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Xenium Spatial Transcriptomics Protocol for Human Kidney

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We use this protocol and it's working

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Disclaimer

This protocol is still in development and the analysis steps may cahnge

Abstract

This is a protocol for performing spatial transcriptomics using 10X Xenium. Here we are using custom panel designed to target cell types in the healthy and disease kidneys and discover their spatial relationships. The protocol has many steps involving selecting tissue, tissue sectioning and mounting on slides, processing to prepare for hybridization with custom panel and in situ imaging. Posy imaging there are number of analyses can be performed and these will depend on the biological questions where the methods and tools are still evolving, however, we will focus on key QC parameters to evaluate assay performance. Each of the steps from sectioning to data generation have quality assurance and control steps. Post Xenium assay, slides are stained with hematoxylin and eosin for light microscopy and flanking tissue sections are typically used for light microscopy evaluation or other technologies.

Image Attribution

The slide template is from 10X Genomics

Guidelines

Panel design: Prior to starting the assay make sure you have all the reagents for Xenium, here V1 kit is used. Also we are using custom panels that target genes of interest and these should be in hand already. These are designed based on specific markers from human kidney atlas, literature and validating them against various spatial transcriptomic technologies. Once this list is finalized, follow 10X guidelines to validate compatibility of probe designs and target genes using 10X tools and adjust for overcrowding based on 10X recommendations (https://www.10xgenomics.com/support/software/xenium-panel-designer/latest). The number of target genes can be modified up to the maximum 10X permits.

Materials

Reagents:

- Epredia™ Modified Harris Hematoxylin **Fisher Scientific Catalog #**22-050-206
- Eosin Y Solution, Alcoholic, with Phloxine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**HT110380
- Ammonium hydroxide solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**221228-500ML-A
- Hydrochloric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H1758-500ML
- Xylene, Fisherbrand™, HistoPrep™ **Fisher Scientific Catalog #**HC-700-1GAL
- Acetic Acid, Glacial **Fisher Scientific Catalog #**A38S-500
- Ethanol (200 Proof) receiving dock Cat. # 11100020S

Tissue Preparation - Tissue selection

1

Note

We adopted the protocol suggested by 10X for tissue mounting on Xenium slides
Detailed protocol here: Xenium FFPE Tissue Prep guide.

Perform the assay on FFPE sections. To ensure proper preservation of tissue ensure warm ischemia time is <4h and tissue is preserved in fixative as soon as possible (preferably within less than an hour from retrieval. For KPMP consortia, this is not a concern as most biopsies are preserved in less than 20 min.

2 Although the assay can work on a few mm of tissue, ideally the tissue should be 5mm or larger but less than 2mm in length to ensure it will fit in the designated area on Xenium slide (QC).

Tissue Preparation - Sectioning

11h 30m

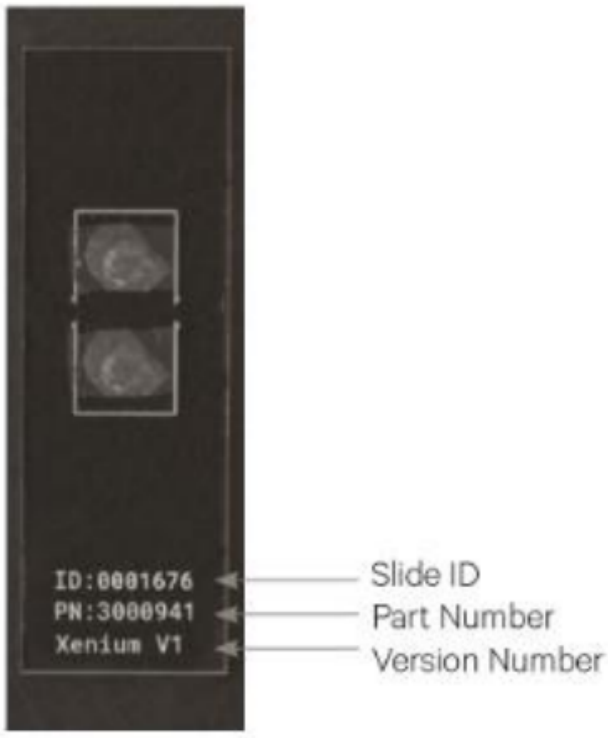
3 Equilibrate blank xenium slides to Room temperature for 00:30:00 . 30m

4 Trim the extra wax to be able to fit as many sections in the designated area on the Xenium slide (see picture 1) in a timely manner so the sections do not disintegrate on the water bath.

5 Cut a few sections from the surface to minimize surface contamination, use one of them for light microscopy to ensure presence of kidney tissue (**QC**). Once confirmed, cut and mount sections at 5uM on the xenium slide. If tissue of interest is already confirmed then can skip.

5.1 Center sections in the outlined scan area (10.45mm x 22.45mm)

- Fiducials just inside white outline



Picture 1

5.2 Do Not overlap tissues or paraffin.

- Do not place tissues over fiducials
- Try not to resubmerge previously mounted tissue when placing additional sections, see 10X tips in the link above.

5.3 Place tissues on etched label side of slide.

5.4 Bake slides at 42 °C for 03:00:00, then at Room temperature Overnight. 11h

5.5 Store slides at Room temperature (with dessicant) until use.

- use within 4 weeks for best results.

Note

QC: Ensure mounted sections are not folded over, or fragmented or have extensive shatter

Assay

16h 37m 40s

6 Here we follow the protocol recommended by Xenium chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://cdn.10xgenomics.com/image/upload/v17
26606313/support
documents/CG000749_XeniumInSitu_GeneExp_CellSegmentation_User_Guide_RevB.pdf

7 **Deparaffinization, hydration and decrosslinking**

Follow xenium protocol. For quality assurance, ensure that sections are completely immersed during these steps.

Note

QC: Check that the tissue has not lifted off the slides, if it has discard and start again

8 **Custom panel addition, morphological marker staining and hybridization**

8h

Probe hybridization. Follow protocol instructions to prepare and add predesigned custom probe panels to the deparaffinized and decrosslinked slides and incubate Overnight .

Note

Tip: Make sure to record custom panel ID and slide ID as it will be used later for setup of the Xenium Analyzer.

8.1 Post hybridization wash.

Note

Tip: Ensure the slide is not cracked and do not let them dry out. Change solutions one at a time. Do not let the slide cool, immediately add wash buffer after removing the hybridization solution.

8.2 Ligation and amplification. Add reagents for ligation, wash and then proceed to rolling circle amplification and wash.

8.3 Morphological marker staining for cell segmentation. 8h 20m

- After series of ethanol washing, incubate with xenium blocking and staining buffer.
- Prepare multi tissue staining mix that harbors cell segmentation staining reagents for cell nuclei, membrane and cytoplasmic boundaries and immune cells for morphology based segmentation.
- Incubate with staining reagents Overnight at 4 °C.
- After washes, treat with stain enhancer for 00:20:00 at Room temperature (start thawing autofluorescence quencher for next step).

8.4 Autofluorescence quenching. 00:10:00 at Room temperature in dark after washes and then post quenching wash in ethanol (normal light) and then in PBS-T in dark as in the protocol. 10m

Note

Break point: can store the slides overnight in dark at 4 °C.

8.5 Nuclei staining. Use nuclei stain reagents provided in the kit. 1m

- after washes incubate with nuclear stain in the dark for 00:01:00 at Room temperature.
- Washes

Note

Brake point: can store slides at 4 °C in dark for less than a week.

Note

QC: check for tissue detachment and folding. These areas will not have signal and regions of fold may be oversaturated. Depending on extent of detachment or folding may determine if usable data will be generated. If the detachment area is less than 25% may proceed, but exceeding 70 % may not be usable. For intermediate may need to manually assess if there are still usable areas. These can be better assessed in the scan view on the analyzer in the next step.

9 **Imaging on Xenium Analyzer.** Here we follow the protocol recommended by 10X chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://cdn.10xgenomics.com/image/upload/v1731971531/support-documents/CG000584_Xenium_Analyzer_UserGuide_RevG_update.pdf

Note

Ensure the correct modes are selected for the kit being used (v1 or prime).

9.1 Make sure the file for custom panel or other panel used are uploaded on the instrument and selected during run setup.

9.2 Perform readiness test to verify all systems are working and the instrument is ready for use.

9.3 Sample scan.

- After scan is complete, assess the scanned image for adequacy of staining as stated above in B6 above. Toggle different channels to ensure morphological markers have stained as expected.

Note

QC: Check for debris and detachments; if there is significant debris causing blurry scan do not proceed.

- Make sure to select at least 1 field of view (FOV) containing tissue. For multiple samples, specify each sample as separate region.

9.4 Transfer data after run is complete.

10 **Xenium Data Quality.** There are several levels of checking the quality of the Xenium in situ experiment. Some of these features should be directly visualized on the Xenium Explorer that can be freely downloaded from 10X https://www.10xgenomics.com/support/software/xenium-explorer/latest

- The initial QC tutorial is here https://www.10xgenomics.com/support/software/xenium-explorer/latest/tutorials/xe-checking-xenium-data-quality

10.1 **Imaging quality.** Check DAPI staining to assess tears, folds or detachment (**QC**).

10.2 **Segmentation.** Check quality of segmentation by examining cell segmentation polygon boundaries and nuclei. The cells contributed by each of the morphological marks can be seen. No segmentation method is perfect, if there are cells that are not segmented these can be exported and evaluated for their identity. Overcrowded areas such immune dense areas may have overlapping cells nuclei and may not segment accurately. Similarly if some cells are not desired to be analyzed, they can be selected using the lasso tool and exported, and these IDs can be removed, for example blood cells may not be desired, or non kidney tissue cells etc. For multimodal segmentation, 5um is used for the DAPI channel. Check if interior segmented cells have RNA stain and nuclei and the boundary segmented cells have boundary stain. Several cell types can be spot checked. Can also manually count select areas and compare with Xenium multisegmentation.

10.3 **Cell clusters and appropriate region quality assurance.** Can use Xenium default clustering or import own file and check on the cluster colors on the tissue to select cells and assess if the marker genes identify the cell type selected expected from the region. For example, in the kidney glomerulus check for parietal epithelial cells, podocytes, glomerular capillaries or adjacent macula densa or renin expressing cells.

10.4 **Transcript quality.** For select gene check **Q score >20**. Check different cell type marker genes to assess separation. Density map of all gene transcripts should show uniform coverage of the tissue.

10.5 **Assay performance.** Check for reproducibility if same sections are repeated and measure count of cells identified, transcript per cell and count correlation of genes among the two samples.

11 **Analytical pipelines.** Additional QC for rigor and reproducibility can be performed using custom analytical pipelines.

We use a combination of SquidPy, TACCO and scVI for pre and post QC analysis, integration, batch correction and clustering. A variety of other tools are used for neighborhood analysis including SquidPy, Banksy, MonkeyBread and NiCo. For cell annotation, KPMP-HuBMAP kidney atlas is used at different levels of annotation. We usually avoid using the degenerative tubular states for initial annotations as they show mismapping due to reduced expression of mature markers and common injury markers. These can be individually checked after subsetting the main clusters.

11.1 **Pre-processing:**

- Starts with **Instrument Output**, converted into **Initial Data** and then **Flat Files**.
- Tools like **Squidpy** are used to ensure data quality.

Quality Control:

- Identifies negative probes and calculates False Discovery Rate (FDR) and other QC metrics. FDR <1%
- Filters cells and genes to create a **Final QC Object. Remove cells with Genes < 5 count. Remove genes not expressed in any cell.**

11.2 **Dimensionality Reduction and Integration:**

- Natural log Normalization, PCA, neighbors, highly variable genes and UMAP clustering are performed using **Squidpy** (Theis Lab).
- **scVI** used for integration or batch correction if the goal is to perform global analysis.
- Annotation is supported by organ-specific atlases (e.g., **TACCO** from Broad Institute). This can be done at level 1 with main cell types or deeper levels 2 and 3 for more granular subtypes.

11.3 **Knowledge-Based Downstream Analysis** (can be done at specific region of a sample, whole sample of an individual sample or a group of samples)

- Focuses on:

1. **Spatial Clustering** to identify niches.
2. **Neighborhood Analysis** to assess cell interactions. Neighboranalysis in spatially resolved ST will depend on parameters chosen such as distance and this may depend on biological goals of how expansive microenvironment is desired. When more granular cell type levels are chosen, due to overlap in closely related cells, one may see cell neighbors that may not be physically plausible based on their regional location in the tissue. These should be easy to point to detect in the output and taken into account.
3. Discovery of **Spatially Variable Genes**.

- Tools like **Squidpy, Monkeybread, NiCo, GraphCompass, and BANKSY** support this step.

12 **Post Xenium H&E staining.** Follow Xenium instructions to remove the cassette and reagents and perform routine H&E staining.

12.1 Remove Xenium Cassette

12.2 H&E Staining 6m 40s

- Milli-Q water – 00:02:00
- Hematoxylin – 00:03:30
- Rinse until clear with running water
- 0.5% HCl/70% EtOH (differentiation step) – 10-15 dips
- Rinse with running water
- 1% Ammonia water (bluing step) – 10-15 dips
- Rinse with running water
- 70% Ethanol – 20 dips
- Eosin (Add ~ 2 mL acetic acid to new eosin after it has been changed) – 00:00:10
- 95% Ethanol – 20 dips
- 95% Ethanol – 20 dips
- 100% Ethanol – 00:00:30
- 100% Ethanol – 00:00:30

- Xylene – 20 dips
- Xylene – 20 dips
- Xylene – store while cover slipping

12.3 Coverslip

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