Samples preparation guidelines for 10X Genomics Visium/VisiumHD

Contents

Submission to GECF

A **submission form**, found on our website, needs to be filled and sent to GECF before the experiment.

Tissue sectioning, fixation, staining, and imaging are typically performed at the EPFL Histology core facility, which you should contact in advance. If you are planning to perform any of these steps yourself, contact us in advance since these guidelines assume you do that with HCF.

The interactions between you/HCF and the GECF will depend very much on which workflow is used. We will give you more details once details of the experiment are discussed.

General information – Cytassist or non-Cytassist? HD or non-HD?

We recommend **VisiumHD** for all projects, apart when it cannot be used (non-human/mouse tissue).

All methods ultimately employ a **Visium slide** containing **Capture Areas** (one area per tissue section) composed of **spots of spatially barcoded oligonucleotides** that capture either gene expression probes (Cytassist, both non-HD and HD) or polyA mRNAs (non-Cytassist). The original Visium slide contains spots of 55 µm in diameter, with a 100 µm centre-to-centre distance between spots. The newer VisiumHD contains spots of 8um with no space in between (these spots are actually composed of 2um sub-spots that can be analysed individually, but with lower sensitivity).

Visium Cytassist (non-HD or HD)

- Visium Cytassist is our method of choice. Advantages are higher sensitivity, more robustness, and the usage of standard histology slides for the tissue sections positioning.

- It is available both in HD version (recommended) and in non-HD version (cheaper).

- It is a **probe-based** method, which improves sensitivity, but comes with a few caveats:

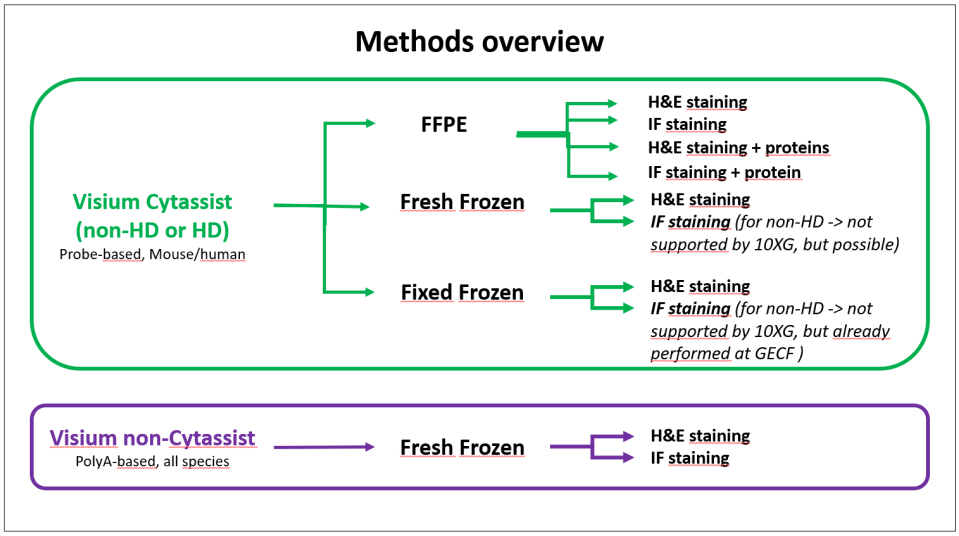
- Detection of exogenous genes (GFP, reporters, viral genes....) requires designing custom probes before starting the experiment.
- It gives no information on SNPs or isoforms (anyway very scarce with non-Cytassist too).
- It can be performed only on **human and mouse** tissues

- It can be performed on **FFPE**, **Fixed Frozen** or **Fresh Frozen** tissues. How to choose if you have the choice?:

- FFPE is the best for preserving morphology and is the most robust.
- Fresh frozen is the best for getting highest UMIs (but its RNA is also the most fragile).
- Fixed frozen is typically chosen only when it is the only available option, as it can be more challenging. See https://kb.10xgenomics.com/hc/en-us/articles/29981279172237 .

Visium non-Cytassist

- Visium non-Cytassist, which is less robust since the slides used are not standard histology slides, should be employed only if Visium Cytassist cannot be used (typically if tissue is human or mouse).



- It is a **polyA-based** method, which can be performed on **any species,** but only on **Fresh Frozen** tissues.

Visium Cytassist workflows details

VISIUM CYTASSIST SLIDE AND CAPTURE AREAS

- Each Visium Cytassist Slide can hold **2 tissue sections**.
- The **capture area** in a Visium non-HD Cytassist Slide can be either **6.5x6.5 mm** (around 5'000 barcoded spots) or **11x11 mm** (around 14'000 barcoded spots). VisiumHD only comes with **6.5x6.5 mm** capture areas (around 700'000 spots of 8um).
- In case of **tissues larger than the selected Capture Area**, a microscope image of the full tissue, with a square delimiting the region of interest, needs to be provided. Include a safety margin when designing the squares (6x6mm and 10.5x10.5mm). The square needs to be parallel to the slide (it cannot be rotated, otherwise it will not fit into the instrument). If a region is especially crucial to include, highlight it.

PROBE-BASED METHOD

- Visium Cytassist is a based on **probes** targeting the **whole mRNA transcriptome**. The human probes panel consists of ~3 probes both for non-HD and HD versions. The mouse probes panel consists of ~3 probes for HD version but only 1 probe for non-HD version.
- Because of this probe-based nature:
    - detection of exogenous genes (GFP, reporters, viral genes....) requires designing **custom probes** in advance. See technical note CG000621 for guidance.
    - no information regarding SNPs or isoforms

TISSUE TYPES AND SPECIES

- Visium Cytassist is available only for **human** and **mouse** (for samples other than human and mouse, Visium-non Cytassist protocol (CG000239) can be used instead).
- It was demonstrated with **several tissue types**. **Additional optimization** may be required for the preparation of specialized tissues, such as tissue with high fat content.
  Consult **compatibility of tissues** in 10X Genomics Support website (https://kb.10xgenomics.com/hc/en-us/articles/7776229391373-What-tissue-types-are-compatible-with-the-Visium-CytAssist-Spatial-Gene-Expression-solution-).

EMBEDDING AND STORAGE

Embedding and storage must be performed according to the latest versions of the 10XG Tissue Preparation Guides. In case these guidelines were not followed we would not be able to guarantee the results. Please inform us in advance and we can discuss how to proceed.

ASSESSMENT OF RNA QUALITY

- It can be done by GECF on demand.
- **DV200** is **strongly recommended** for **FFPE** and **Fixed Frozen** tissues (it is **mandatory** for **archived slides**). **RIN** is **optional** for **Fresh Frozen**. If DV200 was not done beforehand, we suggest collecting some sections for DV200 already during the cut of the Visium protocol itself. *If the experiment gives poor results and RNA quality hasn't been checked, the assessment will be done afterwards and, if quality is not sufficient, the GECF will not be held responsible.*

- RNA quality recommendations:
  - **Cytassist FFPE** -> tissue blocks with a **DV200 ≥30%** are recommended.
  - **Cytassist Fixed Frozen** -> tissue blocks with a **DV200 ≥50%** are recommended.
  - **Cytassist Fresh Frozen**-> tissue blocks with **RIN ≥4** are recommended.

SECTION THICKNESS

Section thickness for:

- **Cytassist FFPE** -> 3-10 um. Most validation was performed by 10XG with sections of 5 um.
- **Cytassist Fresh Frozen**-> 10-20 um. Recommended for most tissue types is 10 µm. Tissues with higher fat content (e.g., breast tissue) may require sections closer to 20 µm.
- **Cytassist Fixed Frozen** -> 10-20 um. Recommended for most tissue types is 10 µm.

Sections outside these specifications may result in reduced performance.

SLIDE PREPARATION

- Tissue sectioning, fixation, staining and imaging are typically performed at the EPFL Histology core facility.
- If you are planning to perform any of these steps yourself, please contact us in advance.
- The tissues must be prepared, sectioned and positioned on slides according to the latest version of the 10XG Tissue Preparation Guides.

TIPS FOR SAMPLE PREPARATION

Some tips for samples preparation for:

- Visium Cytassist FFPE https://kb.10xgenomics.com/hc/en-us/sections/7622802025869-Tissue-Preparation-FFPE
- Visium Cytassist Fresh Frozen and Fixed Frozen https://kb.10xgenomics.com/hc/en-us/sections/12233648909325-Tissue-Preparation-Fresh-Frozen-Fixed-Frozen

DIVERSE NOTES

Labelled antibodies panels for concomitant protein detection are available from 10XG. These are only available for human tissues, and only one panel exist for now, specific for immune markers. This technology is only validated for Cytassist for FFPE.

Visium non-Cytassist workflow

Visium non-Cytassist is a **polyA-based** method, which can be performed on **all species, but** only on **Fresh Frozen** tissues.

GENERAL INFORMATION, VISIUM CYTASSIST SLIDE AND CAPTURE AREAS

- Visium non-Cytassist is performed on slides with **4 capture areas**. All capture areas must be used.
- Each capture area is **6.5 x 6.5 mm** and contains ca 5'000 spots.
- **Permeabilization optimization is mandatory** prior to the experiment (see Visium Optimization Slide paragraph below).
- You can run 2 tissues with **different permeabilization times on the same gene expression slide**.

- This method was demonstrated with **several tissue types**. Consult compatibility of tissues in 10X Genomics Support website https://support.10xgenomics.com/spatial-gene-expression/tissue-optimization/doc/specifications-visium-spatial-gene-expression-optimized-tissues
- Good quality of starting tissue is critical for optimal results.

VISIUM PERMEABILIZATION TIME OPTIMIZATION SLIDE

- The cost of this optimization is much lower than a true Visium experiment (refer to our price list).
- The optimization must be done in the **exact experimental conditions** that will be used later for the real experiment: exact tissue type, development stage, dissection, freezing, storage method and duration, sectioning, fixation and staining. On user side, preparing the samples for the optimization or the real experiment is identical.
- In case of doubt when assessing optimization results, it is better to opt for the slightly longer time.
- **Thickness of tissues section could also be optimized** if initial results are unsatisfactory.

EMBEDDING AND STORAGE

They must be performed according to the latest version of the 10XG Tissue Preparation Guide (protocol CG000240).In case these guidelines were not followed we would not be able to guarantee the results. Please inform us in advance and we can discuss how to proceed.

ASSESSMENT OF RNA QUALITY

Tissue blocks with **RIN ≥7** are recommended.

- It is **optional** but if the experiment gives poor results and RNA quality hadn't been checked beforehand, the assessment will be done afterwards and, if quality is not sufficient, the GECF will not be held responsible.
- It can be done by GECF if provided with the necessary amount of tissue.

SECTION THICKNESS

- **Between 10-20 um**. Recommended for most tissue types is 10 µm (sections outside these specifications may result in reduced performance).
- **To determine optimal tissue thickness** check whether your tissue of interest is mentioned in this list: https://support.10xgenomics.com/spatial-gene-expression/tissue-optimization/doc/specifications-visium-spatial-gene-expression-optimized-tissues.

If not, 10XG say: "Section thickness for the Visium slides will depend upon tissue type. Customers should try to achieve a good quality section at the minimum section thickness for their tissue type. 5 - 35 µm sections have been tested in-house; however, 10 µm is used for most tissue types. Fatty tissues generally require thicker sections."

SLIDE PREPARATION

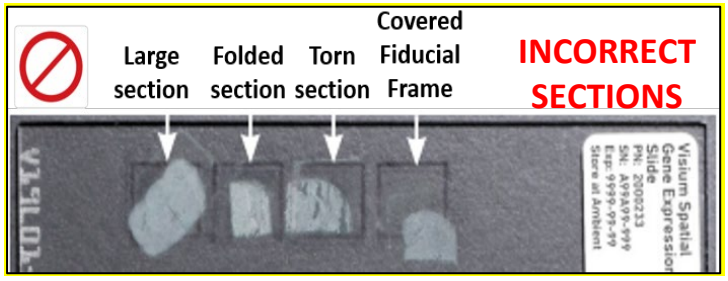
Tissue sectioning, fixation, staining and imaging are typically performed at the **EPFL Histology core facility**. If you are planning to perform any of these steps yourself (not recommended), contact us in advance and we will give you further details.

- Briefly, the tissues must be prepared, sectioned and positioned directly on Visium slides according to the latest version of the 10XG Tissue Preparation Guide (Visium non-Cytassist: **protocol CG000240**).
- The fixation and imaging must be done according to either one of these protocols:

  - Methanol Fixation, H&E Staining Protocol (CG000160)
  - Methanol Fixation, Immunofluorescence Staining Protocol (CG000312)

CAUTION: obtaining good data requires that the tissue is properly sectioned, without cracks, or other freezing/conservation/cutting/staining artefact. This is likely the most important and often most tricky step of the whole 10XG Visium procedure. Therefore, we strongly recommend that you test and optimize the histology procedure beforehand on similar but dummy samples and slides.

- If **sections are incorrectly placed** on Visium slide or quality is poor (folds, breaks, see image below), it is possible to do a **slide reset** prior to tissue permeabilization (see guide CG000332).



DIVERSE NOTES FOR VISIUM NON_CYTASSIST

- Some **tips for samples preparation** for Visium non-Cytassist can be found here: https://kb.10xgenomics.com/hc/en-us/sections/360007223212-Sample-Preparation
- If you want to **monitor a GFP expression**, here are comments by 10XG: "Based upon results from limited in-house testing, fluorescent GFP reporters show compatibility with the Visium assay, but we have not yet fully validated this technique.
  - we recommend performing the fluorescent imaging immediately after the isopropanol drying step and before the H&E staining to avoid background autofluorescence from the H&E stain. Keep the imaging time to a minimum.
  - An alternative solution is to perform the fluorescent imaging on a subsequent section on a plain glass slide and overlay this with the H&E image generated from the Visium slide. Unfortunately, with this method you will only get a general GFP+ cell distribution, as opposed to a direct 1:1 relationship."

Sequencing and analysis

- **Sequencing depth**:
  - **Visium Cytassist HD**: a minimum of **300mio reads per tissue section** is required for a section covering the full area. More reads will give better sensitivity.

  - **Visium Cytassist nonHD**: a minimum of **25'000 reads per tissue covered spot** are required. More reads will give better sensitivity.
  - **Visium non-Cytassist**: a minimum of **50'000 reads per tissue covered spot** are required. More reads will give better sensitivity.

  So, for example, for Visium Cytassist nonHD, at least 100 million reads will be necessary for a sample covering around 80% of the Capture Area of a 6.5 mm slide (0.8 x 5'000 x 25'000).
- SpaceRanger results of single samples coming from different biological conditions of the same sample or from consecutive sections of the same tissue block can be aggregated into a single feature-barcode matrix by running SpaceRanger aggr.
- SpaceRanger v2.1 has introduced a reference-free spot deconvolution function. This allows the deconvolution for cell-typing, as described here: https://www.10xgenomics.com/support/software/space-ranger/algorithms-overview/gene-expression#lda-based-spot-deconvolution-7e5466 . The results can be explored through the Spot Deconvolution feature of Loupe Browser. 10XG provide a tutorial for this method: https://www.10xgenomics.com/analysis-guides/exploring-your-visium-data-a-spot-deconvolution-story

Results

Once the experiment is finished, user will get a folder of the sequencing data and a folder of the Spaceranger analysis.

For the Spaceranger results, we recommend to inspect in particular the **websummary.html** file and the **cloupe.cloupe** file (for the latter, Loupe Browser software is needed).

- For **Visium Cytassist non HD** and for **Visium non Cytassist**, these files are located in the "out" folder inside each sample's results
- For **Visium HD**, the websummary.html file is in the "out" folder, while the cloupe.cloupe file is in outs\binned_outputs\square_008um

Binned outputs (only Visium HD)

For Visium HD, by default, the binned_outputs directory has three subdirectories (square_002um, square_008um, and square_016um). Each subdirectory contains:

  - filtered_feature_bc_matrix,
  - raw_feature_bc_matrix,
  - spatial,
  - filtered_feature_bc_matrix.h5,
  - raw_feature_bc_matrix.h5.

The analysis directory is only provided at 8 and 16 µm bin size. The **cloupe.cloupe is only provided at 8 µm bin size**. The raw_probe_bc_matrix.h5 is only provided at 2 µm resolution.

It is possible to bin Visium HD data to a specified bin size in addition to the standard binning size (2 µm, 8 µm, 16 µm). This also produces a Loupe file and secondary analysis outputs at the specified bin size in addition to the standard analysis bin size (8 µm) if it is larger than 8 µm.

If user wants a different bin size than the standard one, they need to specify it in the submission file, in the comment section.

Bioinformatics tools such as *bin2cell* allow to bin the 2um spots smartly into cells shapes based on the H&E image, getting one step closer to true single-cell resolution.

Visium integration with single cell data

Visium data can be combined with single cell data in order to get additional information on the sample (https://www.10xgenomics.com/analysis-guides/integrating-single-cell-and-visium-spatial-gene-expression-data).

Starting from the same blocks used for the Visium experiment it is possible to isolate nuclei/cells for Flex single cell gene expression (protocol for FFPE samples can be found here, while protocol for Fresh frozen OCT embedded samples can be found here).

Versions log

- vA.02-03 (15.08.2024): Added guidelines regarding Visium non-cytassist samples preparation. Mentioned to include safety margins when supplying the ROI squares on the microscope images. Added references to VisiumHD.
- vA.04 (30.09.2024): added FrFr and FxFr for HD, removed mandatory DV200 for general FFPE. Added Results section. Mentioned bin2cell tool.
- vA.05 (07.11.2024): Added that square showing the ROI needs to be parallel to the slide + added possibility to do Flex on the same block used for Visium