Visium Spatial Gene Expression for FFPE Imaging Guidelines

Introduction

The Visium Spatial Gene Expression for FFPE workflow assays RNA levels by using probes against the whole transcriptome in intact formalin fixed paraffin embedded (FFPE) tissue sections and maps the location(s) where gene activity is occuring. Immuno-staining 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 Gene Expression for FFPE – Tissue Preparation Guide for complete information (CG000408).
- Wear a clean pair of gloves when handling slides.
- Do not touch the active surface of the slide.
- Ensure slides are clean prior to imaging. Use a laboratory wipe to clean the bottom of the slide (the side without tissue) if necessary.
- Place slides gently and evenly on the imaging stage.
- Dry objectives can be used for image acquisition. Do not use immersion media. Use a combination of a high numerical aperture for high resolution images and low magnification for minimal scan time, i.e. 10X (NA 0.45), 20X (NA 0.75), or 40X (NA 0.95).
- Use a coverslip appropriate for the chosen objective, e.g. a #1.5 (0.17 mm) coverslip. If using an inverted system, use an objective with a correction collar that can be adjusted to image through the slide.
- 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 precise and accurate imaging of the tissue section and the 8 x 8 mm 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, 2,424 x 2,424 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.

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

A TRITC filter cube is required for the immunofluorescence staining protocol to image the fiducial frame. Filter cube choice will depend on fluorophore selection. Fluorophore conjugated primary antibodies may require specific filter sets. Ensure filter sets are compatible with fluorophore choice.

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.

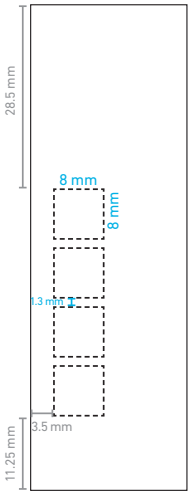
Supplier	Description	Configuration
Thermo Fisher Scientific	EVOS M7000	Inverted
Leica	Aperio Versa 8 Leica DMi8	Upright Inverted
MetaSystems	Metafer	Upright
Nikon	Nikon Eclipse Ti2	Inverted
BioTek	Cytation 7	Inverted or Upright
Keyence	Keyence BZX800	Inverted

All samples are loosely mounted in glycerol (H&E/brightfield) or SlowFade Diamond (immunofluorescence) with a coverslip, which means that slides cannot be inverted for imaging. When using an inverted system, image through the slide from below. Imaging through the slide requires an appropriate objective with a correction collar. For example, the EVOS M7000 should be used with an Olympus Plan Fluorite 20x; NA 0.7 objective. Consult manufacturer recommendations for further information.

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.

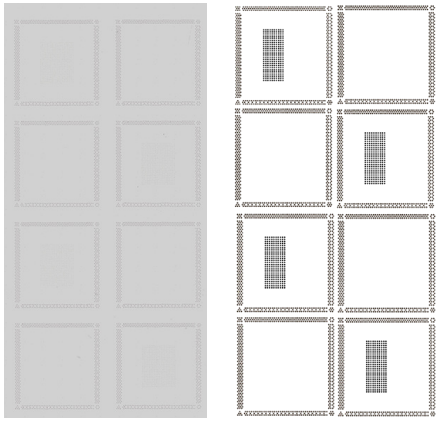
Capture Areas – Gene Expression Slide



Visium Imaging Test Slide

Slide Information

- Use the Visium Imaging Test Slide to verify imaging settings prior to the Visium Spatial Gene Expression workflow.
- 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 spot columns that are detectable with TRITC and Cy5 filter cubes.
- The positioning of fiducial frames matches the Visium Spatial Gene Expression (A1, B1, C1, D1) .
- The Visium Imaging Test Slide should be used in advance to create imaging macros for Visium protocols.
- After creating imaging macros, ensure that images can be stitched together without distorting fiducial frames or fiducial markers.



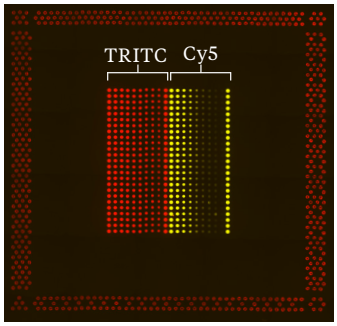
Capture Area labels are illustrative and are not visible under magnification.

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.

Fluorescence Imaging

- Fluorescent spot columns 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 below.
- 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.
- Visium Imaging Test Slide fiducial frame may appear brighter than 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.

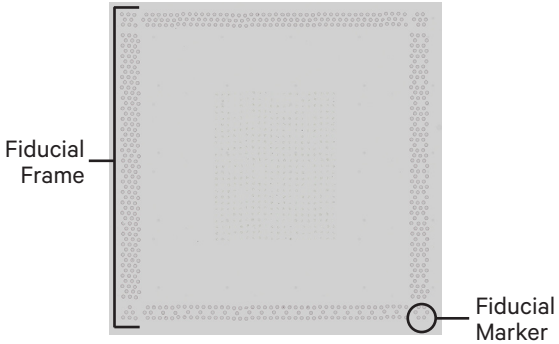


Image captured with BF settings.

Immunofluorescence Imaging

General Information

- Immunofluorescence staining can be performed prior to the Visium Spatial Gene Expression for FFPE workflow and is only required if simultaneous protein detection is desired.
- Consult the Deparaffinization, Decrosslinking, IF Staining & Imaging - Visium Spatial for FFPE Demonstrated Protocol for the full staining protocol and for information on antibody optimization (CG000410).
- 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.

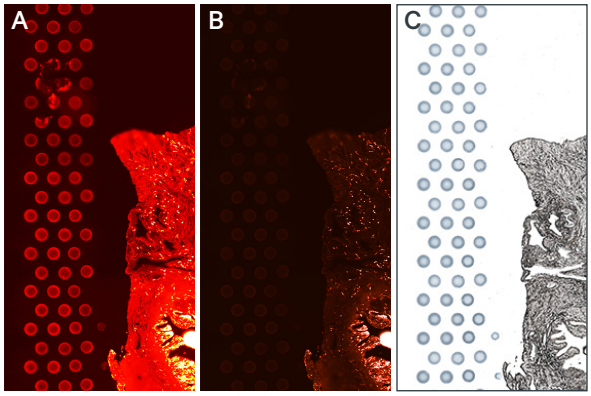
Image Acquisition

- 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.
- The signal from the fiducial frame in the TRITC/Cy3/555 channel can be significantly weaker than the signal from an immunofluorescence staining. The dynamic range of the imaging system may not allow simultaneous acquisition of both TRITC immunofluroescence and the fiducial frame. To avoid the saturation of the immunoflurescent signal, either:
  - Acquire an ancillary brightfield image of the fiducial frame using the same camera as the one used for the fluorescent acquisition to avoid misalignment (preferred choice).
  - Create two TRITC channels with different exposure times adjusted for the fiducial frame and the immunofluorescent signal respectively. Images can either be saved as multi-page TIFFs or split individual channels. Ensure capture of exactly the same field of view at the same magnification for all images.
- Fluorescent imaging requires manual alignment in Loupe Browser before entering the Space Ranger count pipeline.

Image Export

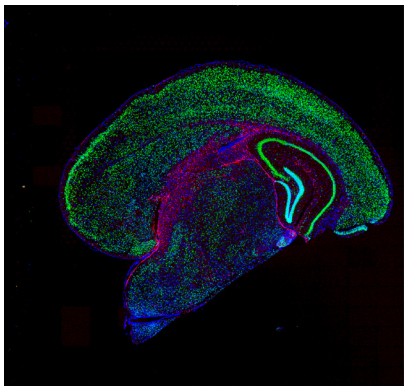
- Export images in one of the following formats: multi-page tiff, individual monochrome tiff/jpeg, or merged RGB color tiff/jpeg.
- Multi-page tiff: export stitched images as multi-page (or multi-stacked) tiff, where each filter set or fluorophore is assigned a single page within the file. Space Ranger 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.

Fiducial Images

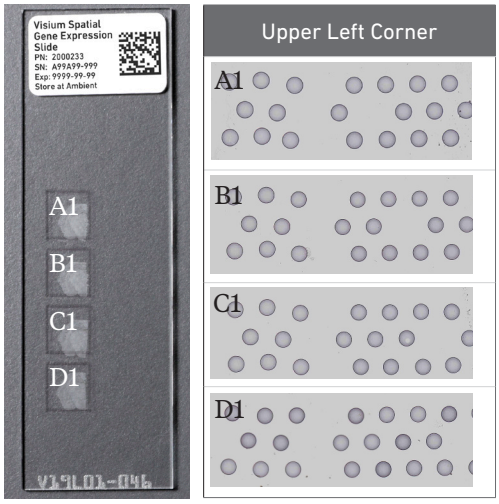


Images with exposure optimized for the fiducial frame **A** and antibody **B**. An ancillary brightfield image is shown in **C**.

Gene Expression Slide Capture Area



Imaging Channels: DAPI (Thermo Scientific, PN: 62248), Cy3 (GFAP, Sigma, PN: C9205-.2ML), and Cy5 (NeuN, Abcam, PN: ab190565



Visium Spatial Gene Expression for FFPE

Slide Information

- The Visium Spatial Gene Expression slides are used to generate libraries from FFPE tissue sections.
- Store unused slides at room temperature in their original container and packaging and keep sealed. DO NOT remove desiccant.
- The Visium Spatial Gene Expression Slide has four Capture Areas 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 (see image above, right).
- Fiducial frames are used by Space Ranger to align the image.
- Consult the Visium Spatial Gene Expression Reagent Kit for FFPE User Guide for complete protocol (CG000407).

Imaging Guidelines

- Image each capture area individually, as shown on the right. Minimize inclusion of adjacent Capture Areas.
- Imaging area should be ~1-2 mm beyond the fiducial frame for optimal imaging alignment and prevention of unintentional cropping.
- 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.

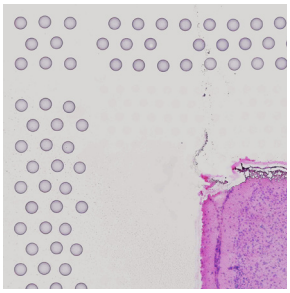
Image Capture Areas individually.

Imaging Examples

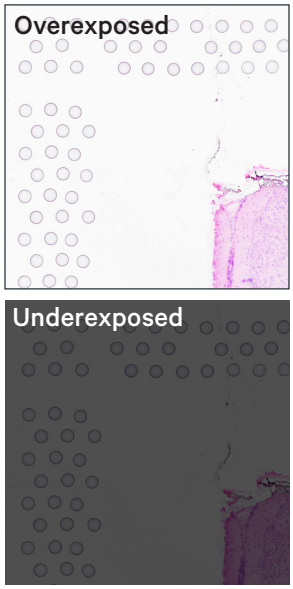
The following imaging artifacts may cause image analysis failure. Ensure that optimal imaging settings are verified prior to beginning the Visium Spatial Gene Expression workflow.

Image Exposure

Correct

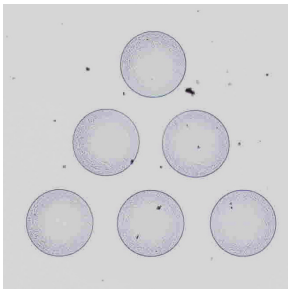


Incorrect



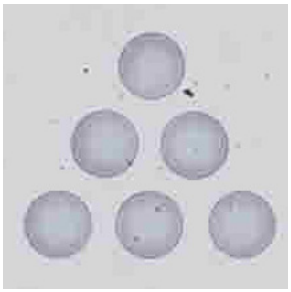
Capture Resolution

Correct



Capture resolution: 0.74 µm/pixel

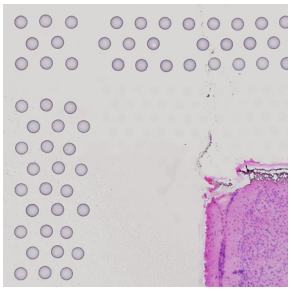
Incorrect



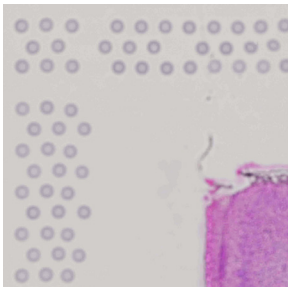
Capture resolution: 5.5 µm/pixel

Focus

Correct

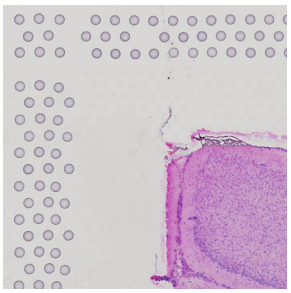


Incorrect

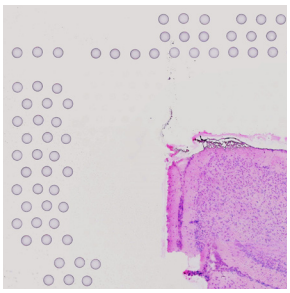


Stitching

Correct

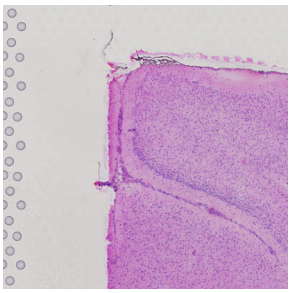


Incorrect

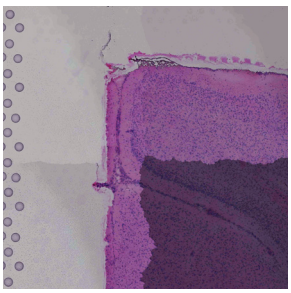


Shade Correction

Correct

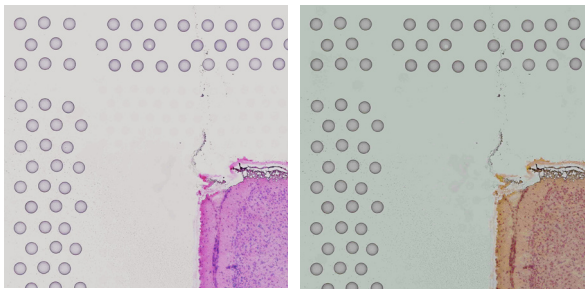


Incorrect



White Balancing

Correct Incorrect

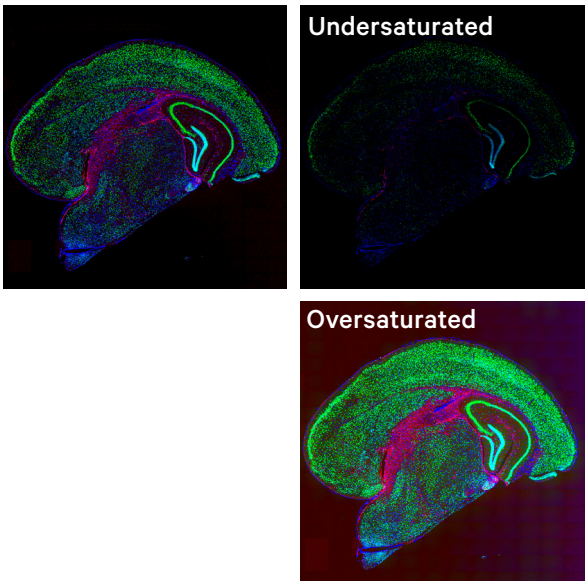


Fiducial Frame Signal

Correct Incorrect

Fluorescence Signal

Correct Incorrect



References

- Visium Gene Expression for FFPE – Tissue Preparation Guide (CG000408).
- Deparaffinization, H&E Staining, Imaging, & Decrosslinking - Visium Spatial for FFPE Demonstrated Protocol (CG000409).
- Deparaffinization, Decrosslinking, IF Staining & Imaging - Visium Spatial for FFPE Demonstrated Protocol (CG000410).
- Visium Spatial Gene Expression Reagent Kit for FFPE User Guide (CG000407).

Document Revision Summary

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