

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 fresh frozen tissues 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.

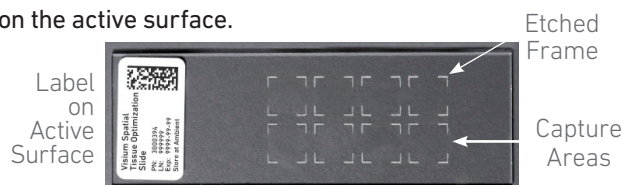
- Use a plain glass slide if optimizing antibody concentrations. Refer to the Antibody Optimization section for more information.
- Use a Visium Spatial Tissue Optimization Slide if proceeding with tissue optimization.
- Use a Visium Spatial Gene Expression Slide or Visium Gateway Gene Expression Slide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

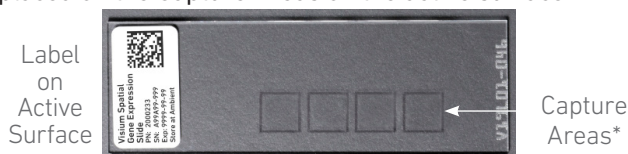
Visium Spatial Tissue Optimization Slide (PN-3000394)

- 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) Visium Gateway Gene Expression Slide (PN-2000363)

- Used with Visium Spatial Gene Expression Reagent Kits User Guide (CG000239) to generate Visium Spatial Gene Expression libraries.
- Includes 2 or 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 Gateway Gene Expression Slide contains 2 Capture Areas


Visium Spatial Reagent Kits

Perform this Demonstrated Protocol in each step on tissue sections as listed.

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 Gateway Tissue Optimization Slide Kit PN-1000313 (store at ambient temperature)

Visium Gateway Tissue Optimization Slide Kit	PN
Visium Spatial Tissue Optimization Slide	3000394
*Slide Seals	3000279
Slide Cassette	3000406
Slide Gasket	3000426
*Tissue Removal Buffer	2000378
*Tissue Removal Enzyme	3000387
*Not used in this protocol	

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

Visium Spatial Gene Expression Slide Kit	PN
Visium Spatial Gene Expression Slide	2000233
*Visium Slide Seals, 40-pack or 20-pack (may come in varying configurations in different lots)	2000284/ 3000279
Visium Slide Cassette & Gasket, 4-pack	2000282/ 2000281
*Not used in this protocol	


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Visium Spatial Reagent Kits

Perform this Demonstrated Protocol in each step on tissue sections as listed.

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

Visium Spatial Gene Expression Slide Kit	PN
Visium Spatial Gene Expression Slide	2000233
*Visium Slide Seals, 12-pack or 5-pack (may come in varying configurations in different lots)	2000283 3000279
Visium Slide Cassette & Gasket, 1-pack	2000282/ 2000281
*Not used in this protocol	

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Visium Gateway Gene Expression Slide Kit, 2 rxns PN-1000312 (store at ambient temperature)

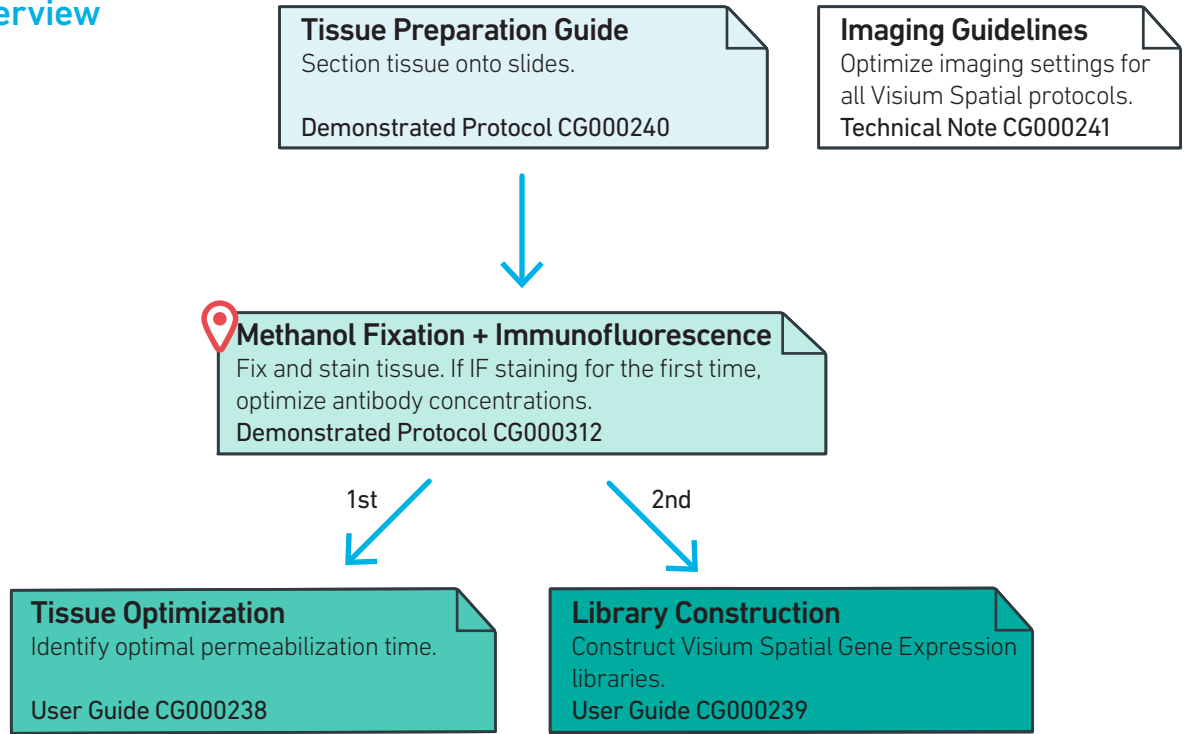
Visium Gateway Gene Expression Slide Kit	PN
Visium Gateway Gene Expression Slide	2000263
*Visium Slide Seals, 5-pack	3000279
Visium Slide Cassette & Gasket, 1-pack	2000281
*Not used in this protocol	

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10x Genomics Accessories

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

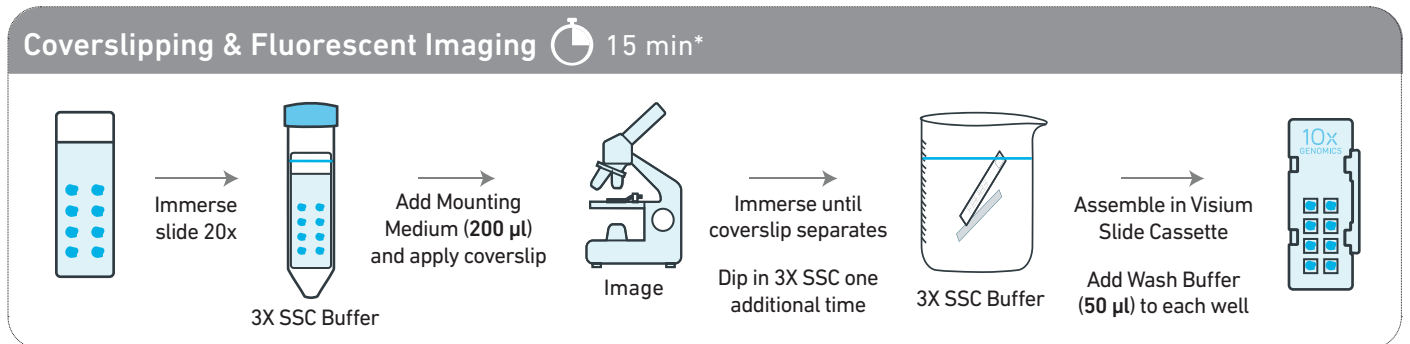
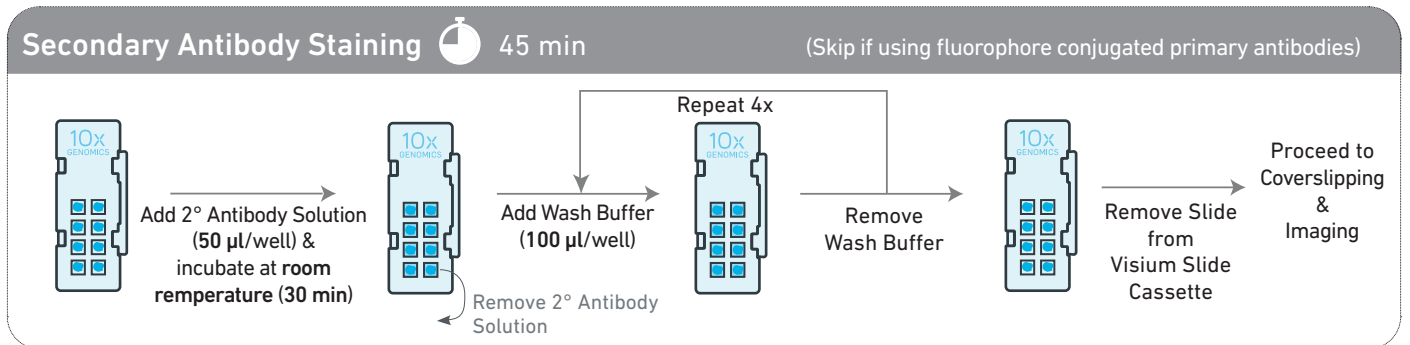
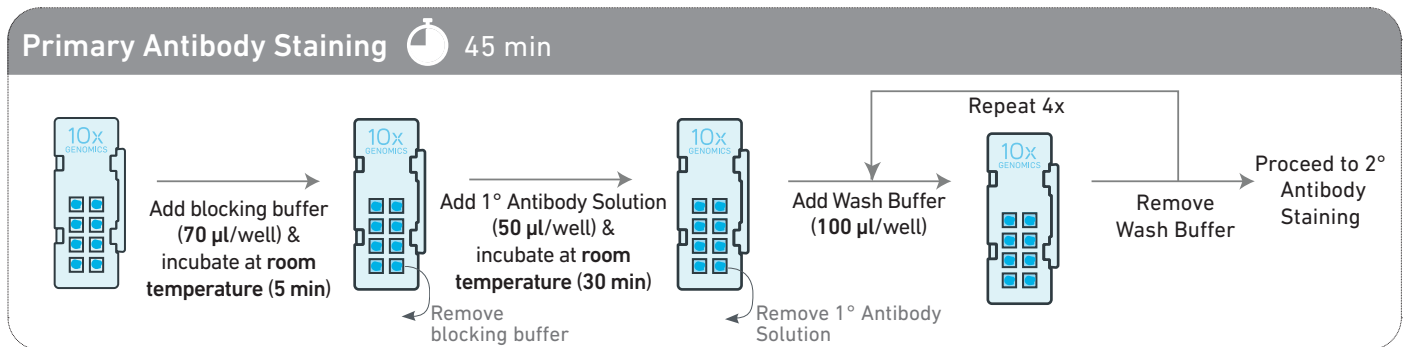
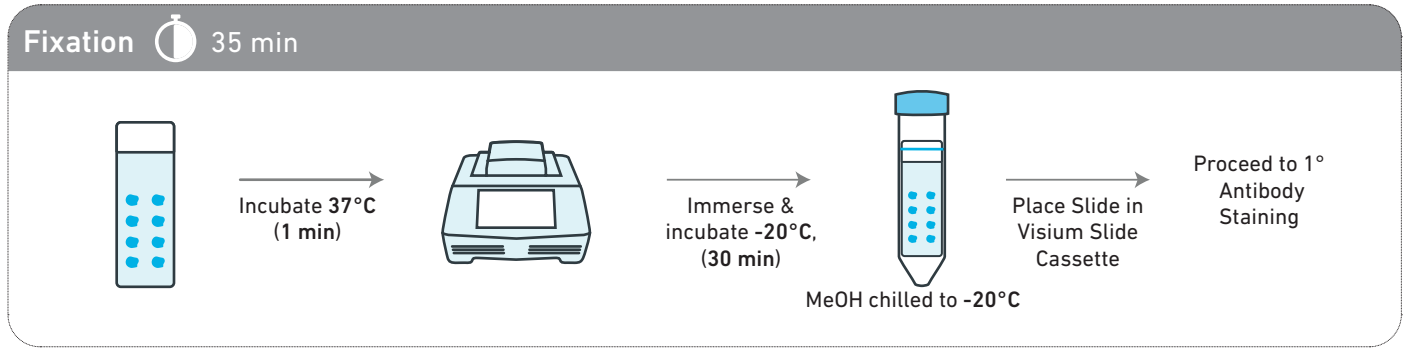
Workflow Overview



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

Protocol Overview

Perform this Demonstrated Protocol in each step on tissue sections as listed.



*Time excludes imaging steps

Proceed to 10x Genomics Visium Spatial Protocols

Tips & Best Practices

Icons



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 dry environment at room temperature in their original packaging and keep sealed. **DO NOT** remove desiccant.
- After tissue placement, store slides in a sealed container. If using an unsealed slide mailer, store in a secondary sealed container, such as a resealable bag.
- Store the sealed container containing slides with tissue at -80°C for up to four weeks.

Store Unsealed Slide Mailers in a Secondary Sealed Container



Slide Handling

- Always wear gloves when handling slides.
- Exercise caution when handling slide edges to prevent injury.
- 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

Correct

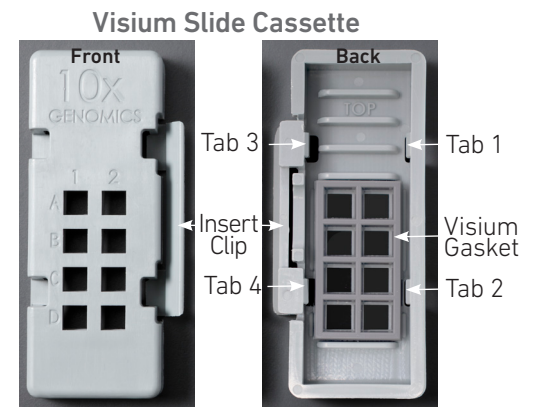


Incorrect



Visium Slide Cassette

- The Visium Slide Cassette encases the slide and creates leakproof wells for adding reagents.
- Place the slides in the Visium Slide Cassette only when specified.
- The Visium Slide Cassette may be reused once. Refer to the Visium Slide Cassette and Visium Gasket Cleaning section for instructions.
- An Insert Clip and four tabs at the back of the Visium Slide Cassette are used for holding the slide in the Visium Slide Cassette, as shown.
- The Visium Slide Cassette includes a reusable Visium Gasket corresponding to the Capture Areas on the slides.
- The Visium Slide Cassette may be assembled using the Slide Alignment Tool or manually. Instructions for both are provided in the following section.
- See Visium Slide Cassette Assembly & Removal instructions for details.
- Ensure that the back of the Visium 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.



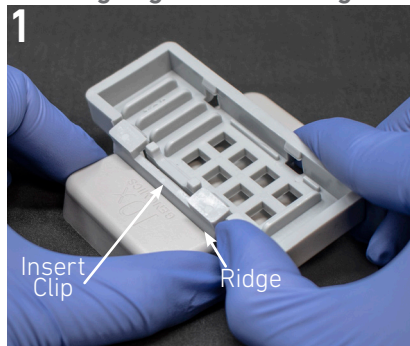
Slide Alignment Tool



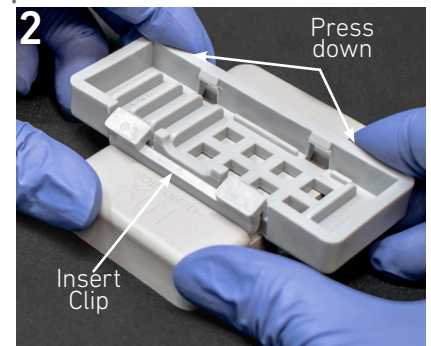
**Visium Slide
Cassette
Assembly**

! Exercise caution when handling slide edges to prevent injury.

Position Visium Slide Cassette along alignment tool ridges



Push Insert Clip along the ridge & press Visium Slide Cassette down



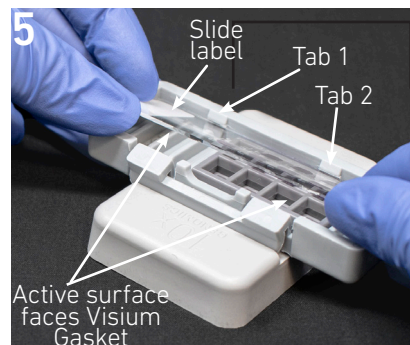
Visium Slide Cassette secured on alignment tool



Position Visium Gasket to align with Visium Slide Cassette cutouts



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



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



Remove Visium Slide Cassette while pressing slide against the Visium Gasket



! Slide insertion may push Visium Gasket out of alignment with slide cutouts. Adjust if necessary.

Visium Slide
Cassette
Removal

Position Visium Slide Cassette
along alignment tool ridges



Push Insert Clip along the ridge
& press down



Visium Slide Cassette sits securely
on alignment tool



Lift slide at Visium Slide Cassette
groove



Manual Visium Slide Cassette Assembly & Removal

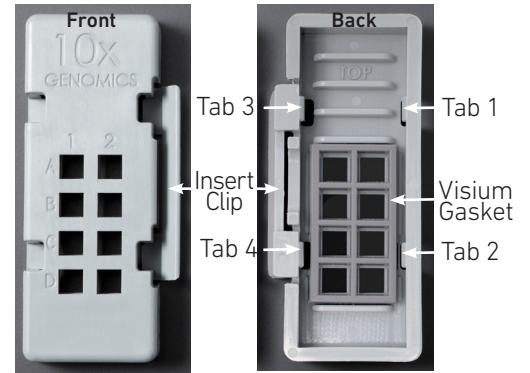
Assembly

- i. Remove the Visium Gasket from the Visium Slide Cassette and re-insert the Visium Gasket, ensuring that the Visium Gasket and Visium Slide Cassette cutouts are aligned.
- ii. Align the label on top of the slide to the top of the Visium 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 Visium 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 Visium Slide Cassette, and one finger between tab 4 and the bottom of the Visium Slide Cassette. Press down on the slide evenly until the slide is under each tab and release the insert clip.

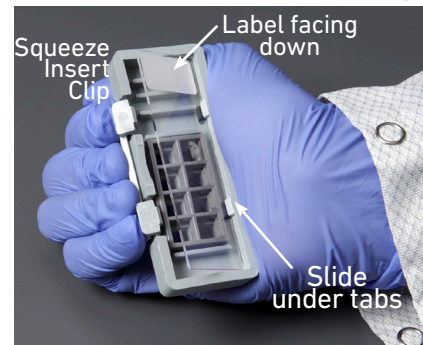
Removal

- i. Press the insert clip **very firmly** to release the slide from the Visium Slide Cassette.
- ii. Lift slide at Visium Slide Cassette groove between tabs 3 and 4 until the slide can be removed.

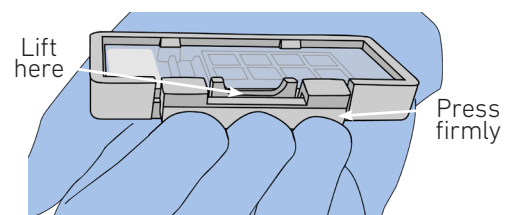
Visium Slide Cassette



Visium Slide Cassette Assembly

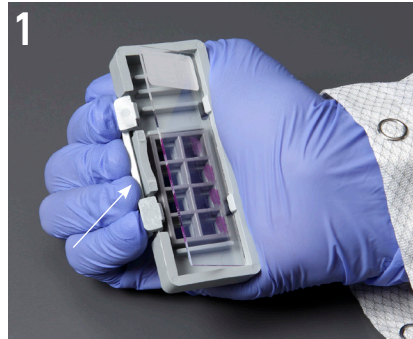


Insert Clip - Press Firmly

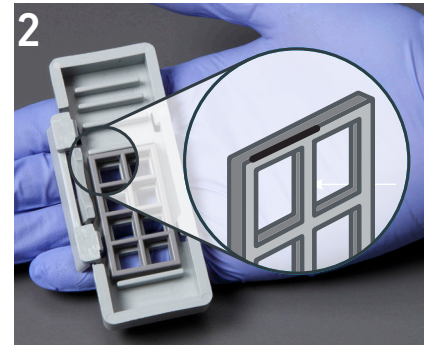


Visium Slide
Cassette &
Visium Gasket
Cleaning

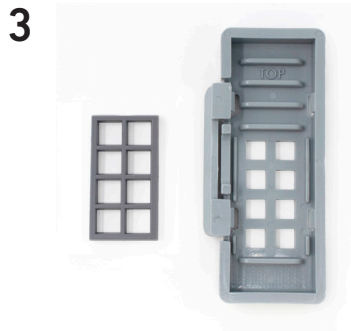
Remove slide from Visium
Slide Cassette



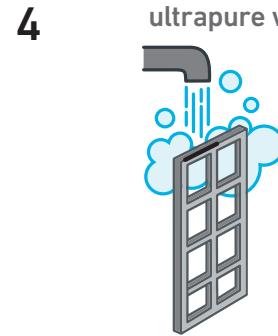
Mark the top portion of the Visium
Gasket that faced the slide with an
alcohol resistant marker



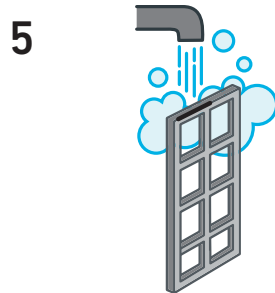
Remove Visium Gasket



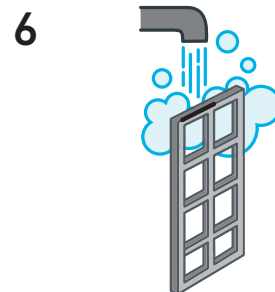
Rinse Visium Slide Cassette
and Visium Gasket with
ultrapure water



Spray with 70% isopropanol, then
rinse with ultrapure water

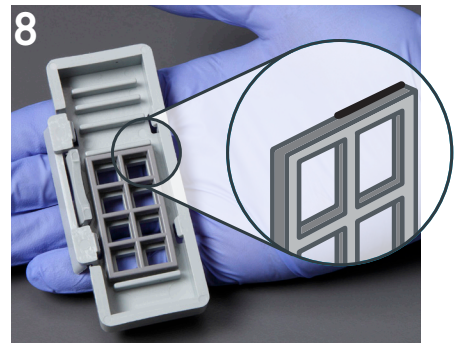


Spray with 70% isopropanol a second
time, then rinse with ultrapure water



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

Air dry



Reagent Addition & Removal from Wells

- Place the assembled slide in the Visium 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

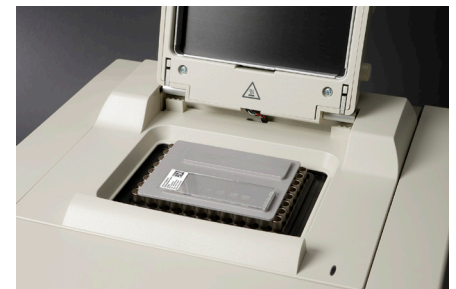
- Position a Thermocycler Adaptor on a thermal cycler that is set at the incubation temperature.
- Ensure that the Thermocycler Adaptor is in contact with the thermal cycler surface uniformly.
- When incubating a slide, position the slide on the Thermocycler Adaptor with the active surface facing up.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



Place Thermocycler Adaptor



Incubate Slide



Incubate Assembled Visium Slide Cassette

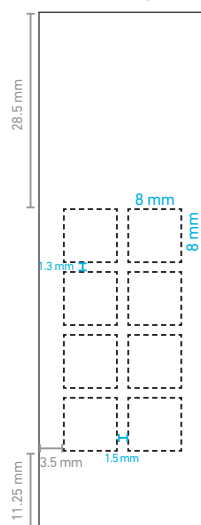


Antibody Optimization

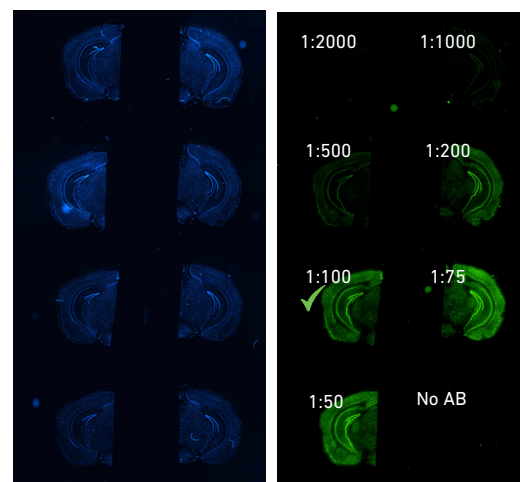
Optimization of antibody concentration is critical for executing this Demonstrated Protocol.

- 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 Visium 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. 10x Genomics recommends performing an antibody dilution series with a starting concentration of **0.01 $\mu\text{g}/\mu\text{l}$ (0.5 $\mu\text{g}/\text{sample}$)**. **0.01 $\mu\text{g}/\mu\text{l}$** corresponds to the 1:50 dilution in the image below.
- 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 Visium Slide Cassette and Visium 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). A 1:100 dilution (0.25 $\mu\text{g}/\text{sample}$) was considered optimal in this example.

Example Slide Layout



Example fluorophore conjugated primary antibody dilution series.



Tissue Fixation & Immunofluorescence Staining

1.0 Overview

1.1 Specific Reagents & Consumables

1.2 Tissue Fixation

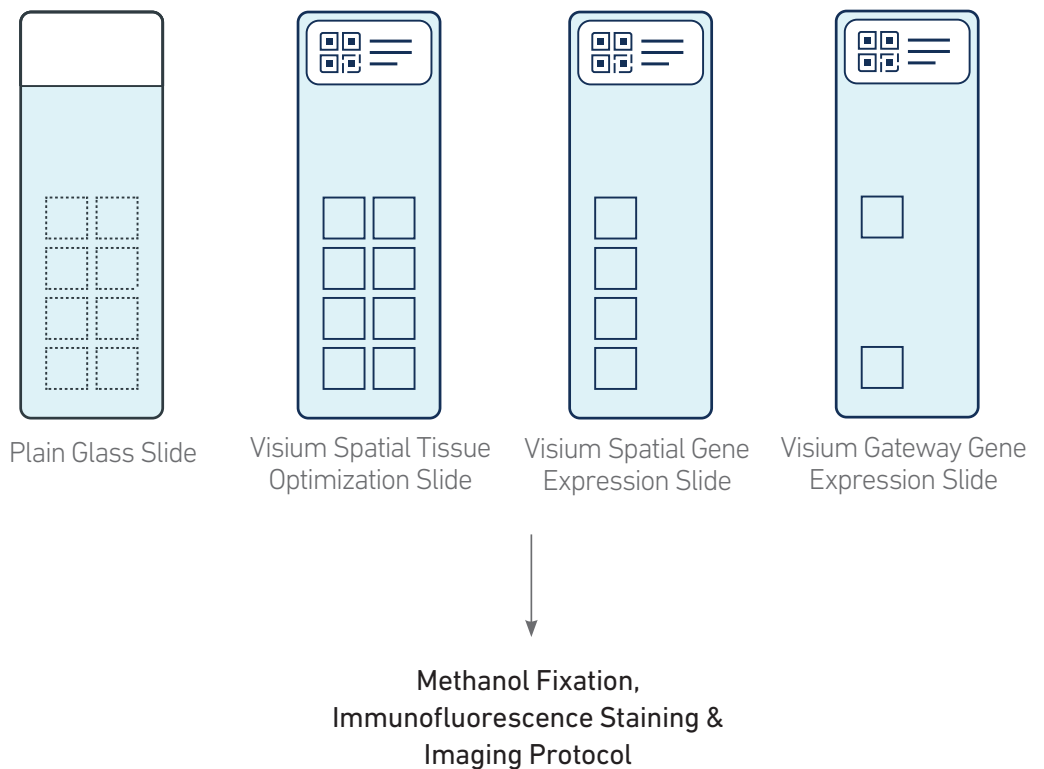
1.3 Immunofluorescence Staining - Primary and Secondary Antibodies

1.4 Immunofluorescence Staining - Fluorophore Conjugated Primary Antibodies

1.5 Slide Mounting & Coverslip Application

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 Solution, ~10% in H ₂ O	93443-100ML
Thermo Fisher Scientific	Thermo Scientific Signature Series Cover Glasses	22-050-233
	Shandon ColorFrost Plus Slides	6776214
	(Alternatively, use any 75 x 25 x 1 mm slide)	
	Fisherbrand Superfrost Plus Microscope Slides	12-550-15
	(Optional - Alternatively, use any 75 x 25 x 1 mm slide)	
	DAPI Solution (1 mg/ml)	62248
	RiboLock RNase Inhibitor	E00382
	(Alternative to Millipore Sigma product)	
	Tween 20 Surfact-Amps Detergent Solution	28320
	(Alternative to Sigma Aldrich Triton X-100)	
Millipore Sigma	SSC Buffer 20x Concentration	S6639L
	Protector RNase Inhibitor	3335402001
	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free	126615-25ML
	(Alternative to Miltenyi product)	
Miltenyi Biotec	MACS BSA Stock Solution	130-091-376
BioLegend	Human TruStain FcX (Fc Receptor Blocking Solution)	422301
	(Alternatively, use any species appropriate Fc blocking solution)	
	TruStain FcX (anti-mouse CD16/32) Antibody (discontinued)	101309
	TruStain FcX PLUS (anti-mouse CD16/32) Antibody	156603
New England Biolabs	Ribonucleoside Vanadyl Complex	S1402S
	RNase Inhibitor, Murine	M0314L
	(Alternative to Millipore Sigma product)	
Invitrogen	Alexa Fluor 488 Phalloidin (Optional)	A12379
	Alexa Fluor 594 Phalloidin (Optional)	A12381
	Alexa Fluor 647 Phalloidin (Optional)	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 (discontinued)	4375786

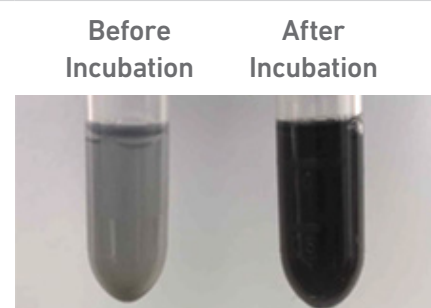
Preparation - Buffers

Prepare

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

Ribonucleoside Vanadyl Complex

- Incubate sealed vial at 65°C for **10 min** or until solution is reconstituted to a dark green solution with no visible particulates. **DO NOT** exceed **15 min**. Aliquot into single-use tubes (**550 μl** per slide including secondary staining). Store tubes at -20°C . **DO NOT** exceed 2 freeze-thaw cycles per aliquot. If using an aliquot immediately for step 1.3, no freezing is necessary. Store at room temperature until use. If precipitation is observed, incubate at 65°C for **5 min**.



	2X Blocking Buffer (prepare fresh on ice)	Stock	Final	2X + 20% (μl)	4X + 20% (μl)	8X + 20% (μl)
2X Blocking Buffer (prepare on ice)	Nuclease-free Water			336.7 μl	673.4 μl	1,346.9 μl
	SSC Buffer	20X	6X	439.2 μl	878.4 μl	1,756.8 μl
	BSA	10%	4%	585.6 μl	1,171.2 μl	2,342.4 μl
	Triton X-100 or Tween 20	10%	0.2%	29.3 μl	58.6 μl	117.7 μl
	RNase Inhibitor	40 U/ μl	2 U/ μl	73.2 μl	146.4 μl	292.8 μl
	3X SSC	Stock	Final	550 ml (1 slide)		
3X SSC	SSC Buffer	20X	3X	82.5 ml		
	Ultrapure Water	-	-	467.5 ml		
	Mounting Medium	Stock	Final	200 μl (1 slide)		
Mounting Medium	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



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

! Ribonucleoside Vanadyl Complex stock solution will precipitate if left at ambient or cool temperatures for extended periods of time. Once the solution has cooled, prepare 1X Blocking Buffer and Wash Buffer immediately.

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

- a. Place the slide in the Visium Slide Cassette. See Tips & Best Practices for assembly instructions. Practice assembly with a blank slide.
- b. Remove Ribonucleoside Vanadyl Complex aliquots from **-20°C** and place at **65°C** for **5 min** until the solution is reconstituted to a dark green solution with no visible particulates. **DO NOT** exceed **10 min**. Allow the solution to cool to ambient temperature prior to use and proceed immediately to the next step.

- c. Prepare 1X Blocking Buffer on ice.

1X Blocking Buffer <i>Vortex.</i>	1X (μ l)	2X + 15% (μ l)	4X + 15% (μ l)	8X + 15% (μ l)
2X Blocking Buffer	35.0	80.5	161.0	322.0
Nuclease-free Water	26.0	59.8	119.6	239.2
Human or Mouse TruStain FcX*	2.0	4.6	9.2	18.4
Ribonucleoside Vanadyl Complex	7.0	16.1	32.2	64.4
Total	70.0	161.1	322.0	644.0

*Choose FC blocking reagent based on sample species.

- d. Prepare Wash Buffer on ice.

Wash Buffer <i>Vortex.</i>	1X (μ l)	2X + 15% (μ l)	4X + 15% (μ l)	8X + 15% (μ l)
2X Blocking Buffer	525.0	1,207.5	2,415.0	4,830
Nuclease-free Water	420.0	966.0	1,932.0	3,864
Ribonucleoside Vanadyl Complex	105.0	241.5	483.0	966
Total	1,050.0	2,415.0	4,830.0	9,660

- e. Vortex 1X Blocking Buffer and add **70 μ l** along the side of the wells to uniformly cover the tissue sections, without introducing bubbles. Tap gently to ensure even coverage.
- f. Incubate for **5 min** at **room temperature**.
- g. Prepare Primary Antibody Solution on ice. Pipette mix.

Primary Antibody Solution. <i>Vortex, centrifuge briefly.</i>	1X (μ l)	2X + 10% (μ l)	4X + 10% (μ l)	8X + 10% (μ l)
2X Blocking Buffer	25.00	55.00	110.00	220.00
Nuclease-free Water	Variable	Variable	Variable	Variable
Primary Antibody #1	Variable	Variable	Variable	Variable
Primary Antibody #2 (optional)	Variable	Variable	Variable	Variable
Primary Antibody #3 (optional)	Variable	Variable	Variable	Variable
RNase Inhibitor	6.75	14.85	29.70	59.40
Total	50.00	110.00	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.

- h. Remove 1X Blocking Buffer.

- i. Add **50 µl** Primary Antibody Solution along the side of each well. Tap gently to ensure uniform coverage.
- j. Incubate for **30 min** at room temperature.
- k. Prepare Secondary Antibody Solution on ice. Pipette mix. Avoid light exposure.

Secondary Antibody Solution. <i>Vortex, centrifuge briefly.</i>	1X (µl)	2X + 10% (µl)	4X + 10% (µl)	8X + 10% (µl)
2X Blocking Buffer	25.00	55.00	110.00	220.00
Nuclease-free Water	Variable	Variable	Variable	Variable
Secondary Antibody #1	Variable	Variable	Variable	Variable
Secondary Antibody #2 (optional)	Variable	Variable	Variable	Variable
Secondary Antibody #3 (optional)	Variable	Variable	Variable	Variable
DAPI	0.17	0.38	0.75	1.50
Phalloidin (optional)	0.05	0.11	0.22	0.44
RNase Inhibitor	6.75	14.85	29.70	59.40
Total	50.00	110.00	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.

- l. Remove the Primary Antibody Solution.
- m. Add **100 µl** Wash Buffer along the side of each well.
- n. Remove Wash Buffer.
- o. **Repeat** steps m and n four more times for a total of five washes.
- p. Add **50 µl** Secondary Antibody Solution along the side of each well. Tap gently to ensure uniform coverage.
- q. Incubate for **30 min** at room temperature. Avoid light exposure.
- r. Remove the Secondary Antibody Solution.
- s. Add **100 µl** Wash Buffer along the side of each well.
- t. Remove Wash Buffer.
- u. **Repeat** steps s and t four more times for a total of five washes. Save **50 µl** Wash Buffer per sample for step 2.2.
- v. 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



- Place the slide in the Visium Slide Cassette. See Tips & Best Practices for assembly instructions. Practice assembly with a blank slide.
- Remove Ribonucleoside Vanadyl Complex aliquots from -20°C and place at 65°C for 5 min until the solution is reconstituted to a dark green solution with no visible particulates. DO NOT exceed 10 min. Allow the solution to cool to ambient temperature prior to use and proceed immediately to the next step.

 Ribonucleoside Vanadyl Complex stock solution will precipitate if left at ambient or cool temperatures for extended periods of time. Once the solution has cooled, prepare 1X Blocking Buffer and Wash Buffer immediately.


- Prepare 1X Blocking Buffer on ice.

1X Blocking Buffer <i>Vortex.</i>	1X (μl)	2X + 15% (μl)	4X + 15% (μl)	8X + 15% (μl)
2X Blocking Buffer	35.0	80.5	161.0	322.0
Nuclease-free Water	26.0	59.8	119.6	239.2
Human or Mouse TruStain FcX*	2.0	4.6	9.2	18.4
Ribonucleoside Vanadyl Complex	7.0	16.1	32.2	64.4
Total	70.0	161.1	322.0	644.0

*Choose FC blocking reagent based on sample species.

- Prepare Wash Buffer on ice.

Wash Buffer <i>Vortex.</i>	1X (μl)	2X + 15% (μl)	4X + 15% (μl)	8X + 15% (μl)
2X Blocking Buffer	275	632.5	1,265	2,530
Nuclease-free Water	220	506.0	1,012	2,024
Ribonucleoside Vanadyl Complex	55	126.5	253	506
Total	550	1,265.0	2,530	5,060

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

- 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.
- Incubate for 5 min at room temperature.
- Prepare Primary Antibody Solution on ice. Pipette mix. Avoid light exposure.

Primary Antibody Solution. <i>Vortex, centrifuge briefly.</i>	1X (μl)	2X + 10% (μl)	4X + 10% (μl)	8X + 10% (μl)
2X Blocking Buffer	25.00	55.00	110.00	220.00
Nuclease-free Water	Variable	Variable	Variable	Variable
Primary Antibody #1	Variable	Variable	Variable	Variable
Primary Antibody #2 (optional)	Variable	Variable	Variable	Variable
Primary Antibody #3 (optional)	Variable	Variable	Variable	Variable
DAPI	0.17	0.38	0.75	1.50
Phalloidin (optional)	0.05	0.11	0.22	0.44
RNase Inhibitor	6.75	14.85	29.70	59.40
Total	50.00	110.00	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.



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

- h. Remove 1X Blocking Buffer.
- i. Add **50 µl** Primary Antibody Solution along the side of each well. Tap gently to ensure even coverage.
- j. Incubate for **30 min** at **room temperature**. Avoid light exposure.
- 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 l and m four more times for a total of five washes. Save **50 µl** Wash Buffer per sample for step 2.2.
- o. Proceed **immediately** to step 1.5

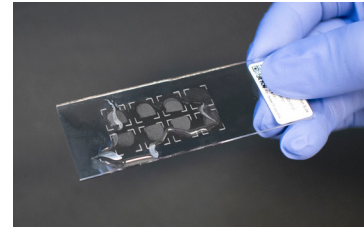
1.5 Slide Mounting & Coverslip Application

- 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 Visium 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 μ l** 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.

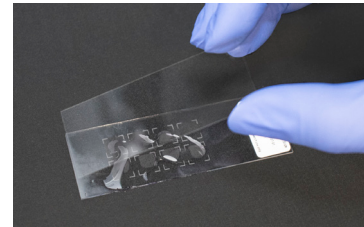


DO NOT let the attached coverslip dry.
 DO NOT use CytoSeal, nail polish, or any other permanent mounting medium for securing the coverslip.

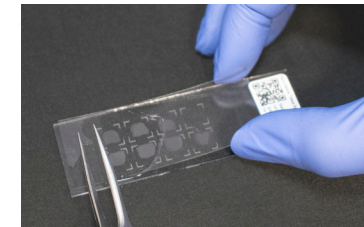
Cover uniformly with Mounting Medium



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) (Optional - dependent on antibody selection)
TRITC filter cube (Excitation 542/20, Emission 620/52)
Cy5 filter cube (Excitation 618/50, Emission 698/70) (Optional - dependent on antibody selection)
2.18 µm/pixel minimum capture resolution
Exposure times 100 milli sec-2 sec

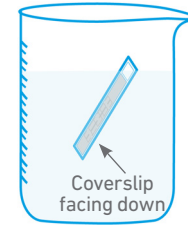
2.1 Tissue Imaging

- 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.

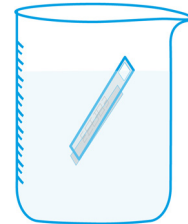
2.2 Coverslip Removal

- a. Wash Visium Slide Cassette and Visium 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. Wipe excess liquid from the back of the slide, without touching the tissue sections.
- f. Place slide in a clean Visium Slide Cassette.
- g. 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.
-  h. Proceed immediately to the Visium Spatial Tissue Optimization User Guide (CG000238) or the Visium Spatial Gene Expression User Guide (CG000239).

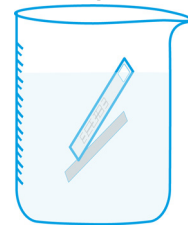
Immerse in 3X SSC Buffer



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.

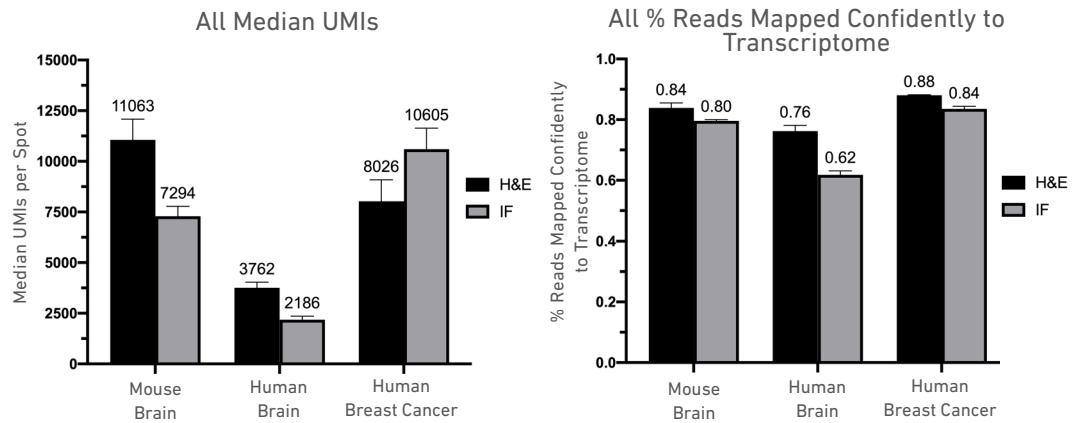


Figure 1. Median UMI counts and % reads mapped confidently to transcriptome for mouse brain, human brain, and human breast cancer tissue. Values indicate the average of 4 replicates per condition. UMI metrics are based on samples downsampled to 20K raw reads per spot.

Troubleshooting

STEP	NOTES
<p>2.1 Weak or no signal</p>	<ul style="list-style-type: none"> • 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.
<p>2.1 High background</p>	<ul style="list-style-type: none"> • 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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