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Human Lung MERFISH Protocol for Fresh Frozen Tissue

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We use this protocol and it's working

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Abstract

This protocol details a comprehensive workflow for applying MERFISH to fresh frozen human lung tissue, enabling spatially resolved transcriptomic analysis. The method encompasses tissue sectioning, fixation, and permeabilization, followed by encoding probe hybridization and gel embedding. Subsequent steps include tissue clearing, autofluorescence quenching, and imaging preparation using the Vizgen MERSCOPE platform. Detailed instructions on reagent preparation, incubation conditions, and imaging parameters ensure that RNA integrity and spatial context are maintained throughout the process.

Guidelines

The protocol should be performed in an RNase-free environment to preserve RNA integrity.

Materials

MERSCOPE Slides (Vizgen, 10500001)

MERSCOPE Sample Prep Kit (Vizgen, 10400012)

MERSCOPE 500 Gene Imaging Kit (Vizgen, 10400006)

Paraformaldehyde (EMS, 15714)

Phosphate-Buffered Saline (10X) pH 7.4, RNase-free (Fisher, AM9625)

GelSlick Solution (Lonza, 50640)

RNaseZap RNase Decontamination Solution (Fisher, AM9782)

RNase inhibitor (NEB, M0314L)

Proteinase K ((NEB, P8107S)

Ammonium persulfate (Sigma, A3678)

N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma, T9281)

Mineral oil (Sigma, 330779)

Tissue Fixation and Permeabilization

- 1 Section samples of fresh-frozen samples embedded with OCT at 12 μm thick on MERSCOPE slides (Vizgen, 10500001) and then allow the sample to dry at -20°C for 2 hours and at RT for 20 min. For detailed tissue block sectioning guidelines, refer to the protocol at dx.doi.org/10.17504/protocols.io.3byl4j91jlo5/v1.
- 2 Prewarm 4% PFA in 1x PBS (Fisher, AM9625) at 47°C for 15 min. Add 4 mL of prewarmed 4% PFA in 1xPBS and incubate at 47°C for 30 min.
- 3 Prewarm 1x PBS. Wash with 5 mL 1X PBS at 47°C for 10 min. Aspirate 1x PBS and dry at RT for 10 min.
- 4 Add 5 mL 70% ethanol, seal the petri dish with parafilm and place at 4°C overnight to permeabilize the tissue.

Encoding Probe Hybridization

- 5 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.
- 6 Add 5 mL Formamide Wash Buffer, incubate at 37°C for 30 min in an incubator.
- 7 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.
 - Formamide Wash Buffer is hazardous. Perform these steps in a fume hood. Bring wash buffers up to room temp before use.
 - Return unused reagents to -20°C storage but minimize freeze-thaw cycles
- 8 Use scissors to cut a piece of parafilm to size of tissue. Add 50 μL Encoding Probe Set Gene Panel onto the center of the tissue section. Use tweezers to peel off the parafilm backing and place the side previously protected by the backing onto the solution. Avoid introducing air bubbles.
- 9 If the Encoding Probe Set is not spread across the tissue section, lift and then lower the parafilm with tweezers until the Encoding Probe Set is spread across the tissue section.



- 10 Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.
- 11 Place in a humidified 37°C cell culture incubator for at least 36-48 hrs. DO NOT let the sample dry out.

Post Encoding Probe Hybridization Wash

- 12 Aliquot 10mL Formamide Wash Buffer with RNase inhibitor 1:2000 (NEB, M0314L) and let warm up while pulling out other buffers. Remove the parafilm and add 5 mL Formamide Wash Buffer. Incubate at 47°C for 30 min in a mini-incubator in a fume hood
 - Formamide Wash Buffer is hazardous, perform these steps in fume hood. Bring all buffers to room temp.
- 13 Aspirate the Formamide Wash Buffer. Add 5 mL Formamide Wash Buffer.
- 14 Incubate at 47°C for 30 min in a mini-incubator in a fume hood.
- 15 Wash 1x with 5 mL Sample Prep Wash Buffer, incubate ~2 min.
- 16 Proceed immediately to the next step.

Gel Embedding

- 17 Clean slide (PN 2947-75X50) with H₂O and RNase-ZAP (Fisher, AM9782) and wipe with Kimwipe.
- 18 Spray with 70% EtOH (RNase-free) and wipe clean.
- 19 Add 400 µl of GelSlick (Lonza, 50640) to the slides.
- 20 Spread evenly with a Kimwipe to coat the entire surface. Wipe dry with Kimwipe.

- 21 Aliquot 5 mL of Gel Embedding Premix into 15ml conical tube. Place it in the vacuum chamber with the screw cap or lid of tubes open. De-gas for ~10 mins
- 22 Aliquot 1 mL of Gel Embedding Premix into a new 1.5 ml tube and leave residual 4 ml in the 15ml tube.
- 23 Prepare the 10% Ammonium persulfate (APS) (20 mg with 200 μ l nuclease-free H₂O, Sigma, A3678) and diluted 1:10 of TEMED soln. (5 μ l + 45 μ l nuclease-free H₂O, Sigma, T9281) freshly.
- 24 Aspirate the Sample Prep Wash Buffer from the samples. Wash with 4ml with added TEMED/APS (7ul 1:10TEMED, 7ul 10% APS to 4ml) for at least 5 min.
- 25 While the coverslip is washing, prepare the polymerizing agents into 1 mL of Gel Embedding Premix (contains beads) solution by adding 5 μ l diluted TEMED and 7 μ l of 10% APS soln.
- 26 Mix thoroughly and quickly drop the 300 μ l of 4% acrylamide gel soln onto the center of each coated glass slide from STEP 4.
- 27 Transfer the coverslip from dilute gel solution using forceps and dry thoroughly but quickly by touching sides to Kimwipe. Invert directly onto the center of 4% acrylamide gel solution as evenly as possible. Avoid Air Bubbles!
- 28 Use Kimwipe along edges of the slide to wick away gel soln until very little soln comes off on Kimwipe. This step will affect the gel thickness. Incubate at RT for 1.5 hrs. (Keep the remaining 4 % acrylamide gel soln. at RT to ensure polymerization occurs during the incubation.)
- 29 After 1.5 hours add fresh 2xSSC on top of the glass and allow to sit for 5 min to rehydrate gel.
- 30 Carefully use a razor to remove coverslip from glass and after inspecting gel place in new dish with 2x SSC. Wash then immediately proceed to digestion and clearing.

Clearing – Resistant Fresh Frozen

- 31 Prepare Digestion Mix:
 - Pull Digestion Premix ~5 mins before use to thaw.
 - 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.

Digestion Mix	1 sample	5 samples	10 samples
Digestion Premix	200 µL	1 mL	2 mL
RNase inhibitor (NEB, M0314L)	5 µL	25 µL	50 µL

32 Aspirate to dry the MERSCOPE Slide without touching the gel. Add 200uL Digestion Mix onto the gel.

33 Incubate at room temperature for a minimum of 2 h and a maximum of 6 h.

34 Warm Clearing Premix 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	5 mL	25 mL	50 mL
Proteinase K (NEB, P8107S)	50 µL	250 µL	500 µL

35 Aspirate the Digestion Mix. Add 5 mL Clearing Solution.

36 Place the lid on the petri dish and spray the outside with 70% ethanol to sterilize.

37 Seal the petri dish with parafilm very well (twice) and place in a humidified 47°C cell culture incubator overnight. DO NOT incubate at 47°C >24 h otherwise the RNA will begin to degrade.

38 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.

39 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



- 40 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

Autofluorescence Quenching

- 41 Do this step in the clearing solution.
Place the parafilm-sealed petri dish under the MERSCOPE Photobleacher. ENSURE there are no labels/writing/other items on the lid that may block the light.
- 42 Turn on the MERSCOPE Photobleacher and leave at room temperature for 3 h after 24hr 47C clearing.

Prepare for Imaging

- 43 Run Instrument Wash following Vizgen protocol.
- 44 Prepare a 37°C water bath with 2 cm of water in the bath. Remove a 500 gene MERSCOPE Imaging Cartridge (PN 10400003) from -20°C storage, remove the outer packaging, and thaw in the 37°C water bath for at least 1.5 hrs. DO NOT allow the valve to come into contact with the water.
- 45 Note down the MERSCOPE Imaging barcode number in case it must be entered manually.
- 46 Ensure the applicable panel-specific MERSCOPE Codebook is available. It may be imported from local storage or the Vizgen Cloud.
- 47 Ensure the MERSCOPE Instrument waste bottle is empty before starting a run.

Stain the MERSCOPE Slide with DAPI and PolyT Stain

- 48 Aspirate the Clearing Solution (from sample preparation). Wash 2x with 5 mL Sample Prep Wash Buffer.
- Residual SDS in the gel will cause strong autofluorescence and therefore adequate washing is important.
- Each wash allowed to sit for 5 minutes, DAPI and PolyT warmed while washing occurred

- 49 Warm up the DAPI and PolyT Staining Reagent for 10 min in a 37°C water bath. Gently shake the tube to ensure the reagents are well mixed, and no precipitate is visible.
- 50 Add 5 mL DAPI and PolyT Staining Reagent, incubate 10 min on a rocker.
 - Protect from light at all times during staining and after the sample is mounted in the MERSCOPE Flow chamber, until loaded in the MERSCOPE Instrument.
- 51 Wash 1x with 5 mL Formamide Wash Buffer, incubate 10 min.
 - Formamide Wash Buffer is hazardous. Perform these steps in a fume hood.
- 52 Wash 1x with 5 mL Sample Prep Wash Buffer, incubate 5 min.
- 53 Proceed immediately to the next step.

Configure the Experiment - Start MERFISH

- 54 Enter experimental details (name, description).
- 55 Select the applicable panel-specific MERSCOPE Codebook (may be imported from local storage or the Vizgen Cloud).
- 56 Specify sample thickness. If sample thickness is unknown, 10 µm should be selected.
- 57 Enter details of any additional auxiliary staining that was performed on the sample that should be imaged in addition to the MERFISH measurement.
 - DAPI and PolyT stains are always imaged automatically.

Load - MERSCOPE Imaging Cartridge Activation and Loading

- 58 Remove the MERSCOPE Imaging Cartridge from the previous run from the MERSCOPE Instrument. Discard the old imaging cartridge. The imaging cartridge contains formamide and should be discarded in accordance with applicable institutional hazardous waste procedures.
- 59 Prepare Imaging Activation Mix. Keep on ice and spin down on benchtop centrifuge before use:



Imaging Activation Mix	1 sample
Imaging Activation Premix	225 µL
RNase inhibitor	90 µL

- 60 Prior to inserting the MERSCOPE Imaging Cartridge into the MERSCOPE Instrument, invert to make sure that the cartridge is thawed (difference between sound of ice and sound of foil crinkling), and wipe the cartridge to remove excess water from the bottom and sides.
- Pull off the top and wipe away any water/condensation inside the cartridge.
- 61 Prior to inserting the MERSCOPE Imaging Cartridge into the MERSCOPE Instrument, pierce the foil at the designated Cartridge Activation Port with a pipette tip.
- 62 With a 1-mL pipette, add all the Imaging Activation Mix into the MERSCOPE Imaging Cartridge by inserting the pipette tip below the level of the liquid in the cartridge. Without removing the pipette tip, change the pipette setting to 1 mL and lower and raise the pipette plunger 10 times at moderate speed to thoroughly mix the solution in the cartridge, but without introducing air bubbles.
- 63 Carefully layer 15 mL mineral oil (Sigma, 330779) in the same port, slowly adding.
- 64 Ensure the barcode on the MERSCOPE Imaging Cartridge is free of any liquid. Insert the activated imaging cartridge into the MERSCOPE Instrument with the valve toward the back and the barcode toward the front.
- 65 Close the imaging cartridge lid and click Scan Barcode.
- The MERSCOPE Instrument scans the MERSCOPE Imaging Cartridge barcode for compatibility with the imported MERSCOPE Codebook. If the barcode is valid, the MERSCOPE Instrument will automatically prime the fluidics from the MERSCOPE Imaging Cartridge.
 - If the MERSCOPE Instrument cannot read the MERSCOPE Imaging Cartridge barcode, the barcode number may be entered manually.

Load - MERSCOPE Flow Chamber

- 66 Unlock the MERSCOPE Flow Chamber from the previous run and remove from its housing in the MERSCOPE Instrument. Disconnect the fluid lines from the instrument and disassemble the flow chamber.

- 67 Clean the Gasket and Aqueduct by spraying with RNaseZap solution and wiping with a Kimwipe, followed by spraying 70% ethanol and wiping with a Kimwipe.
- 68 Place the MERSCOPE Flow Chamber base on the bench close to the sample petri dish. Gently pick up the MERSCOPE Slide with tweezers or gloved fingers and place into the base (sample gel facing up).
- 69 Assemble the MERSCOPE Flow Chamber per the diagram. Twist the top until a loud click is heard to ensure secure assembly.
- 70 Once assembled, spray the bottom of the MERSCOPE Slide with H₂O first, followed by 100% ethanol and wipe clean with a lens paper (Vizgen, 30400006). Repeat 2x more (3x total) to ensure the optical imaging surface is clean.
- 71 Connect the assembled MERSCOPE Flow Chamber fluid lines to those in the MERSCOPE Instrument per the diagram below. First connect the output line (to the left). Then connect the input line (to the right).
- 72 Wet the MERSCOPE Flow Chamber. Hold the flow chamber vertically with the output lines upwards as flow starts to pull the bubbles through the flow chamber and fluid lines.
- 73 If air bubbles are visible in the MERSCOPE Flow Chamber or input fluid line, click Pull more liquid. If/when no air bubbles remain, click Next.
 - There is enough liquid for 4 flushes, but the software will cut you off after 3. Can get around by relaunching the program.
- 74 Insert the MERSCOPE Flow Chamber into the MERSCOPE Instrument. First insert the back edge of the flow chamber under the overhangs in the stage adapter. Then, lower the chamber to lie flat within the stage adapter.
- 75 Lock the MERSCOPE Flow Chamber into place, close the flow chamber lid, and click Acquire Mosaic to acquire a low-resolution mosaic.

Select Regions of Interest - Low-Magnification Obje

- 76 Select the regions of interest to be included in the experiment using the touchscreen. Draw boundaries on the image to define the region of interest for MERFISH imaging. Once one boundary is drawn it is saved and a summary appears on the right-hand side of the screen. Drawing another boundary automatically creates a new region.
- 77 Select an existing region by clicking on it on the right-hand side of the screen. When a region is selected, hold and drag a boundary dot to change its location (to redefine the boundary). Click Done to exit out of a selected region.
- 78 The regions can be renamed at any time by touching the name.

- 79 Up to 10 regions can be selected with a total area of up to 100 mm² (1 cm²). When selections are complete, click Next.

Switching to High-Magnitude Objective

- 80 Unlock and remove the MERSCOPE Flow Chamber from its housing in the instrument but DO NOT DISCONNECT the fluid lines.
- 81 Clean the immersion oil from the high-magnification objective with lens paper.
- 82 Add 50 µL fresh immersion oil (Vizgen, 30400007) to the high-magnification objective using a pipette. Ensure there are no air bubbles in the immersion oil. If air bubbles are present, clean off the immersion oil using lens paper and repeat the application of fresh immersion oil.
- 83 Place the MERSCOPE Flow Chamber back into the MERSCOPE Instrument, lock into place, and close the flow chamber lid.
- 84 After the run completes you will get a segmentation parameter screen and there select
- Algorithm: "Watershed segment"
 - Seed Channel: "DAPI"
 - Watershed channel: "PolyT"

Cleaning

- 85 Click Clean Instrument to proceed to cleaning the MERSCOPE Instrument. The Clean process and a new experiment can begin on the instrument while imaging processing analysis of previous experiments is running in the background.
- 86 The user interface guides users through (1) fluidic line washes, (2) emptying the waste container (outlined below), and (3) cleaning the immersion oil off the high-magnification objective (with lens tissue, outlined below) after an experiment is complete. Click Next to advance through screens as operations are performed. The MERSCOPE Flow Chamber may be stored in the locked or unlocked position in the stage adapter.
- 87 Discard the waste container contents per applicable institutional hazardous waste procedures.
- The waste container must be below a certain weight for the instrument to proceed with the next run.



- 88 At the end of cleaning, click Done to return to the home page and start the next experiment.
- 89 After the experiment is completed run a wash by selecting "Maintenance" and then "Start instrument wash"
- 90 As prompted, remove the imaging cartridge, confirm the 60x objective is clean of immersion oil, and run a wash using the correct wash cartridge, filling the back right well with RNase-Free water.

Data Analysis: Filtering and Annotation

- 91 Load .h5ad file from MERSCOPE/output/{experiment_name} using SCANPY¹
- 92 Load scRNA-seq reference data.
- 93 Filter both the MERFISH and scRNA-seq cell by gene data as follows:
 1. Remove cells with less than 5 counts (raw counts/no normalization)
 2. Remove cells with less than 3 genes
- 94 Concatenate both MERFISH and scRNA-seq data together, hence "combined data". We used the function `scanpy.AnnData.concat()`
- 95 Using scanpy (as sc) on combined data:
 1. `sc.tl.nearestneighbors()` with 30 neighbors, and 'euclidian' distance metric
 2. `sc.tl.pca()` with default parameters
 3. (optional for visualization) `sc.tl.umap()` with default parameters
- 96 Use Harmony² integration on the combined data, using the PCA representation of the data.
- 97 Train K Nearest Neighbors Classifier (we used *scikit-learn*) on harmonized scRNA-seq data. Use the trained classifier to predict cell types of MERFISH cells.
- 98 Save predicted labels to preharmonized MERFISH data, and continue with downstream analysis as needed.

Data Analysis: BANKSY Neighborhood Analysis



- 99 Initialize BANKSY³ (*banksy.initialize_banksy.initialize_banksy()*) neighborhood analysis with the following parameters:
- `k_geom = 50`
 - `max_m = 1`
 - `nbr_weight_decay = "scaled_gaussian"`
- 100 Run *banksy.embed_banksy.generate_banksy_matrix()* with the following parameters:
- `max_m = 1`
 - `lambda` as desired
- 101 Run *banksy_utils.umap_pca.pca_umap()* with the following parameters:
- `pca_dims = 50`
 - `add_umap = True`
- 102 Run *banksy.cluster_methods.run_Leiden_partition()* with the following parameters:
- `resolutions` as desired
 - `num_nn = 50`
 - `num_iterations = -1`
 - `match_labels = True`

Data Analysis Citation:

- 103 1.) Wolf, F., Angerer, P. & Theis, F. SCANPY: large-scale single-cell gene expression data *Genome Biol* **19**, 15 (2018). <https://doi.org/10.1186/s13059-017-1382-0>
- 104 2.) Korsunsky, I., Millard, N., Fan, J. *et al.* Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat Methods* **16**, 1289–1296 (2019). <https://doi.org/10.1038/s41592-019-0619-0>
- 105 3.) Singhal, V., Chou, N., Lee, J. *et al.* BANKSY unifies cell typing and tissue domain segmentation for scalable spatial omics data analysis. *Nat Genet* **56**, 431–441 (2024). <https://doi.org/10.1038/s41588-024-01664-3>