EPC Genomics Core

Visium HD guidelines C – Fixed Frozen (FxF)

The Emory Winship CTPSR and EPC Histology and Molecular Pathology Laboratories do not currently support the section placement, staining (H&E or IF) and imaging components of the Visium HD FxF workflow. Partnership with an alternative histology/imaging core is required or preparation of tissue slides must be performed by the researchers themselves.

For planning of experiments, please refer to:

CG000698 Visium HD Spatial Gene Expression Protocol Planner (Rev B, Addendum 1)

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

CG000764 Visium HD Fixed Frozen Tissue Preparation Handbook (Rev B)

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

CG000730 Visium Cassette S3 for Assembly & Disassembly (Rev A)

CG000688 Visium HD Spatial Applications Imaging Guidelines (Rev B)

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 intact, stopping at the following steps in CG000764 (Rev B) -

- H&E workflow stop at step b**, 2.4 Imaging**, page 54
- IF workflow stop at step c**, 3.7 Imaging**, page 86

Please make sure the Stopping Point Mounting Medium (which contains RNase Inhibitor) is used.

Once the slides have been imaged, the Genomics Core is limited to a **one-week window** (where slides are stored in the dark at 4°C) 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 3 days) after coverslip mounting and before imaging, but if this is used, the Genomics Core is required to proceed to Coverslip Removal 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. In addition, the earlier stopping points may only be used if staining guidance for stopping points was followed.

RNA quality assessment:

- Requires 20-30 mg of tissue sections from the OCT-embedded tissue block (~4 sections at 25 µm thickness). If tissues contain extensive connective or adipose tissue, cryosection up to 50 mg of tissue.
- Tissue sections with DV200 ≥ 50% are optimal for the Visium HD FxF assay

Thickness of sections for Visium HD:

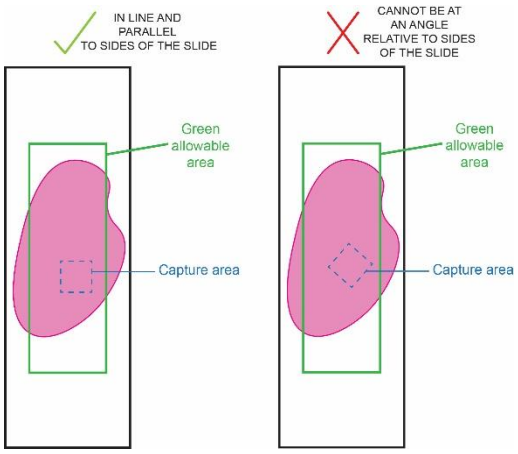
Recommended section thickness for most tissue types is 10 µm, but tissues from 10-20 µm are compatible with the assay.

Placement of sections and Area of interest (AOI):

Please refer to CG000698 for guidance on slides that have been tested with Fixed Frozen 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:

- CG000764 (Rev B), page 50, 2.1 Rehydration – 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 CG000764 (Rev B), page 61)

- □ 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

- CG000764 (Rev B), page 74, 3.1 Rehydration – 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
- CG000764 (Rev B), page 75, 3.2 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
- CG000764 (Rev B), page 75, 3.2 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
- CG000764 (Rev B), page 77-82, 3.3-3.5 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 (CG000764, page 62, **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.

Data analysis supported by Genomics Core – please provide the microscope images to us as soon as it is available (either in the format from the microscope or converted to one of the file types compatible with Space Ranger (see below)).

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 export the images to a suitable format using QuPath as recommended by 10x in the Handbook. For .ndpi images, NDP.view2 can also be used for export to other formats.

If you are performing the exports yourself, please provide the images as one of the following: TIFF (.tif, .tiff), BigTIFF (.tf2, .tf8, .btf), qpTIFF (.qptiff) or JPEG (.jpg, .jpeg).

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 but QuPath can be used for conversion if not.

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

Please review the requirements for images compatible with Space Ranger (summarized below) in CG000688 (Table 3) 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

10x Genomics has not provided clear guidelines for how images should be exported (whether from QuPath or other software), but the goal is for the output to retain the highest resolution possible.

QuPath

The instructions below are what we have used in the GenCore to export JPEG or TIFF images (from .ndpi) in QuPath –

File > **export images** > **original pixels** >
Export format: JPEG
Downsample factor: Choose the smallest number that does not give the "Output image size: XXXXXxXXXX pixels (too big!)" flag, which has often been '**4**' or '**6**' for our projects

File > **export images** > **original pixels** >
Export format: TIFF (Image J)
Downsample factor: Choose the smallest number that does not give the "Output image size: XXXXXxXXXX pixels (too big!)" flag, which has often been '**4**' or '**6**' for our projects

NDP.view2

The instructions below are what we have used in the GenCore to export JPEG or TIFF images (from .ndpi) in NDP.view2 –

Right click > Export > Export Image >

The same window can be brought up with **CTRL + E** or **COMMAND + E**

Do not change anything in **Lens** or **Scale**

In the **Print Size** section, increase the Resolution to highest possible number (usually 4800 dpi), and increase the **Height** to largest possible number which does not cause the "XXXXX x XXXXX pixels" text to change to red. This has often been around **7.6 inches** for **JPEG** and **5.9 inches** for **TIFF** for the images we have worked with. The **Width** can also be changed instead of **Height**.

Select **OK.** In the window that pops up, you can choose to save as type **"JPEG Image"** or **"Tagged Image File Format".**

Some of the exported JPEG or TIFF files are not viewable in the Photo app (Windows) or Preview app (Mac) but can be opened in ImageJ for review. The JPEG exported from NDP.view2 are CMYK and not able to be viewed in **Image J**. **Image J** can also be used for checking the bit/color depth of the images and for adding a scale bar.