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FFPE Tissue Processing for Multimodal Imaging Assays (Phenocycler-Fusion + H&E) following Xenium In Situ Gene Expression

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ABSTRACT

Xenium In Situ Gene Expression assay from 10X Genomics enables multiplexed imaging of RNA transcripts *in situ*. There are several antibody based multiplexed imaging technologies that enable multiplexed protein detection. Hematoxylin and Eosin (H&E) staining is a gold standard method for observing tissue morphology. In order to generate a highly multimodal imaging dataset, we developed a protocol to prepare tissue slides for multiplexed imaging on the Phenocycler-Fusion platform and H&E staining following a Xenium run.

Although the Xenium protocol includes a decrosslinking step which is akin to antigen retrieval, a second round of antigen retrieval, as outlined in this protocol, is necessary for epitope unmasking and accurate protein detection by antibody based methods.

The steps for slide storage and tissue processing for multiplexed imaging can also be applied to imaging assays on other platforms.

Protocol status: Working

We use this protocol and it's working

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Keywords: Xenium, Phenocycler Fusion, multiplexed imaging, multiomics, spatial transcriptomics, spatial proteomics, codex, hematoxylin, eosin, H&E

MATERIALS

Specialty Reagents:

- Xenium assay kits and reagents as detailed in 10X User Guide
- Phenocycler buffers and reagents as detailed in Akoya User Guide
- Validated and barcoded antibodies for Phenocycler assay

General Reagents and Materials:

- 1X PBS, pH 7.4 (Gibco; 10010023, ThermoFisher Scientific)
- Glycerol (G5516, Sigma Aldrich)
- DI water or Milli-Q water
- Antigen retrieval buffer (pH6, Abcam ab93678 or pH9, Abcam ab93684)
- Xylene (Sigma cat# 534056) or Histo-Clear (National Diagnostics cat# HS-200)
- 100% (200 proof) ethanol - Sigma cat# E7023
- Hematoxylin Solution, Mayer's - Sigma cat# MHS32-1L
- Epredia Shandon Bluing Reagent - Thermo cat# 6769001
- Eosin Y Solution; Alcoholic - Sigma cat# HT110116-500ML
- DPX Mountant - Sigma cat# 06522-100ML
- Coplin jars
- Slide mailers

Instrumentation:

- Xenium Analyzer (10X Genomics)
- Phenocycler-Fusion (Akoya Biosciences)
- De-cloaking chamber (Biocare Medical or BioSB TintoRetriever) or pressure cooker (Instant Pot)
- Chemical fume hood
- Weighing balance

Tissue Processing for Xenium In Situ Gene Expression

1 FFPE tissue on Xenium slides is processed according to the protocols in 10X User Guides:

Please refer to the linked 10X User Guides for all materials and detailed instructions. The key steps are noted below:

- Tissue deparaffinization: Step 1.2 of **CG000580 (Rev C)**
- Decrosslinking: Step 1.4 **CG000580 (Rev C)**. We selected the  80 °C condition that is recommended for all tissues other than skin.

- Probe hybridization: step 1 of **CG000582 (Rev F)**
- Post hybridization wash: step 2 of **CG000582 (Rev F)**
- Ligation: step 3 of **CG000582 (Rev F)**
- Amplification: step 4 **CG000582 (Rev F)**
- Autofluorescence quenching and nuclear staining: step 5 of **CG000582 (Rev F)**

After processing, the slide was loaded onto the Xenium Analyzer instrument and run according to instructions in User Guide **CG000584 (Rev E)**

User Guides:

[Link: CG000580 \(Rev C\): Xenium In Situ for FFPE Tissues – Deparaffinization & Decrosslinking](#)



[Link: CG000582 \(Rev F\): Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification](#)



[Link: CG000584 \(Rev E\): Xenium Analyzer UserGuide](#)



1.1 At the end of the Xenium run, remove the slide cassette assembly from the Xenium Analyzer.



Xenium slide cassette assembly

Note

The cassette assembly can be retained for use in future incubation steps

- 1.2** The slide can be processed immediately or stored at  4 °C for up to **2 weeks**.

Slide Storage

- 2** For slide storage:

- 2.1** After the Xenium run, remove the liquid covering the slide. Add  1 mL PBS and incubate  5m
 00:05:00 mins  Room temperature

2.2 Remove PBS. Add  storage solution (50% glycerol in PBS) to the slide. Replace the lid to seal the cassette and store for up to **2 weeks** at 

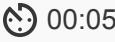
Tissue processing for Phenocycler-Fusion assay

1h 1m

3 Tissue processing can start directly after Xenium run or from a previously stored slide

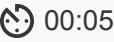
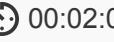
3.1 Processing slide directly after Xenium run:

7m

- After the Xenium run, remove the liquid from the slide and remove slide from the cassette assembly
- Wash slide in PBS for  Room temperature . Repeat for a total of two washes
- Wash slide in DI or Milli-Q water for  Room temperature
- Proceed to step 4

3.2 Processing slide from storage:

7m

- Remove storage solution from the tissue and remove slide from the cassette assembly
- Wash slide with PBS for  Room temperature . Repeat for a total of four washes
- Wash slide in DI or MilliQ water for  Room temperature
- Proceed to step 4

4 Perform antigen retrieval to de-crosslink and reveal epitopes. Antigen retrieval can be performed in citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0). Antigen retrieval buffers are readily available from several vendors. Choose the retrieval buffer that is compatible with the entire panel of antibodies used in the multiplexed imaging assay.

4.1 Prepare the requisite amount of 1X antigen retrieval buffer by diluting in Milli-Q water. Ensure the vessel holding the slides contains enough buffer to cover the sample slide

4.2 Immerse the slides in the vessel and cover with aluminum foil.

4.3 Fill the pressure cooker with approximately 500 mL DI water and place the vessel containing the slides inside. Close the pressure cooker and set to the low-pressure setting (90 °C - 110 °C) for 00:15:00 mins 15m

4.4 After incubation, release the pressure, remove the vessel from the cooker and rest on the bench for 00:30:00 mins 30m

4.5 Wash slide in DI or Milli-Q water for 00:02:00 mins Room temperature 2m

5 After this point, continue processing by following the protocol from *Chapter 3, page 50, Step 4* in the PhenoCycler-Fusion User Guide **PD-000011 Rev L**

PhenoCycler-Fusion User Guide_2.1.0.pdf 15.3MB

Note

To continue immunostaining via other assays, transfer slide to PBS and follow the corresponding protocol steps.

6 **Expected result:**

Human pancreas tissue was processed for Xenium in situ gene expression assay using a custom probe set. Following Xenium run, the tissue was processed for multiplexed imaging on Phenocycler-Fusion using a panel of 40 antibodies following the steps listed in this protocol.

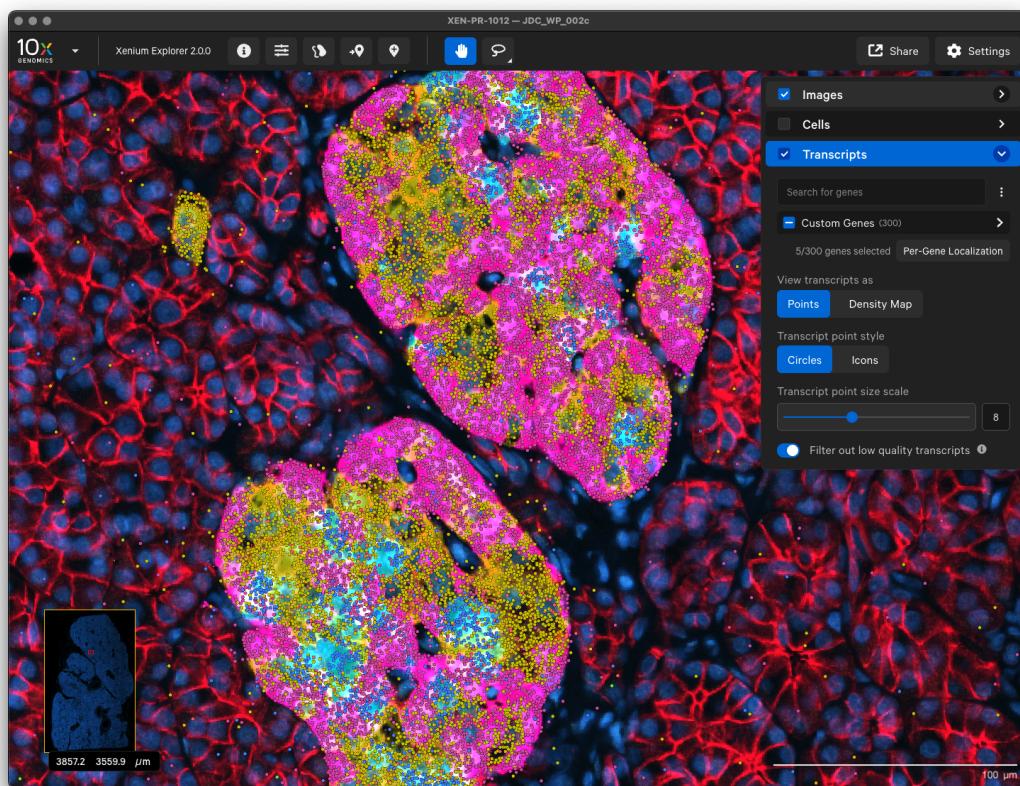
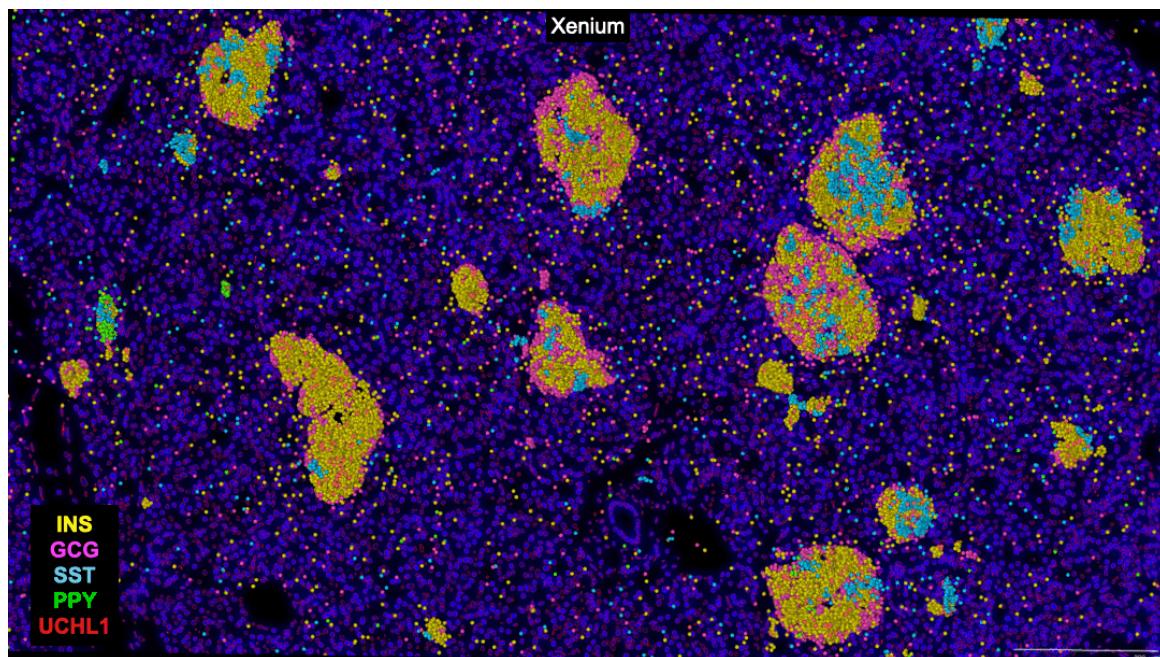


Image of human pancreas tissue visualized in Xenium Explorer showing RNA transcript expression overlaid on top of protein expression data. (*Data courtesy of Prof. Paul Robson, The Jackson Laboratory*)



Animation demonstrating transcript expression (Xenium) and protein expression (Phenocycler-Fusion) data in normal human pancreas tissue (*Data courtesy of Prof. Paul Robson, The Jackson Laboratory*)

H&E staining

- 7 After a successful Phenocycler run, slides with attached flow cells can be stored in the Akoya Storage Buffer at 4 °C or used immediately for H&E staining.

Note

Tissues that have been processed for Xenium assay have undergone autofluorescence quenching. 10X user guides recommend quencher removal before proceeding to H&E staining. In our tests, we found that autofluorescence quencher removal is not critical for good quality H&E staining.

If desired, autofluorescence quencher removal can be performed (Step 12)

Flow Cell Removal

1h 1m

- 8 The flow cell needs to be removed from the slide to continue with H&E staining. Retrieve tissue slides with attached flow cells either from the storage buffer or directly after Phenocycler run

- 8.1 Fill a coplin jar with xylene or Histo-Clear. There must be sufficient volume to cover the entire flow cell.
- 8.2 Place slides in the coplin jar containing xylene or Histo-Clear and incubate  24:00:00 hours  1d at  Room temperature
- 8.3 Following incubation, gently remove the flow cell from the slide.

Staining Procedure

1h 1m

- 9 Place the slides in 100% ethanol for  00:02:00 mins . Raise and dip the slides 10 to 15 times to fully cover the tissue. 
- 10 Place the slides in 95% ethanol for  00:02:00 mins . Raise and dip the slides 10 to 15 times to fully cover the tissue. 
- 11 Place the slides in DI water for  00:02:00 mins . Raise and dip the slides 10 to 15 times to fully cover the tissue. 

OPTIONAL: Autofluorescence Quencher Removal

- 12 If autofluorescence quencher removal is desired, it can be performed at this stage before proceeding to Step 13 for staining.

For full details, check 10X Genomics User Guide [CG000613](#)
[CG000613_Demonstrated_Protocol_Xenium_H_EStaining_RevB](#)

 10x(CG000613)_Demonstrated_Protocol... 2MB

- 12.1 Prepare  10 mL fresh quencher removal solution:
- In a fume hood, weigh  17.4 mg of sodium hyrosulfite
 - Add the sodium hyrosulfite into  10 mL of Milli-Q water in a  15 mL conical tube.
- Close the tube and vortex briefly until dissolved

Note

The quencher removal solution is only effective for  00:10:00 mins and must be used **immediately**. If the solution turns white, prepare fresh.

- 12.2 IMMEDIATELY transfer the quencher removal solution to a slide mailer and place the slides into  10m the solution. Incubate for  00:10:00 mins
- 12.3 Wash three times in Milli-Q water for  00:01:00 min each  1m

Staining Procedure 1h 1m

- 13 Place the slides in the following reagent series:

- 13.1 Mayer's Hematoxylin -  00:04:00 mins  4m

Note

Lymphoid tissues or tissues with dense cellularity should be stained for only  00:03:00 mins. Rinse the slide in DI water to observe the stain. Return the slide to Mayer's hematoxylin if staining is not adequate.

- 13.2 DI water -  00:01:00 min 1m
- 13.3 Bluing Reagent -  00:01:00 min 1m
- 13.4 DI water -  00:01:00 min 1m
- 13.5 Alcoholic Eosin -  00:02:00 mins 2m
- 13.6 95% ethanol -  00:01:00 min without agitation or 20-30 dips to remove all excess reagent from previous step 1m
- 13.7 100% ethanol -  00:01:00 min without agitation or 20-30 dips to remove all excess reagent from previous step 1m
- 13.8 100% ethanol -  00:01:00 min without agitation or 20-30 dips to remove all excess reagent from previous step 1m
- 13.9 Xylene/Histo-Clear -  00:01:00 min without agitation or 20-30 dips to remove all excess reagent from previous step 1m

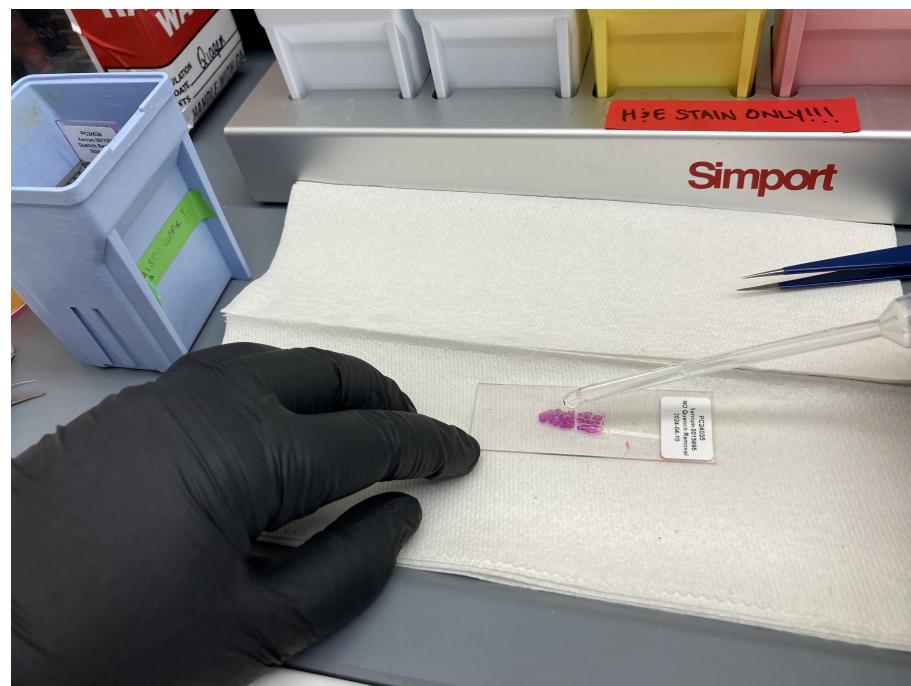
13.10 Xylene/Histo-Clear - Hold in this solution as slides are mounted one by one**Slide Mounting**

20m

- 14** To mount the slides, remove slides one at a time on to a paper towel inside the fume hood. Leave other slides in the last xylene/Histo-Clear coplin jar to prevent over-drying the tissue.

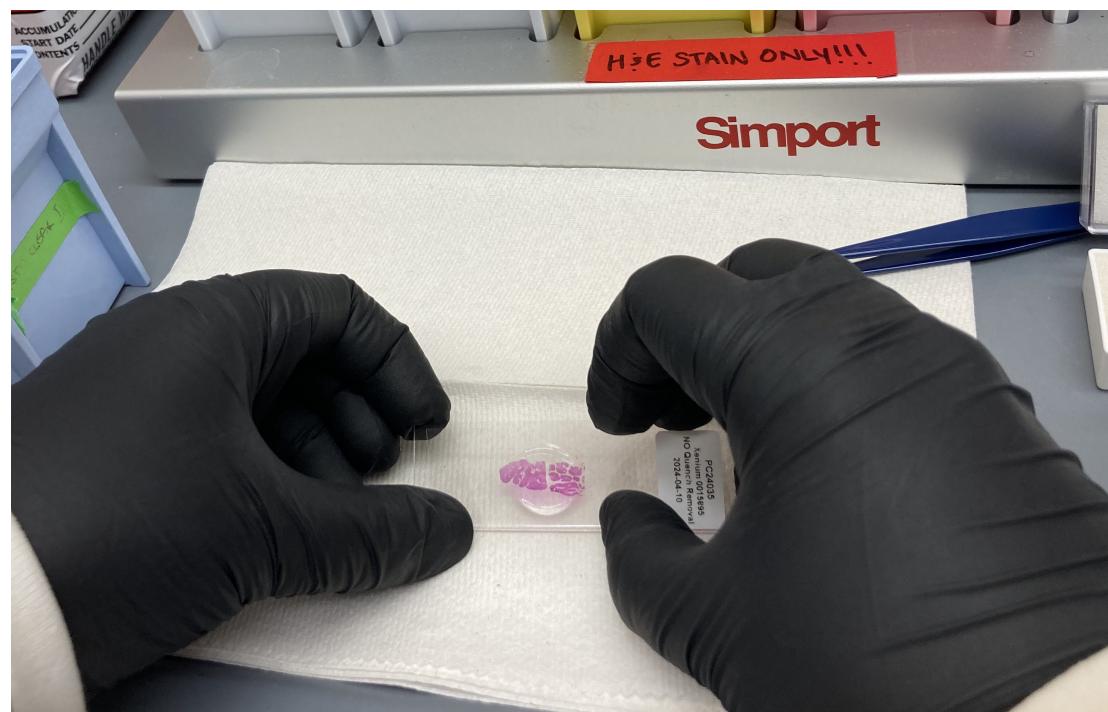
Dry the back of the slide and tilt to remove excess xylene, but do not let the tissues dry.

- 14.1** Using a disposable transfer pipette add enough DPX mountant (or other xylene-based mountants) to cover desired tissue area (1 to 2 drops)



- 14.2** Clean a glass coverslip quickly and remove any particles on its surface. Place the long edge of the coverslip on the edge of the slide closest to you, tip the **slide** towards yourself so the

mountant begins to reach the coverslip, and gently push down until the mounting media has spread evenly across the slide area containing tissue.





- 14.3** Remove excess mountant by placing the edges along the paper towel. Avoid mountant covering the top of the coverslip.



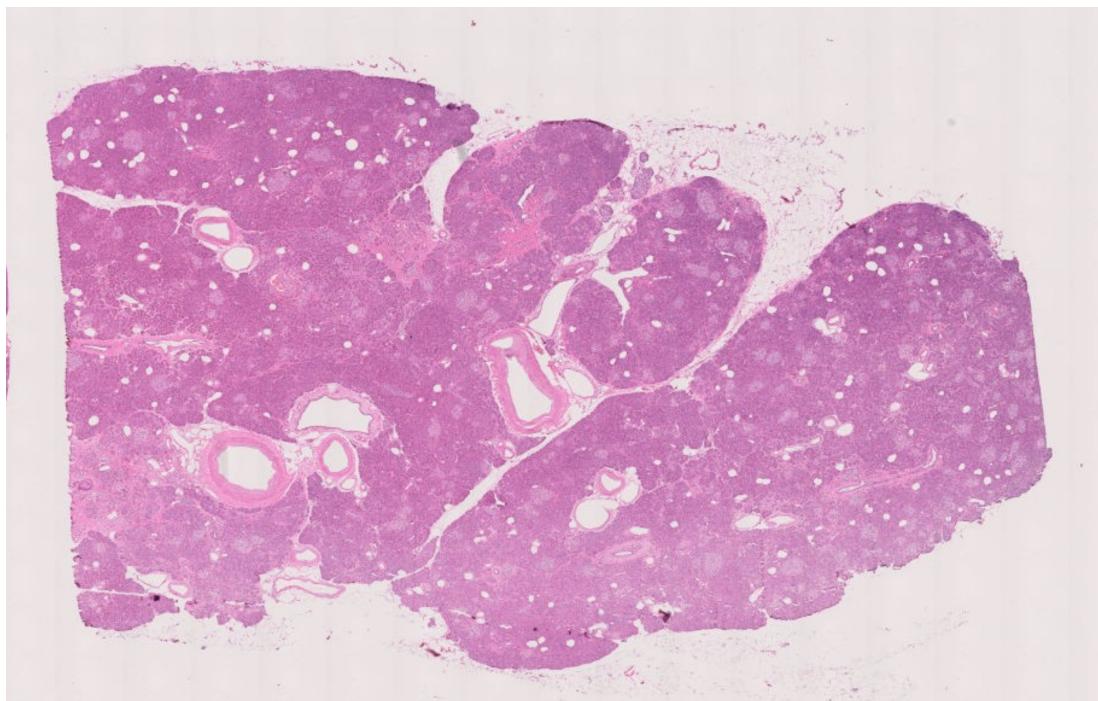
14.4 Gently press down on the coverslip to remove any bubbles that remain above the stained tissue.

15 Allow mountant to cure inside the hood for at least  00:20:00 mins before moving and imaging. Xylem-based mountants take up to 24 hours to cure completely. 

16  go to step #14 and repeat mounting procedure for remaining slides.

17

Mounted slides can be imaged and stored in a slide storage box at  Room temperature

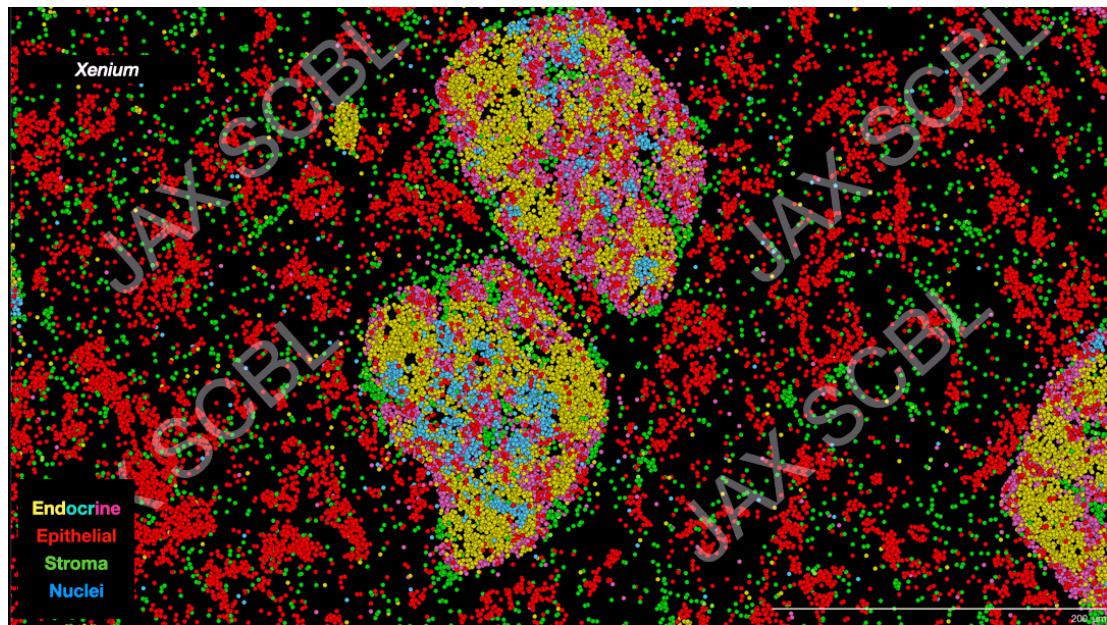


H&E staining of human FFPE pancreas tissue following Xenium and Phenocycler-Fusion assays (*Data courtesy of Prof. Paul Robson, The Jackson Laboratory*)

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Expected Result:

Animation showing the expected result following each assay- Xenium, followed by Phenocycler-Fusion, followed by H&E staining on the same FFPE pancreas tissue.



(Data courtesy of Prof. Paul Robson, The Jackson Laboratory)