Xenium Prime 5K Gene Expression Workflow, Analysis & Data Highlights

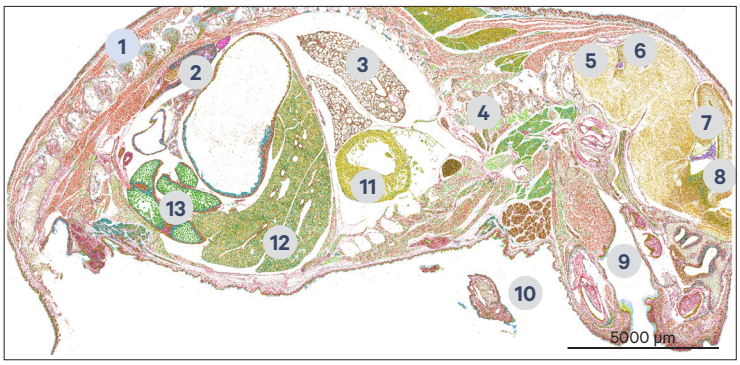
Introduction

The Xenium Prime 5K assay on the Xenium Analyzer offers the ability to analyze ~5,000 genes with optional cell segmentation staining on FFPE and FF tissue samples. The new Xenium Prime chemistry and updated analysis software provide exceptional sensitivity, specificity, and spatial fidelity for assessing in situ gene expression. The highly reproducible assay is compatible with Xenium Prime 5K Human and Mouse Pre-designed Panels, with up to 100 custom add-on genes. This Technical Note provides an overview of the Xenium Prime workflow, compatible reagents, and analysis pipeline. It also includes several Xenium Prime data highlights, including comparisons with Xenium v1 and Visium HD.

Xenium Prime In Situ Gene Expression assays RNA with high sensitivity and specificity at the subcellular level by using targeted priming oligos and probe panels. The Xenium Prime 5K pre-designed probe panels, along with custom add-on panels are available for human and mouse species and enable high-plex exploration of genes to interrogate pathways, biomarkers, and cell-cell interactions.

Xenium Prime is compatible with formalin fixed & paraffin embedded (FFPE) and fresh frozen (FF) tissue sections. Similar to Xenium In Situ Gene Expression (Xenium v1), Xenium Prime relies on probe hybridization, ligation, and enzymatic amplification to generate multiple copies of a gene-specific barcode for each RNA target. Additionally, cell segmentation staining (optional) enables labeling of cell nuclei, membranes, and interiors that are inputs for automated morphology-based cell segmentation analysis.

Xenium Prime 5K Gene Expression in Neonatal Mouse



1. Vertebrae
2. Kidney
3. Lung
4. Trachea
5. Medulla
6. Cerebellum
7. Dentate gyrus
8. Hippocampus
9. Tongue
10. Upper limb
11. Heart
12. Liver
13. Small intestine

Figure 1. Xenium Prime 5K assay on neonatal mouse reveals defined organs. Cells are colored by graph-based clustering results, visualized using Xenium Explorer v3.0. Scale bar: 5000 µm.

High-quality, Reproducible In Situ Gene Expression Data with Xenium Prime 5K

The Xenium Prime assay, along with parallel improvements to the Xenium Onboard Analysis (XOA) software enables high-quality in situ gene expression data with confidence in each transcript assignment. Updates to the assay chemistry and decoding codebook result in increased transcript density and improved spatial resolution in Xenium Prime 5K for both FF and FFPE fixed human and mouse samples (Figure 2A and 2B). Transcript density is also influenced by fixation method and tissue thickness. New image processing and decoding algorithms in XOA v3.0 enable transcript detection and localization at Xenium Prime 5K's higher transcript densities (see Appendix).

Additionally, the Xenium Prime assay is highly reproducible across different tissue types (Figure 2C and Figure 3). Adjacent sections of FFPE human pancreas and kidney and FF mouse liver and brain tissue were processed on different Xenium slides using the protocol described in the Workflow section below.

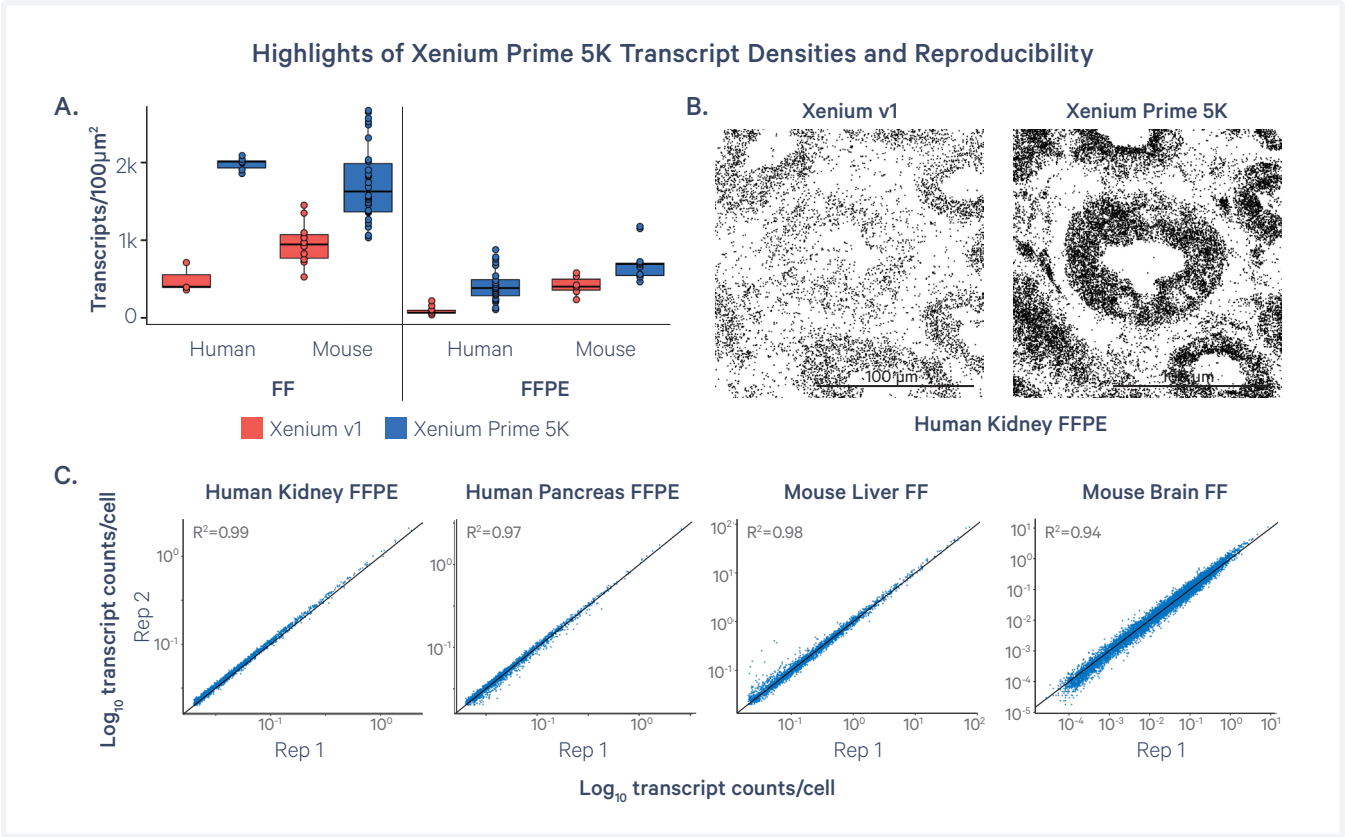


Figure 2. **(A)** Xenium Prime 5K assay results in higher transcript density per 100 µm² across a broad range of tissue types from human and mouse FF and FFPE samples compared to Xenium v1. Consult the 10x Genomics Support Website for a list of tested tissue types. Xenium v1 data were generated with the pre-designed Xenium Human Multi-Tissue and Cancer Panel (377 genes) or Xenium Mouse Tissue Atlassing Panel (379 genes). All samples were analyzed on-instrument with XOA v3.0. See Appendix for more information about the impact of XOA pipeline version on Xenium v1 samples. **(B)** Example of human kidney FFPE sample processed with the Xenium v1 and Xenium Prime 5K workflow, and visualized with Xenium Explorer v3.0. The Xenium Prime 5K sample shows significantly higher transcript density. **(C)** Xenium Prime 5K is highly reproducible. Log10 transcript counts per cell (>= Q20) for Rep 1 vs. Rep 2. Per-gene sensitivity correlation between the two replicates is greater than R^2 = 0.94 for all sample types tested, highlighting the reproducibility of the Xenium Prime assay. Replicates were adjacent sections on different slides. Scale bar: 100 µm.

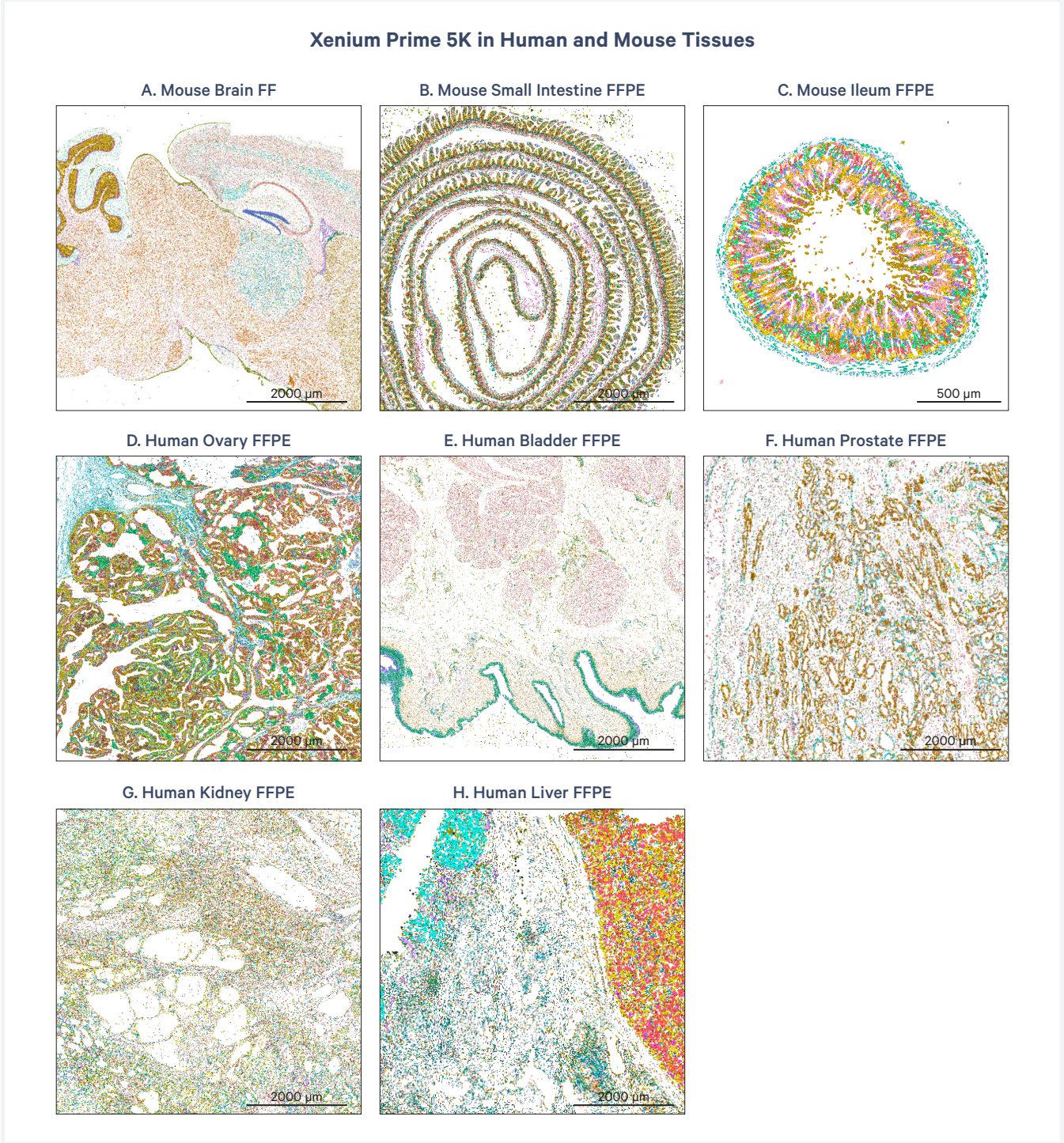


Figure 3. Cells colored by graph-based clustering results for Xenium Prime 5K data in a variety of human and mouse FF and FFPE tissues visualized in Xenium Explorer v3.0.

Xenium Prime Workflow

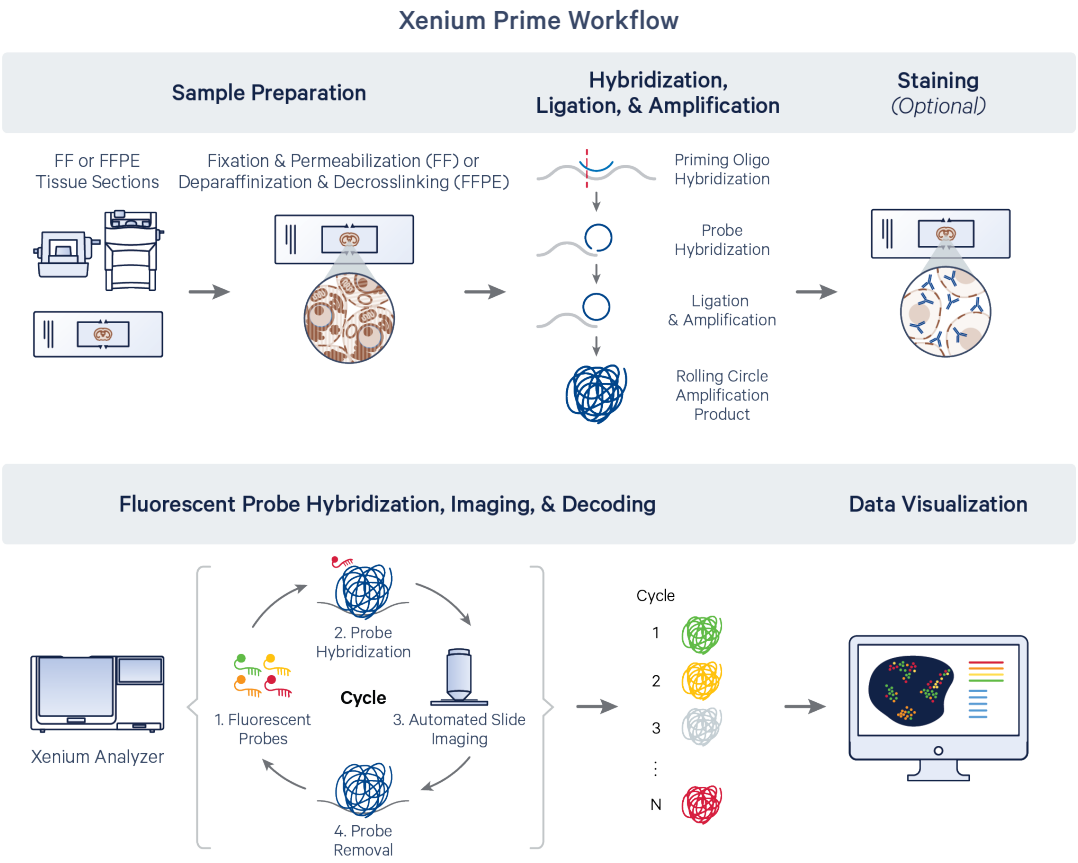


Figure 4. Xenium Prime assay workflow overview.

Figure 4 depicts the high-level workflow steps of the Xenium Prime assay and are described in more detail below.

Tissue and Sample Preparation

The Xenium Prime assay is compatible with both FFPE and FF tissues and is validated for human and mouse tissue types. Tissue and sample preparation can be performed as described in their related specific protocols:

- Tissue & Sample Preparation for FFPE (CG000578)
- Tissue & Sample Preparation for FF (CG000579)

Assay Workflow and Xenium Analyzer

Samples are processed as described in the Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining User Guide (CG000760). Prepared Xenium slides are loaded on the Xenium Analyzer where on-instrument imaging and decoding are performed. Full details on instrument setup are found in the Xenium Analyzer User Guide (CG000584). Multimodal cell segmentation is optional for Xenium Prime and does not require a separate decoding plate (unlike Xenium v1). For detailed information on cell segmentation staining, consult the Xenium In Situ Multimodal Cell Segmentation: Workflow and Data Highlights Technical Note (CG000750).

Samples processed for Xenium v1 assay followed instructions in the Xenium In Situ Gene Expression User Guide (CG000582)/Xenium In Situ Gene Expression with Cell Segmentation Staining User Guide (CG000749).

Pre-designed 5K Panels

The Xenium Prime assay includes the pre-designed Xenium Prime 5K Human Pan Tissue & Pathways Panel and Xenium Prime 5K Mouse Pan Tissue & Pathways Panel. These panels are designed to enable comprehensive cell type and cell state identification using publicaly available single cell RNA sequencing data. The panels also cover canonical signaling pathways as well as genes relevant to developmental biology, immuno-oncology, and genes that are well known in biomedical literature.

Xenium Prime 5K pre-designed and custom panels have a gene split design in which multiple codewords can be assigned to each gene, with 1 probe set per codeword and ~3 probe sets per gene (but up to 6 and as few as 1) (Figure 5). Gene splitting allows for an expansion of the Xenium codebook necessary for the higher plexy seen in Xenium Prime 5K. Consult the 10x Genomics Support Website for more information about panel design options and considerations.

Software

The Xenium In Situ software suite has been updated to v3.0 for Xenium Onboard Analysis (XOA), Xenium Explorer, and Xenium Ranger. This version enables analysis, visualization, and reanalysis of Xenium Prime 5K data.

XOA v3.0 includes changes to output files to accommodate the increase in transcript data. Xenium Explorer v3.0 introduces several improvements for interacting with transcript data. The new scaled transcript view allows for visualization of spatial transcript distribution for high and low expression genes, at any zoom level.

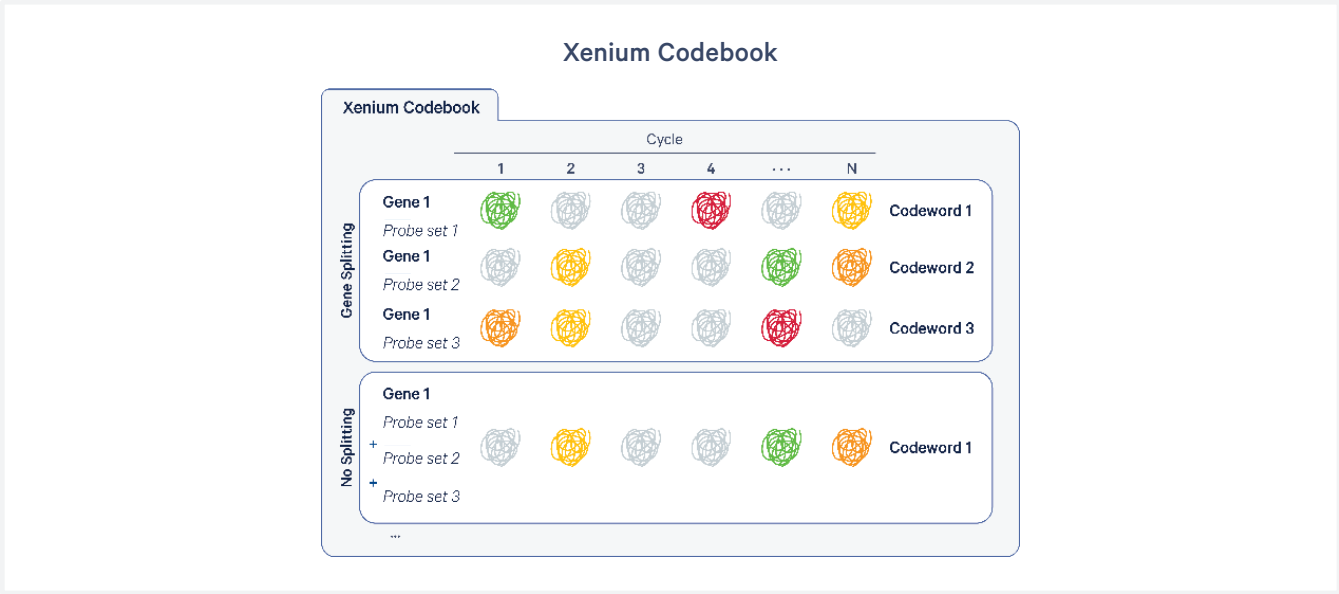


Figure 5. The Xenium codebook contains a collection of codewords that are assigned to genes in a given panel. Each codeword is defined by a pattern of fluorescent signal intensities recorded across channels and cycles. The codebook can either have a 1 codeword to 1 gene relationship ("no splitting", Xenium v1 panels) or a configuration where there are multiple codewords per gene ("gene splitting", Xenium Prime 5K panels). Multiple probes are used per gene to successfully capture biological variation (i.e., multiple isoforms). A probe set is a collection of probes designed to detect as many isoforms of a gene as possible based on GENCODE BASIC annotations. These modes are illustrated for an example gene.

Choosing Between Xenium v1 and Xenium Prime 5K

Xenium Prime 5K and Xenium v1 offer different advantages depending on the experimental needs. Choosing the right Xenium product will depend on a number of factors including the number of genes, species compatibility, and cost. Additional key differences are presented in Table 1. Consult the relevant product-specific documentation on the 10x Genomics Support Website for complete information.

	Xenium v1	Xenium Prime 5K
Tissue & Sample Prep		
	Tissue-agnostic sample preparation recommendations are similar for both Xenium v1 and Xenium Prime. When applicable, sample preparation differences are highlighted in the Demonstrated Protocols. Consult product-specific documentation on the 10x Genomics Support Website.	
Panels		
Genes per panel (#)	Up to 480	Up to 5100
Probe sets per gene	Pre-designed and custom panels: 8 probe sets/gene targeted	Pre-designed panel: 2–3 probe sets/gene targeted Custom panel: 5 probe sets/gene targeted
Codewords per gene	1, no splitting	>= 1, gene splitting
Panel customization options* *Consult the 10x Genomics Support Website for more information.*	• Add-on custom (up to 100) • Standalone custom (up to 480) • Species standalone • Advanced custom targets	• Add-on custom (up to 100) • Advanced custom targets
Species *Base panel species (pre-designed, or standalone if applicable) for custom designs*	Species with single cell reference data	Human or mouse
Assay		
10x Reagents Kits* **Consult the 10x Genomics Support Website for the most current list of available panels and part numbers.*	• Xenium Slides & Sample Prep Reagents, PN-1000460 • Xenium Decoding Consumables, PN-1000487 • Xenium Pre-Designed Gene Expression Panel* • Xenium Add-on Custom Gene Panel* • Xenium Standalone Custom Gene Panel* • Xenium Instrument Accessory Kit Module A PN-1000530 • Xenium Slide Kit, PN-1000659/1000660	• Xenium Prime Sample Prep Reagents, PN-1000720 • Xenium Decoding Consumables v2, PN-1000726 • Xenium Prime 5K Human Pan Tissue & Pathways Panel, PN-1000724 • Xenium Prime 5K Mouse Pan Tissue & Pathways Panel, PN-1000725 • Xenium Prime 5K Custom Add-On Panel (up to 50 genes), PN-1000731* • Xenium Prime 5K Custom Add-On Panel (51 to 100 genes), PN-1000766* • Xenium Thermocycler Adaptor v2 PN-1000739
	Kits are not interchangeable between Xenium v1 and Xenium Prime.	

	Xenium v1	Xenium Prime 5K
Assay protocol time	2 days (3 days if performing cell segmentation)	2 days (3 days if performing cell segmentation)
Multimodal Cell Segmentation (optional)		
10x Reagent Kits	• Xenium Cell Segmentation Add-on Kit, PN-1000662 • Kit includes Xenium Cell Segmentation Staining Reagents and Xenium Cell Segmentation Detection Reagents	• Xenium Cell Segmentation Staining Reagents, PN-1000661 • Detection reagents are included in the Xenium Prime 5K Decoding Reagents Kit (Module B - 5K). No separate plate is needed.
Instrument		
10x Reagent Kits **Consult the 10x Genomics Support Website for the most current list of available panels and part numbers.*	• Xenium Decoding Consumables, PN-1000487 • Xenium Decoding Reagents PN-1000461	• Xenium Decoding Consumables v2 PN-1000726 • Xenium Reagent Bottles PN-1000730 • Xenium Prime 5K Decoding Reagents PN-1000740
Approximate instrument run time **Consult the 10x Genomics Support Website for more detailed information.*	2–4 days	4–6 days
Software Versions		
Xenium Onboard Analysis	XOA v1.5 and higher XOA v2.0 and higher *(if performing multimodal cell segmentation)*	XOA v3.0 and higher
Xenium Explorer	XE v1.0 and higher	XE v3.0 and higher
Xenium Ranger	XR v1.6 and higher	XR v3.0 and higher

Table 1. Key differences between Xenium v1 and Xenium Prime 5K.

Data Highlights

Sensitivity Comparison of Xenium Prime 5K vs. Xenium v1

This highlight compares sensitivity and data quality metrics for Xenium v1 and Xenium Prime 5K generated with XOA v3.0. Xenium Prime data used the human and mouse pre-designed 5K panels. Xenium v1 data used the pre-designed Xenium Human Multi-Tissue and Cancer Panel (377 genes) or Xenium Mouse Tissue Atlassing Panel (379 genes).

The Xenium Prime 5K gene panels are designed to accommodate higher plexy while minimizing optical crowding. Xenium Prime 5K panels average 2-3 probe sets per gene and Xenium v1 panels average 8 probe sets per gene. The reduction in probe sets per gene for Xenium Prime 5K balances per-gene sensitivity with increased panel plexy. Due to differences in panel design, direct sensitivity comparisons are not recommended between Xenium v1 and Xenium Prime 5K datasets.

Depending on tissue type and preservation method, when comparing shared genes between assays, the Xenium Prime 5K assay has on average 20-40% of the per-gene sensitivity observed in the Xenium v1 assay (Figure 6). However, the Xenium Prime 5K assay generally detects a higher number of median transcripts per cell (Figure 2A) and has lower false discovery rates compared to Xenium v1 in human and mouse tissues (Figure 7).

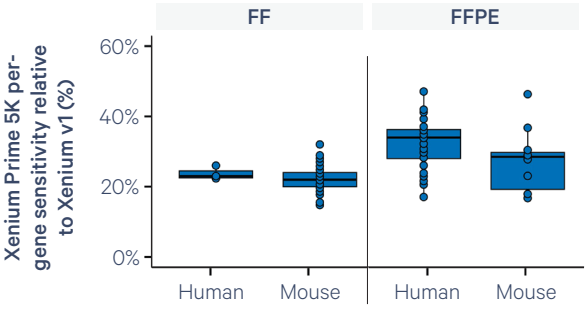


Figure 6. Plots showing the % per-gene sensitivity trade-off for Xenium Prime 5K data compared to Xenium v1 for shared genes between assays across a broad range of tissue types from human and mouse FF and FFPE samples. Consult the 10x Genomics Support Website for a list of tested tissue types. All data was analyzed on XOA v3.0. Per-gene sensitivity is measured by comparing the counts per cell of each gene and taking the median over all genes.

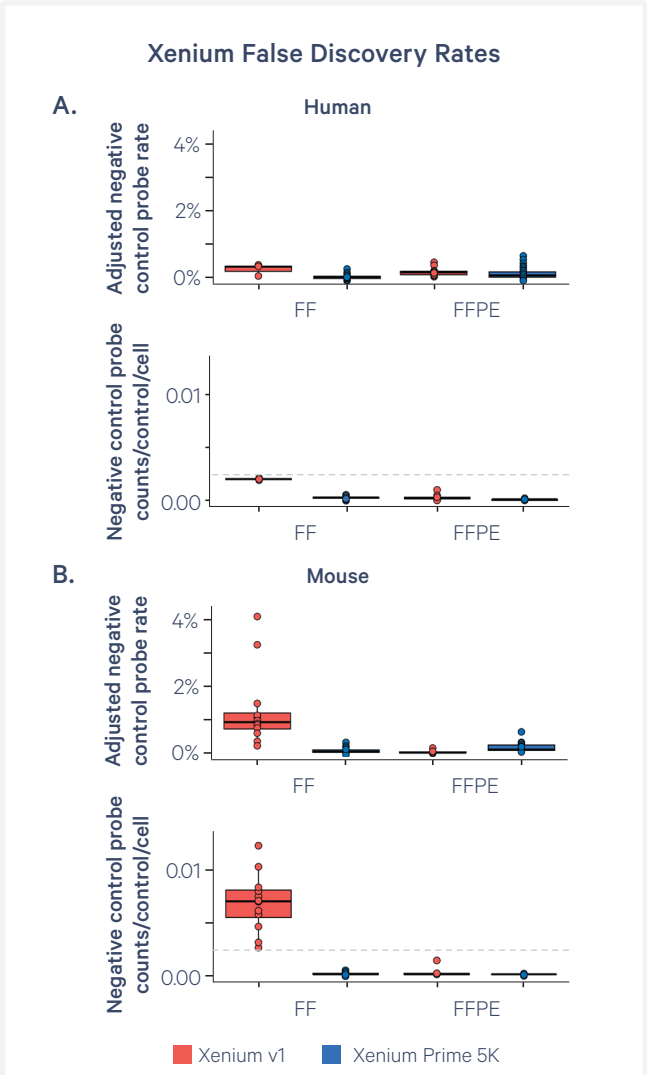


Figure 7. False discovery rates and counts for negative control probes across a broad range of tissue types from human **(A)** and mouse **(B)** FF and FFPE samples. Consult the 10x Genomics Support Website for a list of tested tissue types. XOA will show a warning alert in the analysis summary for negative control probe counts/control/cell if it is above the dotted line. This may indicate potential issues with the assay workflow and/or the input tissue block.

Below are the results for two example tissues. Regardless of variations in sample preparation method, both examples demonstrate good overall quality metrics (Table 2) and clustering (Figure 8).

	Xenium v1	Xenium Prime 5K
Human Kidney FFPE		
Number of cells detected	203,206	177,803
Median transcripts/cell**	29 19*	128 7*
Median genes/cell	17	109
High-quality transcripts	91.0%	90.1%
Total high-quality decoded transcripts	10,052,237	39,740,049
Nuclear transcripts (per 100µm^2)	75.5	330
Adjusted negative control probe rate	0.3%	0.2%
Mouse Liver FF		
Number of cells detected	71,056	65,746
Median transcripts/cell**	1,434 272*	2,352 108*
Median genes/cell	93	792
High-quality transcripts	89.1%	83.3%
Total high-quality decoded transcripts	144,468,586	213,948,519
Nuclear transcripts (per 100µm^2)	1,266.3	1,743.4
Adjusted negative control probe rate	0.5%	0.0%

**For shared genes between Xenium v1 and Xenium Prime 5K assays*

***Similar trends as reported in Figure 2A and Figure 6*

Table 2. Example of Xenium Prime 5K vs. Xenium v1 results for specific tissue types. Overall, the metrics illustrate higher transcript density and quality metrics and lower false discovery rates for the Xenium Prime 5K data. See Appendix for more information about the impact of XOA pipeline version on Xenium v1 samples.

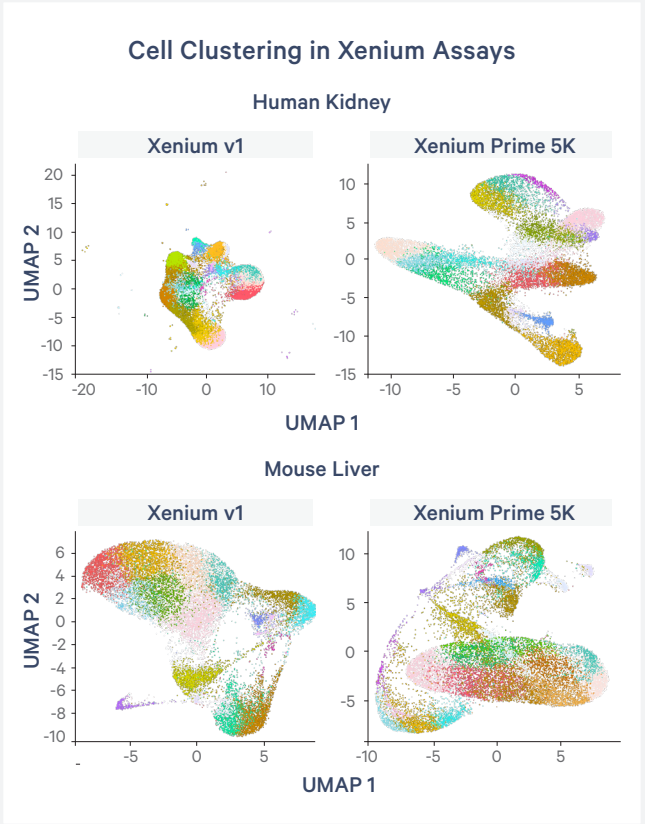


Figure 8. UMAP plots colored based on graph-based clustering results in human kidney and mouse liver samples processed using Xenium v1 and Xenium Prime 5K assays.

Sample condition and quality influence genomic DNA false discovery rate

Xenium Prime includes genomic control probes that target intergenic regions of the genome. Assay specificity is critical with higher plexy pan tissue gene panels, as there are more opportunities for off-target binding with the increase in probes per reaction. Including genomic control probes allows for measurement of probe binding to genomic DNA (gDNA), in addition to erroneous decoding and nonspecific probe binding.

The XOA v3.0 pipeline reports a more complete measure of false discovery rate using the genomic control probes ("adjusted genomic control probe rate"), in addition to the negative control probe and negative control codeword metrics (Figure 7). The rate is calculated as the fraction of high-quality

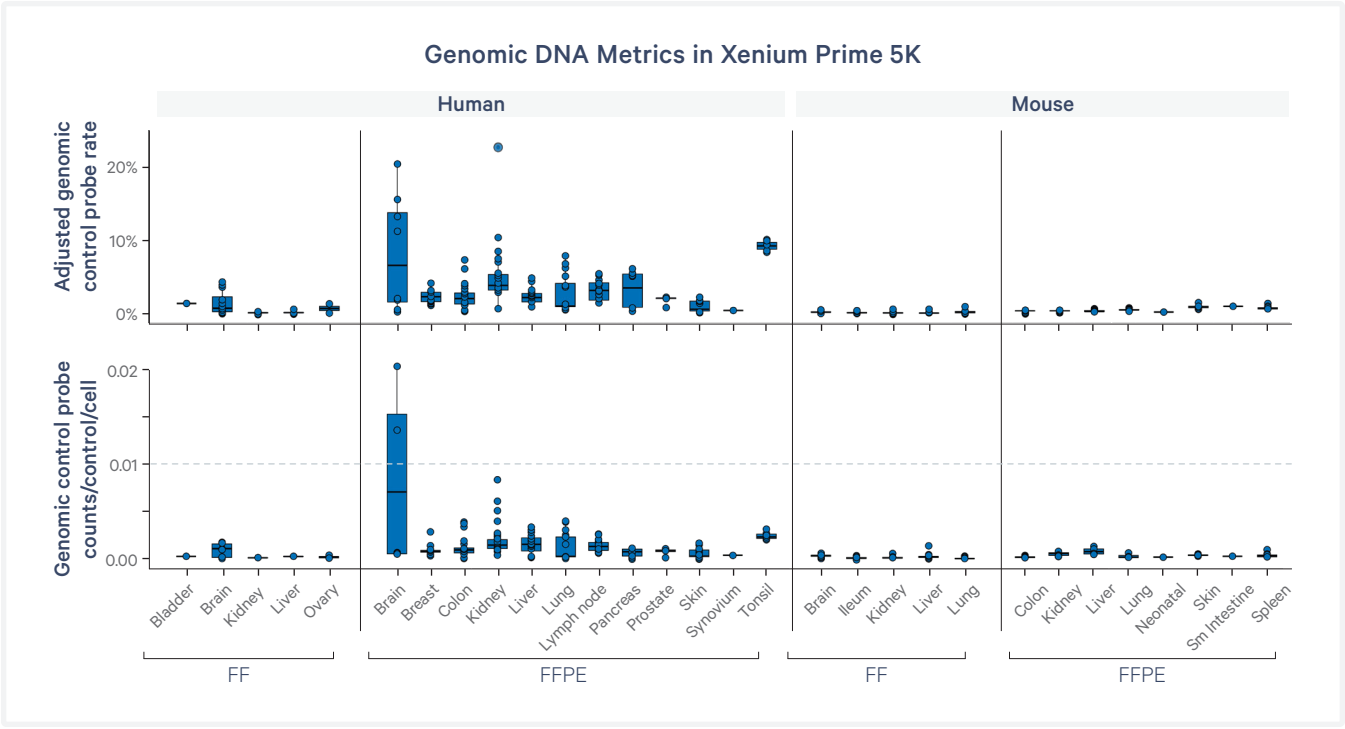


Figure 9. Genomic DNA false discovery rate and counts metrics for a variety of FF or FFPE human and mouse tissue types processed for Xenium Prime 5K. In general, data from human FFPE tissues had higher gDNA false discovery rate, which may be due to sample preparation and biology. Human samples include both healthy and diseased tissues. Mouse samples only include healthy tissues. See Appendix, Figure 13 for comparison to Xenium v1. XOA will show a warning alert in the analysis summary for genomic control probe counts/control/cell if it is above the dotted line. This may indicate potential issues with the assay workflow and/or the input tissue block.

transcripts that were assigned to genomic control probes (Phred quality score >= 20), divided by the fraction of probe-associated codewords in the panel that belong to genomic control probes.

The XOA v3.0 pipeline also reports the "genomic control probe counts per control per cell" and "estimated number of false positive transcripts per cell including genomic counts" in the analysis and metric summary output files. These metrics can help troubleshoot workflow or poor sample quality issues, indicate presence of background noise in the transcript data, and enable better interpretation and decision-making for data analysis.

While the Xenium Prime assay is designed to avoid denaturing gDNA, there are other sources of gDNA that could explain high levels in some Xenium Prime data. These include sample condition (i.e., necrotic regions), sample preparation method (i.e., under fixation for FFPE tissues, tissue arrays) (Figure 9), and/or the biology of the sample (i.e., cancerous tissues) (Figure 10).

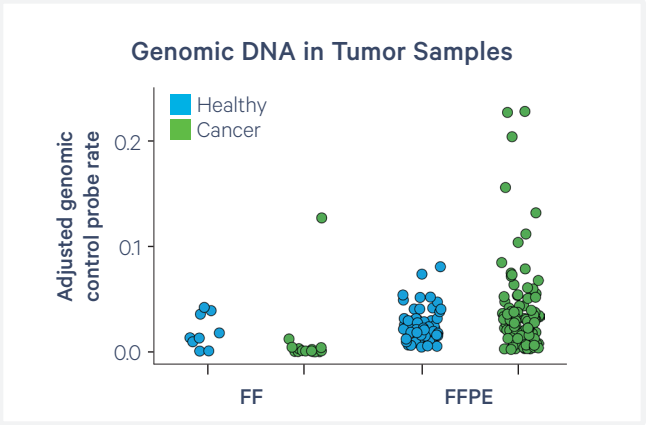


Figure 10. Adjusted genomic control probe rates for Xenium Prime 5K human healthy and cancer tissue samples. Genomic DNA false discovery rate is slightly higher for FFPE cancer tissues.

Sensitivity Comparison of Xenium Prime 5K vs. Visium HD Spatial Gene Expression

The Visium HD Spatial Gene Expression assay uses the Visium CytAssist instrument to process tissues placed on glass slides. First, probes targeting the whole transcriptome are hybridized to complimentary mRNA, followed by probe ligation. In the instrument, ligation products are released from the tissue and captured by spatially-barcoded oligos on the Visium HD slide. Finally, captured ligation products are prepared into libraries that are sequenced downstream. The Visium HD Slide contains a continuous lawn of oligos that allows for probe capture across the entire Capture Area, without gaps (Figure 11A).

Human brain cancer, Human ovary cancer, and mouse intestine FFPE blocks were sectioned onto Xenium Slides for the Xenium workflow and glass slides for the Visium HD workflow. The Xenium and Visium HD assays were performed on serial tissue sections (human brain cancer, human ovary cancer) or non-serial sections from the same tissue block (mouse intestine). For Xenium, the Visium HD H&E and Xenium DAPI images were aligned so that the analyzed area was restricted to the same tissue region covering the paired Visium HD Capture Area. Only genes that are common between the Xenium and Visium HD probe sets were analyzed to compare sensitivity differences between the assays. Visium HD libraries were sequenced to the saturation levels noted in the figure.

The expression of genes shared between assays is highly correlated in normal and cancer tissue (Figure 11B). Per-gene sensitivity for genes in the Xenium 5K pre-designed panel is typically higher than the same genes detected with the Visium HD assay. Generally, transcript density is higher for Visium HD vs Xenium Prime 5K. For example, for the shared region in the human ovarian cancer sample shown in Figure 11, Visium HD total detected molecules are 72,666,265 while Xenium Prime 5K total detected molecules are 59,718,497.

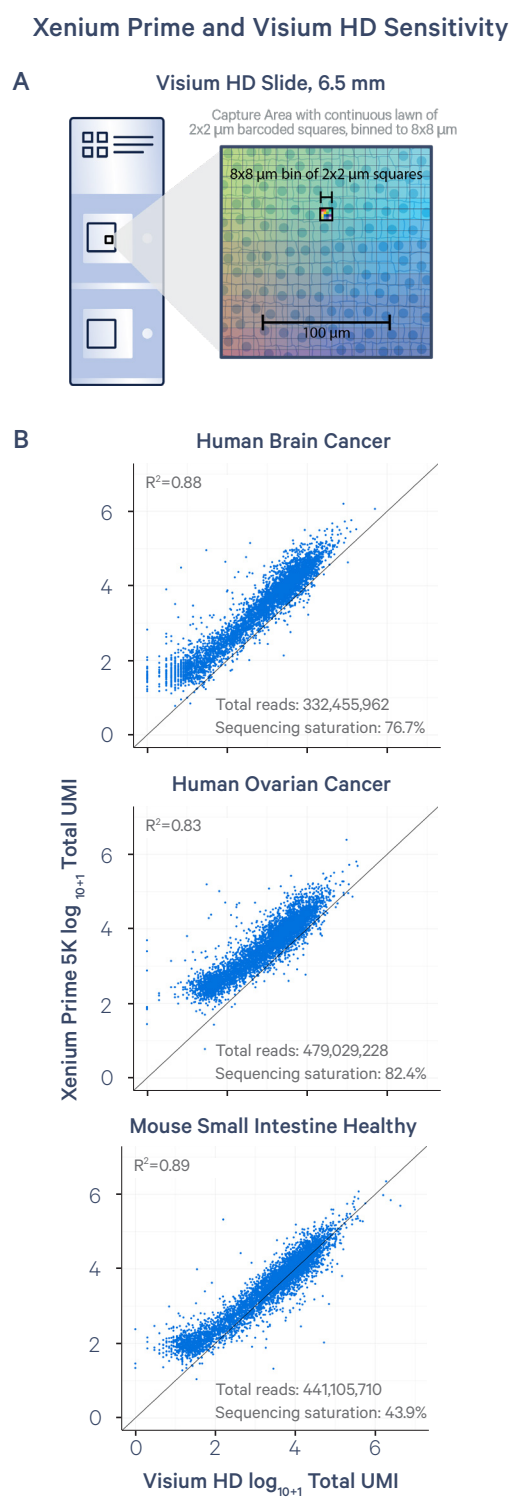


Figure 11. (A) Visium HD slide schematic showing continuous lawn of barcoded squares. **(B)** Spearman correlation of total detected molecules by both the Xenium Prime 5K and Visium HD assays. Each dot represents one gene.

It is important to note that all metrics may vary from sample to sample. For tissues processed with Visium HD that have not hit sequencing saturation, more information can be obtained from the sample with additional sequencing. The mouse small intestine sample, for example, was only sequenced to 43.9% saturation.

Choosing the optimal assay for a given application will depend on experimental goals. For example, the Visium HD assay may be an ideal choice for single cell scale discovery applications (e.g. broad tissue characterization, de novo identification of cell types) as the assay targets the whole transcriptome. Since per-gene sensitivity is generally higher with Xenium Prime 5K but targets a subset of the transcriptome, Xenium Prime 5K may be an ideal choice for more targeted analyses that require subcellular resolution.

Conclusions

The Xenium Prime assay utilizes pre-designed probe panels targeting ~5,000 genes to enable high-fidelity spatial analysis of mouse and human tissues. As noted in this Technical Note, the Xenium Prime assay features:

- Ability to analyze >5,000 genes with up to 100 gene add-on for custom panels
- Analyze data in 4-6 days on average and visualize with Xenium Explorer
- High reproducibility across replicates and compatible with Cell Segmentation Staining workflow
- Includes genomic control probes that enable the Xenium Prime assay to measure gDNA false discovery

Choose the assay that best meets the requirements of the research questions and experimental design.

Appendix

Xenium v1 data comparison analyzed on XOA v2.0 vs. XOA v3.0

XOA v3.0 includes improvements leading to sensitivity gains for both Xenium v1 and Xenium Prime panels. While XOA v3.0 is required for Xenium Prime, users with active studies on Xenium v1 assays who upgrade to instrument software v3.0 can select XOA v2.0 during instrument run setup to avoid batch effects. The XOA v3.0 pipeline resulted in a 1.2-1.9 fold increase in transcript density across human and mouse FF and FFPE samples for Xenium v1 samples compared to XOA v2.0 (Figure 12A). The sensitivity increase in XOA v3.0 is achieved through an adjustment of the optical system's point spread function model that is specifically calibrated to each instrument. This improves the detection and localization of puncta in densely populated areas, such as perinuclear areas. It also allows for increased detection and localization of puncta near the edges of field of views (FOVs), effectively minimizing FOV-related artifacts.

Due to sensitivity gains in the XOA v3.0 software, batch effects may be observed between data analyzed on XOA v2.0 and XOA v3.0. Key metrics are similar between versions for less dense tissues (or tissue regions), such as the human kidney, but can differ more dramatically for dense tissues (or tissue regions), such as mouse liver or brain (Table 3 and Figure 12B). For dense tissue samples, we recommend analyzing data with the same pipeline version or consider batch correction during analysis. Batch correction with 10x software is currently unsupported, however community-developed tools may help.

	XOA v2.0	XOA v3.0
Human Kidney FFPE		
Number of cells detected	203,227	203,206
Median transcripts/cell	28	29
Nuclear transcripts/100µm²	66.7	75.1
Total high quality decoded transcripts	9,670,435	10,052,237
Mouse Brain FF		
Number of cells detected	148,723	148,734
Median transcripts/cell	197	394
Nuclear transcripts/100µm²	334.6	716.0
Total high quality decoded transcripts	64,697,389	111,376,230

Table 3. Key metrics for human kidney FFPE and mouse brain FF analyzed on XOA v2.0 and XOA v3.0.

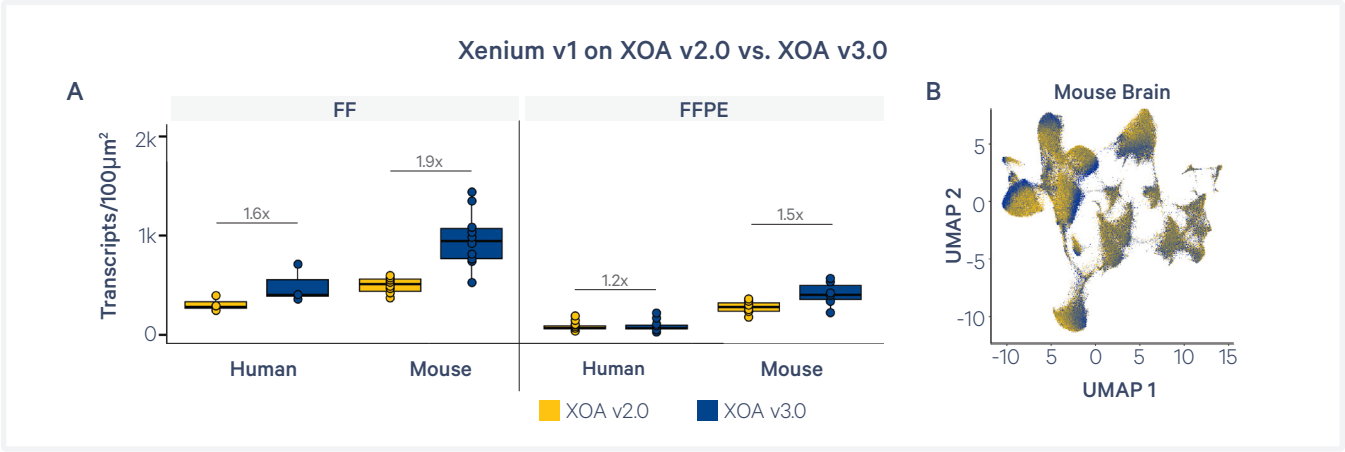


Figure 12. (A) Transcript density across a broad range of tissue types from human and mouse FF and FFPE samples analyzed on XOA v2.0 vs. XOA v3.0. Consult the 10x Genomics Support Website for a list of tested tissue types. Fold increase shown in gray. **(B)** Unsupervised clustering of a mouse brain sample analyzed with XOA v2.0 and XOA v3.0 shows batch effects in highly dense tissue.

Genomic DNA in Xenium v1 vs. Xenium Prime 5K

While not commercially available for the Xenium v1 assay, genomic control probes were used in internal studies to compare genomic DNA in Xenium v1 and Xenium Prime 5K (Figure 13). The analysis shows that elevated genomic control probe rates and counts are more dependent on sample source and quality than assay type, and they are lower in Xenium Prime 5K.

Negative control probes are present in Xenium v1 and Xenium Prime 5K panels. Importantly, negative control rates and probe counts are low overall for both assays, and again lower in Xenium Prime 5K samples (Figure 7).

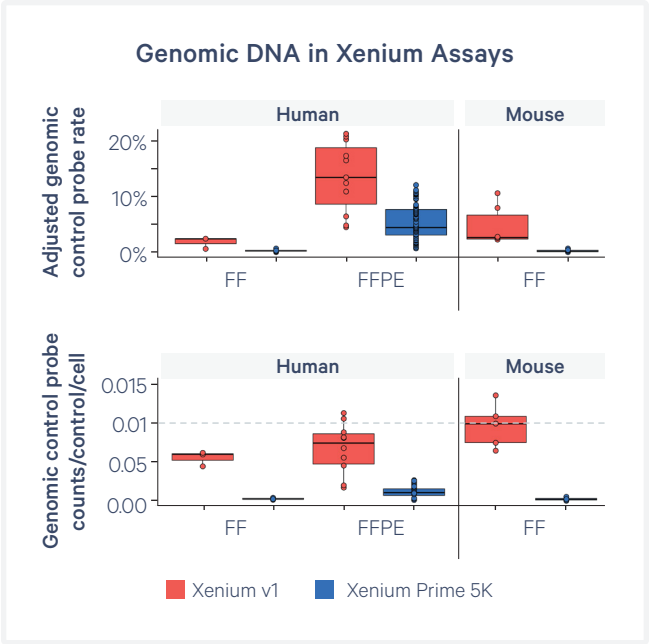


Figure 13. False discovery rates and counts for genomic control probes across a broad range of tissue types from human and mouse FF and FFPE samples. Consult the 10x Genomics Support Website for a list of tested tissue types. XOA will show a warning alert in the analysis summary for genomic control probe counts/control/cell if it is above the dotted line. This may indicate potential issues with the assay workflow and/or the input tissue block.

References

1. Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining User Guide (CG000760)
2. Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578)
3. Xenium In Situ - Fresh Frozen Tissue Preparation Handbook (CG000579)
4. Xenium Analyzer User Guide (CG000584)

Document Revision Summary

Document Number	CG000775
Title	Xenium Prime 5K: Workflow, Analysis & Data Highlights
Revision	Rev B
Revision Date	August 2025
Description of Changes	Updated references for Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578) throughout. Updated references for Xenium In Situ - FF Tissue Preparation Handbook (CG000579) throughout. Updated for general minor consistency of language, format, and terms throughout.

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