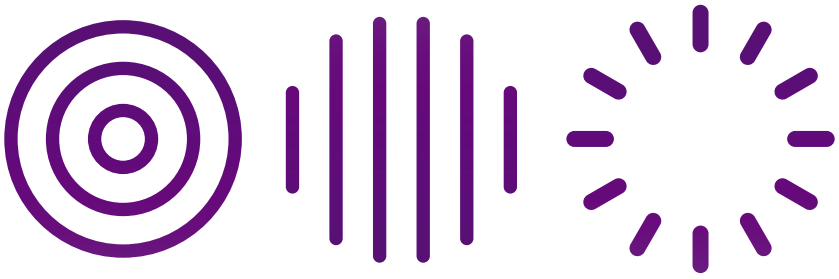
STOmics

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

USER MANUAL



Cat. No.: 211SP11118 (8 RXNs)
Kit Version: V1.1
Manual Version: B_1

STUM-PR002

REVISION HISTORY

Manual Version: A
Kit Version: V1.1
Date: Sep. 2024
Description: Initial release.

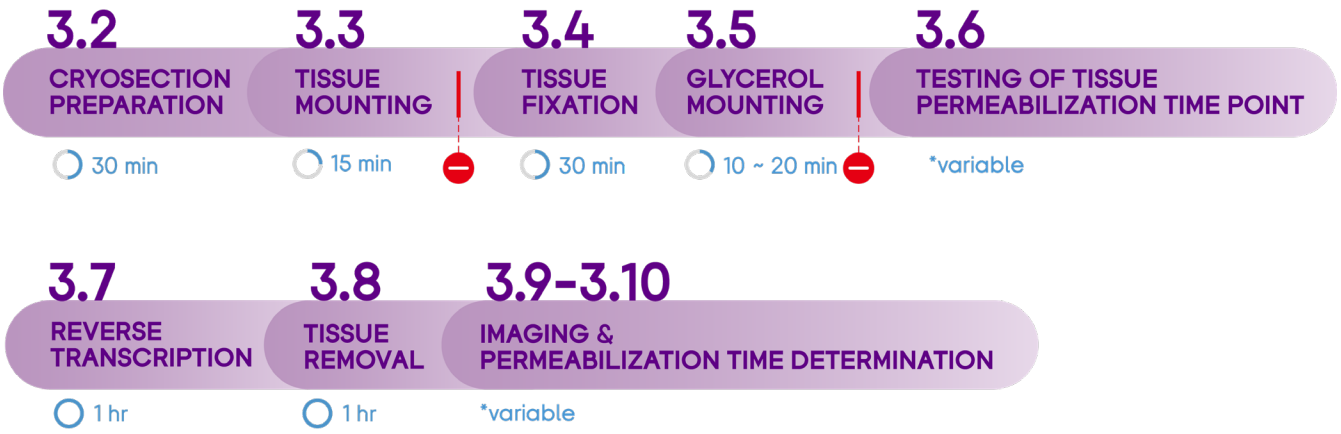
Manual Version: A_1
Kit Version: V1.1
Date: Nov. 2024
Description: Minor error fixation in experiment preparation steps.

Manual Version: B
Kit Version: V1.1
Date: Mar. 2025
Description:
- Minor bugs fixed.
- Extended the transferring and storage temperature.
- Reduced the dosage of RI in H&E staining procedure of Stereo-seq Permeabilization Set.
- Added tissue sectioning and mounting instructions in this manual.

Manual Version: B_1
Kit Version: V1.1
Date: Apr. 2025
Description:
- Corrected typo in chapter 3.2 title.

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

WORKFLOW


3.2
CRYOSECTION PREPARATION
30 min
3.3
TISSUE MOUNTING
15 min
3.4
TISSUE FIXATION
30 min
3.5
GLYCEROL MOUNTING
10 ~ 20 min
3.6
TESTING OF TISSUE PERMEABILIZATION TIME POINT
*variable
3.7
REVERSE TRANSCRIPTION
1 hr
3.8
TISSUE REMOVAL
1 hr
3.9-3.10
IMAGING & PERMEABILIZATION TIME DETERMINATION
*variable
TOTAL TIME: ~5 HRS

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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 a large Field of View (FOV), 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, obtain a gene expression profile of a specific tissue regions, and conduct downstream differential analysis among selected regions of interest.

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

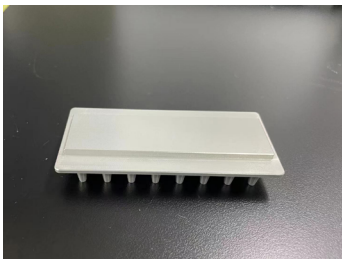
1.2. List of Kit Components

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)

Compatible auxiliary but not included:

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



Catalog numbers, kit components, and specifications are listed below (Table 1-1 to Table 1-4).

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

The 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 the appropriate conditions.

Table 1-1 Stereo-seq Permeabilization Kit Components

Stereo-seq Permeabilization Kit	Cat. No.:211KP11118		
Component	Reagent Cat. No.	Cap Color	Quantity (tube)
RI	1000028499		300 μL × 1
PR Enzyme	1000028500		10 mg × 1
RT QC Buffer Mix	1000047918		792 μL × 1
Glycerol	1000047910		100 μL × 1
H&E Mounting Medium	1000041969		50 μL × 1
RT QC Enzyme Mix	1000047919	(transparent)	88 μL × 1
TR Enzyme	1000028504		71 μL × 1
TR Buffer	1000028505		1725 μL × 2
Storage Temperature: -25°C~-15°C	Transported by cold chain	Expiration Date: refer to label	

This reagent is used solely for coverslip mounting on the H&E-stained tissue section and is not used if the H&E staining workflow is not implemented.

Table 1-2 Stereo-seq Chip P Slide (1cm * 1cm) Kit Components

Stereo-seq Chip P Slide (1cm*1cm)	Cat. No.: 210CP118
Component	Quantity (per kit)
Stereo-seq Chip P Slide (1cm * 1cm)	8 EA
Storage Temperature: 2°C~ 8°C	Transported by cold chain	Expiration Date: refer to label

Table 1-3 STOmics Accessory Kit Components

STOmics Accessory Kit	Cat. No.: 100033700	
Component	**Reagent Cat. No.**	**Quantity (per kit)**
Cassette	1000033699	1 EA
Gasket	1000033698	4 EA
Sealing Tape	1000042970	6 EA
Storage Temperature: 18°C~ 25°C	Transported at 0°C~ 30°C	Expiration Date: refer to label

Table 1-4 Stereo-seq PCR Adaptor Components

Stereo-seq PCR Adaptor	Cat. No.: 301AUX001-02	
Component	**Quantity (per kit)**	
Stereo-seq PCR Adaptor	2 EA	
Storage Temperature: 18°C~ 25°C	Transported at 0°C~ 30°C	Expiration Date: refer to label

1.3. Additional Equipment and Materials

The table below lists the equipment and materials needed for this protocol. The user is expected to have access to common laboratory equipment not named in the document (equipment such as an ice maker, biological safety cabinet, freezers, and so on). For specific microscope requirements, refer to the **Stereo-seq Imaging Requirements and Guidelines**.

Table 1-5 Additional Equipment and Materials

Equipment		
Brand	**Description**	**Cat. No.**
-	Cryostat	-
-	Benchtop Centrifuge	-
-	Pipettes	-
Leica*	Fluorescence Microscope	DM6M
STOmics*	Fluorescence Microscope	900-000586-0
-	Vortex Mixer	-
-	Metal Bath (or equivalent instrument)	-
Bio-Rad^	T100 Thermal Cycler	1861096
Thermo Fisher Scientific^	ProFlex 3 x 32-well PCR System	4483636

*Choose either one of the listed brands (marked with *).

^Choose either one of the listed brands (marked with ^). A suitable PCR Adaptor will be needed.

Table 1-6 Addition reagents required

Reagent		
Brand	**Description**	**Cat. No.**
Ambion	Nuclease-free Water	AM9937
	20X SSC	AM9770
Sigma Aldrich	Hydrochloric Acid, HCl (0.1N)	2104-50ML
	Methanol	34860-1L-R
SAKURA	SAKURA Tissue-Tek® O.C.T. compound	4583
Sangon Biotech (or other brands)	Eosin Y, free Acid	A600190-0025
Sigma Aldrich	Hematoxylin Solution (filter before use)	51275
Agilent (or other brands)	Bluing Buffer, Dako	CS70230- 2

The rows highlighted in purple are for Stereo-seq Transcriptomics H&E staining workflow only and will not be used if the H&E staining workflow is not implemented.

Table 1-7 Additional consumables required

Consumables		
Brand	**Description**	**Cat. No.**
-	Aluminum Foil	-
-	Forceps	-
-	Slide Staining Rack	-
-	Glass Slide	-
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	1.5 mL Centrifuge Tubes	MCT-150-A
	1,000 µL Filtered Tips	TF-1000-L-R-S
	200 µL Filtered Tips	TF-200-L-R-S
	100 µL Filtered Tips	TF-100-R-S
	10 µL Filtered Tips	TXLF-10-L-R-S
-	Microscope Glass Coverslip (size: 24 mm × 32 mm)	-
-	Disposable Sterile Syringe	-
Millipore (or other brands)	Millex Syringe Filter, Durapore PVDF, 0.22 µm pore size	SLGV033N

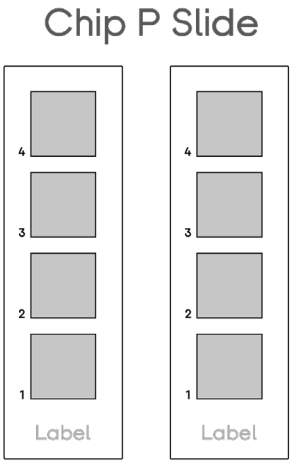
The rows highlighted in purple are for Stereo-seq Transcriptomics H&E staining workflow only and will not be used if the H&E staining workflow is not implemented.

1.4. Stereo-seq Chip Slide Information

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.

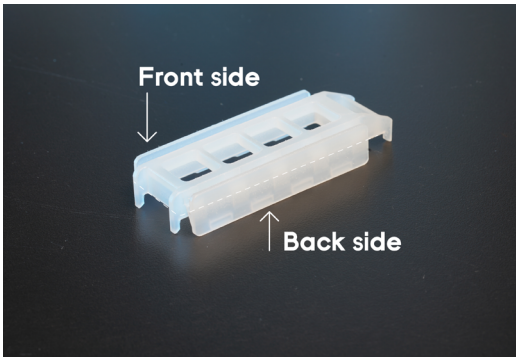


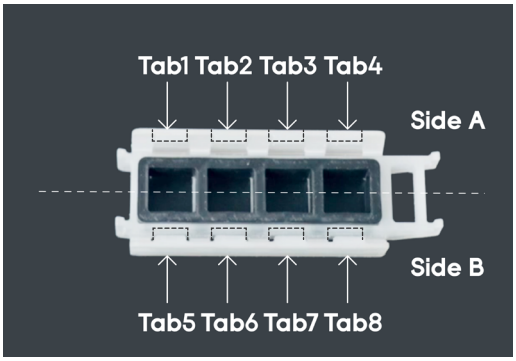
Stereo-seq Chip P Slide Storage

Always store unused slides in their original slide container and keep them sealed in a sealable aluminum bag at 2°C~8°C. Keep sealed with tape or another re-sealable bag. **Always KEEP the desiccant in 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
For assembly instructions, refer to Appendix I: Stereo-seq Slide Cassette Assembly

1.5. Precautions and Warnings

- This product is intended for research use only, not for use in diagnostic procedures. Read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, ensure that you are familiar with all related instruments and operate them according to the manufacturers' instructions.
- Instructions provided in this manual are intended for general use only; 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 use. For other reagents, thaw them first at room temperature, invert several times to mix them properly, and centrifuge them briefly. Place them on ice for future use.
- RNA capture will be compromised or absent for any scratched areas on the front surface of the chip.
- We recommend using filtered pipette tips to prevent cross-contamination. 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, for PCR reaction preparation and PCR product cleanup tests, we recommend working in two distinctly separate working areas in the laboratory. Use designated pipettes and equipment for each area, and perform 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 AND EXPERIMENT PREPARATION

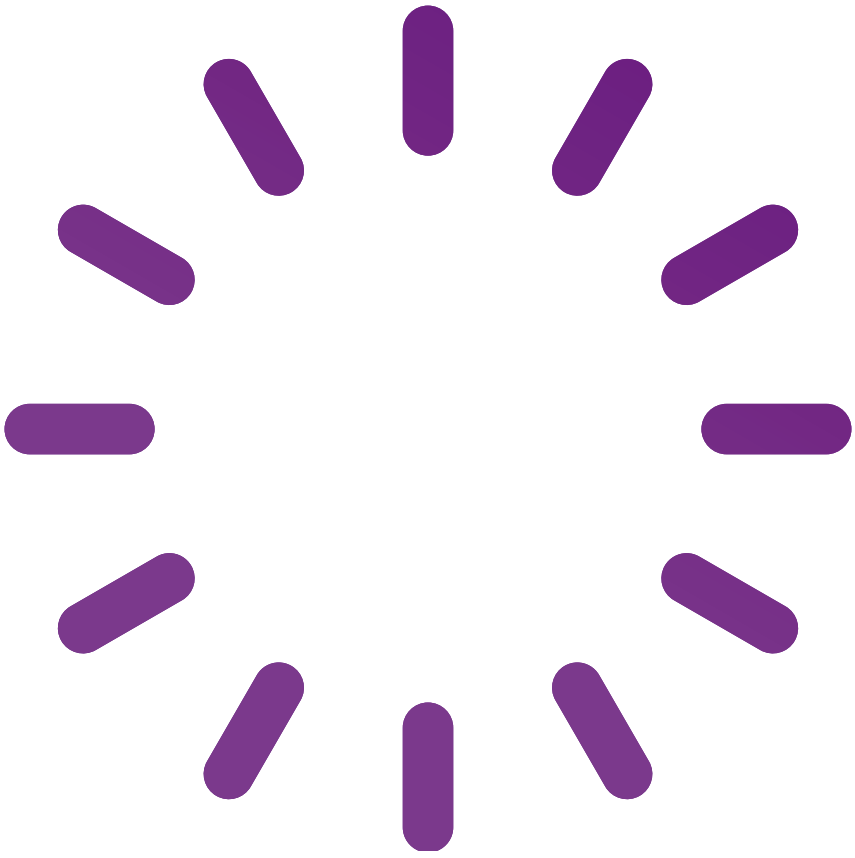
For frozen sample embedding, guides, refer to **Sample Preparation for Fresh Frozen Samples on Stereo-seq Chip Slides (Document No.: STUM-SP001)**.

This guide describes how to check the RIN quality of a fresh frozen tissue sample before proceeding to the Stereo-seq experiment.

It is strongly recommended that you proceed only with tissue samples with a RIN value ≥4.0.

CHAPTER 3

Stereo-seq PERMEABILIZATION SET FOR CHIP-ON-A-SLIDE STANDARD OPERATING PROCEDURE



3.1. Experiment Preparation

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

Table 3-1 Experiment Preparation Steps

Reagent	Preparation Steps	Maintenance
Methanol	Pre-cool at -20°C for no longer than 30 min before use.	-20°C
0.1X SSC	Dilute 250 µL of 20X SSC to 50 mL.	Room temperature
Wash Buffer	Prepare at least 200 µL per chip (190 µL 0.1X SSC with 10 µL RI).	On ice until use
0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Measure and ensure that the pH = 2.	Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. 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 conducting experiments.		
10X Permeabilization Reagent (PR) 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 by pipetting.	On ice until use, up to 1 hr
DO NOT vortex the permeabilization enzyme. Mix by pipetting before use. **Aliquot the 10X stock solution to avoid freeze-thaw cycles and keep it at -20°C for long term storage.**		
1X Permeabilization Reagent Solution	Prepare 1X PR Solution by diluting 10X PR stock solution with 0.01N HCl. Prepare 150 µL per chip.	On ice until use, up to 6 hr
Glycerol	Equilibrate to room temperature 5 min in advance. Prepare 5 µL per chip.	Room temperature
Eosin Solution	Dissolve 0.026g Eosin Y powder in 50 mL methanol and keep it sealed with a parafilm until use.	Room temperature up to 1 month
Hematoxylin (filtered)	Prepare and filter the Hematoxylin Solution using a 0.22 µm pore-sized filter (a needle cartridge filter and a disposable sterile syringe) and seal it with a parafilm until use; use 100 µL per chip.	Room temperature up to 7 days in the dark
H&E Mounting Medium	Equilibrate to room temperature 5 min in advance. Prepare 3.5 µL per chip.	Room temperature

The rows highlighted in purple are for Stereo-seq Transcriptomics H&E staining workflow only and will not be used if the H&E staining workflow is not implemented.

Other Preparation		
Equipment	**Preparation Steps**	**Note**
Cryostat	Set the cryostat chamber temperature to -20°C and the 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: 1. 37°C for slide drying and permeabilization (heated lid at 60°C). 2. 45°C for reverse transcription (heated lid at 60°C). 3. 55°C for tissue removal (heated lid at 60°C).	Check the PCR Thermal Cycler for abnormalities. If necessary, replace it.
Metal Bath (or other equivalent heating instrument)	37°C for preheating of 1X Permeabilization Reagent Solution	Check the instrument for any abnormalities and replace it if necessary.
Fluorescence Microscope	Set the epi-fluorescence channel to TRITC mode.	Check the microscope for any abnormalities and replace it if necessary.

3.2. Cryosection Preparation

a. Set the PCR thermal cycler with a PCR Adaptor to 37°C with heated lid set to 60°C in advance.

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	On	-	-
37°C	**∞**	**1**	**Slide Drying and Permeabilization**
45°C	∞	1	Reverse Transcription
55°C	∞	1	Tissue Removal

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

If the specimen disc is over-cooled, it can result in tissue section cracking during sectioning. When the disc temperature is too high, sections will wrinkle. Optimal specimen disc temperature depends on the tissue type.

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

d. Transfer the OCT-embedded tissue sample from the -80°C freezer and place it in the chamber for 30 min to allow it to equilibrate to the 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. Trim again if necessary to ensure a good fit between the tissue section and the Stereo-seq Chip. Now, the specimen is ready for cryosection.

3.3. Tissue Mounting

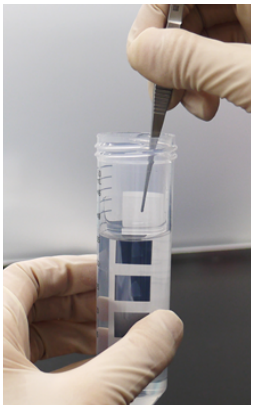
For a demonstration video of tissue mounting onto the Stereo-seq Chip Slide, refer to the link below or scan the QR code:
https://en.stomics.tech/resources/videos/list.html

a. Take the Stereo-seq Chip Slide out of the vacuum-sealed aluminum bag and record the Chip ID (SN) number located on the back of the slide. Do not touch the front of the chip.

After opening the bag, check all Stereo-seq Chip Slides in the slide container and make sure they are oriented front-side up. The front of the chip has a shiny surface that contains DNB-probes for RNA capture. DO NOT scratch the surface.

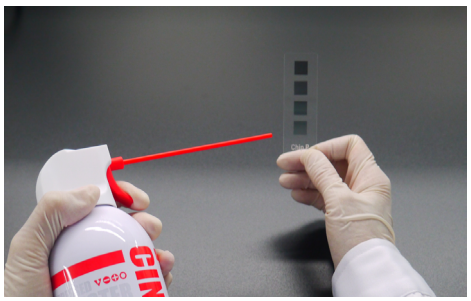
b. Make sure the PCR Thermal Cycler has been turned on and set to 37°C.

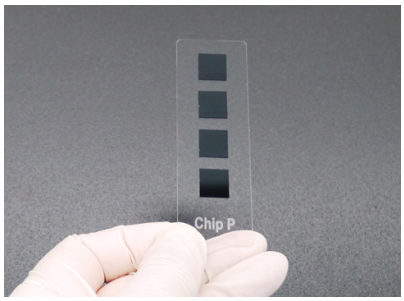
c. Equilibrate the Stereo-seq Chip Slide to room temperature for **1 min** on the bench, then rinse with 100 µL nuclease-free water **twice** using a pipette, or, rinse the slide twice in a 50 mL centrifuge tube by immersing it in sufficient water, using forceps for handling.



Seal unused slides in the original packaging (first in the slide container and then in the sealable aluminum bag) and store at -25°C ~ 8°C. KEEP the desiccant in the aluminum bag.

d. Gently blow off excess water from the chip with a power dust remover. Wipe off excess water from around the edges of the chip and on the slide with dust-free paper.





e. When the chip is completely dry and void of wavy white stains, it is ready for tissue mounting.

f. Prepare enough methanol in a 50 mL centrifuge tube or an empty slide container with sufficient volume for submerging all the chips on the slide. Immerse a regular glass slide in the methanol-containing tube to confirm that there is sufficient volume. Close the lid and pre-cool the methanol for **5-30 min** at -20°C.

[H&E applications only]: Prepare one slide container or a 50 mL centrifuge tube, and add enough eosin solution at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the eosin-containing tube to check if the volume is enough. Close the lid and pre-cool the eosin solution for **5-30 min** at -20°C. This step only applies if the H&E staining is intended for later use in the Stereo-seq workflow.

g. Place the tissue-mounted specimen disc/holder onto the cryostat head and adjust the angle accordingly.

h. Tissue mounting can be achieved using either the cold method (option A) or the warm method (option B). We recommend practicing tissue mounting and section placement on plain glass slides first.

A. Cold Method

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

Prolonged cooling for longer than 6 min may cause mist to form on the chip surface.

2) Perform cryosection, then carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes. Carefully place a tissue section onto the chip center using 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 back 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 the remaining chips.

5) When all tissue mounting is completed, immediately dry the Stereo-seq Chip Slide at 37°C on a PCR thermal cycler with a 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) can 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), and 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 such that the space between each is greater than the chip spacing on the Stereo-seq Chip Slide, avoiding the slide contact with other sections.

3) Flip the Stereo-seq Chip Slide over and aim the tissue section within a chip area on the Stereo-seq Chip Slide by gently touching the section with the front 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) Flip the Stereo-seq Chip Slide over with the front facing up, and immediately dry it in the PCR Thermal Cycler at 37°C with PCR Adaptor for **5 min** (without heated lid).

Stop Point:

- **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 sealable bag, push out as much air as possible and seal the bag tightly. The sealed Stereo-seq Chip Slide can be transferred to a −80°C freezer on dry ice.**
- **Store the sealed bag 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, take out the tissue containing Stereo-seq Chip Slides then immediately incubate at 37°C with PCR Adaptor for 5 min.**

3.4. Tissue Fixation

For tissue samples that are intended for Stereo-seq Transcriptomics H&E workflow, tissue fixation & eosin staining (at -20°C), hematoxylin solution staining & bluing, and coverslip mounting steps need to be completed prior to Tissue Permeabilization Testing to obtain an accurate permeabilization time. Skip sections 3.4 and 3.5, and refer to Appendix II: H&E Staining and Coverslip Mounting Operating Procedures for detailed procedures.

a. Set the PCR thermal cycler to 37°C in advance, and pre-heat the PCR Adaptor in the PCR thermal cycler to the desired temperatures according to Table 3-2. Set aside the pre-cooled methanol that you prepared in **step f.** in Section 3.3 Tissue Mounting.

Table 3-2 PCR Thermal Cycler Program

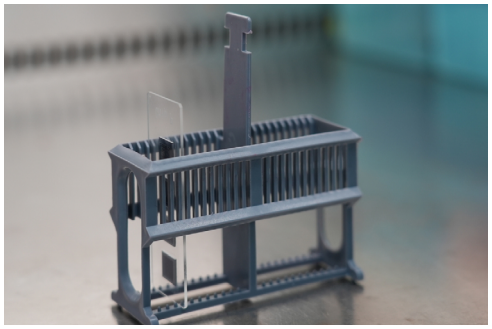
Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	On	-	-
37°C	**∞**	**1**	**Permeabilization Time Testing**
45°C	∞	1	Reverse Transcription
55°C	∞	1	Tissue Removal

b. After drying the tissue-mounted Stereo-seq Chip Slide, immediately immerse it in pre-cooled methanol for a **30-min** fixation at -20°C (do not exceed 1 hr). When immersing the Stereo-seq Chip Slide in methanol, ensure that all tissue sections are completely submerged.

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

d. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that 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 allow the methanol to evaporate completely.



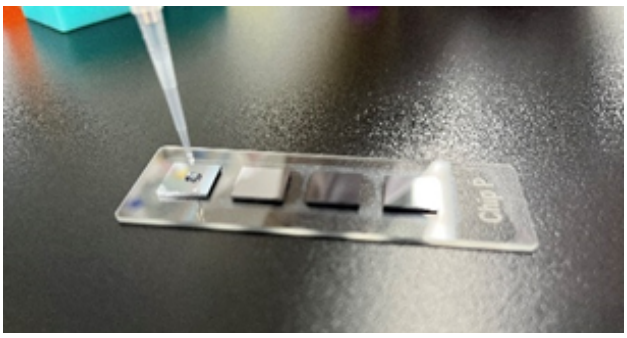
f. When the methanol is completely evaporated, transfer the Stereo-seq Chip Slide onto a flat and clean bench.

3.5. Glycerol Mounting

Ensure that the glycerol has been equilibrated to room temperature for 5 min beforehand.

Ensure that the coverslip is clean and free of any dust or debris. To clean the coverslip, wipe it with an alcohol swab or use a power dust remover.

a. Before using the glycerol tube, centrifuge it to remove any bubbles. Using a pipette, carefully add 5 µL of glycerol to the center of the tissue on the chip without introducing air bubbles.



b. Using clean forceps, place one end of the coverslip onto the tissue edge while holding the other end and then gradually lower the coverslip onto the tissue. Ensure that the tissue is completely covered with glycerol and the coverslip. Let it stand on the bench for **10 min**.

Glycerol mounted chips can not be stored longer than 2 hr at room temperature. For tissues that are prone to RNA degradation, such as pancreas, proceed to next step immediately to avoid RNA degradation.

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

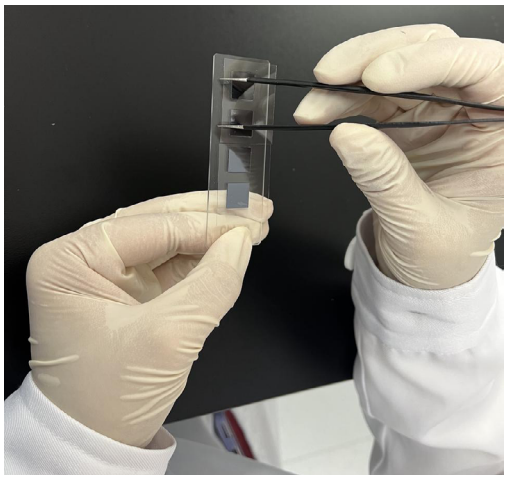
d. Set the temperature of a metal bath or equivalent heating instrument to 37°C, and set the PCR program on hold at 37°C.

e. Take out one gasket and one cassette from the STOmics Accessory Kit and blow any impurities off of the gasket with a power dust remover and then assemble only the Stereo-seq cassette and the gasket.

f. Ensure that the PCR thermal cycler has been switched on and set to 37°C. Pre-warm the assembled cassette and gasket in the PCR thermal cycler for **10 min**.

g. Warm the aliquoted 1X Permeabilization Reagent Solution inside the 37°C PCR thermal cycler or metal bath for **>10 min (no longer than 30 min)**.

h. After 10 min, use clean forceps to grip the coverslip and then pull and slide the coverslip over the Stereo-seq Chip Slide edge slowly until the chips and the coverslip are completely separated.



i. Place the Stereo-seq Chip Slide in a centrifuge tube filled with at least 30 mL 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 the solution.

j. Take out the Stereo-seq Chip Slide and wipe off the excess solution from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that there is no liquid residue around the chips.

3.6. Testing of Tissue Permeabilization Time Point

a. Thaw RT QC Buffer Mix on ice and place the RT QC Enzyme Mix on ice until use.

b. Assemble the cassette and gasket then place the Stereo-seq Chip Slide in the cassette according to the instructions in **Appendix I: Stereo-seq Slide Cassette Assembly**. It is recommended that you practice with a regular blank glass slide. Grip along the Stereo-seq Cassette to ensure that the Stereo-seq Chip Slide has been locked in place.

Do not touch the front of the chip while assembling the Stereo-seq Slide Cassette.

c. Ensure that the PCR thermal cycler has been set to the desired temperature (rows **highlighted in bold** below).

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	On	-	-
37°C	**Hold**	**1**	**Permeabilization Time Testing**
45°C	Hold	1	Reverse Transcription
55°C	Hold	1	Tissue Removal

d. 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 that you use a suggested time course of **6 min, 12 min, 18 min and 24 min (4 time points, 6-min intervals)**.

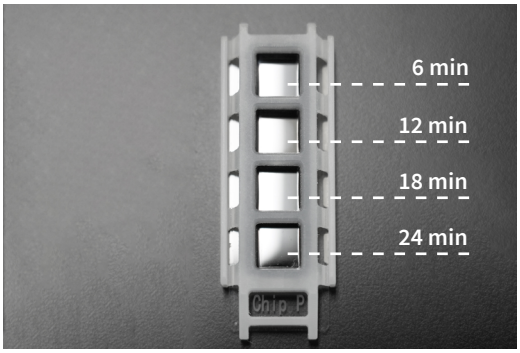
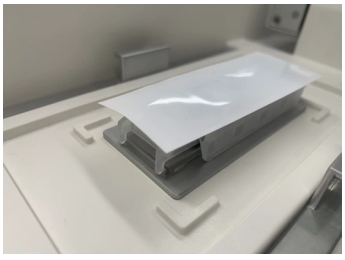


Figure 1. Permeabilization Times (min)

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 droplets.

Ensure that the chip is completely covered with 1X Permeabilization Reagent Solution.

2) Apply **unpeeled** sealing tape on top of the Stereo-seq Slide Cassette, with white backing facing up, 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 on top of the Stereo-seq Slide Cassette, close the lid, and incubate at 37°C.

5) Repeat the process, working backward to the shortest incubation time (chip with **6-min** time point).

e. While waiting for permeabilization to be completed, prepare RT QC Mix according to Table 3-3, wrap the RT QC Mix in aluminum foil, and place RT QC Mix on ice until later use **in the dark**.

Table 3-3 RT QC Mix

Components	1X (μL)	4X + 10% (μL)
RT QC Buffer Mix	90	396
RT QC Enzyme Mix	10	44
Total	**100**	**440**

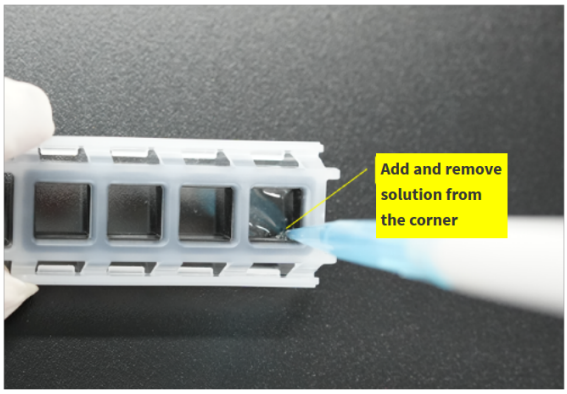
f. When incubation is completed, remove the Stereo-seq Slide Cassette from the PCR Adaptor (37°C).

g. PCR thermal cycler: Skip the 37°C step and continue to the 45°C step (**highlighted in bold**).

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	On	-	-
37°C	∞	1	Permeabilization Time Testing
45°C	**∞**	**1**	**Reverse Transcription**
55°C	∞	1	Tissue Removal

h. Slightly tilt the Stereo-seq Slide Cassette at an angle of less than 20°. Pipette to remove the 1X Permeabilization Reagent Solution from the corner of the well; **DO NOT TOUCH** the chip surface.

i. Add **200 μL** of Wash Buffer per chip and then slightly tilt the Stereo-seq Slide Cassette to remove the solution from the corner of each well.



To prevent RNA degradation, proceed immediately to 3.7 Reverse Transcription.

3.7. Reverse Transcription

a. Ensure that the temperature of the PCR thermal cycler with PCR Adaptor has been set to 45°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 to Stereo-seq Slide Cassette and seal it tightly. Incubate the Stereo-seq Slide Cassette at 45°C for **1 hr** or longer (no longer than 5 hr) **in the dark**.

3.8. Tissue Removal

a. Heat the TR Buffer for 5 min at 55°C to dissolve the precipitate. Equilibrate it to room temperature prior to use.

If white precipitates are visible in the buffer, dissolve them by heating the buffer at 55 °C again and equilibrate to room temperature before mixing.

b. Prepare Tissue Removal Mix according to Table 3-4 and place the mix at room temperature.

Table 3-4 Tissue Removal Mix

Component	1X (µL)	4X + 10% (µL)
TR Buffer	392	1724.8
TR Enzyme	8	35.2
Total	400	1760

c. When incubation is completed, remove the Stereo-seq Slide Cassette from the 45°C PCR Adaptor.

d. PCR thermal cycler: Skip the 45°C step (not highlighted below) and continue to the 55°C step (**highlighted in bold**).

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	On	-	-
37°C	∞	1	Permeabilization Time Testing
45°C	∞	1	Reverse Transcription
55°C	**∞**	**1**	**Tissue Removal**

e. Remove the sealing tape. Slightly tilt the Stereo-seq Cassette and, using a pipette, remove the RT QC Mix 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 force to Side A and Side B of the cassette. This prevents the Stereo-seq Chip Slide from falling off of the cassette.

f. Add **400 µL** 0.1X SSC solution into each well.

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

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

i. Repeat **steps f. through h**.

j. Add **400 µL** of Tissue Removal Mix per well. Ensure that there is uniform solution coverage within each well.

k. Apply sealing tape on the Stereo-seq Slide Cassette and incubate at 55°C on the PCR Adaptor for **1 hr**.

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

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

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

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

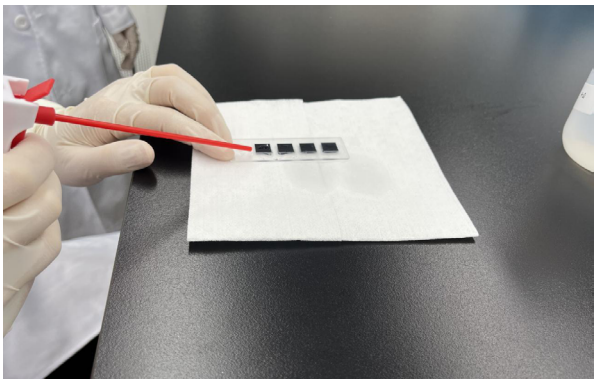
o. Gently pipette 0.1X SSC solution up and down **5 times** at the corner of each well. Use a pipette to remove 0.1X SSC from the corner of each well.

p. Repeat **steps n. and o**.

q. Add **400 µL** of nuclease-free water into each well and pipette up and down to wash the chip surface and remove the salt contained in the SSC solution. Discard the liquid.

r. Remove the slide from the Stereo-seq Slide Cassette according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly.

s. Place the Stereo-seq Chip Slide onto a clean dust-free paper and completely dry the chip surface with a power dust remover.

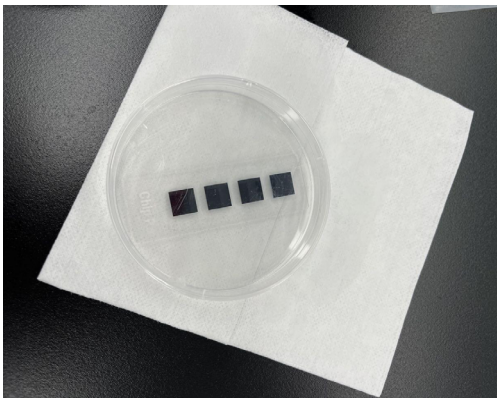


If obvious tissue traces remain on the surface of the chip, wash again by adding 100 µL nuclease-free water and then blow dry. This step can be repeated until there are no visible tissue traces on the chip surface.

Alternative Step:

Remove the slide from the Stereo-seq Slide Cassette after step p. and rinse the Stereo-seq Chip Slide up and down 10 times in a 50 mL centrifuge tube or a slide container filled with sufficient 0.1X SSC (ensure the chips are fully submerged), then rinse up and down 10 times with sufficient nuclease-free water. Dry the chip surface with a power dust remover. This step can be repeated until there are no visible tissue traces 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 ready for imaging.



3.9. Imaging

a. Create a new folder in the microscope imaging software, name it with the chip ID number and other essential information.

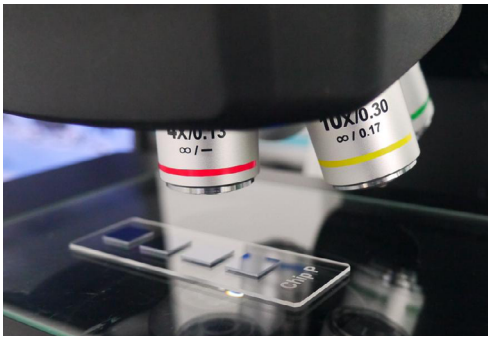
Use only letters, numbers, and underscores in the folder name. Special characters and spaces are not allowed.

Example chip ID number: 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 to 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.

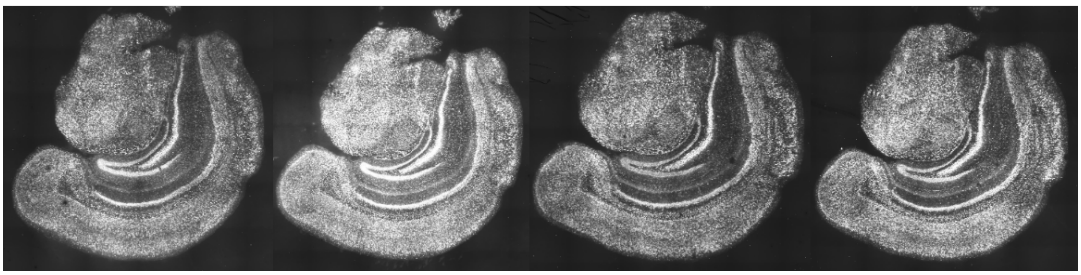
Be sure the desired capturing area is clear and within focus during full scanning.

Chips with different permeabilization times of the same tissue should be scanned under the same imaging conditions, including brightness, exposure, and other parameters.

3.10. Permeabilization Time Determination

The optimal permeabilization time should result in the strongest fluorescence signal with the lowest signal diffusion. However, this is based on complete tissue removal as well as images taken under the same settings.

For example, as shown in Figure 2, for the **6-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 show the strongest signal and finer details among four 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.



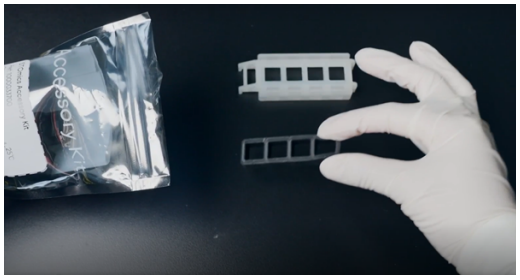
6 min 12 min 18 min 24 min

Figure 2. The optimal permeabilization time determination of a mouse brain coronal section

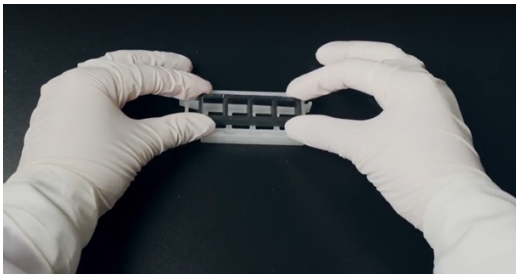
Appendix I: Stereo-seq Slide Cassette Assembly

Stereo-seq Slide Cassette Assembly

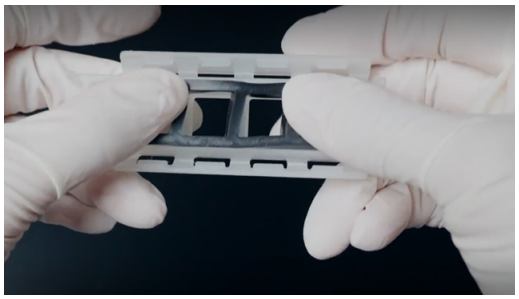
a. Take the Stereo-seq Slide Cassette and Gasket out of the STOmics Stereo-seq Accessory Kit.



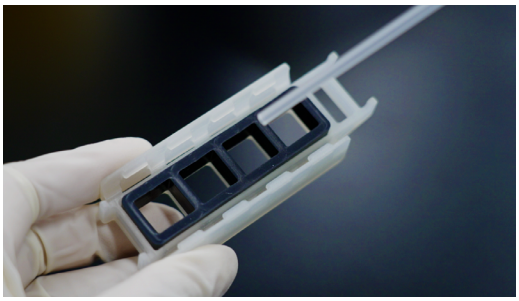
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



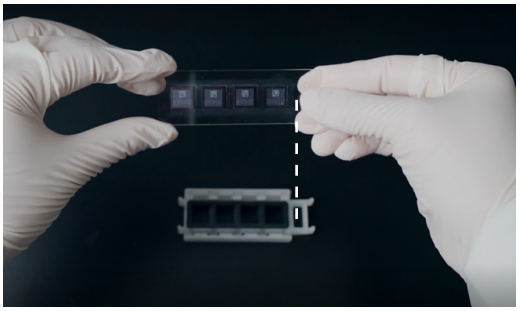
c. Press the gasket down to secure it in the cassette.



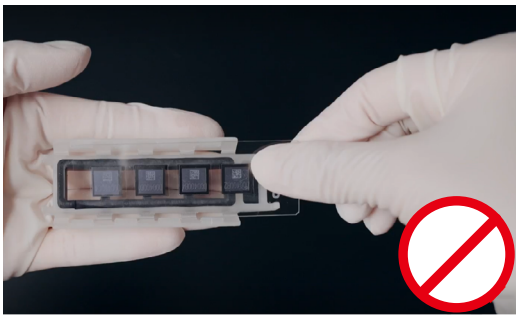
d. Use a power dust remover to blow off any debris on the gasket if necessary.



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

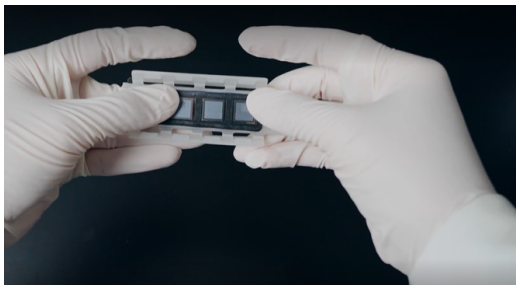


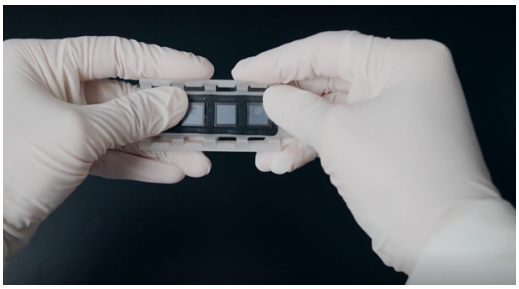
f. Ensure that the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert the Stereo-seq Chip Slide under the bottom 4 tabs.



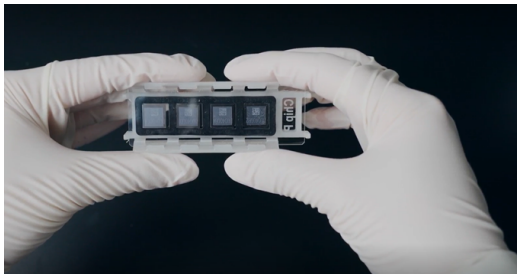
g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2, and place your while right thumb between tab 3 and tab 4.

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

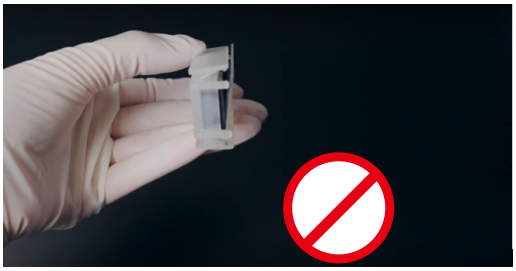




i. Press along both edges of the Stereo-seq Slide Cassette to ensure that the Stereo-seq Chip Slide is locked in place.

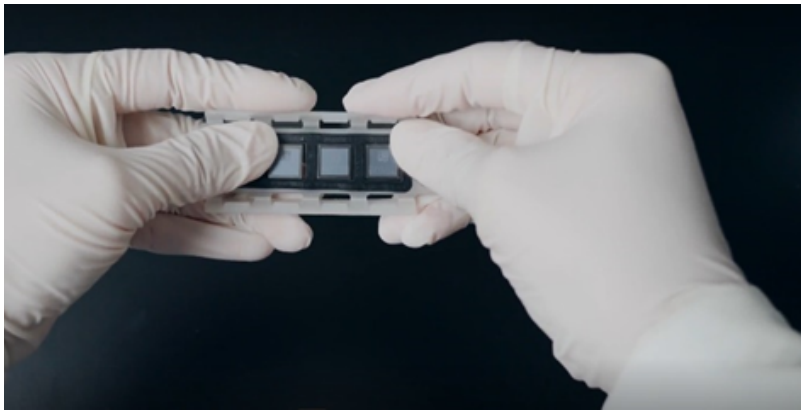


j. Recheck the Stereo-seq Slide Cassette and verify that the slide is clipped in place.

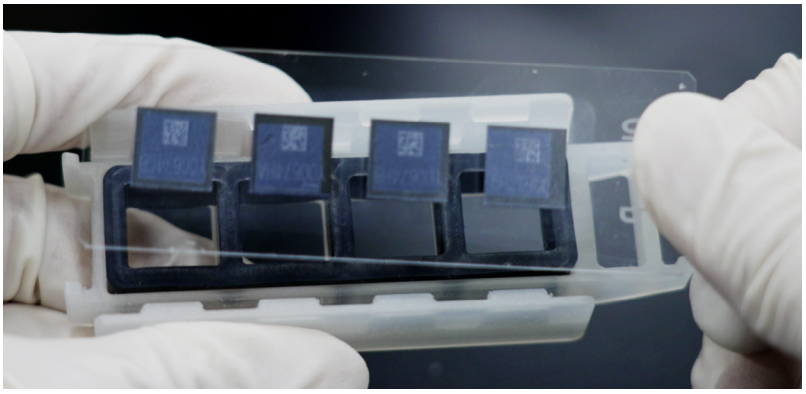


Stereo-seq Slide Cassette Removal

a. To release the slide from the tabs, first flip the cassette over, and then as you gently support the back of the Stereo-seq Chip Slide with both thumbs to prevent the Stereo-seq Chip Slide from falling, firmly press the upper side down.



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



Appendix II: H&E Staining Operating Procedures

Reagent Required for Appendix II	Purpose	Preparation
Methanol	Tissue fixation & eosin staining	Prepare 30-50mL of methanol in a centrifuge tube or a slide container. Pre-cool at -20°C for **5-30 min**.
Eosin Solution (H&E application)	Tissue fixation & eosin staining	Dissolve 0.026g Eosin Y powder in 50 mL methanol and keep sealed with a parafilm. Pre-cool at -20°C for **5-30 min**.
0.1X SSC	Washing	Dilute 250 µL of 20X SSC to 50 mL, then place it at room temperature.
Wash Buffer	Washing	Requires at least 200 µL per chip.
Hematoxylin Solution	Hematoxylin staining	Prepare at least 100 µL per chip. Filter before use.
Bluing Buffer	Bluing	Prepare at least 100 µL per chip.
H&E Mounting Medium	Mounting	Equilibrate to room temperature 5 min in advance. Prepare 3.5 µL per chip.

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

a. After drying the tissue-mounted Stereo-seq Chip Slide, immediately immerse it in pre-cooled methanol for a 30-min fixation at -20°C (do not exceed **1 hr**). When immersing the Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

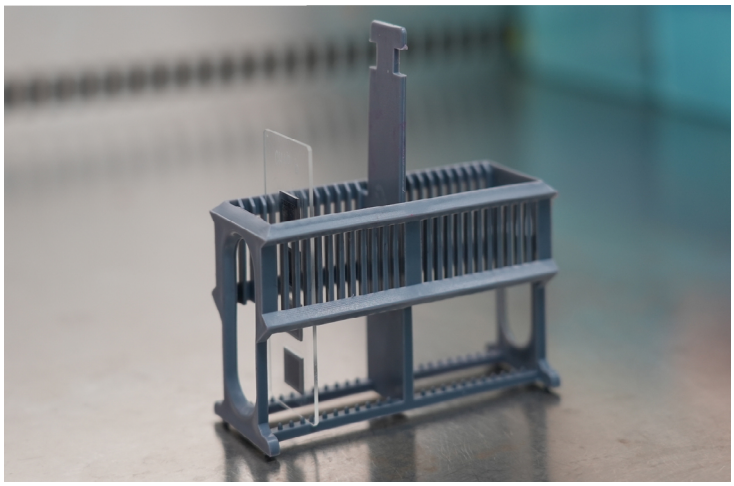
b. Transfer the Stereo-seq Chip Slide to the pre-cooled eosin solution, and ensure that all the tissue sections are completely submerged. Stain for **3 min** at -20 °C.

The staining duration should be adjusted to achieve uniform coloring of the tissue and controlled within a range of 3 to 5min. It is important to maintain a consistent staining time for the same tissue block.

c. When eosin staining is completed, transfer the Stereo-seq Chip Slide back to the methanol-containing tube and incubate at -20°C for another **1 min**.

d. Move the methanol container to a sterile fume hood. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that 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 allow the methanol to evaporate completely.



f. When the methanol is completely evaporated, transfer the Stereo-seq Chip Slide onto a flat and clean bench for further staining.

Hematoxylin Staining and Bluing

a. Set aside the 2 mL 0.01N HCl that you prepared in **3.1 Experiment Preparation**. Prepare the following reagents and DO NOT leave them on ice. Vortex the reagents before use.

Prepare		
Reagent	**Preparation Steps**	**Storage**
Hematoxylin Solution	Filter before use and prepare at least 100 µL per chip.	Room temperature **in the dark** up to 5 min
Bluing Buffer	Prepare at least 100 µL per chip.	Room temperature up to 5 min

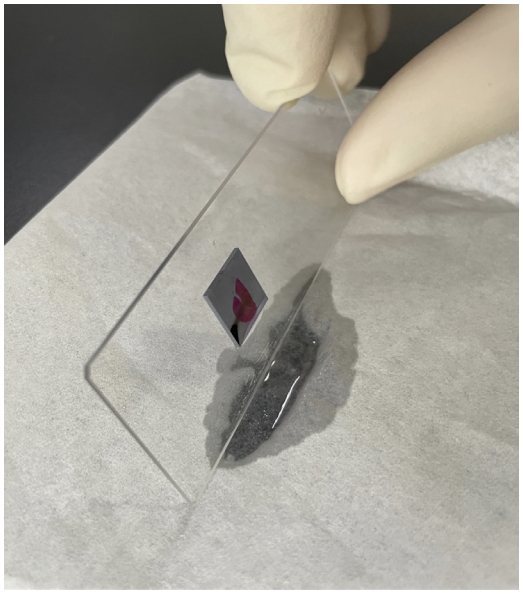
b. Add **100 µL** of Hematoxylin Solution onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets, ensuring uniform solution coverage on the chip. Incubate at room temperature for **7 min** (Hematoxylin Solution from Sigma Aldrich) or **1-2 min** (Hematoxylin Solution from Solarbio).

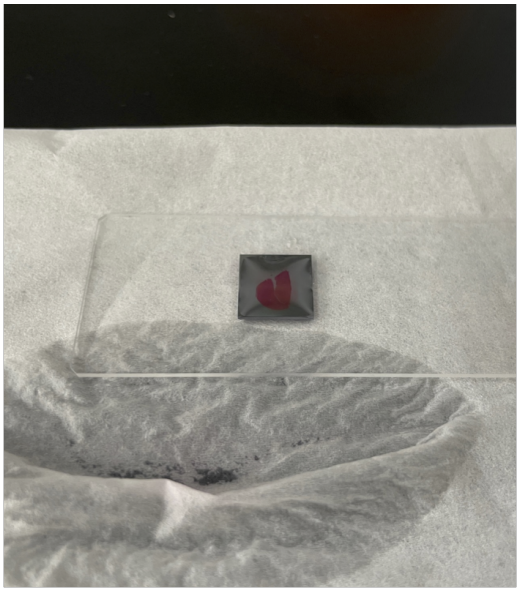
The incubation time needs to be adjusted according to the reagent manufacturer's protocol.

Equilibrate the H&E Mounting Medium to room temperature 5 min prior to use.

c. Discard Hematoxylin Solution by turning the Stereo-seq Chip Slide sideways at an angle of less than 60°, gently touch the edge of the chip with dust-free paper and allow the Hematoxylin Solution to pour onto dust-free paper. Remove as much solution as possible.

d. Add **100 μL** 0.1X SSC per chip and then discard it by turning the Stereo-seq Chip Slide sideways at an angle of less than 60° and allowing the 0.1X SSC to pour onto dust-free paper.





e. Repeat **step d. twice**.

f. Add **100 μL** of Bluing Buffer 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, ensuring uniform staining solution coverage on the chip. Incubate at room temperature for **2 min** (bluing reagent from Agilent).

The incubation time needs to be adjusted according to the reagent manufacturer's protocol.

g. Discard Bluing Buffer by turning the Stereo-seq Chip Slide sideways at an angle of less than 60°, gently touch the edge of the chip with dust-free paper and allow the Bluring Buffer to pour onto dust-free paper. Remove as much solution as possible.

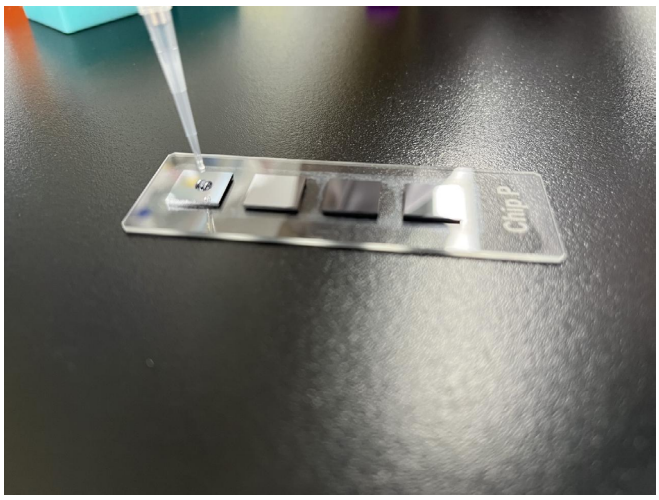
h. Add **100 μL** 0.1X SSC per chip and then discard it by turning the Stereo-seq Chip Slide sideways at an angle of less than 60° and allowing the 0.1X SSC to pour onto dust-free paper. Try to remove as much solution as possible during the final wash: gently touch the edge of the chip with dust-free paper 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 using a power dust remover held in the other hand at an approximate distance of 2-3 cm from the chip surface. Blow quickly from one side of the chip at a 30-degree angle horizontal to the plane of the chip. Ensure that there is no liquid residue around the chips.

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

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

The cap color of the H&E Mounting Medium reagent is identical to that of glycerol. Identify the reagent label with caution before use.



Ensure the coverslip is clean and free of any dust or debris. Wipe with an alcohol swab or blow the debris off with a power dust remover.

k. Using clean 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. Do not image the H&E-stained tissue section. Let it sit on the bench for **10 min**.

H&E Mounting Medium mounted chips can not be stored longer than 2 hrs at room temperature. For tissues that are prone to RNA degradation, such as pancrease, proceed to the next step immediately to avoid RNA degradation.

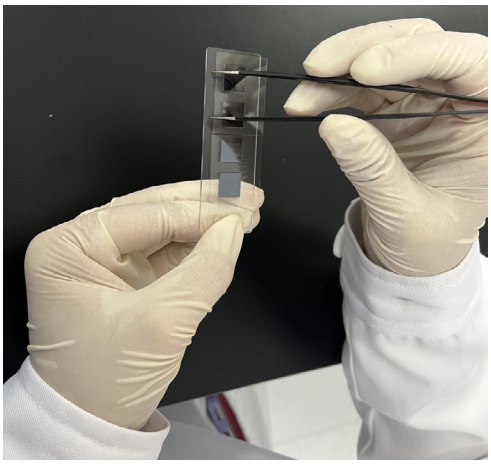
l. Set aside the 2 mL of 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in 3.1 Experiment Preparation.

m. Set the temperature of a metal bath or equivalent heating instrument to 37°C, and set the PCR program on hold at 37°C.

n. Take out one gasket and one cassette from the STOmics Accessory Kit. Blow any impurities off the gasket with a power dust remover, and then assemble only the cassette and the gasket.

o. Ensure that the PCR thermal cycler has been switched on and set to 37°C. Pre-warm the assembled cassette and gasket in the PCR thermal cycler for 10 min. Warm the aliquoted 1X Permeabilization Reagent Solution inside the 37°C PCR thermal cycler or metal bath for **>10 min** (no longer than 30 min).

p. After 10 min of mounting, use clean forceps to grip the coverslip and then pull and slide the coverslip over the Stereo-seq Chip Slide edge slowly until the chips and the coverslip are completely separated.



q. Place the Stereo-seq Chip Slide in a centrifuge tube filled with at least 30 mL 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 the solution.

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

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

Refer to Section 3.6 Testing of Tissue Permeabilization Time Point to continue with the procedure.