

EPC Genomics Core

Visium HD guidelines A – Formalin-Fixed, Paraffin Embedded (FFPE)

The Emory Winship CTPSR and EPC Histology and Molecular Pathology Laboratories can fully support the section placement, staining and imaging components of the Visium HD FFPE and H&E staining workflow. If interested in IF staining, partnership with an alternative histology/imaging core is required or this must be performed by the researchers themselves.

Please find the relevant 10x Genomics documentation at this support page:

<https://www.10xgenomics.com/support/spatial-gene-expression-hd>

For planning of experiments, please refer to:

CG000698 Visium HD Spatial Gene Expression Protocol Planner (Rev E)

For sectioning of tissue, section placement, staining and imaging, please refer to:

CG000684 Visium HD FFPE Tissue Preparation Handbook (Rev D)

CG000548 Visium CytAssist Tissue Slide Alignment Instruction, Quick Reference Cards (Rev F)

CG000730 Visium Cassette S3 for Assembly & Disassembly, Quick Reference Cards (Rev B)

CG000688 Visium HD Spatial Applications Imaging Guidelines (Rev C)

Sample number:

Please submit projects with an even number of samples as each Visium HD slide comes with two capture areas and cannot be reused if only one capture area is used. We will charge the full price of the Visium HD slide/two reactions if you have an odd number of samples.

Format of slides to submit to EPC Genomics Core:

After imaging, slides must be provided with the coverslips removed, stopping at the following steps in CG000684 (Rev D) -

- H&E workflow stop at step h, **3.6 Coverslip Removal**, page 66
- IF workflow stop at step g, **4.9 Coverslip Removal**, page 94

Once this step has been reached, the Genomics Core is limited to a **two-week window** (where slides are stored in slide mailers in the dark at 4°C with desiccant) in which the downstream processing must start. Please coordinate the timing of your stopping point and sample drop-off with us well in advance.

There is a stopping point (up to 24 hours) after coverslip mounting and before imaging, but if this is used, the Genomics Core is required to proceed to Destaining/Probe Hybridization immediately after you complete imaging. Unless we have agreed to this ahead of time, we would prefer that you do not use this stopping point.

RNA quality assessment:

- Requires two 10 µm tissue sections from the FFPE tissue block (four sections for smaller tissues ≤6.5 x 6.5 mm).
- 10x Genomics recommends proceeding only with samples where the DV200 is > 30%

Thickness of sections for Visium HD:

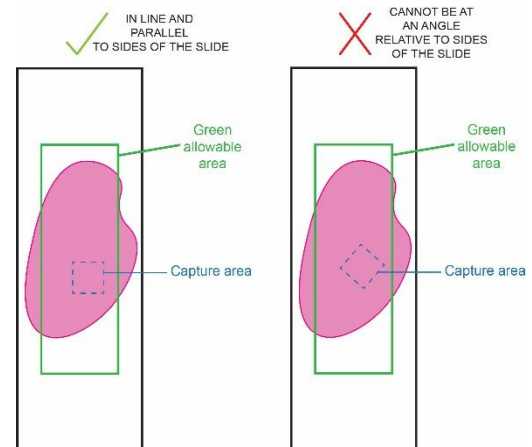
Recommended section thickness is 3-10 µm, but 5 µm sections are most commonly used.

Placement of sections and Area of interest (AOI):

Please refer to CG000698 for guidance on slides that have been tested with FFPE Tissues in the Visium HD assay.

Use CG000548 to determine if the tissue section and your approximate AOI is located in an area that results in successful analyte transfer and imaging (within the green allowable areas in the diagrams). Make sure you are using the diagram that matches the width of the slide you are using and the correct diagram for slides that have frosted ends and/or marks.

The AOI cannot be at an angle relative to the sides of the slide (see diagram on the right).



AOI annotation:

Please annotate your 6.5 mm x 6.5 mm AOI with green marker on the back side of the tissue slide, this helps us with the alignment of the gasket over the AOI as this is very difficult to do without visual cues on the physical slide.

H&E workflow

- annotate AOI using green marker on the back side of the tissue slide based on assessment of tissue morphology from the H&E image
- we will remove the annotations prior to loading tissue slide onto the CytAssist instrument

IF workflow

- before immunostaining, the tissue slide must be assembled in the Tissue Slide Cassette for decrosslinking, and this requires placing of the gasket over the AOI prior to staining
- use tissue morphology information from an adjacent H&E or IF stained section to annotate the back of the tissue slide with green marker
- we will remove annotations prior to loading tissue slide on the CytAssist instrument

10x Genomics accessories and/or reagents required during tissue slide preparation and imaging steps:

H&E workflow:

- CG000684 (Rev D), page 66, 2.5 Coverslip Removal – requires the Low Profile Thermocycler Adapter from the 10x Genomics CytAssist Accessory Kit
 - ▶ Please also consult CG000698 to assess whether your thermal cycler is compatible
 - ▶ These can be borrowed from the Genomics Core, but must be returned when you deliver your slides to us
- No other 10x accessories or reagents required

IF workflow:

One additional Tissue Slide Cassette (Visium Tissue Slide Cassette S3, 6.5 mm, 4 pk PN-1000684) is required for each processed Tissue Slide to perform the assay (see CG000684 (Rev D), page 71)

- ☐ PN-1000684 is \$600 for 4 Cassettes
- ☐ You can purchase this yourself through 10x Genomics or we can provide this and will include this on your invoice when the project is closed
- ☐ One cassette is required during the permeabilization and IF staining steps and a second cassette is required at the end of coverslip removal through to the downstream genomics component of the protocol; 10x technical support confirmed that the same cassette should not be used for both parts of the workflow to avoid cross contamination

- CG000684 (Rev D), page 78, 85, 4.3 Decrosslinking – requires the Decrosslinking Buffer B and Perm Enzyme B from the 10x Genomics Visium HD Spatial Gene Expression Reagent Kit
 - ▶ These can be obtained from the Genomics Core
- CG000684 (Rev D), page 85, 4.3 Decrosslinking, step d, slide is placed in a Tissue Slide Cassette
 - ▶ Refer to CG000730 for assembly instructions – be very careful with the gasket as this can cause the tissue to detach
 - ▶ See above for options to obtain Tissue Slide Cassettes
 - ▶ The extra Tissue Slide Cassette kit that is purchased should come with slide seals, but if not, we have plenty and can provide them
- CG000684 (Rev D), page 87-92, 4.4-4.6 Immunofluorescence staining – this is performed with the slide in the Cassette with the slide taken out for Coverslip Mounting

10x recommends performing prior testing of the IF antibodies on the same tissue block before going ahead with the Visium HD workflow (CG000684 (Rev D), page 73, **Antibody Optimization**)

- This requires a different Cassette, the Visium 8-port Cassette S3, 4 pk (PN-1000685) that must be purchased separately. We can also purchase this for you and include it on the invoice at the conclusion of the project.

High-resolution microscope image:

For analysis of your Visium HD data, the image taken by the Visium CytAssist instrument is used to map gene expression data back to a high-resolution image captured with a microscope. Although this high-resolution image is optional in Space Ranger analysis, we expect most researchers to want to include it.

We have found that images acquired with at least 40X magnification have worked best.

Data analysis supported by Genomics Core – please provide the microscope images to us as soon as they are available (either in the raw format from the microscope or converted to the BigTIFF (.tif2, .tf8, .btf) format).

Data analysis performed by researcher – we will still undertake routine QC (Space Ranger Count on the 10x Cloud Analysis platform) of the sequencing data before releasing the FASTQ files to you. The high-resolution microscope image is not absolutely required for this, but if we do include it in our QC analysis, we will know whether there are any issues with the image formats provided or if the CytAssist to image registration algorithm is working. If the automatic alignment fails, we can let you know ahead of time that a manual alignment using Loupe Browser is required before you start your Space Ranger processing.

If you are providing one of the file types mentioned in CG000688 that is in the format generated by the software associated with the microscope – i.e. .vsi, .ndpi, .svs, .nd2, we can convert the images to BigTIFF for you using the command line tool, `bftconvert` (see below).

Raw microscope images can be viewed with QuPath. For .ndpi images, NDP.view2 can also be used for viewing.

A few of the software associated with the microscope (depending on the supplier) can acquire .tif images or be used to export images as TIFF/BigTIFF – please make sure to acquire and/or export images as BigTIFF.

QuPath is available for free at the following link: <https://qupath.github.io/>

NDP.view2 is available for free at the following link: <https://www.hamamatsu.com/us/en/product/life-science-and-medical-systems/digital-slide-scanner/U12388-01.html>

Low-resolution microscope image with AOI annotated – It is also useful for you to provide the microscope images with the AOIs drawn as this will help with the alignment of the gasket. The raw microscope images can be converted to one of the lower resolution formats (TIFF or JPEG) using QuPath or NDP.view2. The AOI can be drawn with QuPath, NDP.view2 or Image J and these programs can also be used for adding scale bars.

Please review the requirements for images compatible with Space Ranger (summarized below) in CG000688 (Table 3 in Rev A only) as well as at the link: <https://www.10xgenomics.com/support/software/space-ranger/latest/analysis/inputs/image-image-recommendation>

Brightfield Image: 24-bit color TIFF or JPEG; 16-bit grayscale TIFF or JPEG

Fluorescence Image (dark): 8 or 16-bit grayscale single, multi-page TIFF; 8 or 16-bit grayscale multiple single-page TIFF or JPEG

Fluorescence Image (colorized): 24-bit single colored image TIFF or JPEG

Space Ranger v4.0 (released June 12, 2025) introduced a new nucleus and cell segmentation algorithm for Visium HD, enabling the assignment of transcripts to inferred cells instead of bins. This uses the microscope image (H&E only).

- Converted images that preserve as much of the resolution of the raw microscope images as possible is best for input into spaceranger segment, hence we request that you convert your images to BigTIFF only.
- For best performance, 10x Genomics recommends that the pixel size of your converted image be in the range of 0.16 micron to 0.33 micron
- Image downsampling is not recommended as it reduces performance
- 10X magnification is not sufficient for cell segmentation with Space Ranger, but we recommend the use of at least 40X magnification

bfconvert

BigTIFF files can be generated in two ways using bfconvert.

1. **Using the -bigtiff flag:** This explicitly tells bfconvert to create a BigTIFF output file. Examples:

```
# bfconvert -bigtiff input.ndpi output.ome.tif
```

```
# bfconvert -bigtiff /path/to/input output.ome.tif
```

2. **Using a BigTIFF file extension:** bfconvert will automatically write a BigTIFF if you use the file extensions .tf2, .tf8 or .btf. It will also automatically convert to BigTIFF if the uncompressed image data exceeds 4GB. Example:

```
# bfconvert input.ndpi output.ome.btf
```

(using .ome.tf2 OR .tf2, .ome.tf8 OR .tf8, .ome.btf OR .btf in the command will all work)

Download bfconvert here:

<https://docs.openmicroscopy.org/bio-formats/6.0.1/users/comlinetools/conversion.html>