Visium CytAssist Spatial Gene Expression for FFPE Imaging Guidelines

Introduction

The Visium CytAssist Spatial Gene Expression for FFPE assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) 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 both Hematoxylin & Eosin (H&E) and immunofluorescence (IF) stained tissue sections on glass slides.

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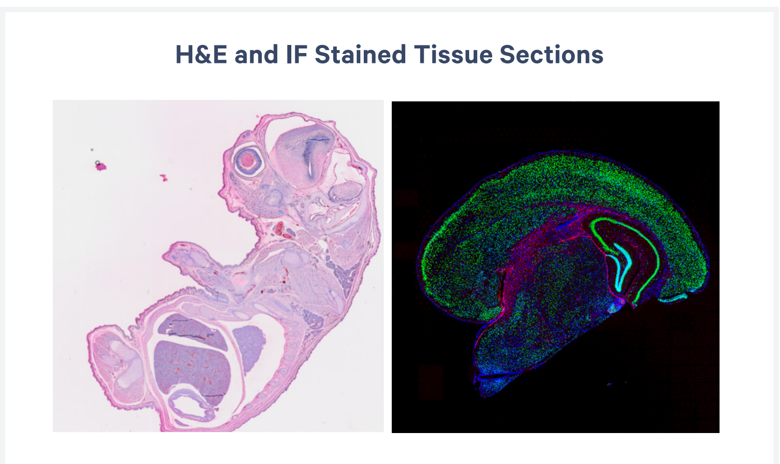
H&E and IF Stained Tissue Sections



General Imaging Guidelines

1.0 Imaging Guidelines Overview

This section provides guidelines on slide handling, 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 sections were recently placed onto a slide, apply a coverslip and mounting medium.
    - 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
    - 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. Refer to Appendix A1: Optional Coverslip Application.
    - 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. Refer to Appendix A2: Coverslip Removal for H&E Stained Sections or Appendix A3: Coverslip Removal for IF Stained Sections.
- 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.

1.2 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 Appendix A4: Imaging Systems Tested.

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	
10X / 0.45 10X / 0.5	10,000 – 20,000	0.4 – 0.8	Tissue compartments with 1 – 10 cells (3 – 10 µm)
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.

1.3 Image Format Requirements

- A compute system with sufficient resources to handle large images (0.5-5 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 and Space Ranger to support manual alignment and tissue selection for IF and H&E stained tissues.
- 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

3.0 Brightfield Imaging Guidelines

- If performing Hematoxylin and Eosin (H&E) staining and imaging, do so prior to the Visium CytAssist Spatial Gene Expression for FFPE workflow. Consult the Deparaffinization, H&E Staining, Imaging & Decrosslinking (Document CG000520) Demonstrated Protocol 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.

3.1 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.

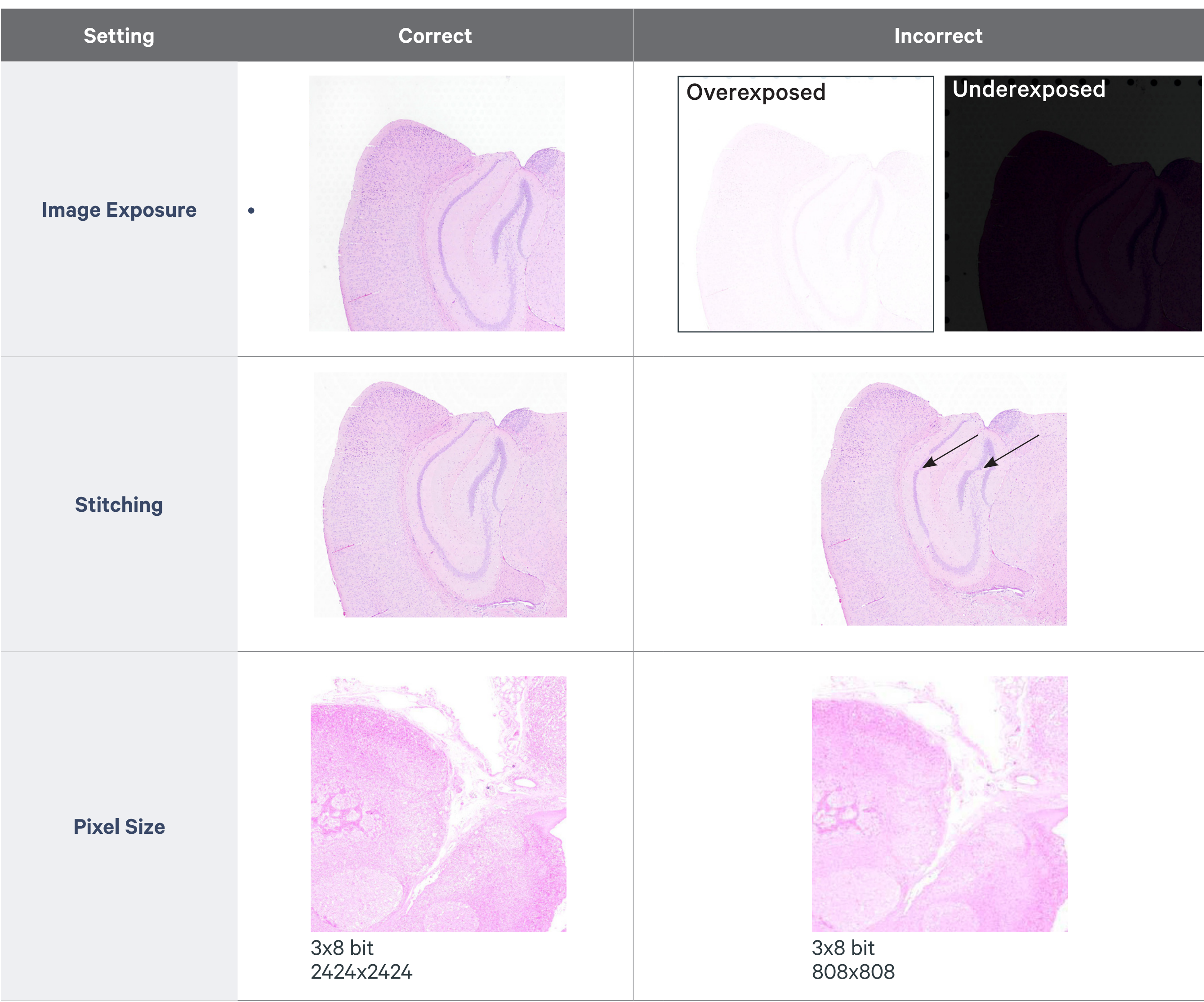
- A color image with merged channels, where individual channels are colored and combined into one image. This format can be a 24-bit RGB color TIFF/bigTIFF or JPEG file. Saving and exporting images in this format will result in the inability to independently adjust each channel after merging.

3.2 CytAssist Imaging Guidelines

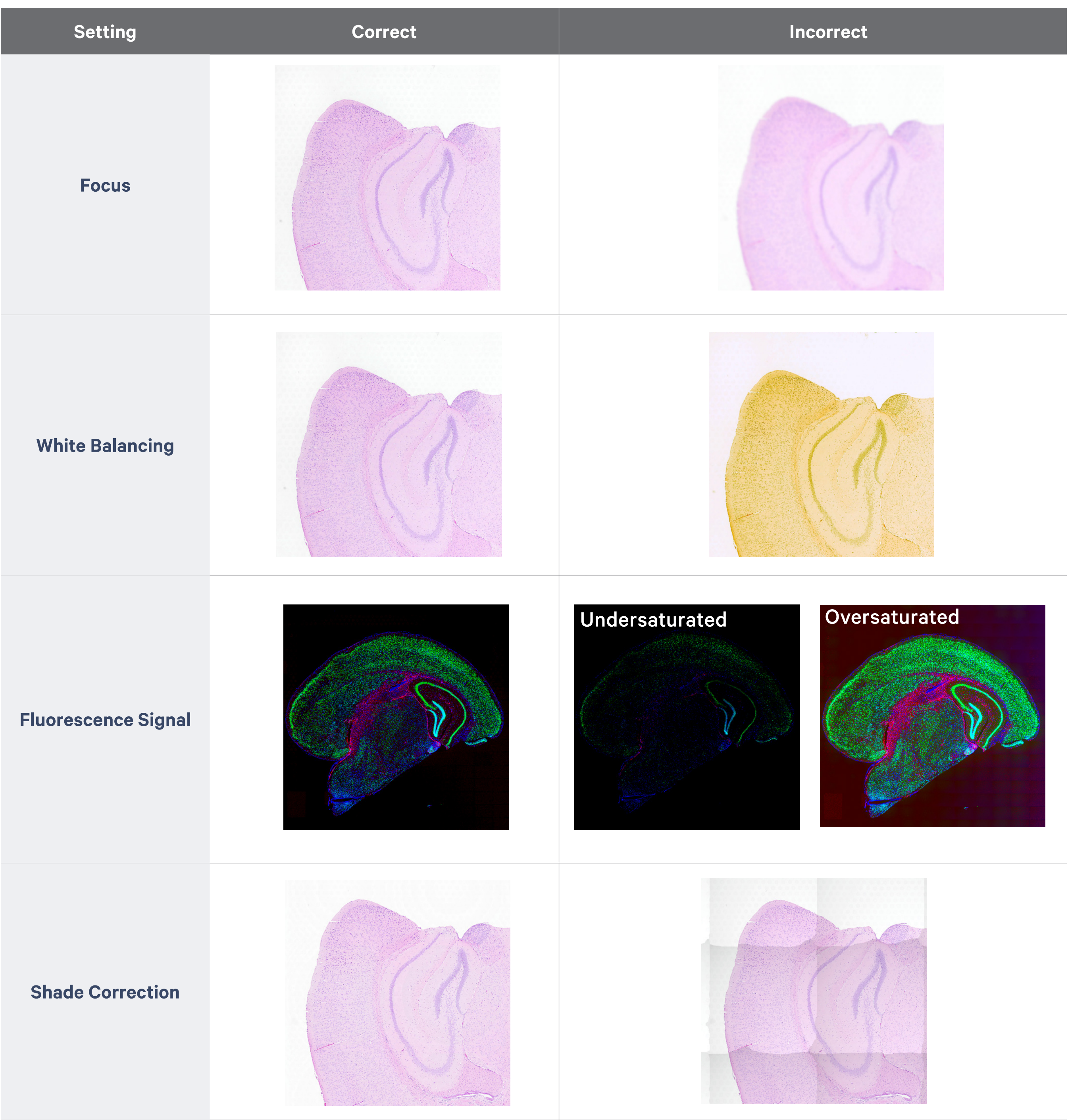
- CytAssist captures a single field of view image of the tissue section, which can be automatically registered to a high resolution H&E or IF image of the same tissue section (if provided) in the Space Ranger analysis pipeline or can be registered using Loupe Browser 6.2.
- If the tissue section is larger than the Visium CytAssist slide Capture Area, the area of interest may be optionally annotated after high resolution imaging using a marker.
- 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.

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 Spatial Gene Expression workflow.



Setting	Correct	Incorrect
Image Exposure	•	Overexposed Underexposed
Stitching		
Pixel Size	3x8 bit 2424x2424	3x8 bit 808x808



Setting	Correct	Incorrect	
Focus			
White Balancing			
Fluorescence Signal		Undersaturated	Oversaturated
Shade Correction			

Appendix

A1: Coverslip Application

- Non-permanent coverslipping of glass slides is recommended if repeatedly imaging slide over time across different imaging systems.
- When using a 10X or higher magnification objective, imaging through the coverslip will provide optimal image quality. Refer to Slide Handling for more information.
- Remove coverslip immediately after imaging and proceed with the appropriate sample preparation workflow.
- Mounting Medium should be pipette mixed to avoid generating bubbles. Centrifuge briefly.

For H&E Staining				
Mounting Medium	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides + 15% (µl)**
Glycerol	100%	85%	85.0	195.5
Nuclease-free Water	-	-	15.0	34.5
Total	**-**	**-**	**100.0**	**230.0**

For IF Staining				
Mounting Medium	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides + 15% (µl)**
Glycerol	100%	80%	80	184
SlowFade Diamond	100%	20%	20	46
Total	**-**	**-**	**100**	**230**

a. Place slide on a flat, clean, non-absorbent work surface. Some residual droplets may remain from previous wash steps.

b. Using a **wide-bore** pipette tip, add **100 µl** of Mounting Medium to uniformly cover all tissue sections on the slide.

c. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, without introducing bubbles. Allow Mounting Medium to spread and settle.

d. If needed, remove any large excess of Mounting Medium by carefully wicking away from the edge of the coverslip with a laboratory wipe. Be careful not to move coverslip and disturb the tissue.

e. Once coverslipping is complete, **immediately** proceed with imaging.

DO NOT let the attached coverslip dry.
DO NOT use Cytoseal, nail polish, or other permanent mounting agent for securing the coverslip.

Slide in image is representative

A2: Loose Coverslip Removal for H&E Stained Sections

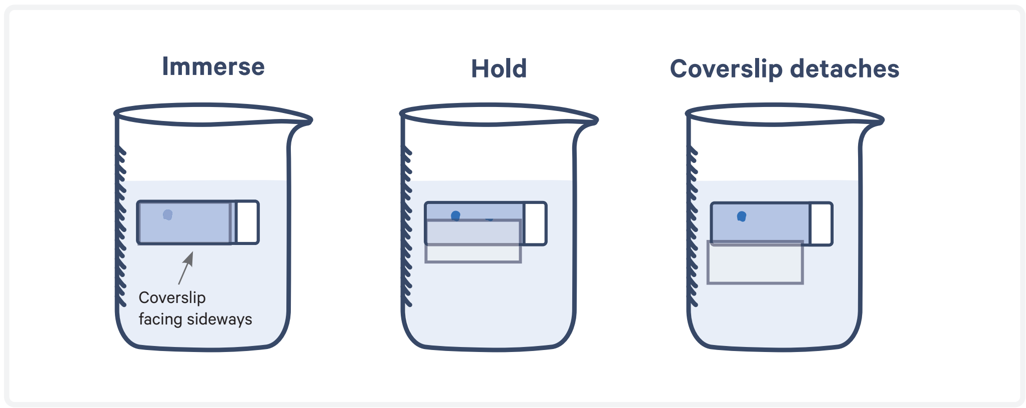
a. Dispense **800 ml** Milli-Q water in a beaker.

b. Immerse the slide sideways/horizontal in the beaker containing **800 ml** Milli-Q water with the coverslipped surface fully sideways.

c. Hold the slide in water until the coverslip slowly separates away from the slide.

To avoid damaging the tissue sections or causing tissue detachment, DO NOT move the slide up and down, shake forcibly or manually move the coverslip.

d. Gently immerse 30x in Milli-Q water to ensure all mounting medium is removed.



e. Wipe the back of the slide with a laboratory wipe. Place on a flat, clean, non-absorbent work surface and air dry.

f. Incubate slide on the Low Profile Thermocycler Adaptor with the thermal cycler lid open for **3 min** at **37°C** to dry the slide.

g. Proceed **immediately** to next step in sample preparation protocol.

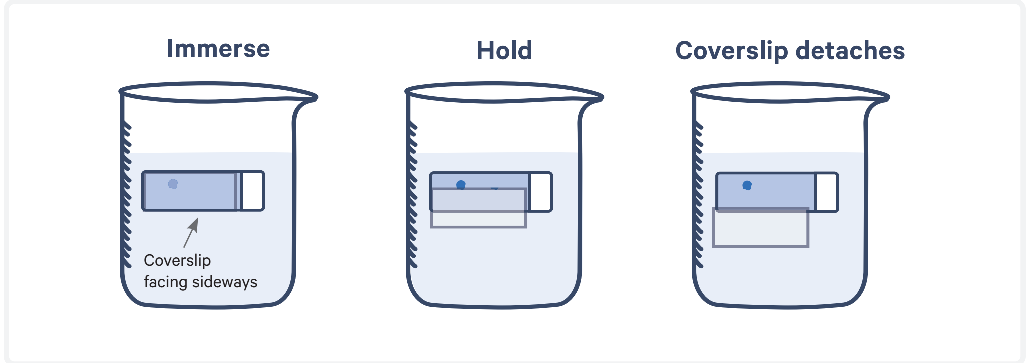
A3: Loose Coverslip Removal for IF Stained Sections

a. Dispense **800 ml** 1X PBS in a beaker.

b. Immerse the slide sideways/horizontal in the beaker containing **800 ml** 1X PBS with the coverslipped surface fully sideways.

c. Hold the slide in water until the coverslip slowly separates away from the slide.

To avoid damaging the tissue sections and Capture Areas or causing tissue detachment, DO NOT move the slide up and down, shake forcibly or manually move the coverslip.



d. Gently immerse 30x in 1X PBS to ensure all mounting medium is removed.

e. Wipe the back of the slide with a laboratory wipe. Place on a flat, clean, non-absorbent work surface and air dry.

f. Incubate slide on the Low Profile Thermocycler Adaptor with the thermal cycler lid open for **3 min** at **37°C** to dry the slide.

g. Proceed **immediately** to next step in sample preparation protocol.

A4: Imaging Systems Tested

The table below 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.

Supplier	Description	Configuration	Image Acquisition Method
Thermo Fisher Scientific	EVOS M7000	Inverted	Semi-automated
Leica	Aperio Versa 8 Leica DMi8	Upright Inverted	Automated 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

References

- Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000518).
- Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging, & Decrosslinking Demonstrated Protocol (CG000520).
- Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, Decrosslinking, IF Staining & Imaging Demonstrated Protocol (CG000519).
- Visium CytAssist Spatial Gene Expression Reagent Kit for FFPE User Guide (CG000495).

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Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA