

Visium CytAssist Spatial Gene Expression Imaging Guidelines

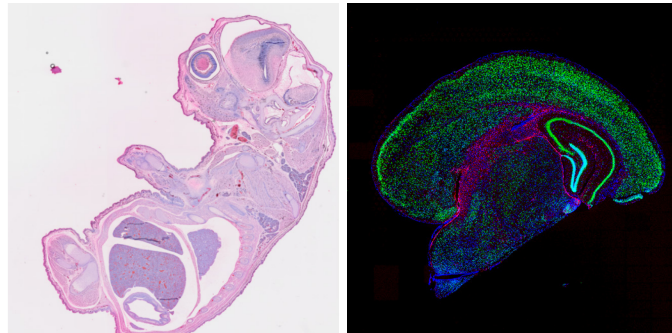
Introduction

The Visium CytAssist Spatial Gene Expression assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) and fresh frozen (FF) tissue samples. Prior to running the Visium CytAssist assay, a high resolution image is captured with a microscope that is combined with the image taken by the instrument to overlay gene expression data. 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. This Technical Note is applicable to Hematoxylin & Eosin (FFPE and FF) and immunofluorescence (FFPE only) stained tissue slides.

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Hematoxylin and Eosin (H&E) and Immunofluorescence (IF) Stained Tissue Sections



General Imaging Guidelines

1.0 Imaging Guidelines Overview

This section provides guidelines on slide handling, coverslip application, imaging system requirements, and output image requirements to ensure good image quality and compatibility with the Space Ranger analysis pipeline.

1.1 Slide Handling

- Wear a clean pair of gloves when handling slides.
 - DO NOT touch or wipe the side with tissue on the slide.
 - Ensure slides are clean. If necessary, use a laboratory wipe or compressed air to clean the bottom (non-active and without tissue) of the slide prior to imaging.
 - Place slides gently and evenly on the imaging stage.
 - If using an inverted system, slides can be imaged without inversion when using a 4X or 5X objective. These slides may also be imaged if using an objective with a correction collar or high working distance that accommodates imaging through 1 mm slides.
- If using a coverslip, samples should be loosely mounted with a non-permanent mounting medium. The coverslip will be removed prior to the assay workflow.
 - It is critical that all excess mounting media be removed prior to imaging, as mounting media may damage the imaging system. Inverted systems are especially prone to this type of damage. The amount of mounting media may be reduced depending on tissue size.
 - If a coverslip is not used for an H&E stained FFPE sample, blackening of hematoxylin may occur.
 - Coverslip application and removal guidance are provided in the Deparaffinization, H&E Staining, Imaging & Decrosslinking of FFPE tissue (Document CG000520) or Methanol Fixation, H&E Staining, Imaging & Destaining of FF tissue (Document CG000614) Demonstrated Protocols for more information.

1.2 Coverslip Use

- Coverslip application is optional for FF samples.
- Imaging through a coverslip will ensure optimal image quality for objectives with a magnification of 10X or higher. Use a coverslip appropriate for the chosen objective. Most objectives are corrected for e.g. a #1.5 (0.17 mm) coverslip. Some specialized objectives are corrected such that a coverslip is not necessary, while objectives with a correction collar (20X or higher) can accommodate other types of coverslips

1.3 Imaging System Requirements and Recommendations

A good quality image depends on even illumination, an objective and camera combination that provides acceptable resolution and image size, correct imaging settings, and automation (i.e. stitching and shading correction) that provides successful image processing. Example image systems tested by 10x Genomics are provided in Table 3

Objectives and Cameras

- The level of detail, clarity, and sharpness of an image depends on the objective's magnification, numerical aperture (NA), and the pixel size of the detection camera.
- Air objectives with magnifications of 4X, 5X, or 10X are recommended for image acquisition. DO NOT use immersion media on air objectives. Choose an objective based on imaging needs. See Table 1 for more information. Objectives with higher magnification and NA may be used, but are not required.
- The resolution, i.e. the smallest discernible detail in the image, is primarily dependent on the objective's NA. Objectives with NA 0.1 can be used to distinguish tissue morphology and subcompartments (resolution of 3-4 μm), while single cell resolution is achieved at NA 0.5 or higher (resolution of 0.7 μm).
- The image pixel size should be equal or 2-3 times lower than the smallest discernible detail in the image. 10x Genomics recommends an image pixel size in the range of 0.8 μm - 4 μm . The pixel size is typically displayed in the imaging software, saved in the image information, or can be calculated by dividing the camera pixel size by the objective's magnification.
- A color camera is required for H&E stained tissue and a monochromatic capable camera/detector for IF stained tissue sections.
- Researchers who wish to perform image-based cell segmentation should use a higher image resolution, e.g. with a 20X or 40X objective.

Filters for Fluorescence Imaging

- Commercially available fluorescently conjugated primary and/or secondary antibodies may require specific filter sets. Ensure filter sets are compatible with imaging system and fluorophore choice.

Objective Magnification / NA	Image Size (pixels) for 8 x 8 mm area	Image Pixel Size (μm)	Image Resolution
4X / 0.1	2,000 – 11,500	0.7 – 4	Tissue morphology and compartments with > 10 cells (50 – 200 μm)
5X / 0.16			
4X / 0.2	4,000 – 11,500	0.7 – 2	Tissue compartments with 3 – 10 cells (20 – 100 μm)
5X / 0.25			
10X / 0.25	5,000 – 20,000	0.4 – 1.6	Tissue compartments with 1 – 10 cells (3 – 10 μm)
10X / 0.45			
10X / 0.5			
20X / 0.8	15,000 – 45,000	0.2 – 0.5	Subcellular resolution, nuclear features (0.5 – 2.5 μm)

Table 1. Image resolution obtained with example objectives and image size settings

Image Acquisition and Processing

- H&E stained tissue sections are imaged with a color camera. Slide scanners and some microscopes are automated for optimal imaging. For microscopes that require manual setting adjustments, verify the following:
 - When imaging with a 10X or higher objective, adjust the microscope for Köhler illumination: This adjustment of the condenser focus and position provides uniform and strong illumination of the tissue and ensures good contrast and clarity. Refer to the manufacturer directions for performing this adjustment.
 - Adjust the illumination power and exposure time to obtain a bright image without saturation. Poorly exposed images cannot be corrected after acquisition.
 - Adjust the color balance on the camera by performing a “white balance” on an empty area.
 - Stitch the individual tiles with shading correction, if available in the software.
- Fluorescence imaging is performed with a monochromatic camera/detector. For microscopes that require manual setting adjustments, verify the following:
 - Adjust the illumination power, detector gain (if available), and exposure time to obtain a clear and non-saturated image. Poorly exposed images cannot be corrected after acquisition.
 - 10x Genomics recommends acquiring images with correction for the field of view flatness. Without this correction, the fluorescence intensity is typically lower at the edge of the field of view compared to its center.
 - Stitch the individual tiles without shading correction that could alter the acquired fluorescence levels.

1.4 Image Format Requirements

- A compute system with sufficient resources to handle large images (0.5-10 GB) and the resulting data should be used for image processing. The compute system should be able to stitch images via the microscope’s native software or third party software such as FIJI/ImageJ. Pre-install the computer system with the most recent version of Loupe Browser to support manual alignment and tissue selection for IF and H&E stained tissues. Space Ranger is a linux pipeline and will be run on a separate linux server.
- Space Ranger and Loupe Browser accept TIFF/bigTIFF (file extension .tif or TIFF) or JPEG (file extension .jpg or JPEG) image formats. Space Ranger and Loupe Browser support pyramid TIFF or multiple single TIFF/bigTIFF images that contain multiple resolutions. Table 2 provides more information. Images in a different format must be converted to TIFF/bigTIFF or JPEG in the native software or other software (i.e. FIJI/ImageJ). Consult the 10x Genomics Support site for recommendations.
- Images have a minimum size requirement of 2,000 pixels (Visium CytAssist Spatial Gene Expression slide v2, 6.5 mm) and 4,000 pixels (Visium CytAssist Spatial Gene Expression slide v2, 11 mm) in at least one direction to be accepted in Space Ranger. This minimum size does not guarantee images with good resolution. Refer to Section 1.2. (Imaging system requirements and recommendations) for imaging best practices.

Image Type	Image Format
Brightfield Image	3 x 8-bit color TIFF/bigTIFF or JPEG
	16-bit grayscale TIFF/bigTIFF or JPEG
	3 x 8-bit color pyramid TIFF and multiple 8-bit TIFF/bigTIFFs at different resolution (Space Ranger 1.2.2 or newer and Loupe Browser 5.0.1 or newer)
Fluorescence Image	8 or 16-bit grayscale single, multi-page TIFF/bigTIFF
	8 or 16-bit grayscale multiple, single-page TIFF/bigTIFF or JPEG
	3 x 8-bit colored image TIFF/bigTIFF or JPEG

Table 2. Acceptable Image Formats for Brightfield and Fluorescence Images

Brightfield, Fluorescence, and CytAssist Imaging Guidelines

2.0 Brightfield Imaging Guidelines

- If performing Hematoxylin and Eosin (H&E) staining and imaging, do so prior to the Visium CytAssist Spatial Gene Expression workflow. Consult the Deparaffinization, H&E Staining, Imaging & Decrosslinking of FFPE tissue (Document CG000520) or Methanol Fixation, H&E Staining, Imaging & Destaining of FF tissue (Document CG000614) Demonstrated Protocols for more information.
- If the tissue is over or underexposed, adjust the illumination power and exposure such that the tissue morphology appears clearly. Poorly exposed images cannot be corrected after acquisition.
- Images intended for automatic processing in Space Ranger should be roughly axis-aligned, although rotations of less than 15 degrees are acceptable.
- After image acquisition, stitch image tiles together with the microscope's native software or third party software such as FIJI/ImageJ.
- Export stitched images as a 24-bit color or 8-bit/16-bit monochrome TIFF/BigTIFF or JPEG images. Pyramid TIFF or TIFF files that contain multiple images at different resolutions are compatible with the latest versions of Space Ranger and Loupe Browser.
- For information on image analysis and tissue alignment, refer to the Visium section of the 10x Genomics Software Support Website.

2.1 Fluorescence Imaging Guidelines

- Immunofluorescence staining and imaging should be performed prior to the Visium CytAssist Spatial Gene Expression for FFPE workflow and is only required if alignment of specific protein staining with gene expression is desired.
- Consult the Deparaffinization, Decrosslinking, IF Staining & Imaging - Visium Spatial for FFPE Demonstrated Protocol (Document CG000519) for the full staining protocol, inclusion of autofluorescence quenching, and information on antibody optimization.

Image Export

- Fluorescence images can be provided to Space Ranger as individual grayscale images or as a single, combined, color image. Save images in one of the following ways:
 - A multi-page TIFF/bigTIFF file that contains one or more 8-bit or 16-bit grayscale images. The file can contain up to six total pages/images with the same bit depth.
- Individual channel images as grayscale, 8-bit or 16-bit TIFF/bigTIFF or JPEG files with up to six total files from the same tissue section. Ensure that each monochrome image has been acquired at the same magnification, has the same bit depth, dimension, orientation, and file format.
- A color image with merged channels, where individual channels are colored and combined into one image. This format can be a 24-bit RGB color TIFF/bigTIFF or JPEG file. Saving and exporting images in this format will result in the inability to independently adjust each channel after merging.
- Table 3 shows imaging systems used or tested by 10x Genomics in the development of this protocol. Any equivalent imaging system can be used as an alternative and is dependent on user needs and applications. For additional information, contact imaging system manufacturer.

2.2 CytAssist Imaging Guidelines

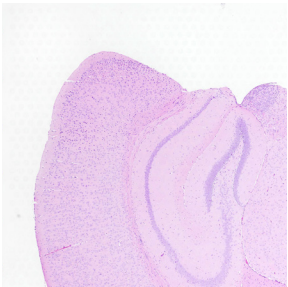

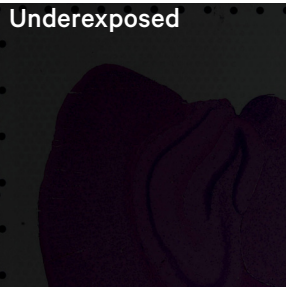
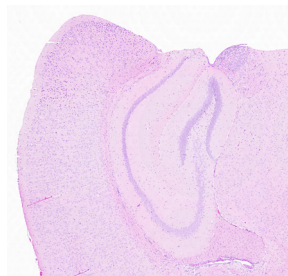
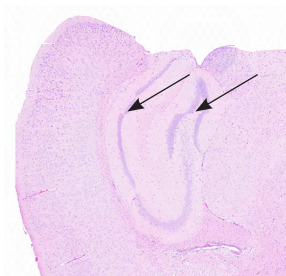
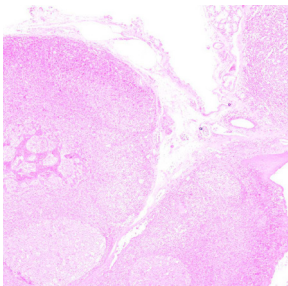
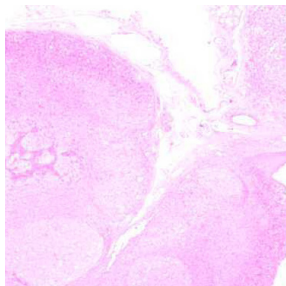
- CytAssist captures a single field of view image of the tissue section, which can be automatically registered to a high resolution H&E or IF image of the same tissue section (if provided) in the Space Ranger analysis pipeline or can be registered using Loupe Browser 6.2 or later.
- If the tissue section is larger than the Visium CytAssist slide Capture Area, the area of interest may be optionally annotated after high resolution imaging using the recommended marker provided in the Demonstrated Protocols.
- If slide is annotated, annotation marks will appear in the CytAssist image, but they will not be transferred on to the data visualization image. Annotation marks interfere with the auto-tissue registration, but it can be fixed by manual tissue registration in Loupe Browser.

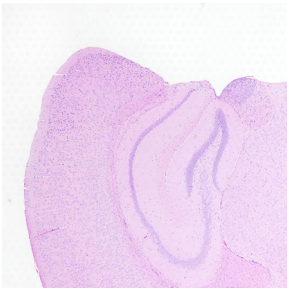
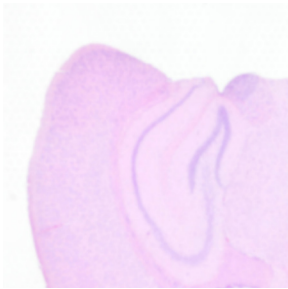
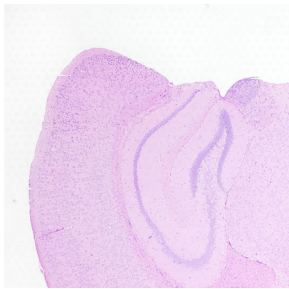

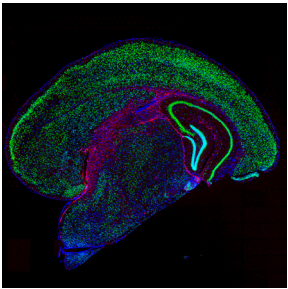
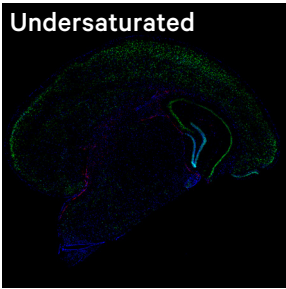
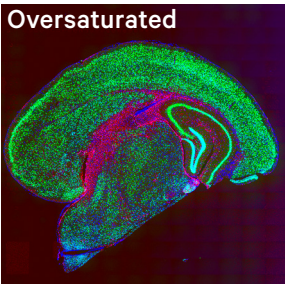
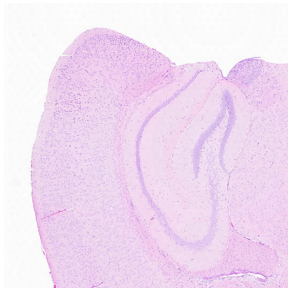
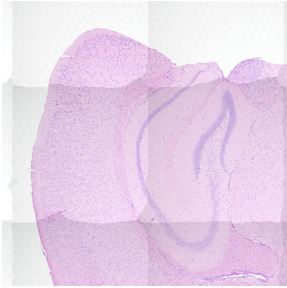
Supplier	Description	Configuration	Image Acquisition Method
Thermo Fisher Scientific	EVOS M7000	Inverted	Semi-automated
Leica	Aperio Versa 8	Upright	Automated
	Leica DMI8	Inverted	Automated
MetaSystems	Metafer	Upright	Automated
Nikon	Nikon Eclipse Ti2	Inverted	Manual
BioTek	Cytation 7	Inverted or Upright	Semi-automated
Keyence	Keyence BZX800	Inverted	Semi-automated
Olympus	VS200	Upright	Automated
Zeiss	Imager.Z2	Upright	Manual

Table 3. Tested imaging systems.

Imaging Examples

The following imaging artifacts may cause poor alignment of the image with gene expression data, reduced image resolution, image analysis failure, and/or difficulty in data interpretation. Ensure that optimal imaging settings are verified with pre-stained tissue prior to beginning the Visium CytAssist Spatial Gene Expression workflow.

Setting	Correct	Incorrect
Image Exposure	<div></div>	<div><div></div><div></div></div>
Stitching	<div></div>	<div></div>
Pixel Size	<div><div>3x8 bit 2424x2424</div></div>	<div><div>3x8 bit 808x808</div></div>

Setting	Correct	Incorrect
Focus		
White Balancing		
Fluorescence Signal		<div><div>Undersaturated</div><div>Oversaturated</div></div>
Shade Correction		

References

- Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging, & Decrosslinking Demonstrated Protocol (CG000520).
- Visium CytAssist Spatial Gene Expression for Fresh Frozen – Methanol Fixation, H&E Staining, Imaging & Destaining Demonstrated Protocol (CG000614).
- Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, Decrosslinking, IF Staining & Imaging Demonstrated Protocol (CG000519).
- Visium CytAssist Spatial Gene Expression Reagent Kit User Guide (CG000495).

Document Revision Summary

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