TECHNICAL NOTE

Visium Spatial Gene Expression Imaging Guidelines

Introduction

The Visium Spatial Gene Expression Solution measures total mRNA in intact tissue sections and maps where gene activity is occurring. Immunostaining tissue sections with fluorescent antibodies enables simultaneous protein detection. Successful gene expression and protein visualization is highly dependent on good imaging practices. This Technical Note provides hardware recommendations, general image acquisition and analysis guidelines, and examples of images that are suitable for downstream analysis with Space Ranger. Individual results may vary depending on the specific imaging system, and/or sample characteristics.

General Imaging Guidelines

- Proper tissue placement is crucial for successful imaging. Consult the Visium Spatial Protocols – Tissue Preparation Guide for complete information (CG000240).
- Wear a clean pair of gloves when handling slides.
- Avoid touching the active surface of the slide.
- Ensure slides are clean and dry. Use a laboratory wipe to clean the side without tissue.
- Place slides gently and evenly on the imaging stage.
- Image setting verification and acquisition may be performed with any of the following objectives: 4X (Plan APO λ; NA 0.20), 10X (Plan APO λ; NA 0.45), and 20X (Plan APO λ; NA 0.75).
- Images have a minimum size requirement of 2000 pixels in at least one direction.

Any imaging system used for this workflow should have tile scanning functionality for clear imaging of the tissue section on each 6.5 x 6.5 mm Capture Area (8 x 8 mm including fiducial frame). Additionally, a computer with sufficient power to handle large images (0.5-5 GB) should be used for image processing. A computer should be able to stitch images via the microscope's native software or third party software such as ImageJ.

Imaging Configuration Recommendations

The table below shows brightfield imaging configurations used by 10x Genomics during protocol development. Any equivalent imaging setup can be used as an alternative.

Brightfield Configuration (Only required for H&E Staining)
Color camera (3 x 8 bit, 2424 x 2424 pixel resolution)
White balancing functionality
2.18 µm/pixel minimum capture resolution
Exposure times 2-10 milli sec

The table below shows fluorescence imaging configurations used by 10x Genomics during protocol development. Any equivalent imaging setup can be used as an alternative.

A TRITC filter cube is required for both the H&E staining and Immunofluorescence staining protocols. Cy5 and DAPI filter cubes may be required for immunofluorescence staining if compatible reagents are used. Filter cube choice will depend on antibody selection. Fluorophore conjugated primary antibodies may require specific filter sets. Ensure filter sets are compatible with fluorophore choice.

Fluorescence 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 filter cube (Excitation 480/40, Emission 535/50)
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

Imaging System Recommendations

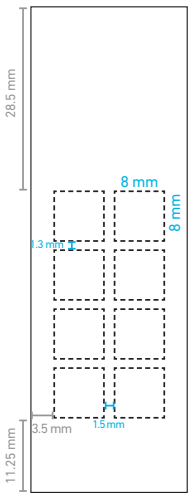
The table below shows imaging systems used by 10x Genomics in the development of this protocol. Any equivalent imaging system can be used as an alternative. Imaging systems should have both brightfield and fluorescence capacity.

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

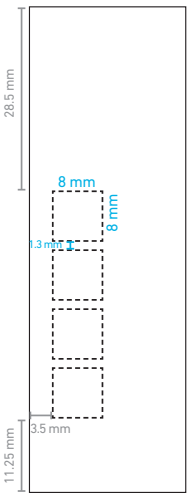
Visium Spatial Slide Layout

A layout of Capture Areas of Visium Spatial slides is shown below and can be used to create representative frames on 75 x 25 x 1 mm plain glass slides or to create imaging macros.

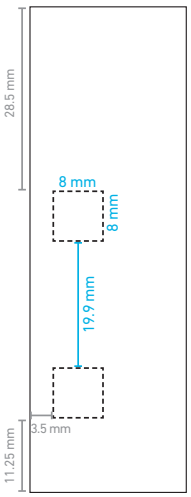
Capture Areas – Tissue Optimization Slide



Capture Areas – Gene Expression Slide



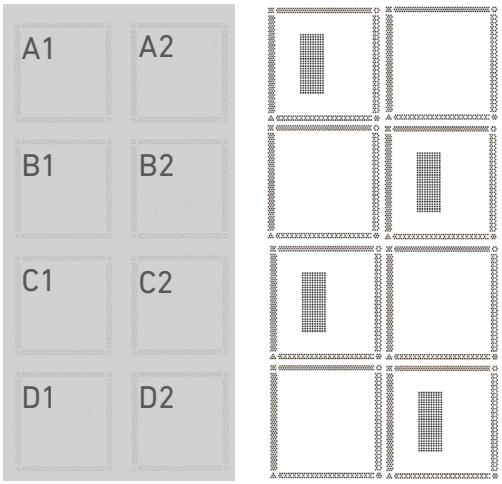
Capture Areas – Gateway Gene Expression Slide



Visium Imaging Test Slide

Slide Information

- Use the Visium Imaging Test Slide to verify imaging settings prior to the Visium Spatial Tissue Optimization and Gene Expression workflows.
- Store the unused slide at **room temperature** in its original packaging and keep sealed. DO NOT remove desiccant. DO NOT touch or wipe the active side. Avoid light exposure.
- The Visium Imaging Test Slide has eight areas surrounded by fiducial frames that are visible under brightfield settings. Each fiducial frame has a fiducial marker at each corner.
- Four areas (A1, B2, C1, D2) have fluorescent spots that are detectable with TRITC and Cy5 filter cubes.
- The positioning of fiducial frames matches the Visium Spatial Gene Expression (A1, B1, C1, D1) and Visium Spatial Tissue Optimization slides (all Capture Areas).
- The Visium Imaging Test Slide should be used in advance to create imaging macros for Visium protocols.



Capture Area labels are illustrative and are not visible under magnification.

Brightfield Imaging

- Fiducial frames and fiducial markers should be clearly visible and in focus using brightfield settings.
- If fiducial frames are not clearly visible, adjust settings accordingly. Refer to the Imaging Examples.
- After creating imaging macros, ensure that images can be stitched together without distorting fiducial frames or fiducial markers.

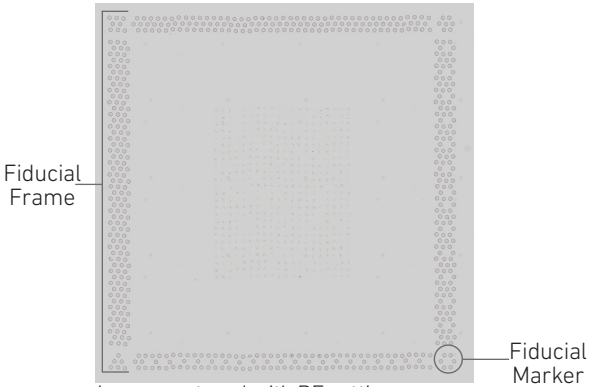
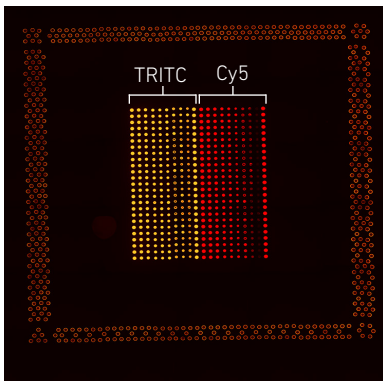


Image captured with BF settings

Fluorescence Imaging

- Fluorescent spots in A1, B2, C1 and D2 should be clearly visible using fluorescence settings.
- Fluorescent spot signal should decrease from left to right, as shown on the image to the right.
- Fluorescent spot columns on the left half can be visualized with TRITC, while the right half is visualized with Cy5.
- Cy5 capability is only required if using compatible antibodies during immunostaining.
- If all fluorescent spots are not clearly visible, adjust settings accordingly. Settings may need further adjustment while running Visium experiments depending on tissue type.
- Fiducial frame intensity is similar to the fiducial frame intensity for the Visium Spatial Gene Expression Slide.
- After verifying brightfield and fluorescence settings, proceed to the desired Visium Spatial Fixation and Staining Demonstrated Protocol.



RGB color image. Imaging channels: TRITC (fiducial frame and left half of fluorescent spots) and Cy5 (right half of fluorescent spots). Image captured with 200 milli sec exposure, 75% light power, 11.4x gain.

Immunofluorescence Imaging

General Information

- Immunofluorescence staining is performed prior to the Visium Spatial Tissue Optimization and Gene Expression workflows and is only required if simultaneous protein detection is desired.
- Consult the Methanol Fixation, Immunofluorescence Staining, & Imaging Demonstrated Protocol for the full staining protocol and for information on antibody optimization (CG000312).
- Optimize imaging settings such that images are acquired with visible fiducials, strong signal, and minimum background. Refer to the Imaging Examples section of this document.

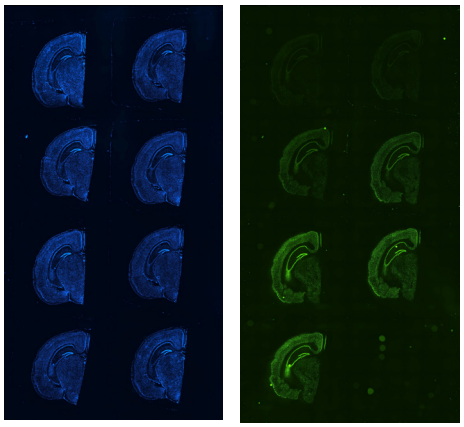
Image Acquisition

- If proceeding with Tissue Optimization after immunofluorescence staining, image all eight Capture Areas at once.
- If proceeding with the Gene Expression workflow after immunofluorescence staining, ensure that each Capture Area is imaged individually.
- Image the fiducial frame in the same orientation and position across individual Capture Area images.
- The fiducial frame is visible using the TRITC filter cube. Optimize and image the fiducial frame as a separate image from the other immunofluorescence channels.
- If using antibodies with a conjugated TRITC compatible fluorophore, optimize and image the fiducial frame and antibodies separately.
- Antibody immunofluorescence will no longer be visible after tissue permeabilization.

Image Export

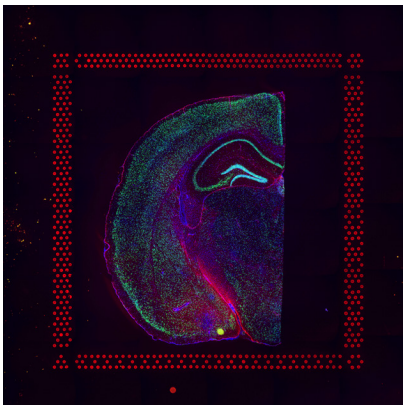
- Export images in one of the following formats: multi-page tiff, individual monochrome tiff/jpeg, or merged RGB color tiff/jpeg.
- Multi-page tiff: export stitched images as multi-page (or multi-stacked) tiff, where each filter set or fluorophore is assigned a single page within the file. Space Ranger can support 8-bit or 16-bit grayscale (single-channel) multi-page tiff images/files.
- Individual monochrome tiff/jpeg: export stitched images as individual monochrome tiff or jpeg files, where each filter set or fluorophore is saved as a separate file. Space Ranger can support 8-bit or 16-bit grayscale single channel single-page tiff (one page per file, up to six files) or jpeg images/files. Ensure that each monochrome channel has the same bit depth, dimension, alignment, and file format.
- Merged RGB color tiff/jpeg: export stitched images as a merged RGB color tiff or jpeg file, where the different channels are merged and converted to an RGB color image (representing the red, green, and blue channels). Space Ranger can support 24-bit (8-bits per channel) RGB color tiff or jpeg images/files. Saving and exporting files as merged RGB color images will result in the inability to independently adjust each filter set or fluorophore.
- Name the file using both the serial slide number and Capture Area identifier in a manner compatible with the user's desktop and cluster environments, i.e. V19L29-033_A1. If saving individual fluorescent channel images, include the channel in the file name i.e. V19L29_033_A1_DAPI.

Tissue Optimization Slide Capture Areas



Imaging Channels: DAPI, FITC (NeuN, Abcam, PN: ab190195)

Gene Expression Slide Capture Area

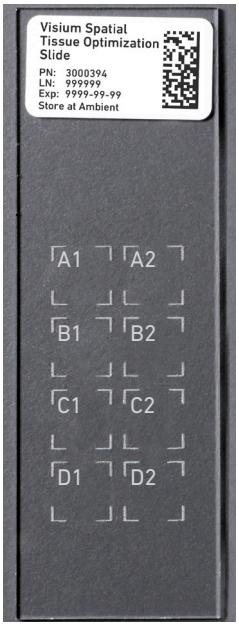


Imaging Channels: DAPI, FITC (NeuN, Abcam, PN: ab190195), and TRITC (GFAP, Abcam, PN:ab201732)

Visium Spatial Tissue Optimization

Slide Information

- The Visium Spatial Tissue Optimization slide is used to identify tissue compatability and the optimal tissue permeabilization time for the Visium Spatial Gene Expression workflow.
- Store unused slides at room temperature in their original container and packaging and keep sealed. DO NOT remove desiccant.
- Store slides with tissue sections in a sealed container at -80°C for up to four weeks.
- The Visium Spatial Tissue Optimization Slide has eight 8 x 8mm Capture Areas surrounded by etched frames.
- Each Capture Area contains oligonucleotides for mRNA capture. Each probe has poly(dT) primers that enable the production of cDNA from poly-adenylated mRNA. These probes do not contain a spatial barcode.
- Fluorescence signal is used as a proxy for permeabilization efficiency. Fluorescence imaging is not required for the Visium Spatial Gene Expression workflow.
- Consult the Visium Spatial Tissue Optimization User Guide for complete information on the tissue optimization workflow (CG000238).

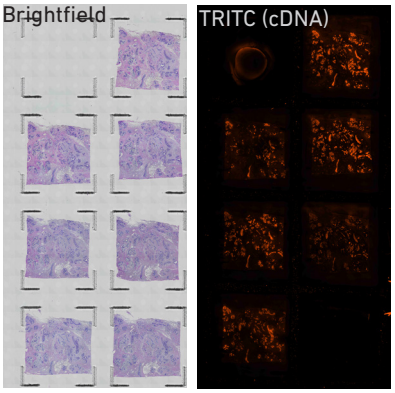


Capture Area labels are illustrative and are not visible under magnification.

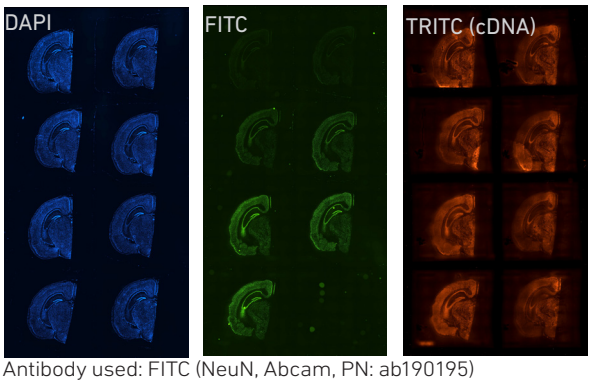
Imaging Guidelines

- During brightfield and fluorescence imaging, image all eight Capture Areas at once without using autoexposure.
- After image acquisition, stitch image tiles together with the microscope's native software or third party software such as ImageJ.
- Compare fluorescence images with brightfield images or DAPI images to ensure that a lack of signal is due to insufficient permeabilization, not missing tissue.
- Select the permeabilization condition that results in the highest fluorescence signal throughout the tissue with the lowest signal diffusion. If the signal is the same at two time points, the longer permeabilization time is considered optimal. See next page for examples.

H&E Staining Protocol Example Images



IF Staining Protocol Example Images



Antibody used: FITC (NeuN, Abcam, PN: ab190195)

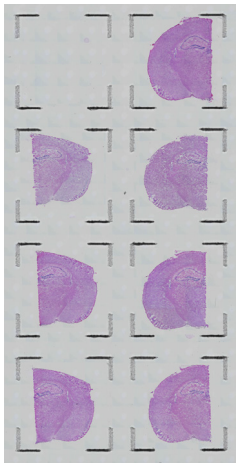
Visium Spatial Tissue Optimization Examples

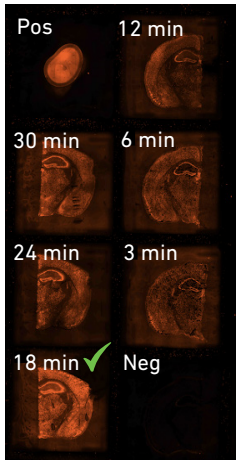
General Settings

- All examples use 10 µm tissue sections.
- All tissue sections were imaged using a Nikon Eclipse Ti2 microscope with the following settings: 10x NA 0.45 objective, 0.73µm/pixel capture resolution, TRITC filter cube, 75% Sola pad.

Mouse Brain

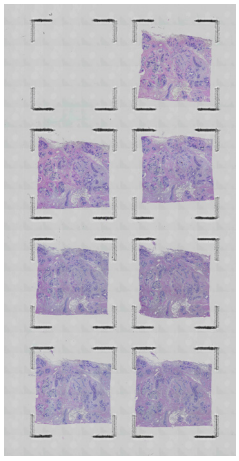
- Exposure: 200 milli sec.
- Time selected: 18 min.
- The negative control shows no fluorescence signal, while the positive control shows a strong signal.
- Low signal after 3-12 min of permeabilization suggest insufficient permeabilization.

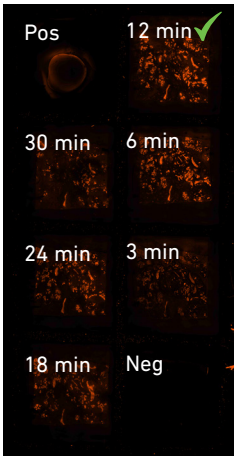




Human Breast

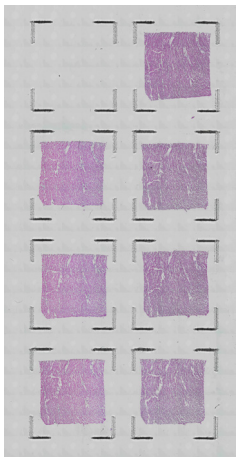
- Exposure: 200 milli sec.
- Time selected: 12 min.
- Assuming uniform reagent coverage, variation in fluorescence signal within the same tissue section is normal and reflects biological variability in transcription.
- Fluorescence signal is similar between 12 min and 6 min, therefore the longer time was selected for the Visium Spatial Gene Expression workflow.

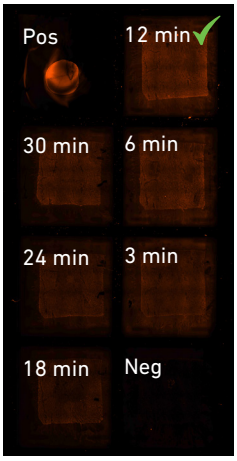




Human Heart

- Exposure: 300 milli sec.
- Time selected: 12 min.
- Although fluorescence signal is dim, this permeabilization time course is considered successful. Dim fluorescence signal is expected due to low RNA content.

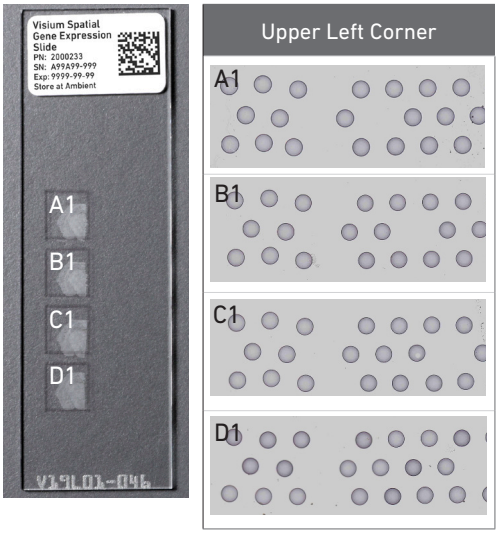




Visium Spatial Gene Expression

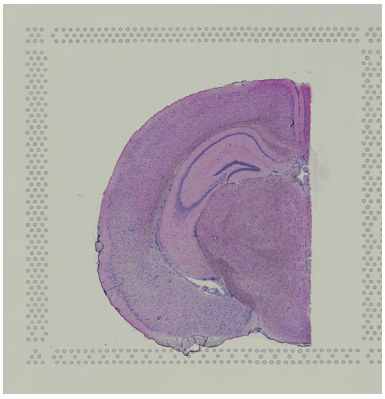
Slide Information

- The Visium Spatial and Gateway Gene Expression slides are used to generate libraries from fresh frozen tissue sections.
- Store unused slides at **room temperature** in their original container and packaging and keep sealed. DO NOT remove desiccant.
- Store slides with tissue sections in a sealed container at -80°C for up to four weeks.
- The Visium Spatial Gene Expression Slide has four Capture Areas surrounded by fiducial frames.
- The Visium Gateway Gene Expression Slide has two Capture Areas (A1 and D1) surrounded by fiducial frames.
- Each Capture Area has ~5,000 unique gene expression spots.
- Each Capture Area can be distinguished from one another by the arrangement of fiducial frame spots in the upper left and bottom left corners of each fiducial frame.
- Fiducial frames are used by Space Ranger to align the image.
- Consult the Visium Spatial Gene Expression Reagent Kit User Guide for complete protocol (CG000239).



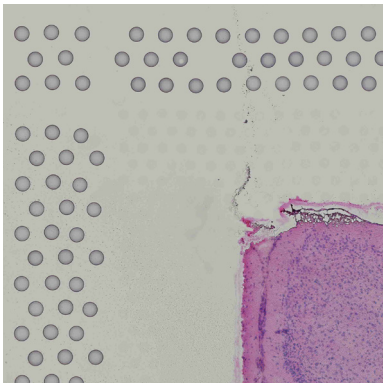
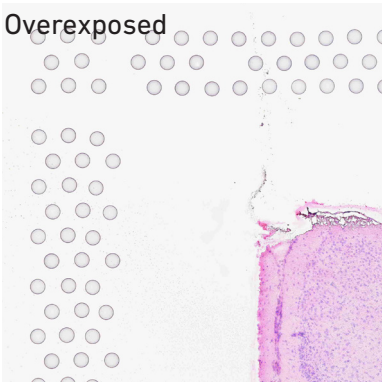
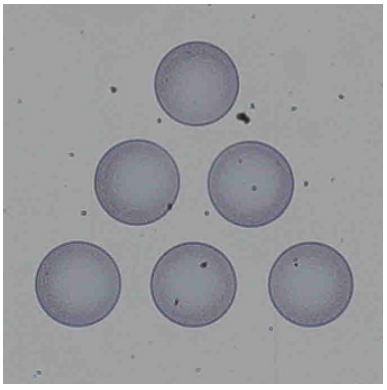
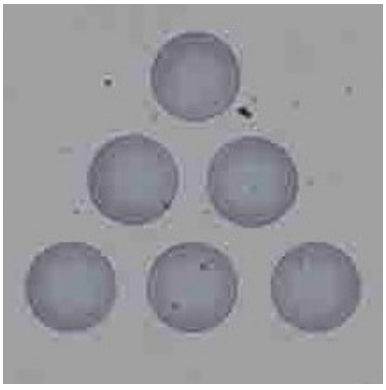
Imaging Guidelines

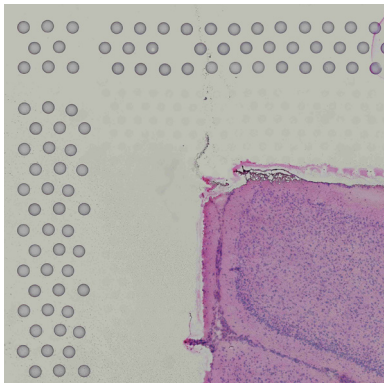
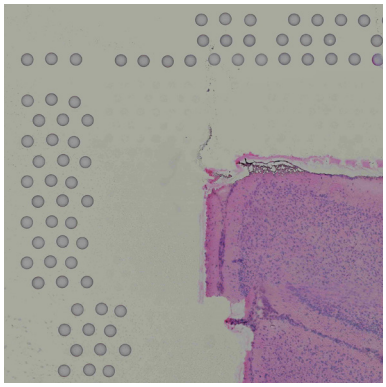
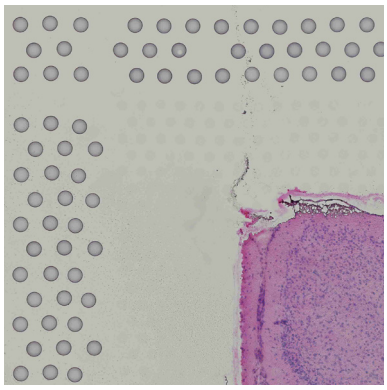
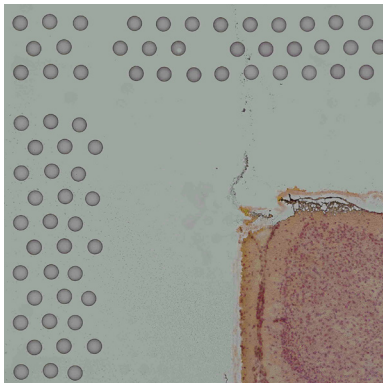
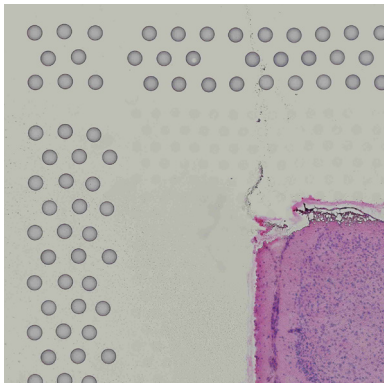
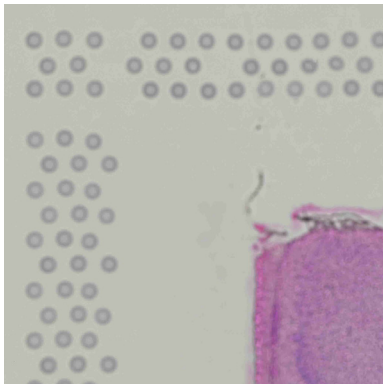
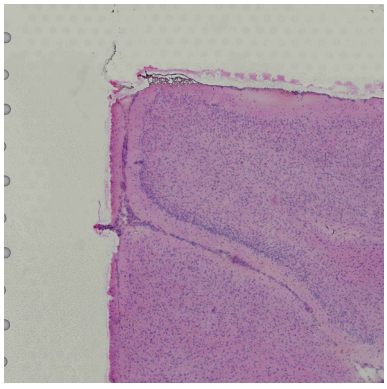
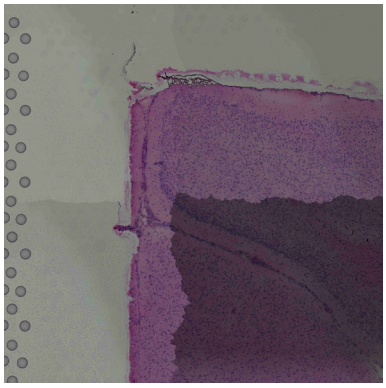
- Image each capture area individually, as shown on the right. Minimize imaging of adjacent Capture Areas.
- Imaging area should be ~1-2 mm beyond the fiducial frame for optimal imaging alignment.
- After image acquisition, stitch image tiles together with the microscope's native software or third party software such as ImageJ.
- Each stitched image should correspond to one Capture Area.
- Export stitched images as a 24-bit color or 16-bit monochrome tiff (preferred) or jpeg image.
- Name the file using both the serial slide number and Capture Area identifier in a manner compatible with the user's desktop and cluster environments, i.e. V19L29-033_A1.
- For information on image analysis and tissue alignment, refer to the 10x Genomics Support Website.

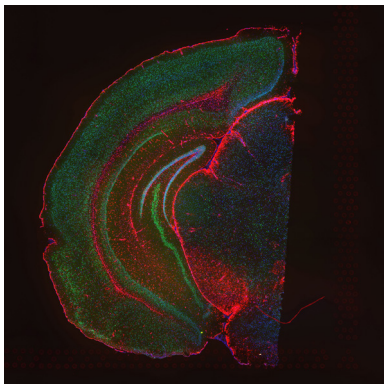
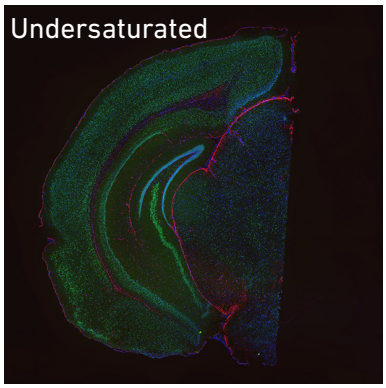
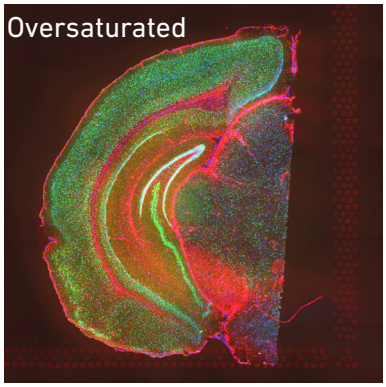
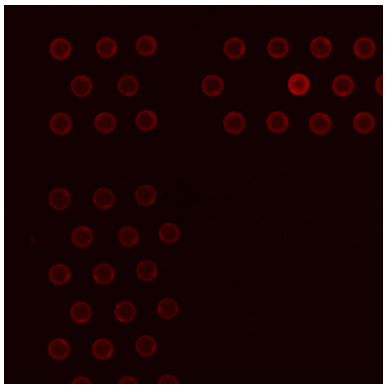
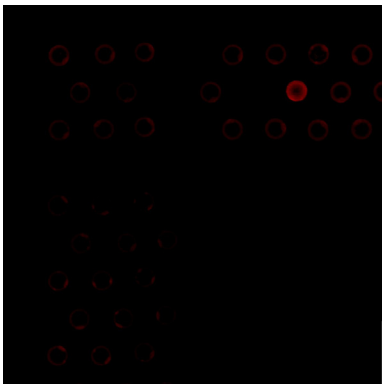


Imaging Examples

The following imaging artifacts may cause image analysis failure. Ensure that optimal imaging settings are verified prior to beginning the Visium Spatial Gene Expression workflow.

CONCEPT	CORRECT	INCORRECT
Image Exposure		 
Capture Resolution	 Capture resolution: 0.74 µm/pixel	 Capture resolution: 5.5 µm/pixel

CONCEPT	CORRECT	INCORRECT
Stitching		
White Balancing		
Focus		
Shade Correction		

CONCEPT	CORRECT	INCORRECT
Fluorescence Signal		
		
Fiducial Frame Signal		

References

- Methanol Fixation, H&E Staining, & Imaging Demonstrated Protocol (Document CG000160)
- Methanol Fixation, Immunofluorescence Staining, & Imaging Demonstrated Protocol (Document CG000312)
- Visium Spatial Gene Expression Reagents Kit - Tissue Optimization User Guide (Document CG000238)
- Visium Spatial Gene Expression Reagent Kits User Guide (Document CG000239)
- Visium Spatial Protocols - Tissue Preparation Guide Demonstrated Protocol (Document CG000240)

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