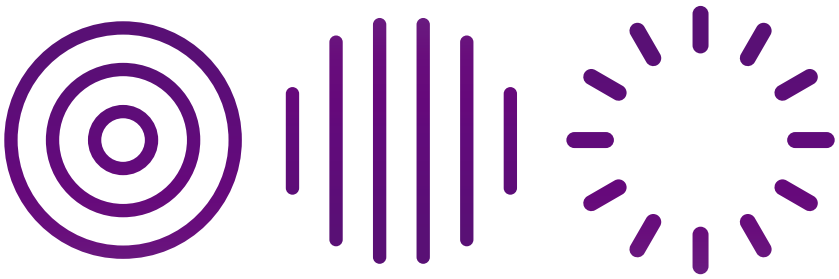
STOmics

STEREO-seq PERMEABILIZATION SET FOR STEREO-CITE PROTEO-TRANSCRIPTOMICS APPLICATION

USER MANUAL



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

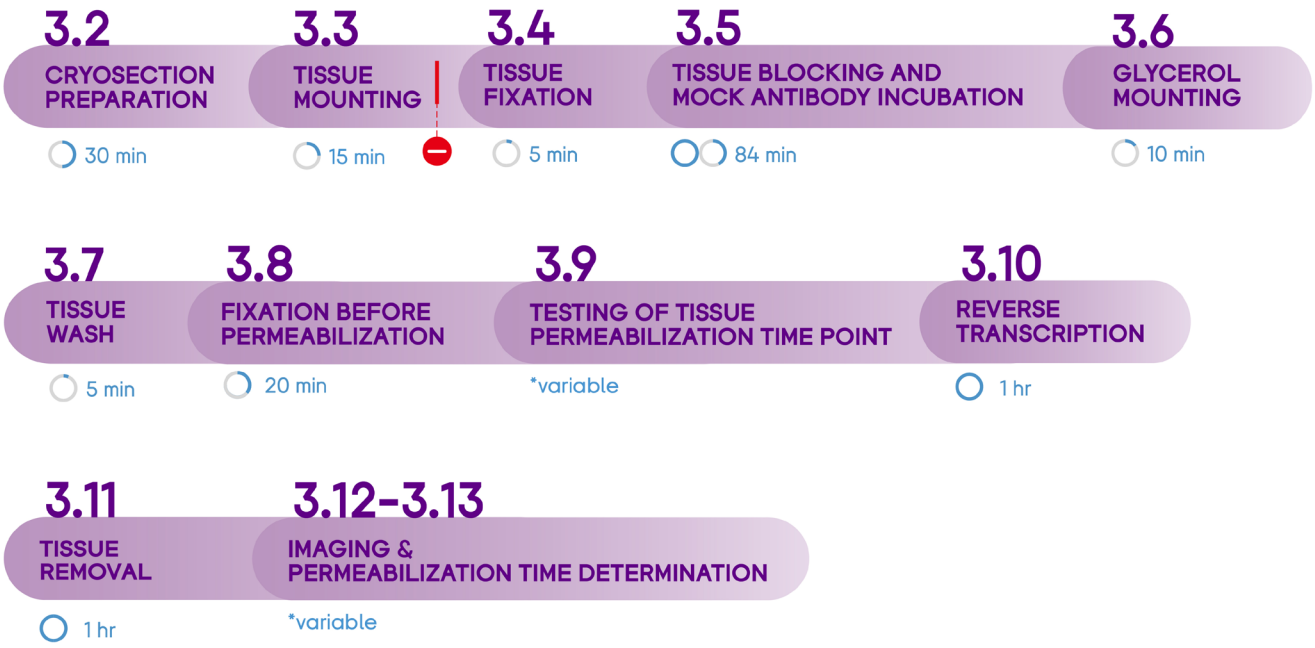
STUM-PR004

REVISION HISTORY

Manual Version:	A
Kit Version:	V1.1
Date:	Mar. 2025
Description:	Initial release

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

WORKFLOW



TOTAL TIME: ~6.5 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

This user manual provides experiment instructions specifically for the Stereo-CITE Proteo-Permeabilization Set V1.1.

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-CITE Proteo-Transcriptomics experiments. Featured with high resolution and a large Field of View (FOV), Stereo-seq Chip P Slides are patterned with capture 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. This manual provides detailed methods of optimal permeabilization time determination for Stereo-CITE Proteo-Transcriptomics Kit V1.1.

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

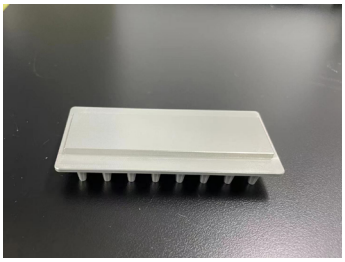
1.2. List of Kit Components

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 Accessory Kit *2 (5 PCs)

Compatible auxiliary not included:

- (Ordered separately) Stereo-seq PCR Adaptor (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 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(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.: 1000033700	
Component	**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 10°C~30°C	Expiration Date: refer to label

Table 1-4 Stereo-seq PCR Adaptor Components

Stereo-seq PCR Adaptor	Cat. No.: 301AUX001
Component	**Quantity (per kit)**
Stereo-seq PCR Adaptor	2 EA
Storage Temperature: 18°C~25°C	Transported at 10°C~30°C
Expiration Date: refer to label	

1.3. Additional Equipment and Materials

Table 1-5 lists 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 **STOmics Microscope Assessment Guideline.**

Table 1-5 Additional Equipment and Materials

Equipment		
Brand	**Description**	**Cat. No.**
Leica	Cryostat	CM1950
-	Benchtop Centrifuge	-
-	Pipettes	-
-	Metal Bath (or equivalent instrument)	-
Leica*	Fluorescence Microscope	DM6M
STOmics*	Fluorescence Microscope	900-000586-00
-	Vortex mixer	-
Eppendorf	Refrigerated Centrifuge (for Stereo-CITE application)	5418 R
Bio-Rad^	T100™ Thermal Cycler	1861096
ABI^	ProFlex™ 3 x 32-well PCR System	4484073

* 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

Reagents		
Brand	**Description**	**Cat. No.**
BOSTER (or other brands)	4% Paraformaldehyde (with DEPC)	AR1068
Invitrogen*	Nuclease-free Water	2186768
Ambion*	Nuclease-free Water	AM9937
	20X SSC	AM9770
Sigma Aldrich	Hydrochloric Acid, HCl (0.1 N)	2104-50ML
	Methanol	34860-1L-R
	Triton X-100 Solution, 10%	93443-100ML
	DMSO	D4540
Thermo Fisher Scientific™	Gibco™ PBS, pH 7.4	10010031
	Gibco™ Horse Serum	26050070
Invitrogen	Salmon Sperm DNA, sheared (10 mg/mL)	AM9680
SAKURA	SAKURA Tissue-Tek® O.C.T. Compound	4583

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

Table 1-7 Additional consumables required

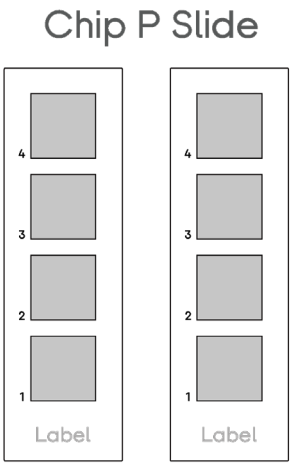
Consumables		
Brand	**Description**	**Cat. No.**
-	Aluminum Foil	-
-	Slide Container	-
-	Forceps	-
-	Microscope Glass Coverslip (size: 24 mm x 32 mm)	-
-	Slide Staining Rack	-
-	Sterilized Syringe	-
Millipore	Millex Syringe Filter, Durapore PVDF, 0.22 μm pore size	SLGV033N
Corning	Corning® 100 mm TC-treated 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	1.5 mL Centrifuge Tubes	MCT-150-A
	2.0 mL Centrifuge Tubes	MCT-200-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

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.

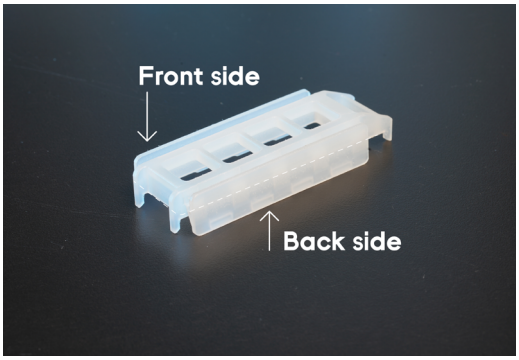


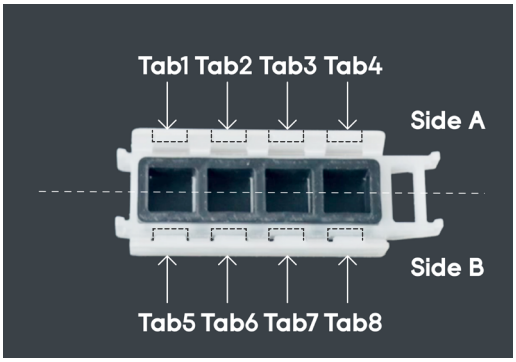
Stereo-seq Chip P Slide Storage

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

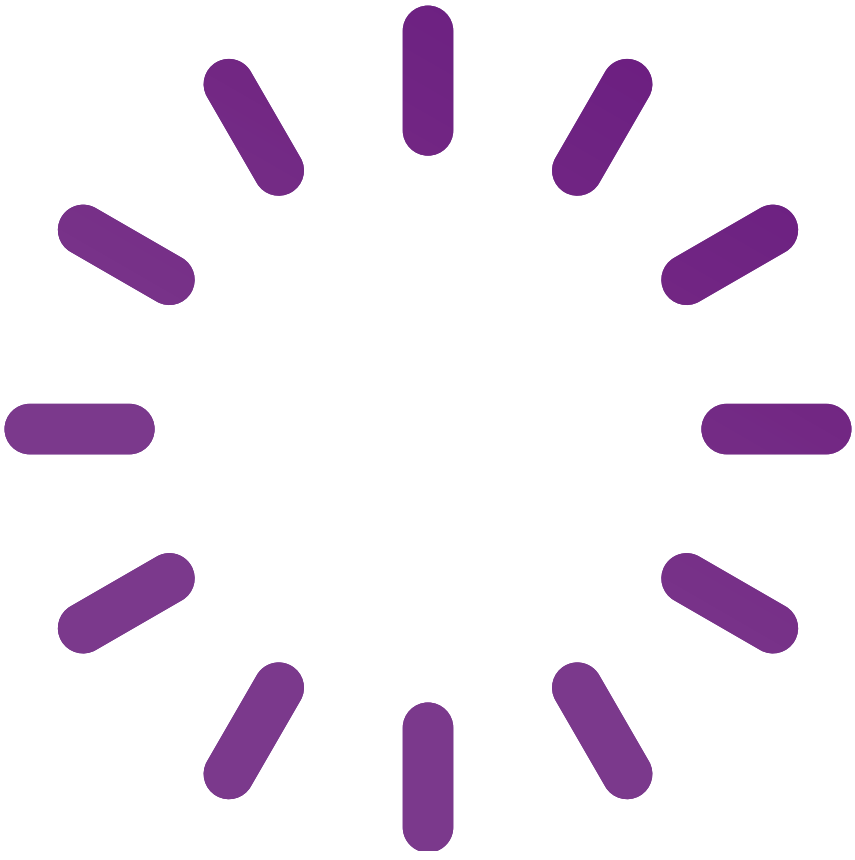
For frozen sample embedding, refer to **Sample Preparation, Sectioning, and Mounting Guide for Fresh Frozen Samples on Stereo-seq Chip Slides (Document No.: STUM-SP001)**.

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

FRESH FROZEN SAMPLE, STEREO-SEQ PERMEABILIZATION SET FOR STEREO-CITE PROTEO-TRANSCRIPTOMICS APPLICATION, STANDARD OPERATING PROCEDURE



3.1. Experiment Preparation

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

Table 3-1 Experiment Preparation Steps

Reagent	Preparation Step	Maintenance
4% PFA	Mix well after thawing at 4°C. Aliquot to 2 mL/tube for storage. Equilibrate to room temperature before use.	4°C up to 1 week
0.1X SSC	Dilute 300 µL of 20X SSC to 60 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, up to 12 hr
5X SSC	Dilute 1 mL of 20X SSC to 4 mL	Room temperature
Methanol	Pre-cool at -20°C for no longer than 30 min before use.	-20°C
Filtered Serum Aliquot	Thaw the horse serum, then filter it with a 0.22 µm pore-sized filter membrane and a sterilized syringe. For convenience, aliquot the filtered serum in 200 µL per tube and store at -20°C. Thaw the aliquoted serum on ice and centrifuge at 14,000 g for 10 min at 4°C. Place on ice until use. Approximately 20 µL of serum is needed for one chip, and any remaining serum can be reused.	On ice until use, up to 6 hr
Do not freeze and thaw the aliquot more than 3 times. Keep the aliquots at -20°C for long-term storage.		
Sheared Salmon Sperm DNA	Thaw on ice before use, 15 µL/chip.	On ice until use, up to 6 hr
RI	Take from -20°C and place on ice until use.	On ice until use, up to 12 hr
10% Triton X-100	Use 10% Triton X-100 or dilute 100% Triton X-100 with nuclease-free water.	Room temperature
Glycerol	Equilibrate to room temperature 5 min in advance.	Room temperature
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. 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 Permeabilization Reagent (PR) Enzyme (red cap, in powder form), and thoroughly mix the reagent through 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.**		
0.5X Permeabilization Reagent Solution	Dilute 7.5 µL of 10X Permeabilization Reagent Stock Solution to 150 µL with 0.01N HCl (at least 150 µL/chip).	On ice until use, up to 6 hr

Other Preparation

Equipments	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 (heating lid at 60°C); 2. 45°C for reverse transcription (heating lid at 60°C); 3. 55°C for tissue removal (heating lid at 60°C).	Check the PCR Thermal Cycler 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.
Metal Bath (or equivalent heating instrument)	37°C for pre-heating of Permeabilization Reagent (PR) Solution	Check the instrument for any abnormalities and replace it if necessary.
Centrifuge	Adjust the temperature to 4°C in advance.	Centrifuge the thawed filtered serum.

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**	**Reverse Transcription**
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 cryostat chamber. Allow it to equilibrate to the cryostat chamber temperature for **30 min**.

e. Meanwhile, add sufficient 4% PFA solution (at least 400 µL 4% PFA solution per chip) and equilibrate to room temperature in a fume hood.

f. While waiting for the cryostat temperature to equilibrate to target temperature, prepare the blocking solution (4X for 4 chips) according to Table 3-2 and leave it on ice.

Table 3-2 Blocking Solution

Component	1X (µL)	1X + 10% (µL)	4X + 10% (µL)
5X SSC	90	99	396
10% Triton X-100	1.5	1.65	6.6
Sheared Salmon Sperm DNA	15	16.5	66
RI	7.5	8.25	33
Filtered Serum Aliquot	15	16.5	66
Nuclease-free Water	21	23.1	92.4
Total	150	165	660

g. 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).

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

i. Trim again if necessary to ensure a good fit between the tissue section and Stereo-seq Chip. Now, the specimen is ready for cryosection.

3.3. Tissue Mounting

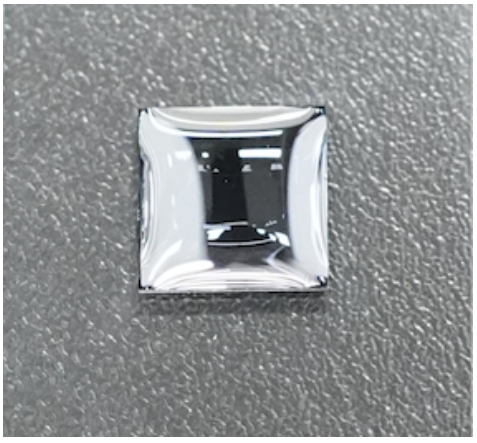
For a demonstration video of tissue mounting onto the Stereo-seq Chip Slide, refer to the link below or scan the QR code:
https://en.stomics.tech/resources/videos/list.html

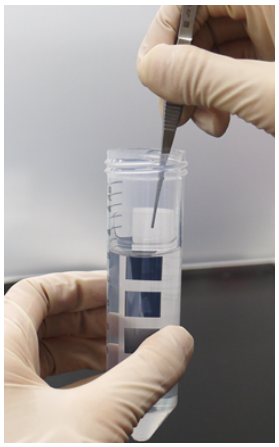
a. Take the Stereo-seq Chip P Slide out of the vacuum-sealed aluminum bag and record the Chip ID (SN) number located on the back of the slide. Do not touch the front of the chip.

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.

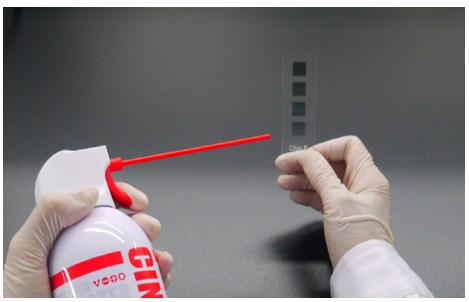
c. Equilibrate the Stereo-seq Chip Slide to room temperature for **1 min** on the bench, then rinse with 100 µL nuclease-free water **twice** using a pipette, or, rinse the slide in a 50 mL centrifuge tube or slide container with sufficient nuclease-free water by holding the slide with forceps and pulling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

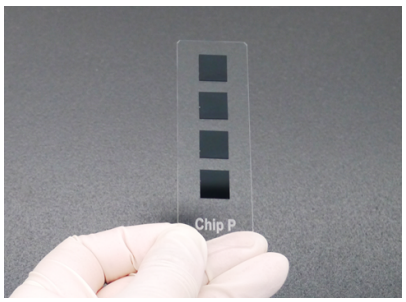




Seal unused slides in the original packaging (first in the slide container and then in the sealable aluminum bag) and store at -25°C ~ 8°C. KEEP the desiccant in the aluminum bag.

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

g. Tissue mounting can be achieved using either the warm method (option A) or the cold method (option B). We recommend practicing tissue mounting and section placement on plain glass slides first. Select proper appropriate section thickness according to the experiment needs; a section thickness of 10 μm is normally used.

A. 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).

B. 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.

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 or a 50 mL tube, 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. Transfer the sealed container 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 21 days.**
- **When retrieving Stereo-seq Chip Slides with tissue from the freezer, transfer out the slide container on dry ice, take out the tissue containing the Stereo-seq Chip Slides, then immediately incubate at 37°C with PCR Adaptor for 5 min.**

3.4. Tissue Fixation

a. Refer to **Appendix I: Stereo-seq Slide Cassette Assembly** for assembling the Cassette and Gasket onto the Stereo-seq Chip Slide.

b. Assemble the Stereo-seq Chip Slide onto the cassette, forming a handheld Stereo-seq Slide Cassette. Make sure the 8 tabs are locked in place and the cassette is tightly securing the sides of the cassette.

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

c. Place the Stereo-seq Slide Cassette in a fume hood, and add **400 µL** of 4% PFA solution per well. Place unpeeled sealing tape on top of the Slide Cassette and incubate for **5 min at room temperature**.

d. After fixation, remove the **unpeeled** sealing tape and place it on the work surface for later use.

e. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20° and remove 4% PFA solution from the corner of the well using a pipette, keeping the chip and tissue surface moist.

f. Immediately add 0.1X SSC solution (**400 µL** per well) and incubate at room temperature for **1 min**.

g. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20° and remove the 0.1X SSC from a corner of the well using a pipette, keeping the chip and tissue surface moist.

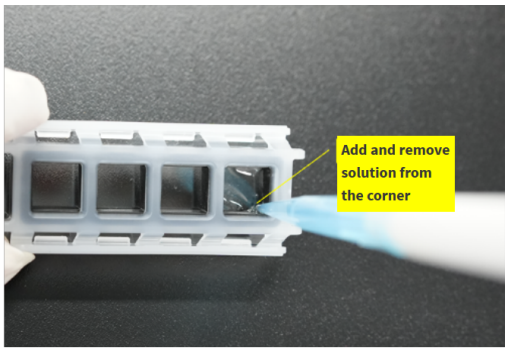
Avoid drying out the tissue during the liquid exchange process. Be sure that all the tissue sections are completely submerged.

h. Repeat **step f**.

i. During the incubation, transfer the Stereo-seq Slide Cassette from the fume hood to a bench. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20° and remove the 0.1X SSC solution from a corner of the well using a pipette. Keep the chip and tissue surface moist.

3.5. Tissue Blocking and Mock Antibody Incubation

a. Immediately add **150 μL** blocking solution into the well and place **unpeeled** sealing tape on top of the Slide Cassette, then incubate at room temperature **for 65 min**.



b. During the incubation, prepare the mock secondary antibody solution according to Table 3-3. Vortex the mixture and place it on ice for later use.

Table 3-3 Secondary Antibody Mock Incubation Buffer

Component	1X (μL)	1X + 10% (μL)	4X + 10% (μL)
5X SSC	90	99	369
RI	7.5	8.25	30.75
Nuclease-free Water	52.5	57.75	215.25
Total	150	165	615

c. After the incubation is completed, remove the unpeeled sealing tape and place it on the bench for later use.

d. Remove the blocking solution from a corner of the well using a pipette, keeping the chip and tissue surface moist.

e. Immediately add **200 μL** 0.1X SSC solution per well and incubate the chip at room temperature for **1 min**.

f. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20° and remove the 0.1X SSC solution from the corner of the well using a pipette, keeping the chip and tissue surface moist.

g. Repeat **steps e.** and **f**.

h. Immediately add **150 μL** secondary antibody mock incubation buffer into the well. Place **unpeeled** sealing tape on top of the cassette and incubate at room temperature **in the dark** for **15 min**.

3.6. Glycerol Mounting

a. When the incubation is completed, remove the mock secondary antibody incubation solution from the corner of the well, keeping the tissue surface moist.

b. Add **200 µL** 0.1X SSC solution per well and incubate the chip at room temperature for **1 min**.

c. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20° and remove the 0.1X SSC solution from the corner of the well using a pipette, keeping the chip and tissue surface moist.

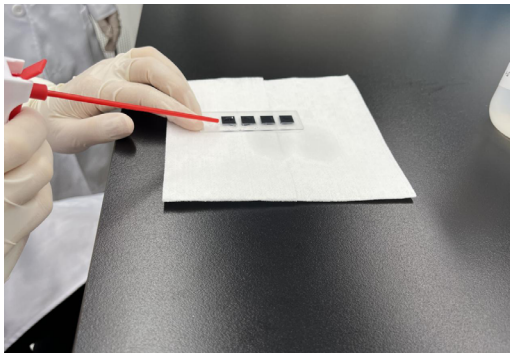
d. Repeat wash **steps b.** and **c.**

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

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

If you are working with multiple slides at the same time, label the Slide Cassettes properly and avoid mixing them up.

f. Hold the slide with one hand and completely dry the chips using a power dust remover held in the other hand at an approximate distance of 2-3 cm from the chip surface. Blow gently from one side of the chip at a 30-degree angle horizontal to the plane of the chip.

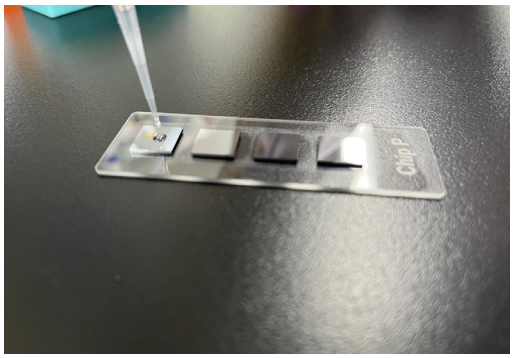


Alternatively, centrifuge the Stereo-seq Chip Slide for 10 sec in a slide spinner to completely dry the chips. Ensure that no residual solution is left on the chip.

Ensure that the glycerol has been equilibrated to room temperature for 5 min before using it in the next step.

Ensure that 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.

g. Centrifuge the glycerol before use to ensure that there are no air bubbles in the glycerol. Pipette **5 µL** glycerol gently onto the center of the tissue on each chip without introducing air bubbles.



h. 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 (no longer than 1 hr)**.

Glycerol mounted chips can not be stored longer than 1 hr at room temperature. For tissues that are prone to RNA degradation, such as pancreas, proceed to next step immediately to avoid RNA degradation.

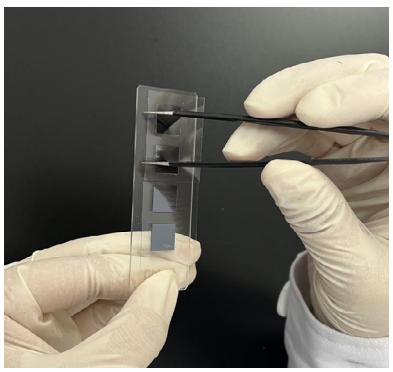
i. Keep the chip covered with the coverslip. Prepare 70% DMSO solution according to Table 3-4 in a fume hood. Mix thoroughly, and equilibrate to room temperature before use. Prepare fresh before each experiment and use within **30 min** after preparation.

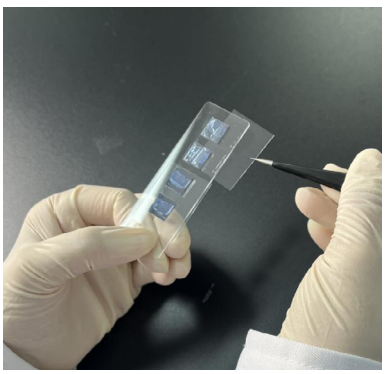
Table 3-4 70% DMSO

Component	1X (μL)	1X + 10% (μL)	4X + 10% (μL)
1X PBS	120	132	528
DMSO	280	308	1232
Total	400	440	1760

3.7. Tissue Wash

a. 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.





b. Wipe off excess solution from around the edges and the back of the slide with dust-free paper without touching the chips. Make sure there is no solution residue.

c. There is no need to replace the gasket. 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.

Do not touch the front of the chip.

d. Add **400 µL** 0.1X SSC solution per well. Incubate for **1 min** at room temperature.

e. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20° and remove the 0.1X SSC from the corner of the well using a pipette, keeping the chip and tissue surface moist.

f. Repeat wash **step d**.

g. Place the Stereo-seq Slide Cassette in a fume hood. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, and remove the 0.1X SSC solution from the corner of the well using a pipette, keeping the chip and the tissue surface moist.

h. Add **400 µL** 70%DMSO solution per well. Place **unpeeled** sealing tape on top of the Slide Cassette, then incubate for **5 min** at room temperature.

i. During incubation, prepare sufficient methanol to a 50 mL centrifuge tube or a slide container, and ensure that all tissue sections are completely submerged.

Immerse a blank microscope slide in the 50 mL centrifuge tube or slide container to ensure that there is sufficient methanol.

j. Keep the tube cap tightly closed and place it in the cryostat chamber. Allow it to equilibrate to the cryostat chamber(-20°C) temperature for **5-30 min**.

3.8. Fixation before Permeabilization

a. Discard the sealing tape and discard the 70% DMSO solution with a pipette.

b. Remove the slide from the Stereo-seq Slide Cassette according to the instructions in **Appendix I: Stereo-seq Slide Cassette Assembly**. Discard the used gasket and place the cassette on the bench for later use.

Do not touch the front of the chip.

c. Wash the slide by immersing it in a 50 mL centrifuge tube or a slide container with sufficient 0.1X SSC for **5 sec**, ensuring that all tissue sections are completely submerged. Take out the Stereo-seq Slide and wipe off excess solution on the slide with Kimwipes to ensure no liquid remaining.

d. After washing, immediately place the Stereo-seq Chip Slide in the -20°C precooled methanol that you prepared in **step i**. in **section 3.7**. and allow it to fix for **20 min**. Ensure that all tissue sections are completely submerged in methanol.

e. During the fixation process, set aside the 2 mL of 0.01N HCl and 0.5X Permeabilization Reagent (PR) Solution that you prepared in **3.1 Experiment Preparation**.

Table 3-5 0.5X Permeabilization Reagent Solution

Component	1X (μL)	1X + 10% (μL)	4X + 10% (μL)
0.01N HCl	142.5	156.75	627
10X Permeabilization Reagent Stock Solution	7.5	8.25	33
Total	150	165	660

f. Prewarm the 0.5X Permeabilization Reagent (PR) Solution in the 37°C PCR Thermal Cycler or metal bath for **10 min (no longer than 30 min)**.

j. After fixation, take the Stereo-seq Chip Slide out of the 50 mL centrifuge tube or the slide container. Wipe off the excess methanol from around the edges and the back of the slide with dust-free paper without touching the tissue. Ensure that 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 allow the methanol to evaporate completely.

i. Assemble a new 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**. Grip along the Stereo-seq Cassette to ensure that the Stereo-seq Chip Slide has been locked in place. Proceed to **3.9 Testing of Tissue Permeabilization Time Point immediately**.

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

3.9. Testing of Tissue Permeabilization Time Point

a. Thaw RT QC Buffer Mix on ice until use.

b. Ensure that the PCR Thermal Cycler has been set to 37°C (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

c. 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)**.

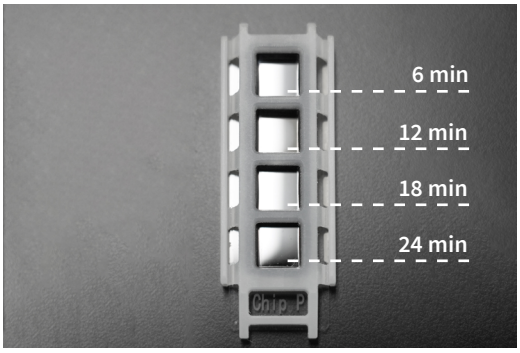
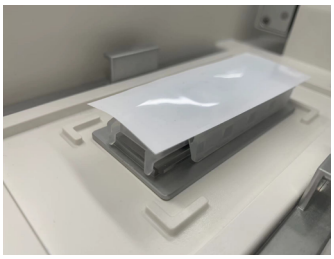


Figure 1. Permeabilization Time Testing (min)

1) Place the Stereo-seq Slide Cassette in the 37°C PCR Thermal Cycler, and add **150 µL** of 0.5X Permeabilization Reagent Solution onto the chip with **24-min** time point. Pipette one droplet at each corner of the chip and then add the rest of the solution to the middle to merge all the droplets.

Make sure the chip is completely covered with 0.5X Permeabilization Reagent Solution.

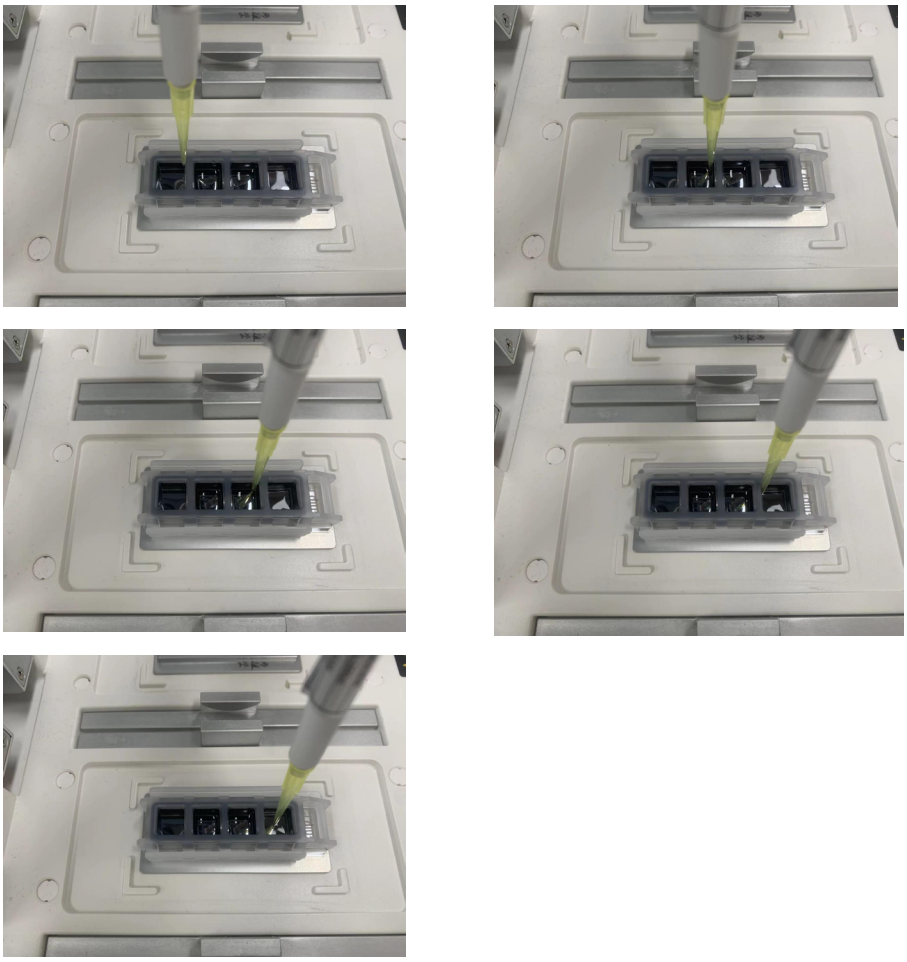
2) Place a new unpeeled sealing tape on top of the Stereo-seq Slide Cassette and let the chip incubate inside the PCR Thermal Cycler at 37°C.



3) After **6 min**, open the lid, remove the unpeeled sealing tape and add **150 μL** of 0.5X 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 above steps until the chip with the shortest permeabilization time (**6 -min**) begins to incubate.



d. While waiting for permeabilization to be completed, prepare RT QC Mix according to Table 3-6, wrap the RT QC Mix in aluminum foil, and place RT QC Mix on ice **in the dark** until later use.

Table 3-6 RT QC Mix

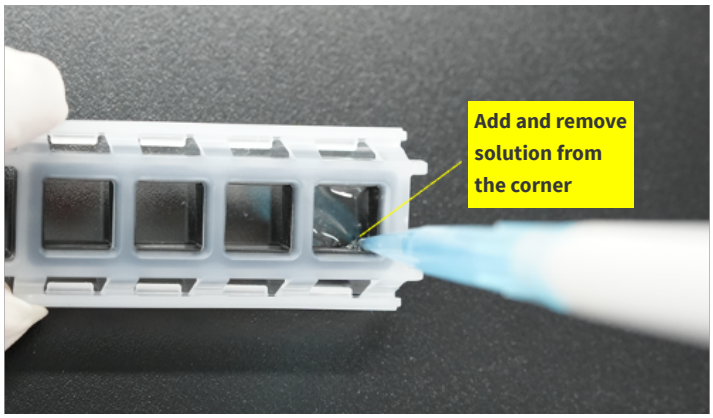
Component	1X (μL)	1X + 10% (μL)	4X + 10% (μL)
RT QC Buffer Mix	90	99	396
RT QC Enzyme Mix	10	11	44
Total	100	110	440

e. When incubation is completed, remove the unpeeled sealing tape from the Stereo-seq Slide Cassette. Then transfer the Stereo-seq Slide Cassette from the PCR Adaptor (37°C) to the bench, skip the 37°C step, and continue to the 45°C step **(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

f. Slightly tilt the Stereo-seq Slide Cassette, and, using a pipette, remove Permeabilization Reagent (PR) Solution from the corner of each well without touching the chip surface.

g. Add **200 µL** of Wash Buffer from the corner of each well.



h. Discard the Wash Buffer with a pipette from the corner of each well, keeping the chip and tissue surface moist.

Do not allow the tissue to dry completely during the wash step. It is recommended that you perform the process on one chip at a time.

To prevent RNA degradation, proceed immediately to 3.10 Reverse Transcription.

3.10. Reverse Transcription

a. Take out the prepared RT QC Mix, mix well by pipetting, and spin down briefly. Immediately add **100 µL** of RT QC Mix per chip along the side of each well, ensuring that the chip surface is uniformly covered with RT QC Mix.

b. Ensure that the temperature of the PCR Thermal Cycler with PCR Adaptor has been set to 45°C in advance.

c. Apply a new sealing tape on top of the Stereo-seq Slide Cassette and make sure it is sealed tightly.

d. Place the Stereo-seq Slide Cassette on the PCR Adaptor of the PCR Thermal Cycler (45°C), close the lid, and incubate the Stereo-seq Slide Cassette at 45°C for **1 hr** or longer (no longer than 5 hr).

3.11. 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 3-7 and place the mix at room temperature.

Table 3-7 Tissue Removal Mix

Component	1X (μL)	1X + 10% (μL)	4X + 10% (μL)
TR Buffer	392	431.2	1724.8
TR Enzyme	8	8.8	35.2
Total	400	440	1760

c. Remove the Stereo-seq Slide Cassette from the PCR Adaptor and skip the PCR program 45°C to 55°C.

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

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

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

g. Slightly tilt the Stereo-seq Cassette and, using a pipette, remove 0.1X SSC solution from the corner of each well.

h. Repeat **steps e. through g**.

i. Add **400 μL** of Tissue Removal Mix per well without introducing air bubbles.

j. Apply a new sealing tape on top of the Stereo-seq Slide Cassette and make sure it is sealed tightly.

k. Incubate the sealed Stereo-seq Slide Cassette at 55°C on the PCR Adaptor for **1 hr**.

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

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

If the tissue removal is incomplete and there is residual tissue, the removal time can be extended to ensure complete removal (no longer than 16 hr). Ensure that all tissue has been removed.

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

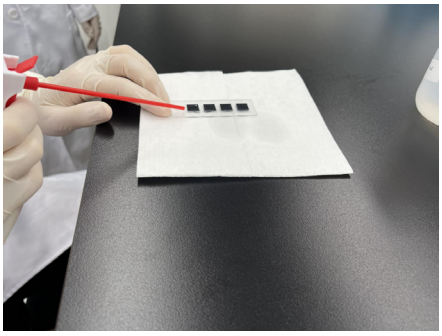
o. Gently pipette 0.1X SSC solution up and down 5 times at the corner of each well. Use a pipette to remove 0.1X SSC solution from the corner of each well.

p. Repeat **steps n. through 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 0.1X SSC solution.

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.

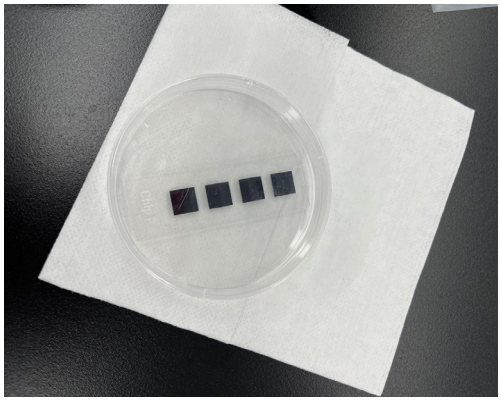


If visible tissue traces remain on the surface of the chip, wash again by adding 100 µL nuclease-free water, then blow dry. If necessary, repeat this step until no visible traces remain on the chip surface.

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 falcon tube filled with 50 mL 0.1X SSC, then rinse up and down 10 times with 50 mL 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 now ready for imaging.



3.12. Imaging

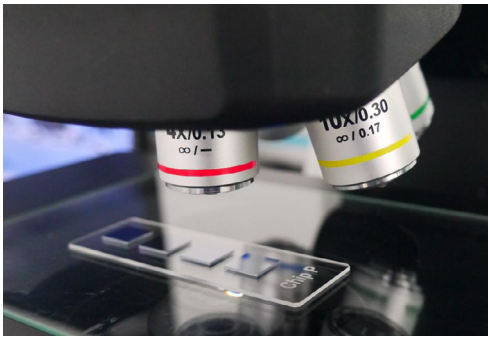
a. Create a new folder in a fluorescent microscope-connected PC, and name the folder with the chip ID number and other essential information.

Use only letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.

Example folder name: 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, and 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.



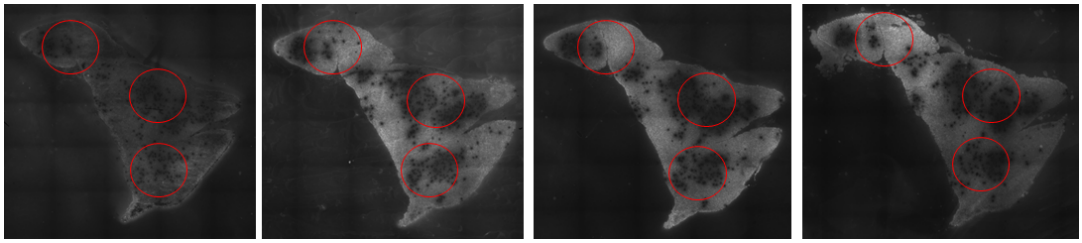
d. Find the desired capturing area with the 4X lens first, then switch to the 10X lens to complete the full scan.

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

3.13. 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.

As shown in Figure 2, for the **6-min** permeabilization time point, the fluorescence signal in some parts of the mouse thymus 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 **18-min and 24-min** permeabilization time points, the signal is uneven, and fluorescence intensity is weak. Based on the 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 thymus section

Depending on the results of the first test, a second test may be needed to determine the permeabilization time.

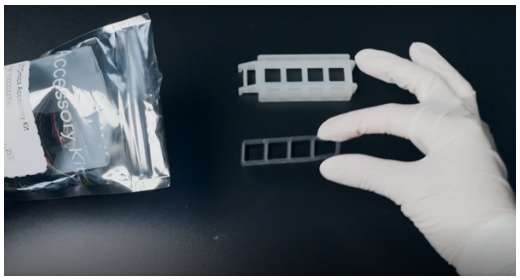
If the signal is dim at all time points tested, but there is a significant gradient difference, it is recommended that you increase the PR Enzyme concentration appropriately in the second test (e.g. 1X Permeabilization Reagent Working Solution).

If multiple time points show similar fluorescence signals and there is an obvious signal diffusion across tissues, we recommend choosing a shorter time point for the second test.

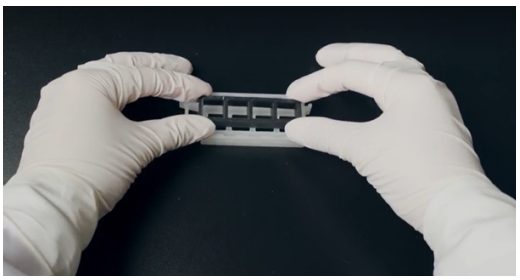
Appendix I: Stereo-seq Slide Cassette Assembly

Stereo-seq Slide Cassette Assembly

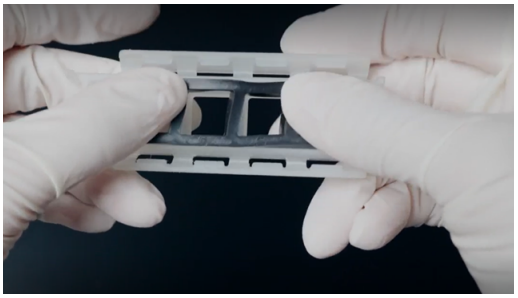
a. Take the Stereo-seq Slide Cassette and Gasket out of the STOmics Stereo-seq Accessory Kit.



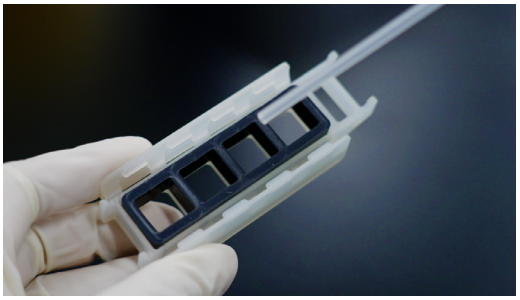
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



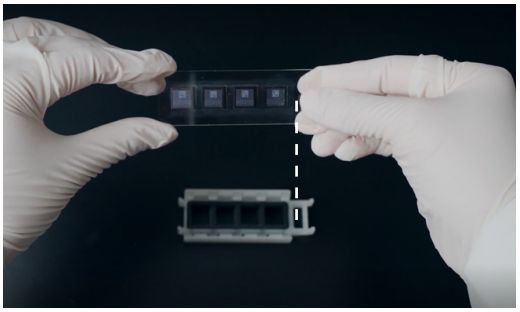
c. Press the gasket down to secure it in the cassette.



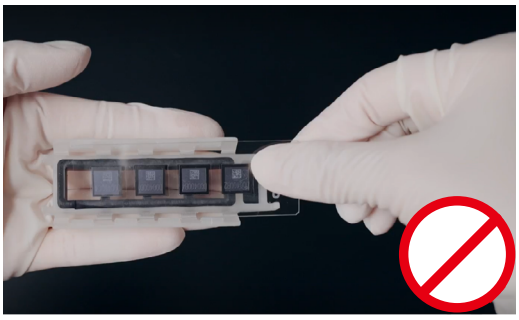
d. If necessary, use a power dust remover to blow any debris off the gasket.



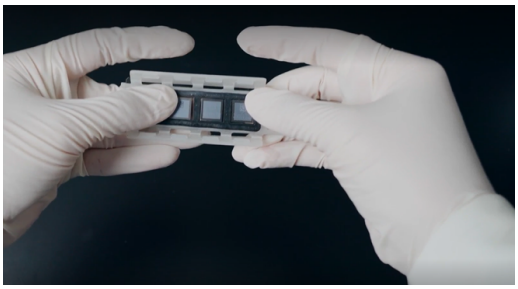
e. Pick up the Stereo-seq Chip Slide and flip it over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.



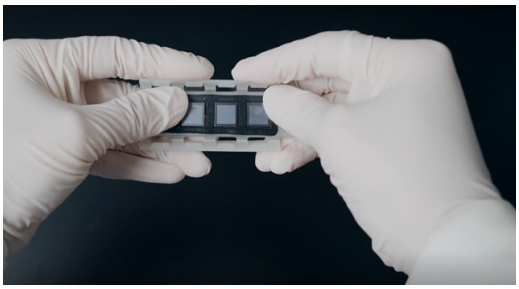
f. Ensure that the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert the Stereo-seq Chip Slide under the bottom 4 tabs.



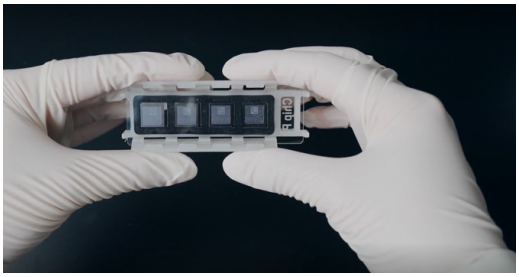
g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2 and place your right thumb between tab 3 and tab 4.



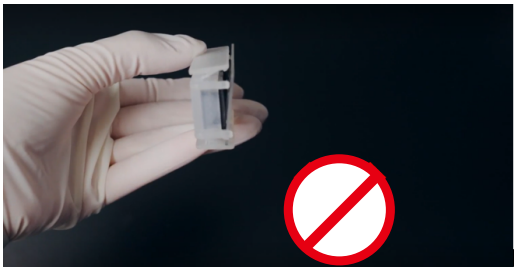
h. Press down evenly on the upper side (A side) of the slide (near the edge) and then simultaneously press the top edge down firmly with both index fingers to clip the slide in place until you hear it click.



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

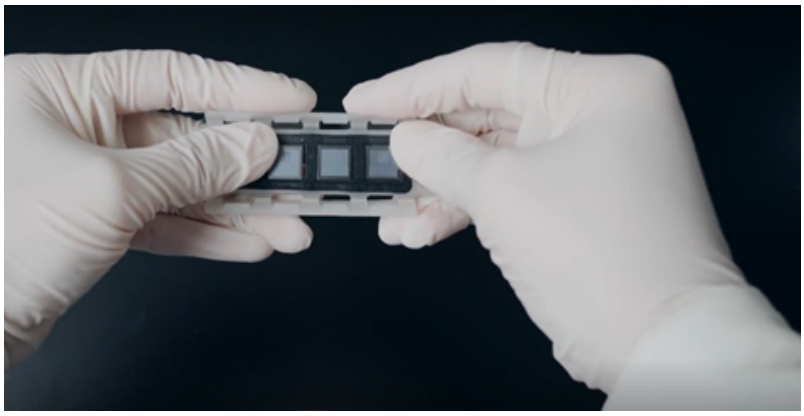


j. Recheck the Stereo-seq Slide Cassette and verify that the slide is clipped in place.



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.