Xenium In Situ Gene Expression - Workflow Planning Guide

Introduction

Xenium In Situ Gene Expression measures gene expression at subcellular resolution for hundreds to thousands of RNA targets in formalin fixed & paraffin embedded (FFPE) or fresh frozen (FF) tissue. Gene expression is measured via the use of pre-designed, add-on custom, standalone custom, or advanced custom probe panels that target genes of interest. Tissue sections placed onto Xenium Slides are analyzed on the Xenium Analyzer for high-throughput, automated in situ analysis. Visualization and interpretation of data outputs using Xenium Explorer allow researchers to identify spatial patterns of gene expression in their original tissues, providing novel insights into cellular structure and function. Reanalyze data in Xenium Ranger with the latest 10x cell segmentation algorithms or import community-developed segmentation results. All outputs are compatible with Xenium Explorer.

Careful planning of a Xenium In Situ experiment is critical for success. This document provides an overview of the key considerations for the various design and execution steps of the Xenium In Situ Gene Expression workflow.

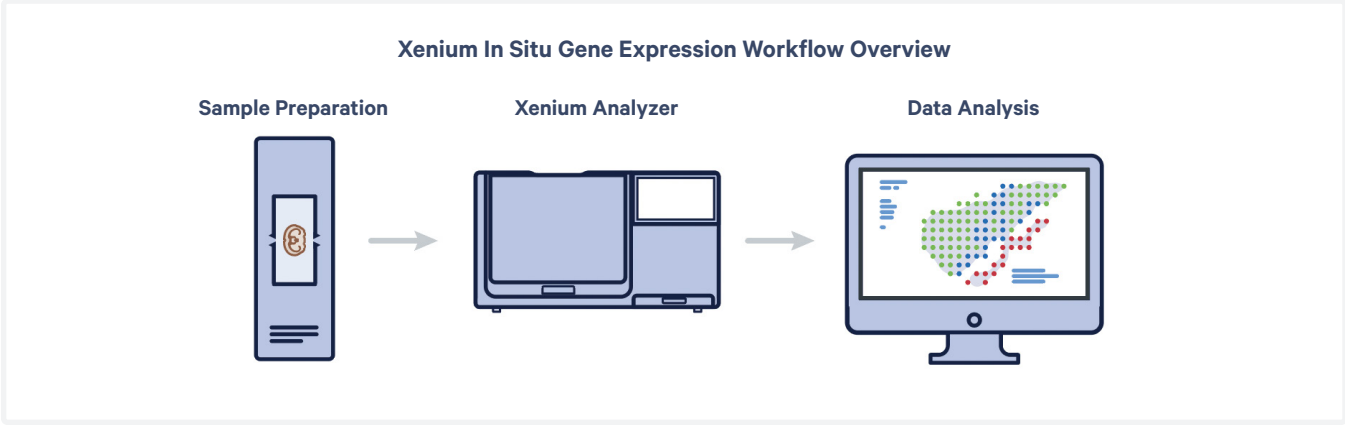


Figure 1. Overview of the Xenium In Situ Gene Expression workflow.

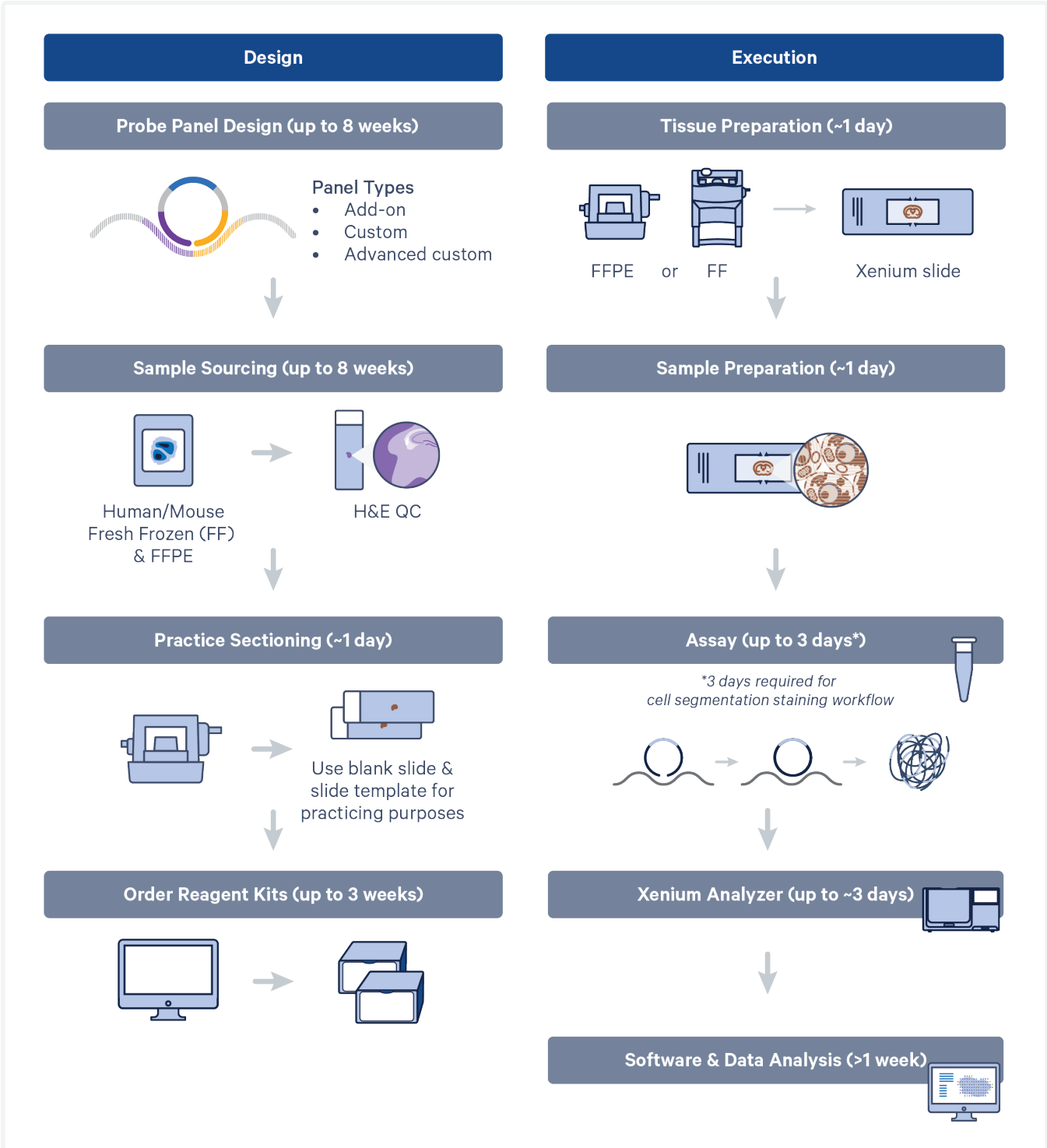


Figure 2. The Xenium In Situ Gene Expression workflow consists of several design and execution steps. The design phase of a Xenium In Situ Gene Expression experiment includes: designing probe panels, sourcing samples, practicing sectioning, and ordering Reagent Kits. The execution phase includes: tissue preparation, sample preparation, probe hybridization, running samples on the Xenium Analyzer instrument, and analyzing output data.

Design & Planning

Design Probe Panel Up to 8 weeks › **Source Sample** Up to 8 weeks › **Practice Sectioning** ~1 day › **Order Reagent Kits** Up to 3 weeks › **Proceed to Execution Phase**

Probe Panel Design

Prior to starting the Xenium In Situ Gene Expression workflow, ensure that a compatible gene panel has been selected.

Pre-designed Panels

Pre-designed panels are designed to cover all major cell types in a tissue for specific species and tissue types and have been extensively tested by 10x Genomics for sensitivity and specificity.

Add-on Custom Panels

Add-on custom panels are used to supplement pre-designed panels and can accommodate up to 100 additional genes. Add-on custom panels are useful for when identification of specific markers for subpopulations of cells or genes expressed under specific conditions (e.g. disease) is desired for your study. Additional guidance for selecting genes to include on Xenium add-on custom panels can be found in the Xenium Add-on Panel Design Technical Note (CG000643).

Standalone Custom Panels

Standalone custom probes are completely customizable and are used independently of pre-designed panels. These panels can be designed for human or mouse.

Advanced Custom Panels

Advanced custom panel designs are any use of the Xenium In Situ platform that goes beyond measuring gene expression for human or mouse. See the Species Standalone Custom and Advanced Custom Panel Design for Xenium In Situ Technical Note (CG000683) for more information about the basic structure of these probes and how advanced users can select their own RNA binding domain sequences for use on the Xenium In Situ platform.

Pre-designed probes are good for two Xenium reactions and custom probes (add-on, standalone, and advanced) are good for four or sixteen reactions. Contact your 10x Genomics Sales Executive for information about designing custom panels. See the Panel Design page on the 10x Genomics Support Website for a complete list of current panels available and information on how to start the design process.

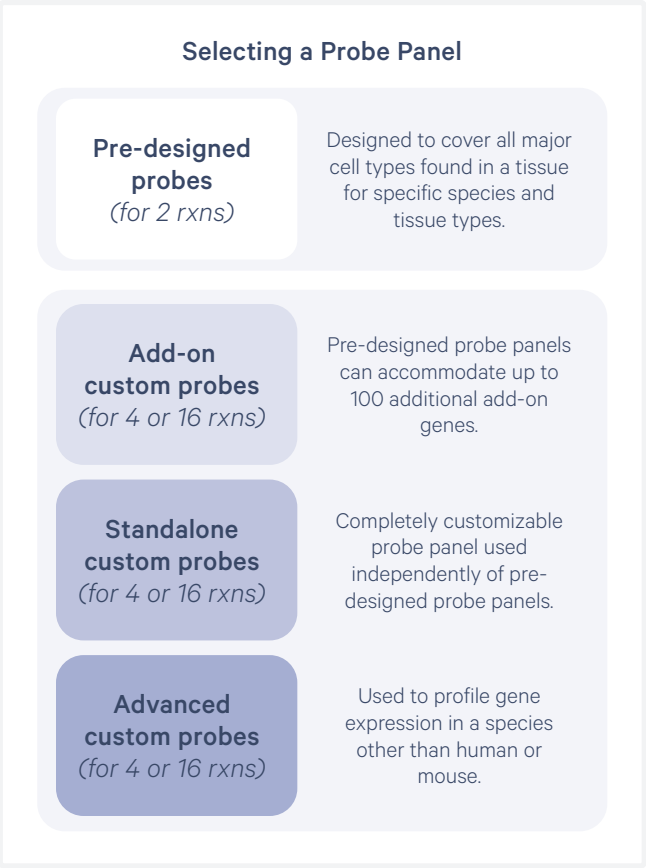


Figure 3. Diagram of criteria for selecting a probe panel.

Design Probe Panel (Up to 8 weeks) › **Source Sample** (Up to 8 weeks) › **Practice Sectioning** (~1 day) › **Order Reagent Kits** (Up to 3 weeks) › **Proceed to Execution Phase**

Sample Sourcing

Sample Types

Xenium probe panels are designed for use in human and mouse tissue. Other species are not supported. Both fresh frozen and FFPE tissue types are compatible with the Xenium In Situ assay.

Practice Sectioning

To practice section placement, draw a frame to represent the Xenium fiducial frame on a blank slide using the Xenium Slide layout. The blank slide should measure 75 x 25 x 1 mm. Draw frames on the back of the slide. Practice correct section placement within the representative frames using non-experimental blocks.

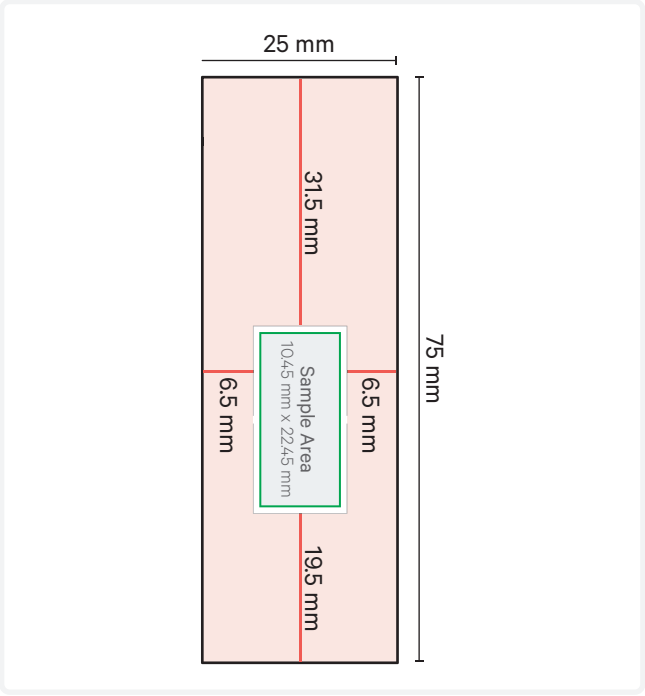


Figure 4. Template to verify that freshly placed tissue sections are compatible with the Xenium slide. Images are to scale if scaling settings are not modified (select "actual size" or "100%" to print to scale).

Order Xenium In Situ Reagents

Below is a list of reagents required for performing workflow (FF and FFPE) and instrument run.

Always refer to the Xenium In Situ Gene Expression User Guide (CG000582) or Xenium In Situ Gene Expression with Cell Segmentation Staining (CG000749) and the Xenium Analyzer Instrument Guide (CG000584) for complete list.

Xenium In Situ Reagent Kits
Xenium Slides & Sample Prep Reagents (2 slides, 2 rxns) PN-1000460
Xenium Slide Kit (4 slides) PN-1000659
Xenium Slide Kit (16 slides) PN-1000660
Xenium Decoding Consumables (1 run, 2 slides) PN-1000487
Xenium Instrument Accessory Kit Module A PN-1000530
Xenium Cassette Kit - (2 cassettes) PN-1000566
Xenium Decoding Consumables (1 run, 2 slides) PN-1000487
Xenium Instrument Accessory Kit Module A PN-1000530
Xenium Cassette Kit - (2 cassettes) PN-1000566
Xenium Cell Segmentation Staining Reagents - (2 rxns) PN-1000661 *(optional, for multimodal cell segmentation)*
Xenium Decoding Reagents (1 run, 2 slides) PN-1000461
Xenium Cell Segmentation Detection Reagents (1 run, 2 slides) PN-1000639) *(optional, for multimodal cell segmentation)*

Order Additional Kits, Reagents & Equipment

Some reagents and consumables not provided by 10x Genomics are necessary for the Xenium In Situ Gene Expression workflow. See the Xenium In Situ Gene Expression Protocol Planner (CG000601) for a complete list.

Execution

Completed Design Phase → **Tissue Preparation** ~1 day → Sample Preparation ~1 day → Probe Hybridization ~2 days → Cell Segmentation ~1 day → Xenium Analyzer ~3 days → Data Analysis >1 week

Tissue Preparation: Fresh Frozen

Proper handling, storage, and preparation techniques are essential to preserve the morphological quality of the tissue sections and integrity of the mRNA transcripts. Freshly obtained tissues are snap frozen to prevent RNA degradation and avoid crystal formation. Once frozen, tissue samples are embedded in Optimal Cutting Temperature (OCT) to preserve tissue structure during cryosectioning. Failure to embed tissue in OCT may compromise Xenium assay performance. Tissue quality is assessed using any preferred H&E Staining protocol. After examining the H&E stained tissue section, tissue blocks are cryosectioned and sections placed onto Xenium slides.

Tissue Quality

Starting material may impact assay performance. Perform H&E staining to assess tissue quality. Inspect the tissue for tissue processing and sectioning artifacts that may contribute to poor assay performance. Proceed with cryosectioning and section placement only if quality is satisfactory.

Tissue Storage

It is recommended that FF tissue blocks be stored at -80°C. If sectioning the same tissue block across multiple days, keep the tissue frozen by placing in a sealed container on dry ice when transporting tissue from -80°C freezer to a pre-cooled cryostat. The tissue block should be capped off with additional OCT before restorage in the -80°C.

Placement of Tissues onto Xenium Slides

Proper tissue placement onto Xenium slides is critical. Practice tissue placement within sample area using nonexperimental, blank slides. When ready, discard the first few of sections after facing the block. Then place sections within the Sample Area on Xenium slides. Avoid covering the fiducials with tissue. Ensure tissue sections are uniform and without cracks, tears, or folds. Do not reposition tissues once sections are placed. This compromises slide integrity and assay performance.

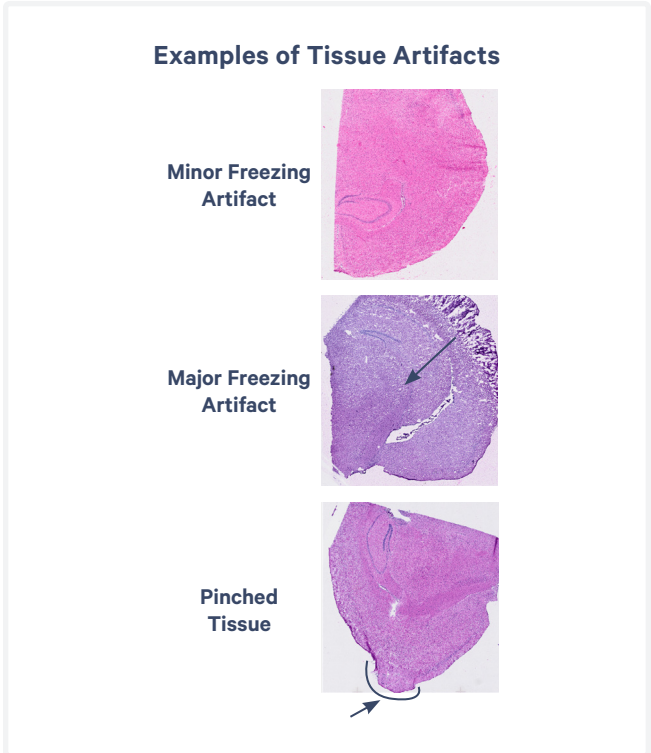


Figure 5. Tissue processing artifacts may include freezing artifacts, squeeze/crush artifacts, ice crystal artifacts, and hemorrhaging.

Completed Design Phase › **Tissue Preparation** ~1 day › Sample Preparation ~1 day › Probe Hybridization ~2 days › Cell Segmentation ~1 day › Xenium Analyzer ~3 days › Data Analysis >1 week

Tissue Preparation: FFPE

Follow proper handling, storage, and preparation techniques to preserve the integrity of the tissue sections. FFPE blocks are placed in a microtome and cut to face the tissue. Block is rehydrated in an ice bath. Sectioned tissue is placed onto Xenium Slides. Assess tissue quality after deparaffinization using preferred H&E Staining protocol.

Fixation

Fix tissue (<5 mm) at 4°C for ~16-24 h to ensure proper fixative penetration. Penetration rate is approximately 1 mm of tissue per hour. Ensure tissue is submerged and receives light agitation. 10% Neutral Buffered Formalin (NBF) is recommended as it balances tissue preservation and molecular integrity. Recommended ratio of fixative to tissue is 20:1. Over or underfixation or not having a large enough fixative volume may impact performance.

Tissue Quality

Starting material may impact assay performance. Perform H&E staining to assess tissue quality. Inspect the tissue for tissue processing and sectioning artifacts that may contribute to poor assay performance. Proceed with section placement only if quality is satisfactory. DV200 assessement is optional; however, running samples with a DV200 score below 30% may result in a decrease in assay performance as measured by lower median transcripts per cell.

Water Bath Temperature & Section Floating Time

Optimal water bath temperature and section floating time are critical for tissue section expansion. 42°C water bath is recommended for most tissues however temperature optimization may be required based on tissue type.

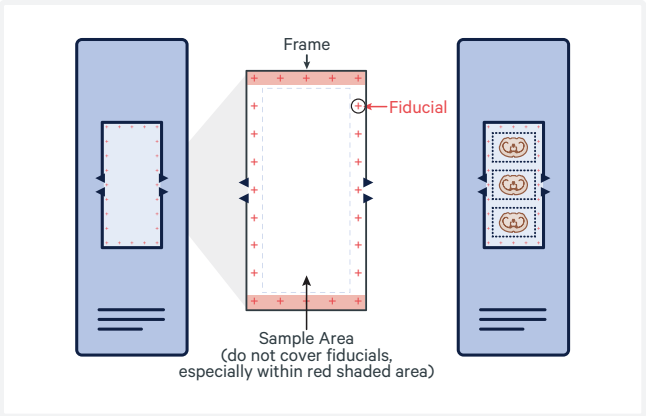


Figure 6. Place tissues within Sample Area of Xenium slides without covering fiducials.

Placement of Tissues onto Xenium Slides

Proper tissue placement onto Xenium slides is critical. Practice tissue placement within sample area using nonexperimental, blank slides. When ready, discard the first few of sections after facing the block. Then place sections within the Sample Area on Xenium slides. Avoid covering the fiducials with tissue. Ensure tissue sections are uniform and without cracks, tears, or folds. Do not reposition tissues once sections are placed. This compromises slide integrity and assay performance.

Drying Time

After sectioning, slides must be dried, first at room temperature to remove excess liquid, and then transferred to either a section dryer oven (preferred) or a thermal cycler. Room temperature drying time is variable depending on liquid and fan use. Consult the Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578) for more information.

Storage

Store FFPE tissue blocks at 4°C and protected from light. Storage of FFPE specimens may influence RNA fragmentation. Storage at higher temperatures, for instance, can lead to increased fragmentation.

Sample Preparation: Fixed Frozen

During Sample Preparation, FFPE tissues on Xenium slides are deparaffinized and decrosslinked, while FF tissues on Xenium slides are fixed and permeabilized, in preparation for the assay workflow.

Prepare all buffers fresh before retrieving tissue sections from -80°C. Use only recommended fixatives for fixation steps (formaldehyde or paraformaldehyde). Alternative fixatives are not supported. Formaldehyde and paraformaldehyde should be handled in a biosafety hood due to their hazardous nature.

Consult Xenium In Situ for Fresh Frozen Tissues - Fixation & Permeabiliztion Demonstrated Protocol (CG000581) for more details.

Sample Preparation: FFPE

Prepare all buffers fresh. Deparaffinization steps should be performed in a fume hood due to the hazardous nature of xylene. Xylene jars should be covered at all times to prevent evaporation. Optional photographs may be taken of the slide during deparaffinization and decrosslinking steps to help identify tissue detachment during the assay workflow.

Consult Xenium In Situ for FFPE - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580) for more details.

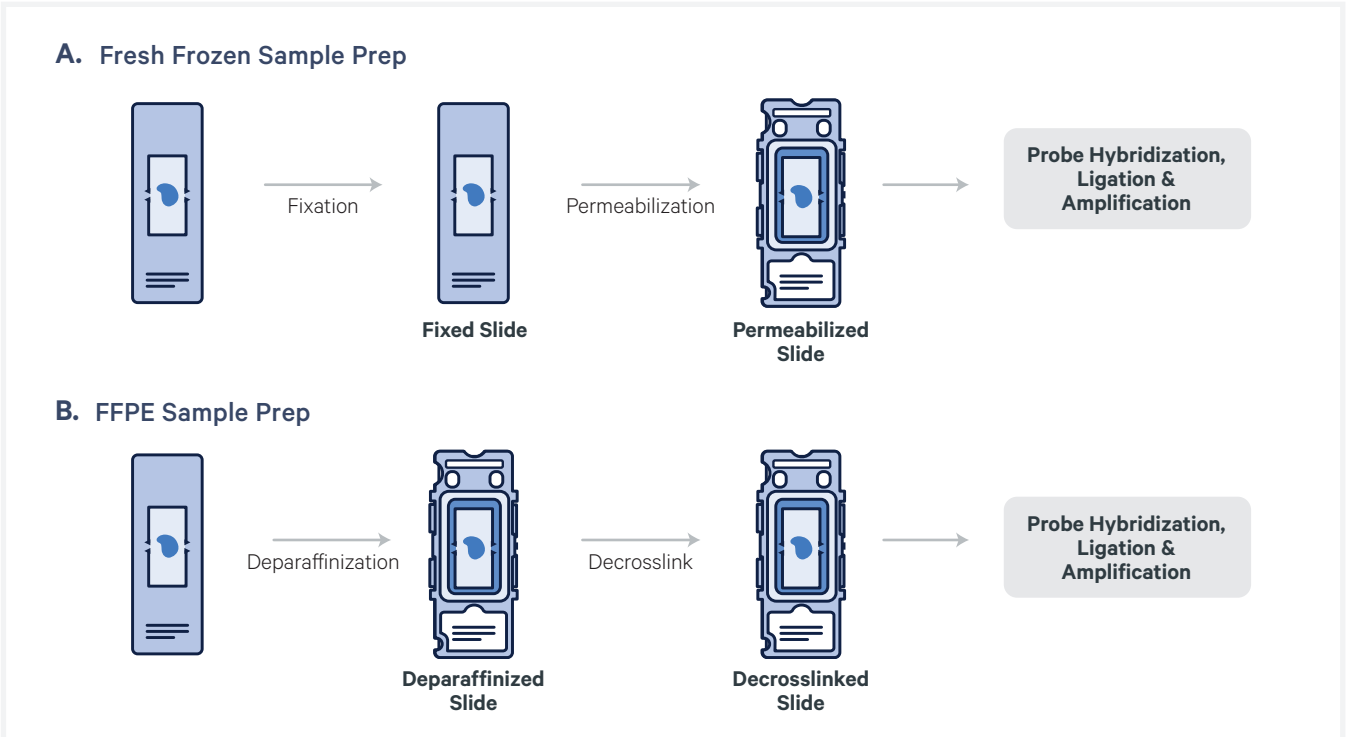


Figure 7. Fixation and permeabilization workflow for fresh frozen tissues (A). Deparaffinization and decrosslinking workflow for FFPE tissues (B).

Probe Hybridization, Ligation & Amplification Workflow

The Xenium In Situ Gene Expression assay workflow begins with probe hybridization, where pre-designed, add-on custom, standalone custom, or advanced custom probes are added to FFPE or FF tissue sections and hybridize to their complementary target RNA in an overnight incubation. Excess, unbound probes are then washed away in the post-hybridization step. After removal of unbound probes, a ligase is added to seal the junction between the probe regions that have hybridized to RNA. Ligation of the probe ends on the targeted RNA region generates a circular DNA probe. The ligation products are enzymatically amplified during the amplification process. Finally, autofluorescence quenching diminishes unwanted autofluorescence, and nuclei are stained with DAPI for visualization of regions of interest during a Xenium Analyzer instrument run.

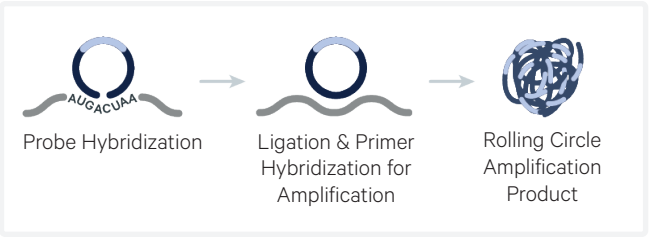


Figure 8. Overview of probe hybridization, ligation & amplification workflow.

Probe Handling & Storage

Add-on custom, standalone custom, and advanced custom probes are delivered lyophilized at room temperature and should be stored at -20°C upon receipt. Custom probes must be resuspended prior to use. Always record the Custom Panel Design ID and Slide Number before starting the Xenium In Situ workflow. This information is critical for identifying the correct electronic decode file when setting up the Xenium Analyzer in downstream steps. Consult Xenium In Situ Gene Expression User Guide (CG000582) for more information.

Xenium Cassette Handling and Storage

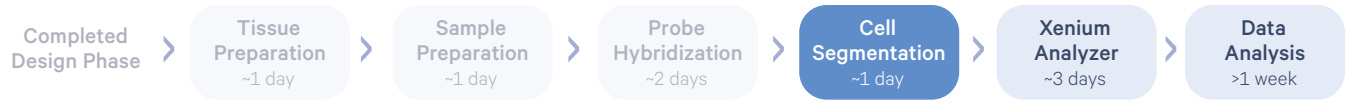
Xenium Cassette Lids are a single-use item and should be discarded after each use. In a few specified instances, Xenium Cassette Lids may be re-used for next steps. Only reuse lids as outlined specifically in the User Guide. Note that PBS-T washes do not require sealing of the cassette with a lid. If a lid is accidentally dropped, it may be washed according to outlined directions in the Xenium In Situ Gene Expression User Guide (CG000582).

Buffer Preparation

Prepare 1X PBS and PBS-T buffers fresh before starting the Xenium In Situ Gene Expression workflow. The volumes of each buffer are sufficient for washes in all subsequent steps of the Xenium In Situ Assay workflow.

Optional Photographs

Optional photos may be taken of the Xenium slide during Autofluorescence Quenching. These images may be compared to images taken earlier in the assay workflow to identify tissue detachment. Consult the Xenium In Situ Gene Expression User Guide (CG000582) for more details.



Multimodal Cell Segmentation

The Xenium In Situ Cell Segmentation Solution utilizes a multimodal approach for cell segmentation. Staining is performed with a single cocktail of Xenium Multi-Tissue Stain Mix (PN-2000991), which provides four different types of labeling that serve as inputs for automated cell segmentation: antibodies labeling membranes, antibodies labeling cell interiors, a universal interior label against Ribosomal RNA, as well as the nuclear label DAPI. Samples stained for cell segmentation are run on the Xenium Analyzer instrument and a custom-deep learning cell segmentation algorithm analyzes the data for cell segmentation.

Xenium Cassette Insert

The Xenium Cassette Insert sits in the Sample Area of the assembled Xenium Cassette and creates a flow cell in the well for adding reagents. Pick up the insert using forceps on the Xenium Cassette Handle. Forceps are needed for all handling steps.

Reagent Addition

Load reagents on the sample by pipetting into the Xenium Cassette Insert Cut-Out. Avoid dispensing reagent too quickly as this may lead to bubble formation.

Slide Seal

Seal the well with provided Slide Seal by firmly pressing down on the seal around the Sample Area to ensure uniform adhesion. Avoid pressing down on the Slide Seal in the middle of the Sample Area (over the handle) as this may damage tissue sections and cause the insert to adhere to the seal. Only Slide Seals, not the Xenium Cassette Lid, should be used with the Xenium Cassette Insert.

Algorithm

Xenium Explorer v2.0 and Xenium Ranger v2.0 are necessary for multimodal cell segmentation.

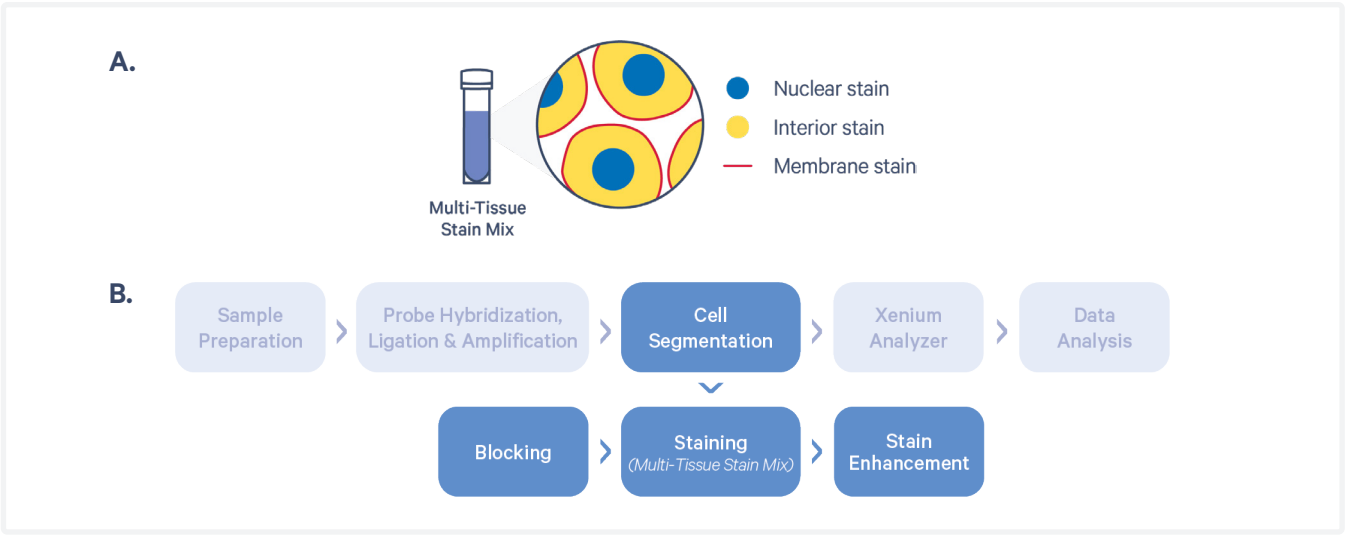


Figure 9. The Xenium In Situ Cell Segmentation Solution utilizes a Multi-Tissue Stain Mix for broad coverage cell segmentation (A). Cell Segmentation within the Xenium workflow (B).

Completed Design Phase › Tissue Preparation ~1 day › Sample Preparation ~1 day › Probe Hybridization ~2 days › Cell Segmentation ~1 day › **Xenium Analyzer ~3 days** › Data Analysis >1 week

Xenium Analyzer Workflow

The Xenium Analyzer performs successive rounds of fluorescent probe hybridization, imaging, and removal to create a unique optical signature revealing the identity of the RNA within each cell of a tissue. Data can be viewed and further analyzed using Xenium Explorer. General guidelines are described below. Always refer to the Xenium Analyzer Instrument User Guide (CG000584) for complete instructions

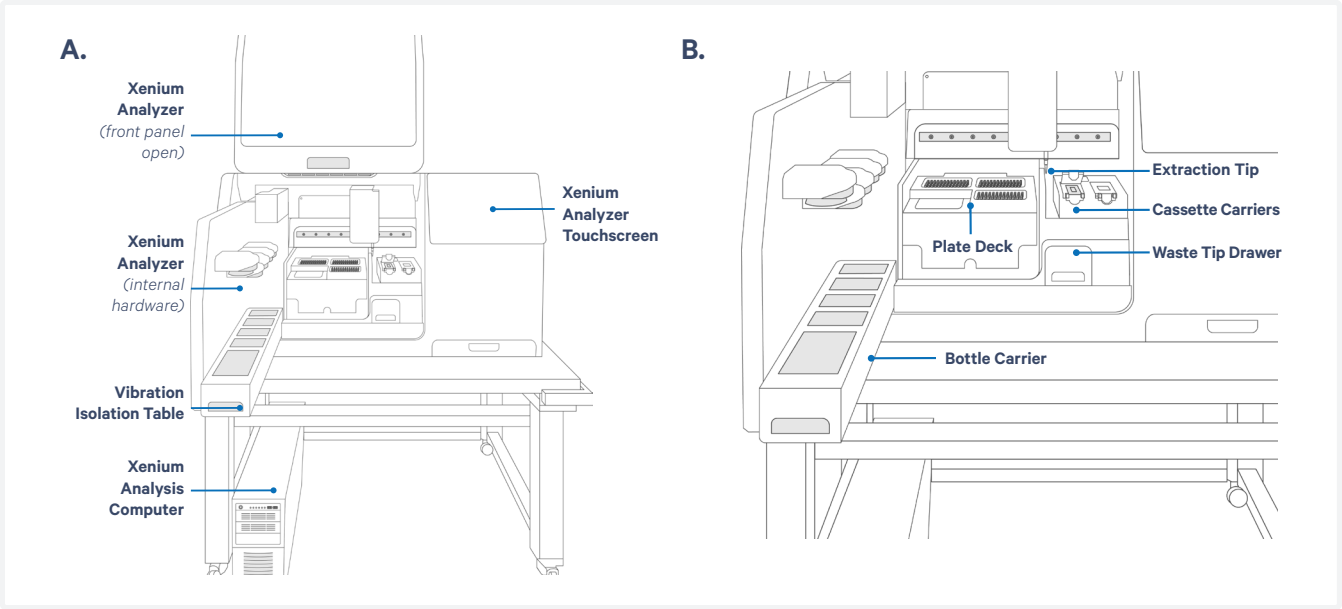


Figure 10. Overview of the Xenium Analyzer instrument layout (A) and instrument hardware (B).

Safety

Before insturment operation, ensure all users have received instruction in general and specific safety practices for the lab and instrument, and have reviewed all Safety Data Sheet (SDS) documents.

Reagent Plate Handling

Up to three reagent plates are required depending on workflow performed. Plates require different storage and handling instructions.

Thaw Decoding Module B and Cell Segmentation Detection Module* overnight at 4°C prior to use. Factor in the overnight thawing step when planning an experiment. Decoding Module A stored at 4°C. It is oxygen sensitive and must be handled carefully. All storing and thawing must be done in the mylar bag for protection. Open the mylar bag and remove the foil-sealed plate only when ready to use, prior to preparation for loading.

Buffer Preparation

Prepare Reagent Buffers fresh prior to filling reagent buffer bottles and loading the instrument.

Cleaning Slides & Cassettes

Clean the bottom of the Xenium slide and cassette prior to loading the instrument. Any fingerprints or lint may interfere with image acquisition and result in a failed run or incomplete or unreliable data generation.

**if performing Cell Segmentation Staining workflow*

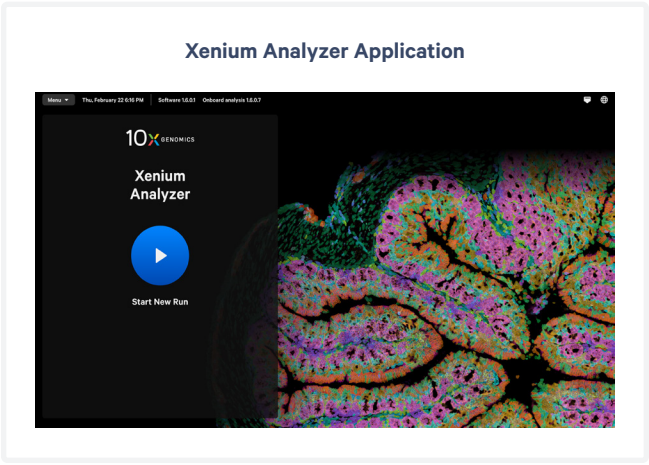


Figure 11. The Xenium Analyzer Application home screen

Instrument UI & Orientation

Ensure user is familiar with all instrument components and how to interact with them. Follow touchscreen instructions throughout workflow. A keyboard is also provided so the user can easily interact with the instrument UI.

Network Connectivity

Monitoring the performance of Xenium Analyzer enables 10x Genomics to optimize instrument performance and throughput by maximizing instrument uptime. This also gives 10x Genomics the ability to respond quickly and troubleshoot any issues that may occur. While the user focuses on processing samples and data collection, 10x Support team will proactively collect data about the instrument to address any potential instrument downtime. Consult the Xenium Analyzer: Network Connectivity Guidelines Technical Note (CG000645) for enabling specific network connectivity guidelines for the Xenium Analyzer.

Readiness Test

The Readiness Test verifies that all systems are working optimally and the instrument is ready for use. No reagents are used (the Readiness Test is the only dry run the instrument utilizes). The Readiness Test is included as a pre-run verification for all instrument runs, but can be initiated as a standalone operation at the discretion of the end user from the Tests Menu option. To initiate the Readiness Test, select Menu (top-left corner of screen) and choose Open Settings on the touchscreen. A successful Readiness Test verifies the instrument is ready for use.

Instrument Operation

A Xenium Analyzer run consists of the following operation steps and estimated time.

1. Initialize Instrument: 10–20 min
2. Load Consumables: 5 min
3. Sample Scan: 1 h
4. Region Selection: 10 min
5. Run: 2-4 days
6. Cleanup: 5 min
7. Unload: 10 min

Data Egress

Data may be exported from the instrument using either a portable USB drive or Local Area Network (LAN). Consult the Xenium Analyzer User Guide (CG000584) for more information about data outputs.

Completed Design Phase › Tissue Preparation ~1 day › Sample Preparation ~1 day › Probe Hybridization ~2 days › Cell Segmentation ~1 day › Xenium Analyzer ~3 days › **Data Analysis >1 week**

Software & Data Analysis

Onboard Analysis

The Xenium Onboard Analysis pipeline occurs on-instrument and involves processing of DAPI images, cell segmentation, decoding, and generation of output data. Review the Onboard Analysis documentation on the 10x Genomics Support Website, which includes an overview of Xenium algorithms and outputs, and Release Notes.

Xenium Explorer & Demo

Download Xenium Explorer, the desktop application that allows for visualization of RNA transcript localization in tissues with subcellular resolution from data generated by the Xenium Analyzer instrument. The Xenium Explorer Web Demo, a modified version of the desktop app, can be used to explore publicly available Xenium In Situ datasets. Features of Xenium Explorer include:

- Organize markers of interest and visualize 3D localized transcripts at any scale
- Examine segmented cells colored by transcript density or clustering result
- Compare gene expression profiles and cell type proportions across tissue regions with a versatile lasso tool

For the latest information on Xenium Explorer, see the 10x Genomics Support website.

Xenium Ranger

Xenium Ranger provides flexible off-instrument reanalysis of Xenium In Situ data. Relabel transcripts, resegment cells with the latest 10x segmentation algorithms, or import your own segmentation data to assign transcripts to cells.

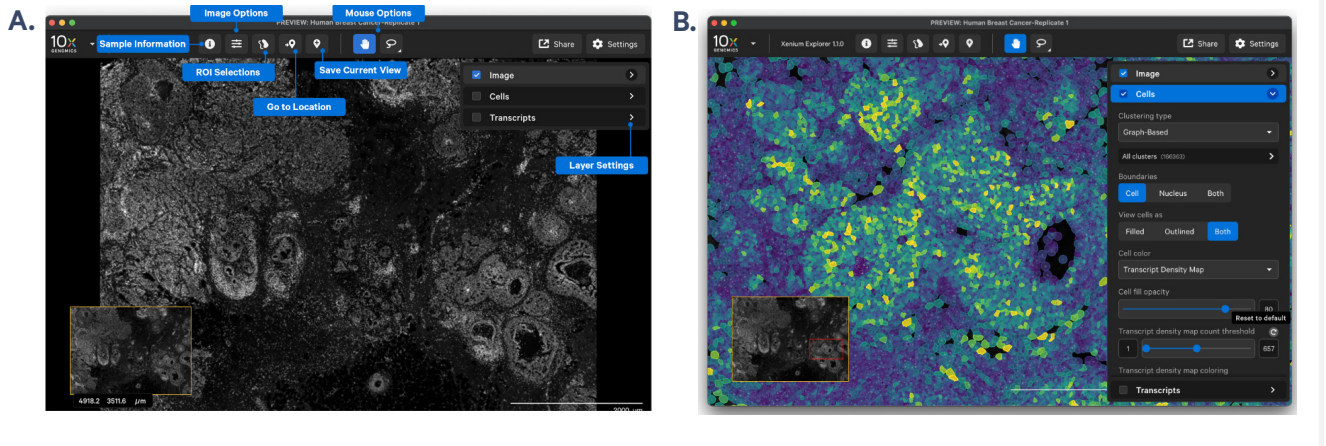
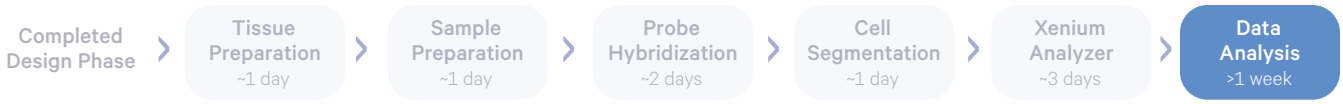


Figure 12. The Xenium Explorer interface includes the following key components: sample information, image options, ROI selections, go to location, save current view, mouse options, and layer settings (A). Cells can be colored by transcript density or cluster affiliation as determined by the genes selected on the Transcripts menu (B).



Datasets

Free, publicly available data are available from a number of different sample types generated with Xenium In Situ solutions.

Data Outputs

Xenium output files include the following: web summary, gene expression metrics, cell-feature matrix, transcript data, cell summary files, gene panel file, secondary analysis results, morphology images, cell and nucleus segmentation files, and Xenium experiment file. For more information, see the At a Glance: Xenium Output Files page of the 10x Genomics Support Website.

Analysis Guide

Read through a collection of tutorials and blogs for data analysis beyond software developed by 10x Genomics.

Completed Design Phase › Completed Execution Phase › **Post-Xenium Applications** ~ 2 days

Post-Xenium Applications

Due to the non-destructive nature of the Xenium In Situ assay, tissue sections remain intact and may be further processed after a Xenium Analyzer instrument run. Listed below are details for the following Post-Xenium applications: H&E staining, immunofluorescence (IF) staining, and Visium CytAssist Spatial Gene Expression workflow. All of these workflows are optional.

H&E Staining

H&E Staining following a Xenium Analyzer instrument run is optional and any desired H&E staining protocol may be used. Autofluorescence Solution must be removed from the tissue during the required Quencher Removal step before proceeding to staining. Quencher Removal Solution should be prepared fresh before each use for optimal results.

Consult the Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613) for more details. If combining H&E staining with additional post-Xenium workflows, see the Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression Technical Note (CG000709) for additional guidance.

IF Staining

Tissue sections may be IF stained following a Xenium Analyzer run. See the Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression Technical Note (CG000709) for more information.

Visium CytAssist Spatial Gene Expression

Tissue sections may also be processed through the Visium CytAssist Spatial Gene Expression workflow following a Xenium Analyzer run. See the Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression Technical Note (CG000709) for additional details.

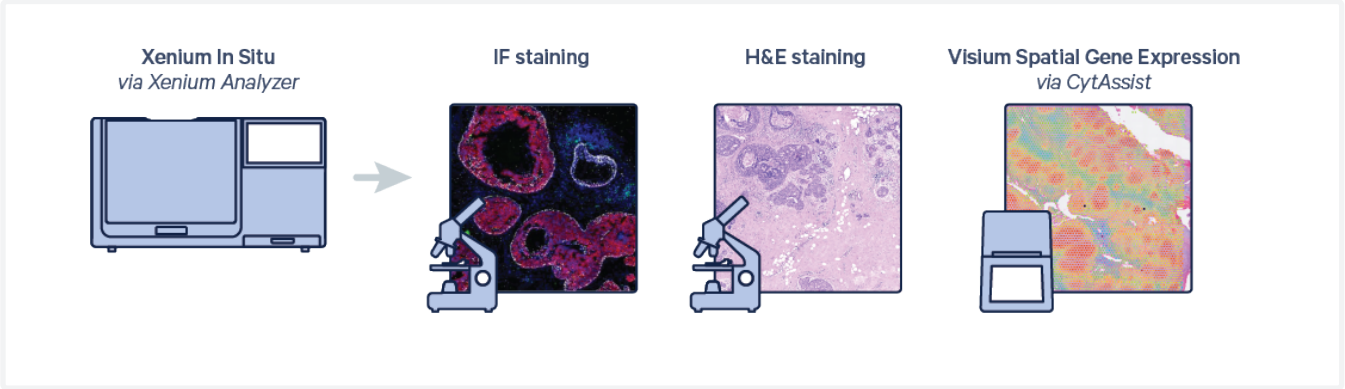


Figure 13. Tissue sections analyzed through the Xenium platform may be used for additional applications, including: protein detection via IF staining, analysis of tissue morphology via H&E staining, or spatial transcriptomics analysis using Visium CytAssist Spatial Gene Expression technology.

References

1. Xenium In Situ Gene Expression User Guide (CG000582).
2. Xenium In Situ for Fresh Frozen Tissues - Tissue Preparation Guide (CG000579).
3. Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578).
4. Xenum In Situ for Fresh Frozen Tissues - Fixation & Permeabilization Demonstrated Protocol (CG000581).
5. Xenium In Situ for FFPE - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580).
6. Xenium Analyzer User Guide (CG000584).
7. Xenium In Situ Gene Expression - Protocol Planner (CG000601).
8. Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613).
9. Xenium Workflow Document Resources (CG000647).
10. Xenium Analyzer Site Preparation Survey (CG000587).
11. Xenium Add-on Panel Design Technical Note (CG000643).
12. Xenium Analyzer: Network Connectivity Guidelines (CG000645).
13. Species Standalone Custom and Advanced Custom Panel Design for Xenium In Situ Technical Note (CG000683).
14. Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression Technical Note (CG000709).
15. Xenium In Situ Gene Expression with Cell Segmentation Staining User Guide (CG000749).
16. 10x Genomics Support Website.

Document Revision Summary

Document Number	CG000754
Title	Xenium In Situ Gene Expression Workflow Planning Guide
Revision	Rev A
Revision Date	March 2024
General Changes	
Specific Changes	

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