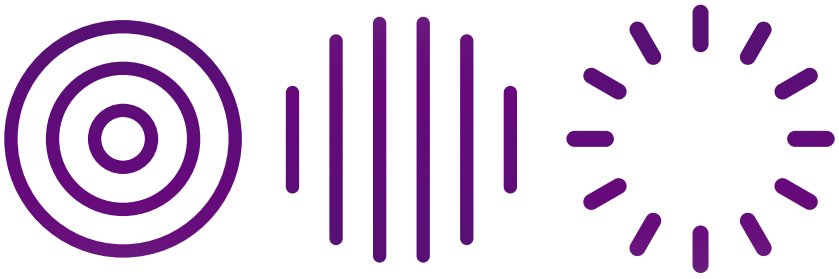
STOmics

Stereo-seq PERMEABILIZATION SET FOR Stereo-seq LARGE-SIZED Chip (≤ 2cm * 3cm)

USER MANUAL

COMPATIBLE WITH 1cm * 2cm, 2cm * 2cm, and 2cm * 3cm chips.



Cat. No.:

111SP124 Stereo-seq Permeabilization Set (1 cm * 2 cm), 4RXN
111SP224 Stereo-seq Permeabilization Set (2 cm * 2 cm), 4RXN
111SP234 Stereo-seq Permeabilization Set (2 cm * 3 cm), 4RXN

Kit Version: V1.0

Manual Version: A

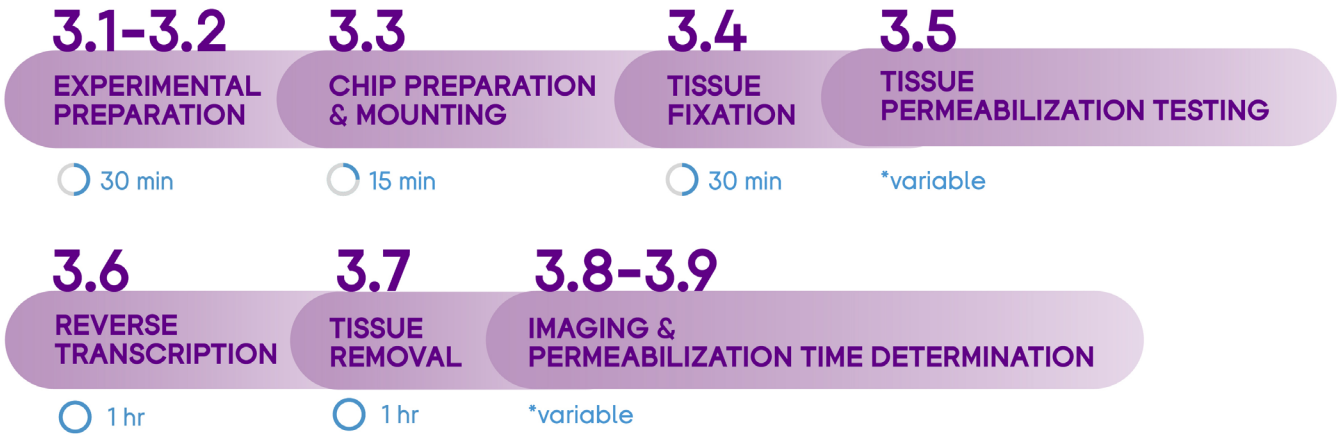
REVISION HISTORY

Manual Version:	A
Kit Version:	V1.0
Date:	Aug. 2023
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: ~4 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 Large Chip 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 Large Chip experiments. Featured with high resolution and large field of view, Stereo-seq Chip P 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 Large Chip experiments.

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

1.2. List of Kit Components

Each Stereo-seq Permeabilization Set for Large-sized Chips consists of:

- 111KP004 Stereo-seq Permeabilization Kit, 4RXN *1 (4 RXN)
- 110CP122 Stereo-seq Chip P (1cm * 2cm) *2 (2 EA) **OR**
  110CP222 Stereo-seq Chip P (2cm * 2cm) *2 (2 EA) **OR**
  110CP232 Stereo-seq Chip P (2cm * 3cm) *2 (2 EA)

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

Upon receiving the Stereo-seq Chip P (1cm * 2cm), Stereo-seq Chip P (2cm * 2cm), or Stereo-seq Chip P (2cm * 3cm), please follow the instructions in Stereo-seq Large-sized Chips (≤ 2cm * 3cm) Operation Guide For Receiving, Handling And Storing to properly store unopened Stereo-seq Chip P.

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

Table 1-1

Stereo-seq Permeabilization Kit Cat. No.:111KP004			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
RI	1000028499		300 μL × 2
PR Enzyme	1000028500		10 mg × 1
RT QC Reagent	1000035168		3900 μL × 1
RT Additive	1000035169	○ (transparent)	250 μL × 1
RT QC Enzyme	1000035260	○ (transparent)	250 μL × 1
TR Enzyme	1000035261		350 μL × 1
TR Buffer	1000039987		3458 μL × 5
Storage Temperature: -25°C~-18°C	Transported by cold chain	Expiration Date: refer to label	

Table 1-2

Kit	Component	Quantity
Stereo-seq Chip P (1cm * 2cm) Cat. No.: 110CP122	Stereo-seq Chip P (1cm * 2cm)	2 EA
Stereo-seq Chip P (2cm * 2cm) Cat. No.: 110CP222	Stereo-seq Chip P (2cm * 2cm)	2 EA
Stereo-seq Chip P (2cm * 3cm) Cat. No.: 110CP232	Stereo-seq Chip P (2cm * 3cm)	2 EA
Storage Temperature: -25°C~-18°C	Transported by cold chain	Expiration Date: refer to label

1.3. Additional Equipment and Materials

Table below lists equipment and materials needed for this protocol. Some common laboratory equipments not named in Table 1-3 are expected to be accessible by the user, for instance, ice maker, biological safety cabinet, freezers, etc. For specific microscope requirements, please refer to **STOmics Microscope Assessment Guideline**.

Table 1-3

Equipment		
Brand	**Description**	**Catalog Number**
-	Cryostat	-
-	Benchtop centrifuge	-
-	Pipettes	-
	Metal heating block dry bath (optional)	-
-	Vortex mixer	-
-	Incubator	-
-	Slide Dryer	-
Bio-Rad*	T100 Thermocycler	1861096
ABI*	ProFlex 3 x 32-well PCR System	4483636

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

Reagents		
Brand	**Description**	**Catalog Number**
Ambion	Nuclease-free water	AM9937
	1X TE buffer, pH 8.0	AM9858
	20X SSC	AM9770
Sigma Aldrich	Hydrochloric acid, HCl	2104-50ML
	Methanol	34860-1L-R
SAKURA	SAKURA Tissue-Tek® O.C.T. compound	4583

Consumables		
Brand	**Description**	**Catalog Number**
-	Stainless-steel base mold	-
-	Aluminum foil	-
-	Forceps	-
-	Sealing tape for well plates	-
-	Plastic wrap	-
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
	6-well cell culture plate	3516
Thermo Scientific™	60 mm Culture Dish	150462
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
PARAFILM	Parafilm	PM996

1.4. Precautions and Warnings

- This product is intended for research use only, not for use in diagnostic procedures. Please read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, users are recommended to ensure that they are familiar with related instruments, and operate them according to manufacturer's instructions.
- Instructions provided in this manual are intended for general use only and optimization may be required for specific applications.
- Thaw reagents in the kits properly prior to use. For enzymes, centrifuge briefly and keep them on ice until further use. For other reagents, thaw them first at room temperature followed by inverting several times to mix them properly, and centrifuge them briefly before placing on ice for further use.
- mRNA capture will be compromised or absent for any scratched areas on the front-side surface of the chip.
- To prevent cross-contamination, we recommend the use of filtered pipette tips. Use a new tip each time for pipetting different solutions.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR products, resulting in data inaccuracy. Therefore, we recommend two distinctly separated working areas in the laboratory for PCR reaction preparation and PCR product cleanup tests. Use designated pipettes and equipments for each area and execute regular cleaning (with 0.5% sodium hydrochloride or 10% bleach) to ensure a clean and sterile working environment.
- Do not consume any sample or reagent, and avoid direct contact of reagents with skin and eyes. In case of an accident, immediately wash the affected area thoroughly with a large amount of water. Seek emergency medical assistance if needed.

CHAPTER 2
SAMPLE PREPARATION

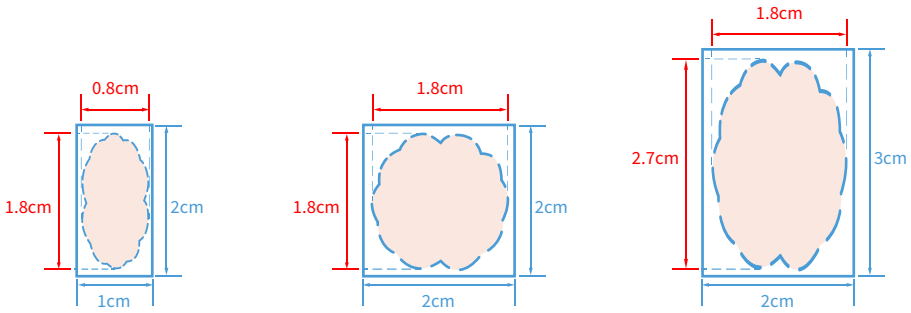
2.1. Sample Requirements for Fresh Frozen Tissue

To avoid RNA degradation, we recommend performing tissue embedding **within 30 min** upon harvesting.

The tissue size should not exceed:

- **0.8 cm x 1.8 cm x 1 cm (for 1cm*2cm Stereo-seq Chip);**
- **1.8 cm x 1.8 cm x 1 cm (for 2cm*2cm Stereo-seq Chip);**
- **1.8 cm x 2.7 cm x 1 cm (for 2cm*3cm Stereo-seq Chip);**

as the tissue section should not exceed 80% area coverage of the chip.



Typically, ~10 tissue slices will be consumed at each step (RIN quality check, Stereo-seq permeabilization optimization, and Stereo-seq Transcriptomics workflow). Please check beforehand whether the embedded tissue thickness can satisfy the need of the experimental design.

Sample Types

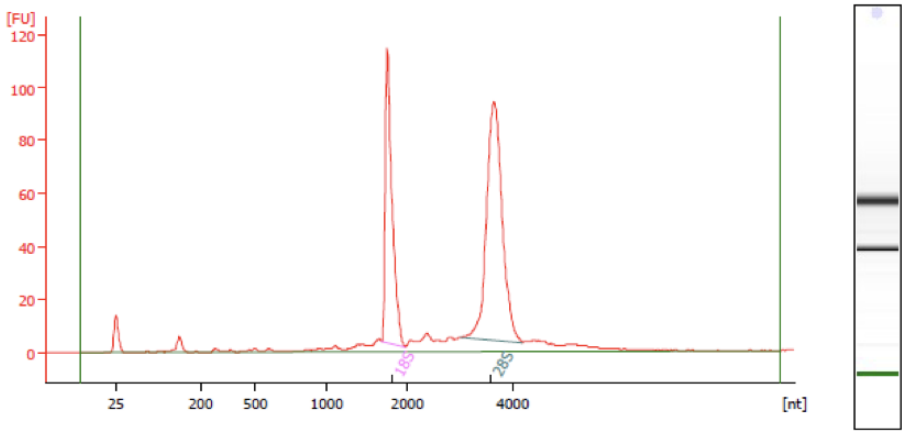
This set of kits can be used for samples from all common animals, including but not limited to human, monkey, and mouse.

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

Fresh Frozen Sample RNA Integrity Number (RIN) Value

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

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



Overall Results for sample 6 : 8522203007050

RNA Area:	568.4	RNA Integrity Number (RIN):	9.8 (B.02.11, Anomaly Threshold(s) manually adapted)
RNA Concentration:	281 ng/µl		
rRNA Ratio [28s / 18s]:	1.6	Result Flagging Color:	
		Result Flagging Label:	RIN: 9.80

Fragment table for sample 6 : 8522203007050

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,675	1,964	151.3	26.6
28S	3,006	4,187	238.4	41.9

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

2.2. Sample Embedding

For a demonstration video of tissue embedding, please refer to the link or by scanning the QR code:
https://drive.google.com/drive/folders/1O138SbfP8lKkYLaScnPkU3pOvwMf0NTW?usp=sharing

a. Prepare these apparatuses/materials in advance:

Materials		
Brand	**Description**	**Quantity**
-	Crushed ice in a box	1
-	Dry ice in a box	1
-	Aluminum foil	1
-	Sealable plastic bag	1
BIOSHARP/Metal Coolbox/ BC032	Metal Block	1
-	Sterile gauze	2
Corning	Corning® 35 mm TC-treated Culture Dish (353001)	1
Sakura/Base Molds/4583	O.C.T	1
Sakura Base Molds 4131/4132/4133 or other brand that's suitable for the tissue size.	Stainless-steel base mold A	1
Sakura Base Molds 4133/4165/4124 or other brand that's suitable.	Stainless-steel base mold B	1
-	Blunt end forceps	1

Materials		
-	Syringe	1
-	Spatula	1
-	Scissors	1
	Stainless Steel Ruler	1

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

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

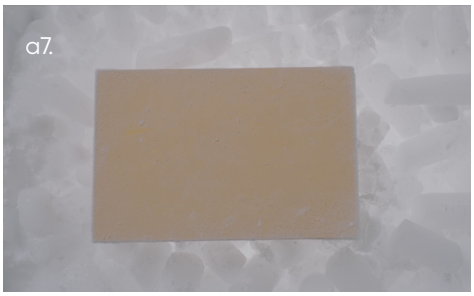
a3. Add a few drops of pre-cooled OCT in the mold A until it reaches approximately 2/3 of the mold and pre-cool on ice for **> 10 min** (remove introduced air bubble using a syringe).

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

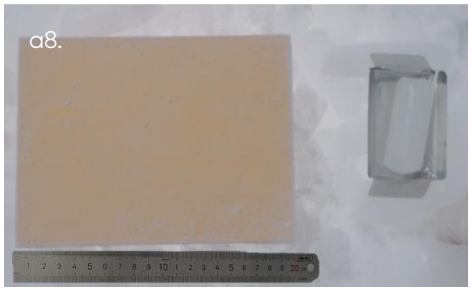
a5. A box of dry ice.

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

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

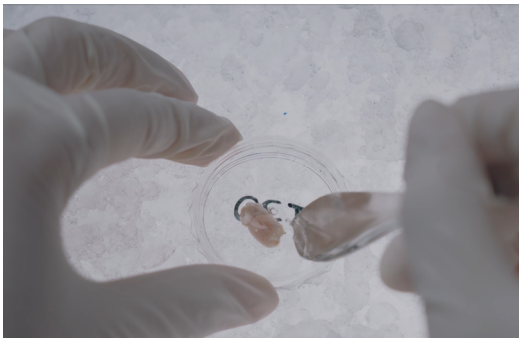


a8. Place mold B and stainless steel ruler on dry ice and pre-cool for **> 5 min**.



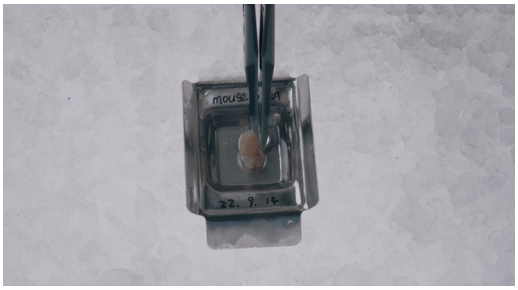
b. Upon harvesting within **30 min**, use sterile gauze or dust-free paper to absorb excess liquid on the tissue surface to avoid ice formation in later steps.

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

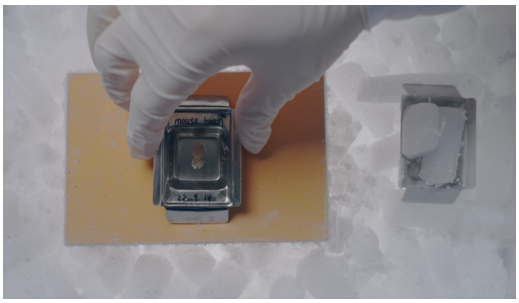


d. Remove any air bubbles using a syringe.

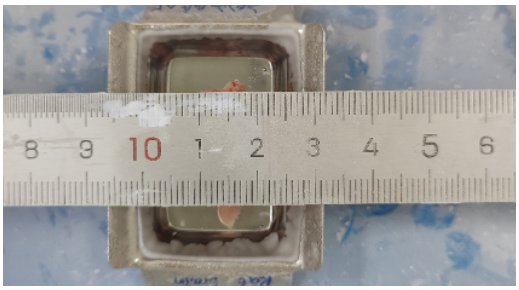
e. Orient the tissue to have the side intended to be sectioned facing downwards and then place into mold A. Make sure the tissue is at the bottom of mold A and fill the mold with chilled OCT without introducing bubbles until the tissue is fully covered.

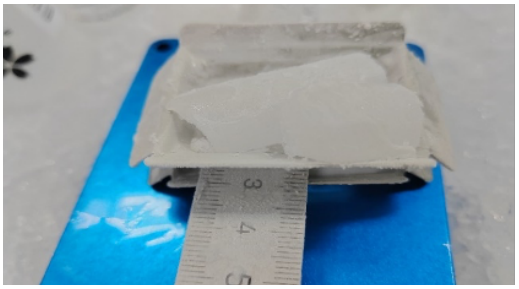


f. Place the tissue containing mold A onto the metal block that was placed on dry ice.



g. Place the pre-cooled stainless steel ruler on top of mold A (place it on the length of the container to prevent compression of the tissue). Use mold B as a lid with opening facing up, place on top of mold A gently and then place a few dry ice cubes on top of mold B. Make sure the two stainless steel base molds can be covered with enough dry ice cubes.





h. After **5 min**, remove mold B and the stainless steel ruler then check if the OCT is completely frozen and turns opaque, otherwise repeat g.

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

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

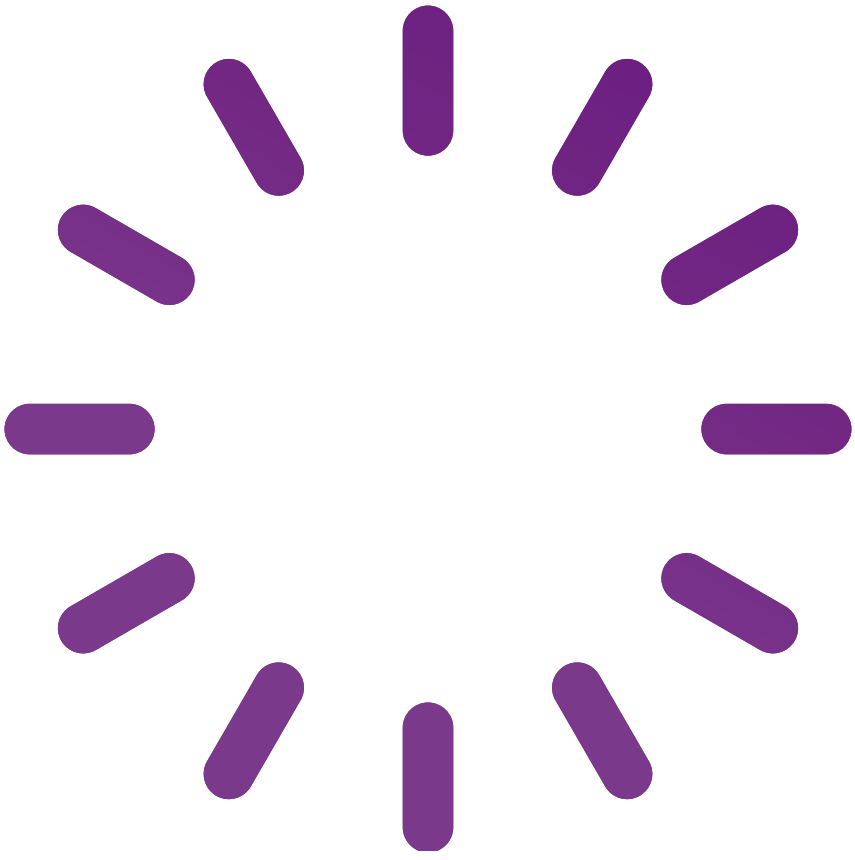
k. Label the tissue block to mark the orientation of the tissue.

2.3. Sample Storage and Transportation

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

CHAPTER 3

Stereo-seq PERMEABILIZATION SET FOR LARGE CHIP STANDARD OPERATING PROCEDURE



3.1. Experimental Preparation

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

Reagent	Preparation Steps for 1cm * 2cm Chip P	Preparation Steps for 2cm * 2cm Chip P	Preparation Steps for 2cm * 3cm Chip P	Maintenance
0.1X SSC	Dilute 100 µL of 20X SSC to 20 mL			Room Temperature
Wash Buffer	Prepare at least **150** µL per chip (**142.5** µL 0.1X SSC with **7.5** µL RI).	Prepare at least **300** µL per chip (**285** µL 0.1X SSC with **15** µL RI).	Prepare at least **400** µL per chip (**380** µL 0.1X SSC with **20** µL RI).	On ice until use
0.01N HCl	Prepare at least **3 mL** of 0.01N HCL per sample. Configure HCL to 0.01N. Measure and make sure the pH = 2.	Prepare at least **3 mL** of 0.01N HCL per sample. Configure HCL to 0.01N. Measure and make sure the pH = 2.	Prepare at least **5 mL** of 0.01N HCL per sample. Configure HCL to 0.01N. Measure and make sure the pH = 2.	Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Please use WITHIN 48 hr of preparation)**
ALWAYS use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.				
10X Permeabilization Reagent Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent through pipetting.			-20°C
DO NOT vortex the permeabilization enzyme. Mix by pipette before using. **Aliquot this 10X stock solution to avoid freeze-thaw cycles.**				
1X Permeabilization Reagent Solution	Make 1X PR solution (at least **200 µL** / chip) by diluting 10X PR stock solution with 0.01N HCl.	Make 1X PR solution (at least **400 µL** / chip) by diluting 10X PR stock solution with 0.01N HCl.	Make 1X PR solution (at least **800 µL** / chip) by diluting 10X PR stock solution with 0.01N HCl.	On ice until use, up to 6 hr

Other Preparation	Consumables Required		
Steps	**1cm * 2cm Chip P**	**2cm * 2cm Chip P**	**2cm * 3cm Chip P**
Chip Placement	100-mm Culture Dish (Place parafilm at the bottom of the culture dish)		
Prepare pieces of trimmed parafilm of suitable size for attaching to the petri dish bottom. Use the back-end of the forceps to gently press and fix the edges of the parafilm to the bottom of the petri dish. Place your chips on the parafilm throughout experiments to prevent chips from sliding and colliding into the wall of the petri dish.			
Chip Washing	6-well plate		60-mm Culture Dish
Methanol Fixation			
Glycerol Removal			
Tissue Removal			
cDNA Release			

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

3.2. Cryosection Preparation

a. Set the slide dryer to 37°C or the PCR thermal cycler to 37°C in advance.

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

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

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

d. Take the OCT-embedded tissue sample out of the -80°C freezer to the chamber and allow it to equilibrate to the cryostat chamber temperature (Equilibration time should vary depending on the size of the tissue sample. In order to ensure a smooth tissue sectioning process, tissue sizes of 2 cm x 3 cm x 1 cm should equilibrate for about **1 hr** as a reference).

e. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block into appropriate size (sectioning area should be smaller than 0.9 cm x 1.8 cm for 1cm*2cm Stereo-seq Chip, 1.8 cm x 1.8 cm for 2cm*2cm Stereo-seq Chip, and 1.8 cm x 2.7 cm for 2cm*3cm Stereo-seq Chip).

f. Use OCT to mount the embedded tissue block onto the specimen disc/holder of the cryostat chamber.

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

3.3. Tissue Mounting

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

The front-side of a chip has a shiny surface which contains DNB-probes for mRNA capture. DO NOT scratch the surface.

b. Equilibrate Stereo-seq Chip P to room temperature for **1 min** within the parafilm attached 100-mm Culture Dish.

c. Rinse Stereo-seq Chip P:

1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
6-well plate	6-well plate	60-mm Culture Dish
Rinse the chip with **3000 µL** nuclease-free water **twice** with a pipette		Rinse the chip with **4000 µL** nuclease-free water **twice** with a pipette

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

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

f. Pre-cool methanol:

1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
6-well plate	6-well plate	60-mm Culture Dish
Add **2-4 mL** of methanol into one well of the 6-well plate for one chip and pre-cool for **5-30 min** at -20°C		Add **3-5 mL** of methanol into the petri dish for one chip and pre-cool for **5-30 min** at -20°C

g. Tissue mounting could be achieved via either **cold method** (option A) or **warm method** (option B).

A. Cold Method

1) Place Stereo-seq Chip inside the chamber with the front-side facing up and pre-cool inside the cryostat chamber for **3~10 min.**

Prolonged cooling for over 10 min may cause mist formation on the chip surface.

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

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

It is recommended to finish tissue section placement within 5 min.

4) Once complete all tissue mounting, immediately dry the Stereo-seq Chip at 37°C for:

Table 3-1 Drying time for Stereo-seq Large-sized Chips

Chip Size:	1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
Chip drying time:	8 min	8 min	10 min

B. Warm Method

1) Perform cryosection, carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes, and move the tissue section to the edge.

2) Hold the chip at one corner with a pair of forceps, turn it around to have its active surface facing downward, and match it onto the tissue section.

3) Place it on top of the tissue section and let the tissue adhere on to the chip.

4) Turn the chip again to make the tissue-mouted front-side facing upward, and immediately dry the Stereo-seq Chip at 37°C according to the time listed in Table 3-1.

3.4. Tissue Fixation

a. After drying, immediately immerse the tissue-mounted chip in pre-cooled methanol prepared in section 3.3-f for a **40-min** fixation at -20°C.

If multiple chips are prepared simultaneously, the 40-min fixation time should be determined by the time of the last chip being added.

b. After fixation, move the 6-well plates or 60 mm culture dish to a sterile fume hood.

c. Take out the chip and absorb excess methanol on the chip from its corner and back with a Kimwipes™ Delicate Task Wipes.

d. Place the chip in the 100mm petri dish with a parafilm attached to the bottom and do not close the lid.

e. Leave the petri dish in the fume hood for **4-6 min** to let the methanol fully evaporate.

f. Once methanol is fully evaporated, transfer the chip-containing petri dish onto a flat and clean bench top surface.

3.5. Tissue Permeabilization Testing

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

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

Table 3-2 1X PR solution addition volume for Stereo-seq Large-sized Chips

Chip Size:	1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
1X PR solution addition volume	**200 μL / chip**	**400 μL / chip**	**800 μL / chip**
Total volume (4X + 10%)	**880 μL for 4 chips**	**1760 μL for 4 chips**	**3520 μL for 4 chips**

b. Make sure your incubator has been switched on and set to 37°C

c. Prewarm the 1X PR solution in the 37°C incubator for **10 min**.

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

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

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

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

2. Let the chip incubate inside the incubator at 37°C.

3. After **6 min**, open the incubator and add 1X Permeabilization Reagent Solution on the chip with **18 min** time point.

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

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

f. For total RNA as positive control:

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

Table 3-3 Total RNA hybridization Mix

Components	1X (μL) for 1cm * 2cm Chip P	1X (μL) for 2cm * 2cm Chip P	1X (μL) for 2cm * 3cm Chip P
Total RNA	X (2μg)	X (2μg)	X (2μg)
Nuclease-free water	105-X	210-X	280-X
20X SSC	37.5	75	100
RI	7.5	15	20
Total	150	300	400

2) Warm up the Total RNA hybridization Mix inside the incubator at 37°C for **10 min**.

3) Add the total RNA hybridization mixture onto the corresponding chip and incubate at 37°C for **15-20 min**.

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

g. Once complete, remove the chip from the incubator.

h. Slightly tilt the Stereo-seq Chip P, remove 1X Permeabilization Reagent Solution or total RNA hybridization mixture from the corner of the chip using a pipette.

i. Add Wash Buffer and then remove the solution to wash the chip:

Table 3-4 Wash Buffer addition volume for Stereo-seq Large-sized Chips

Chip Size:	1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
Wash Buffer addition volume	**150 μL / chip**	**300 μL / chip**	**400 μL / chip**

j. Slightly tilt the Stereo-seq Chip P, remove Wash Buffer from the corner of the chip using a pipette.

k. Prepare RT QC mix according to Table 3-5 and equilibrate to room temperature **in the dark.**

Table 3-5 RT QC Mix

Components	1X (μL) for 1cm * 2cm Chip P	1X (μL) for 2cm * 2cm Chip P	1X (μL) for 2cm * 3cm Chip P
RT QC Reagent	144.5	272	357
RT Additive	8.5	16	21
RT QC Enzyme	8.5	16	21
RI	8.5	16	21
Total	170	320	420
Volume Addition per chip	150	300	400

3.6. Reverse Transcription

a. Make sure the temperature of the incubator has been set to 42°C in advance.

b. Gently add RT QC Mix on to the chip from the corners according to Table 3-5. Make sure the chip is completely covered with RT QC Mix.

c. Seal the petri dish containing the chip tightly with parafilm and then wrap the petri dish with aluminum foil. Incubate at 42°C for **1 hr** or longer (no longer than 16 hr) **in the dark.**

3.7. Tissue Removal

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

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

a. Check and make sure the incubator temperature is set to 55°C.

b. Take the chip with the petri dish out of the 42°C incubator.

c. Slightly tilt the petri dish, remove RT QC Mix from the chip surface with a pipette.

d. Add 0.1X SSC solution on to the chip according to Table 3-6

Table 3-6 0.1X SSC addition volume for Stereo-seq Large-sized Chips post RT

Chip Size:	1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
0.1X SSC addition volume	**150 µL / chip**	**300 µL / chip**	**400 µL / chip**

e. Slightly tilt the petri dish, remove 0.1X SSC with a pipette from the corner of the chip.

f. Repeat step d. - e.

g. Transfer Stereo-seq Chip P into a 6-well plate or 60mm Culture Dish.

h. Prepare Tissue Removal Mix as shown in Table 3-7 and leave at room temperature.

Table 3-7 Tissue Removal Mix

Components	1X (µL) for 1cm * 2cm Chip P	1X (µL) for 2cm * 2cm Chip P	1X (µL) for 2cm * 3cm Chip P
	6-well plate	6-well plate	60-mm Culture Dish
TR Buffer	1470	1960	2940
TR Enzyme	30	40	60
Total	1500	2000	3000

i. Add Tissue Removal Mix onto the chip without introducing bubbles. Ensure uniform solution coverage on the chip.

j. Place a sealing tape on the 6-well plate or wrap the parafilm around the 60-mm Culture Dish, then wrap the 6-well plate of 60-mm Culture Dish entirely with plastic wrap.

k. Incubate the chip at 55°C for **1 hr**.

l. At the end of incubation, remove the Stereo-seq Chip P from the incubator.

m. Slightly tilt the Stereo-seq Chip P, remove Tissue Removal Mix with a pipette from the chip.

n. Add 0.1X SSC on to the chip according to Table 3-8:

Table 3-8 0.1X SSC addition volume for Stereo-seq Large-sized Chips post tissue removal

Chip Size:	1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
0.1X SSC addition volume	**1500 μL / chip**	**1500 μL / chip**	**3000 μL / chip**

o. Gently pipette 0.1X SSC solution up and down around each corner of the chip 5 times.

p. Remove 0.1X SSC with a pipette from the the corner of the well or culture dish.

q. Repeat step n. - p.

r. Add nuclease-free water onto the chip according to Table 3-9:

Table 3-9 Nuclease-free water addition for Stereo-seq Large-sized Chips post tissue removal

Chip Size:	1cm * 2cm Chip P	2cm * 2cm Chip P	2cm * 3cm Chip P
Nuclease-free water addition volume	**1500 μL / chip**	**1500 μL / chip**	**3000 μL / chip**

s. Pipette nuclease-free water up and down to wash the chip as the SSC solution contains salt.

If obvious salt stains were observed on the chips, please wash with additional nuclease-free water.

If tissue remains on the chip after tissue removal step, increase the incubation time (no longer than 16 hr) and then wash with nuclease-free water. Make sure the tissue is completely removed.

3.8. Imaging

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

Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.
Example: B00249A1B2

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 chip onto the water drop. Water surface tension will grab onto the chip and adhere it onto the imaging platform.

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

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

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

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

3.9. Permeabilization Time Determination

The optimal permeabilization time should results 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 **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 showed the strongest signal and finer details among three groups. For the **18 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.

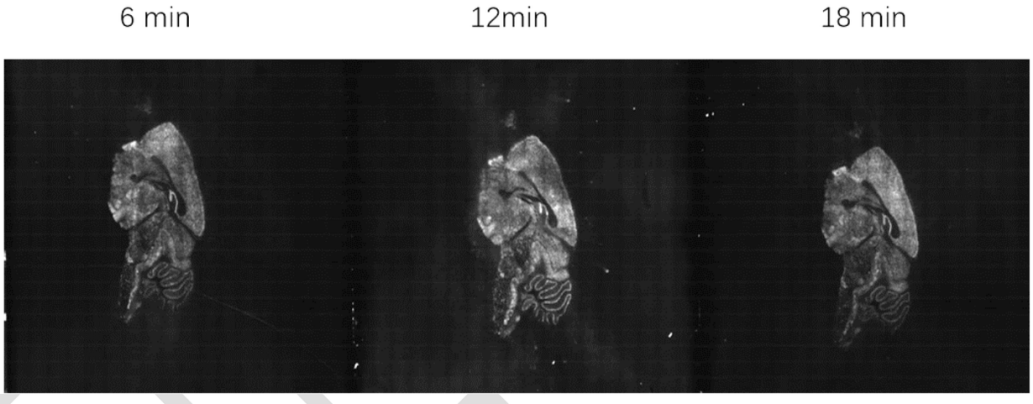


Figure 2. The optimal permeabilization time determination of a rat brain (median sagittal plane).