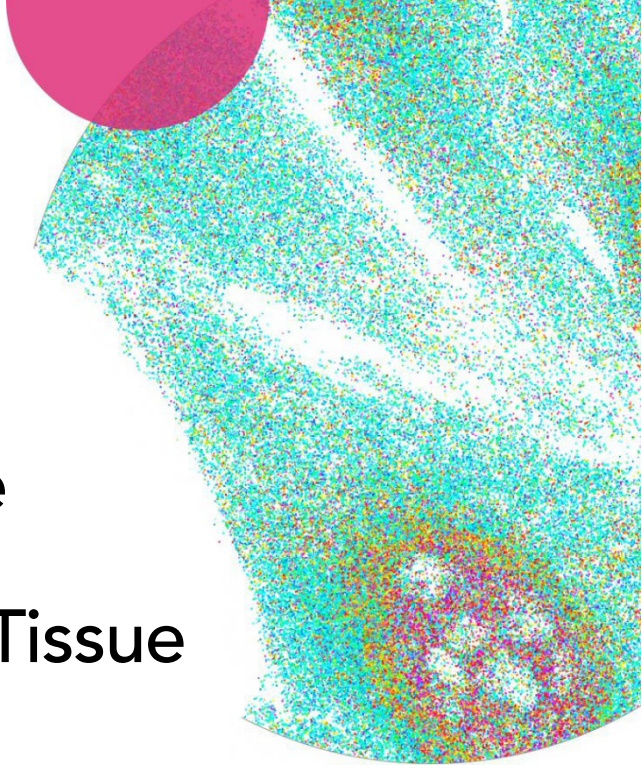
91600002 Rev B



MERSCOPE™ User Guide

Fresh and Fixed Frozen Tissue Sample Preparation

Vizgen™ Materials	
10500001	MERSCOPE Slide Box, 20 samples
10400012	MERSCOPE Sample Prep Kit, 20 samples
10400009	MERSCOPE Cell Boundary Staining Kit, 20 samples – Optional
10400001	MERSCOPE 140 Gene Panel, 20 samples
10400002	MERSCOPE 300 Gene Panel, 20 samples
10400003	MERSCOPE 500 Gene Panel, 20 samples

vizgen

NOTICES

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Other Vizgen references, available online at https://vizgen.com/

Vizgen document	Document number
MERSCOPE Instrument Site Preparation Guide	91500001
MERSCOPE Instrument User Guide	91600001
MERSCOPE Cultured Cells Sample Preparation User Guide	91600003
MERSCOPE Sample Verification Kit User Guide	91600004

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INTRODUCTION

MERFISH Technology

Multiplexed error-robust fluorescence in situ hybridization (MERFISH) is a spatially resolved single-cell transcriptome profiling technology. MERFISH combines the power of single-cell transcriptomics with spatial biology by directly visualizing and counting RNA transcripts from 100s to >10,000 genes in cells or tissue sections.

MERFISH advances the power of single molecule fluorescence in situ hybridization (smFISH) – the gold standard of RNA quantification – with error robust barcoding, combinatorial labeling, and sequential imaging to greatly expand the multiplexing capacity. MERFISH enables researchers to map the molecular, cellular, and functional composition of biological systems with spatial context.

The Vizgen MERSCOPE Platform Solution

The Vizgen MERSCOPE Platform Solution is comprised of the MERSCOPE Gene Panel Design Software, MERSCOPE reagent kits, the MERSCOPE Instrument, the MERSCOPE Analysis Computer, and the MERSCOPE Vizualizer software to streamline the acquisition of high quality MERFISH data and data interpretation.

Reagent kits facilitate sample preparation. The automated MERSCOPE Instrument integrates high-resolution imaging and fluidics to automatically acquire a full MERFISH dataset. The MERSCOPE Vizualizer software automates image processing and offers interactive visualization tools to explore the MERFISH data.

The MERSCOPE Workflow

Design your gene panel. The intuitive MERSCOPE Gene Panel Design Software makes recommendations on your gene panel for optimal performance in a MERFISH measurement. For example, the software highlights genes that are too short or whose abundance may be too high and could cause optical crowding artifacts. Vizgen is currently offering gene panels for up to 140 genes, up to 300 genes, and up to 500 genes.

Order your MERSCOPE Gene Panel. MERSCOPE Gene Panels are delivered in a ready-to-use format. Once the gene panel is finalized, users will be able to download their panel-specific MERSCOPE Codebook to the MERSCOPE Instrument.

Prepare your sample. Vizgen's sample preparation user guides step you through sample preparation on MERSCOPE Slides.

Load and run the MERSCOPE Instrument. The MERSCOPE Slide is assembled into the MERSCOPE Flow Chamber and then loaded into the instrument along with a MERSCOPE Imaging Cartridge. Users define regions of interest on the MERSCOPE Slide and initiate the fully automated instrument run.

Data Processing and Visualization. The MERSCOPE Instrument Software (in combination with the MERSCOPE Analysis Computer) automatically processes the raw images to output spatial genomics measurements in a format ready for immediate downstream analysis. The output includes the list of all detected transcripts and their spatial locations in three dimensions (CSV files), the mosaic images (TIFF), output from the cell segmentation analysis: the transcripts per cell matrix (CSV), the cell metadata (CSV), the cell boundaries (HDF5), and a binary for use with the MERSCOPE Vizualizer software. The MERSCOPE Platform Solution includes the MERSCOPE Vizualizer software for visualizing and analyzing the data. The output files are also compatible with open-source tools for single cell and spatial analysis.

Broad Application

The MERSCOPE Platform Solution has broad application in both fundamental biology and medicine – from basic science, to drug discovery, to clinical pathology. Find out more at https://vizgen.com/applications/.

Oncology | Immunology | Neuroscience | Infectious Disease | Developmental Biology & Regenerative Medicine

SAMPLE PREPARATION OVERVIEW

This user guide **is applicable to fresh frozen and paraformaldehyde (PFA)-fixed frozen tissue**. It is not applicable to formalin-fixed paraffin-embedded (FFPE) tissue. It is also not applicable to cultured cells. Refer to the applicable user guide for other sample types. Vizgen supports mouse and human tissue samples only.

I. Tissue Sectioning, Fixation, Autofluorescence Quenching (If Necessary), Permeabilization

The tissue of interest is sectioned and adhered to a MERSCOPE Slide. MERSCOPE Slides for fresh frozen and fixed frozen samples are supplied with fluorescent fiducials for subsequent imaging. If not already fixed in PFA, the tissue section is fixed with a fixation buffer and made permeable to the hybridization probes by overnight incubation in ethanol. Users may utilize their own fixation and permeabilization protocols in this step and use the MERSCOPE Sample Verification Kit to verify that the sample preparation conditions are compatible with MERFISH imaging with the MERSCOPE Instrument.

Autofluorescence background often occurs in aged brain, heart, intestine, and liver tissues and is often caused by lipofuscin or lipofuscin-like pigments. Human tissues are more likely to have autofluorescence background than mouse tissues. Users may use the MERSCOPE Sample Verification Kit to evaluate whether autofluorescence interferes with MERFISH imaging with the MERSCOPE Instrument. If autofluorescence is prevalent, the sample tissue should be placed under the MERSCOPE Photobleacher to remove the background signal. Autofluorescence quenching can be carried out at the same time as permeabilization (i.e., following tissue fixation) or when the sample is in Clearing Solution.

II. Cell Boundary Staining (If Necessary)

Cell boundary staining conveniently marks cell boundaries to enable individual cell analysis. If cells are adequately dispersed in a sample (e.g., in central nervous system tissue) it may not be necessary to do this step.

III. Encoding Probe Hybridization and IV. Post Encoding Probe Hybridization Wash

Formamide Wash Buffer denatures the RNA to allow encoding probe binding. The custom encoding probes hybridize to the sample. Adequate washing minimizes background.

V. Gel Embedding

Gel embedding immobilizes the RNA (with bound encoding probes) and creates a protective layer so the RNA species cannot escape in subsequent steps.

VI. Clearing

Clearing removes all tissue components other than RNA and DNA and is critical to minimize the autofluorescence background and thereby maximize signal. The optimal clearing protocol depends on the tissue sample type. Users may use the MERSCOPE Sample

Verification Kit to evaluate if their tissue is resistant to clearing or not. Tissues resistant to clearing may require digestion and/or higher-temperature clearing.

Mouse tissues are **generally not resistant** to clearing. The **exception** is mouse skin tissue, which is generally resistant to clearing.

Human tissues are **generally resistant** to clearing. The **exceptions** are human liver, heart, spinal cord, and brain tissues, which are generally not resistant to clearing.

Three clearing protocols are provided for (i) non-resistant fresh frozen tissue, (ii) non-resistant fixed frozen tissue, or (iii) resistant fresh frozen or fixed frozen tissue.

Next Steps

Refer to the *MERSCOPE Instrument User Guide* for next steps. In short, the sample is stained with DAPI and PolyT stain and then MERSCOPE Slide with prepared sample is assembled into the MERSCOPE Flow Chamber and inserted in the instrument. The MERSCOPE Imaging Cartridge is loaded into the instrument and the fully automated instrument run is initiated.

TIMELINE

Day	Step	Bench time	Incubation time	Stopping points / storage
Day 1	I. Tissue Sectioning and Fixation	1.5 h	-	-
	I. Autofluorescence Quenching (If Necessary)		3 h	-
	I. Permeabilization	-	Overnight	70% EtOH at 4°C Up to 1 month
Day 2	II. Cell Boundary Staining (If Necessary)	0.5 h	3 x 1 h	-
	III. Encoding Probe Hybridization	0.5 h	36 - 48 h	-
Day 4	IV. Post Encoding Probe Hybridization Wash	0.25 h	2 x 0.5 h	-
	V. Gel Embedding	0.5 h	1.5 h	-
	VI. Digestion (If Necessary)	0.25 h	1 - 6 h*	-
	VI. Clearing	0.25 h	24 h+**	Clearing solution at 37°C Up to 7 days
Refer to the MERSCOPE Instrument User Guide for next steps				

A total of 5 days for sample preparation is based on the minimum times for Encoding Probe Hybridization and Clearing. If extra time needed for these steps, total days increase accordingly.

*Time depends on resistance to clearing. ** Clearing incubation time depends on resistance to clearing.

TECHNICAL TIPS

Experimental Planning

The MERSCOPE Instrument analyzes one sample at a time and imaging takes 0.5 – 1.5 days.

Sample preparation can be performed in batches and samples can be stored in Clearing Buffer at 37°C for up to 7 days.

Minimize freeze-thaw cycles for reagents/kits stored at –20°C.

Refer to the TIMELINE for stopping points and sample storage conditions.

It is recommended to label samples on the bottom of the petri dish for future identification.

The Clearing step depends on tissue type and fixation. Select step VI Clearing based on the sample tissue type and fixation.

When approaching imaging, ensure the MERSCOPE Imaging Cartridge is thawed and the appropriate MERSCOPE Codebooks are available. Refer to the *MERSCOPE Instrument User Guide* for more information.

Sample Quality

It is recommended to evaluate RNA integrity/quality before doing experiments (e.g., using an Agilent TapeStation System or Agilent Bioanalyzer System).

RIN >7	Optimal sample quality, the higher the better
RIN 5-7	May be used, but detection efficiency may be compromised
RIN <5	Unacceptable sample quality

RIN, RNA Integrity Number

RNase Decontamination

MERFISH measurements are sensitive to RNase activity. RNase contamination of any materials or reagents will degrade data quality.

Samples should be prepared in an area decontaminated with RNaseZap solution.

It is recommended to use RNase-free disposables, e.g., RNAse-free media bottles (VWR PN 82051-594) for preparing buffers.

MERSCOPE Slide Handling

MERSCOPE Slides are fragile, handle with care. MERSCOPE Slides may be handled with tweezers or, if handling with gloved fingers, hold the edges to minimize the potential of touching the sample.

Maintaining Humidity During Long Incubations

Encoding Probe Hybridization and Clearing steps involve long incubation times. It is important that the samples do not dry out during incubation.

If a humidified incubator is not available, fill a small petri dish with nuclease-free water and place it together with the sample (covered in its 60-mm petri dish) within a 150-mm petri dish.

Safety and Hazardous Steps

Safe laboratory practices should be followed at all times.

Formamide Wash Buffer is hazardous. Fixation buffers may contain hazardous materials. Perform steps using these materials in a fume hood. The reagents used in gel embedding also contain hazardous materials.

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

FROZEN TISSUE PREPARATION AND SECTIONING TIPS

Frozen Tissue Care

MERFISH measurements are sensitive to RNA degradation. Fresh frozen tissue minimizes RNA degradation and is the recommended sample type for MERFISH imaging with the MERSCOPE Instrument. Fixed frozen tissue preserves cell morphology and is recommended when the integrity of cell morphology is critical.

Both fresh and fixed frozen tissue blocks should be embedded with optimal cutting temperature (OCT) compound for long term storage at −80°C to prevent tissue drying.

Minimize the number of freeze-thaw cycles of tissue.

Vizgen recommends that users source laboratory supplies and materials for handling fresh and fixed frozen samples from vendors such as Electron Microscopy Sciences (e.g., https://www.emsdiasum.com/microscopy/products/histology/tissue-tek.aspx)

Fresh Frozen Tissue Preparation

There are 2 options to prepare fresh frozen tissue blocks. The speed of freezing is proportional to the size of the tissue block. It is generally recommended to limit the size of tissue blocks to less than 1.5 cm^3.

Option 1:

a. Prepare an isopentane and liquid nitrogen bath.
b. Immerse the tissue into chilled isopentane until completely frozen for at least 1 min.
c. Transfer the tissue to a prechilled tissue embedding plastic mold on dry ice.
d. Add prechilled OCT (4°C) to embed the tissue. Wait until the OCT completely solidifies and turns white.

Option 2:

a. Prepare an isopentane and liquid nitrogen bath.
b. Place the tissue in a tissue embedding plastic mold.
c. Pour prechilled OCT (4°C) into a tissue embedding plastic mold until OCT completely covers the tissue.
d. Using forceps, transfer the plastic mold into the isopentane and liquid nitrogen bath **without submerging**. Wait until the OCT completely solidifies and turns white.

Fixed Frozen Tissue Preparation

The recommended method to prepare fixed frozen tissue blocks:

a. If possible, first perfuse the animal or tissue with freshly prepared 4% PFA in 1X PBS.
b. Dissect the tissue and place the tissue into freshly prepared 4% PFA for 16-24 h at 4°C.
c. Immerse the tissue in 15% sucrose in 1X PBS at 4°C for 6-12 h, and then transfer to 30% sucrose in 1X PBS at 4°C until the tissue sinks to the bottom of the container.

d. Freeze the tissue in OCT with dry ice or an isopentane and liquid nitrogen bath. Wait until the OCT completely solidifies and turns white.

Frozen Tissue Sectioning

Place the OCT compound-embedded tissue block into the cryotome and allow it to sit at −20°C for at least 30 min prior to sectioning.

Use new blades when sectioning tissue.

Trim the bulk of the tissue using one part of the cryotome blade.

The surface of fresh frozen and fixed frozen tissue may have lower RNA quality. When a frozen OCT-embedded tissue block is trimmed to expose the tissue of interest, discard at least the first 50 µm slices of the tissue prior to sectioning for MERSCOPE samples.

When approaching the tissue of interest, move the blade over to use a previously unused section of the blade for sample sectioning.

It is recommended to collect an additional 6-10 tissue sections (from either side of the section of interest) for RNA extraction and RNA quality measurement.

At the end of cyrosectioning, add a few drops of OCT compound onto the top of the exposed tissue to protect the tissue from drying, and store the used samples at −80°C for future use. If the tissue is to be reused, again discard the first 30-50 µm slices of the tissue.

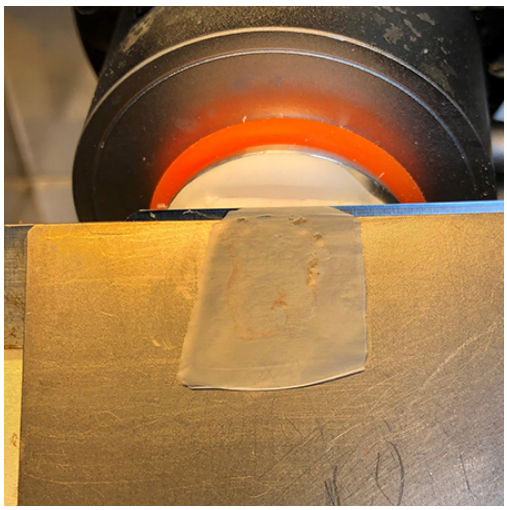
Tissue Adherence to a MERSCOPE Slide

Spraying gloves with 70% ethanol can help prevent additional tissue sections sticking to the MERSCOPE Slide.

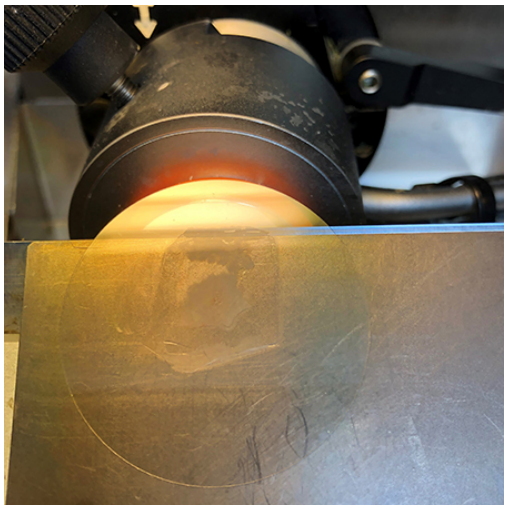
Keep the empty labeled petri dish in the cryostat or at −20°C during sectioning to prevent the tissue section from melting.

After carefully lowering the MERSCOPE Slide onto the tissue section, leave the MERSCOPE Slide on the tissue in the cryostat for ~5 sec to allow the tissue section to refreeze and adhere to the MERSCOPE Slide. The tissue section will turn white as it refreezes – OCT compound is white when frozen.

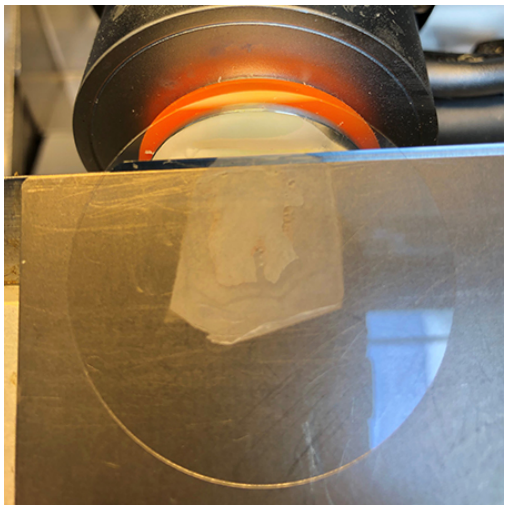
Place the MERSCOPE Slide into a dry petri dish (tissue facing up) and leave at −20°C for 5 min to allow the tissue section to adhere.



Tissue section immediately after sectioning.



Tissue section ~1 sec after lowering the MERSCOPE Slide onto the tissue section.



Tissue section ~3 sec after lowering the MERSCOPE Slide onto the tissue section.

The OCT compound becomes white as the tissue refreezes.

Leave the MERSCOPE Slide with the tissue in the cryostat for ~5 sec to allow the tissue section to refreeze and adhere to the MERSCOPE Slide.

MATERIALS

Vizgen Materials

MERSCOPE Slide Box, 20 samples	10500001	Storage
MERSCOPE Slide, 20 x 1 sample	20400001	–20°C, horizontally

MERSCOPE Sample Prep Kit, 20 samples	10400012	Storage
Sample Prep Wash Buffer	20300001	4°C
Formamide Wash Buffer	20300002	4°C, protected from light
Gel Embedding Premix	20300004	4°C, protected from light
Gel Coverslip	20400003	Room temperature
Clearing Premix	20300003	4°C
Digestion Premix, 5 x 4 samples*	20300005	–20°C upon receipt
*Minimize freeze-thaw cycles. If routinely doing fewer than 4 samples, aliquot a tube upon first use. Sample Prep Wash Buffer and Formamide Wash Buffer are used in the imaging and verification workflows.		

MERSCOPE 140 Gene Panel, 20 samples	10400001	Storage
MERSCOPE 140 Gene Panel Mix, 5 x 4 samples	20300006	–20°C

MERSCOPE 300 Gene Panel, 20 samples	10400002	Storage
MERSCOPE 300 Gene Panel Mix, 5 x 4 samples	20300007	–20°C

MERSCOPE 500 Gene Panel, 20 samples	10400003	Storage
MERSCOPE 500 Gene Panel Mix, 5 x 4 samples	20300008	–20°C

MERSCOPE Cell Boundary Staining Kit, 20 samples	10400009	Storage
Cell Boundary Blocking Buffer Premix, 4 x 5 samples*	20300012	–20°C
Cell Boundary Primary Staining Mix*	20300010	–20°C
Cell Boundary Secondary Staining Mix*	20300011	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Photobleacher	10100003	Supplied with the MERSCOPE Instrument

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

Required Materials and Recommended Suppliers

Item	Vendor	Part number
Buffers and additives		
32% Paraformaldehyde (Formaldehyde) Solution	EMS	15714
Ammonium Persulfate *Refresh stock every 6 months*	Millipore-Sigma	09913-100G
Ethyl Alcohol, Pure (200 proof)	Millipore-Sigma	E7023-6X500ML
N,N,N′,N'-Tetramethylethylenediamine (TEMED) *Refresh stock every 6 months*	Millipore-Sigma	T7024-25ML
RNase Inhibitor, Murine	NEB	M0314L
Proteinase K, Molecular Biology Grade	NEB	P8107S
Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher	AM9625
UltraPure DNase/RNase-free Distilled Water	Thermo Fisher	10977015
Solutions, consumables & small laboratory equipment		
RNaseZap RNase Decontamination Solution	Thermo Fisher	AM9782
Petri Dish, 60 x 15 mm, Sterile	VWR	25382-687
Petri Dish, 150 x 15 mm	VWR	75799-948
Gel Slick Solution	VWR	12001-812
Parafilm M	VWR	102091-164
Hobby Blades *2x are provided with the MERSCOPE Instrument*	VWR	80094-372 and 103301-802
High Precision Tweezers *1x are provided with the MERSCOPE Instrument*	Techni-Tool	758TW462
Serrated Tweezers *1x are provided with the MERSCOPE Instrument*	Techni-Tool	758TW450
Cleaning tissue (Kimwipe or similar)		

EMS, Electron Microscopy Sciences. NEB, New England BioLabs.

General Laboratory Equipment

- Fume hood
- 47°C incubator in a fume hood (e.g., VWR PN 10055-006)
- Cell culture hood with aspirator
- 37°C cell culture incubator
- Benchtop centrifuge
- Benchtop (vacuum) aspirator
- Rocker
- Analytical balance
- Cryotome
- Water bath (e.g., VWR 76308-896)*
- Tube/bottle weight (e.g., VWR 47748-174)
- Benchtop cooler (e.g., VWR 414004-286)

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

Additional Buffer Recipes

These buffers are not provided in Vizgen kits.

Commonly used buffers – make with nuclease-free water
70% Ethanol
1X PBS

Used in Step I and Step II (when included) – 5 mL per sample needed each time			
Fixation Buffer*	1 sample	5 samples	10 samples
10X PBS	0.56 mL	2.8 mL	5.6 mL
32% paraformaldehyde (formaldehyde) solution	0.7 mL	3.5 mL	7 mL
Nuclease-free water	4.34 mL	21.7 mL	43.4 mL

*Make fresh every time used

Used in Step V – 25 µL per sample needed	Prepare fresh aliquots every time*		
10% w/v Ammonium Persulfate Solution	1 sample	5 samples	10 samples
Ammonium persulfate**	100 mg	100 mg	100 mg
Nuclease-free water	1 mL	1 mL	1 mL

*Only 25 µL per sample is needed. Adjust volume of nuclease-free water to mass of ammonium persulfate weighed out in each case to obtain 10% w/v solution.

**Discard any ammonium persulfate that is more than 6 months old.

SAMPLE PREPARATION PROTOCOL

The protocol considers each MERSCOPE Slide a sample.

Each sample is prepared in a separate petri dish.

Incubations are stationary and at room temperature unless stated otherwise. 5-mL buffer and reagent volumes are sufficient to cover a MERSCOPE Slide in a 60-mm petri dish.

I. Tissue Sectioning, Fixation, Permeabilization

MERSCOPE Slides (PN 20400001) are stored at −20°C. Warm up at room temperature for 15 min before use. Unused MERSCOPE Slides should be stored at −20°C.

Refer to FROZEN TISSUE PREPARATION AND SECTIONING TIPS for best practices.

1. Place the optimal cutting temperature (OCT) compound-embedded tissue block into the cryotome, and allow it to sit at −20°C for at least 30 min.
2. Trim the OCT-embedded tissue block until the desired tissue region is exposed.
3. Cut a **10 µm** section from the OCT-embedded tissue block.
4. Ensure the tissue section is flat. Carefully mount the tissue section into the center of the MERSCOPE Slide by carefully lowering the MERSCOPE Slide onto the tissue section using gloved fingers.
5. Place the MERSCOPE Slide into a dry 60-mm petri dish (tissue facing up) and place at −20°C for 5 min to allow the tissue section to adhere.
6. **Fresh frozen tissue**: In a fume hood, add **5 mL** fixation buffer and incubate at room temperature for 15 min. **Fixed frozen tissue**: Skip this step.
7. Wash **3x** with **5 mL** 1X PBS, incubate 5 min each wash.
8. Add **5 mL** 70% ethanol, seal the petri dish with parafilm and place at 4°C overnight to permeabilize the tissue.

The sample can be stored in 70% ethanol in a labeled 60-mm petri dish, sealed with parafilm, at 4°C for up to 1 month.

***IF** autofluorescence quenching is necessary, this can be done prior to placing the petri dish at 4°C overnight. Autofluorescence quenching may also be performed when the sample is stored in Clearing Solution.*

I. Autofluorescence Quenching (If Necessary)

> ***ENSURE*** *the petri dish is sealed with parafilm prior to autofluorescence quenching, otherwise the ethanol will evaporate under the MERSCOPE Photobleacher.*

1. Place the parafilm-sealed petri dish under the MERSCOPE Photobleacher (PN 10100003). **ENSURE** there are no labels/writing/other items on the lid that may block the light.
2. Turn on the MERSCOPE Photobleacher and leave at room temperature for at least 3 h.

> *The sample can be stored in 70% ethanol in a labeled 60-mm petri dish, sealed with parafilm, at 4°C for up to 1 month.*
>
> *Autofluorescence quenching may also be performed when the sample is stored in Clearing Solution.*

II. Cell Boundary Staining (If Necessary)

If the sample does not require cell boundary staining, proceed directly to step III. Encoding Probe Hybridization.

Maintain Cell Boundary Primary Staining Mix, Cell Boundary Secondary Staining Mix, and RNase inhibitor in a benchtop cooler until use.

Thaw Cell Boundary Blocking Buffer Premix. Ensure fully thawed and mixed, and spin down using a benchtop centrifuge before use.

Return unused reagents to −20°C storage but minimize freeze-thaw cycles.

1. Aspirate the 70% ethanol. Add **5 mL** 1X PBS.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Cell Boundary Blocking Buffer Premix (PN 20300012)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL

3. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the tissue section.
4. Add **100 µL** Blocking Solution onto the center of the tissue section. Use scissors to cut a piece of parafilm 2×2 cm. Use tweezers to peel off the parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

The parafilm should fit within the MERSCOPE Slide, otherwise the Blocking Solution may wick away into the petri dish.

5. Incubate at room temperature for 1 h.
6. Prepare Primary Staining Solution:

Primary Staining Solution	1 sample	5 samples	10 samples
Cell Boundary Blocking Buffer Premix (PN 20300012)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL
Cell Boundary Primary Staining Mix (PN 20300010)	1 µL	5 µL	10 µL

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE Slide, leaving just enough liquid to cover the tissue section.

9. Add **100 µL** Primary Staining Solution onto the center of the tissue section. Use scissors to cut a piece of parafilm 2×2 cm. Use tweezers to peel off the parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

   The parafilm should fit within the MERSCOPE Slide, otherwise the Primary Staining Solution may wick away into the petri dish.

10. Incubate at room temperature for 1 h.
11. Use tweezers to remove the parafilm.
12. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.
13. Prepare Secondary Staining Solution:

Secondary Staining Solution	1 sample	5 samples	10 samples
Cell Boundary Blocking Buffer Premix (PN 20300012)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL
Cell Boundary Secondary Staining Mix (PN 20300011)	3 µL	15 µL	30 µL

14. Aspirate the 1X PBS to dry the MERSCOPE Slide, leaving just enough liquid to cover the tissue section.
15. Add **100 µL** Secondary Staining Solution onto the center of the tissue section. Use scissors to cut a piece of parafilm 2×2 cm. Use tweezers to peel off the parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.

    The parafilm should fit within the MERSCOPE Slide, otherwise the Secondary Staining Solution may wick away into the petri dish.

16. Incubate at room temperature for 1 h.
17. Use tweezers to remove the parafilm.
18. Wash **3x** with **5 mL** 1X PBS, incubate 5 min on a rocker each wash.
19. Aspirate the 1X PBS. In a fume hood, add **5 mL** fixation buffer to fix the stained tissue section at room temperature for 15 min.
20. Wash **2x** with **5 mL** 1X PBS, incubate 5 min each wash.
21. Proceed immediately to the next step.

III. Encoding Probe Hybridization

> *Maintain the applicable MERSCOPE Gene Panel Mix in a benchtop cooler until ready for use. Thaw and spin down using a benchtop centrifuge before use.*
>
> *Formamide Wash Buffer is hazardous. Perform these steps in a fume hood.*
>
> *Return unused reagents to −20°C storage but minimize freeze-thaw cycles.*

1. Aspirate the 1X PBS (if continuing from cell boundary staining) or 70% ethanol (if continuing from permeabilization) and wash **1x** with **5 mL** Sample Prep Wash Buffer (PN 20300001).
2. Add **5 mL** Formamide Wash Buffer (PN 20300002), incubate at 37°C for 30 min in an incubator.
3. First aspirate the Formamide Wash Buffer to dry the region of MERSCOPE Slide that does not have tissue section. Then carefully aspirate around the tissue section to remove extra Formamide Wash Buffer without touching the tissue section. The tissue section should not be completely dry for more than 1 min.

> *Aspirate all the residual solution without disrupting the tissue section to avoid diluting the MERSCOPE Gene Panel Mix.*

4. Add **50 µL** MERSCOPE Gene Panel Mix onto the center of the tissue section. Use scissors to cut a piece of parafilm 2×2 cm. Use tweezers to peel off the parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.
5. If the MERSCOPE Gene Panel Mix is not spread across the tissue section, lift and then lower the parafilm with tweezers until the MERSCOPE Gene Panel Mix is spread across the tissue section.

> *The parafilm should fit within the MERSCOPE Slide, otherwise the MERSCOPE Gene Panel Mix may wick away into the petri dish.*

6. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
7. Place in a humidified 37°C cell culture incubator for at least 36 h and a maximum of 48 h. **DO NOT** let the sample dry out.

> *If a humidified incubator is not available, fill a small petri dish with nuclease-free water and place it together with the sample (covered in its 60-mm petri dish) within a 150-mm petri dish.*

IV. Post Encoding Probe Hybridization Wash

Formamide Wash Buffer is hazardous. Perform these steps in a fume hood.

1. Remove the parafilm and add **5 mL** Formamide Wash Buffer (PN 20300002).
2. Incubate at 47°C for 30 min in a mini-incubator in a fume hood.
3. Aspirate the Formamide Wash Buffer. Add **5 mL** Formamide Wash Buffer.
4. Incubate at 47°C for 30 min in a mini-incubator in a fume hood.
5. Wash **1x** with **5 mL** Sample Prep Wash Buffer (PN 20300001), incubate 2 min.
6. Proceed immediately to the next step.

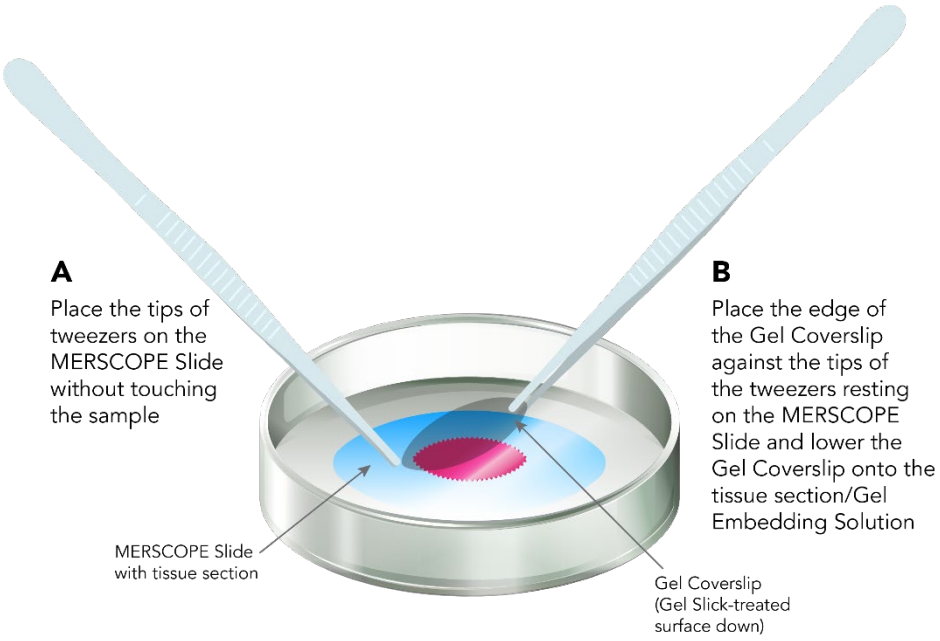
V. Gel Embedding

Gel embedding reagents contain hazardous materials.

1. Clean a Gel Coverslip (PN 20400003) by spraying with RNaseZap solution and wiping with a Kimwipe, followed by spraying 70% ethanol and wiping with a Kimwipe.
2. Add **50 µL** Gel Slick Solution onto the Gel Coverslip, wipe gently with a Kimwipe to spread the Gel Slick.
3. Prepare Gel Embedding Solution:

Gel Embedding Solution	1 sample	5 samples	10 samples
Gel Embedding Premix (PN 20300004)	5 mL	25 mL	50 mL
10% w/v ammonium persulfate solution	25 µL	125 µL	250 µL
N,N,N′,N'-tetramethylethylenediamine	2.5 µL	12.5 µL	25 µL

4. Aspirate the Sample Prep Wash Buffer. **Retain 100 µL** Gel Embedding Solution in a small tube. Add **the remainder of the 5 mL** Gel Embedding Solution, incubate at room temperature for 1 min.
5. Using a pipette, transfer the majority of the Gel Embedding Solution to a waste tube (to monitor the gel formation).
6. Aspirate to dry the MERSCOPE Slide, leaving just enough liquid to cover the tissue section.
7. Add **50 µL** of the retained Gel Embedding Solution on the tissue section.
8. Place the tips of one pair of tweezers on an area of the MERSCOPE Slide without touching the tissue section. Use tweezers to pick up the 20-mm Gel Slick-treated Gel Coverslip. With the Gel Slick-treated side **facing down** toward the tissue, place the edge of the Gel Coverslip against the tweezer tips resting on the MERSCOPE Slide, creating stability, and slowly lower the Gel Coverslip onto the tissue section to spread the Gel Embedding Solution. If needed, adjust the Gel Coverslip so it is positioned in the center of the MERSCOPE Slide. Gently press the Gel Coverslip to squeeze out excess Gel Embedding Solution, and remove the extra Gel Embedding Solution by aspiration.



Air bubbles in the gel solution inhibit gel polymerization. If a bubble forms, lift and then lower the Gel Coverslip to help the air bubbles escape.

Squeeze the Gel Coverslip gently to remove the Gel Embedding Solution that seeps out and leaves only a thin layer of gel between the Gel Coverslip and the MERSCOPE Slide. Avoid squeezing the Gel Coverslip too hard as it may damage the sample and result in gel that is too thin/no gel.

9. Incubate at room temperature for 1.5 h.

Monitor the Gel Embedding process in the waste tube. Gel starts to form within 1 h.

Repeat the Gel Embedding process from step 1 if:

- *no gel forms.*
- *it is not possible to remove the air bubbles. Air bubbles result in an empty area in the gel.*
- *too much Gel Embedding Solution is squeezed out and the gel is too thin and not visible.*

It is not necessary to remove the gel prior to repeating the Gel Embedding process.

10. Ensure **eye protection** is worn during this step. Gently lift the 20-mm Gel Slick-treated Gel Coverslip with the sharp tip of a Hobby Blade and discard the Gel Coverslip appropriately.
11. Proceed immediately to the next appropriate step for the tissue sample/type of fixation.

Choose *step* VI based on *tissue and fixation type. Refer to VI. Clearing in the SAMPLE PREPARATION OVERVIEW for more information.*

VI. Clearing – Non-resistant Fresh Frozen Tissue

1. Warm Clearing Premix (PN 20300003) at 37°C for 30 min before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm until the solution becomes clear. Prepare Clearing Solution:

Clearing Solution	1 sample	5 samples	10 samples
Clearing Premix (PN 20300003)	5 mL	25 mL	50 mL
Proteinase K	50 µL	250 µL	500 µL

2. Add **5 mL** Clearing Solution.
3. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
4. Seal the petri dish with parafilm and place in a humidified 37°C cell culture incubator for 24 h or until the tissue section becomes transparent.

If the tissue is not transparent after 24 h, consider transferring the petri dish to 47°C and incubate for 24 h.

DO NOT *incubate at 47°C >24 h otherwise the RNA will begin to degrade.*

If the tissue is still not transparent, transfer the petri dish back to 37°C until the tissue has cleared.

SAMPLES CAN BE STORED IN CLEARING SOLUTION AT ***37°C*** *FOR UP TO* ***7 DAYS***

REPLENISH *THE CLEARING SOLUTION* ***AFTER 4 DAYS*** *IN CLEARING SOLUTION*

REFER TO THE MERSCOPE INSTRUMENT USER GUIDE FOR NEXT STEPS

Choose *step* VI based on *tissue and fixation type. Refer to* *VI. Clearing* *in the* *SAMPLE PREPARATION OVERVIEW* *for more information.*

VI. Clearing – Non-resistant Fixed Frozen Tissue

1. Warm Clearing Premix (PN 20300003) at 37°C for 30 min before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm until the solution becomes clear. Prepare Clearing Solution:

Clearing Solution	1 sample	5 samples	10 samples
Clearing Premix (PN 20300003)	5 mL	25 mL	50 mL
Proteinase K	50 µL	250 µL	500 µL

2. Add **5 mL** Clearing Solution.
3. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
4. Seal the petri dish with parafilm and place in a humidified 47°C cell culture incubator for 24 h. **DO NOT** incubate at 47°C >24 h otherwise the RNA will begin to degrade.
5. **IF** the tissue is not transparent after 24 h, transfer the petri dish to a 37°C incubator and incubate until the tissue is cleared or until the tissue section becomes transparent.

SAMPLES CAN BE STORED IN CLEARING SOLUTION AT ***37°C*** *FOR UP TO* ***7 DAYS***

REPLENISH *THE CLEARING SOLUTION* ***AFTER 4 DAYS*** *IN CLEARING SOLUTION*

REFER TO THE MERSCOPE INSTRUMENT USER GUIDE FOR NEXT STEPS

> ***Choose*** *step* VI based on *tissue and fixation type. Refer to VI. Clearing in the SAMPLE PREPARATION OVERVIEW for more information.*

VI. Clearing – Resistant Fresh Frozen or Fixed Frozen Tissue

> *Maintain Digestion Premix and RNase inhibitor in benchtop cooler until ready for use. Ensure fully thawed and mixed, and spin down using a benchtop centrifuge before use. Return unused reagents to −20°C storage but minimize freeze-thaw cycles.*

1. Prepare Digestion Mix:

Digestion Mix	1 sample	5 samples	10 samples
Digestion Premix (PN 20300005)	200 µL	1 mL	2 mL
RNase inhibitor	5 µL	25 µL	50 µL

2. Aspirate to dry the MERSCOPE Slide without touching the gel. Add **200 µL** Digestion Mix onto the gel.
3. Incubate at room temperature for a minimum of 1 h and a maximum of 6 h.

> *1 h digestion is suitable for most clearing-resistant tissue.*
>
> *However, if a tissue does not become transparent with 1 h Digestion Mix treatment and 24 h tissue clearing at 47°C, consider extending the Digestion Mix incubation time to facilitate tissue clearing.*

4. Warm Clearing Premix (PN 20300003) at 37°C for 30 min before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm until the solution becomes clear. Prepare Clearing Solution:

Clearing Solution	1 sample	5 samples	10 samples
Clearing Premix (PN 20300003)	5 mL	25 mL	50 mL
Proteinase K	50 µL	250 µL	500 µL

5. Aspirate the Digestion Mix. Add **5 mL** Clearing Solution.
6. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
7. Seal the petri dish with parafilm and place in a humidified 47°C cell culture incubator for 24 h. **DO NOT** incubate at 47°C >24 h otherwise the RNA will begin to degrade.
8. **IF** the tissue is not transparent after 24 h, transfer the petri dish to a 37°C incubator and incubate until the tissue is cleared or until the tissue section becomes transparent.

> *SAMPLES CAN BE STORED IN CLEARING SOLUTION AT **37°C** FOR UP TO **7 DAYS***
> ***REPLENISH** THE CLEARING SOLUTION **AFTER 4 DAYS** IN CLEARING SOLUTION*
> *REFER TO THE MERSCOPE INSTRUMENT USER GUIDE FOR NEXT STEPS*