10x GENOMICS

Xenium Workshop

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Outline

- Overview of Xenium Workflow
  - Supported Tissues, Block Generation, & Sectioning
  - Probe Hybridization Preparation
  - Instrument Operation Interactive Demo
- Pre-Designed Panels
- Overview of Xenium Custom Panel Design
  - Required Information & Files to Design a Custom Panel
  - Understanding the *In Situ O*ptical Detection Budget
  - Understanding Probe Sets in Xenium Panel Design
- Xenium Analysis Overview
- Understanding Xenium Algorithms
  - Decoding
  - Cell Segmentation
- Understanding Xenium Outputs
- Reanalysis with Xenium Ranger
- Continuing Analysis with Community Software

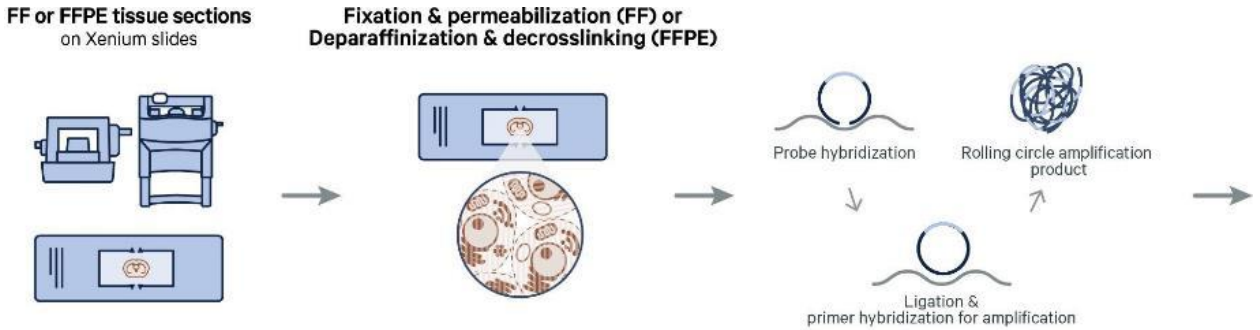
Overview of Xenium Workflow

- Block Generation & Sectioning
- Probe Hybridization Preparation
- Instrument Operation Interactive Demo

A Simple Workflow: 3-6 Hours of Hands-On Time

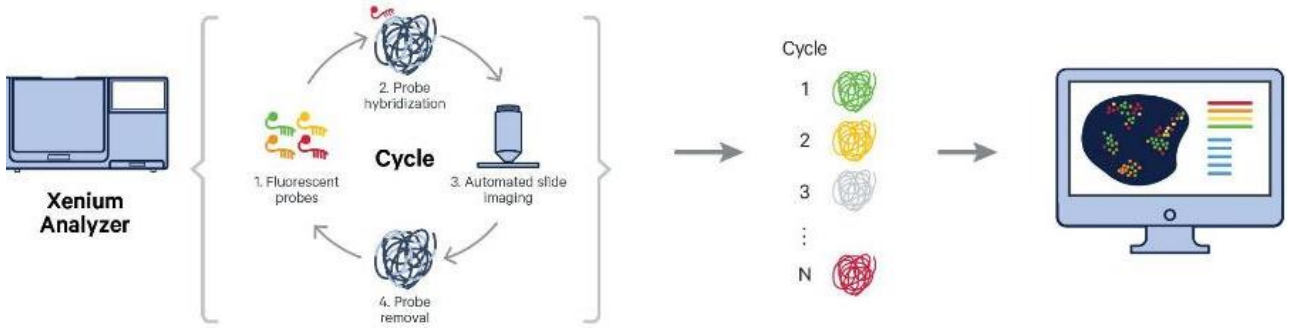
From tissue to instrument start in 2-3 days, from instrument start to data in ~2 days

Sample Preparation → **Probe hybridization, ligation, & amplification**



Simple ~4-6 hours hands on time workflow

Fluorescent probe hybridization, imaging & decoding → **Data visualization**

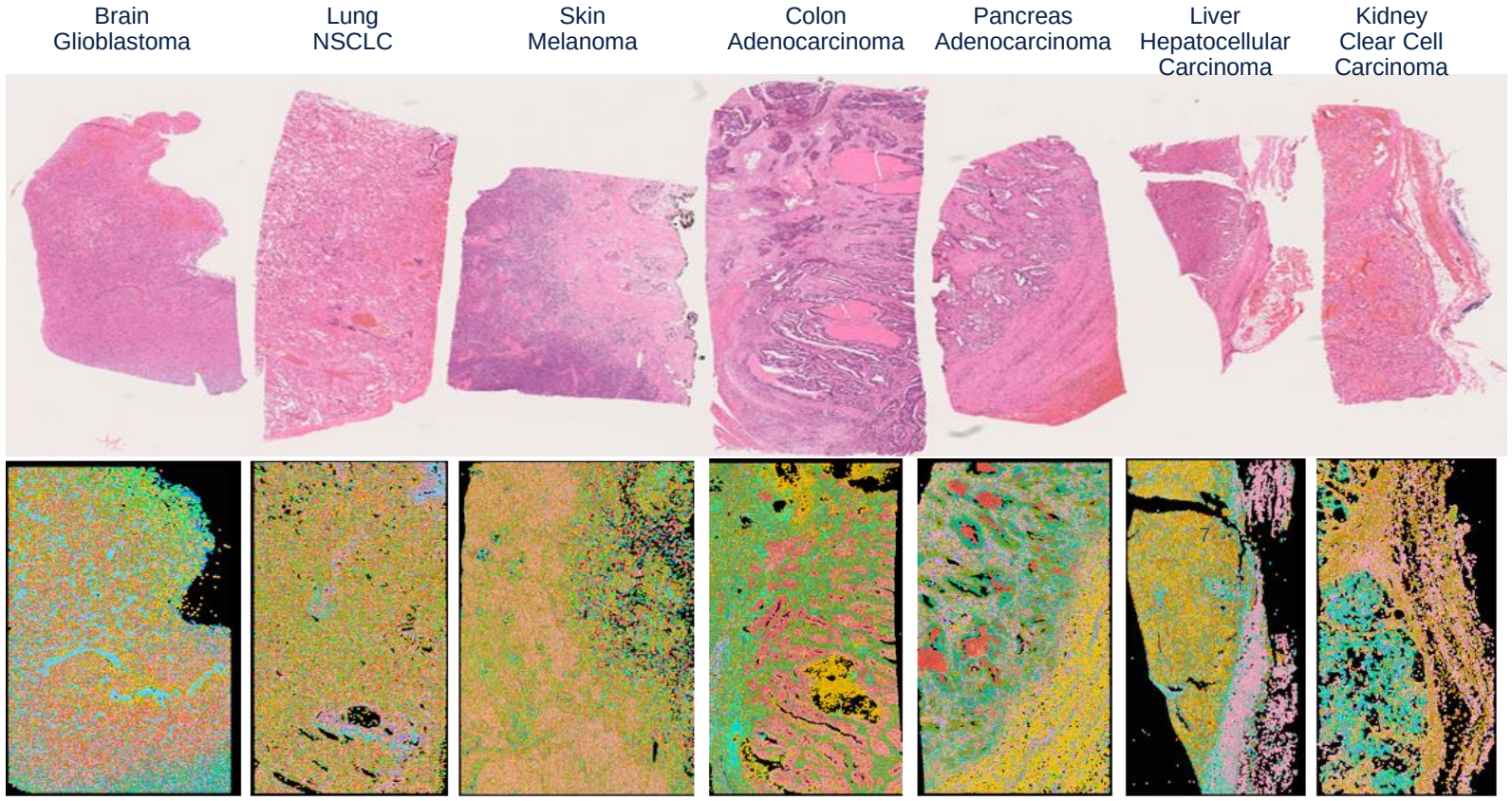


Fully automated decoding and analysis

Supported Tissues, Block Generation, & Sectioning

Xenium is Compatible with a Large Variety Tissues

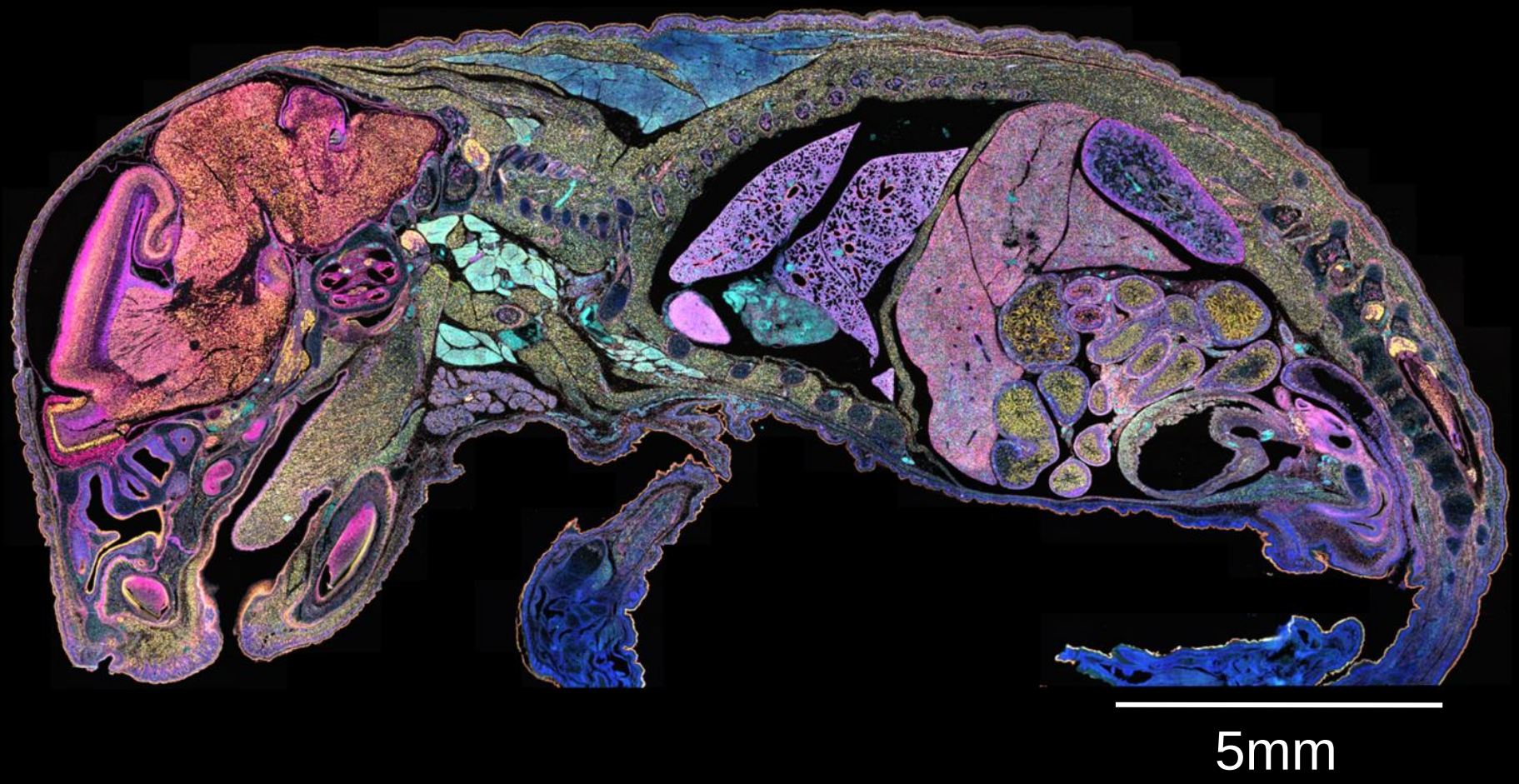
Formal-fixed paraffin-embedded (FFPE)



Tissue array with **7 different** human FFPE tissues run on a single Xenium slide

Xenium is Compatible with a Large Variety of FF and FFPE Tissues

Fresh Frozen (FF)



Generating FFPE Blocks for Xenium

Formal-fixed paraffin-embedded (FFPE)

FFPE Tissue Blocks

Human or Mouse FFPE Blocks

Fixation solution:

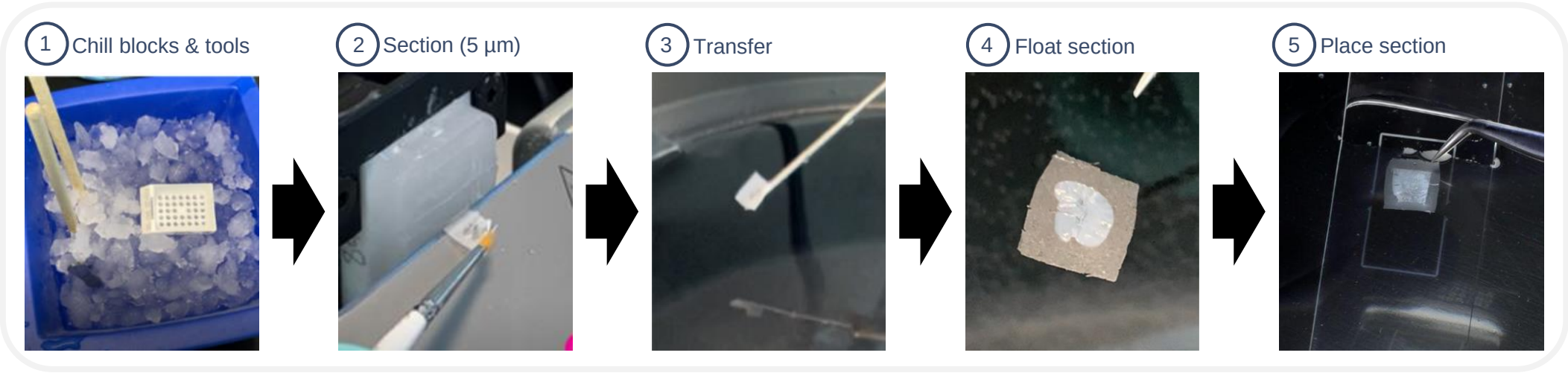
- 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)

Carefully optimize fixation tissue size & time

- Over fixation → decreased RNA accessibility
- Under Fixation → RNA degradation

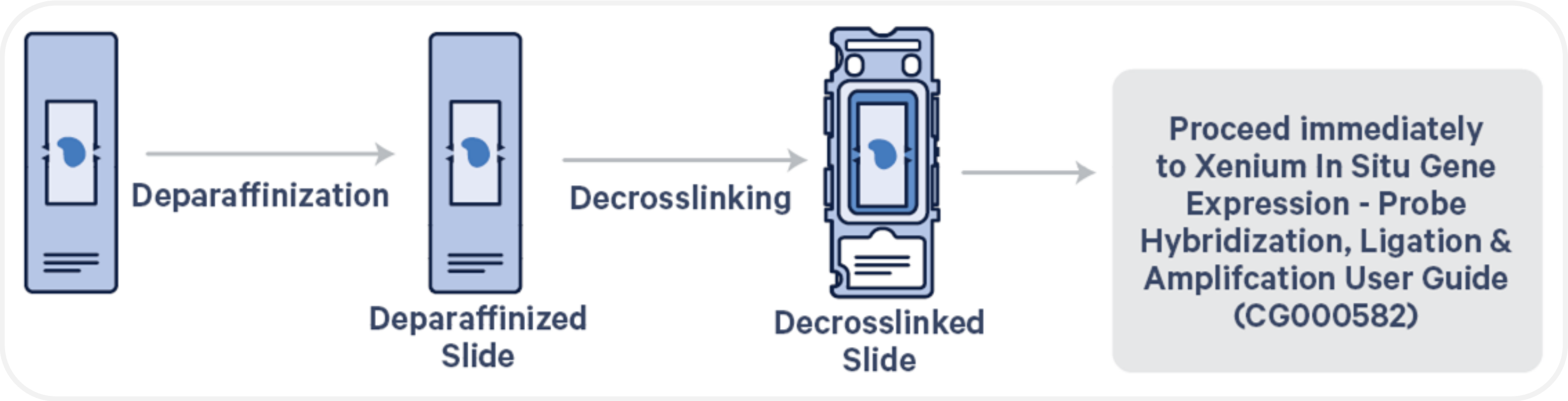
Sectioning FFPE Blocks for Xenium

Formal-fixed paraffin-embedded (FFPE)



Practice tissue placement on blank slide before using Xenium Slides

Xenium Sample Preparation for FFPE slides



- Deparaffinization to remove the wax
- Decrosslinking makes analytes (RNA and/or protein) accessible
  - Has a big impact on tissue adhesion and autofluorescence generation

Generating Fresh Frozen (FF) Blocks for Xenium

Fresh Frozen (FF)

FF Tissue Embedded With OCT

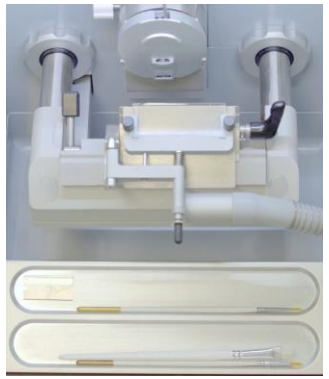
Image is representative; follow guidance in CG000579

Human or Mouse FF Tissue

- Tissue fresh freezing and OCT embedding
  - Fixed-frozen tissue is not supported at this time
- QC (H&E) tissue prior to placing on Xenium slide
- Section on to Xenium slide
  - 10 µm thickness

Sectioning FF Blocks for Xenium

1 Cryostat & tools



2 Trim OCT

3 Place section on stage

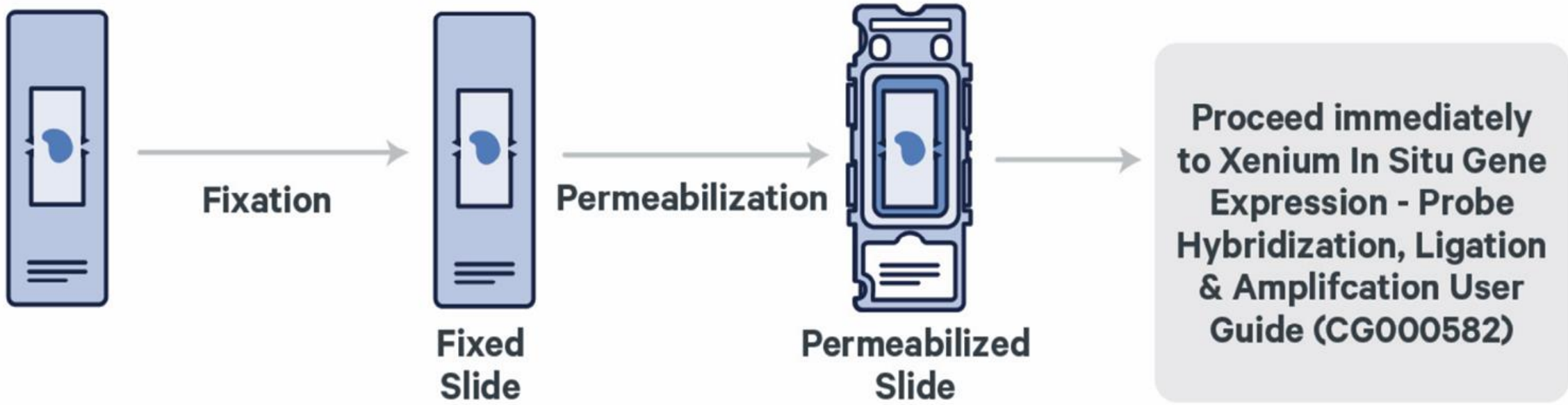
4 Section (10 µm)

5 Flatten and place

Practice tissue placement on blank slide before using Xenium Slides

Xenium Sample Preparation

Fresh frozen sample/slides



- Fresh frozen tissue must be fixed to retain RNA
  - 4% PFA or 3.7% Formaldehyde
- Permeabilization allows RNA to be accessible and probes to enter cells

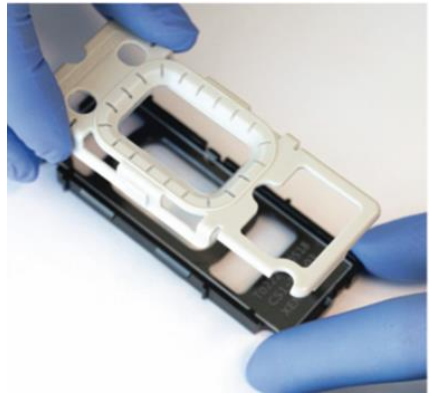
Xenium Cassette Assembly

Inspect cassette and gasket when removing from packing

1 Place *dry* slide in cassette

2 Press down on slide

3 Secure clips



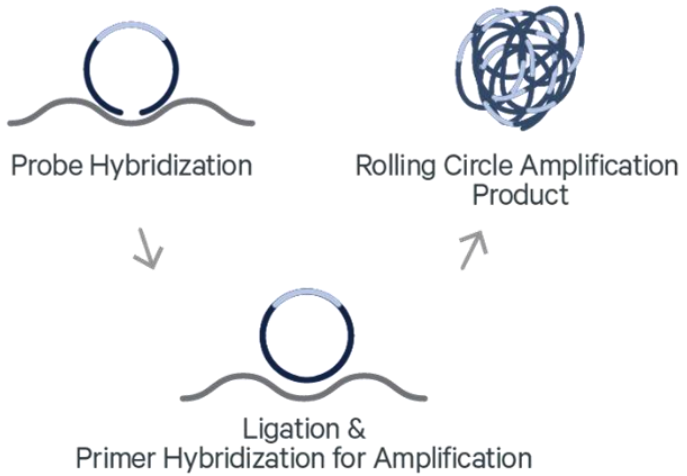
4 Press down on all sides

After assembly, inspect all sides of cassette and gasket for tight seal

Probe Hybridization Preparation

Xenium Assay Workflow: Probe Hybridization Prep Overview

Probe hybridization, Probe Ligation, and Rolling Circle Amplification

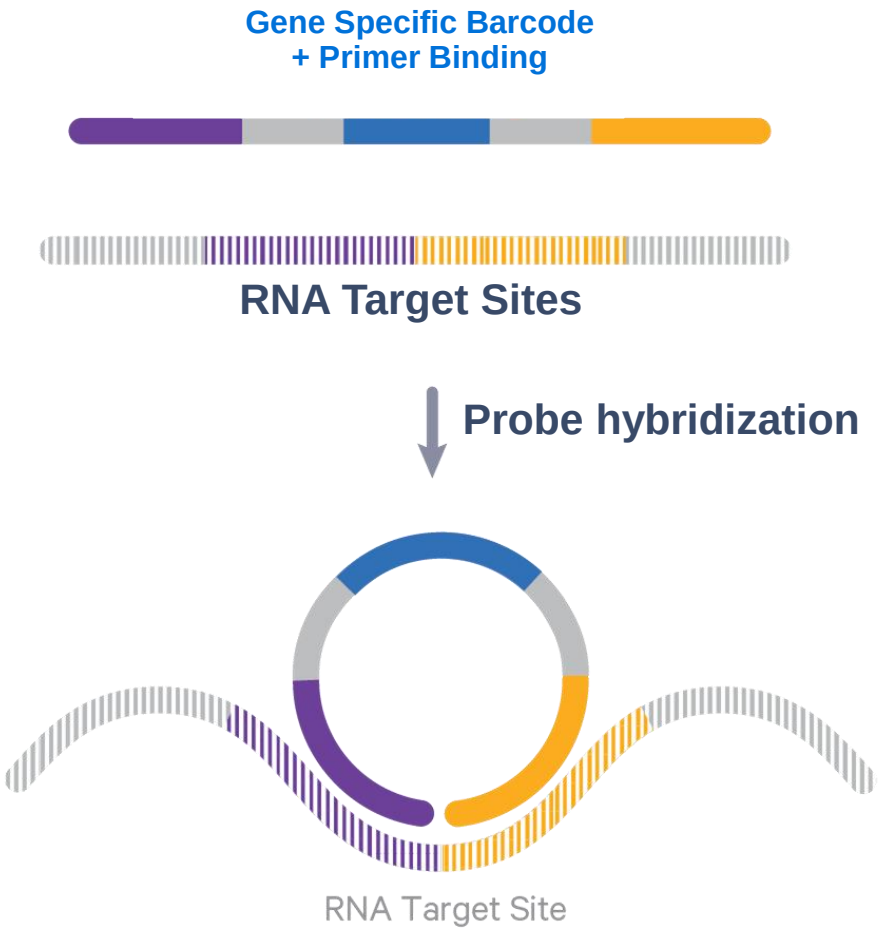


Assay Workflow

- Transforms RNA into detectable signatures through rolling circle amplification (RCA)
- Two slides processed in parallel

Xenium Assay Workflow - Probe Hybridization

Hybridized probe



Probe Hybridization

- Probes target RNA
- Two regions independently hybridize to target RNA
  - Increases specificity
- Gene specific barcode and primer binding site

Xenium Assay Workflow - Ligation

Probe Ligation

Ligation

- Ligation seals junction between probe arms
- Generates circular DNA probe

Xenium Assay Workflow - Amplification

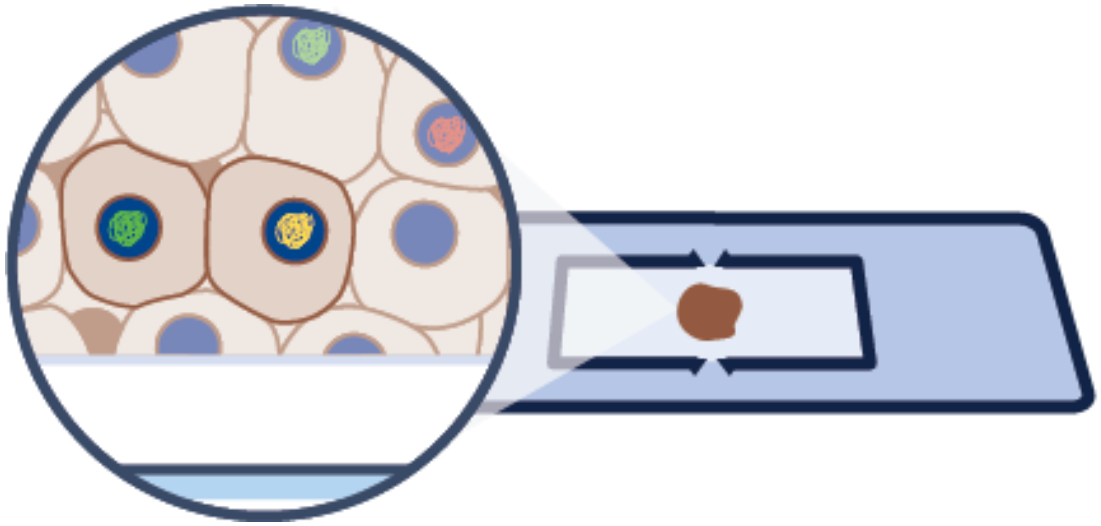
RNA Target Site

Amplification

- Ligation products enzymatically replicated
- Rolling Circle Amplification (RCA)
- 100s of copies of probes generated

Xenium Assay Workflow - Quenching and Staining

Xenium Assay Workflow - Autofluorescence Quenching and Nuclei Staining



Autofluorescence Quenching

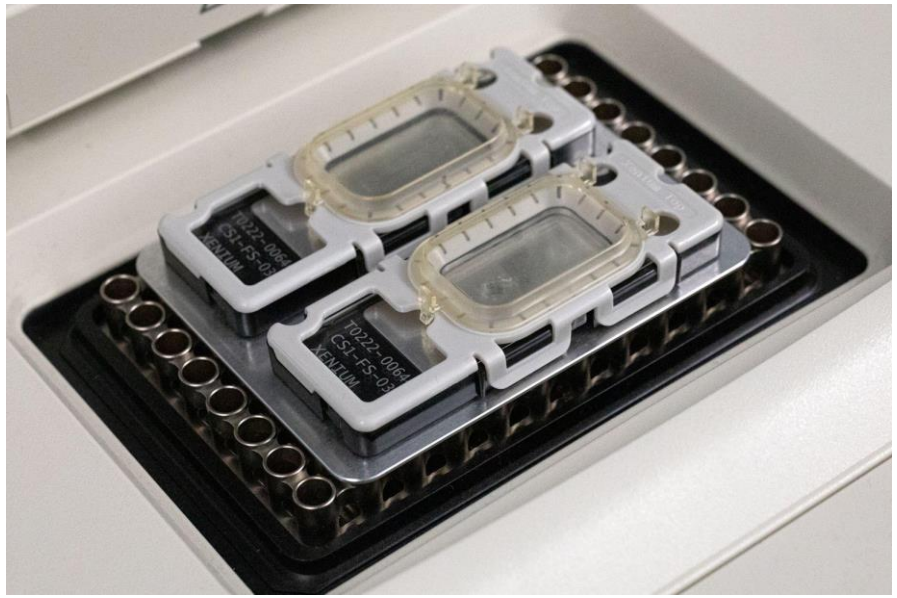
- Proprietary autofluorescence mix
- Improves signal-to-noise ratio
- No tissue optimization required for FFPE and fresh frozen tissue

Nuclei Staining

- Assists in nuclei identification in overview scan

Xenium Assay Workflow - Validated Thermal Cyclers

Two slides on thermocycler adaptor



Which Thermal Cyclers Are Compatible with Xenium?

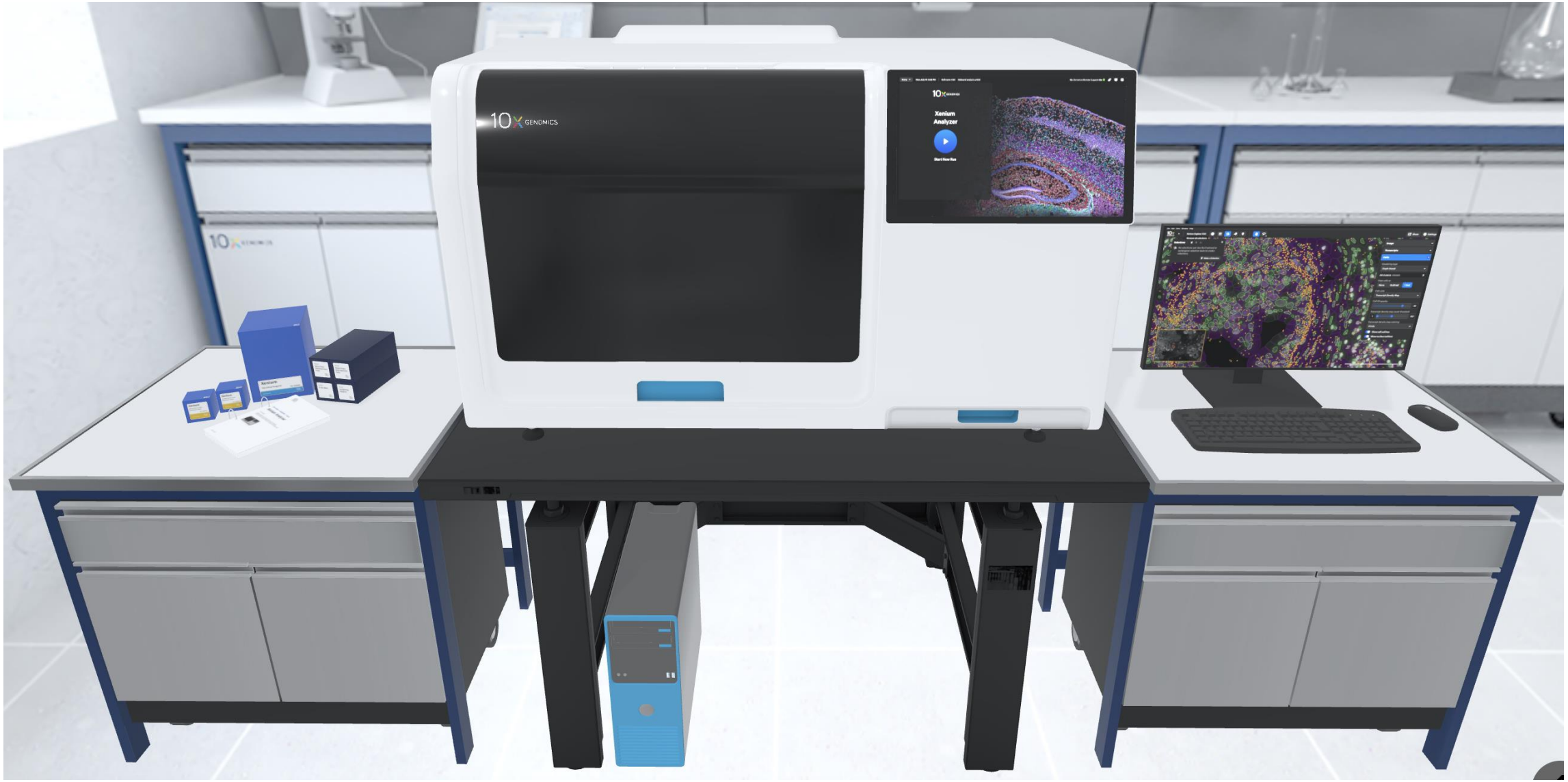
Best practices

- 10x recommends using adjustable lid models
  - Close lid and tighten until click is heard
  - Do not turn past the click
- Pre-equilibrate thermocycler adaptor
- Always run two slides at a time

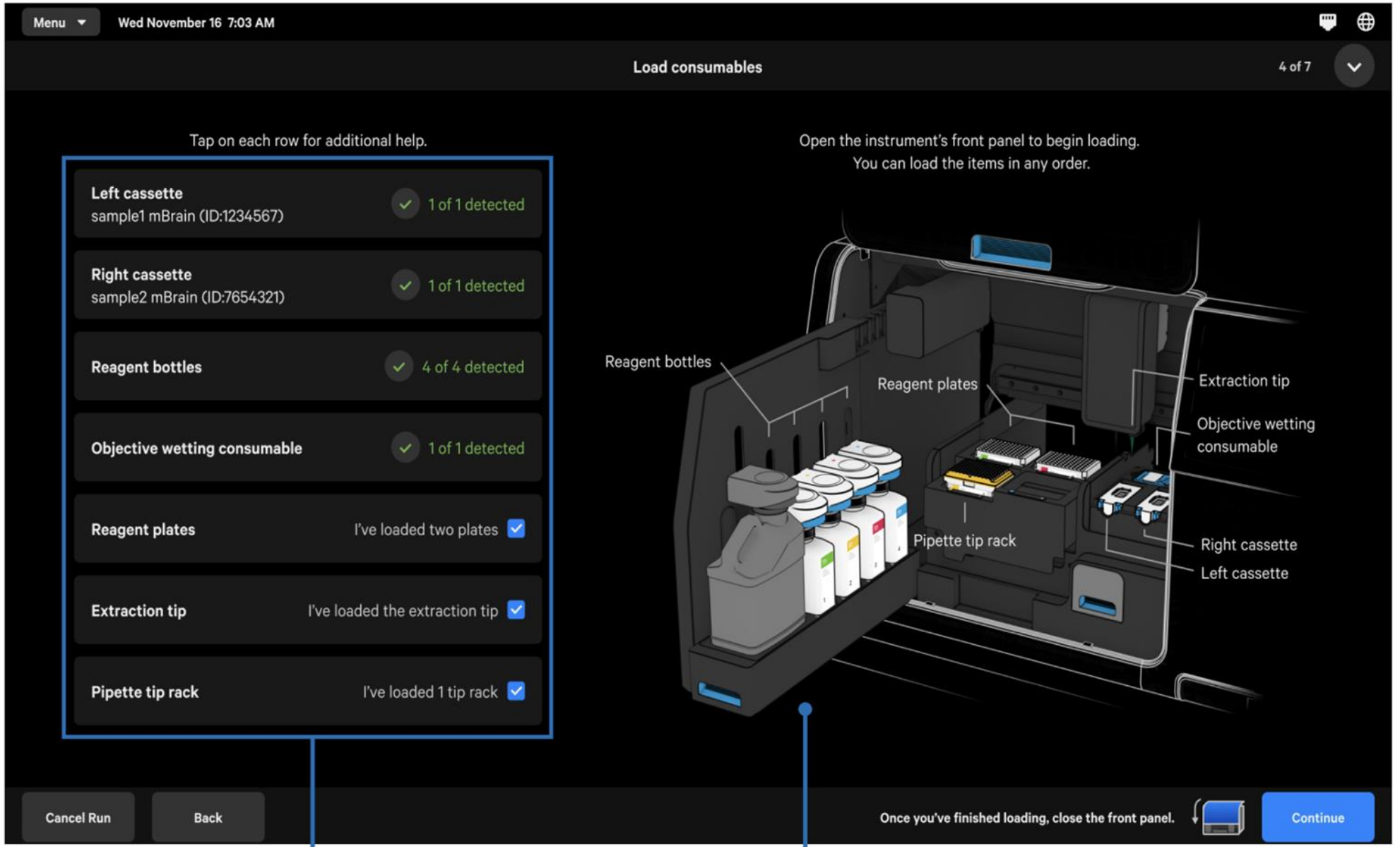
e.g., Analytik Jena Biometra TAdvanced 96 SG (846-5-070-241)

Instrument Operation

Xenium Equipment Setup

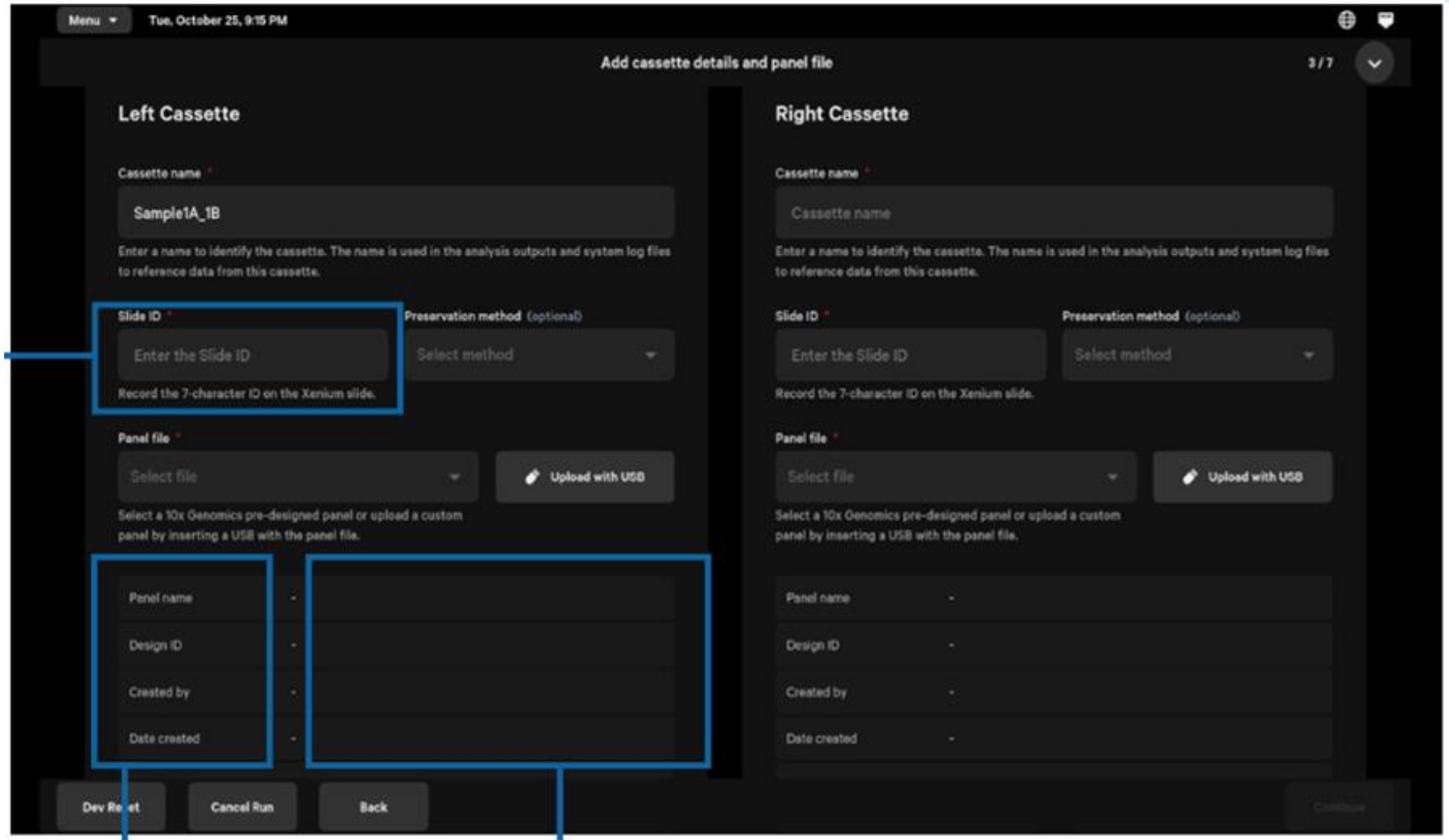


Xenium On-Instrument Workflow



Xenium On-Instrument Workflow

Initialize ~10-20 min › Load ~5 min › Sample Scan 1 h › Region Sel. ~10 min › Run ~27 hrs › Cleanup ~5 min › Unload ~10 min

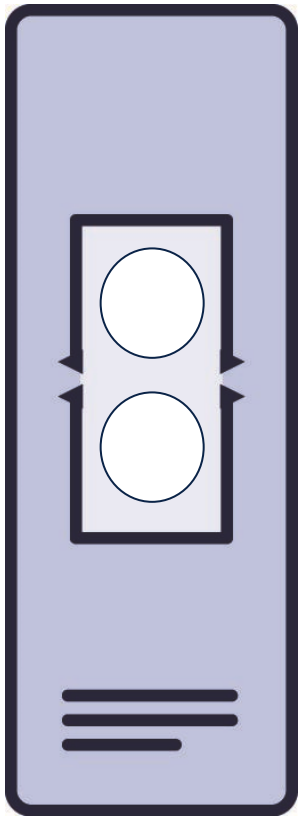


Slide ID

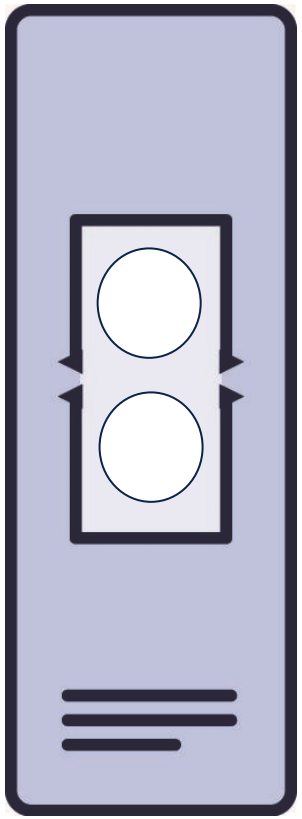
Panel Information

Custom Panel

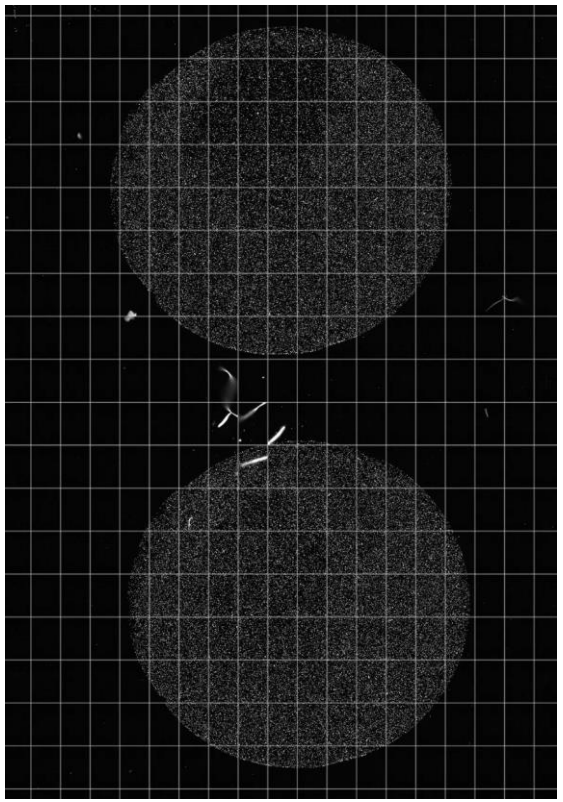
Xenium On-Instrument Workflow



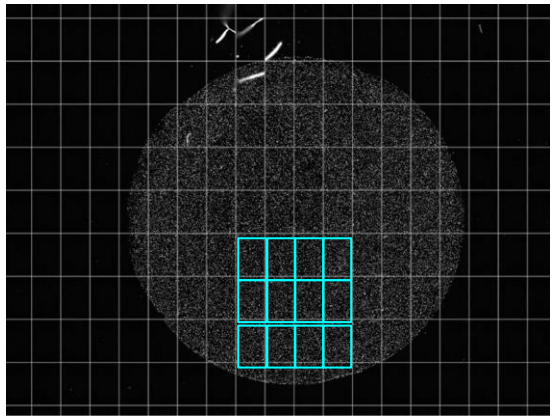
Slide 1



Slide 2



Overview scan
Slide 1

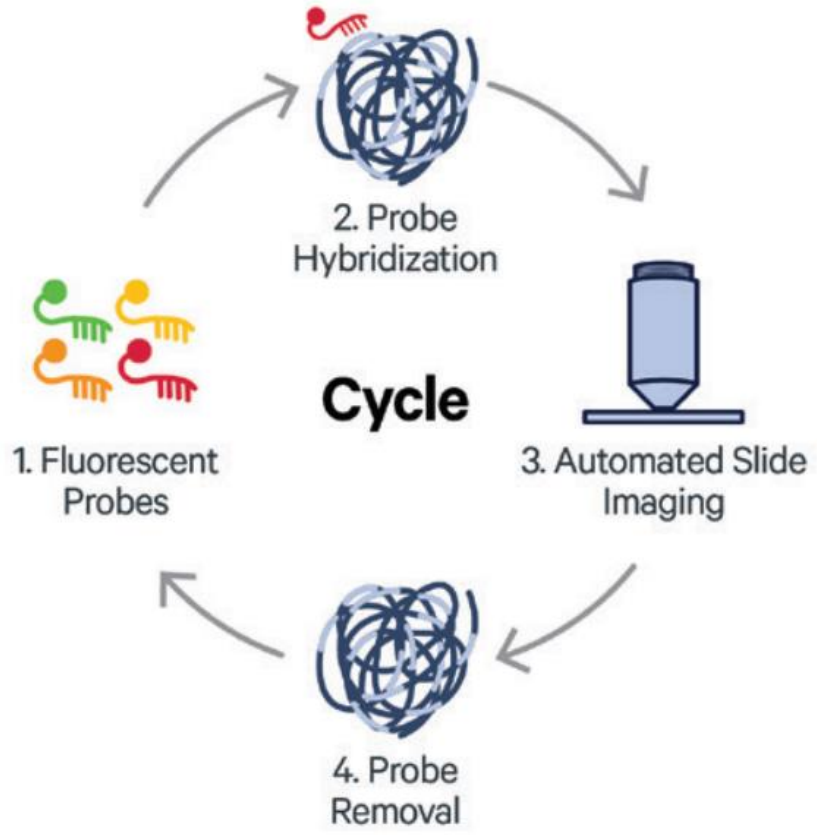


Region selection:

- 12 total FoVs
- Contiguous rectangle
  - (3x4, 4x3, 2x6, 6x2)
- Avoid obvious defect - debris, low cell density, bubble, etc.

Overview scan with FoVs

Xenium On-Instrument Workflow



Xenium Analyzer Run

- During Xenium Analyzer run, instrument goes through a series of cycles
  1) First, rolling circle products are labeled with fluorescent signals
  2) Then, the signals are imaged at each cycle
  3) After imaging is complete, probes are removed to leave Rolling Circle Products available for subsequent cycles of RNA labeling

Pre-Designed Panels

Pre-Designed Panels: Design Philosophy

Expertly Curated, Experimentally Validated, Readily Available 10x In Situ Gene Panels

Data-driven approach to gene curation

- Publicly available scRNA-seq or other large datasets
  - Human Protein Atlas, Tabula Sapiens, Tabula Muris, etc
- Literature curation, especially for disease states

Eight pre-designed panels for **cell typing** using combinations of gene expression that uniquely label cell types

Human & mouse

~ 250 to 380 genes/ panel

Six tissue specific panels

Two multi- tissue panels

Add up to 100 custom genes of interest

Panel and Custom Menu Offers Maximum Flexibility

Customize any panel or build your own standalone panel

Pre-designed & validated panels

Human Breast
280 genes

Human Multi-Tissue & Cancer
377 genes

Human Lung
289 genes

Add up to 100 custom targets

Human Colon
322 genes

Human Brain
266 genes

Mouse Brain
248 genes

Human Skin
260 genes

Mouse Multi-Tissue
379 genes

Coming Soon in 2024

5000 & 2000 gene panels

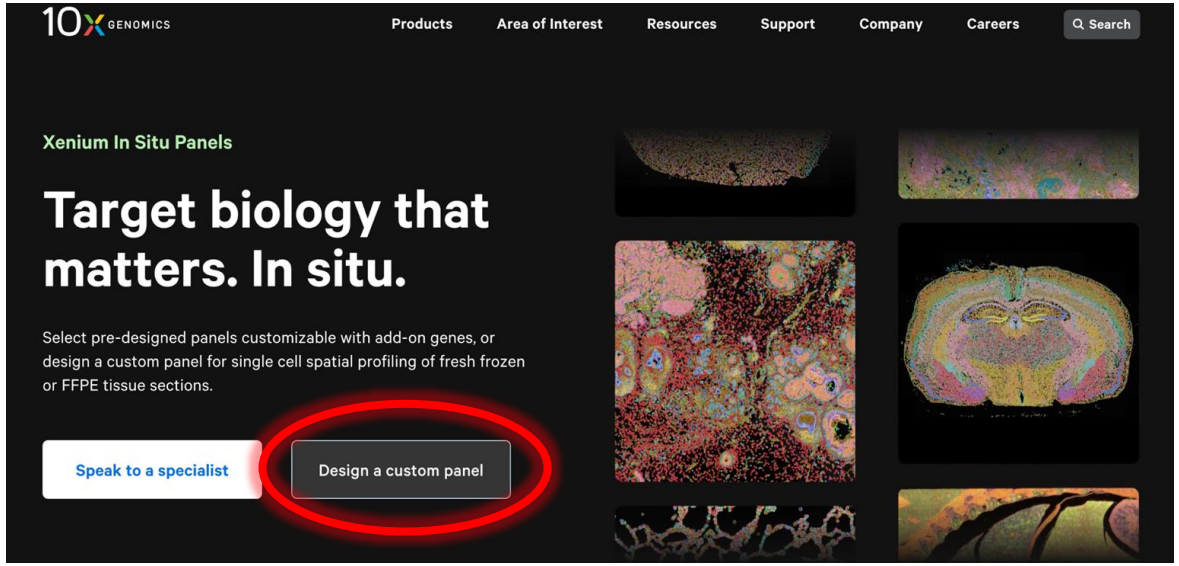
Overview of Xenium Custom Panel Design

- Required Information & Files to Design a Custom Panel
- Understanding the *In Situ O*ptical Detection Budget
- Understanding Probe Sets in Xenium Panel Design

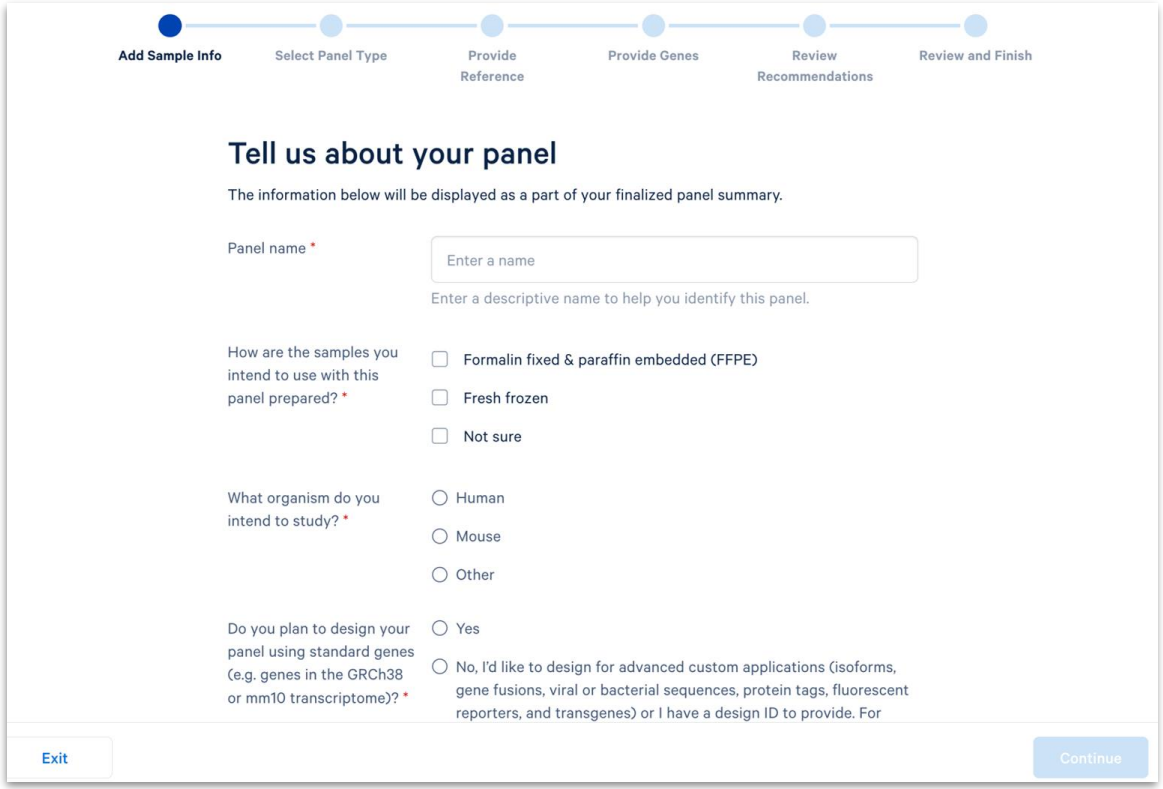
Build your Custom Panel Online with Xenium Panel Designer

Panel design algorithms specifically developed for 10x assays enable breadth of applications

- Design standard human and mouse gene expression **custom panels for up to 480 plex** independently
- **Upgrade to advanced custom panels** to access wide range of applications and species with 10x support



www.10xgenomics.com/products/xenium-panels



Panel and Custom Menu Offers Maximum Flexibility

Customize any panel or build your own standalone panel

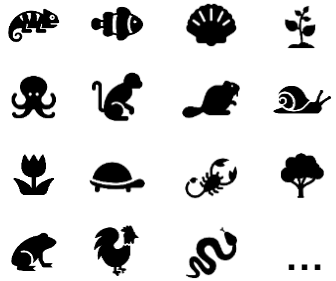
Standalone custom

480 custom genes

300 custom genes

100 custom genes

50 custom genes



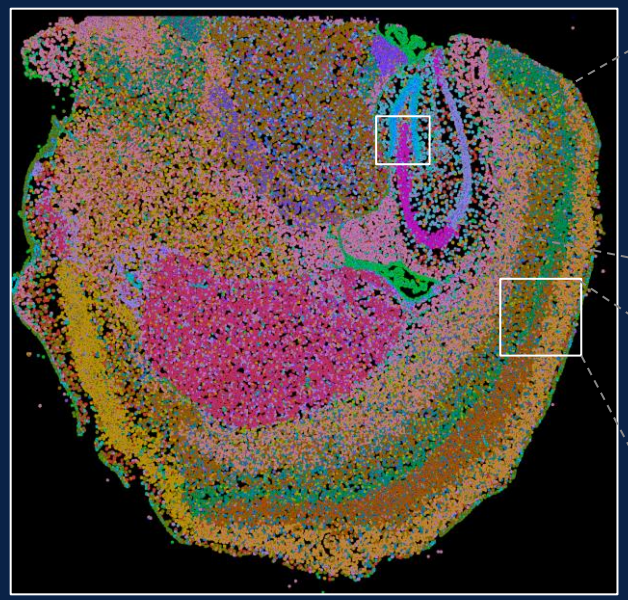
Panels designed or under design:

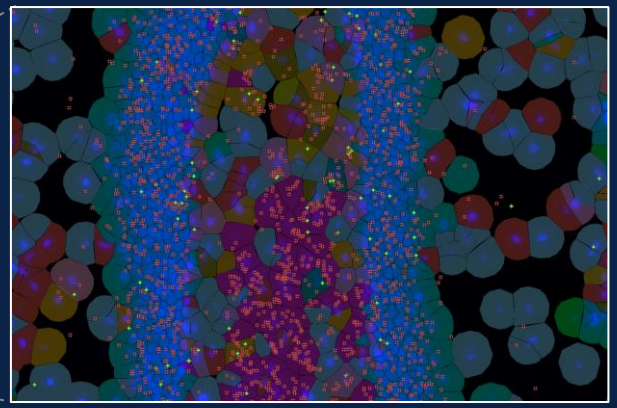
- Rat
- Dog
- Zea mays
- Zebrafish
- Pig
- Mosquito
- And more…

Exclusive to Xenium: Isoform Detection

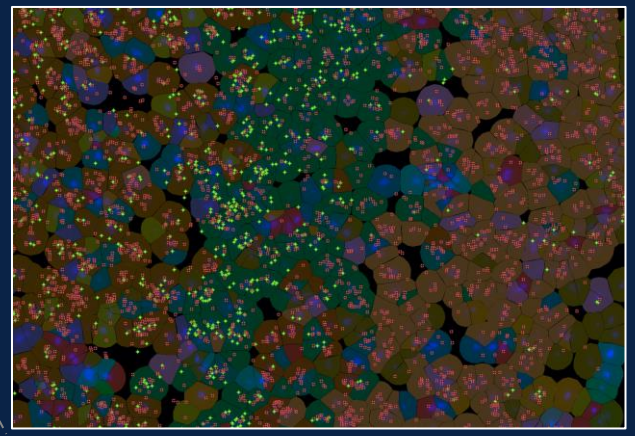
Xenium mBrain panel + custom probes for Isoforms -> Differential cell-type expression of isoforms

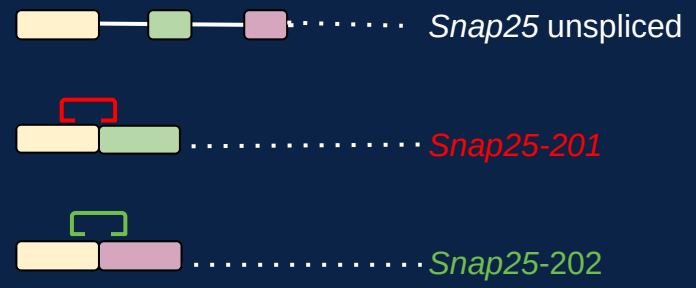
Cell typing with base panel





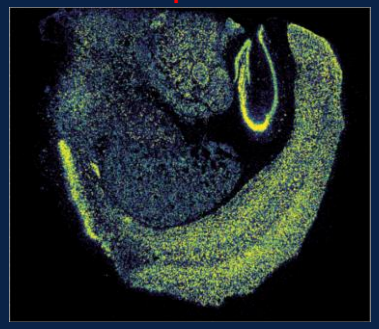
Snap-25 isoforms are differentially localized



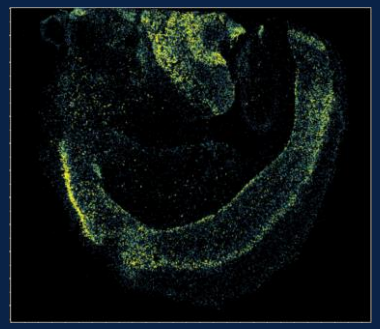


Snap25 isoforms have regional localization (especially during development) involved with plasticity of neurons

Snap25-201



Snap25-202

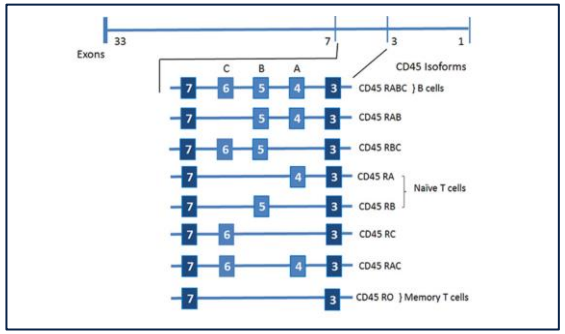


Advanced Customization Enables Broader Applications

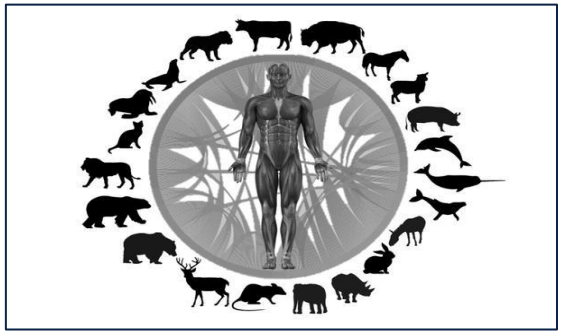
Unique padlock chemistry affords key applications with additional ones in development

Available Now

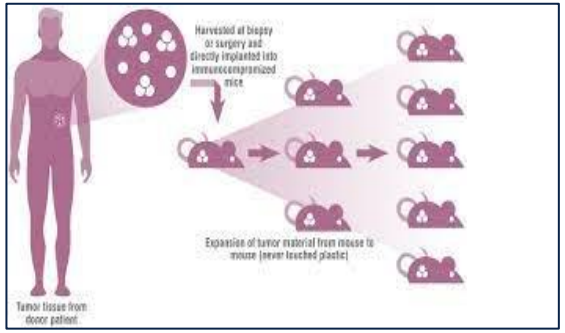
Isoform/Translocations



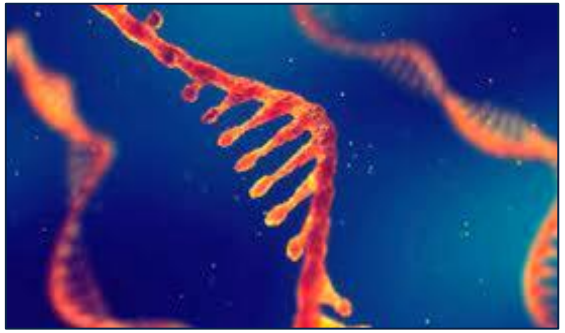
Diverse Species



Xenografts

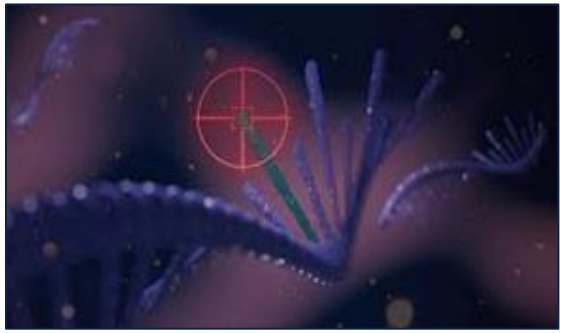


Exogenous Sequences

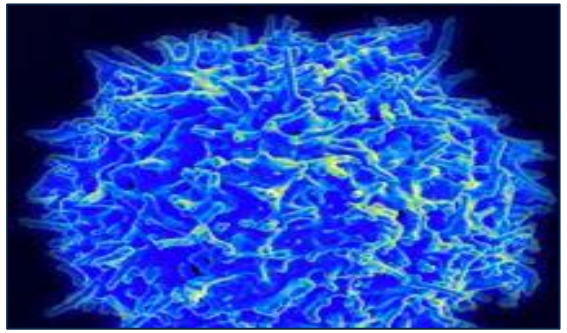


Coming Soon

Expressed SNVs



TCR/BCR Profiling*



*Determine the V and J gene of the heavy and light chains paired for each T/B cell .

Required Information & Files to Design a Custom Panel

What Do You Need To Get Started With Panel Design?

- **A gene list**

  Selection of genes for elucidating biology of interest

  - Panel design app will recommend genes to remove, but will not recommend genes to include

- **A single cell reference**

  The panel design app has a selection of curated references from CELLxGENE that can be used if they work with the experimental model (mouse & human)

  - Can be either Chromium (fresh or Flex) or Visium (with reference-free deconvolution)
  - Matched reference is recommended, but is not required
  - Reference **must** be in 10x MEX or h5 format

- **Additionally for Advanced custom only** (custom targets)

  The sequence of the target transcript

The Importance of Single Cell Reference Data

Having closely matched scRNA-seq data is desirable

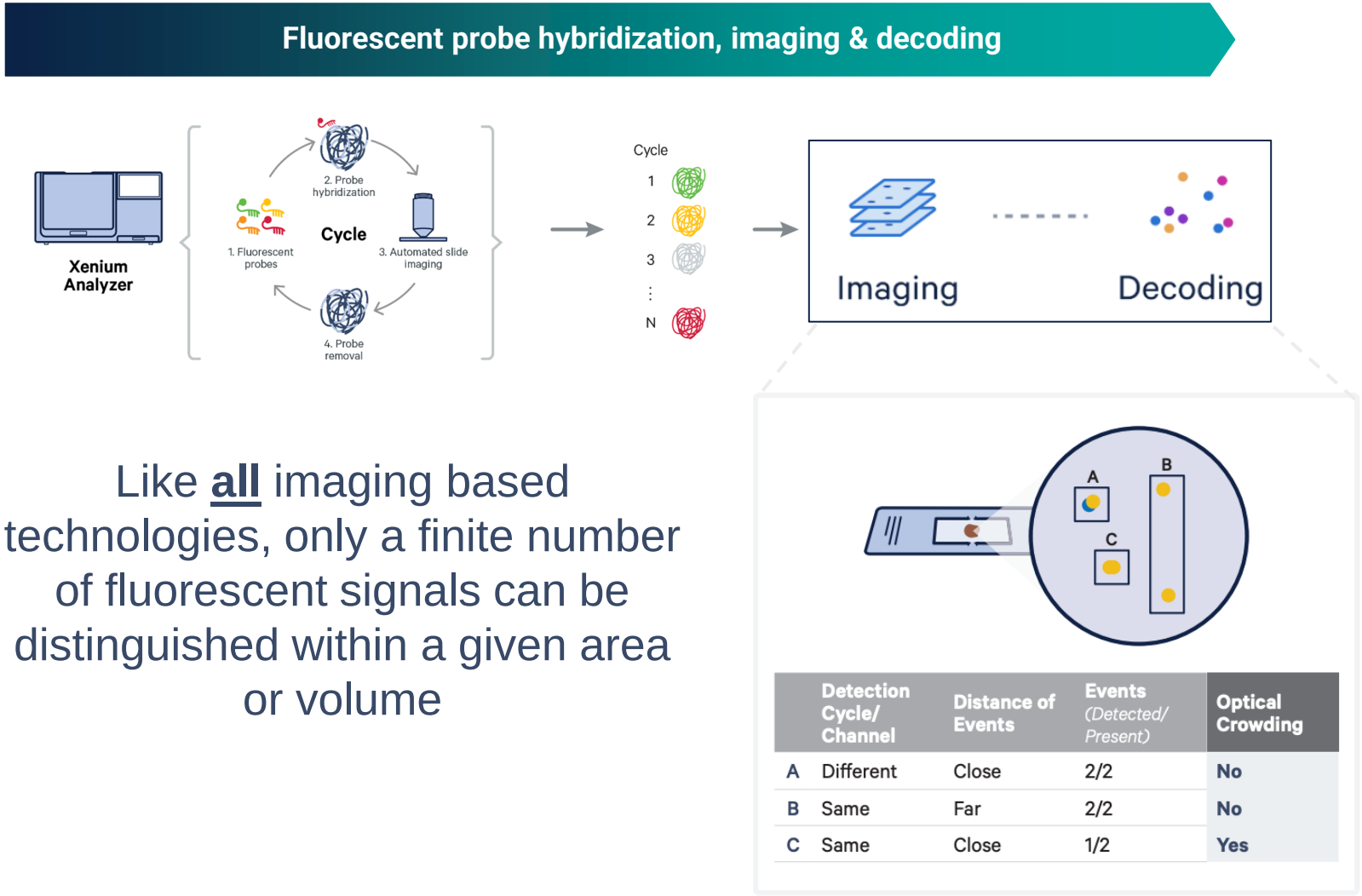
1. Determines how much optical budget the panel is using
2. Place highly expressed genes in the same cell type on optically distant barcodes
3. Provides a basis for choosing probeset coverage reduction

If you don't have scRNA-seq data, you can use publicly available data from sources such as CELLxGENE

1. It is particularly important to find the best scRNA-seq dataset you can when you are working in diseased tissue

What is the In-situ Optical Detection Budget?

Careful panel design for optimal spot detection & high accuracy gene identification



	Detection Cycle/ Channel	Distance of Events	Events *(Detected/ Present)*	Optical Crowding
A	Different	Close	2/2	No
B	Same	Far	2/2	No
C	Same	Close	1/2	Yes

Like **all** imaging based technologies, only a finite number of fluorescent signals can be distinguished within a given area or volume

Optical detection budget

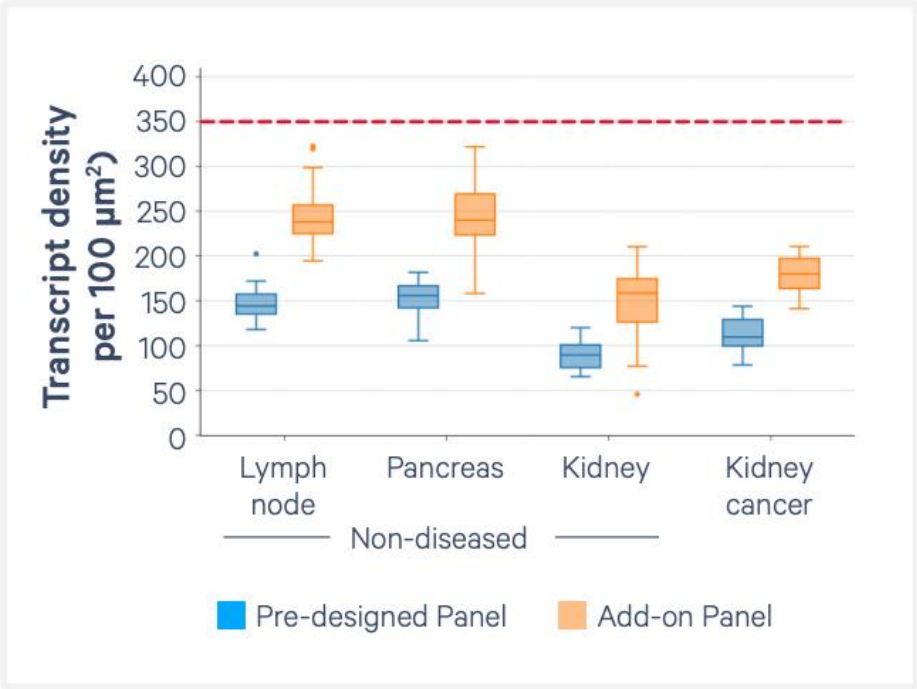
Upper limit of spots that can be resolved in a single image

- Detection of genes is carefully divided in different cycles to avoid optical detection limit

- Gene expression per cell from scRNA-seq is used as a reference to select genes that maximize spot detection efficiency

Optical Budget Considerations For Panel Design

Optical budget is reserved for add-on genes in 10x pre-designed panels

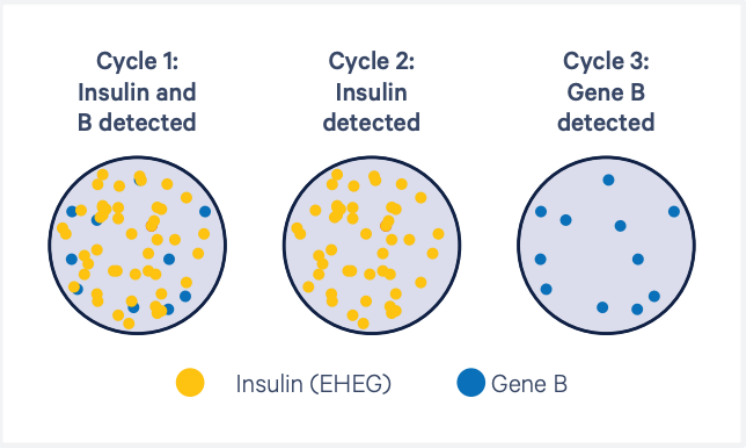


Chemistry	Low Expression Threshold (Mean Transcripts per Cell)	High Expression Threshold (Mean Transcripts per Cell)
Chromium Single Cell 3' v2	<0.1	>50
Chromium Single Cell 3' v3.1		>100

- Single Cell Gene Expression data is used to quantify the expression of a gene in the relevant cell type
- Ideally, select genes with a mean expression of at least 4/2 transcripts per cell

Understanding the Nuance of Optical Detection Budget

Highly expressed genes in an imaging based in situ panel need to be carefully evaluated



Extremely highly expressed genes (EHEGs) can utilize all or most of the allocated optical detection budget for a particular cell, limiting detection of other genes within the cell

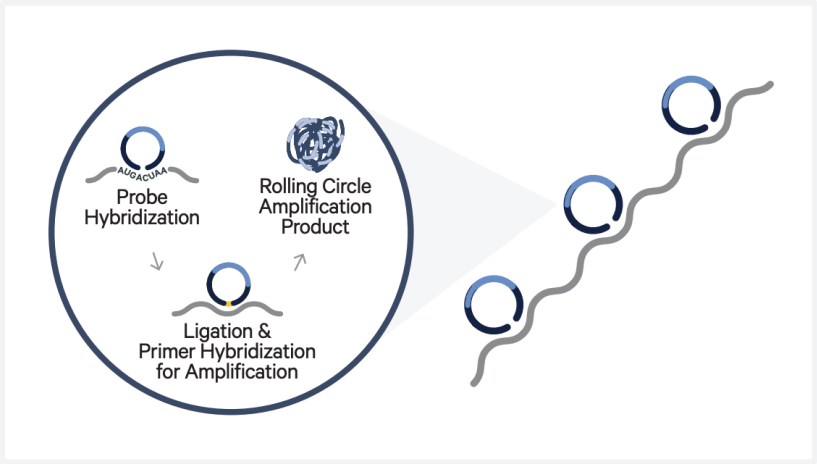
- However, EHEGs can be included in the assay based on the biology of interest
- Consider what cell types they are expressed in, and what information you are hoping to obtain from that cell type

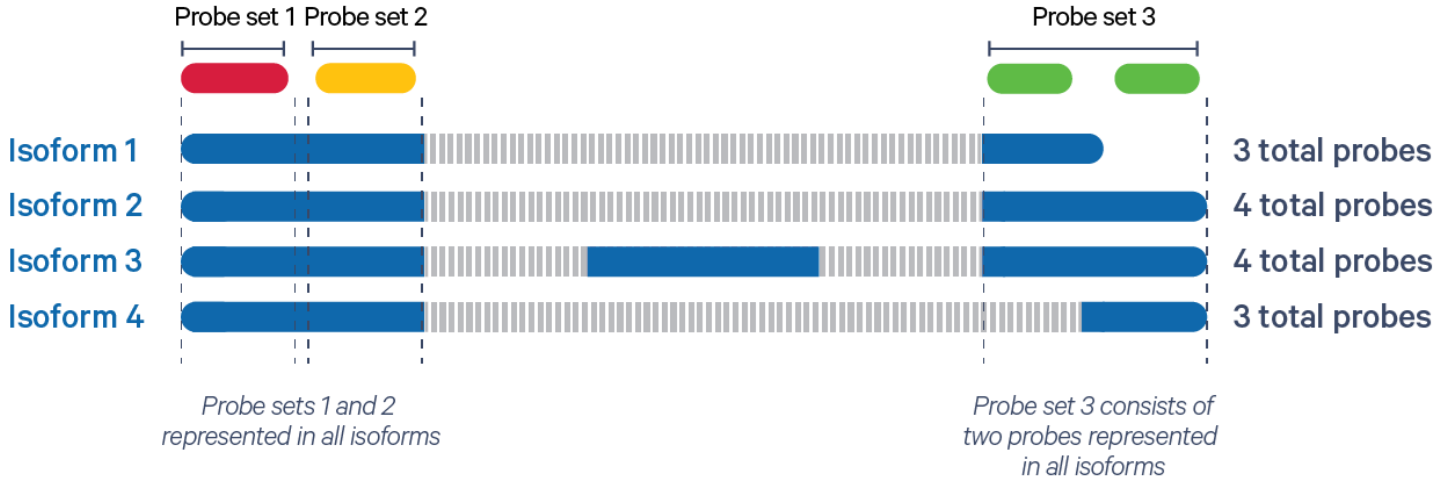
EXAMPLE: Insulin

- Highly expressed only in pancreatic beta cells
- Including INS on your panel will effectively label beta cells
- The remaining expression profile of the beta cells will be hard to determine
- The other cell types in your experiment will be unaffected by the high expression of INS in beta cells

Understanding Probesets in Xenium Panel Design

General info





By default, every gene on a panel has 8 probesets

- A probeset is one or more probes that cover every isoform of a given gene
- Some genes cannot have 8 probesets due to a variety of factors:
  - Gene length
  - Repeat content / GC content
  - Sites of known variation
- Some genes have zero probesets due to the above factors; if this is true for a gene you want on your panel, please contact 10x support

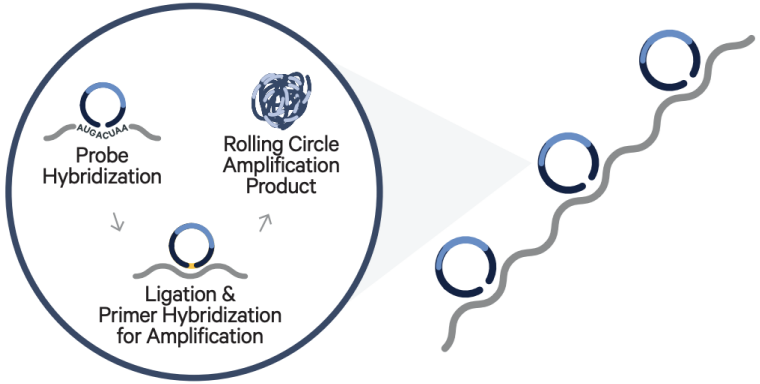
Understanding Probesets in Xenium Panel Design

Assay sensitivity

Assay Sensitivity: Number of transcripts detected per gene

The number of probesets for a gene have a roughly linear relationship with the sensitivity of the gene on the panel

- For highly expressed genes, reducing the probeset count will reduce the sensitivity i.e., fewer transcripts detected
- Recommendation: At least 3 probesets per gene
  - For some applications, single probeset per target can be used



Note:

- *Reducing probeset count enables analysis of highly expressed genes*
- *Increasing probeset count enables analysis of lowly expressed genes*

What to Think About When You Are Picking Your Gene List

1. What is the goal of my panel?
    1. Cell typing or something more?
2. How much am I willing to accept potential optical crowding?
    1. Lower sensitivity isn't necessarily bad, depending on what you said to #1
3. Can I pick genes for my panel that give me the goals I want in #1 without picking highly expressed genes?
4. Can I pick non-redundant genes that represent my biology of interest?
    1. Only a single HLA class I gene
    2. Avoid multiple mitochondrial genes
    3. Representative gene from my pathway of interest
5. Are the genes I am interested in ubiquitously and/or highly expressed (collagen, immunoglobulins)
6. Are the genes I am interested in very lowly expressed?
7. Can I use co-expressed genes to infer my biological question without using a highly expressed gene?

Overview of the Xenium Panel Design Workflow

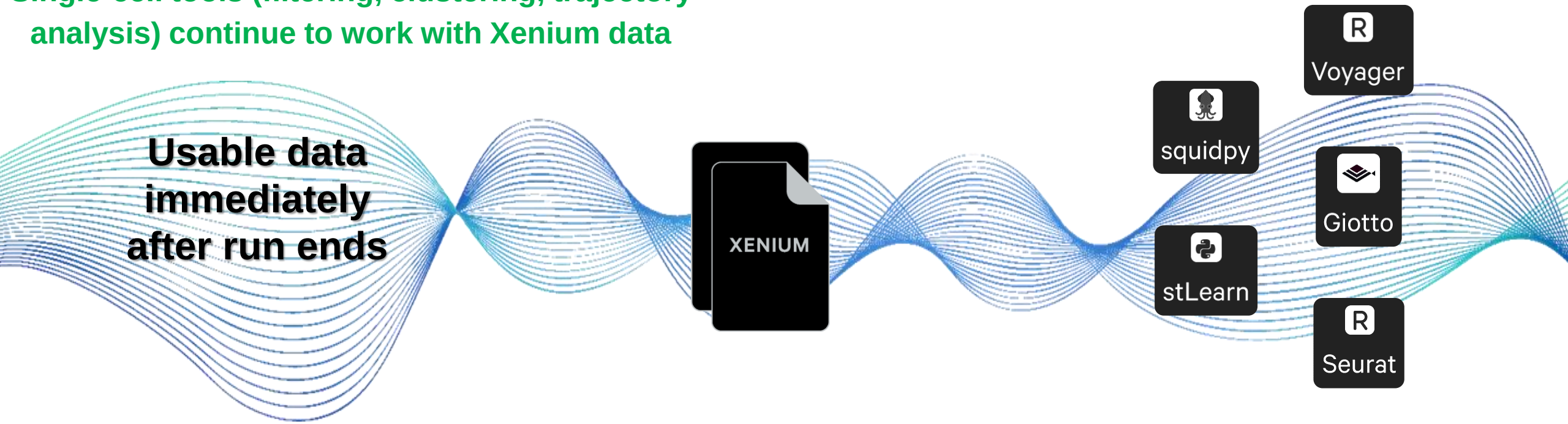
1. You will be asked to select one or more curated single cell references and/or upload your own single cell reference(s)
2. You will provide a gene list which is validated against the 10x Genomics 2020-A reference
3. The application will run the panel design algorithm
    1. Detects genes that are highly expressed and recommends either removing the gene or reducing the number of probesets for that gene
    2. If more genes are provided than the panel specification, recommends genes to drop based on the cell-typing efficiency of the panel
    3. Assigns genes to barcodes based on the expression profile to make the most of the optical budget
4. The application generates a summary report that shows you the recommended panel adjustments and how much of the optical budget is being used
5. Allows you to iterate on the panel by adjusting the gene list, manually change probeset counts, or use different single cell references

Xenium Analysis Overview

Common File Formats for Ease of Use

10x has extensive experience optimizing single cell and spatial data formats

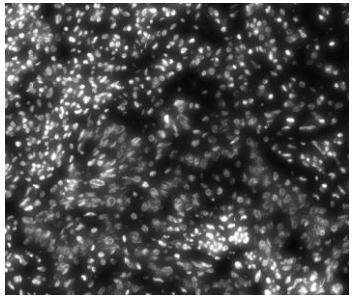
Single-cell tools (filtering, clustering, trajectory analysis) continue to work with Xenium data



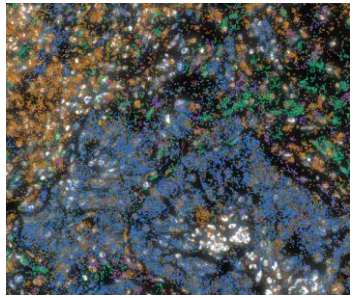
Seamless integration with Seurat, Squidpy, stLearn, Giotto, and Voyager

Usable Data Immediately After Your Run Ends

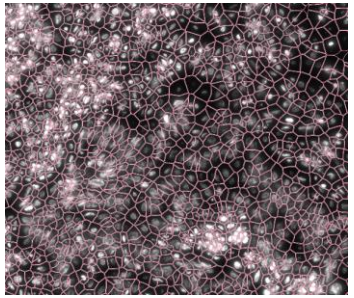
No additional steps of analysis time required to analyze and visualize your data



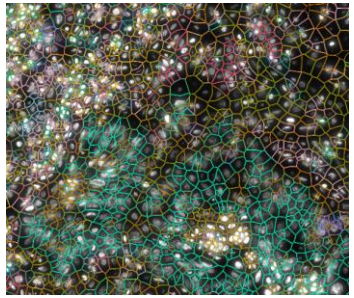
Morphology images



Localized Transcripts



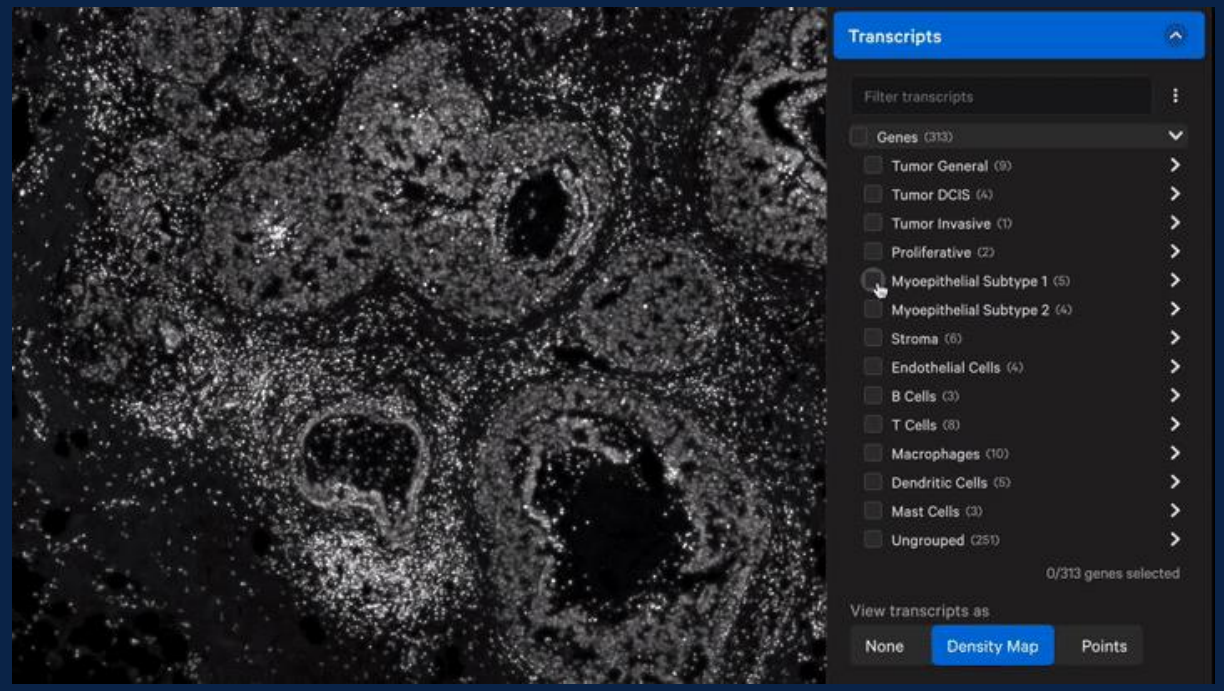
Cell Segmentation

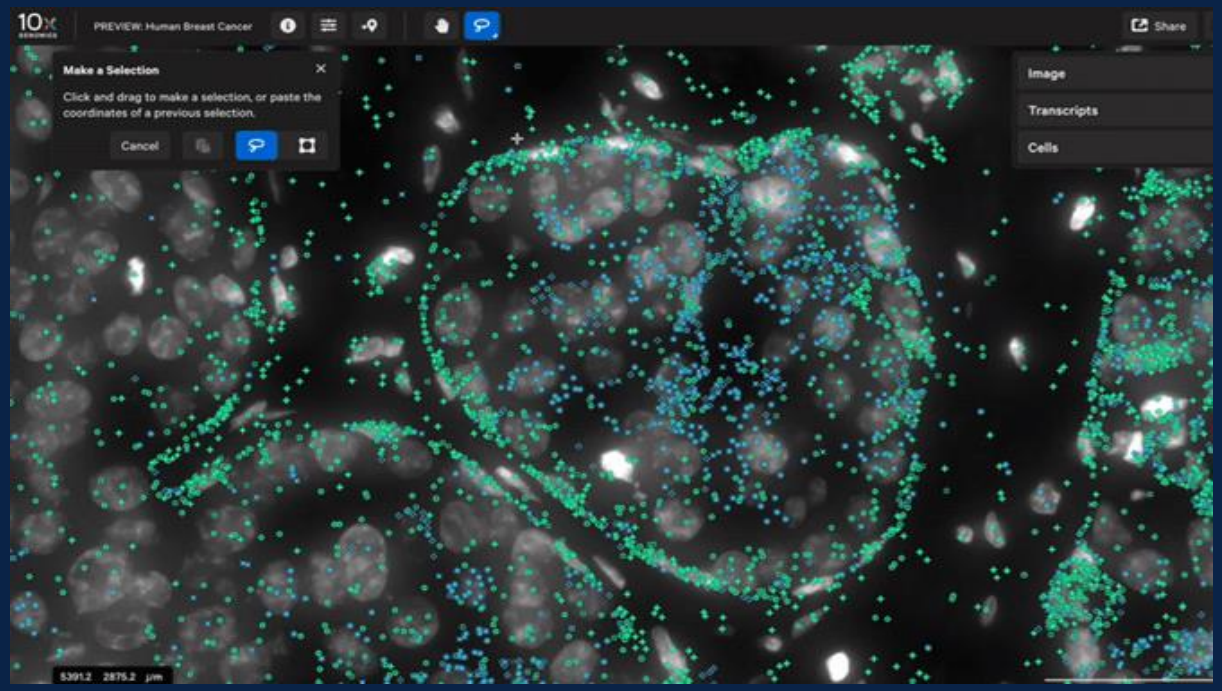


Unsupervised Clustering

Xenium Explorer – Powerful interactive native visualization

Visualization is no longer just static plots





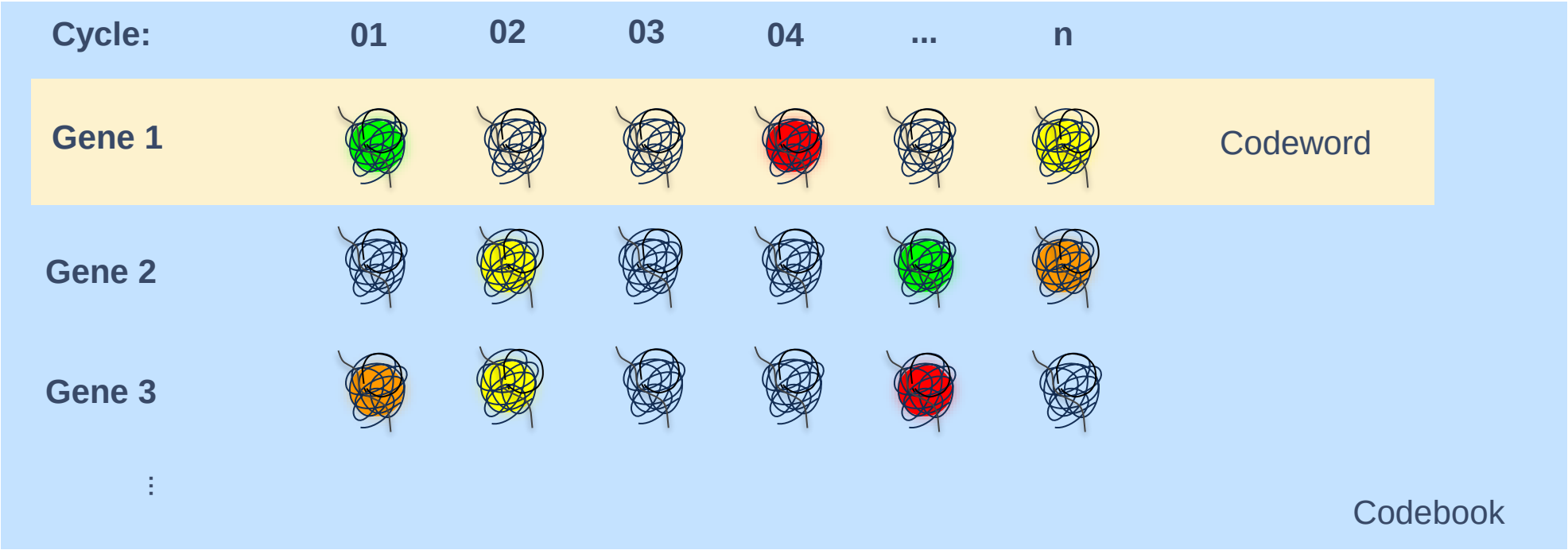
Understanding Xenium Algorithms

- Decoding
- Cell Segmentation

Decoding

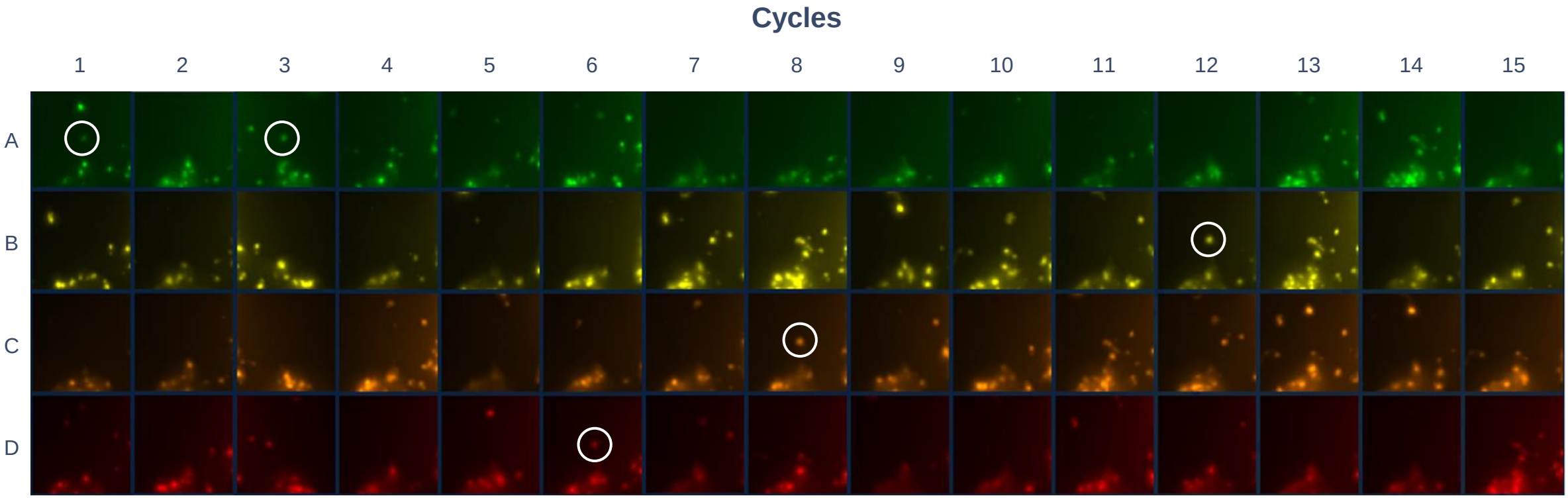
Xenium Encodes Genes Across Cycles & Channels

- A codebook is simply a collection of codewords assigned to genes
- Codewords determine when fluorescent signals (puncta) are expected across cycles and channels
- Each Xenium panel uses a codebook that contains 40 negative control codewords; each panel also includes 20 negative control probe sets (except the Xenium Mouse Brain Gene Expression Panel, which has 27)



Xenium Decoding

Decoding example of a single transcript across cycles and channels



- Example codeword: AEAEEDECEEEBEEE
- Using a probabilistic model that takes signal intensities, similarity to known codewords, and other attributes into account, Xenium can decode the example codeword to sub-pixel accuracy

See more at our Overview of Xenium Algorithms support page

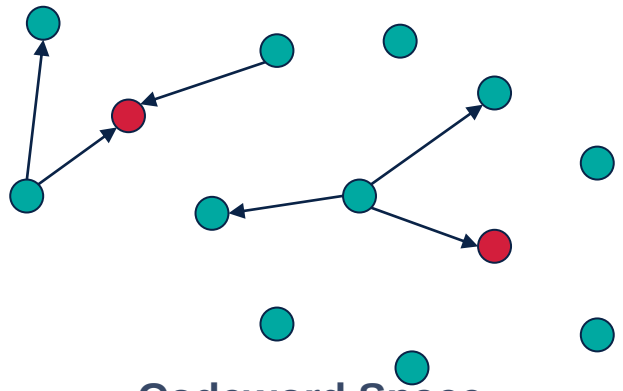
Xenium decoding outputs include calibrated quality scores

- A Phred-scale calibrated quality score (Q-score) is assigned to each decoded transcript to signify the confidence in the decoded transcript identity
- This is just a re-scaling of the probability of error that a reported gene decoding is incorrect
  - **Q-score = $-10 * \log_{10}(P_{err})$**

Q-score	Error probability (P_{err})
10	10%
20*	**1%**
30	0.1%

*__Xenium Q-score threshold >20__ << 1% of reported transcripts are incorrectly decoded

Xenium Measures P_{err} Via Negative Control Codewords



Codeword Space

- Gene Codeword (N_g = 500)
- Control Codeword (N_c = 40)
- Possible decoding errors

Negative Control Codewords are a random subset of codewords, with identical properties to gene codewords

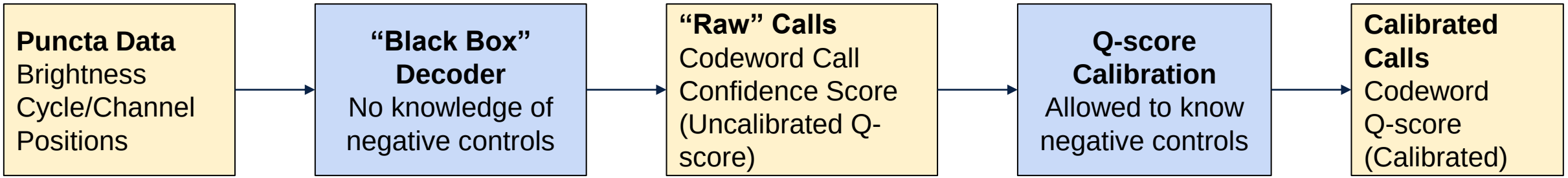
- By definition, a call made to negative control codeword is an error
- A call to a gene codeword might be correct or might be an error
- We observe:
  - D_g - number of puncta that decode to genes
  - D_c - number of puncta that decode to controls
- Some errors are not observed because the error goes to another gene codeword - Correcting for that gives us:
  - **$P_{err} = D_c / D_g * N_g / N_c$**
- So, we have an *in situ* measure of $P_{err,}$ based on negative control codewords counts

Xenium Q-Scores are Based on Empirical Calibration

Q-score calibration procedure:

1. Divide “raw” calls into bins according to confidence score
1. Within each bin, compute empirical P_{err}, based on formula
2. Convert P_{err} to Q-score and assign to all calls in the bin

This guarantees that Xenium Q-scores are always well calibrated



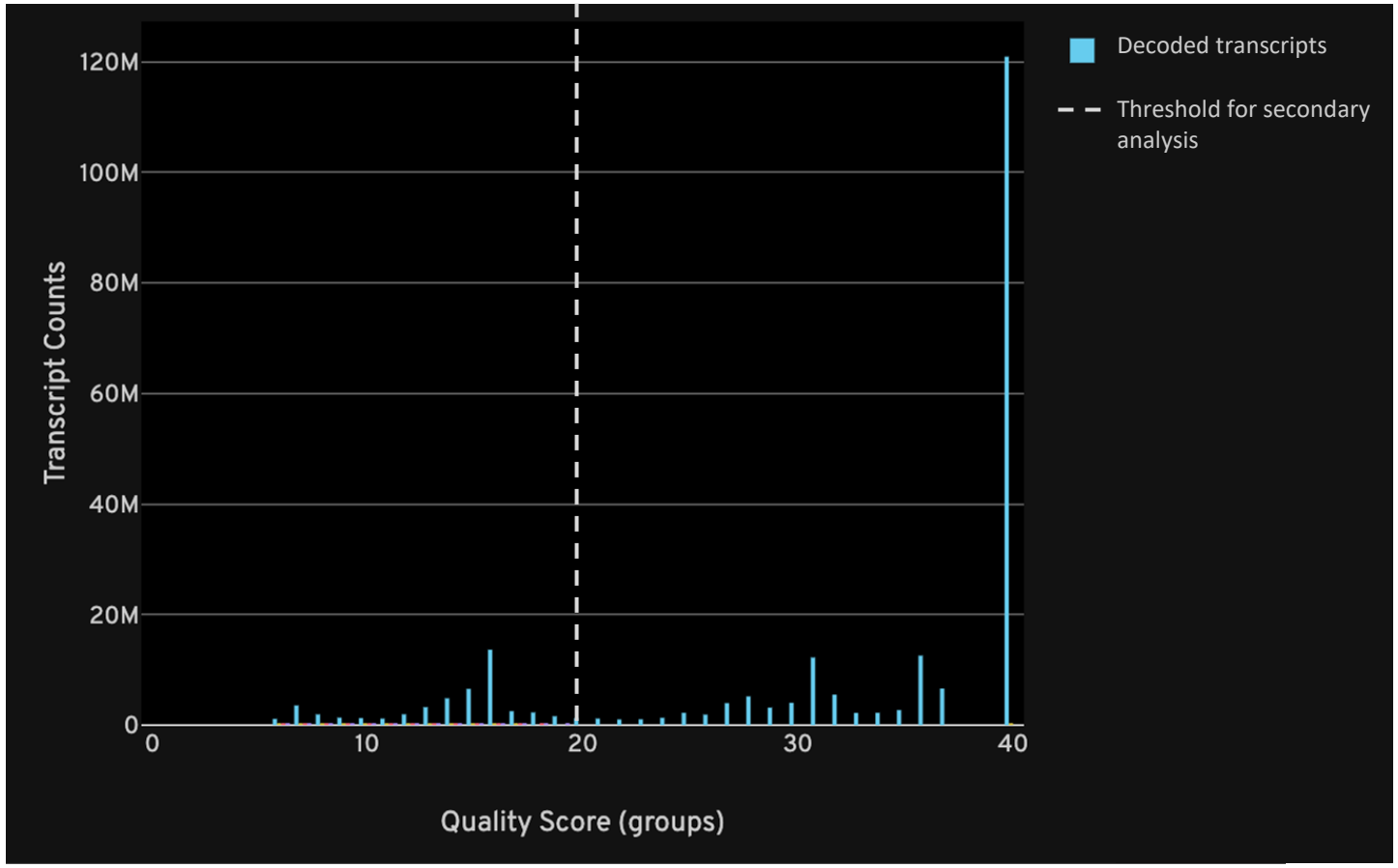
Typical Xenium Q-Score Distribution Shows Low Error Rate

Example from Xenium Mouse Pup FFPE Sample Data

- All counts regardless of Q-score are included in Xenium outputs
- The threshold for inclusion in secondary analysis is Q20
- This threshold is applied *per-transcript*

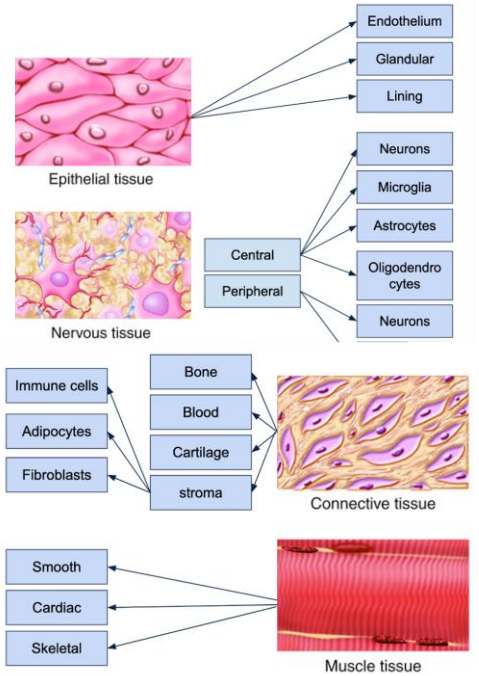
- Overall P_{err} of calls > Q20: 0.05%
  - Only a small fraction of calls are at Q20
  - Most calls are > Q20
  - Therefore, the *average* decoding error rate is < 1%

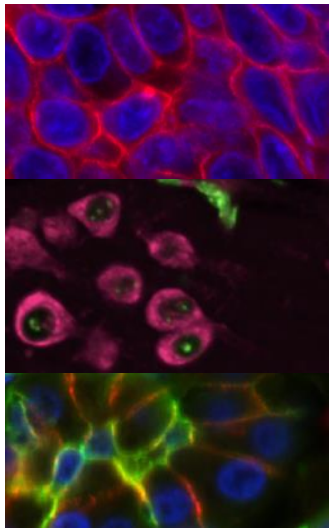
Q-score distribution (Mouse Pup FFPE dataset)

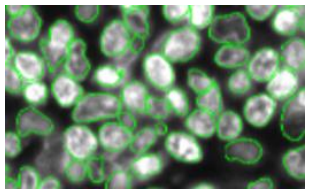


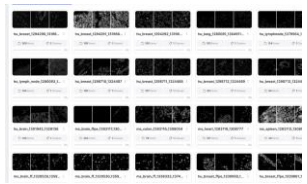
Cell Segmentation

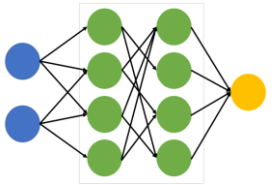
A Comprehensive Approach to Cell Segmentation

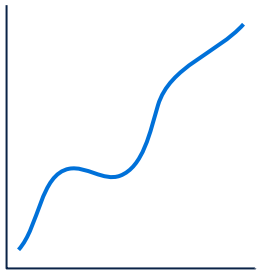


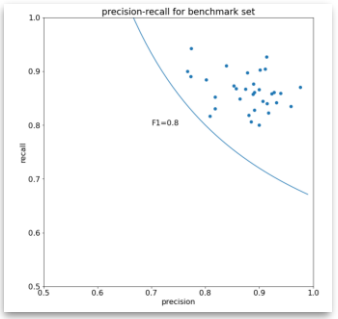








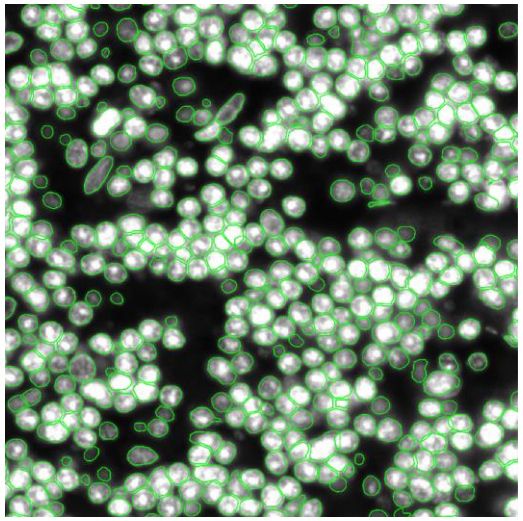




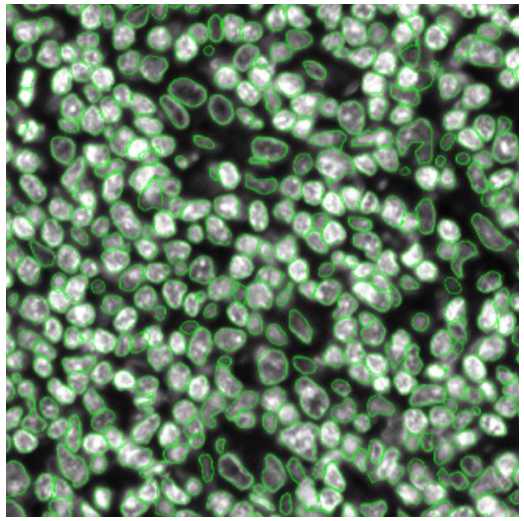
Setting the Foundation with Xenium Nucleus Segmentation

State-of-the-art nucleus segmentation

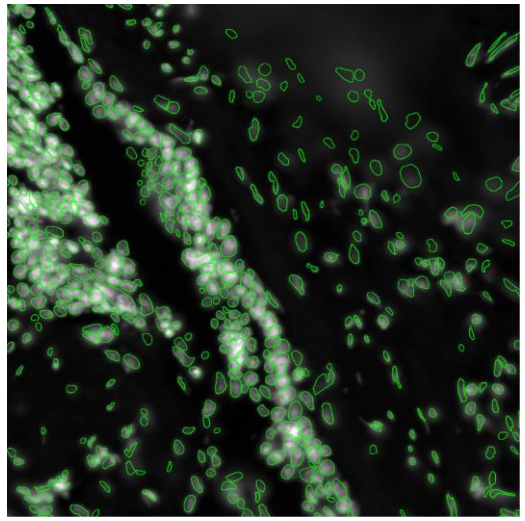
A critical foundation for membrane, cytoplasm, and transcript-based methods



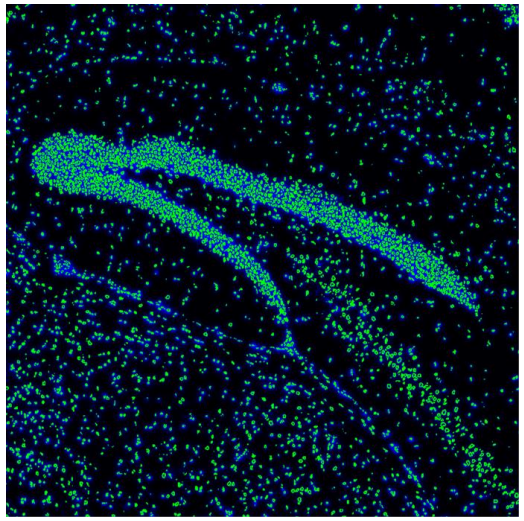
Human Cerebellum



Human Tonsil



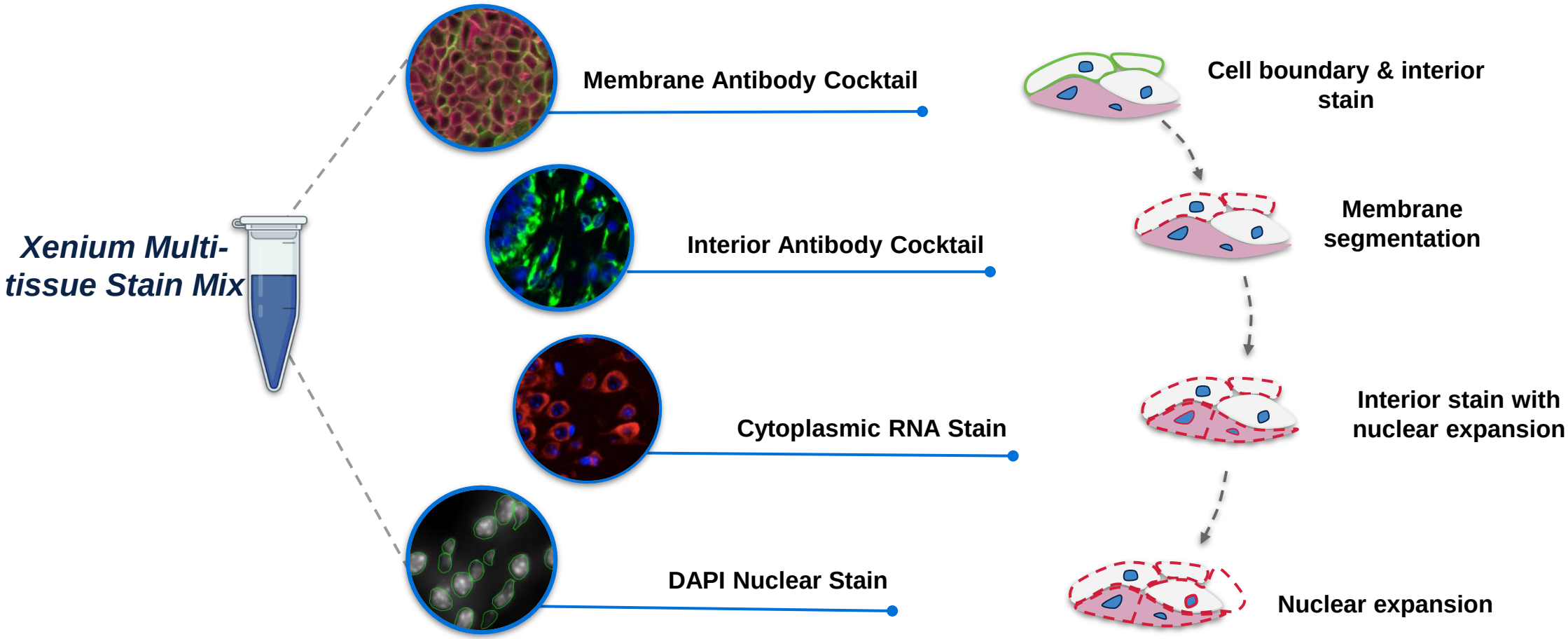
Human Lung



Mouse Brain

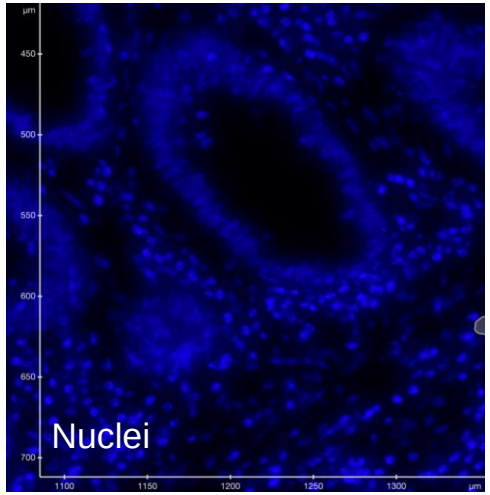
See more in the segmentation section of our Overview of Xenium Algorithms support page

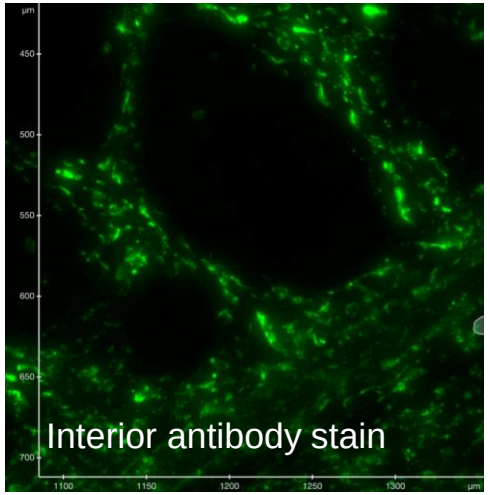
Shipping Q1 2024: Xenium Multi-Modal Segmentation

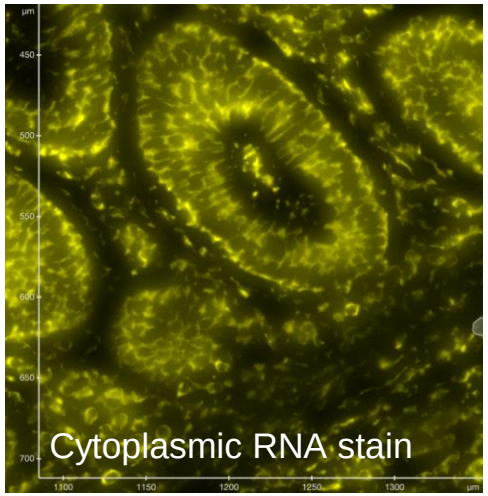


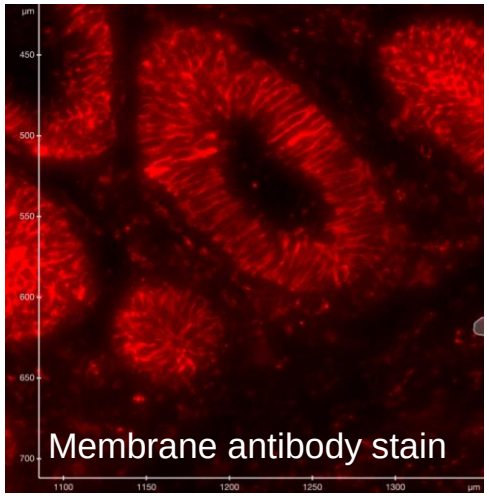
Distinct Advantages of 4 Channel Cell Morphology Images

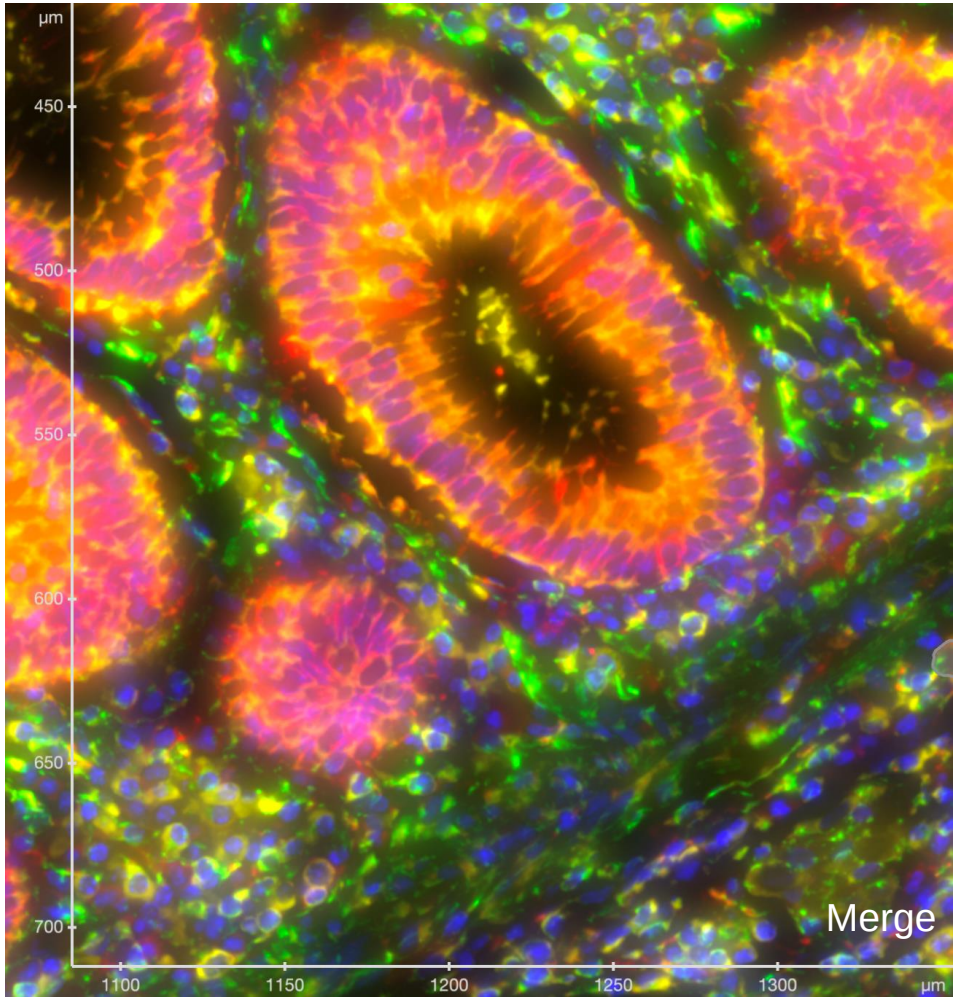
FFPE human colon



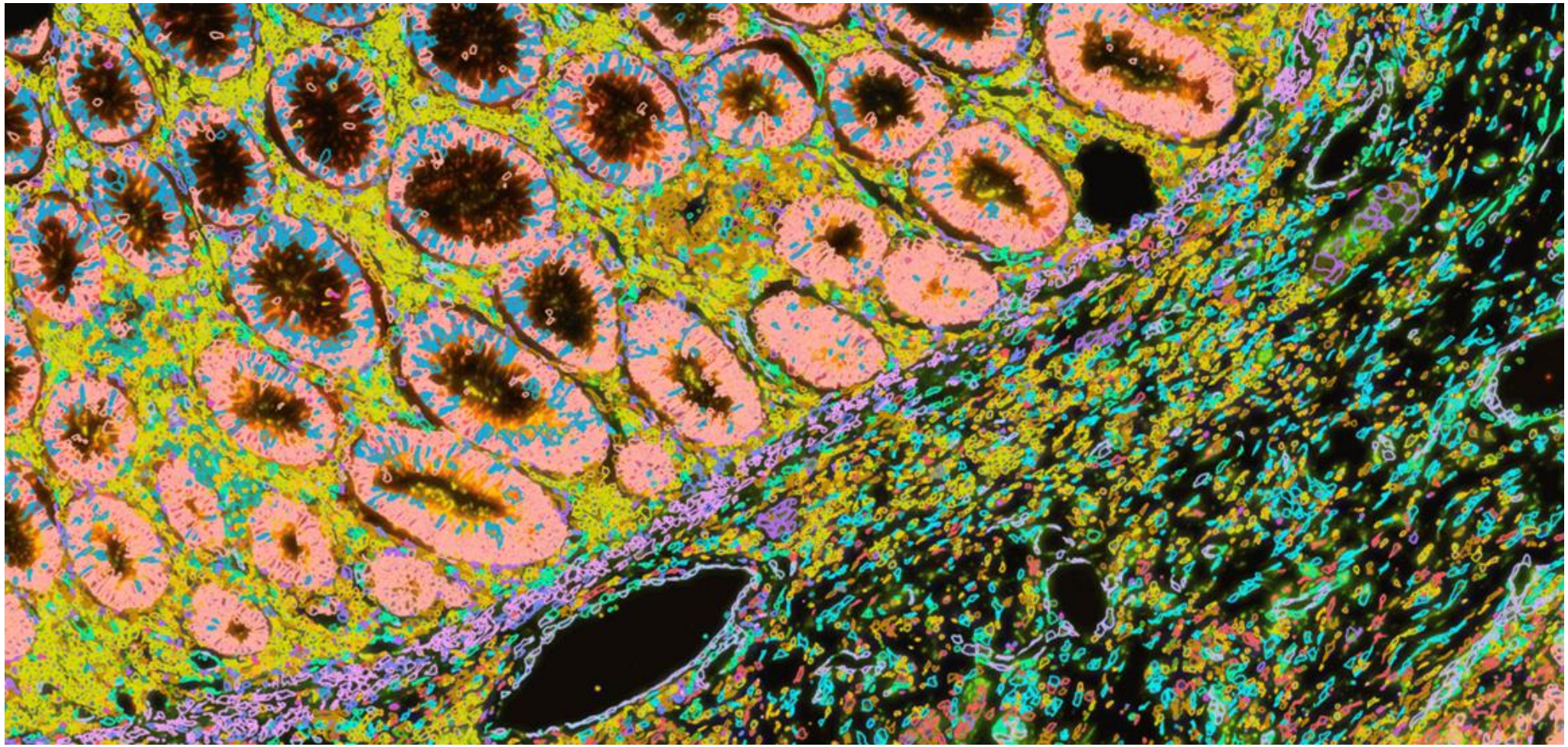








Multi-Modal Segmentation - Built for Broad Tissue Coverage



FFPE human colon with cells colored based on clustering from Human Multi-Tissue panel

Understanding Xenium Outputs

Region Selection Occurs After an Overview Scan

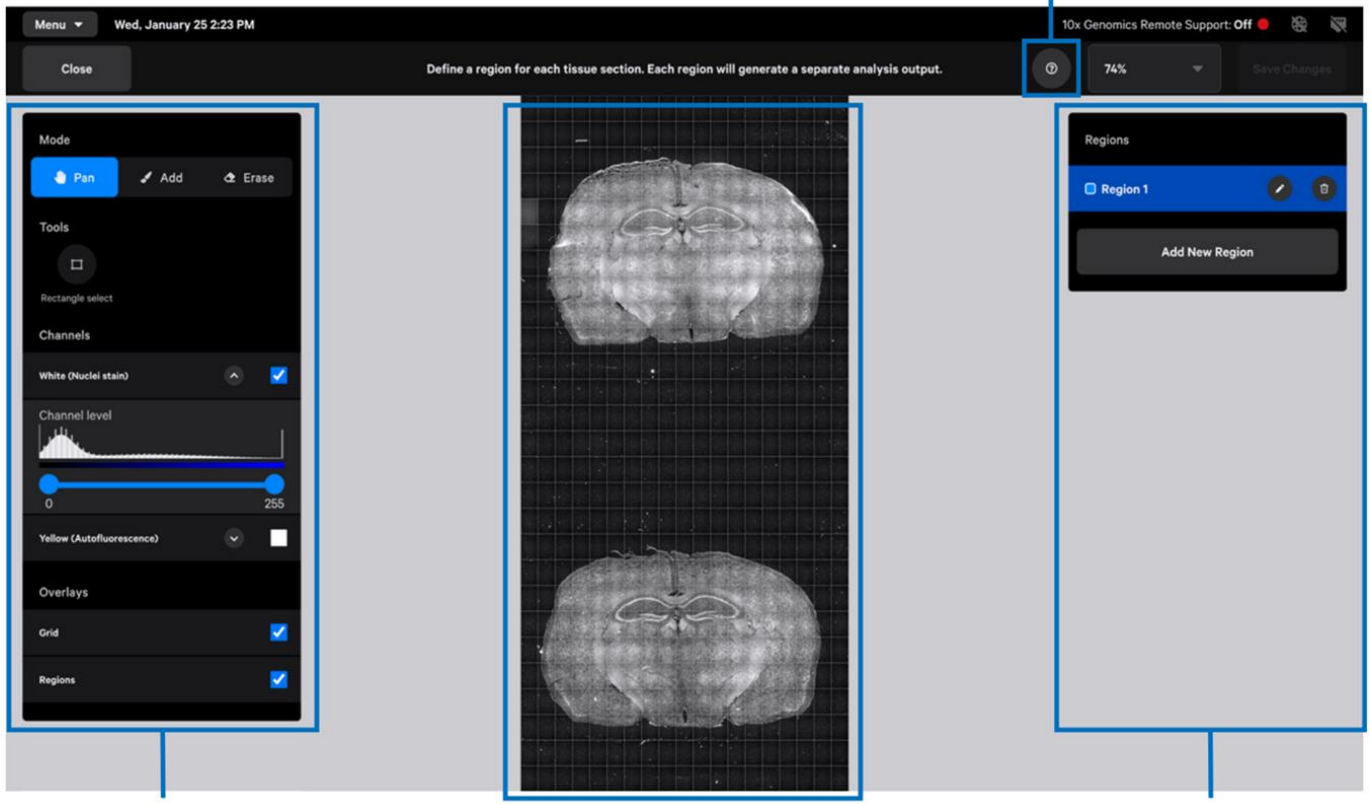
See more in our Xenium Analyzer User Guide

Overview Scan

Layout and features seen following completion

Region Selection Guidance

Instructions to properly select regions



Options Panel

Toggle views on/off and adjust channels

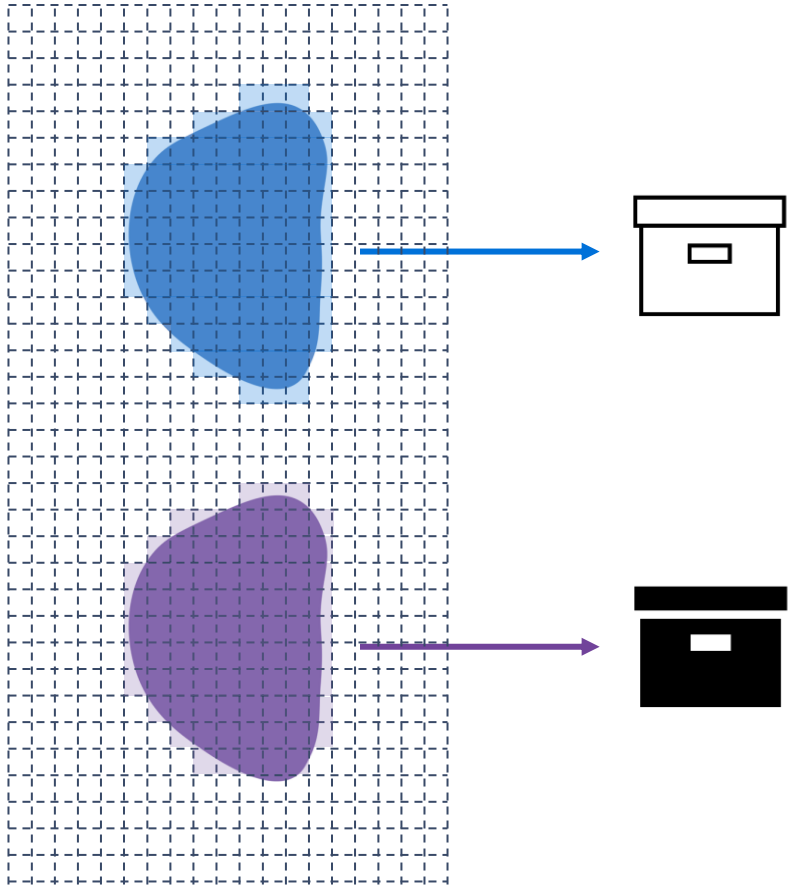
Sample Area

Shows image of scanned sample

Region Information

View, add, edit or delete regions

Each Selected Region Produces a Separate Output Directory



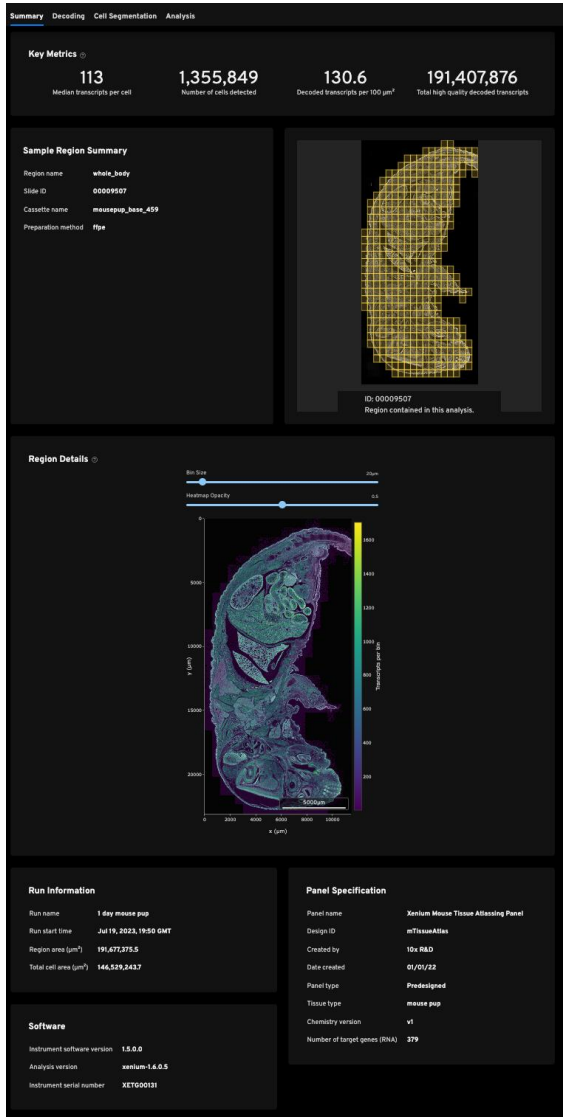
- Each Xenium run can analyze two slides; each slide has an area of 12 x 24 mm divided into approximately 19 x 33 FOV (Fields of View)
- Slides can have multiple regions, which produce separate output bundles
- Output size is a function of tissue area and sample-specific factors like tissue shape, number of cells, number of decoded transcripts, and percent of high-quality transcripts

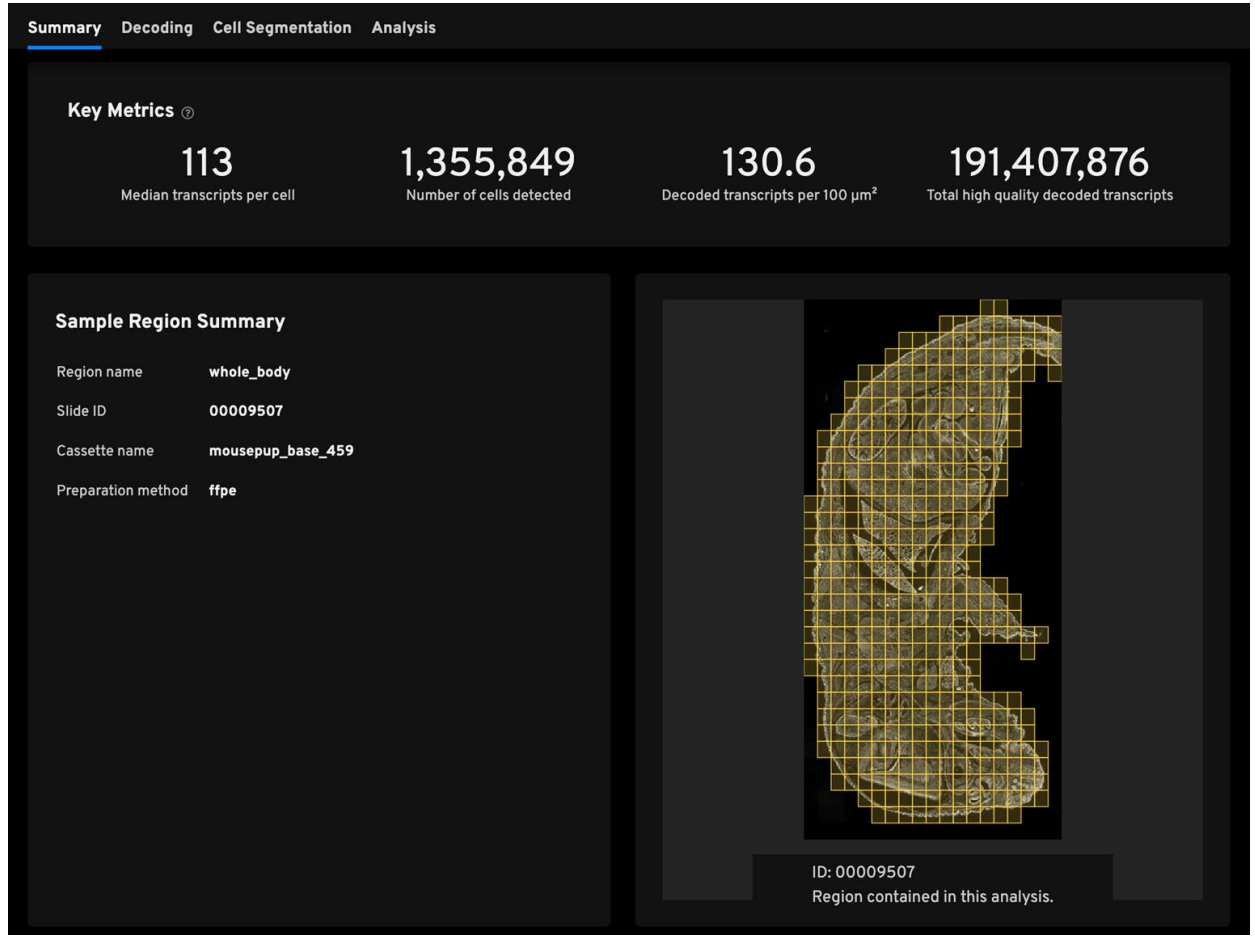
Tissue	Tissue area (cm^2)	Estimated output directory size (GB)
Core needle biopsy	0.01	0.2
Hemisphere of coronal mouse brain	0.5	10
Full coronal mouse brain	1	20
Tissue section covering entire sample area	2.35	60

The table shows *estimated* output directory sizes as a function of tissue area, assuming the sample has similar properties to a model mouse brain coronal section

Analysis Summary Is Available On-Instrument

Gain immediate confidence in your data with key metrics and plots

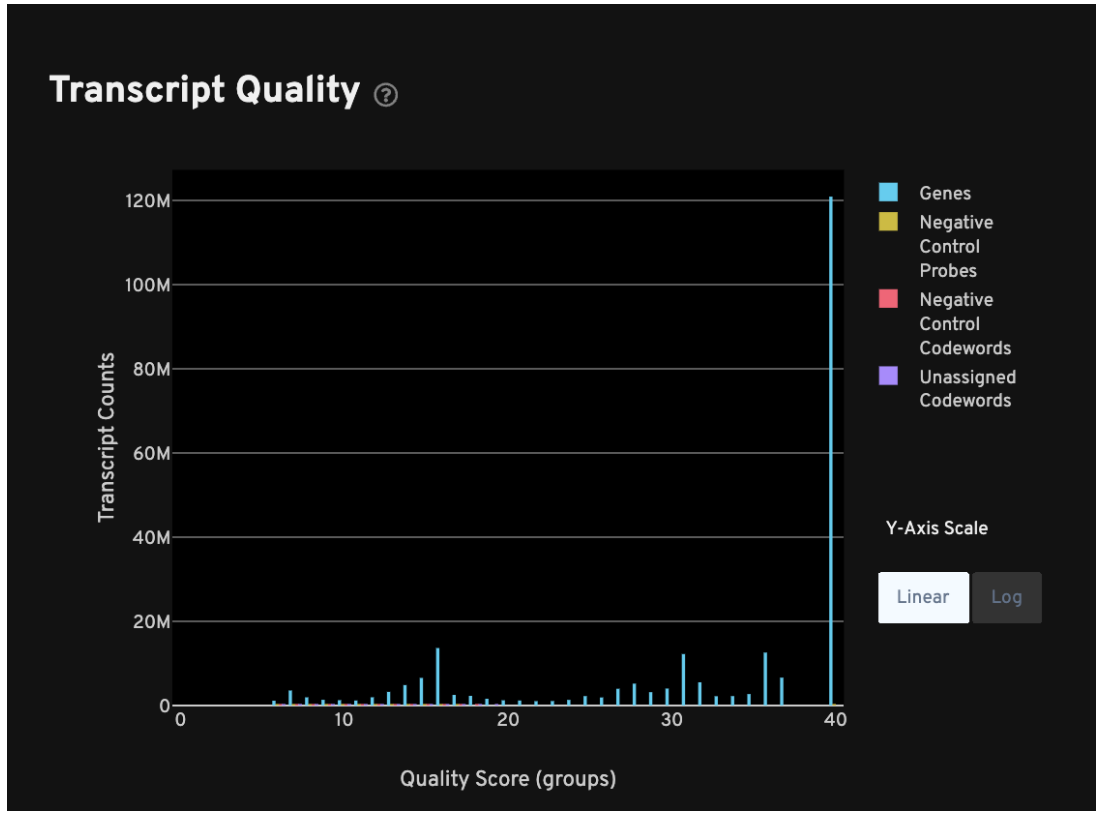




See more at our Overview of the Xenium Analysis Summary support page

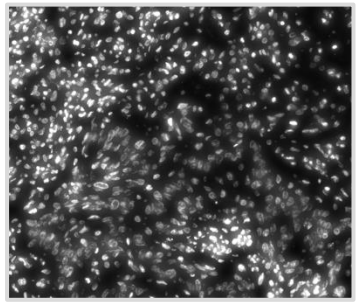
Analysis Summary Is Available On-Instrument

Gain immediate confidence in your data with key metrics and plots



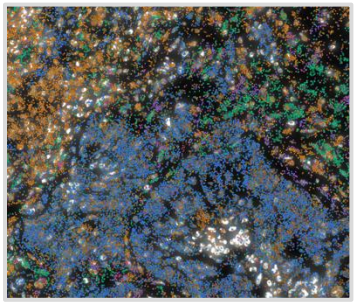
See more at our Overview of the Xenium Analysis Summary support page

Xenium Onboard Analysis Output Formats



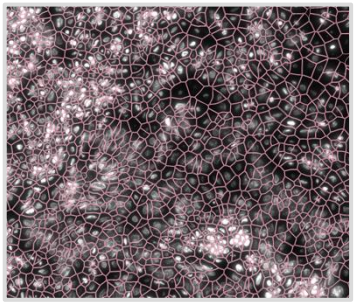
Morphology images

OME-TIFF



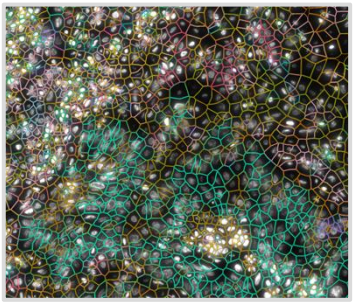
Transcripts w/ calibrated Q-scores

Zarr | Parquet | CSV



Cell segmentation

Zarr | Parquet | CSV



Cell-feature matrix & clustering

Zarr | MEX | HDF5

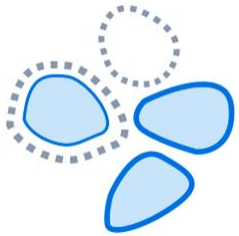
See more at our Understanding Xenium Outputs support page

Reanalysis with Xenium Ranger

Xenium Ranger Enables Reanalysis & Custom Segmentation

Three pipelines with more in development

Xenium Ranger is run on a range of Linux distributions to reanalyze Xenium data and produce an output bundle that can be viewed in Xenium Explorer

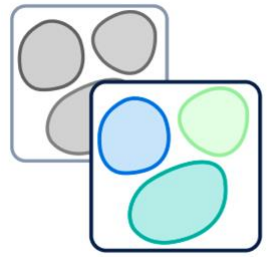


Resegment

Resegment Xenium data by adjusting cell expansion distance or nucleus intensity filter or by using our latest nucleus segmentation model.

Import Segmentation

Reassign transcripts in Xenium Ranger using segmentation results produced by 3rd party tools (Cellpose, Baysor, etc.) and visualize in Xenium Explorer.



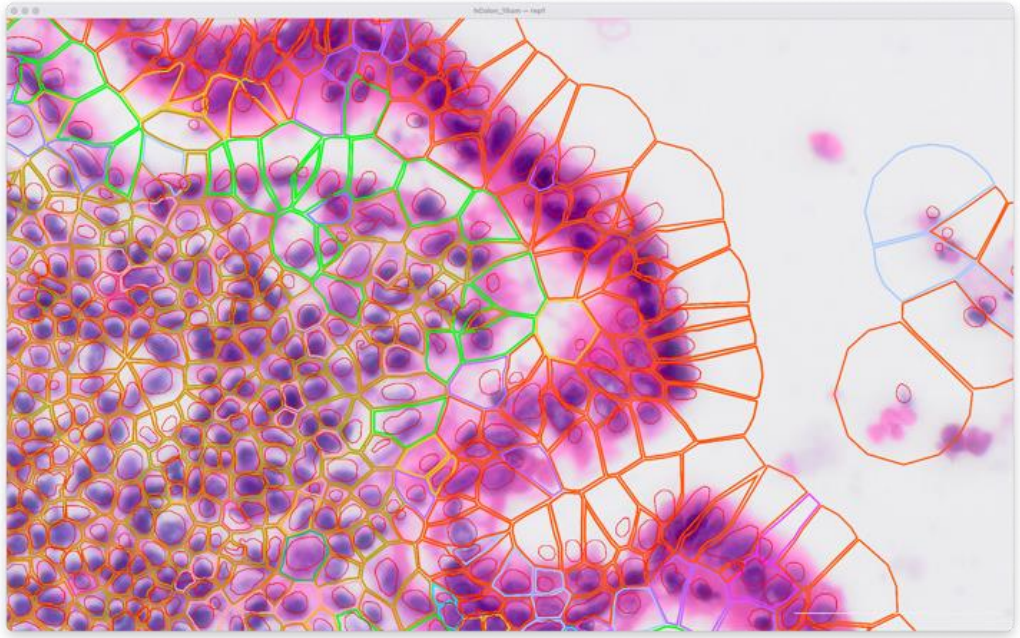
Relabel

Correct the gene panel applied to decoded transcripts so a run doesn't have to be restarted or aborted due to user error.

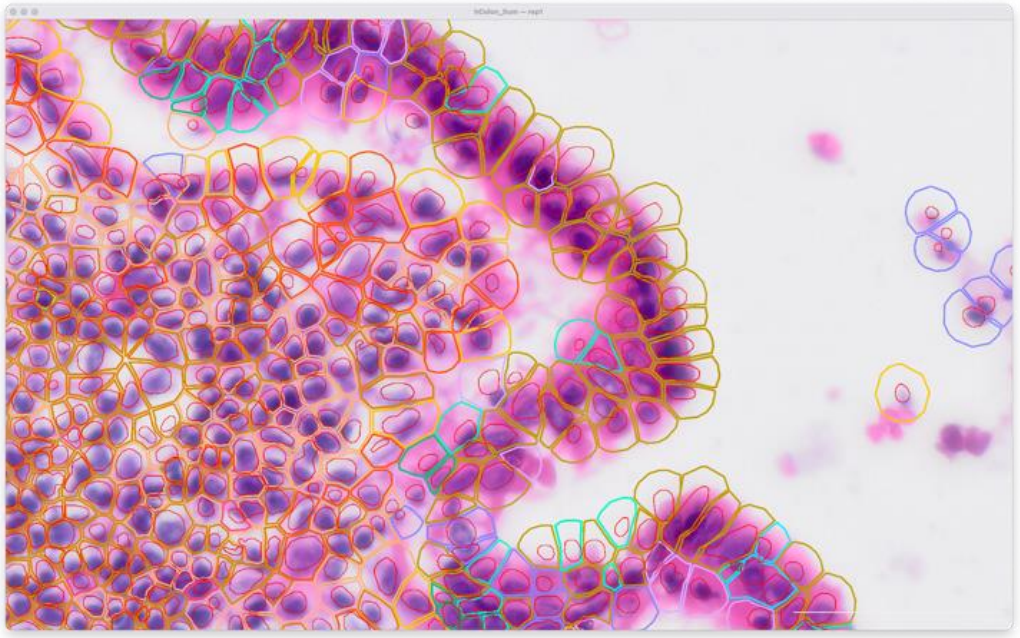
Resegment – Tune Cell Boundary Expansion

Match the size of cells in the tissue

Default 15µm expansion distance



Resegment with 5µm expansion distance



Cell boundaries on an H&E image of epithelial cells in human colon tissue show that reducing expansion distance to 5µm leads to more accurate cell boundaries for this sample

Import-Segmentation: Leverage Alternative Methods

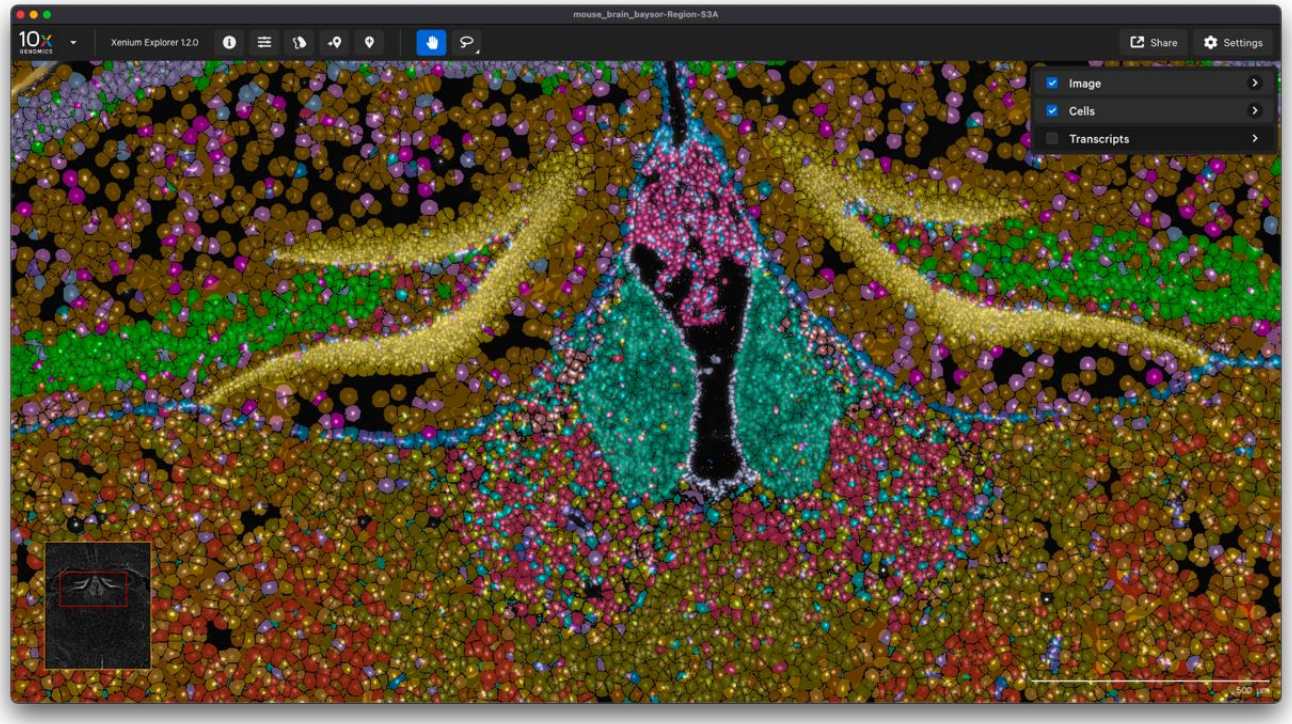
Image and transcript-based segmentation and QC in Xenium Explorer

Use the segmentation method which is best suited to your samples and experimental question and import results in Xenium Ranger

Supported segmentation formats:

- **Cellpose:** labeled mask in TIFF or NumPy NPY format
- **QuPath:** polygons in GeoJSON format
- **Baysor:** transcript-based segmentation outputs

Segmentation on post-Xenium IF images is possible by providing a transformation matrix which can be generated in Xenium Explorer

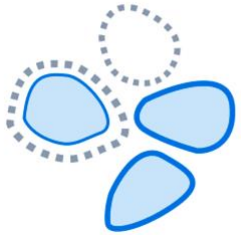


Mouse brain dataset processed with Baysor and import-segmentation, visualized in Xenium Explorer

Continuing Analysis with Community Developed Tools & Software

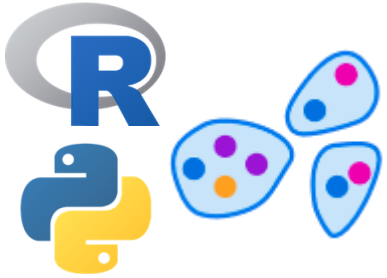
Community Developed Tools Enable Path to Conclusions

An example



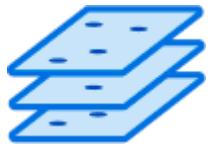
Cell Segmentation Refinement

- Refining cell segmentation based on transcriptional composition with Baysor
- Augmenting segmentation with post-Xenium IF imaging



Single Cell Style Analysis & Data Integration

- Clustering, cell typing, and differential expression
- QC, normalization
- Sample de-array, aggregation, batch correction



Spatial Context Analysis

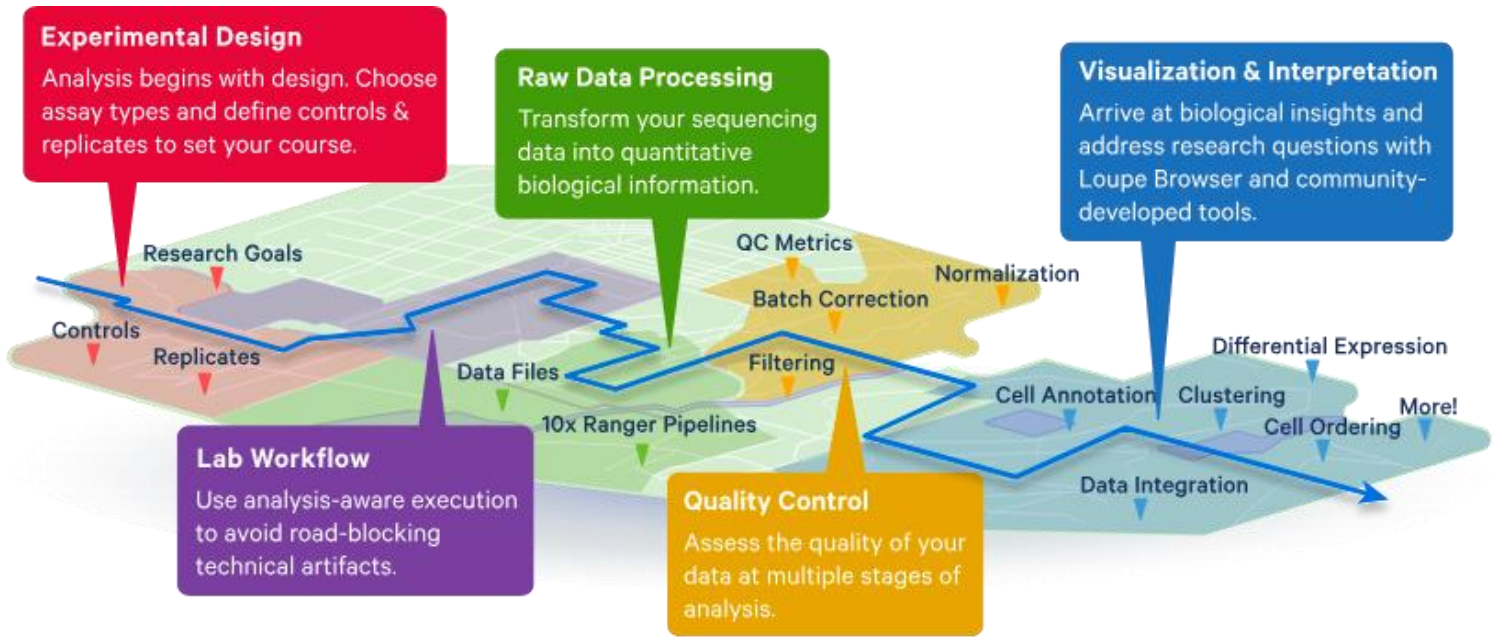
- Layering histopathology annotations
- Spatial trajectory analysis
- Neighborhood enrichment analysis

Xenium Explorer Visualization & Validation

10x Provides Analysis Guides

Facilitate your continued journey with Xenium Analysis

- Continuing Your Journey after Xenium Analyzer: overview of community-developed tools
- Using Baysor to Perform Xenium Cell Segmentation*
- H&E to Xenium DAPI Image Registration with Fiji (alternative to Xenium Explorer alignment)



** Note: Installing and running Baysor requires computational skill and compute infrastructure. The Baysor Analysis Guide was written using a previous version of Baysor - we recommend following Baysor instructions for installation.*

Conclusion

Exceptional Launch Year

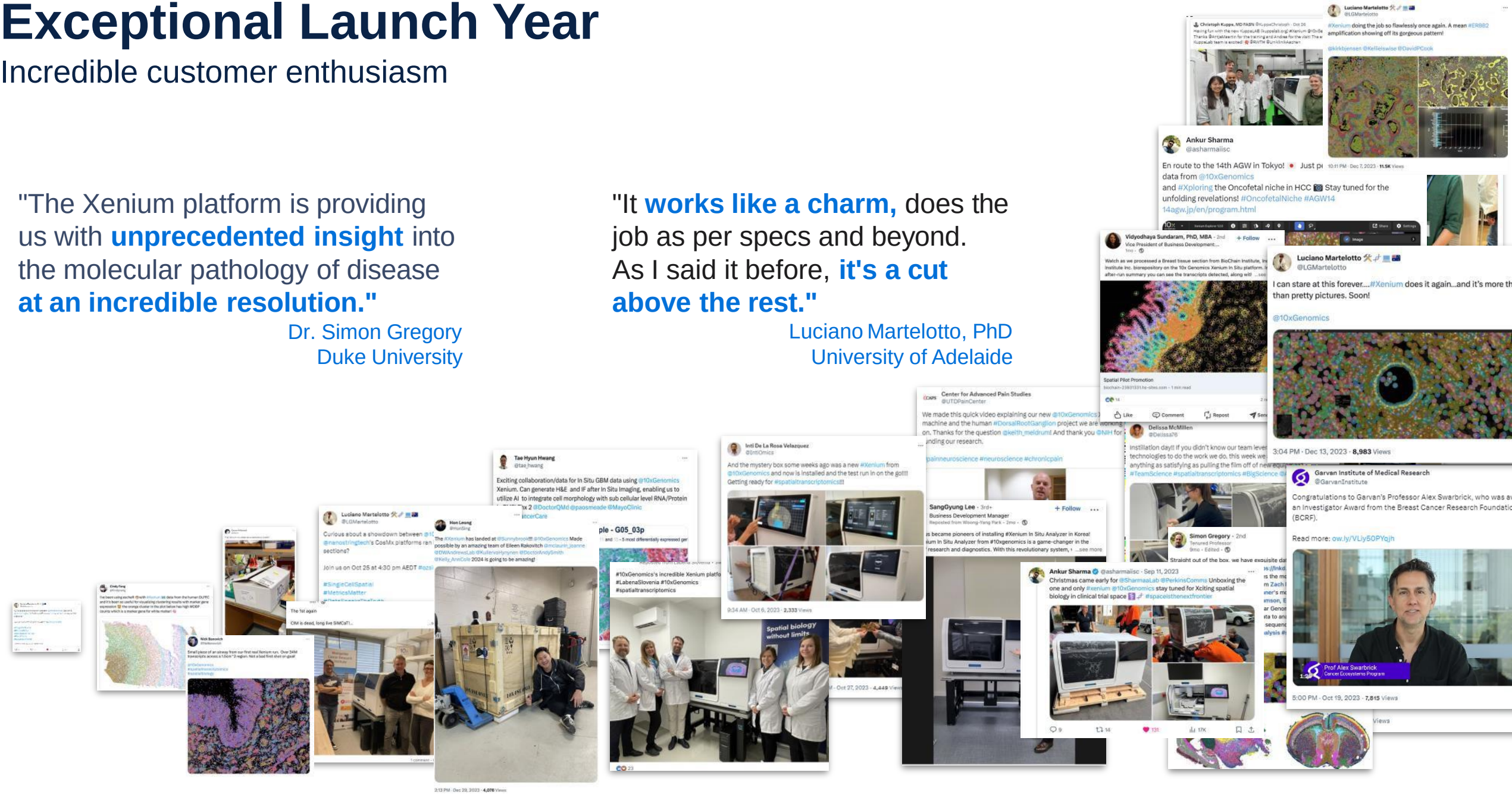
Incredible customer enthusiasm

"The Xenium platform is providing us with **unprecedented insight** into the molecular pathology of disease **at an incredible resolution."**

Dr. Simon Gregory
Duke University

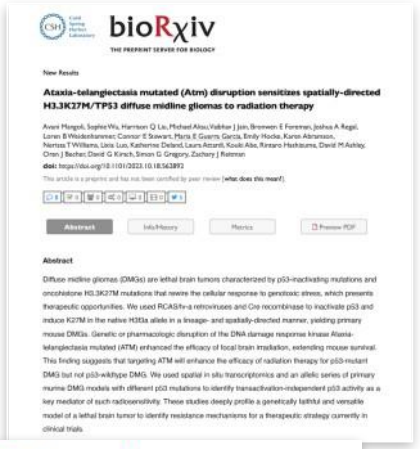
"It **works like a charm,** does the job as per specs and beyond. As I said it before, **it's a cut above the rest."**

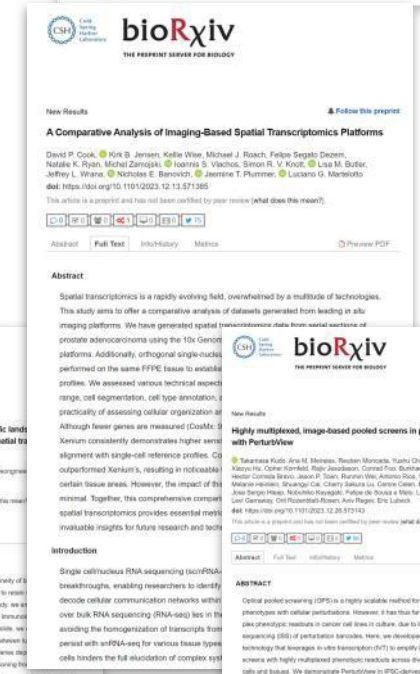
Luciano Martelotto, PhD
University of Adelaide

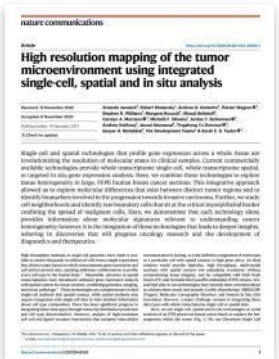


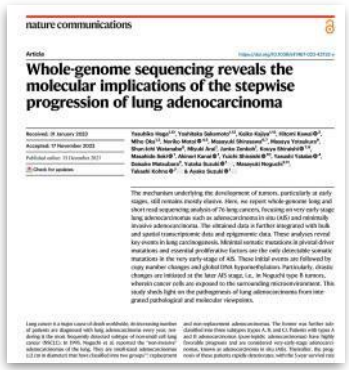
Exceptional Launch Year

Early customer success, from install to insight









Biology's Most Comprehensive Toolkit

Thank you!

Please attend our CytAssist GEX Workshop

Sprague Hall 105 - Jan 24th - 1:30 – 3 PM

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