Xenium Protein: Same-Cell RNA & Protein Detection Workflow, Analysis & Data Highlights

Introduction

Xenium Protein is an extension of the Xenium v1 assay chemistry that brings same-cell multiomics to the Xenium platform by enabling simultaneous RNA and protein detection within the same human FFPE tissue section. Xenium Protein is a fully automated, integrated workflow on the Xenium Analyzer that enables robust identification of major immune cell types, functional states, and the tissue microenvironment. This Technical Note provides an overview of the assay workflow and analysis, as well as the post-Xenium IF workflow. Performance metrics demonstrate assay robustness and data highlights show the advantages of simultaneous RNA and protein detection.

The Xenium In Situ Gene and Protein Expression assay (referred to as Xenium Gene and Protein throughout) detects RNA and protein in human formalin fixed, paraffin embedded (FFPE) tissue sections. It uses targeted probes from either Xenium v1 pre-designed or custom (custom add-on, standalone custom, and advanced custom) panels to assay RNA with high sensitivity and specificity at the subcellular level and utilizes the Xenium In Situ (Xenium v1) chemistry. After probe hybridization, ligation and enzymatic amplification are used to generate multiple copies of a gene-specific barcode for each RNA target.

To detect protein, tissue sections are stained with Xenium Protein antibody subpanels, each containing three to four oligonucleotide-conjugated antibodies. The full protein panel includes 27 immunology markers organized into six subpanels - Immunology, Immune Cell Subpanels A/B/C, Proliferation & Differentiation, Immune Checkpoint, and Tumor - designed to be run collectively for comprehensive cell typing and spatial characterization of immune and tumor microenvironments.

During the instrument run and after RNA decoding, fluorescently labeled oligos specific to protein detection bind to these antibodies, revealing the target protein stain. The subpanels enable identification of major immune cell types and subtypes, as well as immune molecules.

Additionally, Cell Segmentation Staining is integrated into the assay, allowing for nuclei, membrane, and interior labeling that are inputs for automated morphology-based multimodal cell segmentation analysis. After the instrument run is finished, the Xenium software suite facilitates the analysis and visualization of gene and protein expression data on a per cell basis.

Xenium In Situ Gene & Protein Expression Workflow

Tissue & Sample Preparation

The Xenium Gene and Protein Expression workflow is compatible with human FFPE tissues. FFPE tissues were sectioned onto a Xenium slide, and deparaffinized and decrosslinked according to the Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578).

Assay & Instrument

Samples were processed for probe hybridization, ligation and amplification, as well as antibody staining for protein detection and cell segmentation, according to the Xenium In Situ Gene and Protein Expression with Cell Segmentation Staining User Guide (CG000819). Prepared Xenium slides were loaded on the Xenium Analyzer where on-instrument imaging and decoding are performed. Full details regarding instrument setup are found in the Xenium Analyzer User Guide (CG000584). A high-level overview of the Xenium Gene and Protein Expression workflow is depicted in Figure 1.

In the assay performance sections, the Xenium Gene and Protein Expression workflow was compared to the Xenium In Situ Gene Expression with Cell Segmentation workflow (CG000749), referred to as the Xenium Gene Expression workflow throughout.

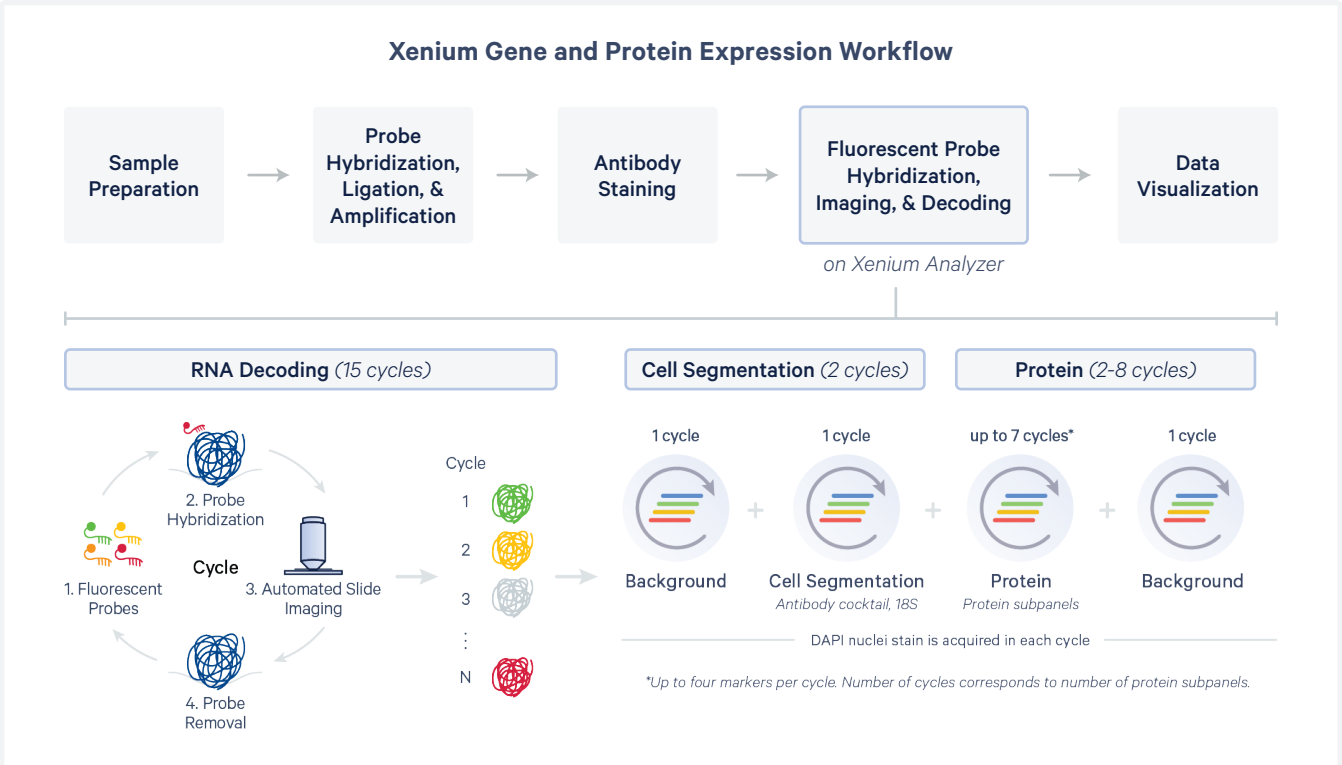

Figure 1. Overview of Xenium Gene and Protein Expression Workflow. The Xenium Gene and Protein Expression workflow utilizes the on-instrument imaging and decoding capabilities of the Xenium Analyzer instrument to perform simultaneous RNA and protein detection within the same instrument run. DAPI nuclei stain is acquired in each cycle for image alignment.

Gene Panels

Xenium Protein subpanels are only compatible with Xenium v1 gene panels, including pre-designed off-the-shelf, custom add-on, standalone custom, and advanced custom panels. See the 10x Genomics Support Website for a detailed description of panel designs.

Protein Subpanels

The Xenium Gene and Protein Expression assay uses a comprehensive set of 27 immunology markers organized into six distinct antibody subpanels. Each subpanel contains 3-4 markers. This modular design allows for user flexibility. Panels require no further optimization and are compatible with a variety of human tissue types. Maximum plex is 27 markers and six subpanels, with the option to select subpanels of interest.

Xenium Protein Subpanel	Markers Included
Immune Cell Subpanel A	CD4, CD20, CD8A, CD3E
Immune Cell Subpanel B	CD138, HLA-DR, CD11c, CD68
Immune Cell Subpanel C	CD16, GranzymeB, CD163
Proliferation & Differentiation Subpanel	CD45RA, PCNA, CD45RO, Ki-67
Immune Checkpoint Subpanel	PD-1, VISTA, PD-L1, LAG-3
Protein Tumor Subpanel	Beta-catenin, CD31, PTEN, PanCK
Cellular Localization *Individually imaged cell segmentation markers*	E-cadherin, Vimentin, alphaSMA, CD45

Table 1. Xenium protein subpanels and associated markers

Potential antibody combinations for creating the protein subpanels were evaluated based on biological relevance and technical considerations. First, antibodies with biologically relevant targets were grouped together into a subpanel. Additionally, each marker was assigned to a specific wavelength to optimize antibody performance and minimize complications from signal crosstalk.

Software & Algorithms

The v4.0 Xenium In Situ software suite enables analysis of gene and protein expression data (Xenium Onboard Analysis (XOA), Xenium Explorer, and Xenium Ranger). In addition to existing XOA gene expression output files, Xenium Gene and Protein Expression datasets include 2D protein and background autofluorescence images, per-cell protein quantification in the cell-feature matrix, and protein clustering and expression analysis. The XOA pipeline automatically performs background subtraction so Xenium 2D protein images only show the stain signal. Autofluorescence is shown separately in the 2D background image outputs. Protein expression is measured as mean fluorescence intensity per cell using pixels from the 2D cell segmentation mask.

XOA v4.0 also includes algorithm improvements for image processing and nucleus segmentation:

- **Consistent segmentation and quantification**: Use the same global focus map in the 2D multifocus image fusion algorithm for all morphology images.
- **Removes out-of-focus light in morphology images**: Images are deconvolved and will appear sharper, which improves image resolution and contrast.
- **Accounts for fluctuations in tissue autofluorescence**: Protein images undergo background subtraction on the instrument using two cycles of autofluorescence imaging.
- **Improves segmentation in nuclei-dense regions**: The nucleus segmentation algorithm performs joint inference on the 2D DAPI and 18S interior stain images and then adds nuclei segmented from the 3D DAPI Z-stack.

Xenium Explorer v4.0 supports visualization of both gene and protein expression in individual cells, in addition to post-Xenium H&E and IF images for the same tissue:

- View up to six protein channels at a time to investigate tissue biology.
- Measure the mean protein fluorescence intensity within cells for selected regions of interest.
- Toggle between gene and protein cluster affiliation based on K-means or graph-based cluster analysis.

Data can be reanalyzed in Xenium Ranger v4.0. It supports selection of protein stains for use in multimodal cell segmentation or importing cell segmentations from third-party tools to requantify transcripts and protein. This version also adds an option for segmenting large cell types, such as human skeletal muscle cells, dorsal root ganglion (DRG) neurons, and adipocytes. See the 10x Support Website for details about all 10x software, outputs, and algorithms.

Post-Xenium Protein Analysis

The Xenium Gene and Protein Expression assay is compatible with post-Xenium immunofluorescence (IF) and H&E staining. Post-Xenium slides should be used immediately after a Xenium Analyzer instrument run or stored in 1,000 µl PBS-T (0.05%) for up to three days at 4°C. Use of anti-rabbit or anti-mouse secondary antibodies for post-Xenium applications will result in cross-detection. To avoid using incompatible secondary antibodies, use a fluorophore-conjugated primary antibody raised in any species or a non-conjugated primary antibody not raised in rabbit or mouse. Some optimization may be required for post-Xenium applications.

For post-Xenium Protein H&E staining, refer to the Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613).

For post-Xenium Protein IF staining (Figure 2), the protocol in CG000709 was followed except for one modification. To align with the Xenium Gene and Protein Expression assay, decrosslinking of IF-only controls was performed using TE buffer, pH 9.0 at 80°C for 20 minutes (instead of Citrate Buffer pH 6.0, 90°C for 30 minutes).

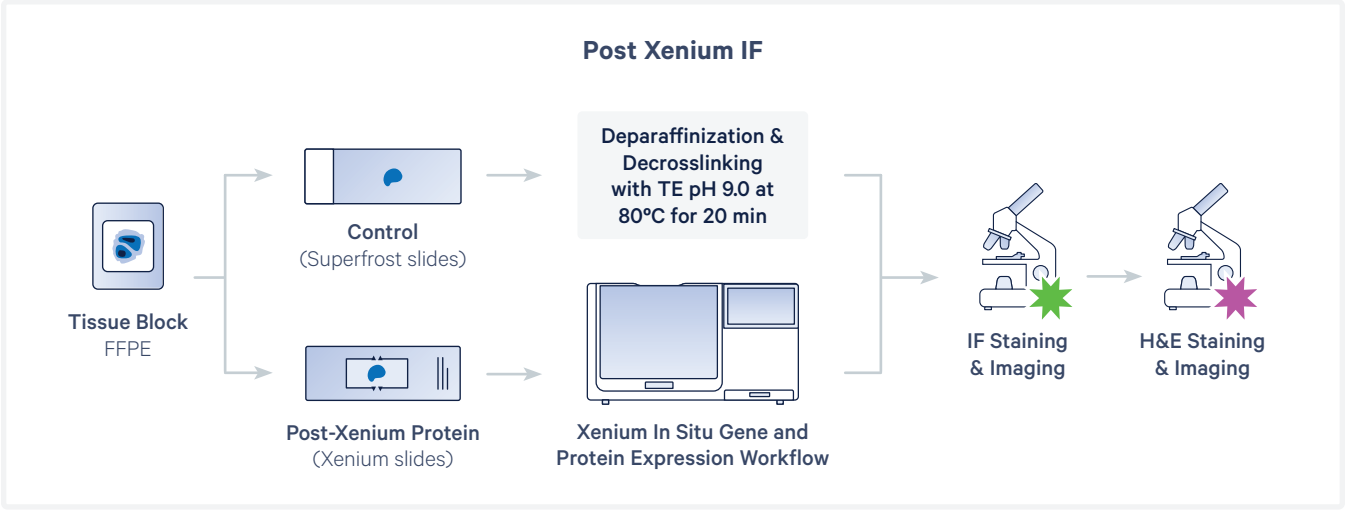

Figure 2. Post-Xenium Protein IF staining overview.

Results – Assay Performance

Antibody Conjugation & Inclusion in a Xenium Subpanel Does Not Impact Individual Antibody Performance

To ensure the conjugation of oligonucleotides to antibodies contained in the Xenium Protein subpanels does not impact staining, human tonsil tissue sections were IHC stained with unconjugated antibodies for CD16, Granzyme B, and CD163. Next, tissue sections generated from the same block were stained with Immune Cell Subpanel C and run on the Xenium instrument as part of the Xenium Gene and Protein Expression workflow. Finally, sections from the same block were stained with the full Xenium Protein panel and run on the Xenium Analyzer as part of the Xenium Gene and Protein Expression workflow.

As shown in Figure 3, IHC staining with a single, unconjugated antibody is comparable to staining with the same antibody when it is conjugated and multiplexed within either the Immune Cell Subpanel C or the full Xenium Protein panel. These results demonstrate that neither conjugation nor multiplexing compromise antibody function or creates steric hindrance that could impact epitope accessibility.

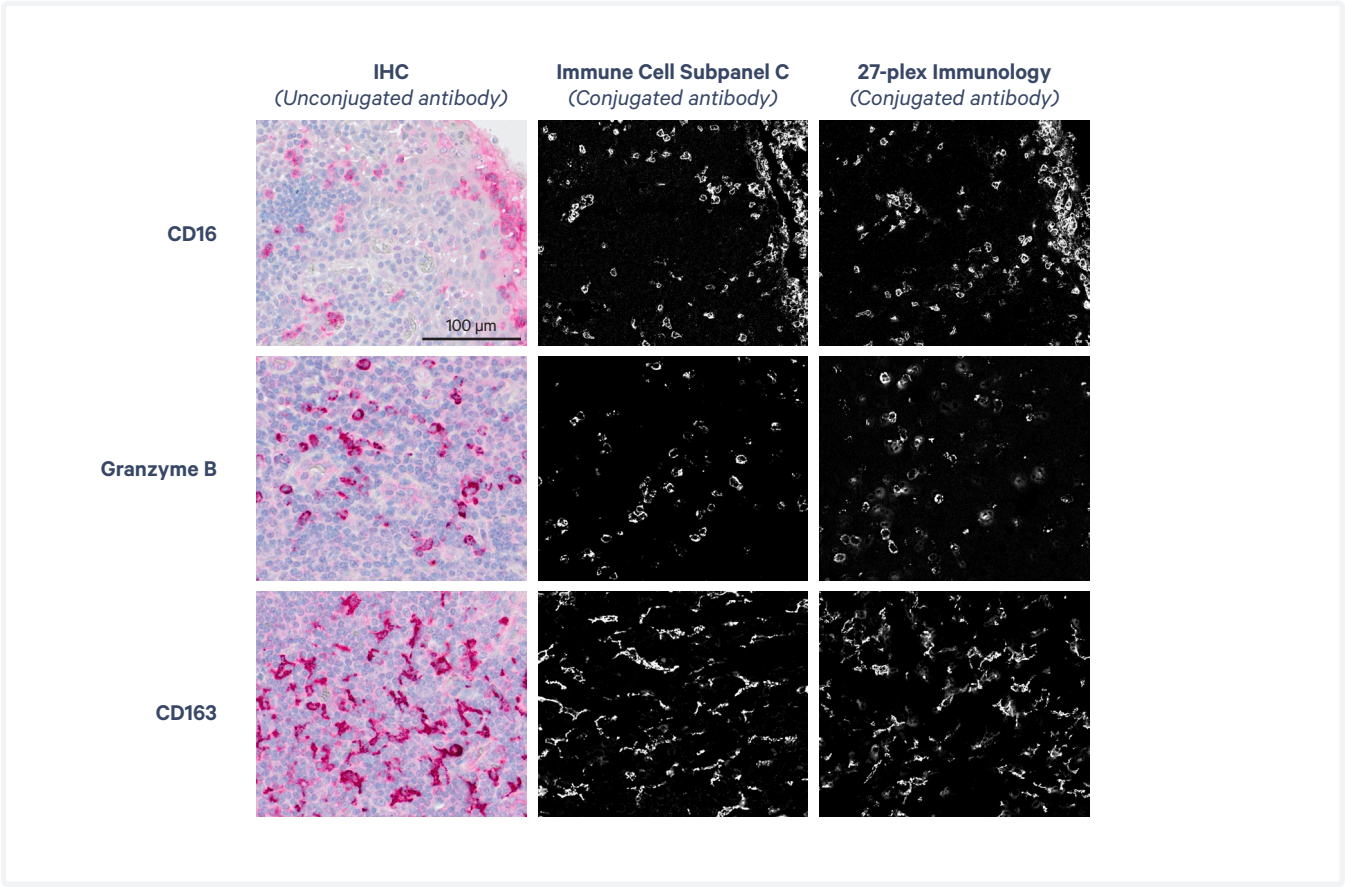

Figure 3. Comparison between IHC staining of unconjugated antibodies, the same antibodies within a Xenium subpanel (conjugated), and within the full panel in human tonsil tissue. Sections are from the same block, but are not serial sections. Differences between IHC staining and Xenium staining reflect expected variability between sections.

Xenium Gene & Protein Expression Exhibits Broad Tissue Compatibility

Xenium Gene and Protein Expression has been tested on 15+ human FFPE tissue types, including live, skin, kidney, breast, lung, colon, appendix, and tonsil. In addition to healthy tissue, various disease states like cancer, Crohn's, and hyperplasia, were also tested and are shown to be compatible. Representative staining of various tissue types using the Xenium Gene and Protein Expression workflow is shown in Figure 4.

See the 10x Genomics Support Website for a detailed list of tested tissue types.

Figure 4. Staining of the Protein Tumor Subpanel and Cellular Localization panels across several human FFPE tissue types. Disease states for these samples are as follows: liver (hepatocellular carcinoma), skin (melanoma), kidney (clear cell renal carcinoma), breast (IDC triple negative), lung (healthy), colon (healthy), appendix (healthy), and tonsil (healthy). Only some markers from the Protein Tumor Subpanel and Cellular Localization panels are shown.

Xenium Gene & Protein Expression has Comparable RNA Sensitivity to the Xenium Gene Expression Assay

Serial sections (or sections within 10-15 microns away from each other) were generated and run through either the Xenium Gene and Protein Expression assay or the Xenium Gene Expression assay. No significant impact on RNA performance (median transcripts/cell) was observed across 12 different tissue types between the two assays. Variation in median transcripts between tissues are due to differences in transcript density (Table 2).

Xenium Gene & Protein Expression is Reproducible Across Instrument Runs, Slides, and Users

Tonsil benign lymphoid hyperplasia samples prepared with the Xenium Gene and Protein Expression workflow and imaged twice in separate instrument runs (run immediately after one another) showed a high degree of reproducibility. Visual inspection revealed nearly identical staining patterns and intensities. Furthermore, qualitative metrics (mean intensity/cell, positive vs. negative ratio) showed little to no difference between replicates for both RNA (not shown) and protein (Figure 5).

Xenium Gene and Protein Expression reproducibility, as assessed by the metrics above, was also consistent across users and regardless of the chosen gene expression pre-designed panel (data not shown).

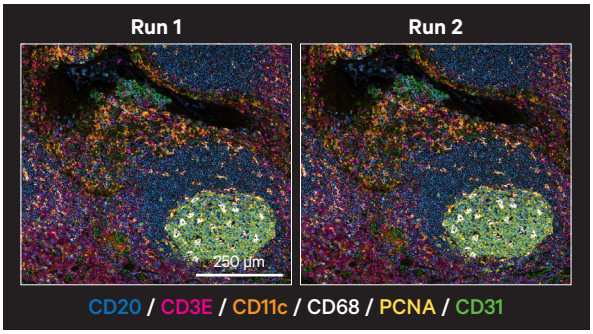

Figure 5. Comparable protein stain images for CD20, CD3E, CD11c, CD68, PCNA, and CD31 in the same human tonsil benign lymphoid hyperplasia samples across two Xenium instrument runs.

	Xenium Gene Expression			Xenium Gene & Protein Expression		
Tissue	**Number of cells detected**	**Median transcripts/cell**	**Total high-quality decoded transcripts**	**Number of cells detected**	**Median transcripts/cell**	**Total high-quality decoded transcripts**
Appendix	170,246	72	20,926,853	174,328	68	20,111,387
Breast	230,096	62	24,278,318	224,928	80	30,182,868
Colon	132,333	43	10,151,909	131,026	45	11,071,514
Kidney	454,477	74	46,640,874	450,995	74	45,861,842
Liver	250,219	135	46,956,473	224,913	142	45,084,448
Lung	278,440	108	49,140,062	296,204	98	49,236,156
Ovary	371,485	262	115,035,359	366,154	237	108,770,333
Skin	154,106	139	38,952,322	142,137	135	36,275,602
Tonsil	515,113	146	91,188,010	467,271	133	80,960,301

Table 2. Median transcripts per cell, as a measure of RNA sensitivity, is at comparable levels between Xenium Gene and Protein Expression and Xenium Gene Expression assays.

Xenium Gene and Protein Expression Performance is Consistent Regardless of Imaging Cycle & Plexy

Users can choose to run up to 6 subpanels in one Xenium Gene and Protein Expression run. Increasing subpanel number will increase the number of imaging cycles the instrument performs. To test the effect of the number of imaging cycles on protein stain intensity and specificity, data from runs using all panels vs. selection of panels on human FFPE liver tissue were compared. Additionally, the same markers were imaged at either three or seven imaging cycles.

Consistent staining was observed in all conditions, confirming consistent stain quality irrespective of the number of subpanels imaged or at what imaging cycle the images are taken (Figure 6). This flexibility allows users to select only the subpanels of interest without sacrificing data quality.

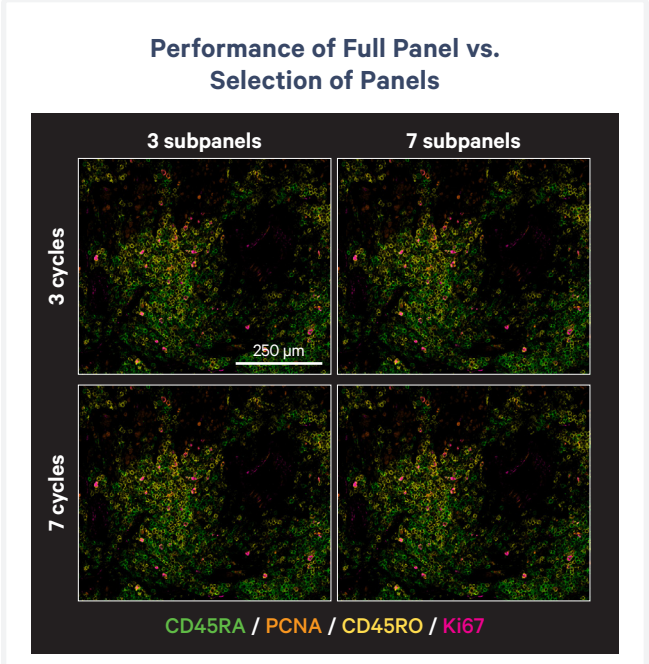

Figure 6. As shown in these human FFPE liver tissue samples, performance of the Proliferation and Differentiation subpanel is consistent, regardless of the number of subpanels included in the Xenium run or at what imaging cycle the subpanels are imaged in.

Cell Segmentation Performance is Comparable between Xenium Gene Expression & Xenium Gene & Protein Expression

Cell segmentation performance was unaffected by the Xenium Gene and Protein Expression workflow. Samples were prepared with either the Xenium Gene and Protein workflow or the Xenium Gene Expression workflow, and the fraction of cells segmented by boundary and interior stains was compared. Across all tissue types tested, there was no significant difference (Figure 7).

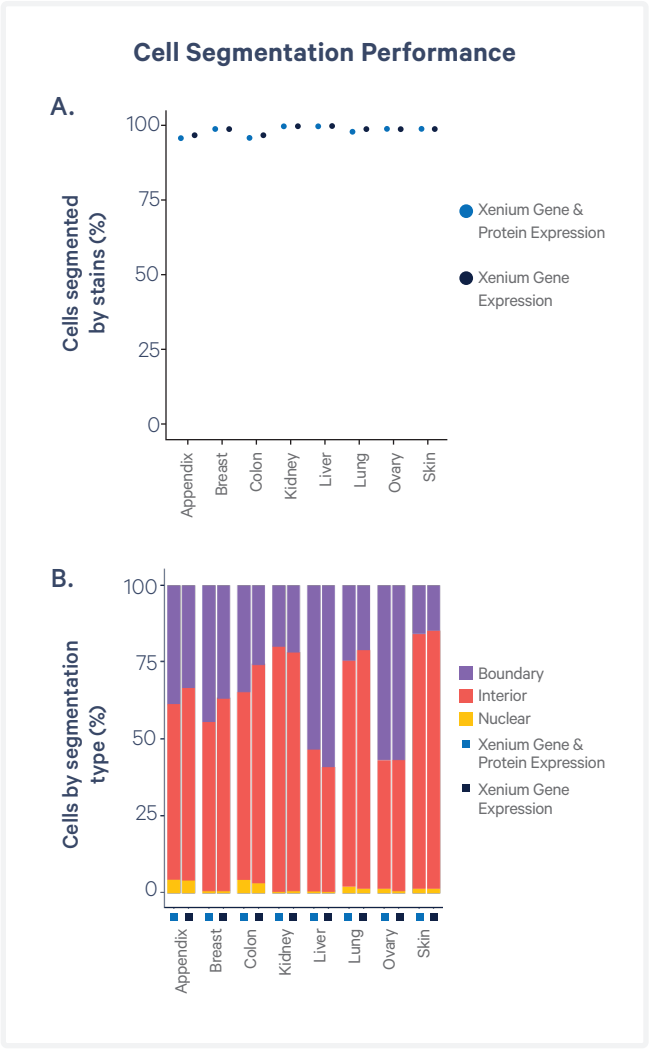

Figure 7. The fraction of cells segmented by boundary and interior stains was consistent across tissue types in samples processed with the Xenium Gene and Protein workflow compared with Xenium Gene Expression.

Post-Xenium H&E Staining is Equivalent between Xenium Gene Expression & Xenium Gene and Protein Expression

To test whether there are differences in H&E staining post Xenium Gene and Protein Expression assay, H&E staining was compared between breast tissue samples processed with either Xenium In Situ Gene Expression or Xenium In Situ Gene and Protein Expression. Tissue morphology and H&E staining were comparable between the two assays (Figure 8). Importantly, tissue detachment incidence was not affected.

Figure 8. Post-Xenium H&E staining in human breast tissue processed with either Xenium Gene Expression or Xenium Gene and Protein Expression. H&E staining was comparable in both groups.

Differences between IF & Xenium Protein images Do Not Affect Biological Interpretation

Conventional immunofluorescence (IF) is typically optimized for visual interpretation and does not usually employ background subtraction. These assays generally include only 1–4 markers, are manually acquired with exposure, optimized per channel, and are interpreted by the experienced human eye. These images are used by researchers to make a binary assessment of marker presence, positive or negative. In this context, background signal is either tolerated or disregarded, and there is no need for standardized computational correction.

In contrast, high-plex spatial proteomics platforms involve automated, multiplexed imaging across dozens of markers and often multiple cycles. These systems generate high-dimensional data used for quantitative analyses such as clustering, phenotyping, and machine learning. Because this data is subject to visual interpretation and is processed computationally rather than only visually, background signal - whether from tissue autofluorescence or nonspecific staining - is actively corrected to ensure accurate interpretation. Background subtraction improves signal-to-noise, normalizes intensity across markers, and enhances marker specificity. All of these are valuable for reliable downstream analysis.

As a result, spatial proteomics images may appear different from traditional IF images in terms of intensity and clarity. Spatial proteomics images are typically cleaner and lower in diffuse background. Importantly, this difference does not reflect a loss of biological relevance. The staining patterns and subcellular localization of markers should remain consistent, and traditional IF or IHC can serve as orthogonal validation methods to confirm the biological specificity of spatial data.

Figure 9 illustrates these concepts in colon and spleen samples. The first row shows the traditional IF stain, and the third row shows the Xenium Protein image. These Xenium Protein images have had background signal (second row) removed from the final image.

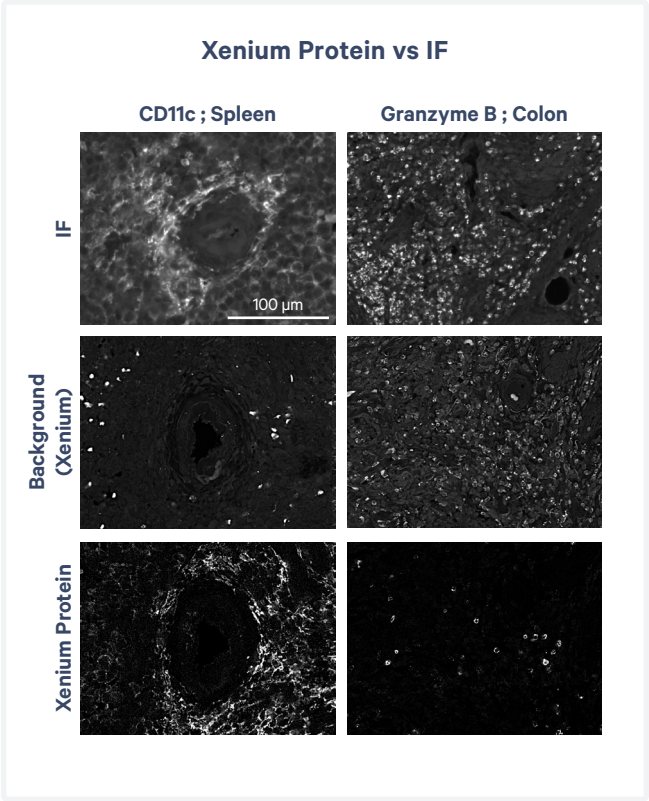

Figure 9. Two examples of an IF stain image compared to the equivalent Xenium Protein assay protein image (middle: intermediate processing step image illustrating difficulty in distinguishing true signal from background; right: final processed protein image), for GranzymeB stain of human colon and CD11c stain of human spleen.

Post-Xenium IF Staining in Xenium Gene & Protein Expression samples

Tissue sections were processed through the Xenium Gene and Protein Expression workflow using either the full protein panel or a panel excluding the Xenium Protein Tumor Subpanel. Those processed without the Tumor Subpanel underwent post-Xenium IF staining. Controls were samples not processed through the Xenium workflow prior to IF staining (staining-only control), with modifications as noted in the Methods section.

Post-Xenium IF staining of PanCK in Xenium Gene and Protein Expression samples was comparable to the protein expression data for PanCK from the Xenium instrument run within the tonsil tissue (Figure 10). Patterning of PanCK was also comparable between post-Xenium IF and IF-only controls. For some markers or tissue types, staining may vary and be dimmer compared to the staining-only control.

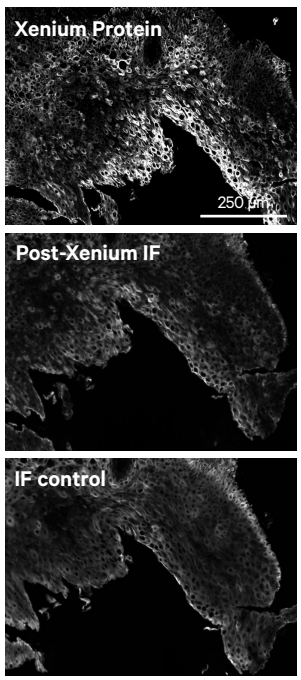

Figure 10. Comparison of PanCK staining in tonsil tissue between Xenium Protein, Post-Xenium IF, and IF controls.

Results – Data Highlights

Xenium Gene & Protein Expression Captures Post-Transcriptional Regulation

Though gene expression is often used as a proxy for protein expression, they are not necessarily correlated with one another due to numerous post-transcriptional and post-translational mechanisms that regulate protein abundance. For example, mRNA can be degraded before translation. Once synthesized, proteins can also become misfolded and subsequently degraded by systems such as the ubiquitin-proteosome system. Therefore, direct detection and quantification of proteins provide a more accurate understanding of the functional molecules present in a cell or tissue.

Figure 11 depicts three examples of varying RNA and protein correlation in human colon cancer. *PECAM1* transcript density shows a strong correlation with the level of CD31 protein stain. On the other hand, *PTEN* transcripts are absent, suggesting lack of protein, however when stained with the PTEN antibody, PTEN protein is detected. Finally, *CTNNB1* transcripts show heterogeneous correlation with Beta-catenin protein.

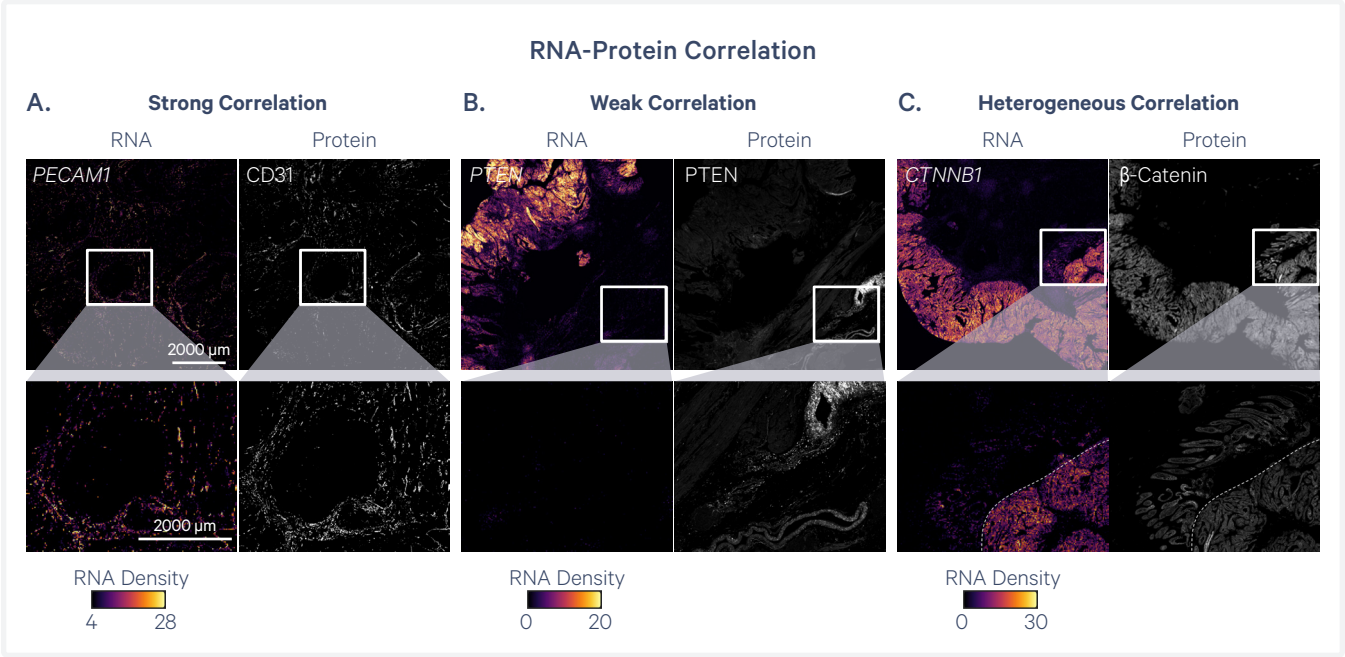

Figure 11. Variance in correlation between RNA and protein in human colon cancer. Post-transcriptional and translational modifications may result in a strong **(A)** weak **(B)** or heterogenous correlation **(C)** between RNA levels and protein.

Xenium Protein Improves Cell Type Identification

Another factor that may impact correlation of gene and protein detection is in probe design. This can be seen when developing isoform specific probes of certain genes. In the case of the CD45RA and CD45RO isoforms of the *PTPRC* gene in human tonsil tissue, transcript detection was minimal, and captured predominantly the *CD45RO* transcripts. However, the protein stain, that distinguishes between the two isoforms, reveals a robust, cell-specific expression of either protein (Figure 12). *PTPRC*, whose expression is expected throughout the tissue, is shown as a control.

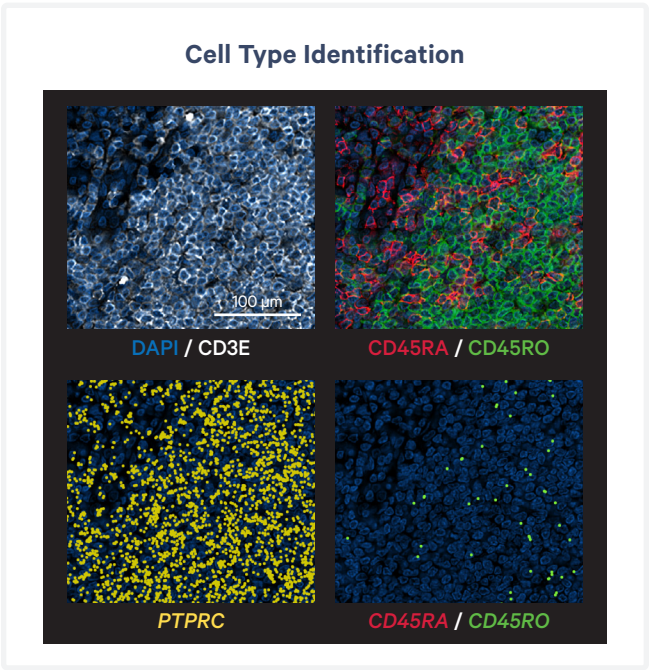

Figure 12. Protein staining of CD45RA and CD45RO in human tonsil tissue reveals clearer cell subtypes than relying on gene detection due to challenges in probe design.

Xenium Protein Enhances Spatial Context

Examining protein expression also allows for investigation of the localization of the protein throughout the tissue. E-Cadherin is a transmembrane protein that plays an important role in cell-cell adhesion, and is typically localized at cell-cell junctions. Disruption in E-cadherin localization is often seen in certain pathological conditions or can indicate a diseased state. The human melanoma sample in Figure 13 shows that in some cells, E-Cadherin is present in the cytoplasm instead of its usual place at the cell-cell junction. This difference would not have been captured when looking at gene expression alone and demonstrates how enhancing spatial context with simultaneous protein detection can provide a clearer understanding of tissue state.

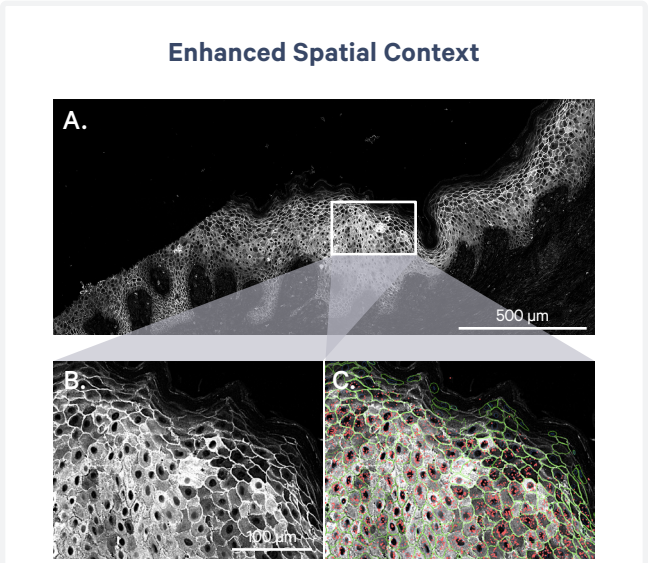

Figure 13. Xenium Protein Enhances Spatial Context **(A)** E-Cadherin staining in a melanoma sample. **(B)** Zoomed-in view of E-Cadherin staining. Some cells show staining localized to the cell membrane, while others show cytoplasmic staining or a combination of nuclear and cytoplasmic staining. **(C)** Cell segmentation (green), nuclear boundaries (blue), and *CDH1* transcripts (red).

Conclusion

The Xenium In Situ Gene and Protein Expression assay can simultaneously detect gene and protein expression on the Xenium In Situ platform. The six protein subpanels are designed to target key immunology markers, along with markers of tissue architecture. Combined, this facilitates a thorough interrogation of the various cell types, spatial organization, and phenotype of the tumor microenvironment, giving detailed insights into tumor biology. As demonstrated in this Technical Note, the Xenium Gene and Protein assay features:

- Simultaneous detection of RNA and protein within one integrated instrument run.
- Compatibility with a diverse variety of human FFPE tissue types
- High reproducibility across experiments, replicates, and users
- Compatibility with Cell Segmentation and Post-Xenium applications, such as IF and H&E staining

The ability to simultaneously detect RNA and protein targets within the same tissue section in a single integrated instrument run can provide a more comprehensive understanding of molecular interactions and spatial organization within tissues. This powerful multiomic approach can advance understanding of disease mechanisms and enable critical biological discoveries that drive medical research progress.

References

1. Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578).
2. Xenium In Situ Gene and Protein Expression with Cell Segmentation User Guide (CG000819).
3. Xenium Analyzer User Guide (CG000584).
4. Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613).
5. Post-Xenium In Situ Applications: Immunofluorescence, H&E, Visium v2, and Visium HD Technical Note (CG000709).

Document Revision Summary

Document Number	CG000829
Title	Xenium Protein: Same-Cell RNA & Protein Detection Workflow, Analysis & Data Highlights
Revision	Rev A
Revision Date	August 2025
Description of Changes	Correction (9/2025): Changed lymph node to tonsil tissue (pg 10)

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