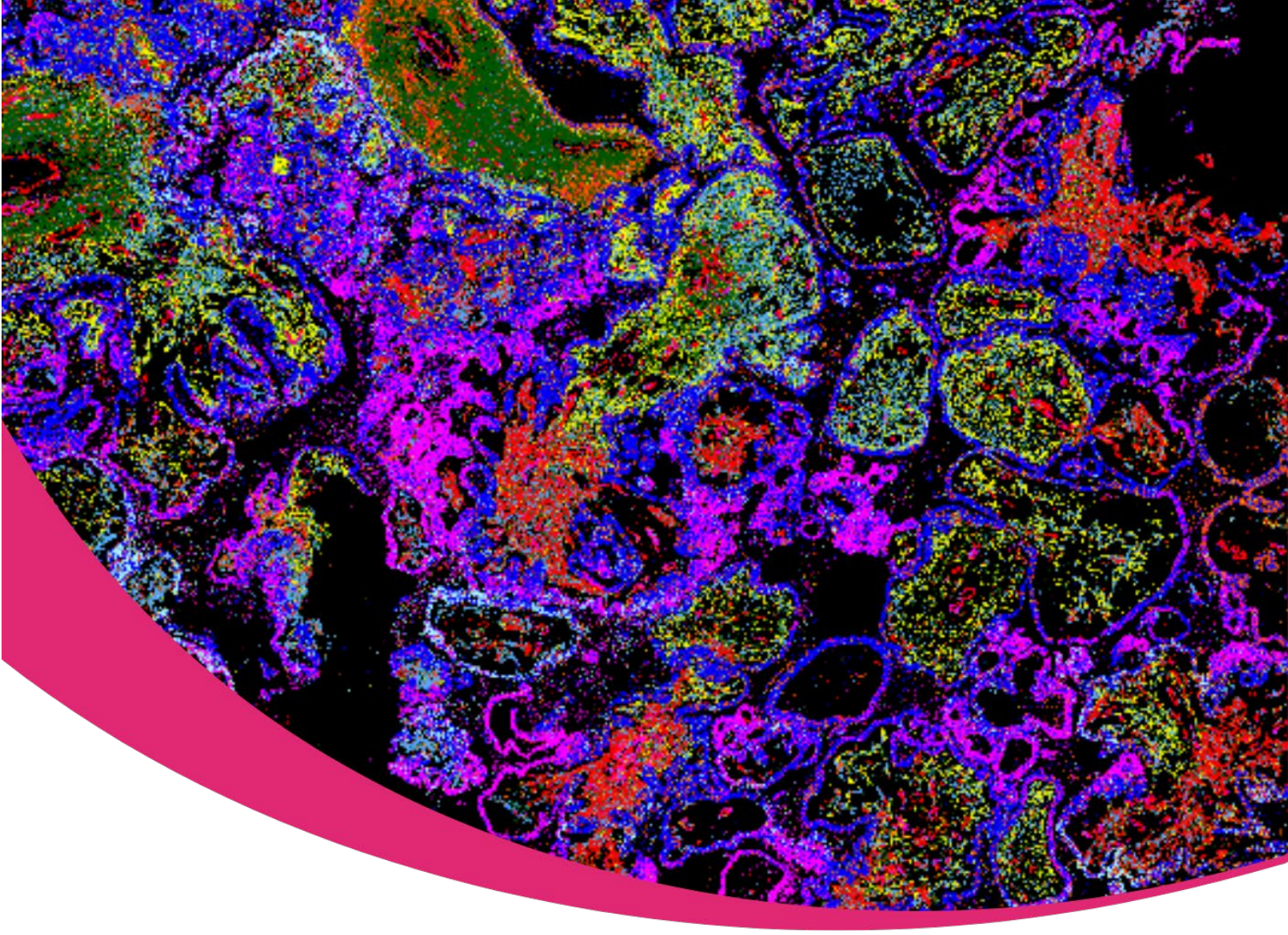

MERSCOPE® Tissue Histology User Guide:

Histology User Guide for Preparing Fresh and Fixed Frozen Tissue Samples for Experiments on the MERSCOPE® Platform

91600129 Rev B

vizgen®

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1 INTRODUCTION

Vizgen MERSCOPE Platform is a spatial transcriptomics imaging platform that can resolve gene expression at the single-cell level. Built based on the multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) technology, MERSCOPE allows massively multiplexed *in situ* RNA imaging with subcellular spatial resolution. It helps to unlock the architecture of complex, multicellular tissues to understand the fundamentals of development and disease. This combination of molecular and morphological characterization provides a previously inaccessible view of tissue biology.

This tissue preparation guide provides recommendations on preparing fresh frozen and paraformaldehyde (PFA)-fixed frozen tissue samples on MERSCOPE slide prior to MERFISH sample preparation, including:

- Tissue placement guidance on the MERSCOPE Slides
- Histology guidelines and tips for preparing frozen tissue samples
- Tissue preparation protocol and tips for frozen tissue samples
- Materials required for tissue preparation on the MERSCOPE platform

Refer to the MERSCOPE Fresh and Fixed Frozen Tissue Sample Preparation User Guide (PN 91600002), MERFISH 2.0 Tissue Sample Preparation User guide (91600132) and MERSCOPE instrument guide (PNs 91600001 and 91600131)) for additional information or contact technical support or your local field application scientist for more information on experimental planning.

2 MATERIALS

2.1 Vizgen Materials

MERSCOPE Standard FF Slide Box, 10 Slides	**10500116**	**Storage**
MERSCOPE Standard FF Slide, 10 x 1 slides	10500116	–20°C, horizontally
MERSCOPE Standard FF Slide Box, 20 Slides	**10500125**	**Storage**
MERSCOPE Standard FF Slide, 20 x 1 slides	20400106	–20°C, horizontally
MERSCOPE Large FF Slide Box, 10 Slides	**10500120**	**Storage**
MERSCOPE Large FF Slide, 10 x 1 slides	20400112	–20°C, horizontally
MERSCOPE Standard Slide Box V 2.0, 10 Slides	**10500132**	**Storage**
MERSCOPE Standard Slide V 2.0, 10 x 1 slides	20400117	–20°C, horizontally
MERSCOPE Large Slide Box V 2.0, 5 Slides	**10500131**	**Storage**
MERSCOPE Large Slide V 2.0, 5 x 1 slides	20400118	–20°C, horizontally
MERSCOPE Sample Placement Guide	**10700252**	**Storage**
MERSCOPE Sample Placement Guide, pack of 5	10300104	Room temperature

Safety Data Sheets are available online at https://vizgen.com/

2.2 Required User Supplied Materials

Table 1. Recommended reagents and equipment for working with fresh tissues that will be analyzed on the MERSCOPE Platform.

Item	Description	Vendor	Catalog Number
Leica CM3050 S Cryostat	Cryostat	Leica	CM3050 S
Leica-Style Cryostat Specimen Chuck Kit	Cryostat specimen chucks	VWR	10756-296
Epredia™ MX35 Premier™ Disposable Low-profile	Microtome Blade	Thermo Fisher	3052835
2-Methylbutane	Isopentane solution	Sigma Aldrich	277258
Epredia™ Peel-A-Way™ Disposable Embedding Molds	Embedding plastic mold	Thermo Fisher	12-20
Ribonucleoside Vanadyl Complex	RNase inhibitor	NEB	S1402S

Tissue-Tek* O.C.T. Compound	Embedding medium for frozen tissue	VWR	4583
Dissecting Needle	Freezing and embedding	Taylor Scientific	12-2973
60 mm Petri Dish*	VWR Tissue Culture Dish	VWR	10062-890
90 mm Petri Dish**	VWR Tissue Culture Dish	VWR	10062-890
Dissecting Cushing Delicate Thumb Forceps	Forceps	Thermo Fisher	08-953C
Leica-brush with Magnet	Tissue section debris brush	Leica	14018340426
Dynasty brushes	Micron Brushes for frozen tissue sectioning	Dynasty	SC2157 R Series
StatMark Pen	Marker	Electron Microscopy Services	72109-12
Razor blades	Embedding and sectioning	VWR	55411-050
32% Paraformaldehyde Solution EM Grade	Fixative	Electron Microscopy Sciences	15714S
Synergy® UV Water Purification System	Milli-Q Water	Millipore-Sigma	CDUFBI001
Ethyl Alcohol, 200 Proof, anhydrous	Ethanol	Millipore Sigma	E7023
Sucrose	Sucrose	Sigma Aldrich	S9378
Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	10X PBS	Thermo Fisher	AM9625
RNaseZap™ RNase Decontamination Solution	RNaseZap™	Thermo Fisher	AM9780
Parafilm M	Slide storage	VWR	102091-164
Direct-zol RNA Miniprep (Product Supplied w/ 200 ml TRI Reagent)	RNA Extraction	Zymo	R2053
Qubit™ 4 Fluorometer	RNA quality verification	Thermo Fisher	Q33238

Qubit™ RNA Broad Range (BR)	RNA quality verification	Thermo Fisher	Q10210
Qubit™ Assay Tubes	RNA quality verification	Thermo Fisher	Q32856
4150 TapeStation System	RNA quality verification	Agilent	G2992AA
RNA ScreenTape	RNA quality verification	Agilent	5067-5576
RNA ScreenTape Sample Buffer	RNA quality verification	Agilent	5067-5577
Kimwipes	Sample and instrument cleaning	Thermo Fisher	06-666

*For use with Standard MERSCOPE Slides

**For use with Large MERSCOPE Slides

2.3 General Laboratory Equipment

General laboratory equipment should be used per manufacturer's instructions.

Fume hood

4°C fridge

-20°C freezer

-80°C freezer

Liquid nitrogen

Vacuum trap system	(e.g., VWR 76207-602)
Vacuum pumps	(e.g., Thomas Scientific 1162B24)
Rocker	
Vortexer	
Analytical balance	
Water bath[a]	(e.g., VWR 76308-896)
Tube/bottle weight	(e.g., VWR 47748-174)

a. If using an alternate make/model, it should be large enough to accommodate the MERSCOPE Imaging Cartridge: 8 × 11 in (20 × 28 cm).

3 OVERVIEW OF MERSCOPE SLIDES

For MERFISH measurement, tissue samples will first need to be sectioned and placed on a MERSCOPE slide prior to sample preparation. Two types of MERSCOPE slides are available, allowing samples to be processed either on the MERSCOPE Instrument or the MERSCOPE Ultra Instrument. The MERSCOPE Instrument enables users to image up to 1 cm^2 of tissue section and the MERSCOPE Ultra Instrument allows users to choose between imaging tissue sizes of up to 1.25 cm^2 or tissue sizes up to 3 cm^2.

Table 2 summarizes the compatible slides and tissue sizes for each Instrument:

Table 2. MERSCOPE instrument and compatible MERSCOPE Slides.

Instrument	Compatible Slides	Maximum Slide Tissue Size Compatibility
MERSCOPE Instrument	MERSCOPE Beaded FF Slide, standard MERSCOPE Standard Slide V 2.0	1 cm^2
MERSCOPE Ultra Instrument	MERSCOPE Beaded FF Slide, standard MERSCOPE Standard Slide V 2.0	1.25 cm^2
	MERSCOPE Beaded FF Slide, Large MERSCOPE Large Slide V 2.0	3 cm^2

Users should determine which Instrument they will be using, and if applicable what size tissue section will be analyzed, before determining which MERSCOPE slide to use for the measurement.

3.1 Selecting MERSCOPE Slides

The MERSCOPE Standard Slide is a round 40 mm diameter slide and supports up to 1 cm^2 area on the MERSCOPE Instrument and 1.25 cm^2 area on the MERSCOPE Ultra Instrument (Figure 1).

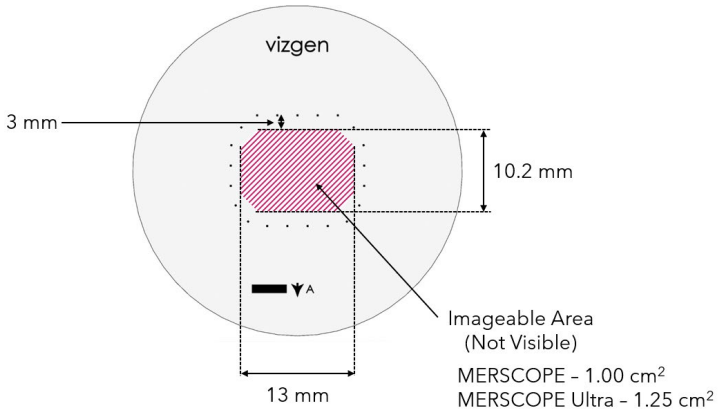


Figure 1. MERSCOPE Standard slides imageable area and distance from imageable area guides marks.

The MERSCOPE Large Slide is a rectangular slide that supports up to 3 cm^2 area on the MERSCOPE Ultra Instrument (Figure 2).

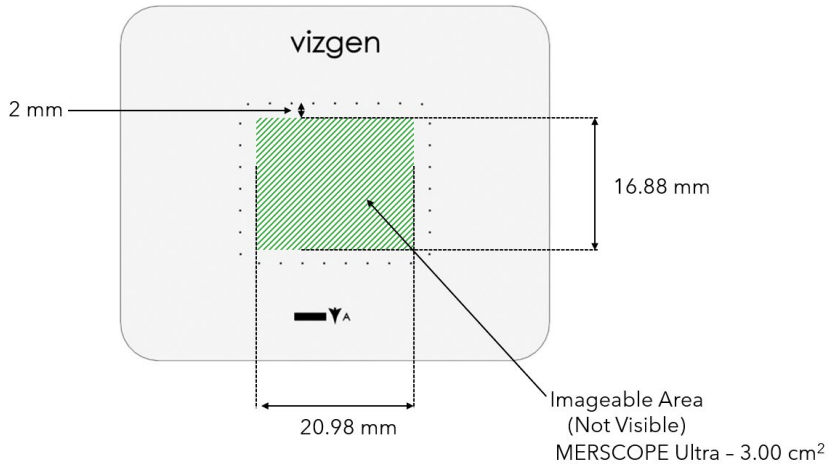


Figure 2. MERSCOPE Large slides imageable area and distance from imageable area guide marks.

3.2 MERSCOPE Marked Slides

MERSCOPE Standard and Large slides contain marking that assist in mounting tissue correctly within the imageable area and loading slides correctly into the MERSCOPE Instrument Flow Chambers. The markings include a Vizgen Logo Mark, Imageable Area Guide Marks, and an Orientation Mark (Figure 3).

- The Vizgen Logo Mark allows easy identification of the correct side of the slide to ensure tissue is mounted correctly.
- The Imageable Area Guide Marks enable users to visually identify the center of the MERSCOPE slide so that tissue can be placed in the imageable area of the slide. The Imageable Area Guide Marks do not represent the exact imageable area but function as guides to help center tissue. The Imageable Area Guide Marks are 3 mm away from the Imageable Area for the "Standard" slides and the Imageable Area Guide Marks are 2 mm away from the Imageable Area for the "Large" slide.
- The Orientation Mark allows users to properly load the sample in the correct orientation within the appropriate flow chamber.

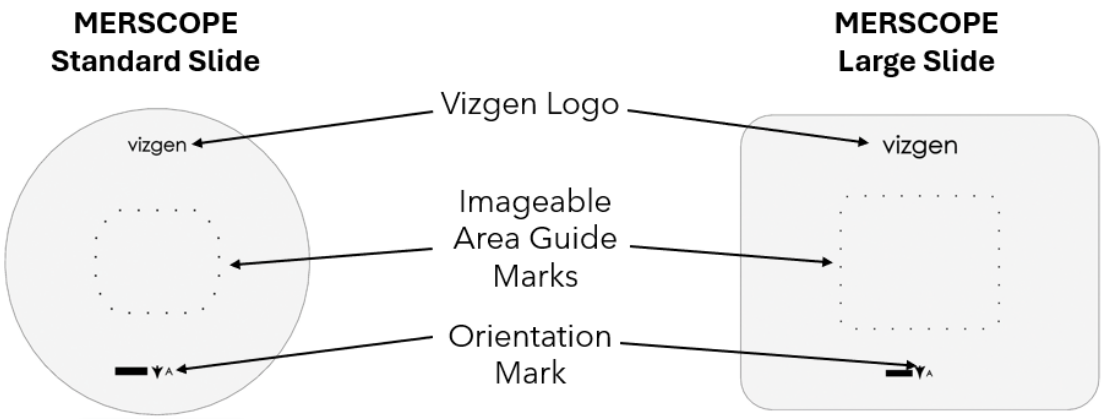


Figure 3. MERSCOPE slides markings.

3.3 Handling MERSCOPE Slides

MERSCOPE Slides are stored at −20°C and must be warmed for at least 15 minutes at room temperature or by other means (warm gloved fingertips) to collect frozen tissue slices (a frozen section will not adhere to a chilled MERSCOPE Slide).

MERSCOPE Slides are fragile and must be handled with extra care. If any cracks or chips are observed, discard them immediately. Chipped or cracked MERSCOPE Slides will not seal in the flow chamber, and cracks will cause the glass coverslip to shatter in the MERSCOPE flow chamber during an imaging run. Both phenotypes will result in a failed MERSCOPE run.

MERSCOPE Slides are coated for tissue attachment and may be handled with forceps; take care to avoid touching adhered tissue sections with forceps. The slides can also be handled with gloved fingers, using care to only touch the edges of the slide and avoid damaging the coated surface of the slides. MERSCOPE Slides should not be cleaned prior to section placement.

3.4 Mounting Tissue on the Correct Side

The two sides of a marked MERSCOPE slide are distinct and tissue must be mounted on the proper side for successful MERSCOPE experiments. Tissue should be mounted on top of the slide where the Vizgen text is readable (Figure 4).

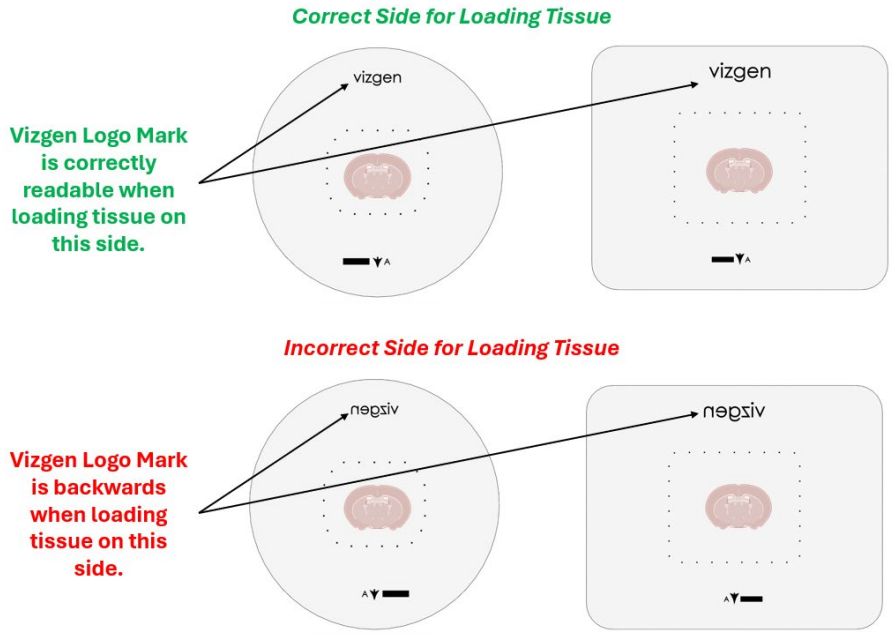


Figure 4. Examples of correct and incorrect slide sides for tissue mounting.

3.5 Correctly Placing Tissue Within the Imageable Area Guide Marks

Vizgen also offers Imageable Area Guide Marks that help center the tissue within the slide's imageable area for MERSCOPE Standard and Large Slides.

For the MERSCOPE Standard slides mount tissue within the Imageable Area Guide Marks ensuring that tissue is at least 3 mm away from any of the Imageable Area Guide Marks (Figure 5).

For the MERSCOPE Large slides mount tissue within the Imageable Area Guide Marks ensuring that tissue is at least 2 mm away from any of the Imageable Area Guide Marks (Figure 5).

Mounting tissue too close to the Imageable Area Guide Marks will result in tissue being outside of the Imageable Area.

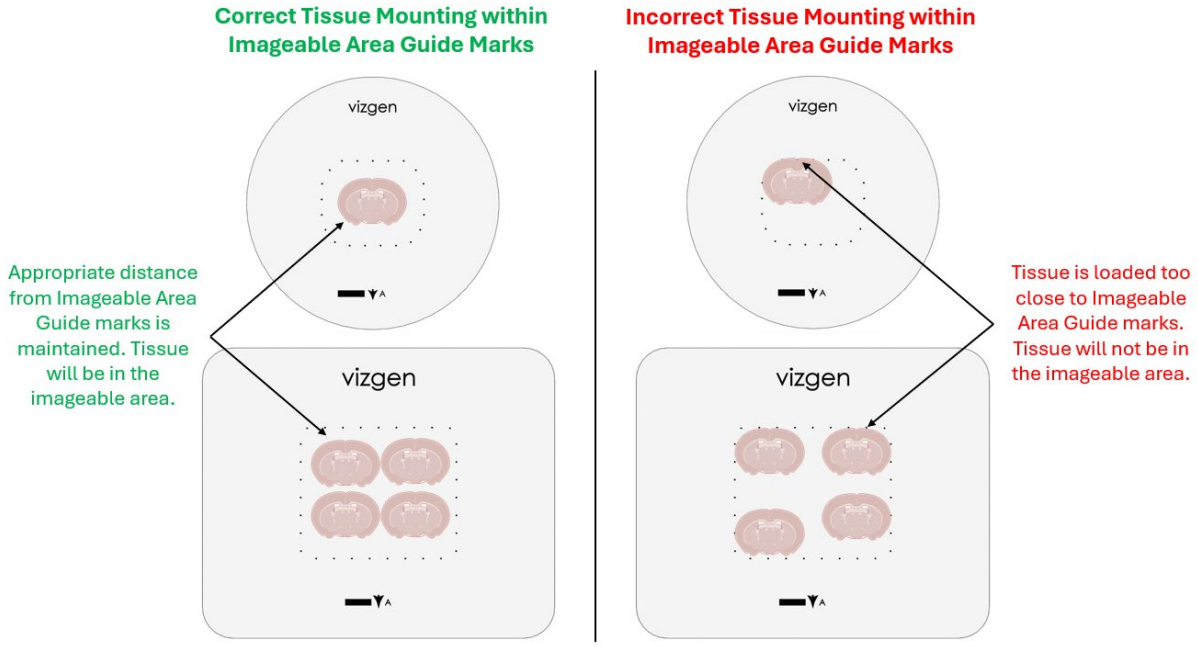


Figure 5. Examples of Correct and incorrect tissue mounting within the imageable area guide marks on MERSCOPE Standard and Large slides.

4 FRESH AND FIXED FROZEN TISSUE PRESERVATION OVERVIEW

RNA is a biomolecule sensitive to degradation. Many factors can contribute to the variation of RNA quality in frozen biospecimens, including sample origin, disease state of samples, time from death or tissue excision to tissue sectioning, sample procurement procedures, storage conditions, and downstream tissue processing workflows. The histological processing, collection, freezing, embedding, and sectioning steps can all impact the sample's morphology and RNA quality. Therefore, careful histological practices aimed at minimizing RNA degradation before imaging are critical to ensure reproducible and high-quality RNA imaging from the MERSCOPE platform.

When working with a sample that is prone to RNA degradation, it is worth considering fixed frozen tissue preparation to harvest and process the sample while implementing histological practices that can facilitate tissue adhesion on MERSCOPE Slides. Fixed frozen tissue allows the user to maintain RNA integrity but will face more adherence issues than fresh frozen tissue.

There are several commonly used methods for frozen tissue preservation. Optimal cutting temperature compound (OCT) provides structural support for sectioning and is the preferred embedding method for frozen tissue sectioning for MERFISH measurement. Tissue blocks can be first embedded in (OCT) compound and then frozen on dry ice in a liquid nitrogen bath, or super-chilled isopentane. Non-embedded snap frozen samples are highly recommended to be embedded in OCT before tissue sectioning.

Alternatively, samples can be submerged in super-chilled isopentane or directly snap frozen in a liquid nitrogen bath (or on dry ice) and then stored at low temperatures (−80°C). Alternatively, fixed frozen preservation is an option for preserving fresh tissues. Tissue is fixed in paraformaldehyde (PFA) first and dehydrated with a sucrose gradient to preserve cell morphology before freezing. This option enables longer term preservation of samples and does not require immediate freezing. In general, fixed frozen tissue contains less RNase contamination as compared to fresh frozen tissue but is prone to tissue detachment after sectioning due to dehydration of the tissue related to the method of processing.

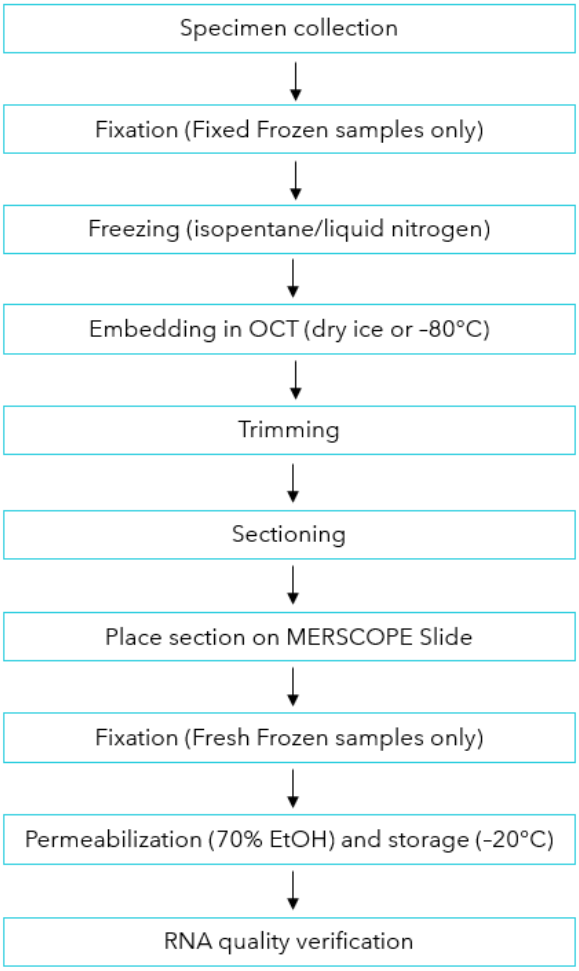


Figure 6. Workflow overview for processing fresh and fixed frozen tissue for measurement on the MERSCOPE Platform.

Figure 6 outlines the general histology workflow for preparing frozen tissue slices for MERSCOPE measurement. The tissue of interest is sectioned and adhered onto a MERSCOPE Slide. If not already fixed in PFA, the tissue section is fixed with fixation buffer and later made permeable to the encoding probes by overnight incubation in nuclease-free 70% ethanol. For this step, users may utilize their preferred fixation and permeabilization protocols. When optimizing conditions such as sectioning, fixation, and permeabilization, it is recommended to use the MERSCOPE Sample Verification Kit to optimize sample

preparation conditions for optimal compatibility with MERFISH imaging using the MERSCOPE Instrument.

5 SPECIMEN COLLECTION, FIXATION, FREEZING, AND EMBEDDING

Tissue specimens will come from various sources and RNA quality can easily be compromised during removal from the patient or experimental animal. It is critical that tissues are handled carefully – fresh frozen tissues should be frozen as soon as possible, and fixed frozen tissues should be fixed as close as possible to the time of extraction or biopsy (see Figure 7A and Figure 7B for recommendations). Improper technique during the specimen collection and processing stages can greatly impact the success of a MERFISH experiment. Below we describe important factors that can influence the quality of RNA in a sample and ways to mitigate impact.

It is important to maintain an RNase-free environment and use RNase-free lab supplies for specimen collection. RNases are conformationally and thermodynamically stable which makes them difficult to permanently inactivate[2-4]. Furthermore, RNases are found nearly everywhere, including on the skin, so lab equipment and glassware can be easily contaminated with these enzymes[5]. It is important to decontaminate the bench and lab supplies with RNaseZap solution before specimen collection.

5.1 Collection

The time from death or tissue excision to tissue freezing and embedding plays a major role in RNA degradation. Enzymatically active tissues and tissues prone to RNA degradation such as the liver, brain, tonsil, salivary gland, pancreas, stomach, spleen, and gastrointestinal (GI) tract should be removed first and either directly frozen or placed in fixatives as soon as possible.

The speed of freezing and fixation is proportional to the size of the tissue block collected. An oversized tissue may experience uneven freezing and fixation throughout the tissue, and the center of the tissue is more likely to have poorer RNA quality due to the longer time needed for freezing and fixation. It is generally recommended to limit the size of tissue blocks to less than 1.5 cm^3 and the volume of fixative should be 5-10x the volume of the tissue for preparing fixed frozen samples.

Furthermore, tissue origin plays an important role in RNA quality during specimen collection. Certain tissue types, such as skin, GI tract, and lung, are more likely to be contaminated by RNase due to their exposure to the exterior environment. Other tissues such as the pancreas may contain more enzymatic activity. To minimize RNA degradation, it is recommended to add an RNase inhibitor to the tissue collection medium before freezing and embedding. For example, freshly harvested tissue can be first immersed in RNase inhibitor (4 mM ribonucleoside vanadyl complex) in 1X PBS or harvesting medium at 4°C for 30 minutes before embedding. Alternatively, the tissue can be prepared as fixed frozen samples, as fixative can also inhibit RNase activity.

Research has demonstrated that RNases can reactivate in thawed fresh frozen samples[6], so it is highly recommended to embed frozen tissue blocks in OCT and store at −80°C for a long term. It is also recommended to minimize the number of freeze-thaw cycles to prevent RNA degradation through the cumulative action of transient RNase reactivation events at higher temperatures.

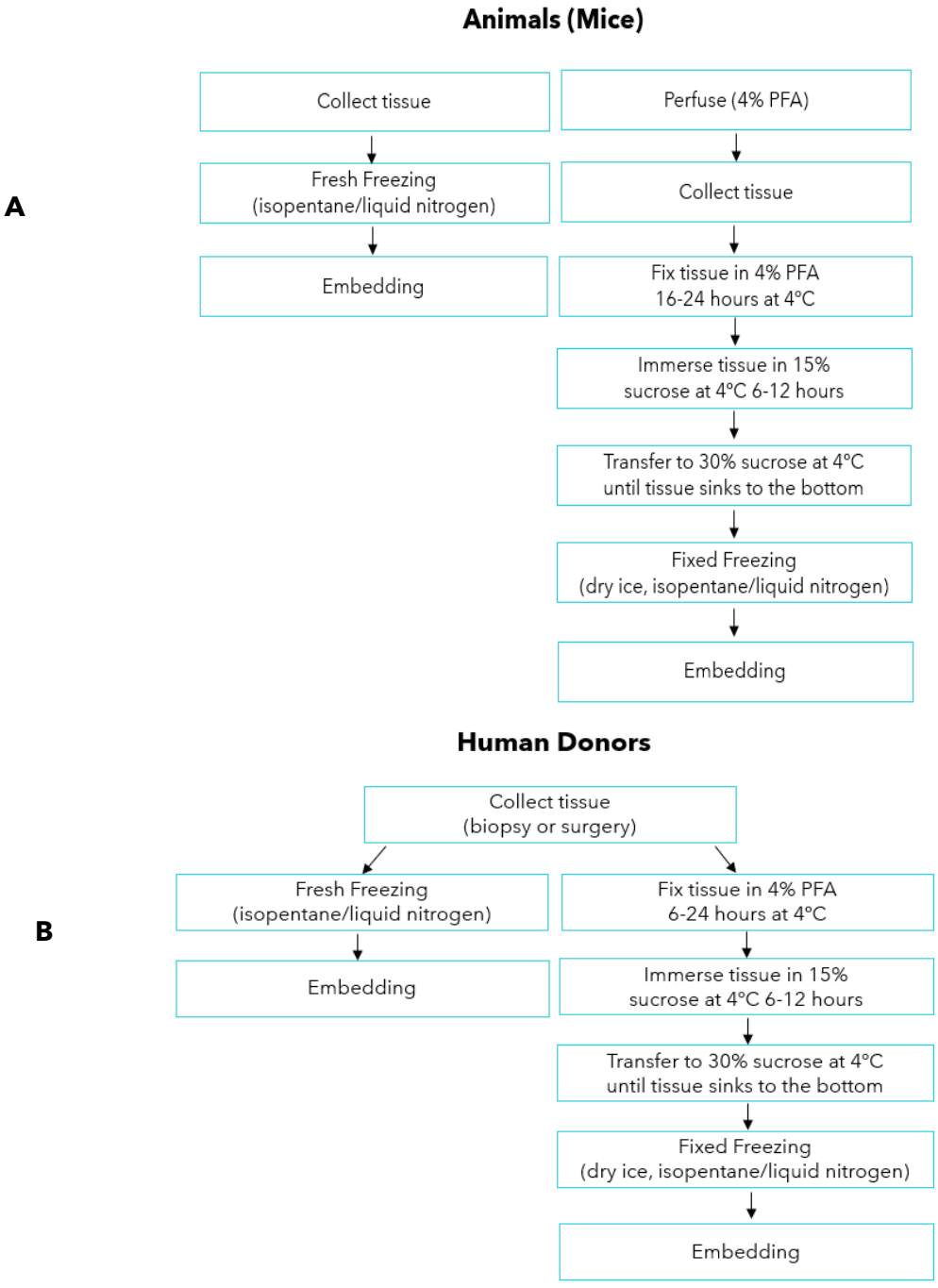


Figure 7. Workflow for collecting, freezing, and embedding tissues from **A)** mice and **B)** human donors.

5.2 Fixation

Ideally, fixation should take place at the site of removal (in the operating theater) or, if this is not possible, immediately following transport to the laboratory (Figure 7A and Figure 7B).

For experimental animals that need to be perfused with a fixative, it is generally recommended to first perfuse the animal or tissue with freshly prepared 4% PFA in 1X PBS. Then the tissue can be harvested and further fixed in 4% PFA at 4°C. For biospecimens that cannot be perfused, after specimen collection, tissues can be placed in a fixative (such as 4% PFA) for fixation (Figure 7A and Figure 7B). PFA is a monofunctional crosslinker and will polymerize over time. Therefore, fresh 4% PFA in 1X PBS should be prepared each time for fixation during specimen collection.

Fixative slowly permeates the tissue, ensuring its preservation and protection from damage during subsequent processing steps[7]. PFA penetrates tissues at a rate of 1 mm/h, and thus tissue specimens should remain in fixative long enough for the solution to fully penetrate the tissue and the chemical reactions of fixation to reach equilibrium (fixation time). Incubation time will vary depending on tissue type; 16-24 hours is recommended for mouse tissue (Figure 7A) and 6-24 hours is recommended for human tissue (Figure 7B). It is important to note that over-fixation should be avoided as PFA-mediated crosslinking may make it challenging for tissue clearing or block MERFISH probes from binding to the RNA transcripts of interest during MERSCOPE sample preparation.

5.3 Freezing

Freezing samples quickly (flash freezing) is important to avoid RNase activity and ice crystal formation. The expansion of ice crystals during freezing tears and distorts the tissue, resulting in sections that appear damaged under the microscope (multiple artifactual holes in the tissue).

The standard method for freezing fresh tissues involves submerging tissue in super-chilled isopentane (Figure 8). The isopentane should be decanted or pipetted into a metal dish, which is in turn floating in liquid nitrogen (Figure 8). The tissue can either be frozen before OCT embedding or frozen in OCT during embedding. Freezing with super-chilled isopentane allows tissues to freeze quickly, preventing air bubbles from forming in the tissue and minimizing tissue damage that may occur from ice crystal formation. If a super-chilled isopentane bath is not feasible, it is possible to directly freeze tissue in liquid nitrogen or on dry ice[7].

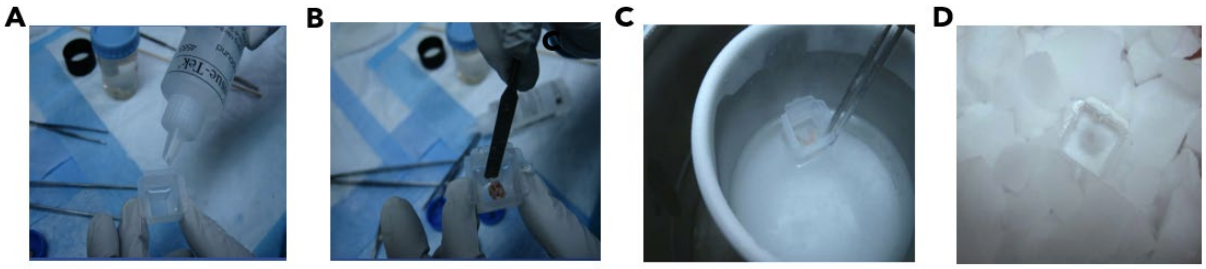


Figure 8. Demonstration of fresh tissue freezing and embedding using isopentane and liquid nitrogen. **A)** Pour the OCT compound into a plastic embedding mold. **B)** Embed tissue in a plastic embedding mold. **C)** Freeze in isopentane by submerging the tissue using forceps. **D)** Temporarily store the embedded samples on dry ice.

For fixed tissue, it is generally recommended to use sucrose to dehydrate the sample after fixation and before freezing. The addition of sucrose or sucrose gradients in these steps prevents the excessive formation of ice crystals in fixed frozen tissues during the cryopreservation process and can better preserve tissue morphology.

5.4 Embedding

For frozen tissue sectioning, it is essential to create a frame of OCT around the tissue. This frame provides support to the tissue and when sections come off the blade, the frame creates a handle that can be gently pulled with a brush to securely direct the section to the stage of cryostat.

It is recommended to embed tissue in a mold to create even edges of the frozen block. Disposable plastic molds should be used regardless of the freezing method. If embedding with the liquid nitrogen-based method, use molds that are compatible with liquid nitrogen so that the mold does not shatter. Choose an appropriately sized embedding mold for the tissue being embedded. It is highly recommended to come up with a system to store tissue blocks with an identifying label, such as in a small resealable bag or a mold that has an attached lid.

Orientation of the tissue in the mold is important as it will determine the plane of the section. Before embedding, identify how the final images should look, including which structures and orientation of those structures would give the most information for MERFISH measurement; keep this in mind when embedding the tissue sample. When cutting a tissue block, ensure it is positioned such that it produces the desired section orientation (Figure 9). When embedding multiple tissues, the orientation of each tissue must be kept in mind.

When placing tissue in the mold, tissue placed at the bottom will be the front face of the block and will be sectioned first. Always ensure the orientation of the tissue block is marked.

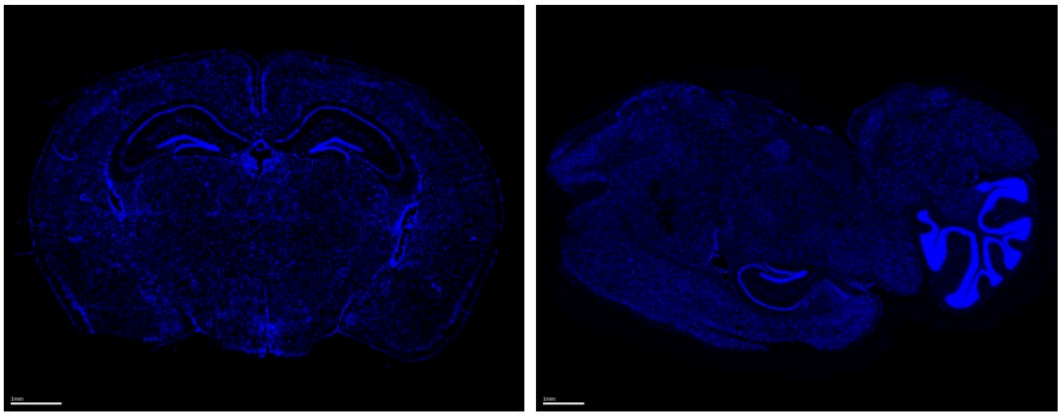


Figure 9. Example images of the mouse brain from MERSCOPE run with different embedding orientations. Left, coronal section; right, sagittal section. Scale bar: 1 mm.

Finally, during embedding, avoid creating multiple layers of OCT in a tissue block (i.e. allowing a layer of OCT to freeze before adding the next layer). The interface between layers of OCT that freeze at different times will result in block-fracturing and tissue loss.

5.5 Embedding Multiple Tissues in a Tissue Microarray

Tissue microarrays (TMAs) enable the analysis of multiple tissue samples simultaneously by co-embedding multiple samples in one OCT block. A few important tips for TMAs are provided below. Please refer to the "A Practical Guide to Frozen Section Technique," Chapter 3 for more detailed information on embedding multiple tissues[8].

Planning the orientation of the tissues in the block is essential before making a TMA block. When embedding, use whole or large portions of tissues and make sure that the tissues are lined up so that the short edges will hit the blade first and the tougher tissue elements get sectioned last. If using tissue cores, products such as the ArrayMold Kits (ArrayMold LC) can be helpful in making the block. Tissues should be separated by at least 1 mm to allow the OCT compound to fully envelop each sample.

TMAs can be embedded in different sizes. When embedding multiple tissue samples into one block, the maximum imageable area on the MERSCOPE Slides should be taken into consideration when arranging the tissues. Only samples within the imageable area can be measured on the MERSCOPE Instrument.

During embedding for multiple tissues, place tissues in planned arrangements on the stage and carefully add chilled OCT to cover the tissue while trying to avoid disturbing the tissue placements; slow and steady dispensing of the OCT is critical. After dispensing OCT, some manipulations can still be made to the tissue position, but movement will be limited due to the viscosity of the OCT. Ensure the original OCT layer does not solidify before adding more OCT, as the interface between layers of OCT frozen at different times is prone to block fracturing and tissue loss.

6 FREEZING AND EMBEDDING PROTOCOLS

Freezing and embedding protocols for fresh and fixed frozen tissues are provided in the subsections below.

6.1 Fresh Frozen Tissue Freezing and Embedding Protocols

Two protocols for preparing fresh frozen tissue blocks are presented below.

6.1.1 Option 1 (Preferred)

1. Prepare chilled isopentane in a metal dish floating in a liquid nitrogen bath.
2. Place the tissue in a labeled tissue embedding plastic mold. Tissue at the bottom of the mold will be the front face of the block.
3. Pour pre-chilled OCT (4°C) into the mold until OCT completely covers the tissue.
4. Gently use a dissecting needle to guide the tissue back to the middle of the mold and to press it down slightly.
5. Using forceps, transfer the plastic mold into the isopentane and liquid nitrogen bath without submerging. Wait until the OCT completely solidifies and turns white.
6. The OCT should be completely frozen before attempting to remove the block from the mold. Depending on the size of the mold and the amount of OCT used, this can take anywhere from 5 minutes to 45 minutes (OCT is white when frozen). Frozen blocks must be stored at −80°C for long-term storage.

6.1.2 Option 2

1. Prepare chilled isopentane in a metal dish floating in a liquid nitrogen bath.
2. Immerse the tissue into chilled isopentane until completely frozen for at least 1 minute.
3. Transfer the tissue to a prechilled, labeled tissue embedding plastic mold on dry ice. Tissue at the bottom of the mold will be the front face of the block.
4. Add pre-chilled OCT (4°C) to embed the tissue.
5. Gently use a dissecting needle to guide the tissue back to the middle of the mold and press it down slightly.
6. The OCT should be completely frozen before attempting to remove the block from the mold. Depending on the size of the mold and the amount of OCT used, this can take anywhere from 5 minutes to 45 minutes (OCT is white when frozen). Frozen blocks must be stored at −80°C for long-term storage.

6.2 Fixed Frozen Tissue Freezing and Embedding Protocol

The recommended method to prepare fixed frozen tissue blocks is:

1. If possible, first perfuse the animal or tissue with freshly prepared 4% PFA in 1X PBS.
2. Dissect the tissue and place the tissue into freshly prepared 4% PFA at 4°C. Incubation time will vary depending on tissue type; 16-24 hours is recommended for mouse tissue and 6-24 hours is recommended for human donor tissue.
3. Immerse the tissue in 15% sucrose in 1X PBS at 4°C for 6-12 hours, and then transfer to 30% sucrose in 1X PBS at 4°C until the tissue sinks to the bottom of the container.
4. Freeze the tissue in OCT with dry ice or an isopentane and liquid nitrogen bath. Wait until the OCT completely solidifies and turns white before removing it from the mold. Frozen blocks must be stored at −80°C for long-term storage.

7 TISSUE SECTIONING

For fresh and fixed frozen samples, equilibrate tissues to −20°C for at least 30 minutes from −80°C storage. Secure the samples in the chuck on the cryostat, section, and carefully adhere the sections to a MERSCOPE Slide. If not already fixed in PFA, the tissue section should be fixed with fixation buffer.

As with any histological technique, tissue sectioning requires practice; we recommend using non-precious samples and slides to test techniques to become comfortable with the technique prior to starting your experiment. Users should practice sectioning and section placement before preparing a sample for MERSCOPE imaging (Figure 10). Draw a placement guide frame on the central area of the back of non-experimental slides to represent the useable area on the MERSCOPE slides. Alternatively, users can trace the MERSCOPE slide markers onto a non-experimental practice slide, or for Standard slides without markings, use the (10300104) tissue placement guide on the back of a slide for practice.

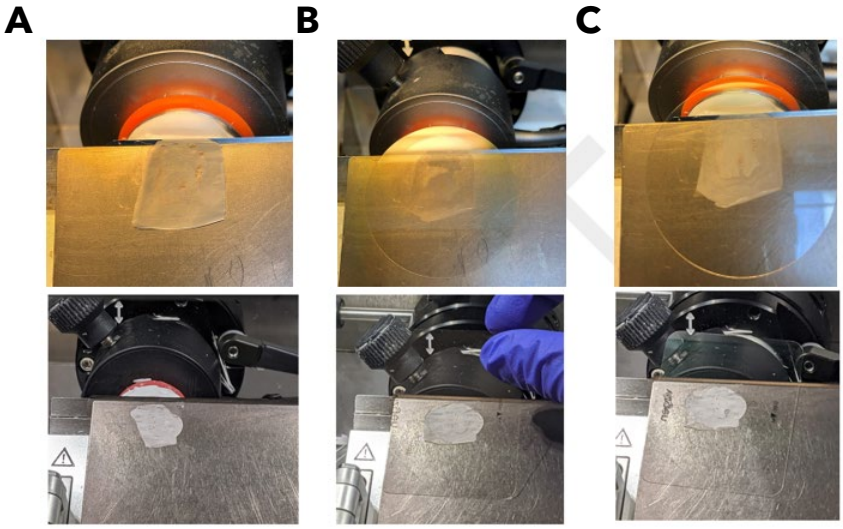


Figure 10. Frozen tissue block sectioning using Standard unmarked slides (top) and Large marked slides (bottom). **A)** Tissue section immediately after sectioning. **B)** Tissue section ~1 second after lowering the MERSCOPE Slide onto the tissue section. **C)** Tissue section ~3 seconds after lowering the MERSCOPE Slide onto the tissue section. The OCT compound becomes white as the tissue refreezes. Leave the slide with tissue in the cryostat for ~5 seconds to allow the tissue section to refreeze and adhere to the MERSCOPE Slide. Note the orientation of the markings on the Large slide, ensuring the tissue is placed on the correct side of the slide.

7.1 Preparing for Tissue Sectioning

7.1.1 Cryostat Maintenance

It is essential to keep the cryostat well-maintained and follow annual preventative maintenance requirements. The compressor must have adequate airflow as overheated

compressors can fail. The compressor is the most expensive and time-consuming part to replace or repair on the cryostat.

If tissue gets stuck beneath the blade, the blade will not properly cut sections. This will result in striations and/or tears in the sections. In this case, the blade should be carefully removed and replaced.

Freeze-lock can occur when frost and ice buildup on the rotors, preventing the cryostat from moving. This can occur if the cryostat is turned off, allowed to warm up to room temperature, and is then turned back on without drying the chamber out first. If freeze-lock occurs, turn off the cryostat and allow it to come to room temperature. Before turning it back on, dry out the chamber completely. This requires taking out anything that can be removed and drying the bottom of the chamber and the rotors, as even a small piece of ice behind the microtome will cause continued freeze-lock issues. When completely dried, turn the cryostat back on and allow the temperature to stabilize for 24 hours. If the cryostat is used before the temperature stabilizes, the temperature will fluctuate while in use. Allowing the temperature to fully stabilize in the cryostat helps prevent such temperature swings.

7.1.2 Decontaminate the Work Environment

RNase contamination must be avoided to obtain high quality MERFISH data. Clean the cryostat, brushes, forceps, and the surrounding area with a Kimwipe. Spray with both RNaseZap solution and 70% ethanol to ensure an RNase-free sectioning environment. Before decontamination, make sure there is no blade in the blade holder. Remove RNaseZap residue by wiping with 70% ethanol. Spraying gloves with 70% ethanol can also help prevent additional tissue sections from sticking to the MERSCOPE Slide but be careful not to get the ethanol on the MRESCOPE slides. All Kimwipes or paper towels used in the cryostat are to be disposed of as biohazardous waste.

7.1.3 Prepare Petri Dishes and Sectioning Tools

Label 60 mm (Standard slides) or 90 mm (Large slides) Petri dishes for the slides. Place the dishes (up to 10 at a time) in the cryostat or at −20°C to cool down. Pre-chill any sectioning tools inside the cryostat (Figure 11).

7.2 Equilibrate Tissue Blocks

Tissue blocks are stored at −80°C and must be brought to the temperature of the cryostat before sectioning. If the tissue block is too cold, a section cannot be obtained easily as the OCT and tissue will fracture. To equilibrate the tissue blocks, place at −20°C for at least 30 minutes prior to sectioning (Figure 11).

7.2.1 Prepare Fixation Buffer

Fresh frozen samples that have not already been fixed must be incubated in a fixation buffer. It is recommended to freshly prepare 4% PFA each time and pre-warm to 37°C or 47°C before being used (Table 3).

Table 3. Fixation buffer recipe.

Slide type	Standard			Large		
Sample number	1	5	10	1	5	10
10X PBS	1 mL	3 mL	6 mL	2 mL	6 mL	12 ml
32% paraformaldehyde solution	1.25 mL	3.75 mL	7.5 mL	2.5 mL	7.5 mL	15 ml
Nuclease-free water	7.75 mL	23.25 mL	46.5 mL	15.5 mL	46.5 mL	93 ml
*Make fresh every time used						

7.2.2 Pre-warm MERSCOPE Slides (Optional)

MERSCOPE Slides are stored at −20°C. Pre-warm slides at room temperature for 15 minutes before use if collecting tissue slices on warm MERSCOPE Slides. Alternatively, cold MERSCOPE Slides can be warmed with a gloved finger to adhere tissue. Unused MERSCOPE Slides should be stored at −20°C.

7.3 Setting Up the Cryostat

7.3.1 Attach Tissue Block to a Chuck

For fresh and fixed frozen tissues, attach the tissue block to a cold chuck by adding OCT onto the chunk and then place the tissue on top of OCT (Figure 11A). Allow the OCT to freeze completely before sectioning. If the OCT is not completely frozen, the block will get knocked off the chuck by the blade during a sectioning event. Fix the OCT tissue block on the block holder of the cryostat (Figure 11B). Ensure the chuck is flush with the chuck holder to avoid vibration, which can cause variable section thickness and quality.

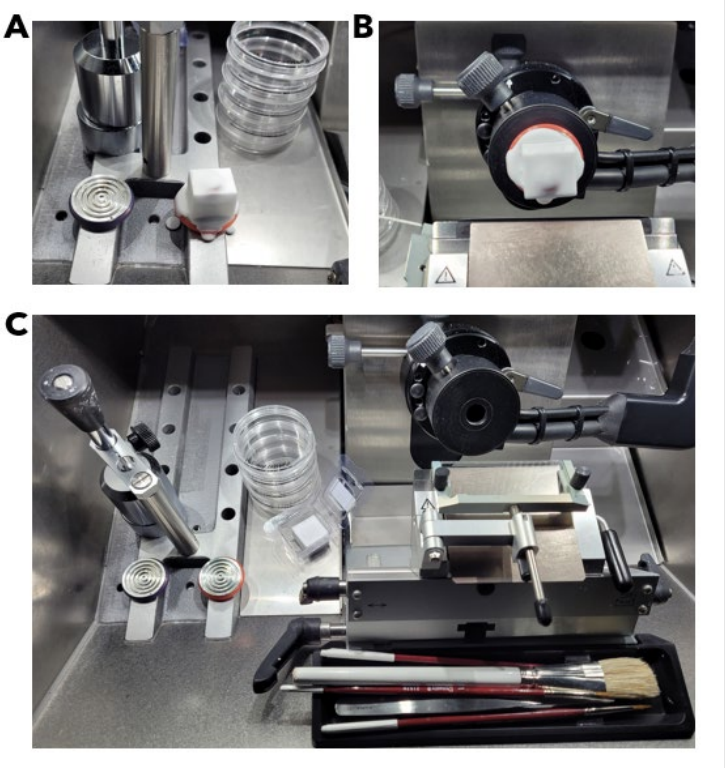


Figure 11. Tissue block mounting and cryostat setup. **A)** The OCT tissue block is mounted on a cryostat specimen chuck using OCT compound. **B)** The OCT tissue block is fixed on the block holder of the cryostat. **C)** Demonstration of cryostat setup; labeled 60 mm Petri dishes, OCT tissue block, cryostat specimen chuck, and sectioning tools are pre-chilled inside of the cryostat.

7.3.2 Cryostat Blade

Always use a new blade during cryosectioning, as nicks on a used blade can cause damage to sections and the tissue block. Set the blade angle at 5 degrees. Set the section thickness to 10µm for most tissues. If tissue sections appear to be thinner, consider increasing tissue section thickness up to 15µm. For example, it is recommended to section mouse brain samples at 14µm, as shrinkage occurs often in this tissue type during the cryosectioning process.

Ensure the cryostat and blade are secured for tissue section uniformity; all levers and knobs must be completely tightened. When sectioning tissue, do not turn the flywheel too fast. The instrument may make "thunking" sounds if the wheel is turned too fast. Moving too quickly can damage both the tissue block and the instrument. When the blade hits the tissue too hard, the block could come off the chuck or a piece of tissue could pop out of the block. Turning the wheel too fast can also cause holes to appear in the tissue.

7.4 Tissue Block Trimming

Trim the entire surface of the tissue block to ensure that all desired tissue is present in the section (Figure 12). Position the block by adjusting the chuck holder so that the blade hits

the tissue at the desired orientation. If this adjustment is not made, some parts of the tissue will be wasted before other parts are exposed. If the tissue is embedded and oriented correctly, this should be easy to do. If a tissue was not embedded and oriented completely flat, misorientation can result in tissue pieces missing on the slide, in which case either deeper sectioning or re-embedding is recommended. When approaching the tissue of interest, move the blade over to a previously unused section for sample sectioning.

Each time a block is used, it is recommended to trim off 50 µm before using any sections, even if the block has been sectioned before. Additional sections should be taken for RNA quality verification. For some precious samples, trimming 50 µm may not be possible. In this case, process the sample at the user's risk. It is also highly recommended to use the MERSCOPE Sample Verification Kit to evaluate and compare the sample quality of adjacent sections for all samples.

Figure 12. Tissue block trimming. Trim until the whole surface of the tissue of interest is exposed, then the tissue is ready for sectioning. An example of a fresh frozen human kidney tissue block is shown here.

7.5 Tissue Block Sectioning

7.5.1 Cryostat Temperature

For each tissue block, it is important to establish the ideal temperature to achieve uniform sections. Cutting sections from a block that is too cold will result in shattered sections (Figure 13), while cutting sections from a block that is too warm can cause tissue to crumple[9]. The ideal sectioning temperature must be determined empirically and depends on several factors, including tissue type and disease state, cryostat model, and the conditions within the lab. When cutting a tissue block, the first few sections can vary in thickness due to temperature fluctuation if the temperature of the block is not well equilibrated, but once this stabilizes, the section thickness normalizes. Experienced histologists can visually confirm section thickness; thin sections will be nearly translucent, and thick sections will lack flexibility and look opaque, while the ideal sections will be flexible and have the thickness of printer paper[9-10].

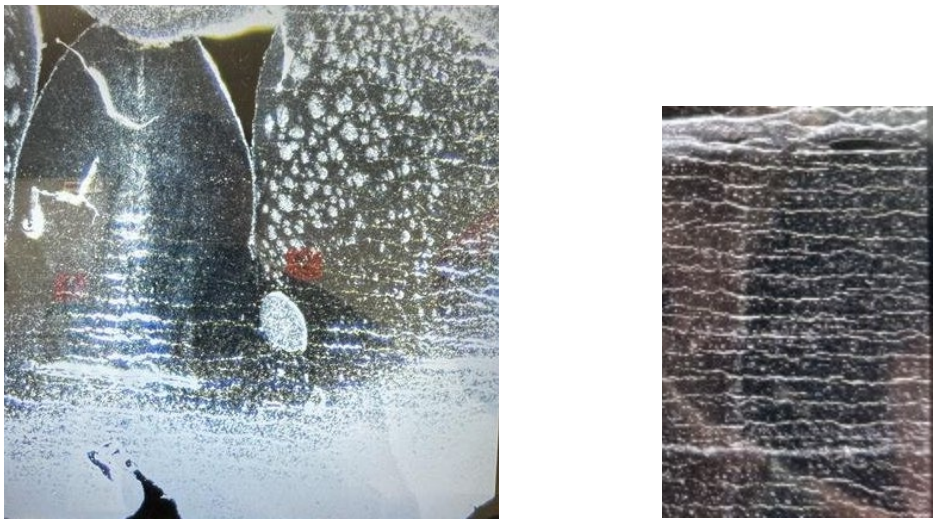


Figure 13. Examples of tissue shattering (note horizontal lines).

7.5.2 Sectioning Speed

When sectioning tissue blocks, the user will need to adjust both the speed and the width for sectioning based on the size of the block, the tissue sample, and the requirements for the tissue of interest. Pausing too long between sections may cause the tissue face to freeze, resulting in the need to discard thinner, smaller sections to obtain tissue at the desired thickness. Speed is essential to the process in order to avoid wasting tissue.

7.5.3 Section Handling

A brush or antiroll device may be used to help hold curling tissue flat during sectioning and to stabilize the section as the blade crosses it (if using a brush, avoid cutting brush bristles on the blade). The leading edge of the OCT may be used to pull the section over the blade and onto the stage. Once sectioning is complete, cover the exposed surface of the tissue block with OCT for storage.

7.5.4 Section Orientation

The orientation of the tissue block to the blade is important to achieve high quality sections[11] (Figure 14). Dense tissues such as skin epidermis and mucosal-lined tissues (GI tract, bladder, uterus, and cervix) should be oriented perpendicular to the blade to prevent bunching of the tissue within the section. To achieve quality sections of hard and fatty elements of a tissue, orient the block so that these areas will be the last areas cut. For very hard tissues such as decalcified bone[12], the blade should be oriented across the longest axis of the tissue. This will cause less stress on the blade and result in smoother sectioning. This orientation also prevents too much force on the bone, which could result in tissue popping out from the block.

If parts of the tissue are "peeling" away from the edge of the OCT, re-orient the chuck so that the blade hits that area from a direction perpendicular to the previous direction. Heart, muscle, and kidney tissues commonly need re-orientation due to peeling.

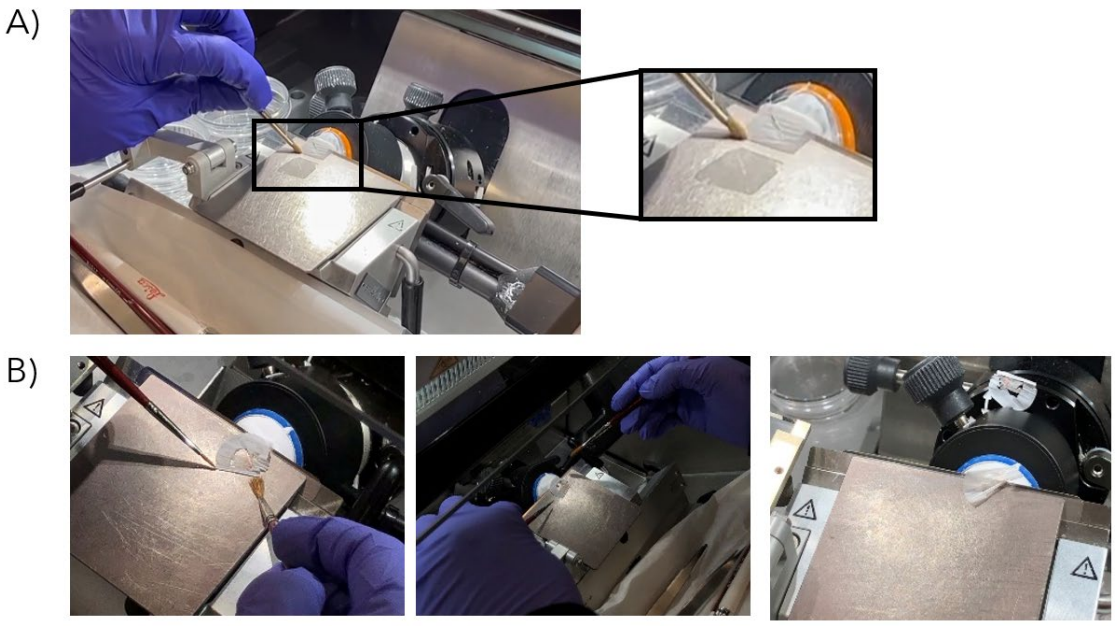


Figure 14. Lipids and fats within the adipose portions of the tissue block will remain in a semisolid waxy state that can melt or smear across the cutting surface of the cryostat blade. Due to this melting effect on the blade and structural differences across tissue regions, the sections often catch, tear, and smear.

7.6 Fresh and Fixed Frozen Tissue Sectioning Protocol

1. Maintain a RNase-free environment. RNase contamination must be avoided to obtain high quality MERFISH data. Clean the cryostat stage, forceps, dissecting needle, other sectioning tools, and surrounding area with RNaseZap™ solution to ensure an RNase-free sectioning environment. Remove RNaseZap™ residue by wiping with 70% ethanol.
2. Place the optimal cutting temperature (OCT) compound-embedded tissue block into the cryostat and allow it to sit at −20°C for at least 30 minutes.
3. Label the petri dish for sectioned slide storage.
4. Warm freshly made 1XPBS and 4% PFA at the desired temperature (room temperature, 37°C or 47°C) for 15 minutes. Separately, consider pre-warming the MERSCOPE slides at room temperature for 15 minutes if collecting tissue sections by using pre-warmed MERSCOPE Slides.
5. Set up the cryostat properly. Install a new blade, adjust the blade angle to 5 degrees, and set the tissue thickness at 10 µm.

NOTE: Generally, the thickness of tissue section should match the height of a single cell. Consider increasing or decreasing tissue thickness if tissue appears thinner or thicker for MERSCOPE imaging. For example, mouse brain samples can be cut at 14 or 15 µm thickness, while tissues such as spleen can be cut at 7 or 8 µm thickness due to the small cell size.

6. Trim the OCT-embedded tissue block until the desired tissue region is exposed.
7. Cut a 10 µm section from the OCT-embedded tissue block.

NOTE: Refer to Section 8.1 Placing Multiple Samples on One MERSCOPE Slide.

8. Ensure the tissue section is flat. Mount the tissue section into the center of the MERSCOPE Slide.

NOTE: If using marked MERSCOPE Slides, tissue sections must be placed on the side of the slide where the Vizgen text is correctly readable. Since the slide will be lowered onto the tissue section, start with the MERSCOPE Slide where the Vizgen text is backwards, then slowly lower the MERSCOPE slide onto the tissue section from above. Once flipped for sample preparation the Vizgen text will be readable with the tissue on the correct side. Ensure that the tissue is centered as much as possible within the Imageable Area Guide Marks. Avoid placing the tissue section within 3 mm of the Imageable Area Guide Marks of MERSCOPE Standard Slides, or 2 mm of the Imageable Area Guide Marks of MERSCOPE Large Slides to ensure that the tissue will be within the imageable area.

9. Place the MERSCOPE Slide into a dry petri dish with tissue facing up (60 mm petri dish for MERSCOPE Standard Slides, 90 mm petri dish for MERSCOPE Large Slides) and place at −20°C for 5 minutes to allow the tissue section to adhere.

NOTE: Incubation at -20°C for up to 30 minutes or longer improves tissue adherence to the slide and reduces tissue lifting during fixation.

10. Fixation:
    a. *If fixed frozen tissue: skip this step.*
    b. *If fresh frozen mouse brain samples: in a fume hood, add 5 mL fixation buffer immediately within seconds and incubate tissue in fixation buffer at room temperature for 15 minutes.*
    c. *If other fresh frozen tissue samples: in a fume hood, add 5 mL 47°C prewarmed fixation buffer immediately within seconds and incubate tissue in fixation buffer at 47°C for 30 minutes.*

NOTE: For fresh frozen samples that are prone to RNA degradation, or have compromised RNA quality, 47 °C fixation protocol is recommended. If the tissue section is sensitive to 47 °C fixation and prone to detachment during fixation, repeat the fixation step with a new section on a new slide using pre-warmed 4% PFA in PBS at 37°C for 1 hour.

11. Wash 3x with 5 mL 1X PBS, incubate 5 minutes each wash.

NOTE: If tissue is prone to detachment after fixation and 1XPBS wash, dry the slide at RT for 1hour.

12. Add 5 mL 70% ethanol, seal the petri dish with parafilm and place at 4°C overnight to permeabilize the tissue.

NOTE: The sample can be stored in 70% ethanol in a labeled petri dish, sealed with parafilm, at 4°C for up to 1 month.

8 TISSUE PREPARATION BEST PRACTICES AND TIPS

8.1 Placing Multiple Samples on One MERSCOPE Slide

Placing multiple samples on one MERSCOPE Slide can reduce overall experimental time as well as batch effects, especially when studying multiple conditions within the same experiment. The MERSCOPE Large Slide, with an imaging area of up to 3 cm^2, is particularly well suited for placing multiple sections. There are a variety of options for placing multiple sections on one MERSCOPE Slide. Users should follow their own histology best practices, personal preferences, and tissue-specific guidelines when deciding which method to use. As with all histological methods, practice with a non-precious sample until comfortable with the technique.

Several options are described below:

Option 1: Mount multiple tissues together in OCT and cut the block to achieve a single section containing multiple tissues. This is the most conservative and safest option for preserving tissue quality. This is also the optimal method for performing MERFISH. An example of embedding multiple tissues together in OCT is shown below (Figure 15).

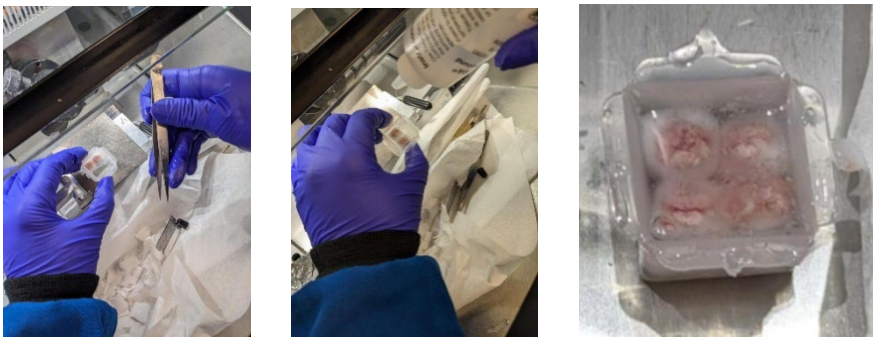


Figure 15. Embedding multiple tissues together in OCT.

Table 4. Pros and cons of Option 1

Pros	Cons
Sectioning and adherence of multiple tissues can be easier with this method.	Embedding multiple tissues in one block may not be feasible for precious sample types.
Processing multiple tissues at once saves time.	Orientation of multiple tissues can be difficult.
Minimizes heat/freeze events on multiple tissues.	It may be difficult to section all tissues in one block evenly.

Protocol:

1. Harvest multiple tissue samples and create an OCT embedded multi-tissue block.
2. Section the tissue block using a cryostat.
3. Tissue collection on MERSCOPE Slides:
    a. If collecting the tissue slice by dropping a MERSCOPE Slide to the tissue section, pre-warm the MERSCOPE slides at room temperature for 15 minutes first and carefully hold the MERSCOPE slide edge by gloved finger or forceps, and then gently drops the MERSCOPE slide on top of the tissue slices. The tissue slice will melt and turn transparent on the MERSCOPE slide within 3-5 seconds. Allow the tissue slice to refreeze and turn white on the cold stage of cryostat before placing the sample into the petri dish.
    b. If collecting the tissue slices by warming up a cold MERSCOPE slide, place the MERSCOPE slide on the cold stage of a cryostat and pre-chill to -20˚C for 3-5 minutes first. Then use brushes to move and arrange the tissue slice to the usable area of MERSCOPE slide. Carefully lift up the MERSCOPE slide by holding the edge of MERSCOPE slide using gloved finger, and then using warmed fingertip, rub against the back of MERSCOPE slide to heat up the tissue slice till it melts and becomes transparent. Then place the MERSCOPE slide on the cold stage to allow the tissue refreeze and turn white before transferring to a petri dish.

Option 2: Sequentially adhere tissues one by one to a MERSCOPE Slide. This option is ideal for users who cannot embed multiple tissues in one block. There are multiple methods for sequentially adhering tissue sections, and users should follow their own histology best practices, personal preferences, and tissue-specific guidelines after practicing. Figure 16 shows how to sequentially section and place multiple sections using a gloved finger to adhere each section.

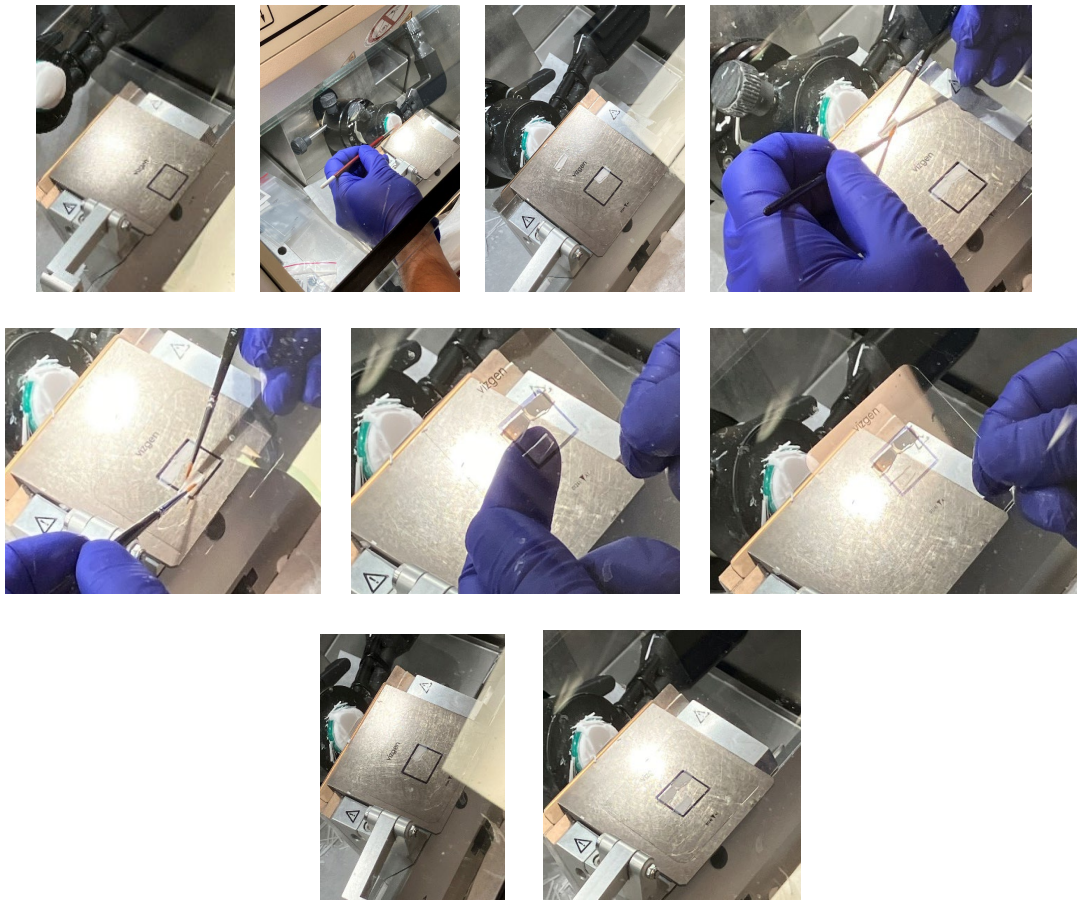


Figure 16. Mounting multiple sections sequentially on to a pre-chilled the MERSCOPE slide (black square drawn to improve contrast).

Table 5. Pros and cons of Option 2

Pros	Cons
Technique may provide more control to some users in tissue placement.	Not as efficient as other techniques.
	Technique heats and freezes each slice already adhered to slide – may cause RNA degradation.

Protocol:

1. Harvest the tissue sample and create an OCT embedded tissue block.
2. Section the tissue block using a cryostat.
3. Tissue collection on MERSCOPE Slides:
    a. If collecting the tissue slice by dropping a MERSCOPE Slide to the tissue section, pre-warm the empty area of a MERSCOPE slide using fingertips first and carefully hold the MERSCOPE slide edge by gloved finger or forceps, and then gently drops the MERSCOPE slide on top of the tissue slice. The tissue slice will melt and turn transparent on the MERSCOPE slide within 3-5 seconds. Allow the tissue slice to refreeze and turn white on the cold stage of cryostat before picking up and warming other empty regions again. Repeat this step till multiple tissue slices are collected. Transfer the sample into a petri dish.

    b. If collecting the tissue slices by warming up a cold MERSCOPE slide, place the MERSCOPE slide on the cold stage of a cryostat and pre-chill to -20˚C for 3-5 minutes first. Then use brushes to move and arrange the first tissue slice to the usable area of MERSCOPE slide. Carefully lift up the MERSCOPE slide by holding the edge of MERSCOPE slide using gloved finger, and then using warmed fingertip, rub against the back of MERSCOPE slide to heat up the tissue slice till it melts and becomes transparent. Then place the MERSCOPE slide on the cold stage, arrange the subsequent tissue slice, melt the tissue and let it refreeze, and repeat this step till multiple tissue slices are collected. Transfer the sample into a petri dish.

Option 3: Section multiple sections and collect them to a single MERSCOPE Slide in one step. This technique is similar to Option 2. It is useful to note that adhering all tissues at the same time may be more difficult than doing so sequentially but is more efficient if the user is well practiced.

Table 6. Pros and cons of Option 3.

Pros	Cons
Technique may provide more control to some users in tissue placement.	Not as efficient as other techniques.
Reduction of heat/freeze events on multiple tissues.	Static charge may destroy the tissue arrangement before the sections adheres to the slide.

Protocol:

1. Harvest the tissue sample and create an OCT embedded tissue block.
2. Tissue collection on MERSCOPE Slides:
    a. If collecting the tissue slice by dropping a MERSCOPE Slide to the tissue section, pre-warm the MERSCOPE slides at room temperature for 15 minutes first, and then section multiple tissue slices using a cryostat, and arrange them to the desired orientation using brushes. Carefully hold the MERSCOPE slide edge by gloved finger or forceps, and then gently drop the MERSCOPE slide on top of the multiple tissue slices. The tissue slice will melt and turn transparent on the MERSCOPE slide within 3-5 seconds. Allow the tissue slice to refreeze and turn white on the cold stage of cryostat before placing the sample into the petri dish.
    b. If collecting the tissue slices by warming up a cold MERSCOPE slide, place the MERSCOPE slide on the cold stage of a cryostat and pre-chill to -20˚C for 3-5 minutes first. Then cut multiple tissue slices and use brushes to move and arrange the first tissue slice to the usable area of MERSCOPE slide. Carefully lift up the MERSCOPE slide by holding the edge of MERSCOPE slide using gloved finger, and then using warmed fingertip, rub against the back of MERSCOPE slide to heat up the tissue slices until all slices melt and become transparent. Then place the MERSCOPE slide on the cold stage and let the sections refreeze before transferring the sample into a petri dish.

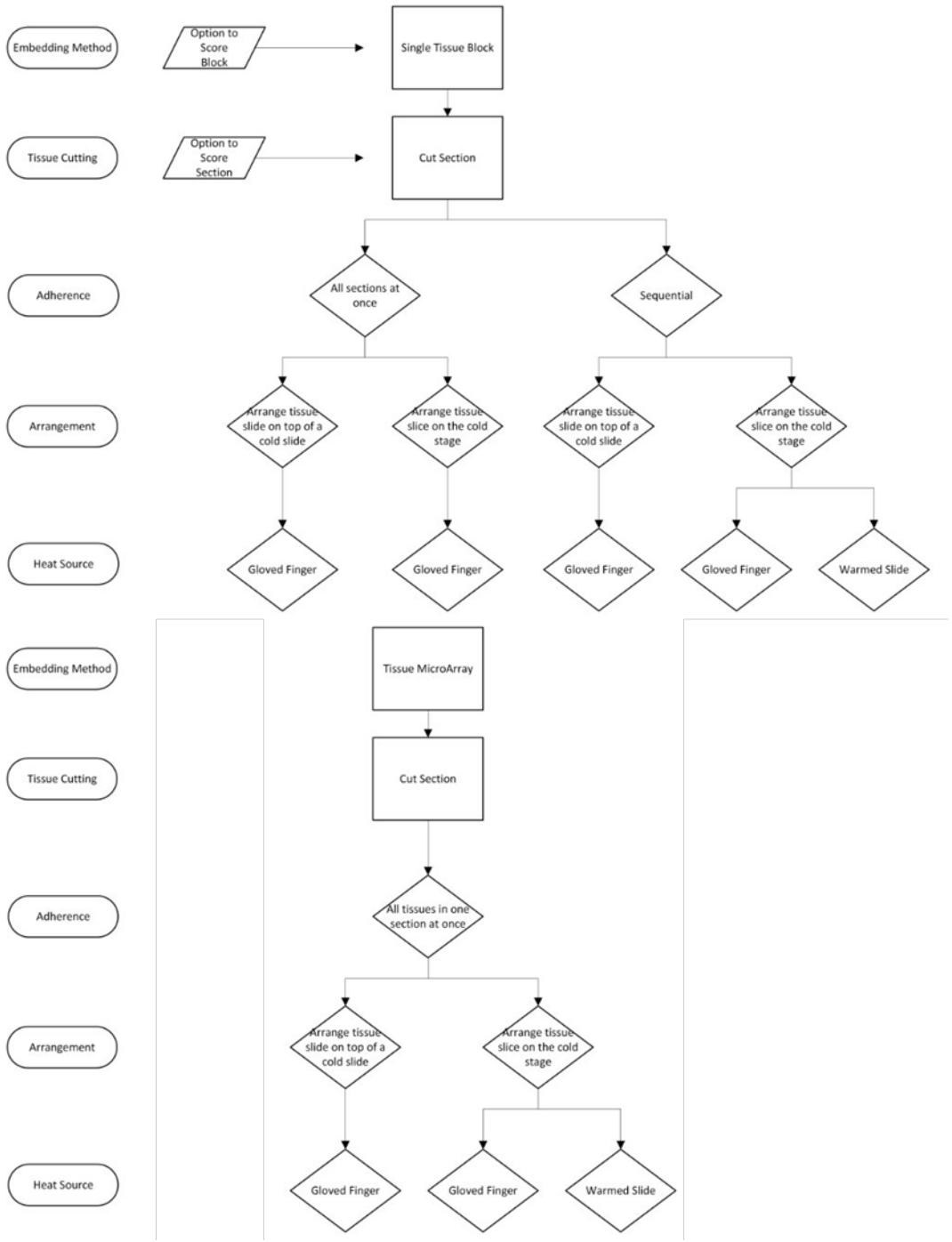


Figure 17. Choosing a method for placing multiple sections.

8.2 Tissue-Specific Sectioning Tips

Due to the properties associated with tissue type, different samples may have specific challenges during sectioning (Figure 14).

For example, fresh frozen adipose tissue is especially prone to processing issues as fat does not freeze in the same way as other solid tissues (Figure 14B). Temperatures sufficient for

sectioning standard solid tissue may be too warm to section fat or fatty tissues, causing high adiposity regions in a block of tissue to smear across the cutting blade. These partially destabilized areas of smeared fat can cause the blade to catch and rip the tissue section or cause the entire section to crumple up on the blade, making it difficult to acquire quality sections from fresh frozen tissue blocks with high adiposity (Figure 14B). A clean stage, sharp blade, continuous motion of the blade, and thicker sections may increase the likelihood of obtaining quality fatty tissue sections[9].

Bone or bony tissue may damage the blade as the tissue is cut (Figure 14A). It is preferred to perform decalcification prior to sectioning bone or bony tissue.

For tissue that contains small cells (embryonic tissue, lymph node, spleen, thymus, etc.), a 10 µm section may contain more than one layer of cells, making downstream cell segmentation for MERSCOPE measurement challenging. It is recommended to reduce the thickness when sectioning tissues of smaller cell sizes to ensure one layer of cell is sectioned in each slice.

Furthermore, the tissue section thickness may vary by a few microns due to the cryostat model used. If the actual tissue section thickness is thinner than the displayed thickness on the cryostat, the top of the tissue section will be imaged in earlier frames as MERSCOPE performs an optical z-scan from the bottom to the top of the sample. This may lead to a loss of RNA transcripts in higher z frames in MERSCOPE images. If this is observed, consider increasing the sectioning thickness on cryostat to ensure a cell is included in one section. The table below provides sectioning advice specific to certain tissues (Table 7).

Table 7. Tissue-specific sectioning advice for mouse and human tissues. Recommendations are provided for sectioning various difficult tissues.

Tissue	Advice
Liver	• Cuts at a higher temperature (−20°C to −13°C). • Usually requires surface warming of the tissue block.
Bone	• If considerable amounts of calcified bone are present (such as in normal cortical bone), do not attempt sectioning. • Fixed frozen or FFPE tissue is recommended as decalcification processes are likely to impact RNA quality. • To section tissue blocks containing decalcified bone, the blade should section into the bone across the longest axis.
Adipose	• It may be possible to section fresh or fixed frozen adipose-rich tissue, but it is necessary to adjust the cryostat temperature to below -20°C and optimize the temperature for sectioning the tissue block by trial and error. • FFPE-preserved samples are recommended to remedy challenges associated with sectioning fresh and fixed frozen adipose-rich tissue.
Gastrointestinal (GI)	• GI tissue may contain fat that can disrupt sectioning. If possible, orient the fat to the side during embedding and orientation. This should reduce the likelihood that the tissue of interest is affected by the fat.

	• GI samples are easily contaminated by RNase. Consider collecting the tissue in 1X PBS or harvesting medium supplemented with RNase inhibitor (4 mM ribonucleoside vanadyl complex) at 4°C for 30 minutes prior to embedding.
Breast	• Normal breast tissue is especially high in adipose content. Consider removing the fat from samples before freezing, and sectioning the sample at temperatures lower than -20°C. • As it is difficult to cut sections from frozen specimens with high adipose content, FFPE preservation is recommended.
Lymph Node	• If fat deposits surround the tissue, consider removing the fat from samples before freezing, and sectioning the sample at temperatures lower than -20°C. • As it is difficult to cut sections from frozen specimens with high adipose content, FFPE preservation is recommended. For tissue containing small cells, is recommended to reduce the thickness of tissue to <10 µm to ensure one layer of cell is sectioned in each slice.

8.3 Working with Samples Larger Than the MERSCOPE Imaging Area

Fresh and fixed frozen tissues should be trimmed to fit the imageable area of the MERSCOPE Slides prior to cryopreservation (Figure 1 and Figure 2). It is possible to proceed with a tissue sample area larger than the imageable area without trimming; in this case, the outer regions of the tissue will not be embedded in the gel during sample preparation and tissue outside of the imageable area will not be imaged.

If the tissue is too large to fit on the MERSCOPE Slide, the tissue block can be warmed up briefly, then the block can be cut into smaller sizes. These smaller blocks can be re-embedded into separate tissue blocks. It is important to bring tissue to the temperature of the cryostat before cutting to avoid fracturing and tissue damage. If the tissue sample in a tissue block is smaller than the imageable area, it is not necessary to trim off OCT around the embedded tissue.

8.4 Static Charge During Sectioning

MERSCOPE Slides have a positively charged surface which may cause tissue to be attracted to the slide before collection. Two options can help neutralize the static charge during sectioning:

Option 1: Spray gloves with 70% ethanol to neutralize the charge before collecting a tissue section. Ensure that 70% ethanol does not contact the MERSCOPE Slide.

Option 2: Place multiple frozen tissue slices on a chilled MERSCOPE Slide and then warm up the frozen sections with fingertips to facilitate tissue melting and adhesion. As the tissue section and MERSCOPE Slide are in direct contact in this procedure, tissue slices won't fly up in the air. Detailed procedures are described above in the "Placing multiple samples on one MERSCOPE Slide" section, Option 2 and 3.

8.5 Tissue Detachment on MERSCOPE Slides

It is critical to maintain section adhesion to MERSCOPE Slides to obtain accurate MERFISH measurements. To facilitate tissue adherence, the sectioned tissues are left on the MERSCOPE Slide in the cryostat briefly to allow the tissue section to refreeze and adhere to the slide (Figure 11).

Tissue must be melted on the MERSCOPE Slide and then refrozen for strong adhesion. If melting or re-freezing is not observed, tissue may easily detach at later steps. It is important to use a new blade to cut sections and utilize the brush to receive and flatten the tissue during cutting. Unevenly cut tissue sections are more likely to detach from the slide. It is possible to add weight or press down on the MERSCOPE Slide with a gloved finger to assist in tissue slice collection. Unfixed tissue slices should never be placed at -80°C or dry ice as the tissue section will fragment or detach after the sample is brought back at a higher temperature in fixatives.

The properties of certain tissues make them more vulnerable to tissue detachment during the MERSCOPE sample preparation workflow. Fresh and fixed frozen mouse and human lung tissues have holes due to alveoli, which is unavoidable and can sometimes lead to tissue detachment. Some fresh frozen tissues and all fixed frozen tissues embedded in OCT are prone to detach from the slide during sample preparation, as are adipose, other fatty tissues, tissues with high collagen content, skin tissue, and lung tissue (Table 8). When working with tissues prone to lifting, take special care to maintain cold temperatures to improve tissue adherence. Extended drying steps can also be included after section placement on the slide and after the post-fixation PBS washes as described in Section 7.6. Fresh and Fixed Frozen Tissue Sectioning Protocol.

Table 8. Tissues prone to detachment.

Species	Preservation Method	Tissue Type
Human	Fixed Frozen	All tissue types
Mouse	Fixed Frozen	All tissue types
Human	Fresh Frozen	Adipose, Breast, Fatty tumors
		Aorta
		Colon
		Decalcified bone
		Ovary
		Skin
		Synovium, Joint
	FFPE	Connective tissue
		Skin
		Adipose

8.6 Optimized Fresh Frozen Tissue Fixation After Sectioning Using the Sample Verification Kit

Most fresh frozen tissue sections should be fixed in 4% PFA and later permeabilized with 70% ethanol. Fixation with 4% PFA in PBS at room temperature for 15 minutes is sufficient for most tissue samples. Fixed frozen tissue sections on slides can be washed with 1XPBS first and then placed directly into 70% ethanol without the need for further fixation after sectioning. Since RNase is not active in 70% ethanol, the samples can be stored in 70% ethanol at 4°C for up to one month. Users may wish to use their own fixation and/or permeabilization conditions which should be evaluated using the MERSCOPE Sample Verification Kit (Human or Mouse).

While fixation is performed at room temperature for most samples, higher temperature fixation can help inactivate RNase faster and thus be used to better preserve RNA in fresh frozen samples. Briefly, tissue sections can be fixed at an elevated temperature at 37°C with 4% PFA for 1 hour or 47°C for 30-35 minutes (Section 7.6: Fresh and Fixed Frozen Tissue Sectioning Protocol). Note that fixation is more extensive with higher temperatures such as 47°C, which may cause increased crosslinking, difficulty in clearing the tissue, and small morphology deformations. If excessive crosslinking is observed at 47°C, consider using 37°C fixation with 4% PFA for 1 hour.

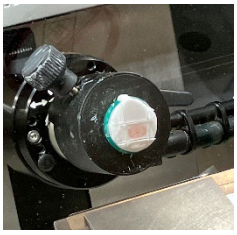
Different fixatives can be used to fix sectioned fresh frozen slices. Aldehydes such as PFA can be used for fixation as they are crosslinkers that create intramolecular bridges. Organic solvents such as methanol and ethanol can also be used for fixation. They do not cause crosslinking and thus RNA transcripts may be more accessible. However, they may cause tissue morphology distortion as they permeabilize the tissue by extracting lipids from the cell membrane. Therefore, organic solvents should not be used as fixation reagents if tissue morphology distortion is a concern. Fixation with ethanol and methanol is usually performed at −20°C to prevent enzyme-mediated tissue degradation (autolysis).

The MERSCOPE Sample Verification Kit (Human or Mouse) should be used to verify that sample preparation conditions result in adequate MERFISH imaging quality on the MERSCOPE Instrument.

8.7 Other Tips and Best Practices

8.7.1 Block Scoring

A method to ease the adherence of multiple tissues on a slide involves scoring the tissue block with a razor blade or similar sharp tool to cut away excess OCT. This produces a smaller section, and the user may find it easier to place multiple sections on a slide without overlapping OCT.



8.7.2 Tissue Scoring

A method to ease adherence of multiple sections on a slide involves scoring the section post cutting. Once the histologist sections the block, the surrounding OCT may be gently cut or pulled off the tissue using cold forceps or other similar tools.

9 RNA QUALITY VERIFICATION ON TISSUE SECTIONS

It is important to verify RNA quality before running MERFISH experiments. There are two methods to test RNA integrity, and these methods can be used together or sequentially to best understand the sample quality prior to starting the experiment.

9.1 RNA Quality Measurement

Before performing a MERFISH experiment, Vizgen recommends first measuring RIN values and DV200 values from a few sections to decide if the tissue block is suitable for MERSCOPE imaging or not. It is recommended to collect an additional 60-80 µm total (from either before or after the section of interest) for RNA extraction and quality measurement by RIN and DV200 measurement using a Bioanalyzer or TapeStation System.

Vizgen recommends selecting samples with DV200>30% for MERSCOPE measurement.

9.2 Performing smFISH Verification

Since different sample types may require optimization of certain steps or conditions during the sample preparation process, it is also important to check the tissue quality using the Vizgen Sample Verification Kit to directly evaluate the RNA quality in prepared tissue sections prior to running a MERFISH experiment. Species-specific Verification Kits can be designed in consultation with Vizgen. Since RIN and DV200 measurements require the extraction of RNA from many cells, some clinical samples may be precious and not readily allow such measurements; however, these precious samples should still be tested with the Sample Verification Kit before running on the MERSCOPE Platform. These reagent kits verify that the sample will result in adequate MERFISH imaging quality with the MERSCOPE Instrument.

Refer to the MERSCOPE User Guide: Sample Verification Kit (PN 91600004) for more information.

10 SAFETY CONSIDERATIONS

Liquid nitrogen is an ultra-cold liquid that can cause frostbite without adequate skin and eye protection. As liquid nitrogen boils into gas, it is an inhalation hazard because the gas

displaces oxygen in the local environment. In small rooms with inadequate ventilation, this can lead to serious health hazards and even asphyxiation.

Similarly, dry ice can melt and turn into carbon dioxide and is considered a serious hazard in a small space that is not well-ventilated.

Isopentane is flammable and can have negative health effects if inhaled; PFA is carcinogenic and is toxic upon skin contact or inhalation.

The blade in a cryostat is a laceration hazard. Since the operator's hands are inside the cabinet while sectioning, it is very important to take note of the blade's position at all times and remain aware of this hazard.

A facility's Bloodborne Pathogen program applies to cryosectioning, as fresh frozen tissue (especially human tissue) is considered potentially infectious. Until the sections are fixed in 4% PFA or any other fixative that is known to inactivate pathogens, all tissues and instruments (including the cryostat itself) are considered biohazards. All Kimwipes or paper towels used in the cryostat are to be disposed of as biohazardous waste. Since fresh frozen tissues are not fixed, any cuts to the skin that occur should be treated in accordance with the facility's Bloodborne Pathogen program.

Freeze sprays are not recommended for use inside the cryostat. These sprays can cause potentially infectious discarded sections and shavings to fly out of the cryostat and at the operator.

Operators should maintain proper posture while using the cryostat to minimize skeletomuscular issues. Also, the cold of the cryostat chamber can cause issues with hand movements. Frequent breaks are encouraged if cryosectioning for long periods of time.

11 SUPPORT

Preparing tissue specimens for MERFISH imaging requires care, skill, and sound procedures. This guide provides practical advice on best-practice techniques and ways to avoid common errors.

Please contact our team at support@vizgen.com for additional support.

Other Vizgen resources are available online at https://vizgen.com.

12 VIZGEN RESOURCES

- MERSCOPE User Guide: Fresh and Fixed Frozen Tissue Sample Preparation 91600002
- MERFISH 2.0® Sample Preparation User Guide for Sectioned Tissue Samples 91600132
- MERSCOPE User Guide: Sample Verification Kit 91600004

13 REFERENCES

1. Cunningham, C. (2023, July 26). Freezing Biological Samples. Leica Biosystems. https://www.leicabiosystems.com/us/knowledge-pathway/freezing-biological-samples/
2. Green, M.R. & Sambrook, J. (2019). How to Win the Battle with RNase. Cold Spring Harb Protoc. doi:10.1101/pdb.top101857

3. Klink, T.A., Woycechowsky, K.J., Taylor, K.M, & Raines, R.T. (2001). Contribution of disulfide bonds to the conformational stability and catalytic activity of ribonuclease A. European Journal of Biochemistry, 267(2): 566-572. https://doi.org/10.1046/j.1432-1327.2000.01037.x
4. Miyamoto, T., Okano, S., & Kasai, N. (2009). Irreversible thermoinactivation of ribonuclease-A by soft-hydrothermal processing. Biotechnology progress, 25(6), 1678–1685. https://doi.org/10.1002/btpr.267
5. Butler, A. E., Matveyenko, A. V., Kirakossian, D., Park, J., Gurlo, T., & Butler, P. C. (2016). Recovery of high-quality RNA from laser capture microdissected human and rodent pancreas. Journal of histotechnology, 39(2), 59–65. https://doi.org/10.1080/01478885.2015.1106073
6. Botling, J., Edlund, K., Segersten, U., Tahmasebpoor, S., Engström, M., Sundström, M., Malmström, P. U., & Micke, P. (2009). Impact of thawing on RNA integrity and gene expression analysis in fresh frozen tissue. Diagnostic molecular pathology: the American journal of surgical pathology, part B, 18(1), 44–52. https://doi.org/10.1097/PDM.0b013e3181857e92
7. Vincek, V., Nassiri, M., Knowles, J., Nadji, M., & Morales, A. R. (2003). Preservation of tissue RNA in normal saline. Laboratory investigation; a journal of technical methods and pathology, 83(1), 137–138. https://doi.org/10.1097/01.lab.0000047490.26282.cf
8. Peters, S.R. (2010). Embedding of tissue for frozen section. In S.R. Peters (Eds.), A practical guide to frozen section technique (pp 37-74). Springer.
9. Pathology Innovations. (2023, July 30). Frozen Section Technique III. https://www.pathologyinnovations.com/frozen-section-technique-3
10. Peters, S. R. (2023, July 30). A Method for Preparation of Frozen Sections. https://biomedizin.unibas.ch/fileadmin/user_upload/biomedizin/core_facilities/histology/Guides/Frozen_Section_Methods.pdf
11. Yeabyo, G. (2022). Factors that Impact Tissue Embedding. https://www.nsh.org/blogs/jemal-clarke/2022/11/05/factors-that-impact-tissue-embedding
12. Pathology Innovations. (2023, July 31). Frozen Section Technique II. https://www.pathologyinnovations.com/frozen-section-technique-2
