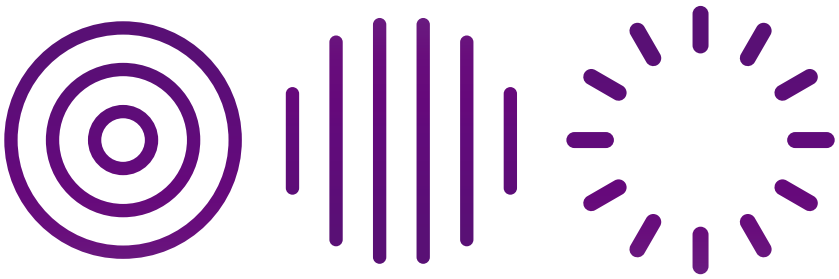
STOmics

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

USER MANUAL



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

STUM-PR004

REVISION HISTORY

Manual Version:	A	**Description**:
Kit Version:	V1.0	
Date:	Mar. 2024	Initial release

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

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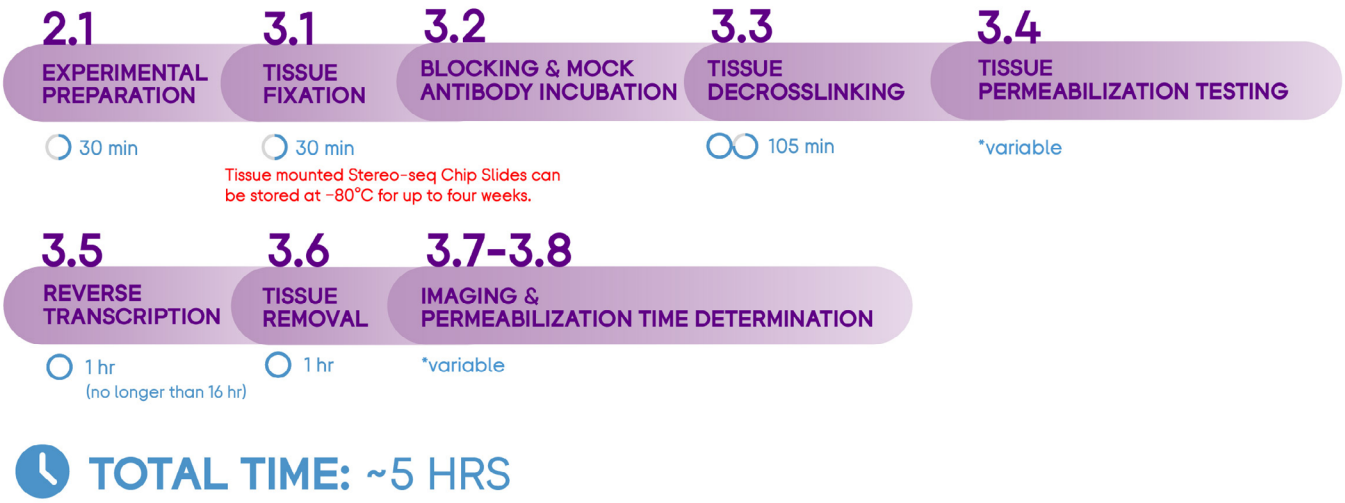
WORKFLOW



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 Stereo-CITE Proteo-Transcriptomics application enables in situ capture of whole transcriptome and proteomic information simultaneously 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 large Field of View (FOV), Stereo-seq Chip P Slides are patterned with capture probes for capturing mRNA and Antibody Derived Tags (ADT). 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-CITE Proteo-Transcriptomics Set for Chip-on-a-slide experiments.

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)

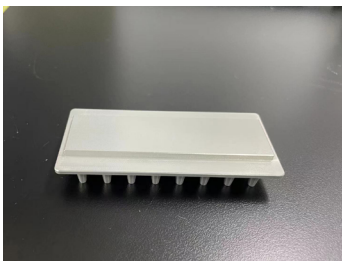
Additional reagents required:

- Decrosslinking Reagents from Stereo-seq Protein Assisted Kit (212KA11)

For more information, refer to Stereo-CITE PROTEO-TRANSCRIPTOMICS SET USER MANUAL

Compatible auxiliary not included:

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



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

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

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

Table 1-1 Stereo-seq Permeabilization Kit

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

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

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

Table 1-3 STOmics Accessory Kit

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

Table 1-4 Stereo-seq PCR Adaptor

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

Table 1-5 Stereo-seq Protein Assisted Kit

Stereo-seq Protein Assisted Kit	Cat. No.:212KA114		
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
Decrosslinking Reagents	1000043548	●	1725 μL × 5
ADT Amplification Kit	1000043547	●	220 μL × 1
ADT Primer Mix	1000043548	●	36 μL × 1
Blocking Reagents	1000044666	○ (transparent)	60 μL × 1
Storage Temperature: -25°C~-18°C	Transported by cold chain		Expiration Date: refer to label

Reagents in gray are not used in this protocol.

1.3. Additional Equipment and Materials

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

Table 1-6 Additional equipment required

Equipment		
Brand	**Description**	**Cat. No.**
-	Cryostat	-
-	Benchtop centrifuge	-
-	Pipettes	-
	Metal heating block dry bath (optional)	-
-	Vortex mixer	-
Eppendorf	Microcentrifuge (for Stereo-seq mIF application, refrigeration function required)	5418 R
Bio-Rad*	T100 Thermal Cycler	1861096
ABI*	ProFlex 3 x 32-well PCR System	4484073

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

Table 1-7 Addition reagents required

Reagents		
Brand	**Description**	**Cat. No.**
BOSTER (or other brands)	4% Paraformaldehyde (with DEPC)	AR1069
Ambion	Nuclease-free water	AM9937
	1X TE buffer, pH 8.0	AM9858
	20X SSC	AM9770
Sigma Aldrich	Hydrochloric acid, HCl (0.1N)	2104-50ML
	Methanol	34860-1L-R
Thermo Fisher Scientific™	RiboLock RNase Inhibitor (40 U/μL)	EO0382
	Gibco™ Horse Serum	26050070
	Gibco™ Goat Serum	16210064
Invitrogen	Salmon Sperm DNA, sheared (10mg/mL)	AM9680
SAKURA	SAKURA Tissue-Tek® O.C.T. compound	4583

Table 1-8 Additional consumables required

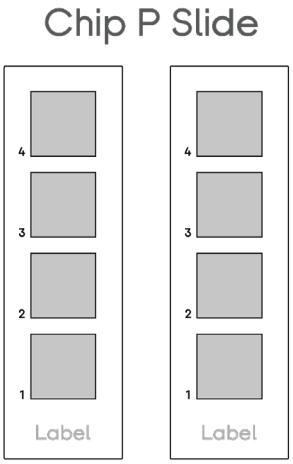
Consumables		
Brand	**Description**	**Cat. No.**
-	Stainless-steel base mold	-
-	Aluminum foil	-
-	Forceps	-
-	Slide staining rack	-
-	Sterilized Syringe	-
-	Microscope glass coverslip (area: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm)	-
Millipore	Millex Syringe Filter, Durapore PVDF, 0.22 μm pore size (for Stereo-seq mIF application)	SLGV033N
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
BBI	5.0 mL Centrifuge Tubes	F611888-0001
Kimtech	KimWipes™ delicate task wipes	34155
MATIN	Power Dust Remover	M-6318
Axygen	1,000 μL filter tips	TF-1000-L-R-S
	200 μL filter tips	TF-200-L-R-S
	100 μL filter tips	TF-100-R-S
	10 μL filter tips	TXLF-10-L-R-S
Invitrogen	Qubit Assay Tubes	Q32856
BIOSHARP	Metal Block	-

1.4. Practice Tips

Stereo-seq Chip P Slide

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

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

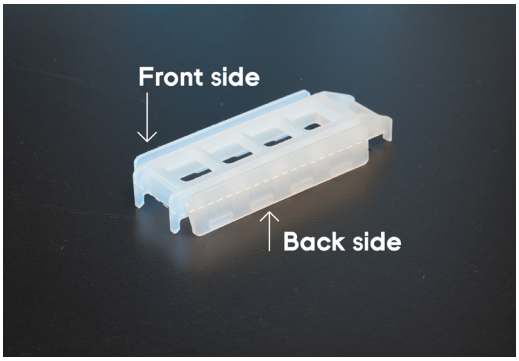


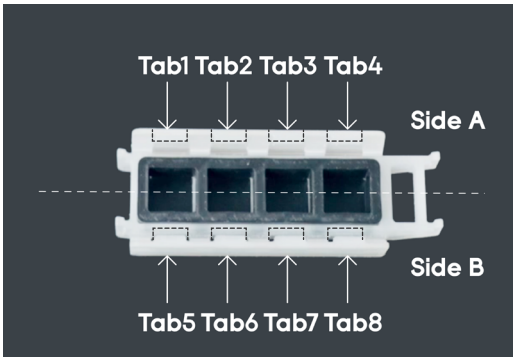
Stereo-seq Chip P Slide Storage

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

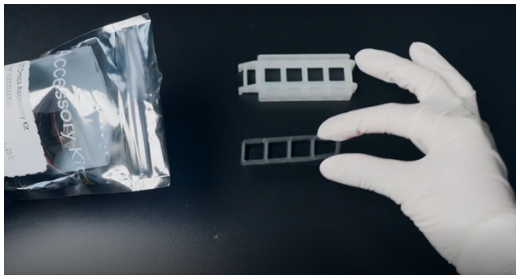




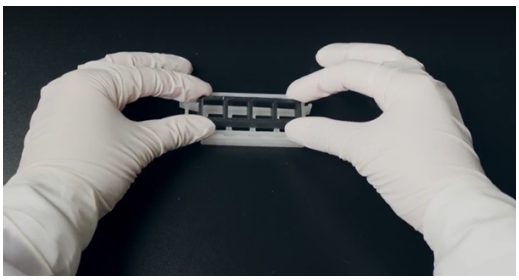
For a demonstration video of Stereo-seq Slide Cassette assembling and removal, please refer to the link or by scanning the QR code:
https://en.stomics.tech/resources/videos

Stereo-seq Slide Cassette Assembly

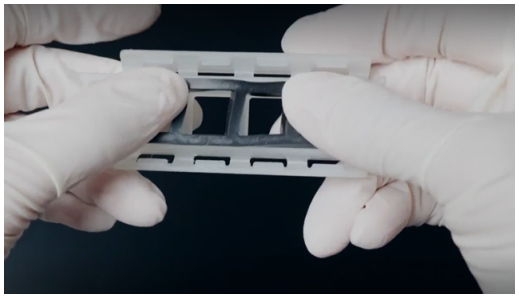
a. Take the Stereo-seq Slide Cassette and gasket out of the STOmics Stereo-seq Accessory Kit.



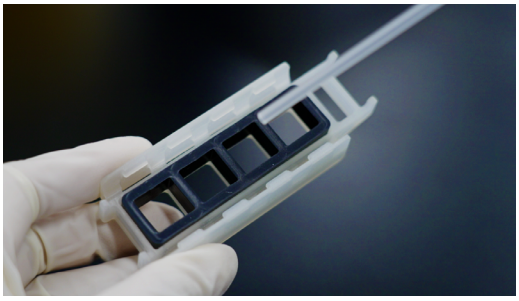
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



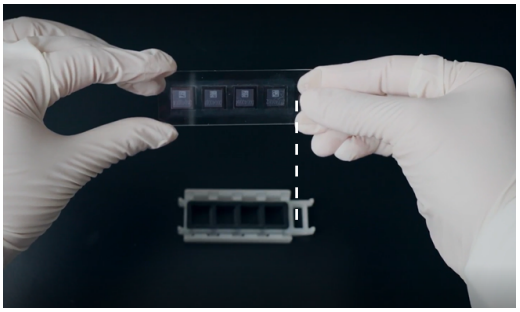
c. Press the gasket down to secure it in the cassette.



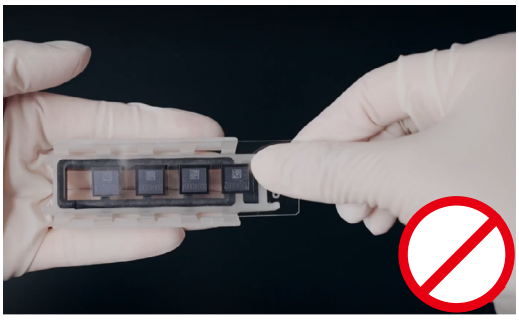
d. Use a power dust remover to blow off any debris on the gasket if necessary.



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.

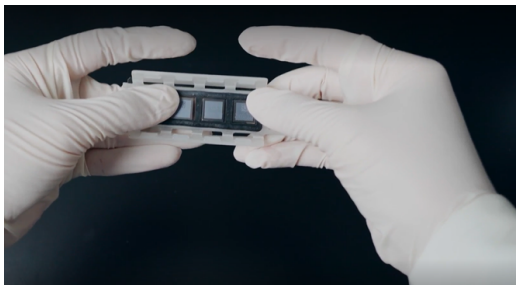


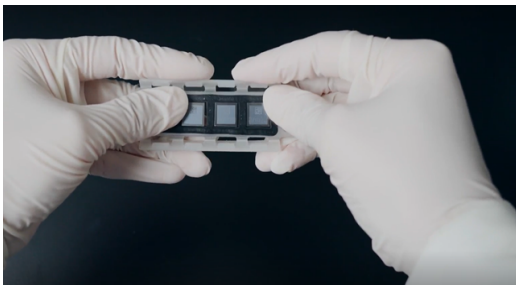
f. Ensure that the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert the Stereo-seq Chip Slide under the bottom 4 tabs.



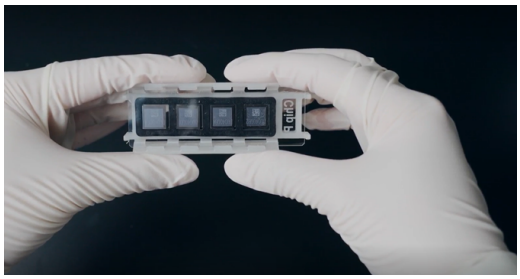
g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2, and place your while right thumb between tab 3 and tab 4.

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.

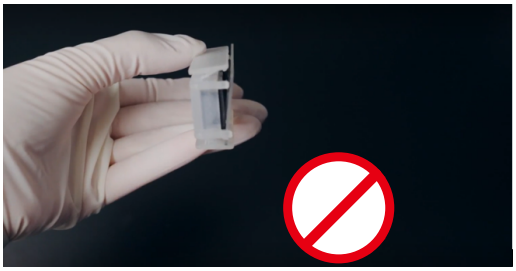




i. Press along both edges of the Stereo-seq Slide Cassette to ensure that the Stereo-seq Chip Slide is locked in place.



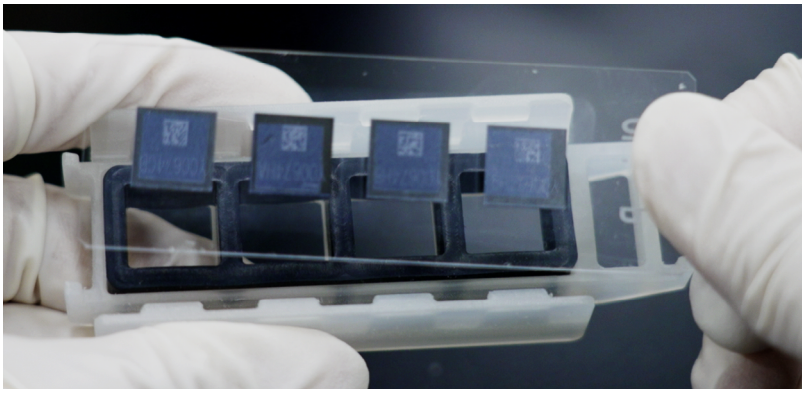
j. Look closely at the Stereo-seq Slide Cassette and confirm that the slide is clipped in place.



Stereo-seq Slide Cassette Removal

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

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



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, users are recommended that you ensure that they are familiar with related instruments, and operate them according to manufacturer's instructions.
- Instructions provided in this manual are intended for general use only, and optimization may be required for specific applications.
- Thaw the reagents in the kits properly prior to use. For enzymes, centrifuge briefly and keep them on ice until further use. For other reagents, thaw them first at room temperature followed by inverting several times to mix them properly, and centrifuge them briefly before placing on ice for further use.
- mRNA capture will be compromised or absent for any scratched areas on the front surface of the chip.

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

CHAPTER 2
SAMPLE AND EXPERIMENT PREPARATION

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

2.1. Experiment Preparation

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

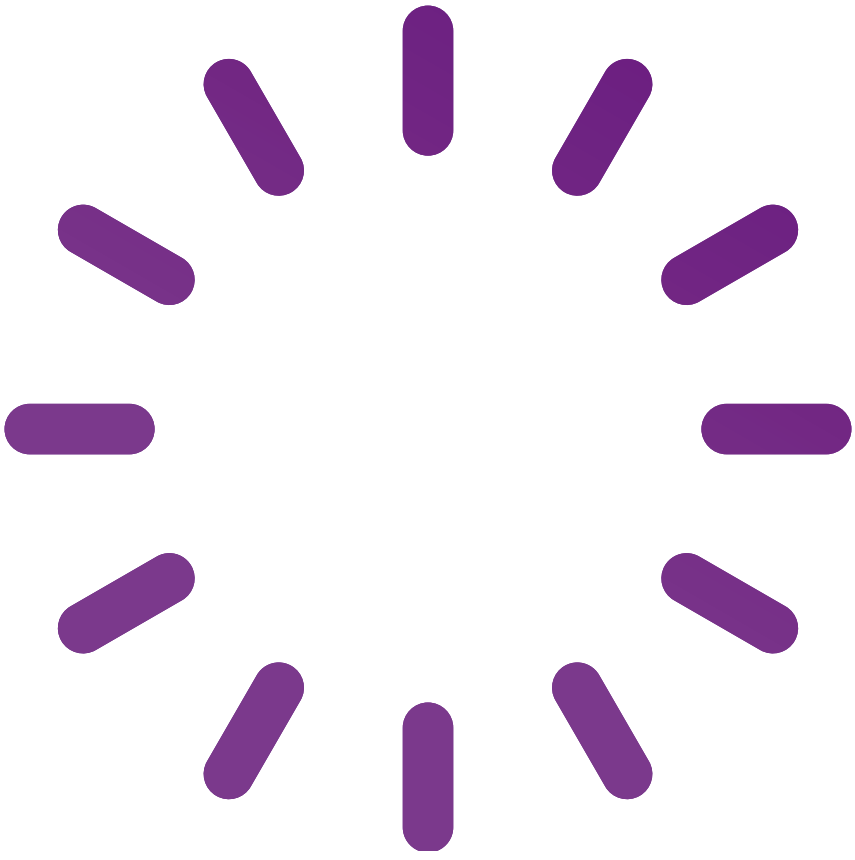
Reagent	Preparation Steps	Maintenance
4% PFA	Mix well after thawing and aliquot to a 2 mL/tube for storage. Equibrate to room temperature before use.	-20°C for no more than 1 month
5X SSC	Dilute 5 mL of 20x SSC to 20 mL.	Room Temperature
0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL; dilute 250 µL of 20x SSC to 50 mL.	Room Temperature
Wash Buffer	Prepare at least 100 µL per chip (95 µL 0.1X SSC with 5 µL RI), and prepare at least 500 µL for each permeabilization optimization experiment (475 µL 0.1X SSC with 25 µL RI).	Place on ice until ready to use.
Filtered Serum Buffer	Take the serum out in advance and thaw it. Mix horse serum and goat serum in a 1:1 ratio. Filter the mixture using a 0.22 µm filter membrane (compatible with a syringe-style sterile disposable syringe). Recommend aliquoting 200 µL per tube (for one round of permeabilization and proteo-transcriptomics workflow). Before the experiment, thaw the aliquoted serum at -20°C. Centrifuge at 4°C, 14000g for 10 min and keep it for later use. Approximately 20 µL of serum is needed for one chip, and any remaining serum can be reused.	-20°C
Do not freeze and thaw the aliquot more than 3 times.		
RI	Thaw on ice before use.	Place on ice until ready to use.
10% Triton X-100	Dilute 100% Triton X-100 into 10% Triton X-100 with nuclease-free water if there is no ready-to-use 10% Triton X-100	Room Temperature

0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Configure HCl to 0.01N. 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 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.	-20°C
DO NOT vortex the permeabilization enzyme. Mix by pipetting before use. **Aliquot this 10X stock solution to prevent freeze-thaw cycles.**		
1X Permeabilization Reagent Solution	Make 1X PR solution (150 µL / chip) by diluting 10X PR stock solution with 0.01N HCl.	Place on ice until ready to use. Can be on ice for up to 6 hr.
Decrosslinking Reagent	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature

Other Preparation		
PCR Thermal Cycler	Set the temperature in the following order: 37°C for slide drying and permeabilization (heating lid at 42°C); 42°C for reverse transcription (heating lid at 47°C); 55°C for tissue removal (heating lid at 60°C).	Check the PCR Thermal Cycler for abnormalities. If necessary, replace it.
Fluorescence Microscope	Set the epi-fluorescence channel to TRITC mode.	Room Temperature
Metal Bath	37°C for pre-warming of PR Enzyme; 70°C for pre-warming of Decrosslinking Reagent	Adjust the temperature accordingly
Centrifuge	Cool the centrifuge to 4°C in advance.	-

CHAPTER 3

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



3.1. Tissue Fixation

a. Refer to **1.4 Practice Tips** instructions on 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. Ensure that the 8 tabs are locked in place and that 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. Seal the cassette with sealing tape and incubate for **10 min at room temperature**.

d. After fixation, remove the sealing tape, 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 while keeping the chip and tissue surface moist.

e. Immediately add Wash Buffer (**400 µL** per well) and incubate at room temperature for **1 min**.

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

Do not allow the tissue to dry out during the liquid exchange process. Ensure that all the tissue sections are completely submerged.

g. Repeat steps e. and f. for one more wash..

3.2. Blocking and mock antibody incubation

a. Blocking: Transfer the Stereo-seq Slide Cassette out of the fume hood to a benchtop and add **150 μL** blocking buffer per chip onto the tissue surface immediately. Incubate the chip at room temperature for **20 min**.

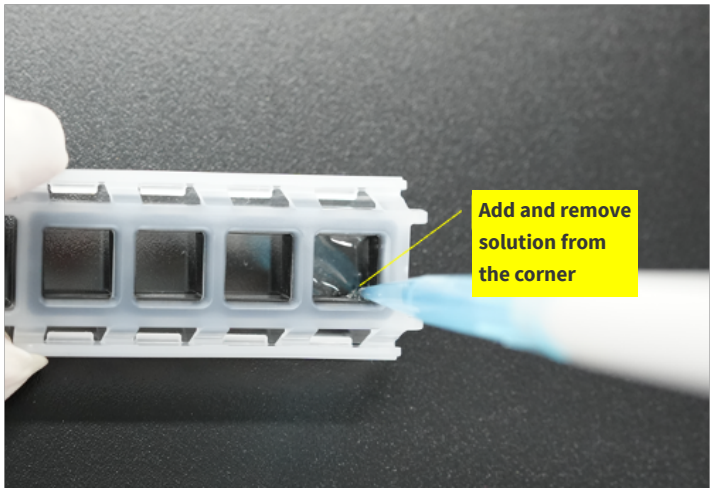


Table 3-1 Blocking Buffer

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
5X SSC	180	378	558	738
10% Triton X-100	3	6.3	9.3	12.3
RI	15	31.5	46.5	61.5
Filtered Serum	30	63	93	123
Nuclease-free water	72	151.2	223.2	295.2
Total	300	630	930	1230

b. Mock incubation of the primary antibody: remove the blocking buffer solution from a corner of the well using a pipette, keeping the chip and tissue surface moist. Add **150 μL** of blocking buffer per well onto the tissue surface immediately. Incubate the chip at room temperature for **45 min**.

Do not allow the tissue to dry out during the liquid exchange process. It is recommended that you operate the procedure one chip at a time.

c. Mock incubation of the secondary antibody: During the mock primary antibody incubation step, prepare the mock secondary antibody solution according to Table 3-2. Vortex the mixture and place it on ice for later use.

Table 3-2 Secondary Antibody Mock Incubation Buffer

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
5X SSC	90	189	279	369
RI	7.5	15.75	23.25	30.75
Nuclease-free water	52.5	110.25	162.75	215.25
Total	150	315	465	615

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

e. Immediately add **200 μL** Wash Buffer 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°, remove the Wash Buffer from the corner of the well using a pipette, keeping the chip and tissue surface moist.

g. Repeat **steps e. and f.** for one more wash..

h. Immediately add **150 μL** secondary antibody mock incubation buffer per well and incubate the chip **in the dark** at room temperature for **15 min**.

i. Prepare the decrosslinking reagents during the secondary antibody incubation step.

3.3. Tissue Decrosslinking

a. Thaw the Decrosslinking Reagent in advance according to the **2.1 Experiment Preparation** section and bring it to room temperature.

b. Set one module of the PCR Thermal Cycler to 70°C and the lid temperature to 75°C. Place the PCR Adaptor in the thermal cycler to equilibrate in advance.

c. Incubate the Decrosslinking Reagent at 70°C for at least **10 min**.

d. Remove the mock secondary antibody incubation solution from the corner of the well while keeping the tissue surface moist.

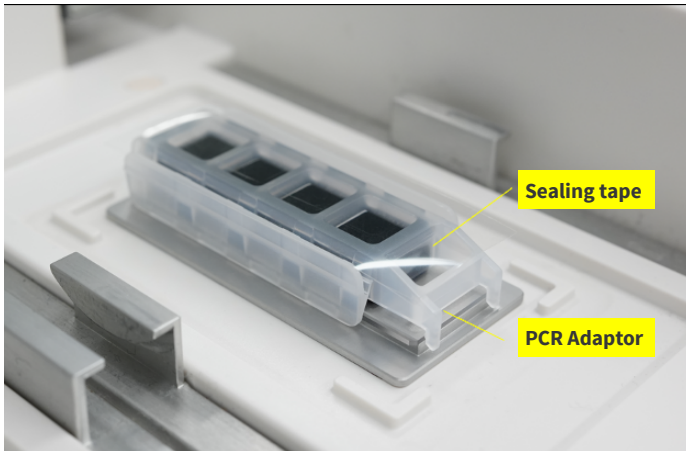
e. Add **200 μL** of Wash Buffer per well and incubate at room temperature for **1 min**.

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

g. Repeat **steps e.-f.** for one more wash.

h. After the final wash, add **400 μL** Decrosslinking Reagent per well.

i. Seal with sealing tape and then place the Stereo-seq Slide Cassette on the PCR Adaptor (70°C). Close the lid of the PCR Thermal Cycler and perform decrosslinking at 70°C for **15 min**.



j. During the decrosslinking process, set aside the 2 mL of 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in 2.1 Experimental Preparation.

k. Ensure that the PCR Thermal Cycler has been switched on and set to 37°C and the heating lid has been set to 42°C. Place the PCR Adaptor in the PCR thermal cycler, then close the heated lid to pre-heat the adaptor for 3 min.

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

l. Warm the aliquoted 1X Permeabilization Reagent Solution inside the 37°C PCR Thermal Cycler or on a Metal Block for **>10 min (no longer than 30 min)**.

m. After decrosslinking, transfer the Stereo-seq Slide Cassette to the lab bench, remove the sealing tape, and cool to room temperature for **10 min**.

3.4. Tissue Permeabilization Testing

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

a. After cooling, slightly tilt the Stereo-seq Slide Cassette at an angle of less than 20°. Discard the decrosslinking reagent from the corner of the well using a pipette, keeping the chip and tissue surface moist. Add **400 µL** of Wash Buffer per well.

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

c. Tissue sections on the Stereo-seq Chip P Slide are incubated for different lengths of time in the range of 0-30 min. For the first trial, it is recommended that you use a suggested time course of **8 min, 14 min, 20 min, and 26 min along with a positive control group (mouse thymus tissue or total RNA).**

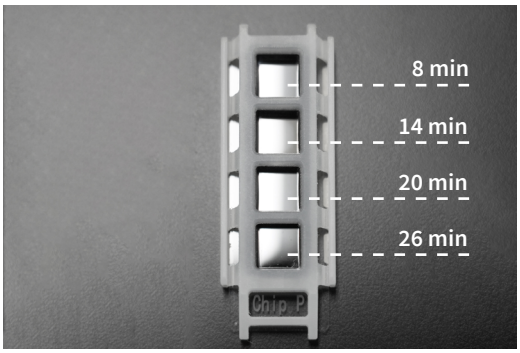
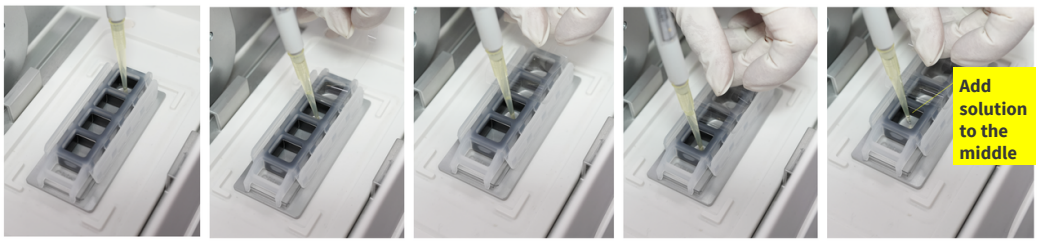


Figure 1. Permeabilization Times (min)

Keep the Stereo-seq Slide Cassette in the PCR Thermal Cycler during the permeabilization and reverse transcription steps. Do not move the cassette.



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 to the Stereo-seq Slide Cassette and let the chip incubate inside the PCR thermal cycler at 37°C.

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

4) Apply unpeeled sealing tape to the Stereo-seq Slide Cassette, close the lid and incubate at 37°C.

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

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

d. For total RNA as positive control:

1) Prepare the Total RNA Hybridization Mix as indicated in Table 3-3.

Table 3-3 Total RNA hybridization Mix

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

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

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

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

e. When incubation is completed, remove the Stereo-seq Slide Cassette from the PCR Adaptor.

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

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

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

i. Prepare RT QC Mix according to Table 3-4 and equilibrate to room temperature **in the dark.**

Table 3-4 RT QC Mix

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

3.5. Reverse Transcription

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

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

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

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

3.6. Tissue Removal

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

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

a. Ensure that the PCR Thermal Cycler with PCR Adaptor has been set to 55°C and the heated lid has been set to 60°C.

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

When removing the sealing tape, hold the Stereo-seq Slide Cassette with one hand without applying force to Side A and Side B of the cassette. Doing so prevents the Stereo-seq Chip Slide from falling off of the cassette.

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

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

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

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

g. Repeat step d.-f.

h. Prepare the Tissue Removal Mix as shown in Table 3-5.

Table 3-5 Tissue Removal Mix

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

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

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

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

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

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

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

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

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

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

p. Repeat step m.-o.

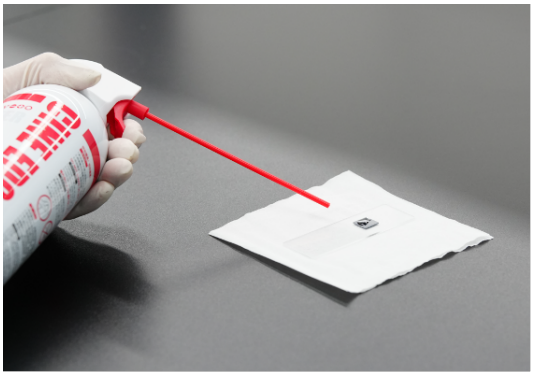
q. Add 400 µL of nuclease-free water into each well and pipette up and down to wash the chip surface as SSC solution contains salt.

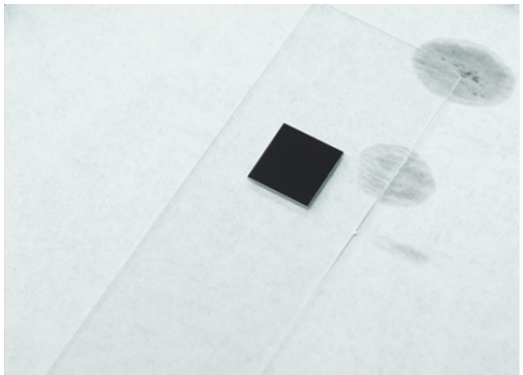
r. Remove the slide from the Stereo-seq Slide Cassette according to the instructions in 1.4 Practice Tips.

Alternative Step:

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

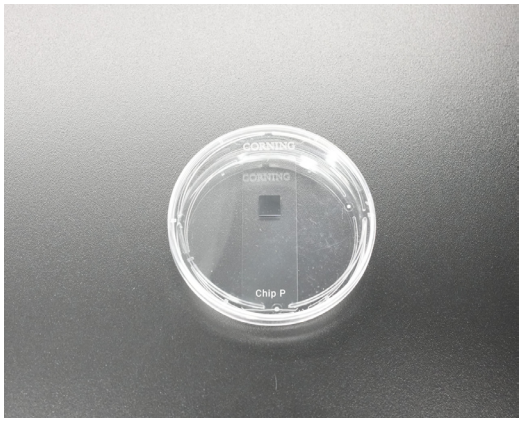
s. Place the Stereo-seq Chip Slide onto a clean dust-free paper and dry the chip surface completely with a power dust remover (MATIN, M-6318).





If visible 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 no visible traces remain on the chip surface.

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



3.7. Imaging

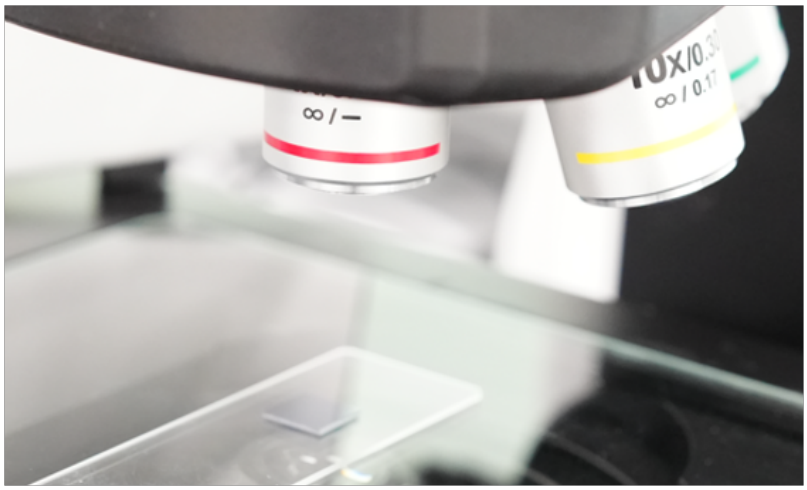
a. Create a new folder in a fluorescent microscope-connected PC, name it with the chip ID number and other essential information.

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

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

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

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



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

Ensure that the desired capturing area is clear and within focus during full scanning.

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

3.8. Permeabilization Time Determination

The optimal permeabilization time should result in the strongest fluorescence signal with the lowest signal diffusion. However, this is based on a complete tissue removal as well as images taken under the same settings. For example, as shown in Figure 2, for the **8 min** permeabilization time point, the fluorescence signal in some parts of the mouse thymus is very low, suggesting insufficient permeabilization. For the **14 min** and **20 min** permeabilization time point, images show the strongest signal and finer details among the 4 time points. For the **26 min** permeabilization time point, the signal is the lowest. Based on this result, the optimal permeabilization time for this tissue is **14 min** and **20 min**. In such case when two permeabilization time points show similar fluorescence signals, we suggest using the shorter time, i.e., **14 min** as the optimal permeabilization time point to avoid potential diffusion.

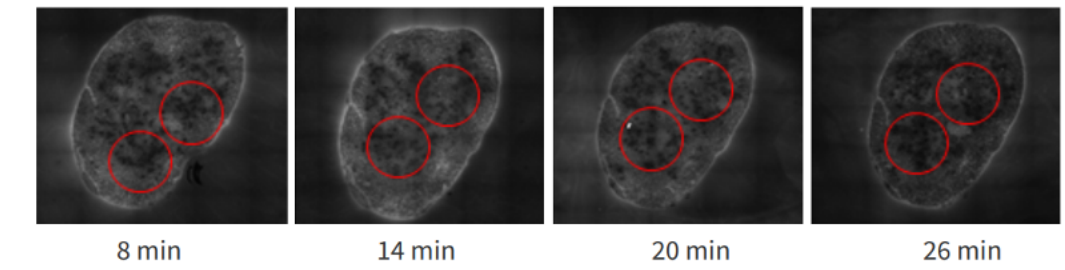


Figure 2. The optimal permeabilization time determination of a mouse thymus section