tips. Use a new tip each time for pipetting different solutions.
- We recommend using a thermal cycler with heated lids for PCR reactions. Unless otherwise stated, pre-heat the thermal cycler to reaction temperature before use.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR products, resulting in data inaccuracy. Therefore, we recommend two distinctly separated working areas in the laboratory for PCR reaction preparation and PCR product cleanup tests. Use designated pipettes and equipments for each area and execute regular cleaning (with 0.5% sodium hydrochloride or 10% bleach) to ensure a clean and sterile working environment.
- Do not consume any sample or reagent, and avoid direct contact of reagents with skin and eyes. In case of an accident, immediately wash the affected area thoroughly with a large amount of water. Seek emergency medical assistance if needed.

CHAPTER 2
SAMPLE PREPARATION

2.1. Sample Requirements for Fresh Frozen Tissue

To avoid RNA degradation, we recommend performing tissue embedding (for fresh frozen tissue) or PFA sample processing (for PFA fixed tissue) **within 30 min** upon harvesting.

The tissue size should not exceed **0.9 cm x 0.9 cm x 2 cm**, as the tissue section should not exceed 80% area coverage of the chip.



Sample Types

This set of kits can be used for samples from all common animals, including but not limited to human, monkey, and mouse.

For details, please refer to the list: **https://en.stomics.tech/resource/stomics-validated-tissue-list?lang=en#**

Fresh Frozen Sample RNA Integrity Number (RIN) Value

It is recommended to check the RNA quality (RIN value) of a tissue sample before proceeding to the Stereo-seq experiment. Total RNA can be extracted from 10-20 slices of 10 μm-thick tissue sections and stored at -20°C in a pre-cooled 1.5mL EP tube. Please refer to the figure below (Figure 1) for the peak of RNA RIN value in mouse brain tissue sections.

It is strongly recommended to proceed only with tissue samples with a RIN value ≥7.

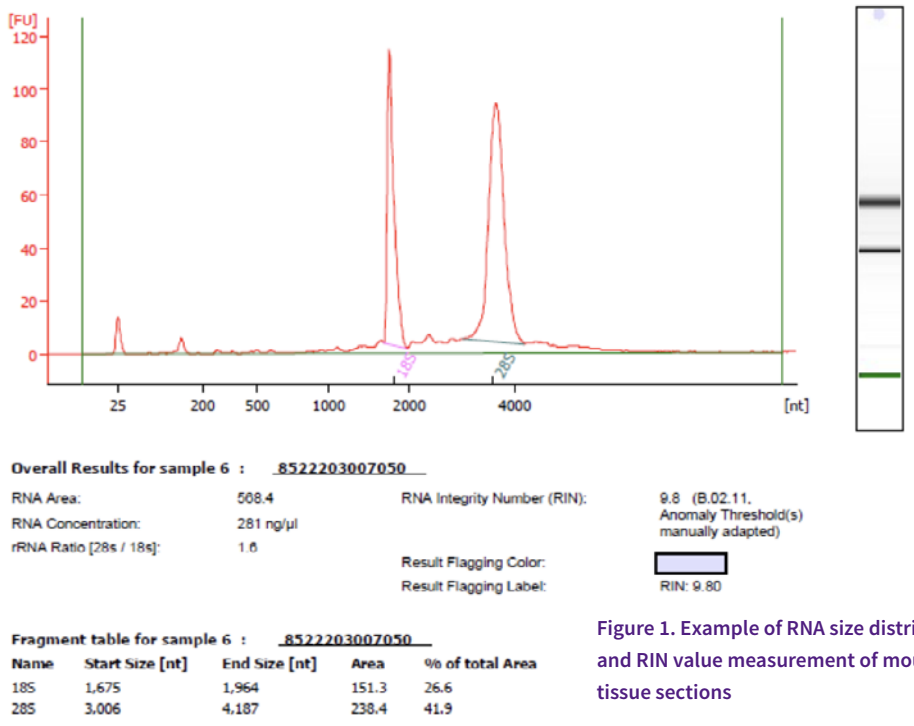


Figure 1. Example of RNA size distribution and RIN value measurement of mouse brain tissue sections

2.2. Sample Requirements for PFA Fixed Tissue

It is recommended to only fix samples using PFA when fresh frozen samples can not be used on the Stereo-seq workflow.

Sample Types

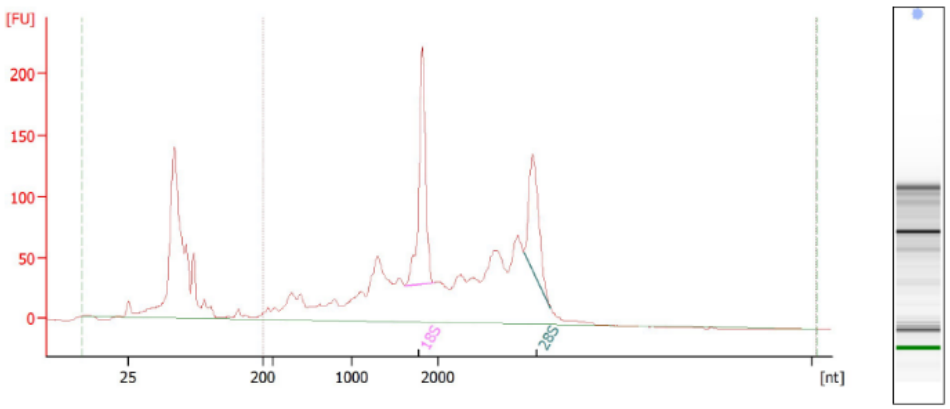
This set of kits can be used for samples from all common animals, including but not limited to human, monkey, and mouse.

For details, please refer to the list: https://en.stomics.tech/resource/stomics-validated-tissue-list?lang=en#

PFA Sample RNA Integrity Number (RIN) Value

It is recommended to check the RNA quality (RIN value) of a PFA tissue sample before proceeding to the Stereo-seq experiment. Total RNA can be extracted from 5-10 slices of 10 μm -thick tissue sections and stored at -20°C in a pre-cooled 1.5mL EP tube using **Qiagen RNeasy FFPE kit (Cat. No.: 73504)** for RNA extraction (follow the protocols but skip the deparaffinization step). Please refer to the figure below (Figure 2) for the peak of RNA RIN value in PFA processed mouse brain tissue sections.

It is strongly recommended to proceed only with tissue samples with a RIN value ≥5.



Overall Results for sample 2 :	Sample 2		
RNA Area:	2,414.8	Result Flagging Color:	
RNA Concentration:	32,832 pg/µl	Result Flagging Label:	RIN: 6.10
rRNA Ratio [28s / 18s]:	0.6	Corr. Area 1:	1,764.2
RNA Integrity Number (RIN):	6.1 (B.02.10)		

Figure 2. Example of RNA size distribution and RIN value measurement of PFA processed mouse brain tissue sections

2.3. PFA Sample Processing

a. Prepare these apparatuses/materials in advance:

- 4% paraformaldehyde, place it at 4°C one day in advance.
- Prepare 1X PBS (with DEPC water, if not available, choose nuclease-free water) and set aside at 4 °C.

Table 1 Preparation of 1X PBS Solution

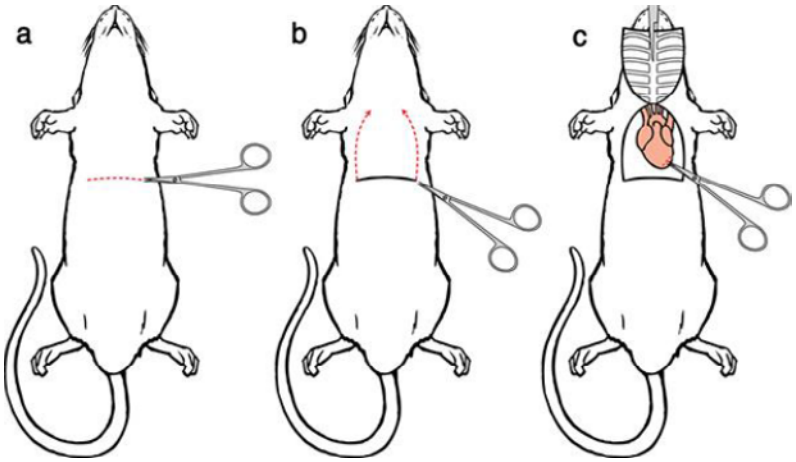
Volume	10mL	100mL
10X PBS	1mL	10mL
DEPC water/ nuclease-free water	9mL	90mL

- According to the number and size of tissues, choose a suitable container to pour the PFA solution with the volume ratio of PFA to tissue greater than 10:1, then place the container on ice ready for use.
- Clean the tips of the forceps and scissors with 75% alcohol spray and dry them with dust-free paper.

b. Rinse the fresh tissue in 1X PBS solution **within 30 min** of tissue harvest.

c. Clean as much blood as possible and transfer the tissue to a container filled with PFA solution; if the tissue type is prone to deformation, such as brain tissues, please perfuse the tissue before harvesting. Here, we take a 5-week-old mouse heart as an example:

After anesthesia, insert the needle in the direction of the cardiac axis and pierced about 3~4 mm into the apical part of the heart. Once the perfusion starts, cut the right auricle, and perfuse with 15 mL of 1 x PBS to clean out the blood, and then perfuse with 25 mL of PFA solution, followed by tissue harvest and fixation.



Reference: http://europepmc.org/article/PMC/3476408#figures-and-tables

Please use an RNase-free container with a flat-bottom and sufficient volume to contain PFA solution and the tissue. If you use a pointed-bottom centrifuge tube, you can place the tube upside down.

d. Place the container with tissue and PFA solution in the refrigerator at 4 °C for **16-18 hr**.

e. Prepare 10%, 20% and 30% sucrose solution with 1X PBS and place them in a 4°C refrigerator until use. Prepare different concentrations of sucrose to be added to the new containers, with a solution to tissue volume ratio greater than 10:1. Here we use 100 mL volume as an example:

Table 2 Sucrose solution preparation at different concentrations

Total Volume	100mL	100mL	100mL
Concentration	10%	20%	30%
Sucrose (g)	10	20	30

When preparing the sucrose solution, make sure to weigh sucrose first, then add 1X PBS to dissolve.

f. Pre-cool 1X PBS. Carefully take the tissue out of the PFA solution in a fume hood and rinse in cold 1X PBS solution. Transfer the tissue to a new container containing 10% sucrose solution with a volume ratio of solution to tissue greater than 10:1 and place in a refrigerator at 4 °C for **6-8 hr**. The tissue should sink to the bottom.

g. Transfer the tissue to the 20% sucrose solution with a volume ratio of solution to tissue greater than 10:1 and leave it overnight in a 4 °C refrigerator. The tissue should sink to the bottom.

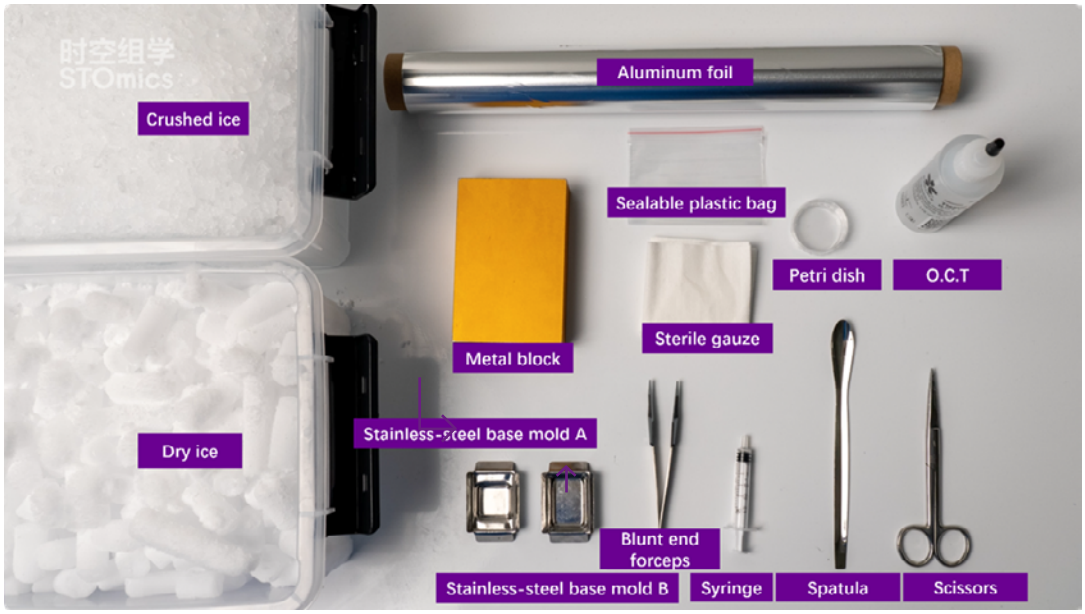
h. Transfer the tissue to the 30% sucrose solution with a volume ratio of solution to tissue greater than 10:1 and place in a refrigerator at 4 °C for **26-30 hr**. During the dehydration process, the tissues could be gently shaken at intervals, and a lab rocker could be used to prevent the tissues from being squeezed and deformed in the container.

Observe the state of the tissue, tissue sedimentation means that the current dehydration step is completed; if the tissue did not sink to the bottom overnight, try to shake it gently and record the details in time, then proceed to the next step.

2.4. Sample Embedding for FF and PFA Samples

For a demonstration video of tissue embedding, please refer to the link or by scanning the QR code: **https://en.stomics.tech/resources/videos/list.html**

a. Prepare these apparatuses/materials in advance:



Materials		
Brand	**Description**	**Quantity**
-	Crushed ice in a box	1
-	Dry ice in a box	1
-	Aluminum foil	1
-	Sealable plastic bag	1
BIOSHARP/Metal Coolbox/ BC032	Metal Block	1
-	Sterile gauze	2
Corning	Corning® 35 mm TC-treated Culture Dish (353001)	1
Sakura/Base Molds/4583	O.C.T	1
Sakura/Base Molds/4162	Stainless-steel base mold A	1
Sakura/Base Molds/7055	Stainless-steel base mold B (slightly larger than mold A)	1
-	Blunt end forceps	1
-	Syringe	1
-	Spatula	1
-	Scissors	1

a1. A box of crushed ice and pre-cool OCT on ice for **10 min** in advance.

a2. **2** pieces of stainless-steel base molds slightly larger than the tissue of your interest - mold A and mold B (slightly larger than mold A).

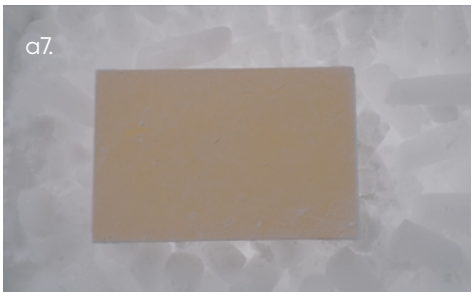
a3. Add a few drops of pre-cooled OCT in the mold A until it reaches approximately 2/3 of the mold and pre-cool on ice for **> 10 min** (remove introduced air bubbles using a syringe).

a4. A petri dish filled with OCT and pre-cool it on ice for **> 10 min** (remove introduced air bubbles using a syringe).

a5. A box of dry ice.

a6. A metal block that has a flat surface to support the stainless-steel base mold when placed on dry ice. The size of the metal block should be larger than the stainless-steel base mold.

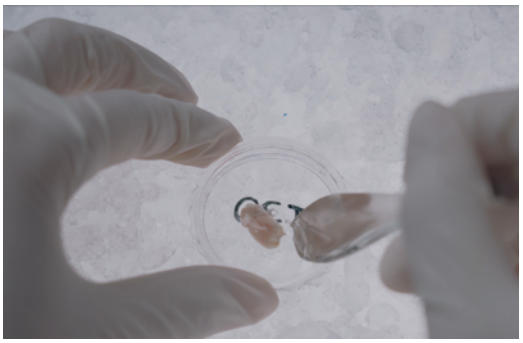
a7. Place the metal block on dry ice and pre-cool for **> 5 min** with the flat surface facing up.



a8. Place mold B on dry ice and pre-cool for **> 5 min**.

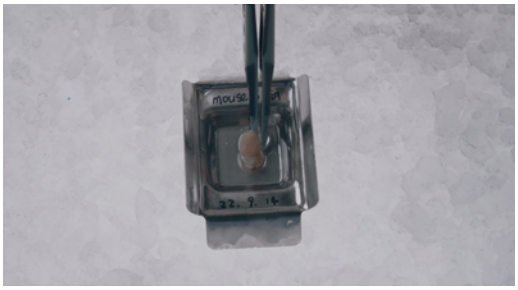
b. Upon harvesting within **30 min**, use sterile gauze or dust-free paper to absorb excess liquid on the tissue surface to avoid ice formation in later steps.

c. Place the tissue in pre-cooled OCT and wrap the tissue evenly with OCT using a spatula without introducing air bubbles.

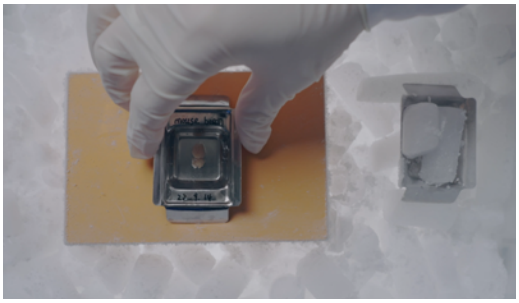


d. Remove any air bubbles using a syringe.

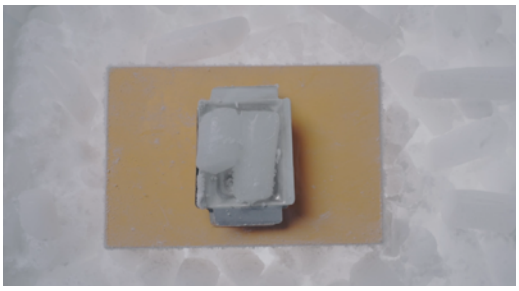
e. Orient the tissue to have the side intended to be sectioned facing downwards and then place into mold A. Make sure the tissue is at the bottom of mold A and fill the mold with chilled OCT without introducing bubbles until the tissue is fully covered.



f. Place the tissue containing mold A onto the metal block that was placed on dry ice.



g. Use mold B as a lid with opening facing up, place on top of mold A gently and then place a few dry ice cubes on top of mold B. Make sure the two stainless-steel base molds can be covered with enough dry ice cubes.



h. After **5 min**, remove mold B and check if the OCT is completely frozen and has turned opaque, otherwise repeat f.

i. If the tissue block has solidified and turned opaque, grip the two edges of mold A and press down the edges to detach the tissue block from the mold.

j. Check if the sectioning side of the tissue has been completely covered by OCT. If not, place the tissue block on the metal block, sectioning side facing up, add a few drops of the OCT and then wait till it solidifies and turns opaque.

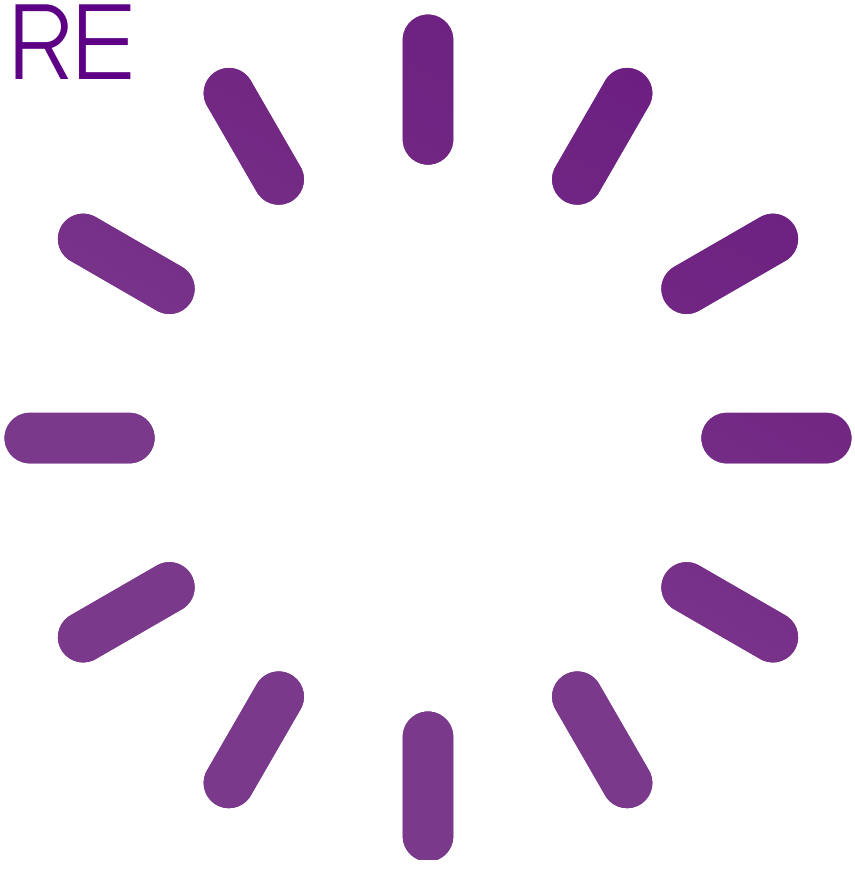
k. Label the tissue block to mark the orientation of the tissue.

2.5. Sample Storage and Transportation

For storing, wrap the tissue block with aluminum foil and keep it in a properly labeled sealable plastic bag to prevent dehydration and damage then store at -80°C. For transportation, please ship samples on dry ice according to local policy.

CHAPTER 3

FRESH FROZEN SAMPLE, Stereo-seq PERMEABILIZATION SET FOR CHIP-ON-A-SLIDE STANDARD OPERATING PROCEDURE



3.1. Experimental Preparation

Unless otherwise specified, nuclease-free water is used for all reagents being prepared prior to this experiment.

Reagent	Preparation Steps	Maintenance
5X SSC (mIF application)	Dilute 5 mL of 20x SSC to 20 mL	Room Temperature
0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL; Dilute 250 µL of 20x SSC to 50 mL	Room Temperature
Wash Buffer	Prepare at least 100 µL per chip (95 µL 0.1X SSC with 5 µL RI), and prepare at least 500 µL for each permeabilization optimization experiment (475 µL 0.1X SSC with 25 µL RI)	On ice until use
0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Configure HCl to 0.01N. Measure and make sure the pH = 2.	Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Please use WITHIN 48 hr of preparation)**
ALWAYS use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.		
10X Permeabilization Reagent Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent through pipetting.	-20°C
DO NOT vortex the permeabilization enzyme. Mix by pipette before using. **Aliquot this 10X stock solution to avoid freeze-thaw cycles.**		
1X Permeabilization Reagent Solution	Make 1X PR solution (150 µL / chip) by diluting 10X PR stock solution with 0.01N HCl.	On ice until use, up to 6 hr
Aliquot Serum (mIF application)	Thaw serum, mix Horse Serum and Goat Serum in a ratio of 1:1, then filter it with a 0.22µm pore-sized filter and a sterilized syringe. Aliquot the filtered serum and store at -20°C.	-20°C
DO NOT freeze and thaw the aliquot more than 3 times.		
Eosin Solution (H&E application)	Dissolve 0.013g Eosin Y powder in 25 mL methanol and keep sealed with a parafilm until use.	Room temperature up to 1 month

Hematoxylin Solution (H&E application)	Prepare and filter the hematoxylin solution using a 0.22μm pore-sized filter.	Room Temperature
H&E Mounting Medium (H&E application)	3.5 μL per chip	Room Temperature

Other Preparation		
Equipments	**Set up**	**Note**
Cryostat	Set the cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.	The specimen disc temperature depends on the tissue type.
PCR Thermal Cycler	Set the temperature in the following order: 37°C for slide drying and permeabilization (heating lid at 42°C); 42°C for reverse transcription (heating lid at 47°C); 55°C for tissue removal (heating lid at 60°C).	Check if there is any abnormality with the PCR Thermal Cycler and replace it if necessary.
Fluorescence Microscope	Set the epi-fluorescence channel to TRITC mode.	Room Temperature

3.2. Cryosection Preparation

a. Set PCR thermal cycler to 37°C with heated lid set to 42°C in advance with a PCR adaptor.

b. Set cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.

If the specimen disc is over-cooled, it could lead to tissue section cracking during sectioning, while sections would wrinkle when the disc temperature is too high. Optimal specimen disc temperature depends on the tissue type.

c. Place forceps, brushes, and razor blades inside the chamber for pre-cooling.

d. Take the OCT-embedded tissue sample out of the -80°C freezer and place it in the cryostat chamber for **30 min** to allow it to equilibrate to cryostat chamber temperature.

e. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block to the appropriate size (sectioning area smaller than 0.9 cm x 0.9 cm).

f. By using OCT, mount the embedded tissue block onto the specimen disc/holder of the cryostat chamber.

g. Do a final trimming if necessary to ensure a good fit between the tissue section and Stereo-seq Chip P. Now, the specimen is ready for cryosection.

3.3. Tissue Mounting

For a demonstration video of tissue mounting onto the Stereo-seq Chip Slide, please refer to the link or by scanning the QR code:
https://en.stomics.tech/resources/videos/list.html

a. Take the Chip P Slide out of the vacuum sealed aluminum bag and record Chip ID (SN) number that is on the back side of the slide. Make sure to not touch the front side of the chip.

Once opened, please check if all the Stereo-seq Chip Slides in the slide container are well orientated with the front-side facing upward. The front-side of a chip has a shiny surface which contains DNB-probes for mRNA capture. DO NOT scratch the surface.

b. Make sure the PCR thermal cycler has been turned on and set to 37°C.

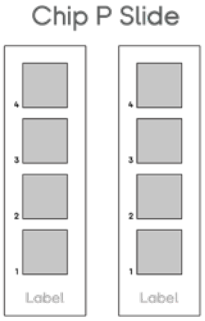
c. Equilibrate Stereo-seq Chip Slide to room temperature for **1 min** on the benchtop, then rinse with 100 µL nuclease-free water **twice** with a pipette or rinse the slide up and down **twice** in a 50 mL corning tube with enough nuclease-free water.

Store unused slides in original packaging (first in the slide container and then the sealable aluminum bag) and keep sealed at -25°C ~ 8°C. KEEP THE DESICCANT IN THE ALUMINUM BAG.

d. Remove excess water on the chip by blowing gently with a power dust remover (MATIN, M-6318) from one side of the chip at a 30~45-degree angle horizontal to the plane of the chip. Wipe excess water around the chip and on the slide with dust-free paper.

e. Only when the chip is completely dry and without wavy white stains is it ready for tissue mounting.

f. Prepare enough methanol in a 50 mL corning tube or an empty slide container at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the methanol containing tube to check if the volume is enough. Close the lid and pre-cool methanol for **5-30 min** at -20°C.



【H&E applications only】: Prepare **three** empty slide containers or **three** 50 mL centrifuge tubes, and add enough methanol to two of the three containers and enough eosin solution to the third container at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the methanol or eosin containing tube to check if the volume is enough. Close the lid and pre-cool the methanol and eosin solution for **5-30 min** at -20°C.

g. Tissue mounting could be achieved via either **cold method** (option A) or **warm method** (option B). We recommend practicing tissue mounting and section placement on plain glass slides first.

A. Cold Method

1) Place Stereo-seq Chip Slide inside the chamber with the front-side facing up and pre-cool inside the cryostat chamber for **1~6 min**.

Prolonged cooling for over 6 min may cause mist formation on the chip surface.

2) Perform cryosection, then carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes. Place the tissue section onto the chip center carefully with forceps and brushes. Make sure the tissue section is complete and without wrinkles.

3) Immediately pick up the Stereo-seq Chip Slide and place a finger on the backside of the Stereo-seq Chip Slide directly under the chip for a few seconds to allow the section to adhere to the chip.

4) Place the tissue mounted Stereo-seq Chip Slide back inside the chamber and move on to the second tissue slicing and mounting. Continue transferring sections on remaining chips.

5) Once all tissue mounting is completed, immediately dry the Stereo-seq Chip Slide at 37°C on a PCR thermal cycler with PCR adaptor for **5 min** (without heated-lid).

When performing cold mounting, mind the time interval between each tissue section placement. Longer time intervals (>5 min) could result in tissue wrinkle formation.

B. Warm Method

1) Perform cryosection and obtain two or four consecutive tissue sections (depending on the number of chips on the Stereo-seq Chip Slide), carefully flatten the tissue sections out by gently touching the surrounding OCT with cryostat brushes.

2) Move the tissue sections to the edge and place each tissue section at a distance greater than the chip spacing on the Stereo-seq Chip Slide.

3) Flip the Stereo-seq Chip Slide and aim the tissue section within a chip area on the Stereo-seq Chip Slide by gently touching the section with the front-side of the chip.

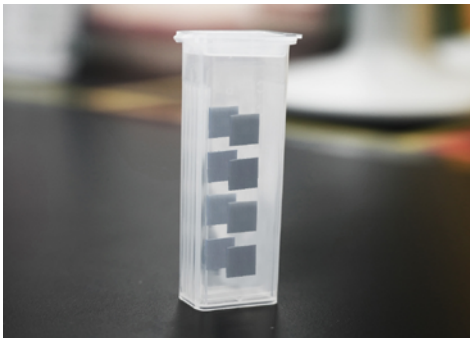
4) Repeat step 3) until all the tissue sections have been mounted on to the chips of the Stereo-seq Chip Slide.

5) Turn the Stereo-seq Chip Slide over, and immediately dry it on a PCR thermal cycler at 37°C with PCR adaptor front-side up for **5 min** (without heated-lid).

If two different tissue blocks need to be cryosectioned and mounted on to the same Stereo-seq Chip Slide, it is recommended to first trim both tissue blocks beforehand. Perform tissue sectioning and mounting for one tissue block first with the warm method, and then place the tissue mounted Stereo-seq Chip Slide on the PCR thermal cycler for no longer than 5 min while preparing for the second tissue block. Perform tissue section and mount the second tissue block using the warm method, then place the tissue mounted Stereo-seq Chip Slide on the PCR thermal cycler to dry for 5 min.

Stop Point:

- **After drying the tissue containing Stereo-seq Chip Slides on a PCR Thermal Cycler, they can be stored in a slide container then transferred to a −80°C freezer on dry ice.**
- **Store the sealed slide container containing Stereo-seq Chip Slides with tissue at −80°C for up to four weeks.**
- **When retrieving Stereo-seq Chip Slides with tissue from the freezer, transfer out the slide container on dry ice, and take out the tissue containing Stereo-seq Chip Slides then immediately incubate at 37°C with PCR Adaptor for 5 min.**



Shipping Guidance for Tissue Mounted Stereo-seq Chip Slide:

- After drying the tissue containing Stereo-seq Chip Slides on a PCR Thermal Cycler, transfer the Stereo-seq Chip Slide into a slide container then place it in a sealable plastic bag. Place one desiccant pack per Stereo-seq Chip Slide into a ziplock bag, push out as much air as possible and seal the bag tightly.
- Prepare a styrofoam shipping container filled with dry ice. Allow 0.5kg of dry ice for every 2 hours in transport. For example, for a 3-day shipping duration, 0.5kg * 12hr * 3 = 18kg of dry ice can be prepared.
- Place the ziplock bag at the bottom layer of the shipping box. If necessary, use sheets of bubble wrap to ensure the slide container remain in a vertical position.
- Fill empty space in the box with bubble wrap or paper. This will help prevent shifting of the slide container when the ice dissipates.
- Close the styrofoam lid. DO NOT tape the styrofoam lid to the box.
- Secure the outer lid of the shipping box with tape. When using dry ice, it is recommended to leave an air gap when taping to ensure that carbon dioxide can be released. This can prevent a buildup of pressure that could rupture the package.
- Label the shipping box with a dry ice sticker and then stick the pre-print label with both recipient address and return address. Contact the recipient lab before shipping to ensure that the staff is prepared to receive the shipment.
- - When retrieving the Stereo-seq Chip Slides with tissue from the carrier, please store at −80°C (less than 4 weeks from mounting) until you are ready to process the slide. Before processing, please incubate the slide at 37°C for 5 minutes, then proceed with tissue fixation.

Shipping guidance is written based on STOmics in-house validation and general shipping guidance provided by CDC www.cdc.gov.

3.4. Tissue Fixation

For tissue samples that will be processed with Stereo-seq Transcriptomics mIF workflow, tissue blocking and mock antibody incubation steps need to be done prior to Tissue Permeabilization Testing for obtaining a more accurate permeabilization time. Please refer to Appendix B for detailed procedures.

For tissue samples that will be stained with H&E during Stereo-seq Transcriptomics H&E workflow, Eosin solution staining, tissue fixation, and Hematoxylin solution staining need to be done prior to Tissue Permeabilization Testing for obtaining a more accurate permeabilization time. Please refer to Appendix D for detailed procedures.

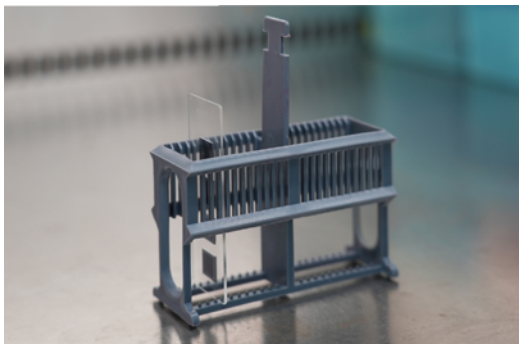
a. After drying, immediately immerse the tissue-mounted Stereo-seq Chip Slide in pre-cooled methanol prepared in section 3.3-f for a **30-min** fixation at -20°C. When immersing the Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

b. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

c. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

d. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to let the methanol fully evaporate.

e. Meanwhile, assemble the Cassette and Gasket (do not assemble the Stereo-seq Chip Slide yet) according to guidance written in 1.4 Practice Tips and pre-warm the assembled Cassette and Gasket on a 37°C PCR thermal cycler for 10 min.



f. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide on to a flat and clean bench top surface.

g. Assemble the Cassette and Gasket then place the Stereo-seq Chip Slide in the Cassette according to guidance written in 1.4 Practice Tips. It is recommended to practice with a regular blank glass slide.

h. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

Make sure to not touch the front-side of the chip while assembling the Stereo-seq Slide Cassette.

3.5. Tissue Permeabilization Testing

For positive control, total RNA or mouse brain tissue (incubate at 37°C for 12 min) can be used. Details are illustrated below:

a. Set aside the **2 mL** of 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in 3.1 Experimental Preparation.

b. Make sure your PCR thermal cycler has been switched on and set to 37°C and the heating lid has been set to 42°C. Place the PCR adaptor in the PCR thermal cycler, then close the heated lid to pre-heat the adaptor for **3 min**.

Temperature	Time	Cycle
(Heated lid) 42°C	on	-
37°C	60 min	1
37°C	Hold	-

c. Warm up the aliquoted 1X permeabilization Reagent Solution inside the 37°C PCR thermal cycler or Metal Block for **>10 min (no longer than 30 min).** Warm up the Stereo-seq Slide Cassette in the 37°C PCR Thermal Cycler for **3 min**.

d. Thaw RT QC Reagent, RT Additive, and RT QC Enzyme on ice.

e. Tissue sections on the Stereo-seq Chip P Slide are incubated for different lengths of time ranging from **0-30 min**. For the first trial, it is recommended to use a suggested time course of **6 min, 12 min, 18 min and 24 min (4 time points, 6-min interval) along with a positive control group (mouse brain tissue or total RNA).**

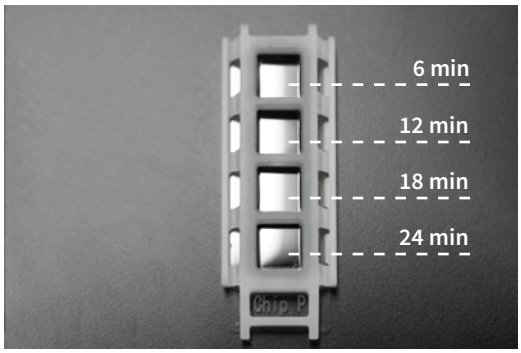


Figure 3. Permeabilization Times (min)

1. Place the Stereo-seq Slide Cassette in the 37°C PCR Thermal Cycler. Add **150 µL** of 1X Permeabilization Reagent Solution onto the chip (with **24-min** time point) by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets.

Make sure the chip is completely covered with 1X Permeabilization Reagent Solution.

2. Place unpeeled sealing tape on to the Stereo-seq Slide Cassette and let the chip incubate inside the PCR thermal cycler at 37°C.

3. After **6 min**, open the lid, remove the unpeeled sealing tape and add **150 µL** of 1X Permeabilization Reagent Solution on the chip (with **18-min** time point) along the side of the well.

4. Place unpeeled sealing tape, close the lid and incubate at 37°C.

5. Repeat the process, working backwards to the shortest incubation time (chip with **3-min** time point).

A second trial of permeabilization time determination (3 more time points) might be required depending on the first trial's results. If multiple time points show similar fluorescent signals, we suggest choosing longer time points for your second trial.

f. For total RNA as positive control:

1) Prepare the total RNA hybridization mixture as indicated in Table 3-1.

Table 3-1 Total RNA hybridization Mix

Components	1X (µL)
Total RNA	X (2µg)
Nuclease-free water	70-X
20X SSC	25
RI	5
Total	100

2) Warm up the Total RNA hybridization Mix inside the 37°C PCR thermal cycler for **>3 min**.

3) Add **100 µL** of total RNA hybridization mixture onto a chip and incubate at 37°C for **15-20 min**.

Tissue removal is not needed for total RNA as positive control group.

g. Once incubation is completed, remove the Stereo-seq Slide Cassette from the PCR Adaptor.

h. Slightly tilt the Stereo-seq Slide Cassette, remove 1X Permeabilization Reagent Solution or total RNA hybridization mixture with a pipette from the corner of each well without touching the chip surface.

i. Add 100 µL of Wash Buffer and then remove the solution from the corner of each well.

j. Place the PCR Adaptor in another PCR Thermal Cycler in advance and set the temperature to 42°C with heated lid set to 47°C.

k. Prepare RT QC mix according to Table 3-2 and equilibrate to room temperature **in the dark.**

Table 3-2 RT QC Mix

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
RT QC Reagent	85	187	280.5	374
RT Additive	5	11	16.5	22
RT QC Enzyme	5	11	16.5	22
RI	5	11	16.5	22
Total	100	220	330	440

3.6. Reverse Transcription

a. Make sure the temperature of the PCR Thermal Cycler with PCR Adaptor has been set to 42°C in advance.

b. Gently add **100 μL** of RT QC Mix per chip along the side of each well, ensuring that the well surface is uniformly covered with RT QC Mix.

c. Apply sealing tape on to Stereo-seq Slide Cassette and make sure it is sealed tightly. Incubate the Stereo-seq Slide Cassette at 42°C for **1 hr** or longer (no longer than 16 hr) **in the dark** with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 47°C	on	-
42°C	60 min	1
42°C	Hold	-

3.7. Tissue Removal

Prepare		
Reagent	**Preparation Steps**	**Storage**
TR buffer	Heat the buffer for **5 min** at 55 °C to dissolve the precipitate. Equilibrate it to room temperature prior to use.	Room Temperature

If white precipitate is observed in the buffer, dissolve them by heating the buffer at 55 °C again and equilibrate to room temperature before mixing.

a. Check and make sure the PCR Thermal Cycler with PCR Adaptor has been set to 55°C and the heated lid has been set to 60°C.

b. Remove the Stereo-seq Slide Cassette from the 42°C PCR Adaptor and then remove the sealing tape.

When removing the sealing tape, hold on to the Stereo-seq Slide Cassette with one hand without applying forces to Side A and Side B of the cassette in order to prevent the Stereo-seq Chip Slide from falling off of the cassette.

c. Slightly tilt the Stereo-seq Cassette, remove RT QC Mix with a pipette from the corner of each well without touching the chip surface.

d. Add 400 µL 0.1X SSC solution into each well.

e. Gently pipette 0.1X SSC solution up and down at the corner of each well for 5 times.

f. Slightly tilt the Stereo-seq Cassette, remove 0.1X SSC with a pipette from the corner of each well.

g. Repeat step d.-f.

h. Prepare Tissue Removal Mix as shown in Table 3-3.

Table 3-3 Tissue Removal Mix

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
TR Buffer	392	862.4	1293.6	1724.8
TR Enzyme	8	17.6	26.4	35.2
Total	400	880	1320	1760

i. Add 400 µL of Tissue Removal Mix per well without introducing bubbles. Ensure uniform solution coverage within each well.

j. Apply sealing tape on the Stereo-seq Slide Cassette and incubate at 55 °C on the PCR Adaptor for **1 hr** with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 60°C	on	-
55°C	60 min	1
55°C	Hold	-

k. At the end of incubation, remove the Stereo-seq Slide Cassette from the PCR Adaptor and remove the sealing tape.

If tissue remains on the chip after tissue removal step, increase the incubation time (no longer than 16 hr). Make sure the tissue is completely removed.

l. Slightly tilt the Stereo-seq Cassette, remove Tissue Removal Mix with a pipette from the corner of each well.

m. Add 400 µL of 0.1X SSC solution into each well.

n. Gently pipette 0.1X SSC solution up and down at the corner of each well for 5 times.

o. Remove 0.1X SSC with a pipette from the corner of each well.

p. Repeat step m.-o.

q. Add 400 µL of nuclease-free water into each well and pipette up and down to wash the chip surface as SSC solution contains salt.

r. Remove the slide from the Stereo-seq Slide Cassette according to instructions in **1.4 Practice Tips**.

Alternative Step:

Remove the slide from the Stereo-seq Slide Cassette after step l. and rinse the Stereo-seq Chip Slide up and down 10 times in a 50 mL falcon tube filled with 50 mL 0.1X SSC, then rinse up and down 10 times with 50 mL nuclease-free water.

s. Place the Stereo-seq Chip Slide onto a clean dust-free paper and completely dry the chip surface with a power dust remover (MATIN, M-6318).

If obvious tissue traces still remain on the surface of the chip, wash again by adding 100 µL nuclease-free water then blow dry. This step can be repeated until no visible traces remain on the chip surface.

t. Place the Stereo-seq Chip Slide in a clean petri dish and wrap it with aluminum foil. The chips are now ready for imaging.

3.8. Imaging

a. Create a new folder in a fluorescent microscope-connected PC, name it with the chip ID number and other essential information.

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.
Example: B00249A1

b. Take fluorescence images from the chip with the following microscope setting: TRITC channel, 4X and 10X objective lenses, with stitching function.

c. Place 1-2 µL of water on the imaging platform first, then transfer and place the Stereo-seq Chip Slide onto the water drop. Water surface tension will grab onto the Slide and adhere it onto the imaging platform.

d. Remove the light shield and select the chip area of interest.

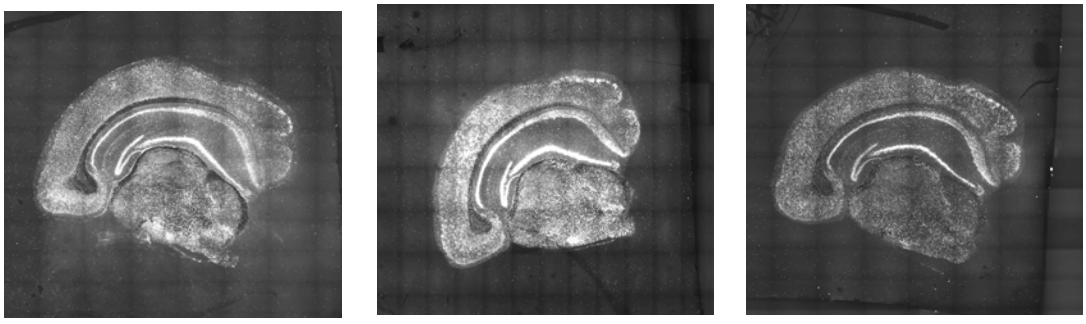
e. Find the desired capturing area with 4X lens first then switch to 10X lens to complete the full scan.

Make sure the desired capturing area is clear and within focus during full scanning.

Positive control with RNA extract should be imaged separately without modifying any parameters.

3.9. Permeabilization Time Determination

The optimal permeabilization time should result in the strongest fluorescence signal with the lowest signal diffusion. However, this is based on a complete tissue removal as well as images taken under the same settings. For example, as shown in Figure 4, for the **3 min** permeabilization time point, the fluorescence signal in some parts of the cortex is very low, suggesting insufficient permeabilization. For the **12 min** permeabilization time point, images showed the strongest signal and finer details among three groups. For the **24 min** permeabilization time point, the signal is lower than the 12 min time point. Based on this result, the optimal permeabilization time for this tissue is 12 min.



3 min 12 min 24 min

Figure 4. The optimal permeabilization time determination of a mouse brain coronal section

As shown in Figure 5 (with mock H&E staining steps), for the **6 min** permeabilization time point, the fluorescence signal has shown uneven brightness throughout the cortex layer, suggesting insufficient permeabilization. For the **12 min** permeabilization time point, images showed the strongest signal and finer details among three groups. For the **18 min** and **24 min** permeabilization time point, the signals are lower than the 12 min time point. Based on this result, the optimal permeabilization time for this tissue is 12 min.

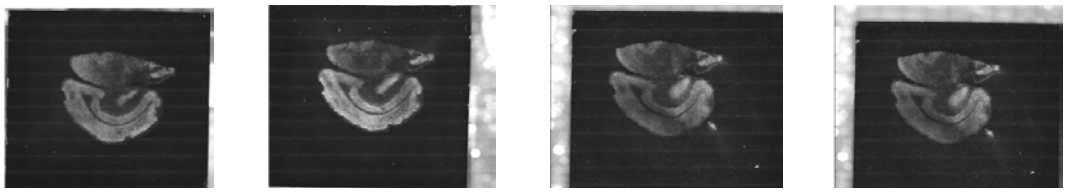


Figure 5. The optimal permeabilization time determination of a mouse brain coronal section (treated with H&E staining)

CHAPTER 4

PFA FIXED SAMPLE, Stereo-seq PERMEABILIZATION SET FOR CHIP-ON-A-SLIDE STANDARD OPERATING PROCEDURE

4.1. Experimental Preparation

For cyrosection preparation and tissue mounting on the Stereo-seq Chip Slide, please refer to section 3.2-3.3 in Chapter 3.

Unless otherwise specified, nuclease-free water is used for all reagents being prepared prior to this experiment.

Reagent	Preparation Steps	Maintenance
5X SSC (mIF application)	Dilute 5 mL of 20x SSC to 20 mL	Room Temperature
0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL; Dilute 250 µL of 20x SSC to 50 mL	Room Temperature
Wash Buffer	Prepare at least 100 µL per chip (95 µL 0.1X SSC with 5 µL RI).	On ice until use
Decrosslinking Buffer	Prepare the decrosslinking buffer by adjusting the pH of TE buffer to 10±0.05 using NaOH. It is recommended to add 155±10 µL of 2M NaOH.	Room temperature up to 1 week
For pre-made decrosslinking buffer, check the pH before each use.		
0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Configure HCl to 0.01N. Measure and make sure the pH = 2	Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Please use WITHIN 48 hr of preparation)**
ALWAYS use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.		
10X Permeabilization Reagent Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent through pipetting.	-20°C
DO NOT vortex the permeabilization enzyme. Mix by pipette before using. **Aliquot this 10X stock solution to avoid freeze-thaw cycles.**		
1X Permeabilization Reagent Solution	Make 1X PR solution (150 µL / chip) by diluting 10X PR stock solution with 0.01N HCl.	On ice until use, up to 6 hr
Aliquot Serum (mIF application)	Thaw serum, mix Horse Serum and Goat Serum in a ratio of 1:1, then filter it with a 0.22µm pore-sized filter and a sterilized syringe. Aliquot the filtered serum and store at -20°C.	-20°C

Other Preparation		
Equipments	**Set up**	**Note**
Cryostat	Set the cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.	The specimen disc temperature depends on the tissue type.
PCR Thermal Cycler	Set the temperature in the following order: 37°C for slide drying and permeabilization (heating lid at 42°C); 70°C for decrosslinking (heating lid at 75°C); 42°C for reverse transcription (heating lid at 47°C); 55°C for tissue removal (heating lid at 60°C).	Check if there is any abnormality with the PCR Thermal Cycler and replace it if necessary.
Fluorescence Microscope	Set the epi-fluorescence channel to TRITC mode.	Room Temperature

4.2. Tissue Fixation

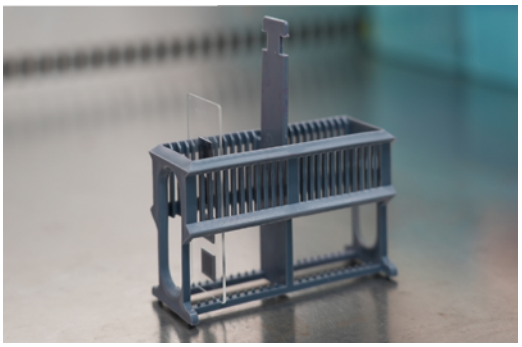
a. After drying, immediately immerse the tissue-mounted Stereo-seq Chip Slide in pre-cooled methanol prepared in section 3.3-f for a **15-min** fixation at -20°C. When immersing Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

b. Make sure your PCR thermal cycler has been switched on and set to 70°C and the heating lid has been set to 75°C. Place the PCR adaptor in the PCR thermal cycler, then close the heated lid to pre-heat the adaptor for **3 min**.

c. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

d. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

e. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to let the methanol fully evaporate.



f. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide on to a flat and clean bench top surface.

g. Assemble the Cassette and Gasket then place the Stereo-seq Chip Slide in the Cassette according to guidance written in 1.4 Practice Tips. It is recommended to practice with a regular blank glass slide.

h. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

Make sure to not touch the front-side of the chip while assembling the Stereo-seq Slide Cassette.

4.3. Tissue Decrosslinking

For PFA tissue samples that will be processed with Stereo-seq Transcriptomics mIF workflow, tissue blocking and mock antibody incubation steps need to be done prior to Tissue Permeabilization Testing for obtaining a more accurate permeabilization time. Please refer to Appendix C for detailed procedures.

a. Add 5% RI to the Decrosslinking Buffer prepared in 4.1 Experimental Preparation (500 µL per chip), make sure the pH = 10. Slowly add the decrosslinking buffer with 5% RI into the wells containing the chip (pH=10).

b. Seal with sealing tape and place the Stereo-seq Slide Cassette on to the PCR Adaptor. Close the lid and incubate at 70°C for **1 hr**.

Temperature	Time	Cycle
(Heated lid) 75°C	on	-
70°C	60 min	1
70°C	Hold	-

c. 【Second Methanol Fixation】 Prepare enough methanol in a 50 mL corning tube or an empty slide container at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the methanol containing tube to check if the volume is enough. Close the lid and pre-cool the methanol for **5-30 min** at -20°C.

Do not reuse the methanol from the previous step after tissue mounting.

d. Remove the Stereo-seq Slide Cassette from the 70°C PCR Adaptor and then remove the sealing tape. Remove decrosslinking buffer (with 5% RI) with a pipette from the corner of each well without touching the chip surface.

When removing the sealing tape, hold on to the Stereo-seq Slide Cassette with one hand without applying forces to Side A and Side B of the cassette in order to prevent the Stereo-seq Chip Slide from falling off of the cassette.

e. Remove the slide from the Stereo-seq Slide Cassette according to instructions in 1.4 Practice Tips and allow the slide to sit at room temperature for **1 min**.

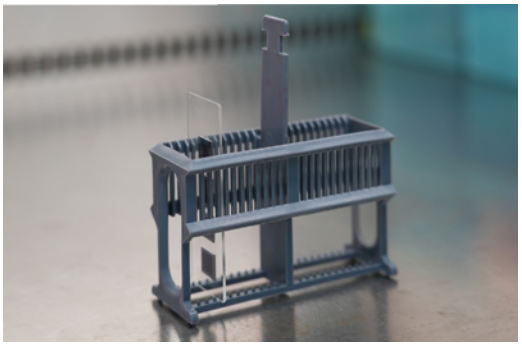
f. Then immerse the Stereo-seq Chip Slide in pre-cooled methanol for a second time fixation at -20°C for **15 min**. When immersing Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

Set aside the removed Stereo-seq Cassette and Gasket for later.

g. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

h. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

i. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to let the methanol fully evaporate.



j. Meanwhile, assemble the Cassette and Gasket (do not assemble the Stereo-seq Chip Slide yet) according to guidance written in 1.4 Practice Tips and pre-warm the assembled Cassette and Gasket on a 37°C PCR thermal cycler for 10 min.

k. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide on to a flat and clean bench top surface.

l. Assemble the Cassette and Gasket then place the Stereo-seq Chip Slide in the Cassette according to guidance written in 1.4 Practice Tips. It is recommended to practice with a regular blank glass slide.

Make sure there is no liquid residual on the Gasket. Blow dry the Gasket with a power dust remover if necessary.

m. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

Make sure to not touch the front-side of the chip while assembling the Stereo-seq Slide Cassette.

4.4. Tissue Permeabilization Testing

For positive control, total RNA or mouse brain tissue (incubate at 37°C for 12 min) can be used. Details are illustrated below:

a. Set aside the **2 mL** of 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in 4.1 Experimental Preparation.

b. Make sure your PCR thermal cycler has been switched on and set to 37°C and the heating lid has been set to 42°C. Place the PCR adaptor in the PCR thermal cycler, then close the heated lid to pre-heat the adaptor for **3 min**.

Temperature	Time	Cycle
(Heated lid) 42°C	on	-
37°C	60 min	1
37°C	Hold	-

c. Warm up the aliquoted 1X permeabilization Reagent Solution inside the 37°C PCR thermal cycler or Metal Block for **>10 min** (no longer than 30 min).

d. Thaw RT QC Reagent, RT Additive and RT QC Enzyme on ice.

e. Tissue sections on the Stereo-seq Chip P Slide are incubated for different lengths of time ranging from **0-30 min**. For the first trial, it is recommended to use a suggested time course of **6 min, 12 min, 18 min and 24 min (4 time points, 6-min interval) along with a positive control group (mouse brain tissue or total RNA).**

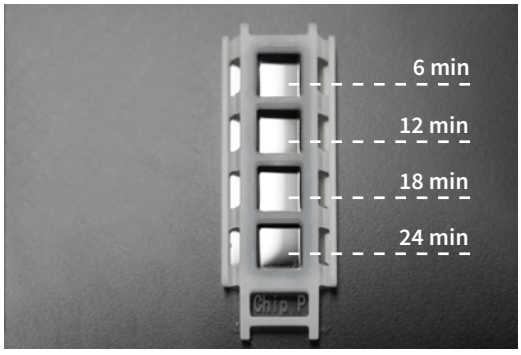


Figure 5. Permeabilization Times (min)

1. Place the Stereo-seq Slide Cassette in the 37°C PCR Thermal Cycler. Add **150 µL** of 1X Permeabilization Reagent Solution onto the chip (with **24-min** time point) by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets.

Make sure the chip is completely covered with 1X Permeabilization Reagent Solution.

2. Place unpeeled sealing tape on to the Stereo-seq Slide Cassette and let the chip incubate inside the PCR thermal cycler at 37°C.

3. After **6 min**, open the lid, remove the unpeeled sealing tape and add **150 µL** of 1X Permeabilization Reagent Solution on the chip (with **18-min** time point).

4. Place unpeeled sealing tape, close the lid and incubate at 37°C.

5. Repeat the process, working backwards to the shortest incubation time (chip with **3-min** time point).

A second trial of permeabilization time determination (3 more time points) might be required depending on the first trial's results. If multiple time points shown similar fluorescent signals, we suggest choosing longer time points for your second trial.

f. For total RNA as positive control:

1) Prepare the total RNA hybridization mixture as indicated in Table 4-1.

Table 4-1 Total RNA hybridization Mix

Components	1X (μL)
Total RNA	X (2μg)
Nuclease-free water	70-X
20X SSC	25
RI	5
Total	100

2) Warm up the Total RNA hybridization Mix inside the 37°C PCR thermal cycler for **>3 min.**

3) Add **100 μL** of total RNA hybridization mixture onto a chip and incubate at 37°C for **15-20 min.**

Tissue removal is not needed for total RNA as positive control group.

g. Once complete, remove the Stereo-seq Slide Cassette from the PCR Adaptor.

h. Slightly tilt the Stereo-seq Slide Cassette, remove 1X Permeabilization Reagent Solution or total RNA hybridization mixture with a pipette from the corner of each well without touching the chip surface.

i. Add 100 μL of Wash Buffer and then remove the solution from the corner of each well.

j. Place the PCR Adaptor in another PCR Thermal Cycler in advance and set the temperature to 42°C with heated lid set to 47°C.

k. Prepare RT QC mix according to Table 4-2 and equilibrate to room temperature **in the dark.**

Table 4-2 RT QC Mix

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
RT QC Reagent	85	187	280.5	374
RT Additive	5	11	16.5	22
RT QC Enzyme	5	11	16.5	22
RI	5	11	16.5	22
Total	100	220	330	440

4.5. Reverse Transcription

a. Make sure the temperature of the PCR Thermal Cycler with PCR Adaptor has been set to 42°C in advance.

b. Gently add **100 µL** of RT QC Mix per chip along the side of each well, ensuring that the well surface is uniformly covered with RT QC Mix.

c. Apply sealing tape on to Stereo-seq Slide Cassette and make sure it is sealed tightly. Incubate the Stereo-seq Slide Cassette at 42°C for **5 hr** or longer (no longer than 16 hr) **in the dark** with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 47°C	on	-
42°C	300 min	1
42°C	Hold	-

4.6. Tissue Removal

Prepare		
Reagent	**Preparation Steps**	**Storage**
TR buffer	Heat the buffer for **5 min** at 55 °C to dissolve the precipitate. Equilibrate it to room temperature prior to use.	Room Temperature

If white precipitate is observed in the buffer, dissolve them by heating the buffer at 55 °C again and equilibrate to room temperature before mixing.

a. Check and make sure the PCR Thermal Cycler with PCR Adaptor has been set to 55°C and the heated lid has been set to 60°C.

b. Remove the Stereo-seq Slide Cassette from the 42°C PCR Adaptor and then remove the sealing tape.

When removing the sealing tape, hold on to the Stereo-seq Slide Cassette with one hand without applying forces to Side A and Side B of the cassette in order to prevent the Stereo-seq Chip Slide from falling off of the cassette.

c. Slightly tilt the Stereo-seq Cassette, remove RT QC Mix with a pipette from the corner of each well without touching the chip surface.

d. Add 400 µL 0.1X SSC solution into each well.

e. Gently pipette 0.1X SSC solution up and down at the corner of each well for 5 times.

f. Slightly tilt the Stereo-seq Cassette, remove 0.1X SSC with a pipette from the corner of each well.

g. Repeat step d.-f.

h. Prepare Tissue Removal Mix as shown in Table 4-3.

Table 4-3 Tissue Removal Mix

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
TR Buffer	392	862.4	1293.6	1724.8
TR Enzyme	8	17.6	26.4	35.2
Total	400	880	1320	1760

i. Add 400 µL of Tissue Removal Mix per well without introducing bubbles. Ensure uniform solution coverage within each well.

j. Apply sealing tape on the Stereo-seq Slide Cassette and incubate at 55 °C on the PCR Adaptor for **1 hr** with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 60°C	on	-
55°C	60 min	1
55°C	Hold	-

k. At the end of incubation, remove the Stereo-seq Slide Cassette from the PCR Adaptor and remove the sealing tape.

If tissue remains on the chip after tissue removal step, increase the incubation time (no longer than 16 hr). Make sure the tissue is completely removed.

l. Slightly tilt the Stereo-seq Cassette, remove Tissue Removal Mix with a pipette from the corner of each well.

m. Add 400 µL of 0.1X SSC solution into each well.

n. Gently pipette 0.1X SSC solution up and down at the corner of each well for 5 times.

o. Remove 0.1X SSC with a pipette from the corner of each well.

p. Repeat step m.-o.

q. Add 400 µL of nuclease-free water into each well and pipette up and down to wash the chip surface as SSC solution contains salt.

r. Remove the slide from the Stereo-seq Slide Cassette according to instructions in 1.4 Practice Tips.

Alternative Step:

Remove the slide from the Stereo-seq Slide Cassette after step l. and rinse the Stereo-seq Chip Slide up and down 10 times in a 50 mL falcon tube filled with 50 mL 0.1X SSC, then rinse up and down 10 times with 50 mL nuclease-free water.

s. Place the Stereo-seq Chip Slide onto a clean dust-free paper and completely dry the chip surface with a power dust remover (MATIN, M-6318).

If obvious tissue traces still remain on the surface of the chip, wash again by adding 100 µL nuclease-free water then blow dry. This step can be repeated until no visible traces remain on the chip surface.

t. Place the Stereo-seq Chip Slide in a clean petri dish and wrap it with aluminum foil. The chips are now ready for imaging.

4.7. Imaging

a. Create a new folder in a fluorescent microscope-connected PC, name it with the chip ID number and other essential information.

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.
Example: B00249A1

b. Take fluorescence images from the chip with the following microscope setting: TRITC channel, 4X and 10X objective lenses, with stitching function.

c. Place 1-2 µL of water on the imaging platform first, then transfer and place the Stereo-seq Chip Slide onto the water drop. Water surface tension will grab onto the Slide and adhere it onto the imaging platform.

d. Remove the light shield and select the chip area of interest.

e. Find the desired capturing area with 4X lens first then switch to 10X lens to complete the full scan.

Make sure the desired capturing area is clear and within focus during full scanning.

Positive control with RNA extract should be imaged separately without modifying any parameters.

4.8. Permeabilization Time Determination

The optimal permeabilization time should result in the strongest fluorescence signal with the lowest signal diffusion. However, this is based on a complete tissue removal as well as images taken under the same settings. For example, as shown in Figure 7, for the **6 min** permeabilization time point, the fluorescence signal in some parts of the tissue is very low, suggesting insufficient permeabilization. Same for the **12 min** permeabilization time point, low fluorescence signal suggesting insufficient permeabilization. For the **24 min** permeabilization time point, the image showed the strongest signal and finer details among three groups. Based on this result, the optimal permeabilization time for this tissue is 24 min.

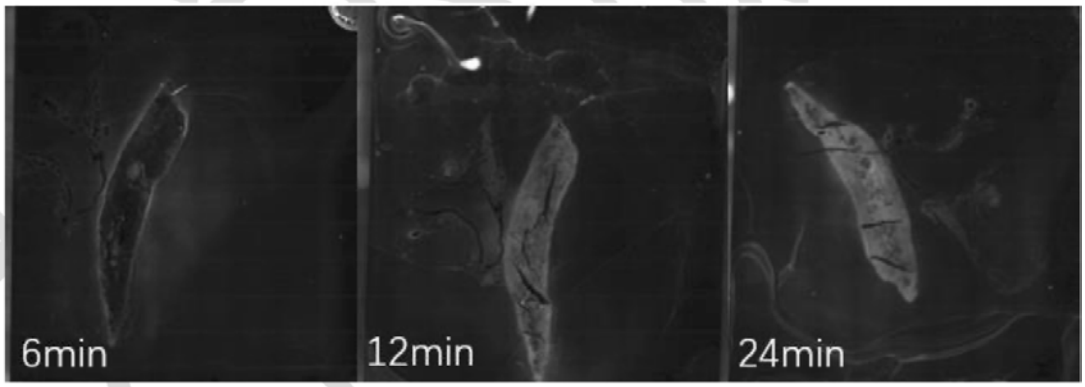


Figure 7. The optimal permeabilization time determination of a PFA mouse thymus section.

Appendix A: Stereo-seq Permeabilization Set for Chip-on-a-slide Experimental Record (Standard FF)

For recording and self-checking experimental procedures, users can access and download the Stereo-seq Permeabilization Set Experimental Record (Standard) we provided here:
https://drive.google.com/drive/folders/18Od8IPO-H1805iycXlZtdTjTtGXIVGex

Appendix B: Tissue Blocking and Mock Incubation Steps for Stereo-seq mIF Application on Fresh Frozen Samples

Tissue Fixation

a. After drying, immediately immerse the tissue-mounted Stereo-seq Chip Slide in pre-cooled methanol prepared in section **3.3-f** for a **30-min** fixation at -20°C. When immersing Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

b. While waiting for the fixation to be done, prepare the reagents required for tissue blocking and mock antibody incubation and prepare the blocking solution in Appendix B Table 1 then leave it on ice.

Reagent	Preparation Steps	Maintenance
RI	Take the aliquot out of the -20°C freezer.	On ice until use
Wash Buffer	For one chip: add 40 µL of RI to 760 µL of 0.1X SSC	On ice until use
10% Triton X-100	If without readily available 10% Triton X-100, prepare by diluting 100% Triton X-100 with nuclease-free water	Room Temperature
Post-centrifuged serum	Take aliquoted serum out of the -20°C freezer and thaw. Then centrifuge at 14,000g, 4°C for 10min, then leave it on ice.	On ice until use

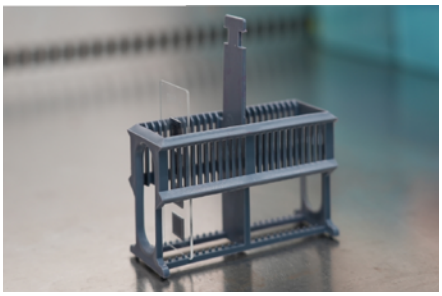
Appendix B Table 1

Components	1X (µL)	2X(µL)	3X (µL)	4X (µL)
5×SSC	120	240	360	480
Post-centrifuged serum (use the supernatant and pipette away from the bottom)	20	40	60	80
10% Triton X-100	2	4	6	8
RI	10	20	30	40
Nuclease-free Water	48	96	144	192
Total	200	400	600	800

c. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

d. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

e. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to let the methanol fully evaporate.



f. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide onto a flat and clean bench top surface.

Tissue Blocking & Mock Antibody Incubation

a. 【Tissue Blocking】Vortex the blocking solution that was prepared in Appendix B Table 1 and add **60 µL per chip** of blocking solution drop wise on the tissue surface then incubate at room temperature for **15 min**.

b. Discard the blocking solution with a pipette.

c. 【Mock Primary Antibody Incubation】Slowly add **60 µL per chip** of the blocking solution from the non-tissue area until the solution covers the tissue section. Incubate at room temperature for **45 min**.

d. Wash by adding **100 µL per chip** of Wash Buffer. Incubate for **3 min** then discard.

e. Repeat **d.** twice for a total three-time wash.

f. 【Mock Secondary Antibody Incubation】Slowly add **60 µL per chip** of the blocking solution to the tissue. Incubate for **25 min** at room temperature.

g. Meanwhile, assemble the Cassette and Gasket (do not assemble the Stereo-seq Chip Slide yet) according to guidance written in 1.4 Practice Tips and pre-warm the assembled Cassette and Gasket on a 37°C PCR thermal cycler for 10 min.

h. Wash by adding **100 µL per chip** of Wash Buffer. Incubate for **3 min** then discard.

i. Repeat **g.** twice for a total three-time wash.

j. Assemble the Cassette and Gasket then place the Stereo-seq Chip Slide in the Cassette according to guidance written in 1.4 Practice Tips. It is recommended to practice with a regular blank glass slide.

k. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

Make sure to not touch the front-side of the chip while assembling the Stereo-seq Slide Cassette.

Please refer back to Chapter 3.5 for Tissue Permeabilization Testing

Appendix C: Tissue Blocking and Mock Incubation Steps for Stereo-seq mIF Application on PFA Samples

Tissue Decrosslinking

a. Make 500 µL per chip of decrosslinking buffer with 5% RI, make sure the pH = 10. Slowly add the decrosslinking buffer with 5% RI into the wells containing the chip (pH=10).

b. Seal with sealing tape and place the Stereo-seq Slide Cassette on to the PCR Adaptor. Close the lid and incubate at 70°C for **1 hr**.

Temperature	Time	Cycle
(Heated lid) 75°C	on	-
70°C	60 min	1
70°C	Hold	-

c. 【Second Methanol Fixation】Prepare enough methanol in a 50 mL corning tube or an empty slide container at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the methanol containing tube to check if the volume is enough. Close the lid and pre-cool the methanol for **5-30 min** at -20°C

Do not reuse the methanol from the previous step after tissue mounting.

d. While waiting for the decrosslinking to be done, prepare the reagents required for tissue blocking and mock antibody incubation and prepare the blocking solution in Appendix C Table 1 then leave it on ice.

Reagent	Preparation Steps	Maintenance
RI	Take the aliquot out of the -20°C freezer.	On ice until use
Wash Buffer	For one chip: add 40 µL of RI to 760 µL of 0.1X SSC	On ice until use
10% Triton X-100	If without readily available 10% Triton X-100, prepare by diluting 100% Triton X-100 with nuclease-free water	Room Temperature
Post-centrifuged serum	Take aliquoted serum out of the -20°C freezer and thaw. Then centrifuge at 14,000g, 4°C for 10min, then leave it on ice.	On ice until use

Appendix C Table 1

Components	1X (μL)	2X(μL)	3X (μL)	4X (μL)
5×SSC	120	240	360	480
Post-centrifuged serum (use the supernatant and pipette away from the bottom)	20	40	60	80
10% Triton X-100	2	4	6	8
RI	10	20	30	40
Nuclease-free Water	48	96	144	192
Total	200	400	600	800

e. Remove the Stereo-seq Slide Cassette from the 70°C PCR Adaptor and then remove the sealing tape. Remove decrosslinking buffer (with 5% RI) with a pipette from the corner of each well without touching the chip surface.

When removing the sealing tape, hold on to the Stereo-seq Slide Cassette with one hand without applying forces to Side A and Side B of the cassette in order to prevent the Stereo-seq Chip Slide from falling off of the cassette.

f. Remove the slide from the Stereo-seq Slide Cassette according to instructions in 1.4 Practice Tips and allow the slide to sit at room temperature for **1 min**.

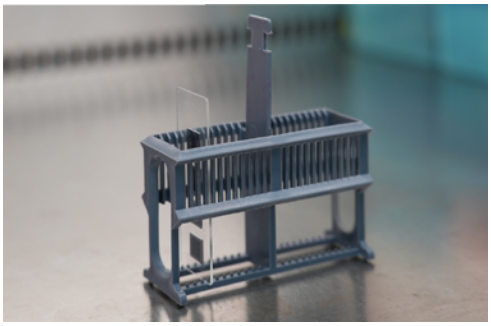
g. Then immerse the Stereo-seq Chip Slide in pre-cooled methanol for a second time fixation at -20°C for **15 min.** When immersing Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

Set aside the removed Stereo-seq Cassette and Gasket for later.

h. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

i. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

j. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to let the methanol fully evaporate.



k. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide on to a flat and clean bench top surface.

Tissue Blocking & Mock Antibody Incubation

a. 【Tissue Blocking】Vortex the blocking solution that was prepared in Appendix B Table 1 and add **60 μL per chip** of blocking solution drop wise on the tissue surface then incubate at room temperature for **15 min**.

b. Discard the blocking solution with a pipette.

c. 【Mock Primary Antibody Incubation】Slowly add **60 μL per chip** of the blocking solution from the non-tissue area until the solution covers the tissue section. Incubate at room temperature for **45 min**.

d. Wash by adding **100 μL per chip** of Wash Buffer. Incubate for **3 min** then discard.

e. Repeat **d.** twice for a total three-time wash.

f. 【Mock Secondary Antibody Incubation】Slowly add **60 μL per chip** of the blocking solution to the tissue. Incubate for **25 min** at room temperature.

g. Wash by adding **100 μL per chip** of Wash Buffer. Incubate for **3 min** then discard.

h. Repeat **g.** twice for a total three-time wash.

i. Assemble the Cassette and Gasket then place the Stereo-seq Chip Slide in the Cassette according to guidance written in **1.4 Practice Tips**. It is recommended to practice with a regular blank glass slide.

j. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

Make sure to not touch the front-side of the chip while assembling the Stereo-seq Slide Cassette.

Please refer back to **Chapter 4.4** for Tissue Permeabilization Testing

Appendix D: H&E staining of tissue sections for Stereo-seq H&E Application on FF Samples

Tissue Fixation & Eosin Staining (performed at -20°C)

a. After drying, immediately immerse the tissue-mounted Stereo-seq Chip Slide in pre-cooled methanol prepared in section 3.3-f for a **17-min** fixation at -20°C **(container 1)**. When immersing Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

If multiple chips are prepared simultaneously, the 17-min fixation time should be determined by the time of the last chip being added. The fixation period should be no more than 27 min.

b. After fixation is done, transfer the Stereo-seq Chip Slide to the eosin solution container **(container 3)** and ensure that all the tissue sections are completely submerged. Stain for **3 min**. Put the methanol containing tube **(container 1)** back into the -20°C freezer.

The amount of staining time can be adjusted according to the uniform coloring of the tissue and should be controlled within the range of 3~5min. Make sure to maintain a consistent staining time for the same tissue block.

c. Once eosin staining is done, transfer the Stereo-seq Chip Slide back to the methanol containing tube **(container 1)** and incubate at -20°C for another **10 min.**

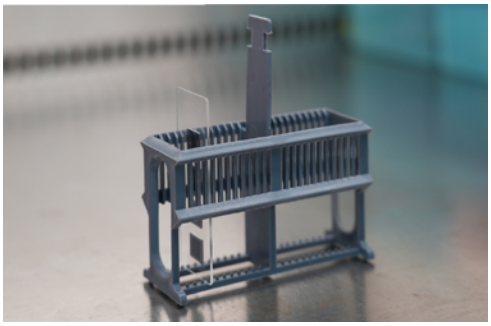
d. Set aside the filtered hematoxylin solution, bluing buffer, and RI.

e. After the second methanol fixation, transfer the Stereo-seq Chip Slide to a new methanol container **(container 2)** and fix the tissue at -20°C for **1 min**.

f. After the third methanol fixation, move the methanol container **(container 2)** to a sterile fume hood.

g. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

h. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to let the methanol fully evaporate.



i. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide onto a flat and clean bench top surface.

Hematoxylin Staining and Bluing

a. Prepare the following reagents. Add RI 5 minutes before the incubation then mix and vortex the reagents right before use.

Appendix D Table 1

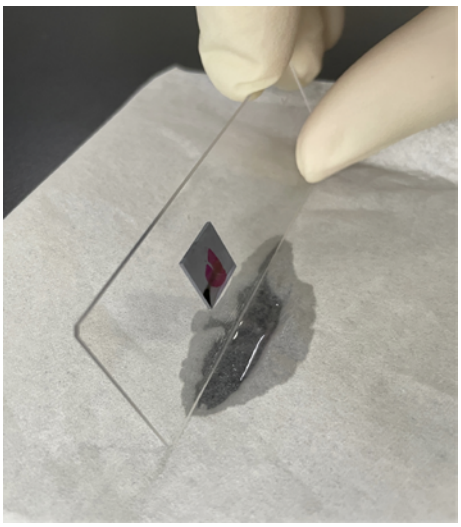
Reagent	Preparation Steps	Maintenance
Hematoxylin Solution (with 5% RI)	Prepare at least 100 µL per chip (95 µL Hematoxylin Solution with 5 µL RI)	Room temperature up to 5 min
Bluing Buffer (with 5% RI)	Prepare at least 100 µL per chip (95 µL Bluing Buffer with 5 µL RI)	Room temperature up to 5 min

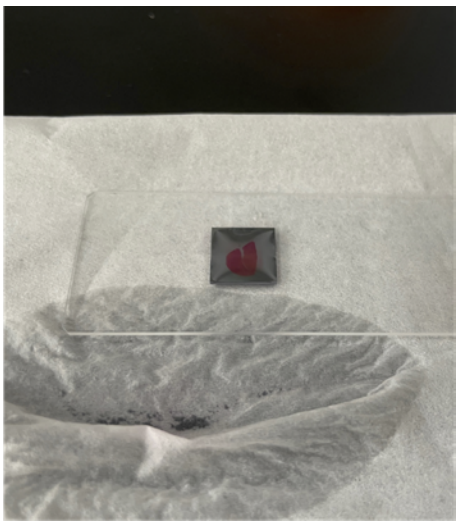
b. Add **100 µL** of Hematoxylin Solution (with 5% RI) onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets. Incubate at room temperature for **7 min**.

c. After the incubation, slightly tilt the Stereo-seq Chip Slide while gently removing the Hematoxylin Solution (with 5% RI) from the corner of the chip using a pipette. Try to remove as much liquid as possible.

d. Add **100 µL** Wash Buffer per chip and then discard it by turning the Stereo-seq Chip Slide sideways at an angle less than 60° while pouring out the Wash Buffer onto a Kimwipe.

e. Repeat d. twice for a total three-time wash. Try to remove as much solution as possible during the last wash by gently touching the edge of the chip with a dust-free paper to absorb the residual liquid.





f. Add **100 µL** of Bluing Buffer (with 5% RI) onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets. Incubate at room temperature for **2 min**.

g. After the incubation, slightly tilt the Stereo-seq Chip Slide while gently removing the Bluing Buffer (with 5% RI) from the corner of the chip using a pipette. Try to remove as much liquid as possible.

h. Add **100 µL** Wash Buffer per chip and then discard it by turning the Stereo-seq Chip Slide sideways at an angle less than 60° while pouring out the Wash Buffer onto dust-free paper. Try to remove as much solution as possible during the last wash by gently touching the edge of the chip with a Kimwipe to absorb the residual liquid.

i. Transfer the Stereo-seq Chip Slide onto dust-free paper. Hold on to the slide with one hand and completely dry the chips further with a power dust remover in the other hand at a distance 2-3 cm away from the chip surface by blowing gently from one side of the chip at a 30-degree angle horizontal to the plane of the chip.

Make sure to quickly dry the chip and the surrounding surfaces completely, especially the crevices between the chip and the slide. If residual liquid remained, eosin staining on the tissue might get "smudgy".

j. Pipette **3.5 µL** H&E Mounting Medium gently onto the center of the tissue on each chip without introducing air bubbles.

k. With a pair of forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chips. Ensure that the chips are completely covered by H&E Mounting Medium and the coverslip.

Make sure the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

l. To mimic the actual imaging steps, leave the Stereo-seq Chip Slide at room temperature for **10 min**.

m. Meanwhile, assemble the Cassette and Gasket (do not assemble the Stereo-seq Chip Slide yet) according to guidance written in 1.4 Practice Tips and pre-warm the assembled Cassette and Gasket on a 37°C PCR thermal cycler for 10 min.

n. Gently push the coverslip with a pair of forceps until it is slightly beyond the edge of Stereo-seq Chip Slide.

o. Grip onto the coverslip with the pair of forceps and pull it to slide over the Stereo-seq Chip Slide slowly until the chips and the coverslip are fully separated.

p. Place the Stereo-seq Chip Slide in a corning tube filled with at least 30mL of 0.1X SSC and immerse it for **3-5 sec**.

Ensure that all the chips on the Stereo-seq Chip Slide have been submerged in SSC solution.

q. Take out the Stereo-seq Chip Slide and wipe off excess solution from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no liquid residue between chips.

r. Add **100 µL** 0.01N HCl solution onto the chip then remove it from the corner of the chip using a pipette.

s. Remove the pre-warmed Cassette and Gasket from the PCR thermal cycler then place the Stereo-seq Chip Slide in the Cassette according to guidance written in 1.4 Practice Tips. It is recommended to practice with a regular blank glass slide.

t. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

Make sure not to touch the front-side of the chip while assembling the Stereo-seq Slide Cassette.

Please refer back to Chapter 3.5 for Tissue Permeabilization Testing