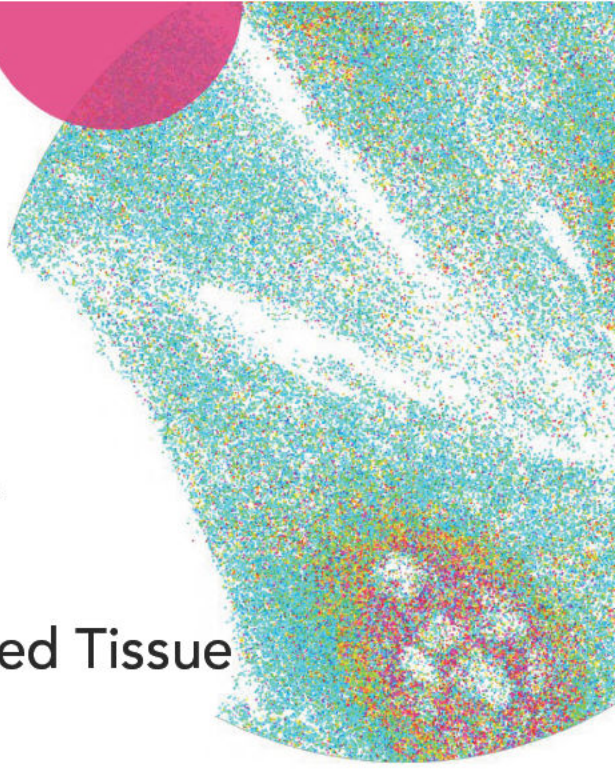
91600112 Rev D



MERSCOPE® User Guide

Formalin-Fixed Paraffin-Embedded Tissue Sample Preparation

Vizgen Materials	
10500102	MERSCOPE FFPE Slide Box, 10 slides
10400114	MERSCOPE FFPE Sample Prep Kit, 10 samples
10400118	MERSCOPE Cell Boundary Stain Kit, 20 FFPE samples – Optional
10400106 - 10400111	MERSCOPE Protein Stain Kits, 20 FFPE samples – Optional *To detect user-provided primary antibodies raised in mouse, rabbit, goat, rat, human, and chicken*
10400001	MERSCOPE 140 Gene Panel, 10 FFPE samples
10400002	MERSCOPE 300 Gene Panel, 10 FFPE samples
10400003	MERSCOPE 500 Gene Panel, 10 FFPE samples
10400125	MERSCOPE 1000 Gene Panel, 10 FFPE samples

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NOTICES

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VERSION CHANGE NOTES

Summary of substantive changes from Rev C to Rev D

Brief description of change	Page(s)
Compatibility note on potential nonspecific binding of MERSCOPE Protein Stains to same-species samples	18
Blue box reminder not to degas Gel Embedding Premix	41
Blue box reminder on the alternative to a humidified incubator for long incubations	47

TABLE OF CONTENTS

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 MERFISH data.

The MERSCOPE Workflow

Begin by either designing a MERSCOPE Custom Gene Panel or ordering a MERSCOPE Predesigned Gene Panel.

Design a custom gene panel. The intuitive MERSCOPE Gene Panel Design Software makes recommendations on your custom 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 custom gene panels for up to 140 genes, up to 300 genes, up to 500 genes, and up to 1000 genes.

Order your MERSCOPE Gene Panel. MERSCOPE Custom Gene Panels and MERSCOPE Predesigned 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), mosaic images (TIFF), experiment metadata (JSON), output from the cell segmentation analysis: transcripts per cell matrix (CSV), cell metadata (CSV), cell boundaries (HDF5 [MERSCOPE Instrument Software v231 or earlier] or PARQUET [v232 or later]), and a binary for use with the MERSCOPE Vizualizer software. The MERSCOPE Platform Solution includes the MERSCOPE Vizualizer software for visualizing and analyzing 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 **formalin-fixed paraffin-embedded (FFPE) tissue**. It is not applicable to fresh frozen and paraformaldehyde (PFA)-fixed frozen tissue. It is also not applicable to tissue or fresh cultured cells. Refer to the applicable user guide for other sample types. Vizgen supports mouse and human tissue samples only.

I. Tissue Sectioning

The tissue of interest is sectioned and adhered to a MERSCOPE FFPE Slide. MERSCOPE FFPE Slides for FFPE samples are supplied with fluorescent fiducials for subsequent imaging. Users should use MERSCOPE Sample Verification Kits and MERSCOPE Protein Stain Verification Kits to verify that the sample preparation conditions are compatible with MERFISH imaging with the MERSCOPE Instrument.

II. Deparaffinization and Decrosslinking

Deparaffinization dissolves the paraffin and decrosslinking reverses the crosslinks originally introduced via formalin fixation.

III. Anchoring Pretreatment

Anchoring pretreatment primes the RNA for RNA anchoring.

IV. Cell Boundary Staining and/or Protein Staining (Optional)

Tissue samples can be:

- Stained with cell boundary stain to mark cell boundaries and enable individual cell analysis, **AND/OR**
- Stained with protein stain for the co-detection of proteins and RNA in a single MERFISH experiment.

Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for **IMPORTANT** information. Users should use MERSCOPE Protein Stain Verification Kits to evaluate whether their primary antibodies are suitable for use with a MERSCOPE Instrument, in addition to optimizing antibody concentrations for protein staining (refer to the *MERSCOPE Protein Stain Verification Kit User Guide*).

V. RNA Anchoring

Formamide Wash Buffer denatures the RNA. Anchoring Buffer prepares RNA for gel embedding.

VI. Gel Embedding

Gel embedding immobilizes the RNA and creates a protective layer so the RNA species cannot escape in subsequent steps.

VII. 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 should use MERSCOPE Sample Verification Kits to optimize tissue clearing protocols, especially for clearing resistant tissue. Tissues resistant to clearing may require digestion.

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.

Clearing protocols are provided for non-resistant FFPE tissue and resistant FFPE tissue.

VIII. Autofluorescence Quenching

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 should use the MERSCOPE Sample Verification Kits to evaluate whether autofluorescence interferes with MERFISH imaging with the MERSCOPE Instrument. If autofluorescence is prevalent, the sample tissue should be placed in the MERSCOPE Photobleacher to remove the background signal when the sample is in Clearing Solution.

IX. Encoding Probe Hybridization and X. Post Encoding Probe Hybridization Wash

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

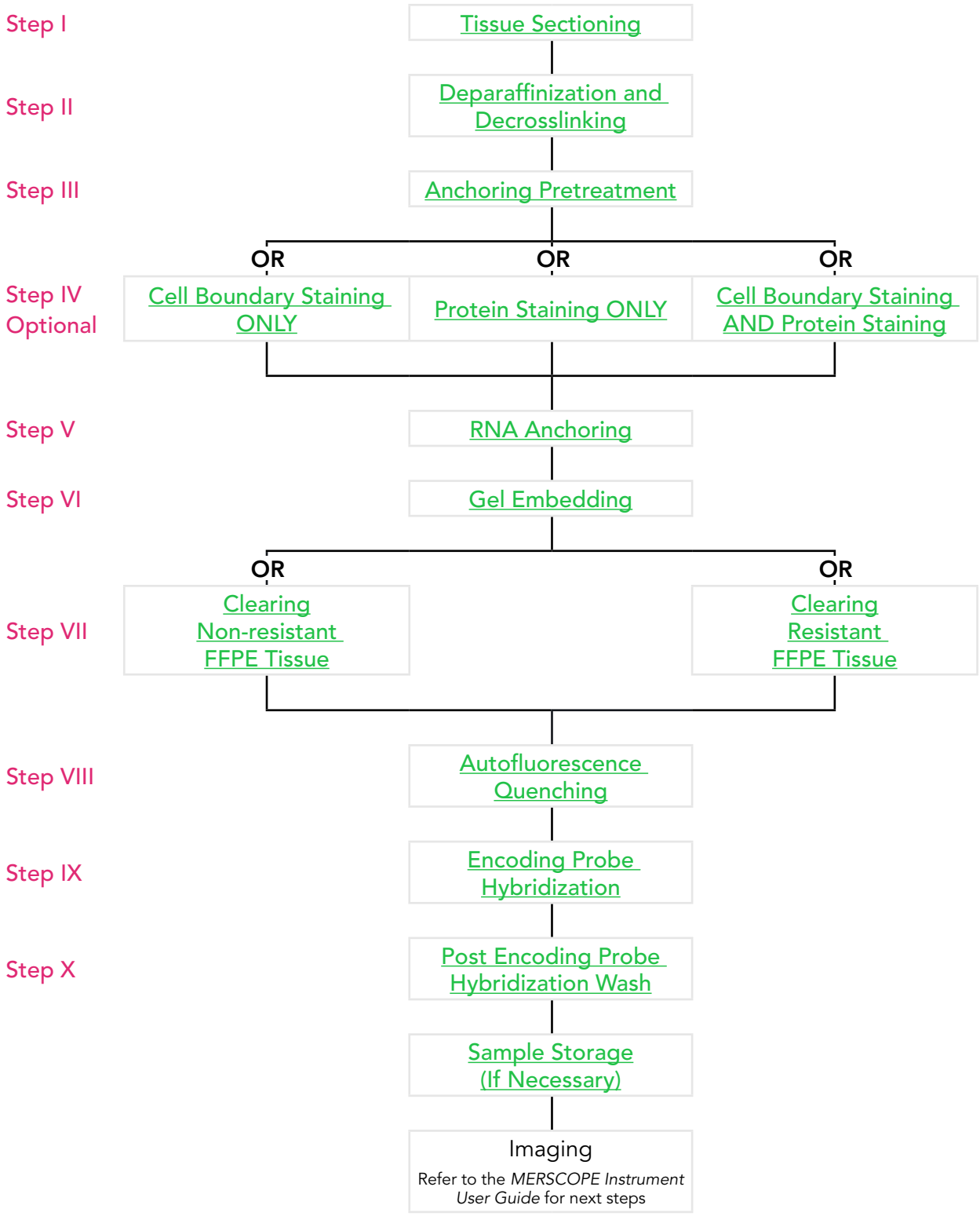
XI. Sample Storage (If Necessary)

Sample preparation can be performed in batches. Return batched samples to Clearing Premix for storage, if necessary. Samples can be stored in Clearing Premix at 37°C for up to 7 days.

Next Steps

Refer to the *MERSCOPE Instrument User Guide* for next steps. In short, any final bench steps are performed and then MERSCOPE FFPE 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.

SAMPLE PREPARATION WORKFLOW OPTIONS



TIMELINE - OPTION 1 (MON-FRI)

Day	Step	Bench time	Incubation time	Stopping points / storage
Day 0	I. Tissue Sectioning	1.5 h	-	Dry at −20°C Up to 1 month
Day 1 MON	II. Deparaffinization and Decrosslinking	0.5 h	1 h	70% EtOH at 4°C 1 day
	III. Anchoring Pretreatment	0.25 h	2.5 h	-
	IV. Cell Boundary Staining and/or Protein Staining	1 h	3 x 1 h	-
	V. RNA Anchoring	0.25 h	0.5 h Then overnight	-
Day 2 TUE	V. RNA Anchoring (Continued)	0.25 h	0.25 h	-
	VI. Gel Embedding	0.5 h	1.5 h	-
	VII. Digestion (If Necessary)	0.25 h	2 h*	-
	VII. Clearing	0.25 h	24 h+‡	Clearing Solution at 37°C Up to 4 days
Day 3 WED	VIII. Autofluorescence Quenching	0.25 h	3 h	-
	IX. Encoding Probe Hybridization	0.5 h	36 - 48 h	-
Day 5 FRI	X. Post Encoding Probe Hybridization Wash	0.25 h	2 x 0.5 h	Clearing Premix 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.

TIMELINE - OPTION 2 (TUE-WED)

Day	Step	Bench time	Incubation time	Stopping points / storage
Day 0	I. Tissue Sectioning	1.5 h	-	Dry at −20°C Up to 1 month
Day 1 **TUE**	II. Deparaffinization and Decrosslinking	0.25 h	0.5 h	70% EtOH at 4°C 1 day
Day 2 **WED**	II. Deparaffinization and Decrosslinking (Continued)	0.25 h	0.5 h	-
	III. Anchoring Pretreatment	0.25 h	2.5 h	-
	IV. Cell Boundary Staining and/or Protein Staining	1 h	3 x 1 h	-
	V. RNA Anchoring	0.25 h	0.5 h Then overnight	-
Day 3 **THU**	V. RNA Anchoring (Continued)	0.25 h	0.25 h	-
	VI. Gel Embedding	0.5 h	1.5 h	-
	VII. Digestion (If Necessary)	0.25 h	2 h*	-
	VII. Clearing	0.25 h	24 h+‡	Clearing Solution at 37°C Up to 4 days
Day 4 **FRI**	VIII. Autofluorescence Quenching	0.25 h	3 h	-
Day 7 **MON**	IX. Encoding Probe Hybridization	0.5 h	36 - 48 h	-
Day 9 **WED**	X. Post Encoding Probe Hybridization Wash	0.25 h	2 x 0.5 h	Clearing Premix at 37°C Up to 7 days
Refer to the *MERSCOPE Instrument User Guide* for next steps				

A total of 9 days for sample preparation (including the weekend) 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 Solution at 37°C for up to 7 days after Post Encoding Probe Hybridization Wash.

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

Refer to TIMELINE - OPTION 1 (MON-FRI) and TIMELINE - OPTION 2 (TUE-WED) 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. Select step VII. Clearing based on the sample tissue type.

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). Higher DV200 scores are associated with better MERFISH data generation.

DV200 >60%	On average, yields high quality MERFISH data
DV200 40-60%	On average, yields quality MERFISH data
DV200 <40%	On average, yields low quality MERFISH data

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 (FFPE) 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

Anchoring Pretreatment, RNA Anchoring, Clearing, and Encoding Probe Hybridization 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. Ensure the small petri dish has sufficient nuclease-free water daily.

Safety and Hazardous Steps

Safe laboratory practices should be followed at all times.

Formamide Wash Buffer is hazardous. Fixation buffers may contain hazardous materials. Pre-Anchoring Activator contains an alkylating agent and dimethyl sulfoxide. 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://portal.vizgen.com

FFPE TISSUE PREPARATION AND SECTIONING TIPS

FFPE Tissue Care

MERFISH measurements are sensitive to RNA degradation.

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

FFPE Tissue Sectioning – RNase-free Environment

Clean the microtome, 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.

FFPE Tissue Sectioning – Preparation

Fill a 150-mm petri dish with nuclease-free water and heat up the water on a hot plate to 45 ± 2°C (the water should be <55°C before sectioning). Tissues with higher fat content use 42 ± 2°C. Samples with higher fat content include sentinel lymph nodes, brain tissue, fatty liver, lipomas, and any tissue with fat attached to it such as skin with fat from the abdomen. If the tissue starts to break apart or expand as it contacts the bath water, a lower temperature water bath should be used.

Chill at least one cold plate in a −20°C freezer overnight prior to sectioning.

FFPE Tissue Sectioning

Place the FFPE tissue block in a nuclease-free water-filled 60-mm petri dish on the cold plate for 10-20 min prior to sectioning. Return the FFPE tissue block to the water-filled petri dish on the cold plate if pausing sectioning to do other tasks (e.g., transferring sections to MERSCOPE FFPE Slides). Maintaining a cold tissue block helps achieve ideal ribbons of tissue sections and minimizes static when laying a section into the water bath. It is recommended to exchange the cold plate for another pre-chilled plate out of the freezer every hour.

If using an FFPE tissue block for the first time, remove excess paraffin by trimming with a razor blade angled away from the tissue.

The surface of an FFPE tissue block may have lower RNA quality. After an FFPE tissue block is fully faced showing all of the tissue of interest, discard at least the first 50 µm of tissue sections prior to sectioning for evaluable 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 60-80 µm total (from either or both sides of the section of interest) for RNA extraction and DV200 quality measurement.

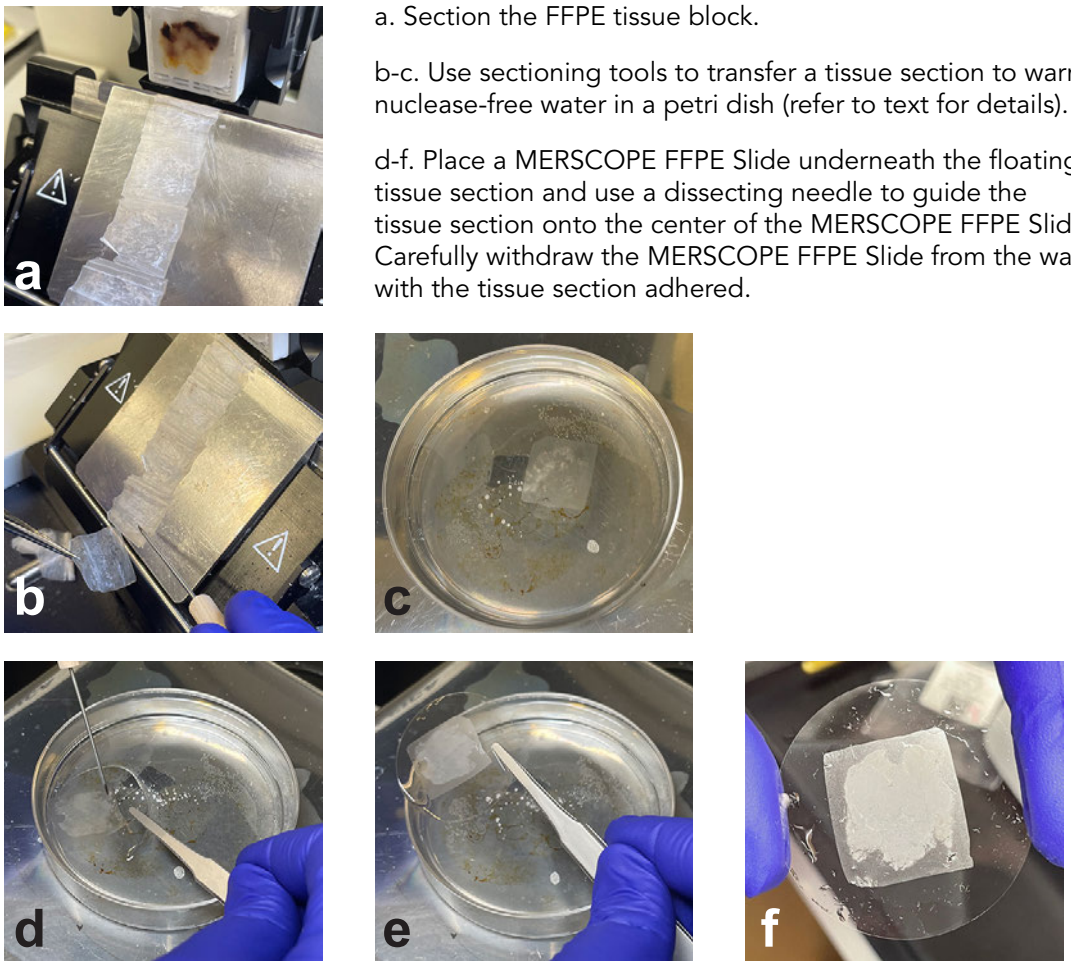
At the end of sectioning, place the tissue block in its own bag and place in a 4°C refrigerator until further use.

Tissue Adherence to a MERSCOPE FFPE Slide

Use sectioning tools to transfer the tissue section into a 150-mm petri dish with warm nuclease-free water on the hot plate (FFPE Tissue Sectioning – Preparation).

Place a MERSCOPE FFPE Slide underneath the floating tissue section in the 150-mm petri dish. Use a dissecting needle to guide the tissue section onto the center of the MERSCOPE FFPE Slide. Carefully withdraw the MERSCOPE FFPE Slide from the water with the tissue section adhered.

Transfer the MERSCOPE FFPE Slide with the tissue section to a drying rack. Use a Kimwipe to absorb extra water. Dry the sample at 55°C in an oven for 15 min and then dry at room temperature for 1-2 h, as necessary. Confirm there are no visible water droplets before transferring to a 60-mm petri dish for further sample preparation or storage. A dry sample can be stored in a labeled 60-mm petri dish, sealed with parafilm, at −20°C for up to 1 month.



a. Section the FFPE tissue block.

b-c. Use sectioning tools to transfer a tissue section to warm nuclease-free water in a petri dish (refer to text for details).

d-f. Place a MERSCOPE FFPE Slide underneath the floating tissue section and use a dissecting needle to guide the tissue section onto the center of the MERSCOPE FFPE Slide. Carefully withdraw the MERSCOPE FFPE Slide from the water with the tissue section adhered.

CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS

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 cell boundary staining.

The MERSCOPE Protein Stain Kits use oligonucleotide-conjugated secondary antibodies to detect user-provided primary antibodies and thereby enable the co-detection of proteins and RNA in a single MERFISH experiment.

Step IV Options

Identify the appropriate protocol for step IV:

- Cell boundary staining **ONLY**
- Protein staining **ONLY**
- Cell boundary staining **AND** protein staining
- No staining (skip the step)

User-provided Primary Antibody Requirements and Verification

User-provided primary antibodies **MUST** be:

- Bovine serum albumin (BSA)-free, **AND**
- Compatible with immunohistochemistry (IHC).

Users should use MERSCOPE Protein Stain Verification Kits to evaluate whether their primary antibodies are suitable for use with a MERSCOPE Instrument, in addition to optimizing antibody concentrations. Refer to the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

MERSCOPE Protein Stains

Vizgen supplies protein stains compatible with user-provided primary antibodies raised in a variety of host species.

Primary Antibody Raised In	Corresponding Protein Stain	Auxiliary Bit	Kit PN
Mouse	Anti-Mouse Aux 4 Protein Stain	Aux 4	10400106
Rabbit	Anti-Rabbit Aux 5 Protein Stain	Aux 5	10400107
Goat	Anti-Goat Aux 6 Protein Stain	Aux 6	10400108
Rat	Anti-Rat Aux 7 Protein Stain	Aux 7	10400109
Human	Anti-Human Aux 8 Protein Stain	Aux 8	10400110
Chicken	Anti-Chicken Aux 9 Protein Stain	Aux 9	10400111

Compatibility

- Rabbit primary antibodies (and therefore the MERSCOPE Anti-Rabbit Protein Stain Kit) are currently **NOT** compatible with concurrent cell boundary staining.
- Gene panels containing sequential gene encoding **MAY NOT** be compatible with certain MERSCOPE Protein Stain Kits. To identify potential conflicts:
  - Locate the auxiliary bit assigned to sequential genes in your gene panel. Navigate to the panel summary page for a constructed gene panel in the MERSCOPE Gene Panel Design Software. Sequential genes are listed along with the assigned auxiliary bits.
  - Compare the auxiliary bits used by the sequential genes in the gene panel to the auxiliary bits of the protein stains (table above). **If the auxiliary bits overlap, the protein stain is not compatible with the gene panel.** E.g., if a sequential gene in the gene panel is assigned to Aux 4, the MERSCOPE Anti-Mouse Protein Stain is not compatible with the gene panel.
- MERSCOPE Protein Stains may display higher levels of non-specific binding to tissue of the same species (e.g., Anti-Mouse Aux 4 Protein Stain may show higher levels of nonspecific binding to mouse tissue).
- Contact Vizgen Support (support@vizgen.com) for more information, if needed.

Primary Staining Solution for Protein Staining – Key Details

- Add the primary antibodies for **EACH** protein to be detected.
  - *E.g., in a single sample, if a protein is detected using a mouse primary antibody and a second protein is detected using a goat primary antibody, add the mouse primary antibody **AND** the goat primary antibody to the Primary Staining Solution.*
- Only 1 primary antibody **PER SPECIES** can be detected in a single MERFISH experiment. **DO NOT** add 2 primary antibodies raised in the same species.
- Optimal primary antibody concentration(s) should be determined during verification. Refer to the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.
- Cell boundary staining **AND** protein staining **ONLY**: Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore should not be included in the Primary Staining Solution.

Secondary Staining Solution for Protein Staining – Key Details

- **ONLY** add the protein stain(s) against **EACH** primary antibody host species used in the Primary Staining Solution.
  - *E.g., if mouse, goat, and rat primary antibodies are used in the Primary Staining Solution, add the required volume of anti-mouse, anti-goat, and anti-rat protein stain to the Secondary Staining Solution.*
- Cell boundary staining **AND** protein staining **ONLY**: Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore Anti-Rabbit Aux 5 should not be included in the Secondary Staining Solution.

MATERIALS

Vizgen Materials

MERSCOPE FFPE Slide Box, 10 slides	10500102	Storage
MERSCOPE FFPE Slide, 10 x 1 sample	20400100	–20°C, horizontally

MERSCOPE FFPE Sample Prep Kit, 10 samples	10400114	Storage
Deparaffinization Buffer	20300112	4°C
Decrosslinking Buffer	20300115	4°C
Conditioning Buffer	20300116	4°C
Sample Prep Wash Buffer	20300001	4°C
Formamide Wash Buffer	20300002	4°C, protected from light
Gel Embedding Premix	20300118	4°C, protected from light
Clearing Premix	20300114	4°C
Gel Coverslip	30200004	Room temperature
Pre-Anchoring Activator*	20300113	–20°C upon receipt, protected from light
Anchoring Buffer	20300117	–20°C upon receipt
Digestion Premix, 3 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, 10 FFPE samples	10400001	Storage
MERSCOPE 140 Gene Panel Mix, 5 x 2 samples	20300006	–20°C

MERSCOPE 300 Gene Panel, 10 FFPE samples	10400002	Storage
MERSCOPE 300 Gene Panel Mix, 5 x 2 samples	20300007	–20°C

MERSCOPE 500 Gene Panel, 10 FFPE samples	10400003	Storage
MERSCOPE 500 Gene Panel Mix, 5 x 2 samples	20300008	–20°C

MERSCOPE 1000 Gene Panel, 10 FFPE samples	10400125	Storage
MERSCOPE 1000 Gene Panel Mix, 5 x 2 samples	20300141	–20°C

MERSCOPE Photobleacher	10100003	

MERSCOPE Cell Boundary Stain Kit, 20 FFPE samples	10400118	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	−20°C
Cell Boundary Primary Stain Mix	20300010	−20°C
Cell Boundary Secondary Stain Mix	20300011	−20°C

*Minimize freeze-thaw cycles

MERSCOPE Anti-Mouse Protein Stain Kit, 20 FFPE samples	10400106	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Mouse Aux 4 Protein Stain	20300101	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Rabbit Protein Stain Kit, 20 FFPE samples	10400107	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Rabbit Aux 5 Protein Stain[a]	20300102	–20°C
*Minimize freeze-thaw cycles		
[a]Not compatible with the MERSCOPE Cell Boundary Stain Kit		

MERSCOPE Anti-Goat Protein Stain Kit, 20 FFPE samples	10400108	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Goat Aux 6 Protein Stain	20300103	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Rat Protein Stain Kit, 20 FFPE samples	10400109	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Rat Aux 7 Protein Stain	20300104	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Human Protein Stain Kit, 20 FFPE samples	10400110	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Human Aux 8 Protein Stain	20300105	–20°C
*Minimize freeze-thaw cycles		

MERSCOPE Anti-Chicken Protein Stain Kit, 20 FFPE samples	10400111	Storage
Blocking Buffer C Premix, 4 x 5 samples*	20300100	–20°C
Anti-Chicken Aux 9 Protein Stain	20300106	–20°C
*Minimize freeze-thaw cycles		

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

Required User Supplied 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	76081-554
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
Cleaning tissue (Kimwipe or similar)	VWR*	21913-214*
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

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

*Alternative to Kimwipe.

General Laboratory Equipment

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

- Fume hood
- 37°C cell culture incubator (humidified)[a,b]
- 47°C cell culture incubator (humidified)[a,b]
- 37°C incubator in a fume hood[a,b] (e.g., VWR 10055-006)
- 47°C incubator in a fume hood[a,b] (e.g., VWR 10055-006)
- 55°C dry oven[a] (e.g., VWR 10055-006)
- 90°C dry oven[a] (e.g., Fisher Scientific, S13043)
- Vacuum trap system (e.g., VWR 76207-602)
- Vacuum pumps (e.g., Thomas Scientific 1162B24)
- Benchtop centrifuge
- Rocker
- Vortexer
- Analytical balance
- Water bath[c] (e.g., VWR 76308-896)
- Tube/bottle weight (e.g., VWR 47748-174)
- Benchtop cooler (e.g., VWR 414004-286)
- Cold plate (e.g., VWR 25608-942)
- Hot plate (e.g., VWR 89090-188)
- Drying rack (e.g., Millipore-Sigma Z743685)
- Forceps, dissecting needle, and sectioning tools
- Microtome

a. Multiple temperatures are needed throughout the protocol. Therefore, it is recommended to have equipment dedicated to each temperature.

b. The 'cell culture incubator' and the 'incubator in a fume hood' may be the same piece of equipment. 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. Ensure the small petri dish has sufficient nuclease-free water daily.

c. 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
90% Ethanol
1X PBS

Used in Step IV (when included) – 5 mL per sample needed each time			
Fixation Buffer*	1 sample	5 samples	10 samples
10X PBS	4 mL	4 mL	8 mL
32% paraformaldehyde (formaldehyde) solution	5 mL	5 mL	10 mL
Nuclease-free water	31 mL	31 mL	62 mL

*Make fresh every time used

Used in Step VI – 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 FFPE 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 FFPE Slide in a 60-mm petri dish.

I. Tissue Sectioning

MERSCOPE FFPE Slides (PN 20400100) are stored at −20°C. Warm up at room temperature for 15 min before use. Unused MERSCOPE FFPE Slides should be stored at −20°C.

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

1. Fill a 150-mm petri dish with nuclease-free water and heat the water on a hot plate to 45 ± 2°C. Tissues with higher fat content use 42 ± 2°C. Ensure the water is <50°C before sectioning.
2. Remove a pre-chilled cold plate from a −20°C freezer and place on the bench.
3. Fill a 60-mm petri dish with nuclease-free water and place on the cold plate.
4. Place the FFPE tissue block in the nuclease-free water-filled petri dish on the cold plate for 10-20 min prior to sectioning.
5. Clean the microtome, 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.
6. If using an FFPE tissue block for the first time, remove excess paraffin and face the tissue block appropriately.
7. Place the FFPE tissue block on the cleaned microtome.
8. Section and discard the top 50 µm to maximize RNA quality.
9. Section the FFPE tissue block at **4 µm or 5 µm** thickness.
10. With sectioning tools, transfer the tissue section into the 150-mm petri dish with nuclease-free water at the appropriate temperature (refer to step 1).
11. Place a MERSCOPE FFPE Slide beneath the floating tissue section in the 150-mm petri dish. Use a dissecting needle to guide the tissue section onto the middle of the MERSCOPE FFPE Slide. The MERSCOPE Sample Placement Guide may be used here. Refer to MERSCOPE Sample Placement Guide Technical Note (91700119) for instructions on how to use it.

12. Transfer the MERSCOPE FFPE Slide with the tissue section to a drying rack. Use a Kimwipe to absorb extra water but **DO NOT** touch the tissue. Dry the sample at 55°C (oven) for 15 min (**DO NOT** dry above 55°C) and then dry at room temperature for 1-2 h, as necessary. Confirm there are no visible water droplets before storing samples.
13. Transfer the sample to a 60-mm petri dish. Label the sample.

The dry sample can be stored in a labeled 60-mm petri dish, sealed with parafilm, at −20°C for up to 1 month.

II. Deparaffinization and Decrosslinking

1. **ENSURE** the sample is completely dry before proceeding.
2. Add **500 µL** Deparaffinization Buffer (PN 20300112) onto the center of the FFPE tissue section. Ensure the Deparaffinization Buffer covers the whole tissue section and try to prevent the Deparaffinization Buffer from flowing beneath the MERSCOPE FFPE Slide. Incubate at 55°C (oven) for 5 min.
3. Aspirate the Deparaffinization Buffer and repeat step 2.
4. Aspirate the Deparaffinization Buffer and add **500 µL** Deparaffinization Buffer onto the center of the tissue section. Ensure the Deparaffinization Buffer covers the whole tissue section and try to prevent the Deparaffinization Buffer from flowing beneath the MERSCOPE FFPE Slide. Incubate at room temperature for 5 min.
5. Aspirate the Deparaffinization Buffer. Wash **3x** with **5 mL** 100% ethanol, incubate 2 min each wash. If Deparaffinization Buffer is underneath the MERSCOPE FFPE Slide, gently lift the slide to ensure oil droplets from underneath are also removed. Transfer to a new labeled petri dish if this facilitates oil droplet removal.
6. **IF** oil droplets are observed after 3x washes, repeat 100% ethanol washes until no oil droplets are observed.
7. Wash **1x** with **5 mL** 90% ethanol, incubate 2 min.
8. Wash **1x** with **5 mL** 70% ethanol, incubate 2 min.

   The sample can be stored in 70% ethanol at 4°C for up to 1 day.

9. Aspirate the 70% ethanol and add **5 mL** Decrosslinking Buffer (PN 20300115).
10. Aspirate and add **5 mL** Decrosslinking Buffer. Place the petri dish onto a heating pan and incubate at 90°C (oven) for 15 min. **DO NOT** heat above 95°C.
11. *The petri dish is hot and should be handled accordingly:* Remove the petri dish from the oven and cool on the bench for 5 min.

III. Anchoring Pretreatment

Pre-Anchoring Activator contains an alkylating agent and dimethyl sulfoxide.

Thaw Pre-Anchoring Activator at room temperature for 30 min before 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. Aspirate the Decrosslinking Buffer.
2. Wash **2x** with **5 mL** Conditioning Buffer (PN 20300116), incubate 1 min each wash.
3. Add **5 mL** Conditioning Buffer, incubate at 37°C for 30 min in an incubator.
4. Prepare Pre-Anchoring Reaction Buffer:

Pre-Anchoring Reaction Buffer	1 sample	5 samples	10 samples
Conditioning Buffer (PN 20300116)	100 µL	500 µL	1 mL
Pre-Anchoring Activator (PN 20300113)	5 µL	25 µL	50 µL
RNase inhibitor	5 µL	25 µL	50 µL

5. First aspirate the Conditioning Buffer to dry the region of MERSCOPE FFPE Slide that does not have tissue section. Then carefully aspirate around the tissue section to remove extra solution without touching the tissue section. The tissue section should not be completely dry for more than 1 min.
6. Add **100 µL** Pre-Anchoring Reaction Buffer onto the tissue to cover the whole tissue. 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.

If the Pre-Anchoring Reaction Buffer is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Pre-Anchoring Reaction Buffer is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE Slide, otherwise the Pre-Anchoring Reaction Buffer may wick away into the petri dish.

8. Seal the petri dish with parafilm and place in a humidified 37°C cell culture incubator for 2 h.
9. Proceed immediately to the next step:
   - **EITHER** the applicable optional step IV:
     - IV. Cell Boundary Staining ONLY,
     - IV. Protein Staining ONLY, **OR**
     - IV. Cell Boundary Staining AND Protein Staining,
   - **OR** V. RNA Anchoring.

Choose *step IV based on the extent of additional staining. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for* ***IMPORTANT*** *information, including antibody requirements and compatibility.*

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step V. RNA Anchoring.

IV. Cell Boundary Staining ONLY

When preparing blocking solutions and primary/secondary staining solutions, use the included Block Buffer C Premix (PN 20300100) that is included with Cell Boundary Stain Kit (PN 10400118).

Maintain Cell Boundary Primary Stain Mix, Cell Boundary Secondary Stain Mix, and RNase inhibitor in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

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

Refer to Additional Buffer Recipes for fixation buffer.

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

1. Use tweezers to remove the parafilm. Add **5 mL** 1X PBS, incubate 2 min.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL

3. Aspirate the 1X PBS to dry the MERSCOPE FFPE 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.

   If the Blocking Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Blocking Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL
Cell Boundary Primary Stain Mix (PN 20300010)	1 µL	5 µL	10 µL

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE FFPE 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.

   If the Primary Staining Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Primary Staining Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	5 µL	25 µL	50 µL
Cell Boundary Secondary Stain Mix (PN 20300011)	3 µL	15 µL	30 µL

14. Aspirate the 1X PBS to dry the MERSCOPE FFPE 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.

> *If the Secondary Staining Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Secondary Staining Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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 (V. RNA Anchoring).

Choose *step IV based on the extent of additional staining. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for* ***IMPORTANT*** *information, including antibody requirements and compatibility.*

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step V. RNA Anchoring.

IV. Protein Staining ONLY

Maintain user-provided primary antibodies per manufacturer's instructions. Maintain Protein Stains and RNase inhibitor in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

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

Refer to Additional Buffer Recipes for fixation buffer.

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

1. Use tweezers to remove the parafilm. Add **5 mL** 1X PBS, incubate 2 min.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL

3. Aspirate the 1X PBS to dry the MERSCOPE FFPE 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.

If the Blocking Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Blocking Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
User-provided primary antibody raised in[a-c]: • Mouse • Rabbit • Goat • Rat • Human • Chicken	1 µL of each	5 µL of each	10 µL of each

a. Add the primary antibodies for **EACH** protein to be detected. E.g., in a single sample, if a protein is detected using a mouse primary antibody and a second protein is detected using a goat primary antibody, add the mouse primary antibody **AND** the goat primary antibody to this Primary Staining Solution.

b. Only 1 primary antibody **PER SPECIES** can be detected in a single MERFISH experiment. **DO NOT** add 2 primary antibodies raised in the same species.

c. Optimal primary antibody concentration(s) should be determined during verification. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS and the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE FFPE 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.

If the Primary Staining Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Primary Staining Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
Protein Stain(s) Select among[a]: Anti-Mouse Aux 4 (PN 20300101) Anti-Rabbit Aux 5 (PN 20300102) Anti-Goat Aux 6 (PN 20300103) Anti-Rat Aux 7 (PN 20300104) Anti-Human Aux 8 (PN 20300105) Anti-Chicken Aux 9 (PN 20300106)	1 µL of each	5 µL of each	10 µL of each

a. **ONLY** add the protein stain(s) against **EACH** primary antibody host species used in the Primary Staining Solution. E.g., if mouse, goat, and rat primary antibodies were used in the Primary Staining Solution, add the required volume of anti-mouse, anti-goat, and anti-rat protein stain to this Secondary Staining Solution.

14. Aspirate the 1X PBS to dry the MERSCOPE FFPE 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.

If the Secondary Staining Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Secondary Staining Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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 (V. RNA Anchoring).

Choose *step IV based on the extent of additional staining. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS for* ***IMPORTANT*** *information, including antibody requirements and compatibility.*

If the sample does not require cell boundary staining and/or protein staining, proceed directly to step V. RNA Anchoring.

IV. Cell Boundary Staining AND Protein Staining

Primary antibodies raised in rabbits (and therefore Anti-Rabbit Aux 5) ***CANNOT*** *be used when protein staining is combined with cell boundary staining.*

For cell boundary staining ***AND*** *protein staining, use Blocking Buffer C Premix (PN 20300100) included in MERSCOPE Protein Stain Kits.*

Maintain user-provided primary antibodies per manufacturer's instructions. Maintain Cell Boundary Primary Stain Mix, Cell Boundary Secondary Stain Mix, Protein Stains, and RNase inhibitor in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

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

Refer to Additional Buffer Recipes for fixation buffer.

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

1. Use tweezers to remove the parafilm. Add **5 mL** 1X PBS, incubate 2 min.
2. Prepare Blocking Solution:

Blocking Solution	1 sample	5 samples	10 samples
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL

3. Aspirate the 1X PBS to dry the MERSCOPE FFPE 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.

If the Blocking Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Blocking Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
Cell Boundary Primary Stain Mix (PN 20300010)	1 µL	5 µL	10 µL
User-provided primary antibody raised in[a-d]: • Mouse • Goat • Rat • Human • Chicken	1 µL of each	5 µL of each	10 µL of each

a. Add the primary antibodies for **EACH** protein to be detected. For example, in a single sample, if a protein is detected using a mouse primary antibody and a second protein is detected using a goat primary antibody, add the mouse primary antibody **AND** the goat primary antibody to this Primary Staining Solution.

b. Only 1 primary antibody **PER SPECIES** can be detected in a single MERFISH experiment. **DO NOT** add 2 primary antibodies raised in the same species.

c. Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore not listed here.

d. Optimal primary antibody concentration(s) should be determined during verification. Refer to CELL BOUNDARY STAINING AND PROTEIN STAINING TIPS and the *MERSCOPE Protein Stain Verification Kit User Guide* for more information.

7. Use tweezers to remove the parafilm.
8. Aspirate the solution to dry the MERSCOPE FFPE 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.

If the Primary Staining Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Primary Staining Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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
Blocking Buffer C Premix (PN 20300100)	100 µL	500 µL	1 mL
RNase inhibitor	10 µL	50 µL	100 µL
Cell Boundary Secondary Stain Mix (PN 20300011)	3 µL	15 µL	30 µL
Protein Stain(s) Select among[a-b]: Anti-Mouse Aux 4 (PN 20300101) Anti-Goat Aux 6 (PN 20300103) Anti-Rat Aux 7 (PN 20300104) Anti-Human Aux 8 (PN 20300105) Anti-Chicken Aux 9 (PN 20300106)	1 µL of each	5 µL of each	10 µL of each

a. **ONLY** add the protein stain(s) against **EACH** primary antibody host species used in the Primary Staining Solution. For example, if mouse, goat, and rat primary antibodies were used in the Primary Staining Solution, add the required volume of anti-mouse, anti-goat, and anti-rat protein stain to this Secondary Staining Solution.

b. Rabbit primary antibodies are currently **NOT** compatible with concurrent cell boundary staining, and therefore Anti-Rabbit Aux 5 is not listed here.

14. Aspirate the 1X PBS to dry the MERSCOPE FFPE 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.

If the Secondary Staining Solution is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Secondary Staining Solution is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE 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 (V. RNA Anchoring).

V. RNA Anchoring

Maintain Anchoring Buffer in a benchtop cooler until use. Spin down using a benchtop centrifuge before use.

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

1. Aspirate the 1X PBS (if continuing from cell boundary staining) or discard parafilm (if continuing from anchoring pretreatment) 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 in a fume hood.
3. Aspirate the Formamide Wash Buffer to dry the region of MERSCOPE FFPE Slide that does not have the 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.
4. Add **100 µL** Anchoring Buffer (PN 20300117) 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.

If the Anchoring Buffer is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Anchoring Buffer is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE Slide, otherwise the Anchoring Buffer may wick away into the petri dish.

5. Seal the petri dish with parafilm and place in a humidified 37°C cell culture incubator overnight (12-18 h).
6. Following overnight incubation, use tweezers to remove the parafilm and add **5 mL** Formamide Wash Buffer.
7. Incubate at 47°C for 15 min in an incubator in a fume hood.
8. Wash **1x** with **5 mL** Sample Prep Wash Buffer, incubate 2 min.
9. Proceed immediately to the next step.

VI. Gel Embedding

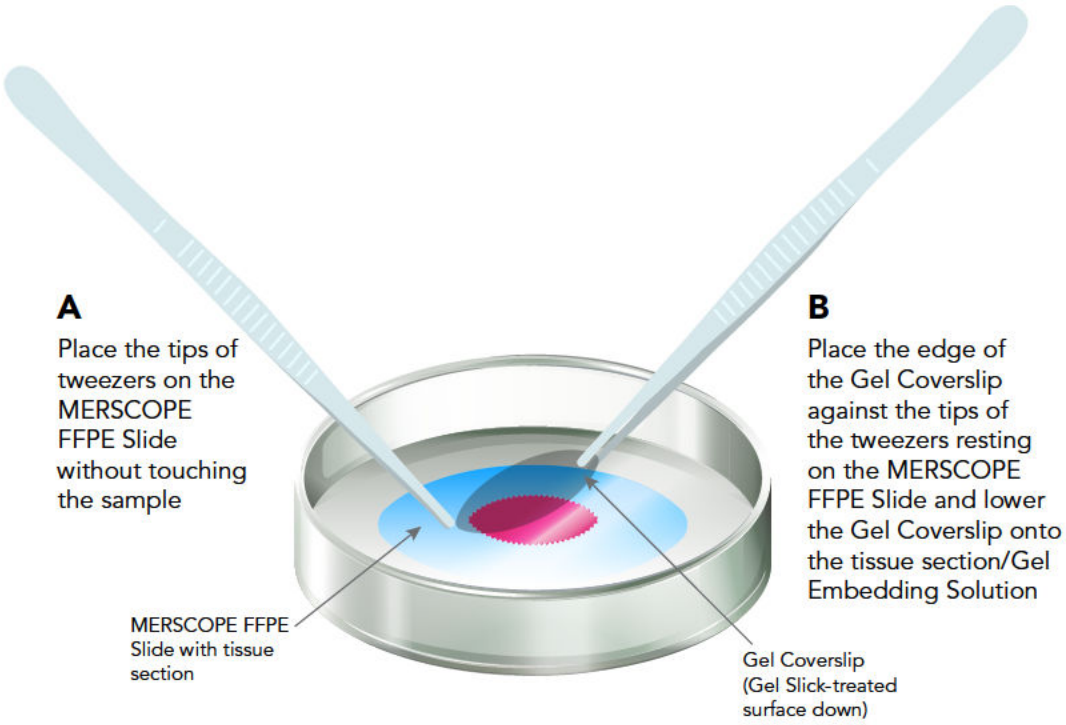
Gel embedding reagents contain hazardous materials.

***DO NOT** degas Gel Embedding Premix.*

1. Clean a Gel Coverslip (PN 30200004) by spraying with RNaseZap solution and wiping with a Kimwipe, followed by spraying 70% ethanol and wiping with a Kimwipe.
2. Add **100 µL** Gel Slick Solution onto the Gel Coverslip. Allow the Gel Slick Solution to evaporate for 10 min at room temperature. Wipe gently with a Kimwipe to remove any remaining film, liquid, or deposition from the glass. Use immediately after preparation.
3. Prepare Gel Embedding Solution:

Gel Embedding Solution	1 sample	5 samples	10 samples
Gel Embedding Premix (PN 20300118)	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
Refer to Additional Buffer Recipes for 10% w/v ammonium persulfate solution			

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 to the sample, ensure the sample is fully covered, and 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 FFPE 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 FFPE 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 FFPE 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 FFPE 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 FFPE 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 brace the Gel Coverslip with tweezers in one hand and 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.

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

VII. Clearing – Non-resistant FFPE Tissue

1. Warm Clearing Premix (PN 20300114) at 37°C for 30 min before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm and mix until the solution becomes clear. Prepare Clearing Solution:

Clearing Solution	1 sample	5 samples	10 samples
Clearing Premix (PN 20300114)	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 - this is important to remember if clearing over the weekend.
5. **IF** the tissue is not transparent after 24 h, transfer the petri dish to a humidified 37°C cell culture incubator and incubate until the tissue is cleared or until the tissue section becomes transparent.

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. Ensure the small petri dish has sufficient nuclease-free water daily.

SAMPLES CAN BE STORED OR CLEARED IN CLEARING SOLUTION

*AT **37°C** FOR UP TO **4 DAYS***

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

VII. Clearing – Resistant FFPE Tissue

Thaw Digestion Premix. Ensure fully thawed and mixed, and spin down using a benchtop centrifuge before use.

Maintain RNase inhibitor in a benchtop cooler until 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 FFPE Slide without touching the gel. Add **200 µL** Digestion Mix onto the gel. 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.

If the Digestion Mix is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Digestion Mix is spread across the tissue section. The parafilm should fit within the MERSCOPE FFPE Slide, otherwise the Digestion Mix may wick away into the petri dish.

3. Incubate at 37°C for 2 h.

2 h digestion is suitable for most clearing-resistant tissue.

However, if a tissue does not become transparent with 2 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 20300114) at 37°C for 30 min before use. The Clearing Premix should be a clear solution before use. If the solution is cloudy, warm and mix until the solution becomes clear. Prepare Clearing Solution:

Clearing Solution	1 sample	5 samples	10 samples
Clearing Premix (PN 20300114)	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 - this is important to remember if clearing over the weekend.
8. **IF** the tissue is not transparent after 24 h, transfer the petri dish to a humidified 37°C cell culture incubator and incubate until the tissue is cleared or until the tissue section becomes transparent.

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. Ensure the small petri dish has sufficient nuclease-free water daily.

SAMPLES CAN BE STORED OR CLEARED IN CLEARING SOLUTION

*AT **37°C** FOR UP TO **4 DAYS***

VIII. Autofluorescence Quenching

> ***ENSURE*** *the petri dish is sealed with parafilm prior to autofluorescence quenching, otherwise the Clearing Solution may evaporate in the MERSCOPE Photobleacher.*
>
> *The sample should still be in Clearing Solution for autofluorescence quenching.*

1. Aspirate bubbles or condensation from the lid of the petri dish to minimize light scattering from above.
2. Place the parafilm-sealed petri dish in the MERSCOPE Photobleacher (PN 10100003). **ENSURE** there are no labels/writing/other items on the lid that may block the light.
3. Turn on the MERSCOPE Photobleacher and leave at room temperature for at least 3 h. Time in MERSCOPE Photobleacher may vary based on sample.

IX. Encoding Probe Hybridization

Maintain the applicable MERSCOPE Gene Panel Mix in a benchtop cooler until use. 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 Clearing Solution and wash **3x** with **5 mL** Sample Prep Wash Buffer (PN 20300001), incubate at room temperature for 5 min on a rocker each wash.
2. Aspirate Sample Prep Wash Buffer and add **5 mL** Formamide Wash Buffer (PN 20300002), incubate at 37°C for 30 min in an incubator in a fume hood.
3. First aspirate the Formamide Wash Buffer to dry the region of MERSCOPE FFPE Slide that does not have gel. Then carefully aspirate around the gel to remove extra Formamide Wash Buffer without touching the gel. The gel should not be completely dry for more than 1 min.

Aspirate all the residual solution without disrupting the gel to avoid diluting the MERSCOPE Gene Panel Mix.

4. Add **100 µL** MERSCOPE Gene Panel Mix onto the center of the gel. 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.

If the MERSCOPE Gene Panel Mix is not spread across the gel, lift and then lower the parafilm with tweezers until the MERSCOPE Gene Panel Mix is spread across the gel. The parafilm should fit within the MERSCOPE FFPE Slide, otherwise the MERSCOPE Gene Panel Mix may wick away into the petri dish.

5. Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
6. Seal the petri dish with parafilm and 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. Ensure the small petri dish has sufficient nuclease-free water daily.

X. 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 an 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 an incubator in a fume hood.
5. Proceed immediately MERSCOPE imaging (refer to the *MERSCOPE Instrument User Guide*) or XI. Sample Storage (If Necessary).

XI. Sample Storage (If Necessary)

1. Warm Clearing Premix (PN 20300114) 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.
2. Wash **1x** with **5 mL** Sample Prep Wash Buffer (PN 20300001), incubate 2 min.
3. Aspirate the Sample Prep Wash Buffer and add **5 mL** Clearing Premix.
4. Seal the petri dish with parafilm and place in a humidified 37°C cell culture incubator.

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

*REPLENISH THE CLEARING PREMIX **AFTER 4 DAYS***

REFER TO THE MERSCOPE INSTRUMENT USER GUIDE FOR NEXT STEPS