DEMONSTRATED PROTOCOL

Methanol Fixation, Immunofluorescence Staining & Imaging for Visium Spatial Protocols

Overview

The Visium Spatial Gene Expression Solution measures the total mRNA in tissue sections and requires a Visium Spatial slide with intact tissue sections as input. Immunostaining tissue sections with fluorescently labeled antibodies enables simultaneous protein detection. This protocol outlines methanol fixation, immunofluorescence staining, and imaging of tissue for use with 10x Genomics Visium Spatial protocols. Fixed and stained tissue sections are inputs for the downstream Visium Spatial Tissue Optimization and Visium Spatial Gene Expression workflows.

Additional Guidance

Consult the Visium Spatial Protocols - Tissue Preparation Guide (Document CG000240) for Tips & Best Practices on freezing, embedding, and cryosectioning tissue and placing sections on Visium Spatial Slides. Consult the Visium Spatial Gene Expression Imaging Guidelines (Document CG000241) to verify imaging settings prior to starting this Demonstrated Protocol. Perform this Demonstrated Protocol on tissue sections placed on the correct slide.

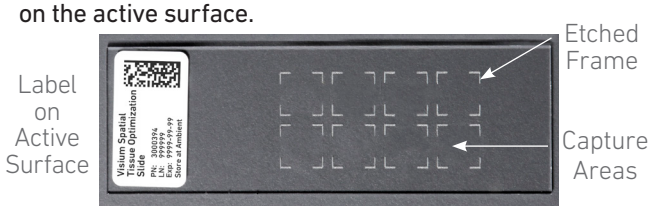
- Use a plain glass slide if optimizing antibody concentrations. Refer to the Antibody Optimization section for more information.
- Use a Visium Spatial Tissue Optimization Slide if proceeding with tissue optimization.
- Use a Visium Spatial Gene Expression Slide if proceeding with library construction.

The Tissue Optimization workflow must be performed prior to the Gene Expression workflow to determine the optimal tissue section permeabilization time. Permeabilization times identified using the H&E staining protocol may not be applicable to immunofluorescence staining. After completing this protocol, proceed with either the Visium Spatial Gene Expression Reagent Kits - Tissue Optimization User Guide (CG000238) or the Visium Spatial Gene Expression Reagent Kits User Guide (CG000239).

Visium Slide Selection

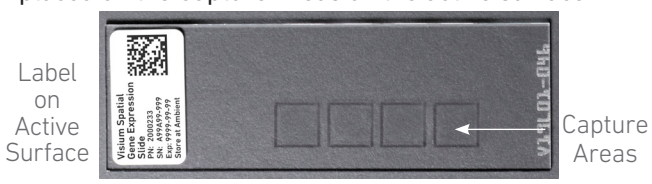
Visium Spatial Tissue Optimization Slide (PN-3000394)

- Used with Visium Spatial Gene Expression Reagent Kits – Tissue Optimization User Guide (CG000238) to identify optimal permeabilization time for a specific tissue type and section thickness.
- Includes 8 Capture Areas, each covered with oligonucleotides for mRNA capture.
- Each Capture Area is 8 x 8 mm and is surrounded by an etched frame.
- A readable label defines the active surface of the slide. Tissue sections are always placed on the Capture Areas on the active surface.



Visium Spatial Gene Expression Slide (PN-2000233)

- Used with Visium Spatial Gene Expression Reagent Kits User Guide (CG000239) to generate Visium Spatial Gene Expression libraries.
- Includes 4 Capture Areas, each with ~5,000 unique gene expression spots.
- Each Capture Area is 6.5 x 6.5 mm and is surrounded by a fiducial frame for a total area of 8 x 8 mm.
- A readable label with a serial number defines the active surface of the slide. Tissue sections are always placed on the Capture Areas on the active surface.



Visium Spatial Reagent Kits

Ensure that tissue sections have been placed onto the appropriate slide prior to starting this Demonstrated Protocol. Consult the Visium Spatial Protocols - Tissue Preparation Guide (CG000240) for more information.

Visium Spatial Tissue Optimization Slide Kit PN-1000191 (store at ambient temperature)

Visium
Spatial Tissue Optimization Slide Kit

	PN
Visium Spatial Tissue Optimization Slide	3000394
*Slide Seals	3000279
Slide Cassette	3000406
Slide Gasket	3000426
*Tissue Removal Buffer	2000221
*Tissue Removal Enzyme	3000387

*Not used in this protocol

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Visium Spatial Gene Expression Slide Kit, 16 rxns PN-1000185, 4 rxns PN-1000188 (store at ambient temperature)

Visium
Spatial Gene Expression Slide Kit

	PN
Visium Spatial Gene Expression Slide	2000233
*Slide Seal	3000279
Slide Cassette	3000406
Slide Gasket	3000426

*Not used in this protocol

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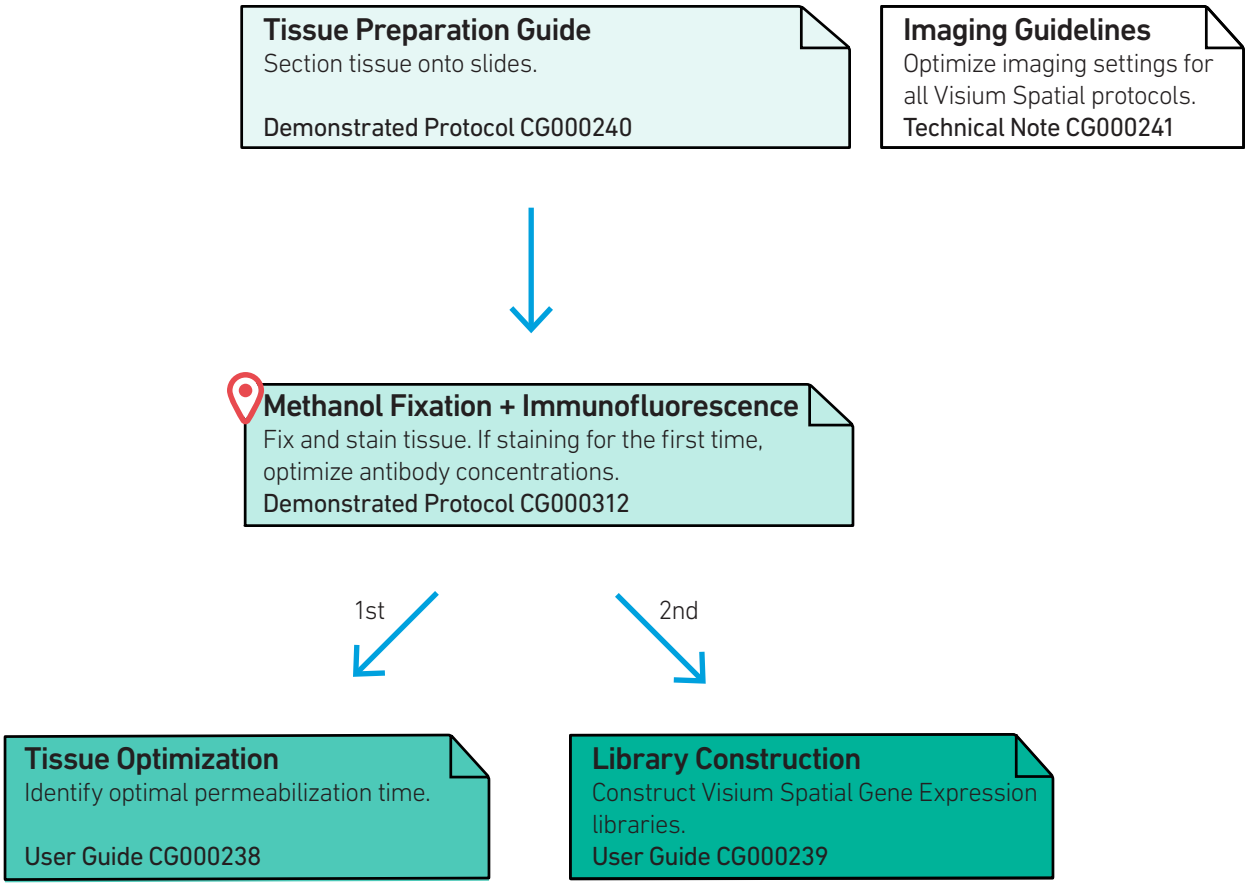
Visium Accessories

Product	Part Number (Kit)	Part Number (Item)
Thermocycler Adaptor	1000194	3000380
Visium Imaging Test Slide		2000235
Slide Alignment Tool		3000433

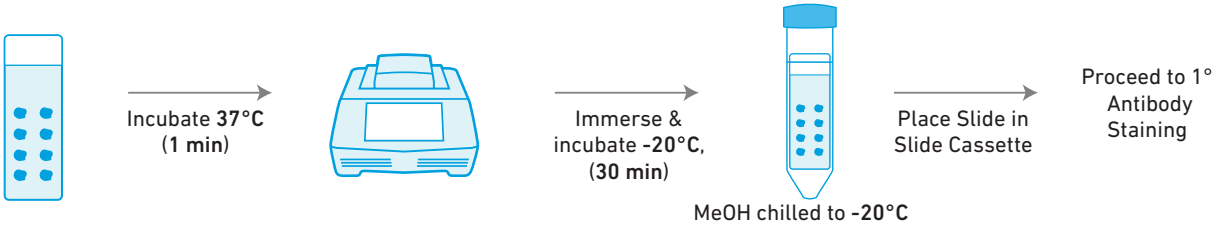
Workflow Overview



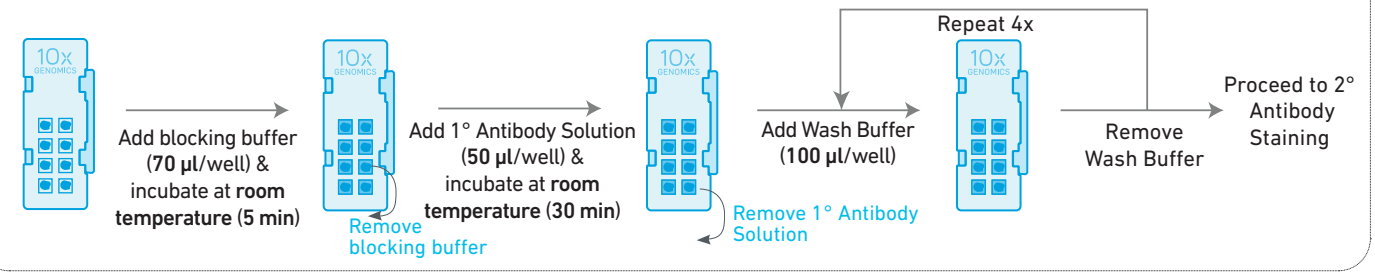
Visit the 10x Genomics Support website for the most current documentation.

Protocol Overview

Fixation 35 min

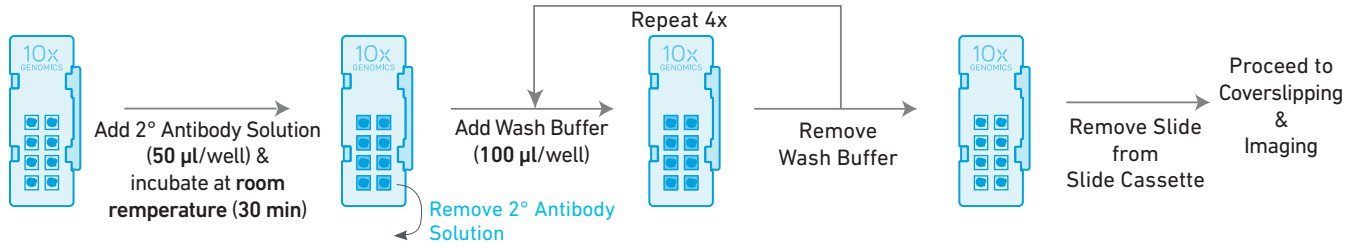


Primary Antibody Staining 45 min

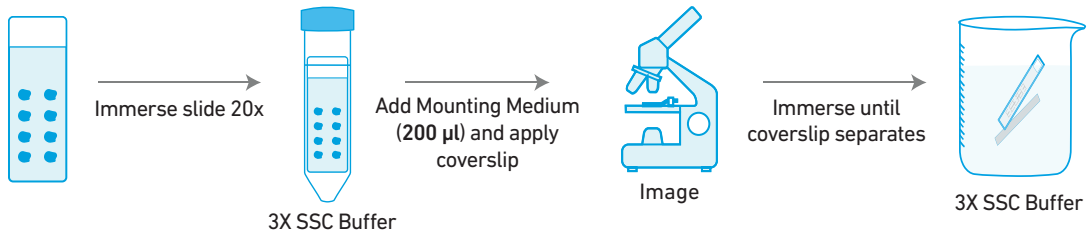


Secondary Antibody Staining 45 min

(Skip if using fluorophore conjugated primary antibodies)



Coverslipping & Fluorescent Imaging 15 min*



*Time excludes imaging steps

Proceed to 10x Genomics Visium Spatial Protocols

Tips & Best Practices

Icons

TIPS		
Tips & Best Practices section includes additional guidance	Signifies critical step requiring accurate execution	Troubleshooting section includes additional guidance

General Reagent Handling

- Thoroughly mix reagents before use.
- Keep all enzymes, buffers and Master Mixes on ice during setup and use. Promptly move reagents back to the recommended storage.

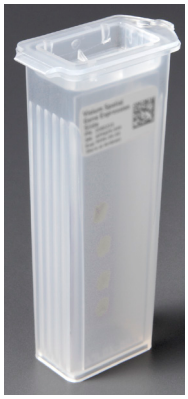
Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Slide Storage

- Always store unused slides in a cool, dry environment.
- Store unused slides in original packaging and keep sealed. DO NOT remove dessicant. If necessary, place the sealed container in a secondary container, such as a resealable bag.
- After tissue placement, store the slides at **–80°C** in a sealed container for up to **4 weeks**.

Slide Storage

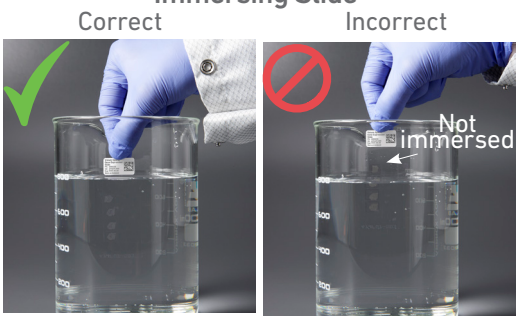


Slide Handling

- Always wear gloves when handling slides.
- Ensure that the active surface of a slide faces up and is never touched. The orientation of the label on the slide defines the active surface.
- The tissue sections should always be on the active surface of the slide. DO NOT touch the tissue sections.
- Minimize exposure of the slides to sources of particles and fibers.
- When immersing slides in 3X SSC, ensure that the tissue sections are completely submerged.
- Keep the slide flat on a clean work surface when adding reagents to the active surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.

Active Surface with Tissue Sections

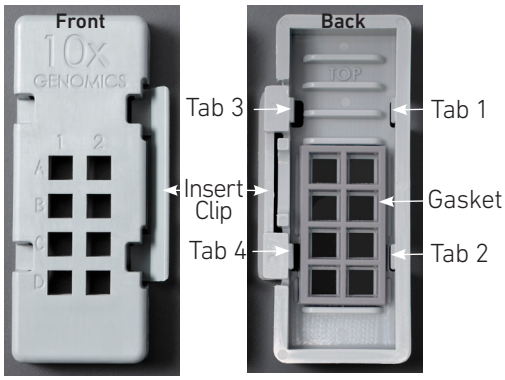
Immersing Slide



Slide Cassette

- The Slide Cassette encases the slide and creates leakproof wells for adding reagents.
- Place the slides in the Slide Cassette only when specified.
- The Slide Cassette may be reused once. Refer to the Slide Cassette and Gasket Cleaning section for instructions.
- An Insert Clip and four tabs at the back of the Slide Cassette are used for holding the slide in the cassette, as shown.
- The cassette includes a reusable gasket corresponding to the Capture Areas on the slides.
- The Slide Cassette may be assembled using the Slide Alignment Tool or manually. Instructions for both are provided in the following section.
- See Slide Cassette Assembly & Removal instructions for details.
- Ensure that the back of the Slide Cassette is facing the user prior to assembly. The active surface of the slide with tissue sections will face down such that the slide label is no longer readable.
- Practice assembly with a 75 x 25 x 1 mm plain glass slide.

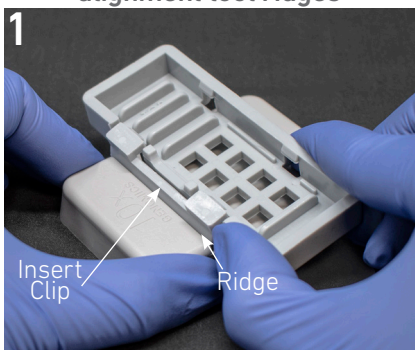
Slide Cassette



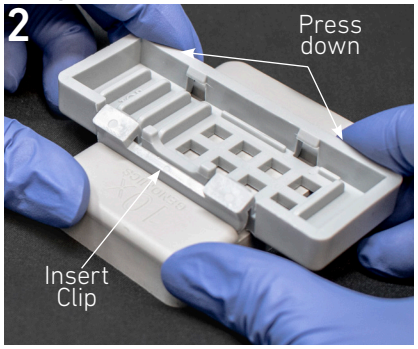
Slide Alignment Tool

Slide Cassette Assembly

Position Slide Cassette along alignment tool ridges

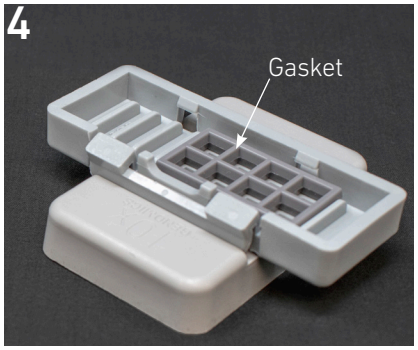


Push Insert Clip along the ridge & press Slide Cassette down

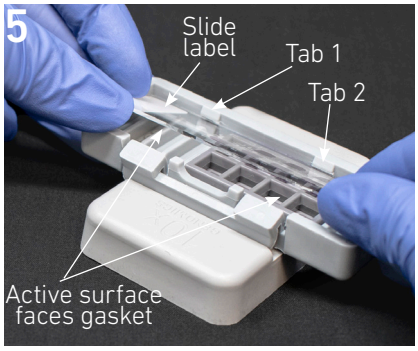


Slide Cassette secured on alignment tool

Position gasket to align with Slide assette cutouts



Insert long edge of slide under tabs 1 & 2; ensure slide is flush



Press slide down until it is flush with the gasket and under tabs 3 & 4

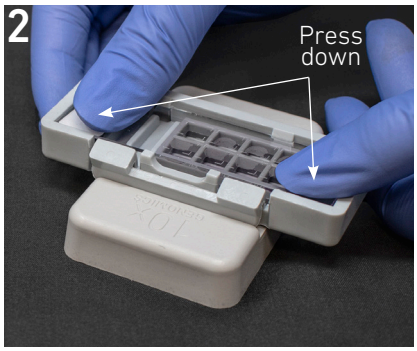
Remove Slide Cassette while pressing slide against the gasket

⚠ Slide insertion may push gasket out of alignment with slide cutouts. Adjust if necessary.

Slide Cassette Removal

Position Slide Cassette along alignment tool ridges

Push Insert Clip along the ridge & press down



Slide Cassette Sits securely on alignment tool

Lift slide at Slide Cassette groove

Manual Slide Cassette Assembly & Removal

Assembly

i. Remove the gasket from the Slide Cassette and re-insert the gasket, ensuring that the gasket and Slide Cassette cutouts are aligned.

ii. Align the label on top of the slide to the top of the Slide Cassette, as shown.

iii. Insert the slide under tabs 1 and 2. Ensure that the long edge of the slide is flush with the side of the Slide Cassette.

iv. Press the insert clip **very firmly** by applying even force on the lower part of the insert clip.

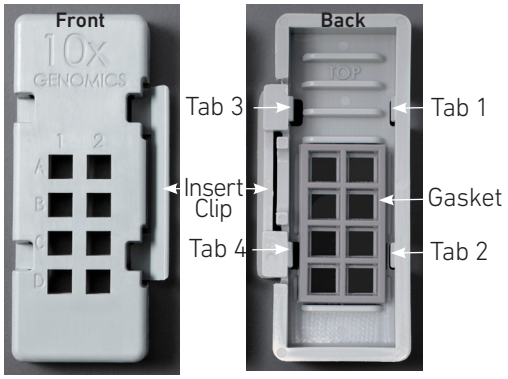
v. Place a finger in between tab 3 and the top of the cassette, and one finger between tab 4 and the bottom of the cassette. Press down on the slide evenly until the slide is under each tab and release the insert clip.

Removal

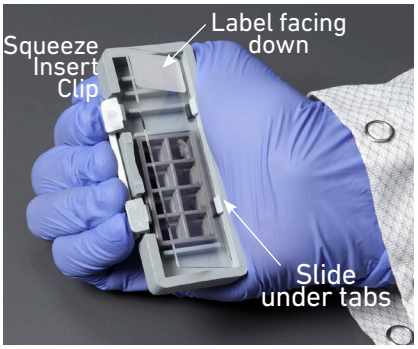
i. Press the insert clip **very firmly** to release the slide from the cassette.

ii. Lift slide at Slide Cassette groove between tabs 3 and 4 until the slide can be removed.

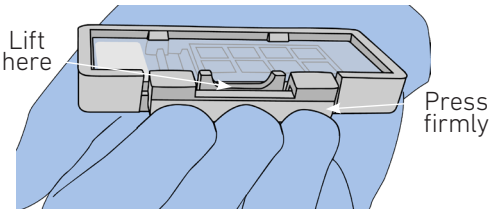
Slide Cassette



Slide Cassette Assembly



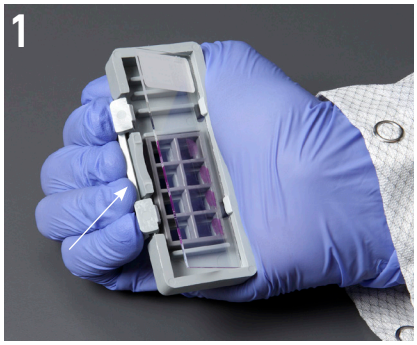
Insert Clip - Press Firmly



Slide Cassette & Gasket Cleaning

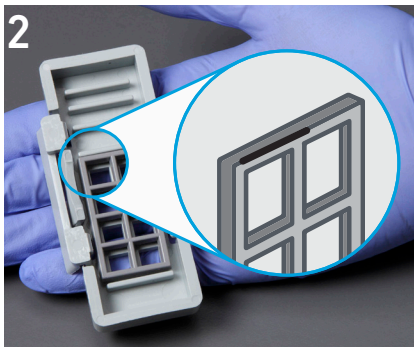
Remove slide from Slide Cassette

1



Mark the top portion of the gasket that faced the slide with an alcohol resistant marker

2



Remove gasket

3

Rinse Slide Cassette and gasket with ultrapure water

4

Spray with 70% isopropanol, then rinse with ultrapure water

5

Spray with 70% isopropanol a second time, then rinse with ultrapure water

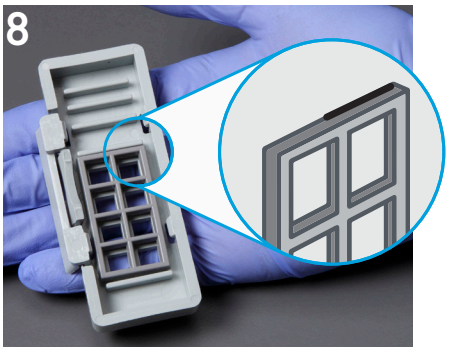
6

Air dry

7

Re-insert the gasket such that the marked portion is at the top of the Slide Cassette and now faces the Slide Cassette.

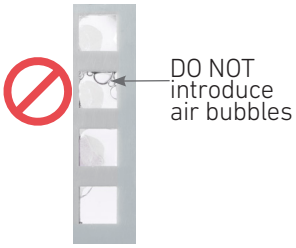
8



Reagent Addition & Removal from Wells

- Place the assembled slide in the Slide Cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the wells without touching the tissue sections and without introducing bubbles.
- Always cover the tissue section completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.
- Pipette reagent carefully so that tissue is not disrupted or dislodged.
- Ensure that no bubbles are introduced in the process.

Reagent Addition/Removal



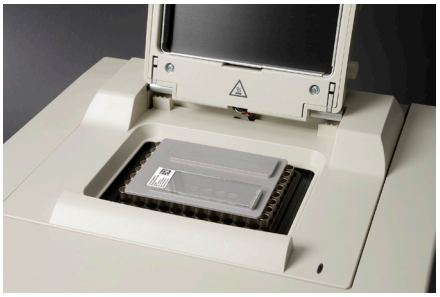
Slide Incubation Guidance

Incubation at a specified temperature

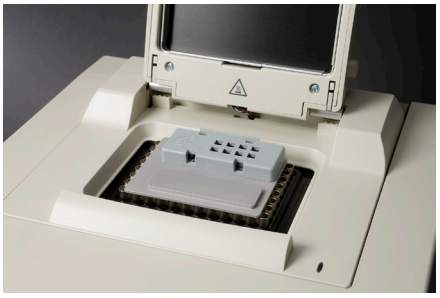
- Position a Thermocycler Adaptor on a thermal cycler that is set at the incubation temperature.
- Ensure that the Thermocycler Adaptor is in contact with the thermal cycler surface uniformly.
- When incubating a slide, position the slide on the Thermocycler Adaptor with the active surface facing up.
- Ensure that the entire bottom surface of the slide is in contact with Thermocycler Adaptor.
- When incubating a slide encased in a Slide Cassette, place the assembled unit on the Thermocycler Adaptor with the wells facing up. The Slide Cassette should always be sealed when on the Thermocycler Adaptor.

Place Thermocycler Adaptor

Incubate Slide



Incubate Assembled Slide Cassette

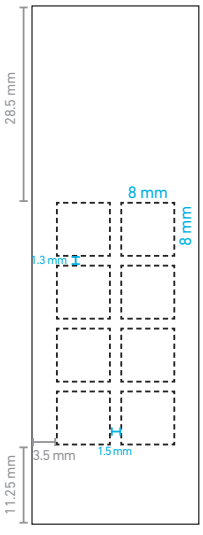


Antibody Optimization

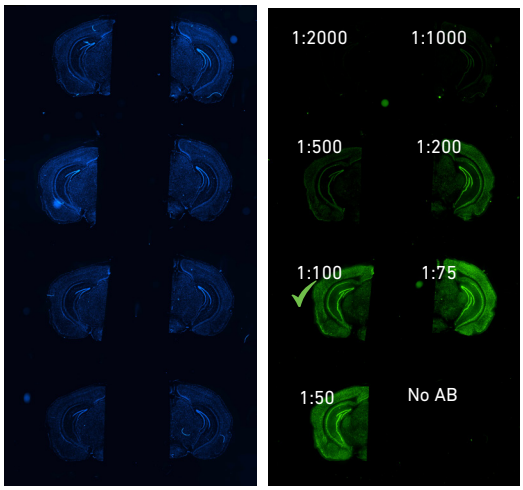
Optimization of antibody concentration is critical for executing this Demonstrated Protocol.

- Optimal antibody concentrations for this Demonstrated Protocol may differ from other applications due to shortened antibody incubation periods. Composition of reagents and fixatives may differ from other immunofluorescence applications.
- To optimize antibody concentration, draw representative frames on the back of a 75 x 25 x 1 mm plain glass slide using the example slide layout.
- Place tissue sections in the frames on the front of the slide for compatibility with the slide cassette. Ensure that tissue sections used during optimization are similar in size to tissue sections used for Tissue Optimization and Gene Expression Protocols
- Primary antibody: Execute the Tissue Fixation & Immunofluorescence Staining protocol (steps 1.0-1.5) using a range of primary antibody concentrations. A starting concentration of **0.01 µg/µl** (**0.5 µg**/sample) is recommended.
- Secondary antibody: Use a similar range of concentrations for secondary antibodies or refer to the manufacturer's recommended dilution.
- Select the antibody concentration that results in the specific staining of desired cells, while minimizing nonspecific background staining.
- Wash Slide Cassette and gasket after antibody staining. See Tips & Best Practices for more information.
- An example dilution layout is provided below. DAPI image is provided to show presence of tissue for each antibody dilution. Dilutions are of recombinant Anti-NeuN antibody conjugated to Alexa Fluor 488 (Abcam, PN:190195, 0.5 mg/ml).

Example Slide Layout



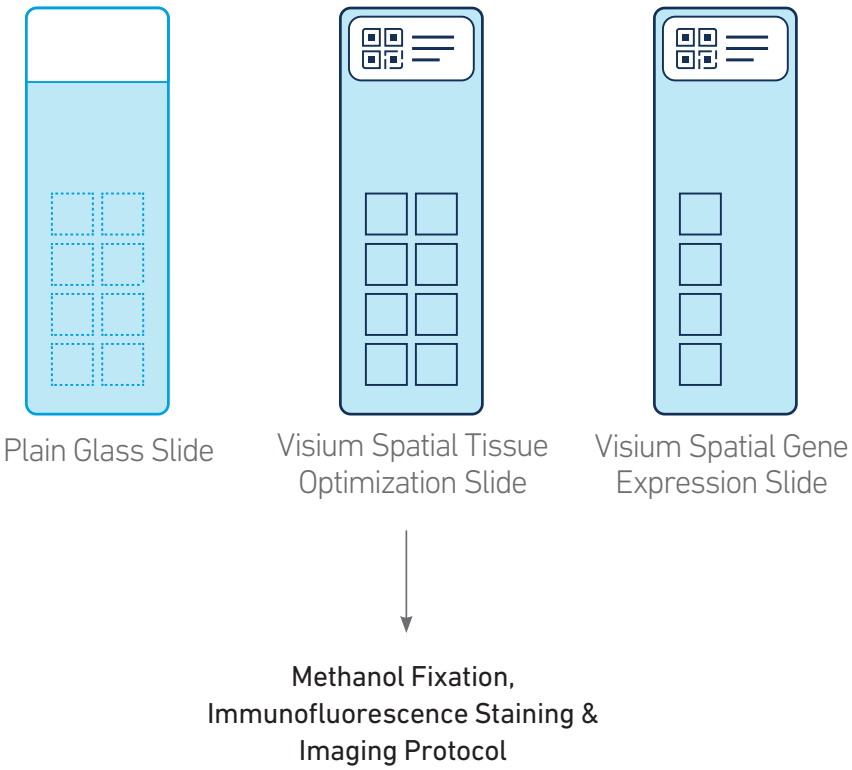
Example fluorophore conjugated primary antibody dilution series.



Tissue Fixation & Immunofluorescence Staining

1.0 Overview

Ensure that this protocol is performed on tissue sections on the correct slide. Refer to the Introduction, Workflow Overview, and Antibody Optimization sections for more information.



Ensure that microscope settings have been verified and imaging programs have been created prior to starting this protocol. Consult the Visium Spatial Gene Expression Imaging Guidelines Technical Note (CG000241) for more information.

If proceeding to Tissue Optimization, DO NOT add buffers or staining reagents to well A1, which is reserved for the positive RNA control. Addition of these reagents to this well may prevent visualization of the positive control.

If staining using fluorophore conjugated primary antibodies, proceed directly to step 1.4 after completing step 1.2.

1.1 Specific Reagents & Consumables

The items in the table below have been validated by 10x Genomics and are highly recommended for Visium Spatial Reagent Kits protocols. Substituting materials may adversely affect system performance. This list does not include standard laboratory equipment, such as water baths, centrifuges, pH meters, vortex mixers, freezers, etc.

Ensure that tissue sections have been placed on the appropriate slide and stored at -80°C. The slide will be retrieved in step 1.2.

Tissue Fixation		
Vendor	**Item**	**Part Number**
Millipore Sigma	Methanol, for HPLC, ≥99.9%	34860
Tissue IF Staining		
Vendor	**Item**	**Part Number**
Corning	Self-Standing Polypropylene Centrifuge Tubes, 50 ml, sterile	430921
Sigma Aldrich	Triton X-100	93443-100ML
Thermo Fisher Scientific	Thermo Scientific Signature Series Cover Glasses DAPI Solution RiboLock RNase Inhibitor (Alternative to Millipore Sigma product)	22-050-233 62248 EO0382
Millipore Sigma	SSC Buffer 20x Concentration Protector RNase Inhibitor	S6639L 3335402001
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution) (Alternatively, use any species appropriate Fc blocking solution) TruStain FcX (anti-mouse CD16/32) Antibody	422301 101309
New England Biolabs	Ribonucleoside Vanadyl Complex RNase Inhibitor, Murine (Alternative to Millipore Sigma product)	S1402S M0314L
Invitrogen	Alexa Fluor 488 Phalloidin (Optional) Alexa Fluor 594 Phalloidin (Optional) Alexa Fluor 647 Phalloidin (Optional)	A12379 A12381 A22287
-	Primary Antibodies	-
-	Secondary Antibodies	-
Additional Materials		
-	Dry Ice	-
-	Glycerol	-
-	Isopropanol	-
-	Ultrapure/Milli-Q water (from Milli-Q Integral Ultrapure Water System or equivalent)	-
-	Forceps	-

Recommended Thermal Cyclers

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197
Eppendorf	MasterCycler Pro (discontinued)	North America 950030010 International 6321 000.019
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler	4375786

Preparation - Buffers

Prepare	
Methanol	• Dispense 40 ml/slide in a 50-ml centrifuge tube. Chill to **-20°C** before use.

2X Blocking Buffer (prepare on ice)

2X Blocking Buffer (prepare fresh on ice)	Stock	Final	4X + 20% (µl)	8X + 20% (µl)
Nuclease-free Water			673.4 µl	1,346.9 µl
SSC Buffer	20X	6X	878.4 µl	1,756.8 µl
BSA	10%	4%	1,171.2 µl	2,342.4 µl
Triton X-100	10%	0.2%	58.6 µl	117.7 µl
RNase Inhibitor	40 U/µl	2 U/µl	146.4 µl	292.8 µl

3X SSC

3X SSC	Stock	Final	550 ml (1 slide)
SSC Buffer	20X	3X	82.5 ml
Ultrapure Water	-	-	467.5 ml

Mounting Medium

Mounting Medium	Stock	Final	200 µl (1 slide)
Glycerol	100%	85%	170 µl
RNase Inhibitor	40 U/µl	2 U/µl	10 µl
Nuclease-free Water			20 µl

1.2 Tissue Fixation

If using a Visium Spatial Gene Expression Slide, note the serial number on the slide label; will be required for downstream analysis.

Ensure that the methanol (40 ml/slide) dispensed in a 50-ml centrifuge tube is chilled to **-20°C**.

a. Place a Thermocycler Adaptor on a thermal cycler set at **37°C** and equilibrate for **5 min**. Heating the thermal cycler lid is not required.

b. Remove slide from **-80°C** and place on dry ice in a sealed container.

Delay in transferring slides to dry ice may result in condensation, which may cause tissue damage and/or shifting of tissue sections on the slide.

c. Place slide on the Thermocycler Adaptor with the active surface facing up and incubate **1 min** at **37°C**. DO NOT close the thermal cycler lid. Maintain thermal cycler at **37°C** for step 1.2.

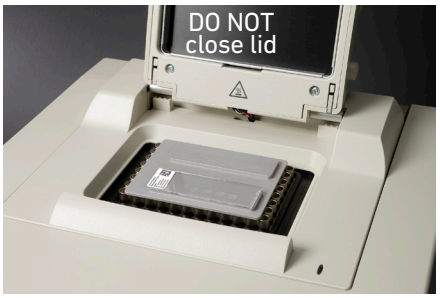
d. Remove slide from Thermocycler Adaptor and if necessary, wipe excess liquid from the back of the slide, without touching the tissue sections.

e. Completely immerse the slide in the pre-chilled methanol. Secure the tube cap to prevent methanol loss.

f. Incubate **30 min** at **-20°C**.

Place Thermocycler Adaptor

Incubate Slide for 1 min at 37°C



Incubate in Methanol for 30 min at –20°C

1.3 Immunofluorescence Staining - Primary and Secondary Antibodies

TIPS

If using fluorophore conjugated primary antibodies, proceed directly to step 1.4

Ensure that tissue sections do not dry out throughout the staining protocol

a. Place the slide in the Slide Cassette. See Tips & Best Practices for assembly instructions. Practice assembly with a blank slide.

b. Prepare 1X Blocking Buffer on ice.

1X Blocking Buffer *Vortex.*	1X (µl)	4X + 15% (µl)	8X + 15% (µl)
2X Blocking Buffer	35.0	161.0	322.0
Nuclease-free Water	26.0	119.6	239.2
Human or Mouse TruStain FcX*	2.0	9.2	18.4
Ribonucleoside Vanadyl Complex	7.0	32.2	64.4
Total	**70.0**	**322.0**	**644.0**

*Choose FC blocking reagent based on sample species.

c. Prepare Wash Buffer on ice.

Wash Buffer *Vortex.*	1X (µl)	4X + 15% (µl)	8X + 15% (µl)
2X Blocking Buffer	525	2,415	4,830
Nuclease-free Water	420	1,932	3,864
Ribonucleoside Vanadyl Complex	105	483	966
Total	**1,050**	**4,830**	**9,660**

d. Vortex 1X Blocking Buffer and add **70 µl** along the side of the wells to uniformly cover the tissue sections, without introducing bubbles. Tap gently to ensure even coverage.

e. Incubate for **5 min** at **room temperature**.

f. Prepare Primary Antibody Solution on ice. Pipette mix.

Primary Antibody Solution. *Vortex, centrifuge briefly.*	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
2X Blocking Buffer	25.00	110.00	220.00
Nuclease-free Water	Variable	Variable	Variable
Primary Antibody #1	Variable	Variable	Variable
Primary Antibody #2 (optional)	Variable	Variable	Variable
Primary Antibody #3 (optional)	Variable	Variable	Variable
RNase Inhibitor	6.75	29.70	59.40
Total	**50.00**	**220.00**	**440.00**

Antibody volumes will depend on concentrations determined during antibody optimization. Add an appropriate volume of nuclease-free water based on the amount of added antibody to achieve the stated total volume.

g. Remove 1X Blocking Buffer.

h. Add **50 µl** Primary Antibody Solution along the side of each well. Tap gently to ensure uniform coverage.

i. Incubate for **30 min** at **room temperature.**

j. Prepare Secondary Antibody Solution on ice. Pipette mix. Avoid light exposure.

Secondary Antibody Solution. *Vortex, centrifuge briefly.*	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
2X Blocking Buffer	25.00	110.00	220.00
Nuclease-free Water	Variable	Variable	Variable
Secondary Antibody #1	Variable	Variable	Variable
Secondary Antibody #2 (optional)	Variable	Variable	Variable
Secondary Antibody #3 (optional)	Variable	Variable	Variable
DAPI	0.17	0.75	1.50
Phalloidin (optional)	0.05	0.22	0.44
RNase Inhibitor	6.75	29.70	59.40
Total	**50.00**	**220.00**	**440.00**

Antibody volumes will depend on concentrations determined during antibody optimization. Add an appropriate volume of nuclease-free water based on the amount of added antibody and phalloidin to achieve the stated final volume.

k. Remove the Primary Antibody Solution.

l. Add **100 µl** Wash Buffer along the side of each well.

m. Remove Wash Buffer.

n. **Repeat** steps k and l four more times for a total of five washes.

o. Add **50 µl** Secondary Antibody Solution along the side of each well. Tap gently to ensure uniform coverage.

p. Incubate for **30 min** at **room temperature**. Avoid light exposure.

q. Remove the Secondary Antibody Solution.

r. Add **100 µl** Wash Buffer along the side of each well.

s. Remove Wash Buffer.

t. **Repeat** steps q and r four more times for a total of five washes. Save **50 µl** Wash Buffer per sample for step 2.2.

u. Proceed **immediately** to step 1.5

Ribonucleoside Vanadyl Complex settles rapidly in solution. Briefly vortex Wash Buffer before each wash.

1.4 Immunofluorescence Staining - Fluorophore Conjugated Primary Antibodies

TIPS

a. Place the slide in the Slide Cassette. See Tips & Best Practices for assembly instructions. Practice assembly with a blank slide.

b. Prepare 1X Blocking Buffer on ice.

1X Blocking Buffer *Vortex.*	1X (µl)	4X + 15% (µl)	8X + 15% (µl)
2X Blocking Buffer	35.0	161.0	322.0
Nuclease-free Water	26.0	119.6	239.2
Human or Mouse TruStain FcX*	2.0	9.2	18.4
Ribonucleoside Vanadyl Complex	7.0	32.2	64.4
Total	**70.0**	**322.0**	**644.0**

*Choose FC blocking reagent based on sample species.

c. Prepare Wash Buffer on ice.

Wash Buffer *Vortex.*	1X (µl)	4X + 15% (µl)	8X + 15% (µl)
2X Blocking Buffer	275	1,265	2,530
Nuclease-free Water	220	1,012	2,024
Ribonucleoside Vanadyl Complex	55	253	506
Total	**550**	**2,530**	**5,060**

Ensure that tissue sections do not dry out throughout the staining protocol

d. Vortex 1X Blocking Buffer and add **70 µl** along the side of the wells to uniformly cover the tissue sections, without introducing bubbles. Tap gently to ensure even coverage.

e. Incubate for **5 min** at **room temperature**.

f. Prepare Primary Antibody Solution on ice. Pipette mix. Avoid light exposure.

Primary Antibody Solution. *Vortex, centrifuge briefly.*	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
2X Blocking Buffer	25.00	110.00	220.00
Nuclease-free Water	Variable	Variable	Variable
Primary Antibody #1	Variable	Variable	Variable
Primary Antibody #2 (optional)	Variable	Variable	Variable
Primary Antibody #3 (optional)	Variable	Variable	Variable
DAPI	0.17	0.75	1.50
Phalloidin (optional)	0.05	0.22	0.44
RNase Inhibitor	6.75	29.70	59.40
Total	**50.00**	**220.00**	**440.00**

Antibody volumes will depend on concentrations determined during antibody optimization. Add an appropriate volume of nuclease-free water based on the amount of added antibody and phalloidin to achieve the stated final volume.

g. Remove 1X Blocking Buffer.

h. Add **50 µl** Primary Antibody Solution along the side of each well. Tap gently to ensure even coverage.

i. Incubate for **30 min** at **room temperature.** Avoid light exposure.

j. Remove the Primary Antibody Solution.

k. Add **100 µl** Wash Buffer along the side of each well.

l. Remove Wash Buffer.

m. **Repeat** steps j and k four more times for a total of five washes. Save **50 µl** Wash Buffer per sample for step 2.2.

n. Proceed **immediately** to step 1.5

Ribonucleoside Vanadyl Complex settles rapidly in solution. Briefly vortex Wash Buffer before each wash.

1.5 Slide Mounting & Coverslip Application

a. Dispense the following volumes of 3X SSC Buffer.
50 ml in one 50-ml centrifuge tube/slide
500 ml in a beaker/2 slides.

b. Remove the slide from the Slide Cassette. See Tips & Best Practices for more information.

c. Immerse the slide 20x in the 3X SSC Buffer in the 50-ml centrifuge tube.

d. Wipe excess liquid from the back of the slide without touching the tissue section. Some droplets may remain.

e. Add **200 µl** Mounting Medium to cover the tissue sections on the slide uniformly. If necessary, hold the slide at an angle for uniform coverage.

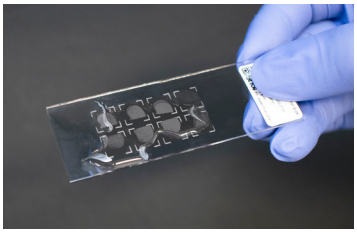
f. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, pressing down gently with forceps, without introducing bubbles.

g. Remove excess Mounting Medium by placing one long edge of the slide on a laboratory wipe, and gently tilt the slide back and forth. Repeat with the second long edge of the slide. Repeat the process until the coverslip is secured.

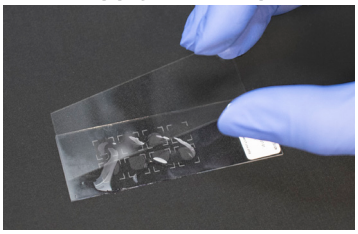
h. After the coverslip is secured, **immediately** proceed with imaging.

DO NOT let the attached coverslip dry.
DO NOT use Cytoseal or nail polish for securing the coverslip.

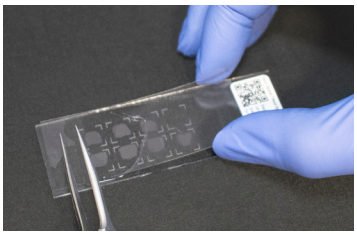
Cover uniformly with Mounting Medium



Apply coverslip



Press down



Remove excess Mounting Medium

2. Tissue Imaging

2.0 Imaging System Recommendations

2.1 Tissue Imaging

2.2 Coverslip Removal

2.0 Imaging System Recommendations

The following table shows imaging systems used by 10x Genomics in the development of this protocol. Any equivalent imaging setup can be used as an alternative. Imaging systems should have both brightfield and fluorescence capacity. Required fluorescence channels will depend on antibody selection. Exposure times will depend on antibody sensitivity and fluorophores.

Supplier	Description
Nikon	Nikon Eclipse Ti2
Molecular Devices	ImageXpress Nano Automated Cell Imaging System
Hamamatsu	NanoZoomer S60
Keyence	Keyence BZX800
BioTek	Cytation 7
Thermo Fisher Scientific	EVOS M7000
Leica	Leica DMi8 Versa 8
Fluorescence Recommended Configuration	
Light source (or equivalent) with a wavelength range of 380-680 nm	
Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution)	
DAPI filter cube (Excitation 392/23, Emission 447/60)	
FITC/GFP filter cube (Excitation 466/40, Emission 698/70)	
TRITC filter cube (Excitation 542/20, Emission 620/52)	
Cy5 filter cube (Excitation 618/50, Emission 698/70)	
2.18 µm/pixel minimum capture resolution	
Exposure times 100 milli sec-2 sec	

2.1 Tissue Imaging

- If imaging a Visium Spatial Tissue Optimization Slide or blank slide for antibody optimization, image all Capture Areas together at the desired magnification using fluorescence imaging settings.
- If imaging a Visium Spatial Gene Expression Slide, image all Capture Areas individually at the desired magnification using fluorescence imaging settings. Ensure that fiducial frames are captured.
- Images should be saved as a multi-channel image (multi-image TIFF), merged RGB image, or as individual single channel images (grayscale TIFF).
- Consult the Visium Spatial Gene Expression Imaging Guidelines Technical Note (CG000241) for additional information.

- Proceed immediately to next step if continuing with the Visium Spatial Tissue Optimization or Gene Expression workflows.

2.2 Coverslip Removal

a. Wash Slide Cassette and gasket. See Tips & Best Practices for more information.

b. Immerse the slide at ~45° angle in the 3X SSC Buffer with the coverslipped surface fully submerged and facing down.

c. Hold the slide in 3X SSC Buffer until the coverslip slowly separates away from the slide.
DO NOT move the slide up and down or shake forcibly to prevent damaging the tissue sections and the Capture Areas.

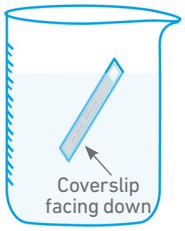
d. Remove the slide from the 3X SSC Buffer and immerse 1x in the 3X SSC Buffer to ensure all Mounting Medium is removed.

e. Place slide in a clean Slide Cassette.

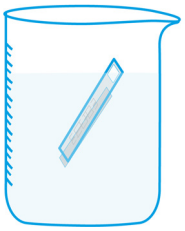
f. Add **50 µl** Wash Buffer along the side of the wells.
DO NOT add Wash Buffer to well reserved for positive RNA control if proceeding to Tissue Optimization.

g. Proceed immediately to the Visium Spatial Tissue Optimization User Guide (CG000238) or the Visium Spatial Gene Expression User Guide (CG000239).

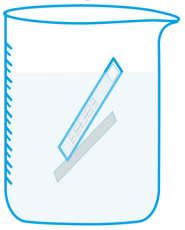
Immerse in 3X SSC Buffer



Hold in 3X SSC Buffer



Coverslip detaches



Results

Performing the Methanol Fixation, Immunofluorescence Staining & Imaging protocol will likely result in a decrease in the number of unique transcripts detected for many tissue types, as compared to the Methanol Fixation, H&E Staining & Imaging protocol (CG000160). However, this should not affect interpretation of experimental results.

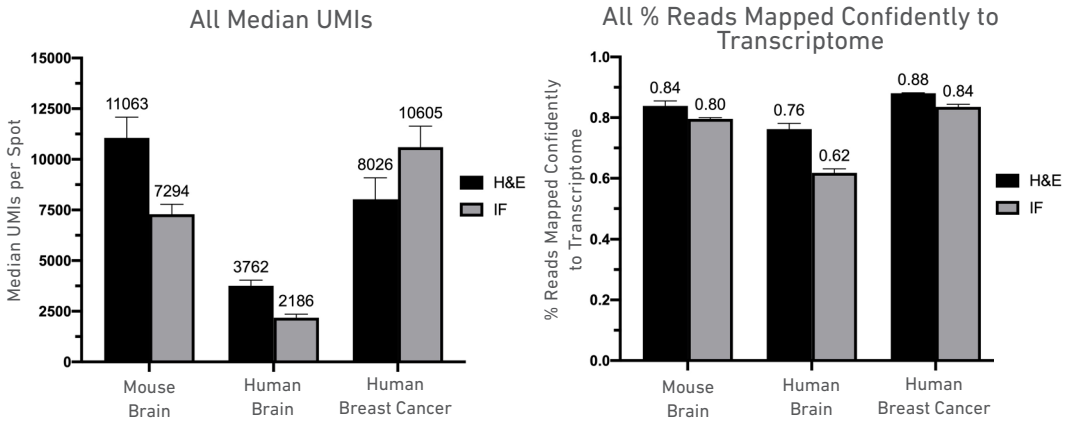


Figure 1. Median UMI counts and % reads mapped confidently to transcriptome for mouse brain, human brain, and human breast cancer tissue. Values indicate the average of 4 replicates per condition. UMI metrics are based on samples downsampled to 20K raw reads per spot.

Troubleshooting

STEP	NOTES
2.1 Weak or no signal	• Verify that samples were not exposed to light after staining with fluorescent antibodies. • Verify antibody dilutions. Ensure that antibody optimization is performed prior to immunofluorescence staining. • Verify that the correct fluorescent secondary antibody was used at the correct dilution, if applicable. • Verify imaging system filter cubes and wavelength. Ensure that fluorophores and filter cubes match. • Protein of interest may have low expression. Test antibodies on tissues of interest prior to working with Visium Spatial slides.
2.1 High background	• Verify that samples did not dry out during the staining protocol. Ensure that samples always remain covered in liquid. • To prevent non-specific antibody binding, compare chosen antibody with antibodies that target the same cell type. If possible, compare staining results to cells known to express higher or lower levels of the target protein.

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