Post-Xenium In Situ Applications: Immunofluorescence, H&E, Visium v2, and Visium HD

Introduction

The Xenium In Situ Gene Expression (Xenium v1) or Xenium Prime In Situ Gene Expression (Xenium Prime 5K) workflows measure gene expression at subcellular resolution in fresh frozen (FF) and formalin fixed & paraffin embedded (FFPE) tissue sections. Gene expression is measured via pre-designed or custom probe panels that target hundreds of genes of interest in a variety of tissue types. Many on-market spatial technologies result in tissue destruction or damage at the end of their workflows, preventing use of these sections for downstream applications. In contrast, tissue sections processed using the Xenium In Situ workflow remain largely intact post-run and can be used for additional applications. Examples of feasible Xenium post-run workflows include immunofluorescence (IF) staining, H&E staining, and whole transcriptome spatial RNA sequencing using the Visium CytAssist Spatial Gene Expression (Visium v2) or Visium HD Spatial Gene Expression (Visium HD) workflows. While tested extensively, 10x Genomics does not guarantee performance of these post-Xenium application workflows.

This Technical Note highlights the experimental steps and key data metrics obtained downstream of the Xenium (post-Xenium) workflow on the same tissue sections, in relation to controls not processed through the Xenium workflow. These data highlight the minimal impact of the Xenium platform on tissue integrity, providing the opportunity to obtain additional information downstream of the workflow on the same tissue sections.

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Experimental Design Overview

After processing tissue sections through either the Xenium In Situ Gene Expression (Xenium v1) or Xenium Prime In Situ Gene Expression (Xenium Prime 5K) workflow, the same sections were used for post-Xenium applications. This document describes the methods used for each post-Xenium application along with data highlights. An overview of the Data Analysis methods is also provided.

Post-Xenium Applications Tested

- IF staining
- H&E staining
- H&E staining followed by Visium CytAssist Spatial Gene Expression (Visium v2) or Visium HD Spatial Gene Expression (Visium HD)

Tissue Preparation

The table below lists the tissues and tissue preservation methods used. Post-Xenium samples were compared to controls (samples not processed through the Xenium workflow) using 10 µm (FF) and 5 µm (FFPE) serial sections.

Organism	Tissue	Type	Vendor
Mouse	Brain	FF *(OCT compound-embedded)*	Charles River Laboratories
Human	Breast	FFPE	Cyence Biopathology
	Colon	FFPE	Avaden Biosciences
	Lung	FFPE	Avaden Biosciences

Prior to staining and CytAssist workflows, sections were either:

- placed on Superfrost Plus Slides (Fisherbrand, PN 12-550-15) and used as controls (not processed with the Xenium workflow prior to staining-only or CytAssist-only workflows)
- placed on Xenium slides for Xenium processing (post-Xenium)

Post-Xenium v1 FF mouse brain and FFPE human breast and human colon sections used for post-Xenium applications were generated using the Xenium Mouse Brain Gene Expression Panel (PN 1000462), the Xenium Human Breast Gene Expression Panel (PN 1000463), and a custom gene panel designed for human colon gene expression profiling.

Post-Xenium Prime 5K FFPE human lung sections used for post-Xenium applications were generated using the Xenium Prime 5K Human Pan Tissue & Pathways Panel (PN-1000724).

Imaging

IF and H&E stained slides were imaged at 20X magnification using a VS200 Slide Scanner. 20X magnification is recommended for accurate pathological assessment of tissue sections. For applications requiring higher resolution, such as cell segmentation, 40x or greater magnification is recommended.

Data Analysis

Visium samples were processed with Space Ranger v2.1 (https://www.10xgenomics.com/support/software/space-ranger/downloads) for Visium v2 comparisons and Space Ranger v3.0 for Visium HD comparisons. Downstream analyses were performed using R version 4.2, with a combination of packages including tidyverse, Seurat, and GenomicRanges. Briefly, filtered_feature_bc_matrix.h5 files were read using Seurat::Read10x_h5. For Visium HD data analysis, Seurat v5.1.0 was used. Non-transformed UMI counts were used for all expression calculations unless otherwise indicated. Graph-based clustering results from Space Ranger v2.1 of v3.0 were used for clustering plots. Overlapping probes were identified between Xenium and Visium using the publicly available .bed files available for each technology and the GenomicRanges::findOverlaps function followed by individual calculation of fraction overlaps for probes that do overlap. Plots were made using ggplot2, ggpubr, ggforce, and patchwork.

Refer to the Xenium Explorer support website for compatible file format details.

Additional Information

Because cell segmentation antibodies in the Xenium Multi-Tissue Stain Mix are raised in rabbit, do not use anti-rabbit secondary antibody for post-Xenium applications as it will result in cross-detection. For all post-Xenium applications after cell segmentation staining, use primary antibodies that are not raised in rabbits. If using a primary antibody raised in rabbit, ensure that it is directly conjugated to a detection tag (such as a fluorescent tag or biotin), thereby negating the use of a secondary anti-rabbit antibody for detection.

IF & H&E Staining Post-Xenium v1

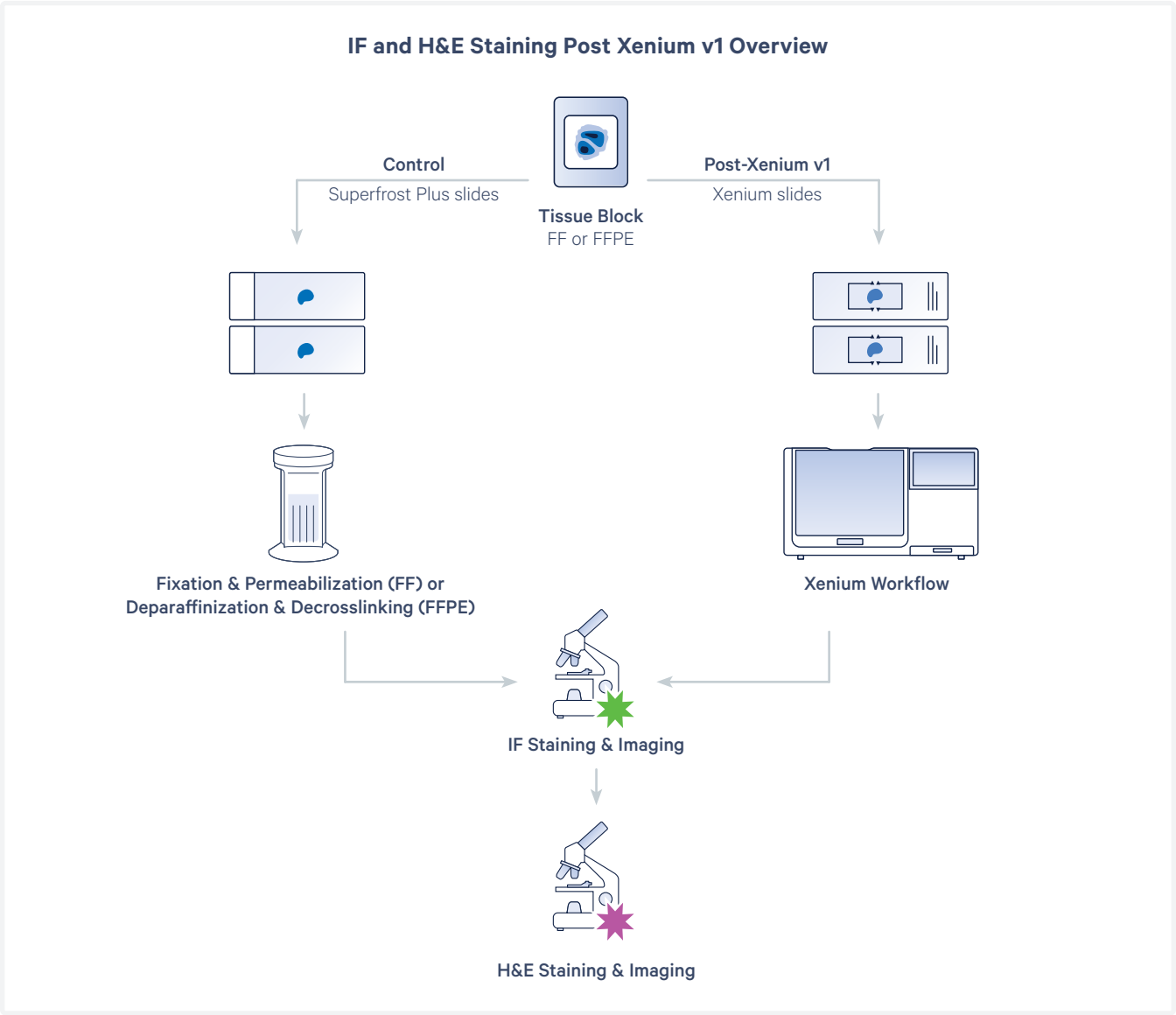

Figure 1. Post-Xenium v1 IF and H&E staining are feasible with both FF (in OCT compound) and FFPE-embedded tissue samples and can be performed in tandem on the same post-Xenium sections. Serial sections from FF and FFPE tissue blocks were prepared. Sections 1 and 4 were placed onto Superfrost Plus slides (staining-only workflow controls) while sections 2 and 3 were placed onto Xenium slides and processed with the Xenium workflow on one Xenium Analyzer instrument run. See Methods section for more details about staining in staining-only control slides and post-Xenium slides.

Methods: *IF & H&E Staining Post-Xenium v1*

FF mouse brain and FFPE human breast samples were in the following experiment.

IF Staining: Post-Xenium Slides

Post-Xenium slides were immediately used or stored in 1,000 µl PBS-T (0.05%) for up to three days at 4°C. PBS-T was removed and slides were washed with 500 µl of PBS-T for a total of three washes, one minute each, before proceeding with the staining workflow. Blocking Buffer was prepared as a solution consisting of 1X PBS pH 7.4, 0.1% of Tween-20, 10% heat-inactivated FBS, and 10 mg/ml dextran sulfate. Staining Buffer was prepared as a solution consisting of fluorescently-labeled primary antibodies in Blocking Buffer.

Optimal staining concentrations were pre-determined by titration using serial sections of the same tissue blocks placed on Superfrost Plus slides and an alternative sample prep workflow similar to the Xenium workflow, as described in the following IF Staining: Control Slides section. This surrogate approach yielded similar results between post-Xenium and control staining-only slides.

Post-Xenium slides were incubated in 500 µl Blocking Buffer for one hour at room temperature. Blocking Buffer was removed and 500 µl Staining Buffer was added. Slides were incubated overnight at 4°C in the dark. Following overnight incubation, slides were washed with 500 µl PBS-T for a total of three washes, ten minutes each. After removal of PBS-T, slides were stained with 500 µl of 5 µg/ml DAPI for one minute. Slides were washed with 500 µl PBS-T for a total of three washes, one minute each.

Slides were mounted with 70 µl of SlowFade Diamond Antifade Mountant (Invitrogen, PN S36963), and imaged at 20X magnification. Alternatively, slides can be mounted using Epredia Cytoseal Mountant XYL (Fisher Scientific, PN 8312-4), for hardset mounting. If continuing with H&E staining a loose mount is preferred.

Staining reagents and conditions used can be found in the Appendix.

IF Staining: Control Slides

FF controls were fixed and permeabilized according to the Xenium In Situ for Fresh Frozen Tissues - Fixation & Permeabilization Demonstrated Protocol (CG000581).

FFPE controls were baked and deparaffinized similarly to the Xenium In Situ for FFPE - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580), except for the decrosslinking step. Following deparaffinization, FFPE IF-control sections placed on Superfrost Plus slides were placed in a Xenium Cassette and decrosslinking was performed in a thermal cycler using 500 µl of 1X Citrate (pH 6.0) at 95°C for 30 minutes.

Control slides (FF or FFPE) were washed, Blocking and Staining Buffers were prepared, and staining incubations and imaging were performed concurrently with post-Xenium slides, following the steps described previously to reduce potential experimental variability between samples.

H&E Staining *(on IF-stained slides)*: **Post-Xenium Slides**

Post-Xenium IF-stained slides were decoverslipped by dipping in Milli-Q Water and dipped 30 additional times to completely remove mounting media. Autofluorescence Quenching Solution added during the Xenium workflow according to Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582) was removed and H&E staining performed following the Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613).

H&E Staining *(on IF-stained slides)*: **Control Slides**

FF mouse brain and FFPE human breast IF-stained control slides were H&E stained following the same steps concurrently with the post-Xenium slides, except that Xenium Quencher Removal was not performed as Autofluorescence Quenching Solution was not used on control slides.

Results: *IF & H&E Staining Post-Xenium v1*

The following post-Xenium and control IF and H&E staining images were obtained from two tissue types. Replicate samples indicate two serial sections placed on separate slides so that each post-Xenium slide has a comparable control slide (sections 2 and 3 were placed on Xenium slides and sections 1 and 4 were placed on Superfrost Plus slides). Figure 1 provides an overview of the post-Xenium Staining workflow. FF mouse brain results are displayed in Figures 2-5 and FFPE human breast results are displayed in Figures 6-9.

Fresh Frozen Mouse Brain

FF mouse brain tissue sections post-Xenium were stained with DAPI and fluorescently-labeled antibodies for glial fibrillary acidic protein (GFAP) and neuronal nuclear antigen (NeuN) on two replicate sections. IF staining across the two post-Xenium replicate tissue sections were consistent (Figure 2).

IF staining in post-Xenium sections, while reproducible across replicates, was dimmer compared to IF staining-only controls (sections not processed with the Xenium workflow prior to IF staining) using the same microscope settings for the two stained target proteins (Olympus VS 200 slide scanner, 20X/NA0.8 objective, 100% LED power; GFAP, channel 532, exposure 1.75 ms; NeuN, channel 488, exposure 50 ms; Figure 3).

Weaker signal in post-Xenium slides could be attributed to the presence of Autofluorescence Quenching Solution on post-Xenium slides or differences in sample preparation (i.e. decrosslinking conditions) between the post-Xenium and staining-only control workflows (Figure 3).

To show that the biological interpretation of a given protein expression is retained post-Xenium, the image contrast was adjusted to similar intensity as the staining-only control following microscope acquisition in a representative post-Xenium section (GFAP marker, Figure 4). Therefore, for optimal imaging outcomes of IF-stained post-Xenium sections, it is recommended that users determine microscope imaging settings that yield the best signal intensities during image acquisition.

The same IF-stained post-Xenium sections were H&E stained to observe their morphological features compared to staining-only controls. Results were consistent between replicates and highly comparable to control staining-only serial sections (Figure 5).

Together, these data highlight that post-Xenium IF and H&E stainings are similar to staining-only controls, supporting that biological interpretations based on the tested markers are retained in post-Xenium slides.

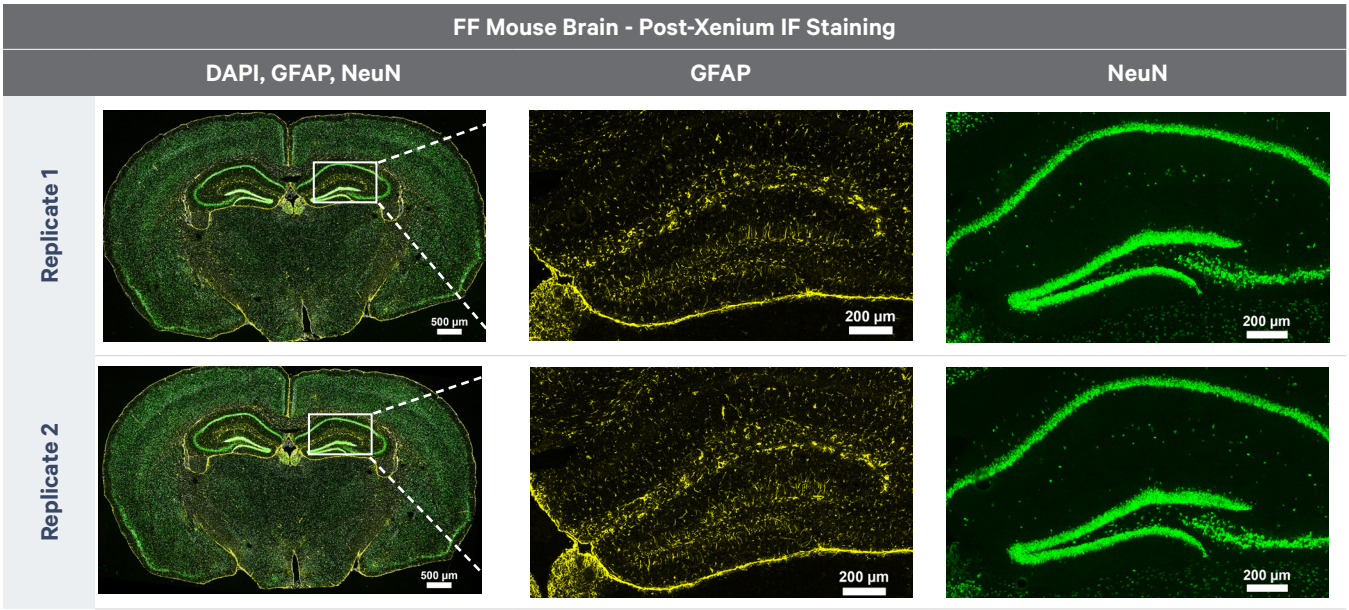

Figure 2. IF staining using DAPI (white) and fluorescently-labeled conjugated antibodies for GFAP (yellow) and NeuN (green) on post-Xenium sections in two replicates (top and bottom). Box highlights detailed images showing GFAP and NeuN staining. DAPI is a marker for nuclei. GFAP is a marker for astrocytes. NeuN is a marker for post-mitotic neurons.

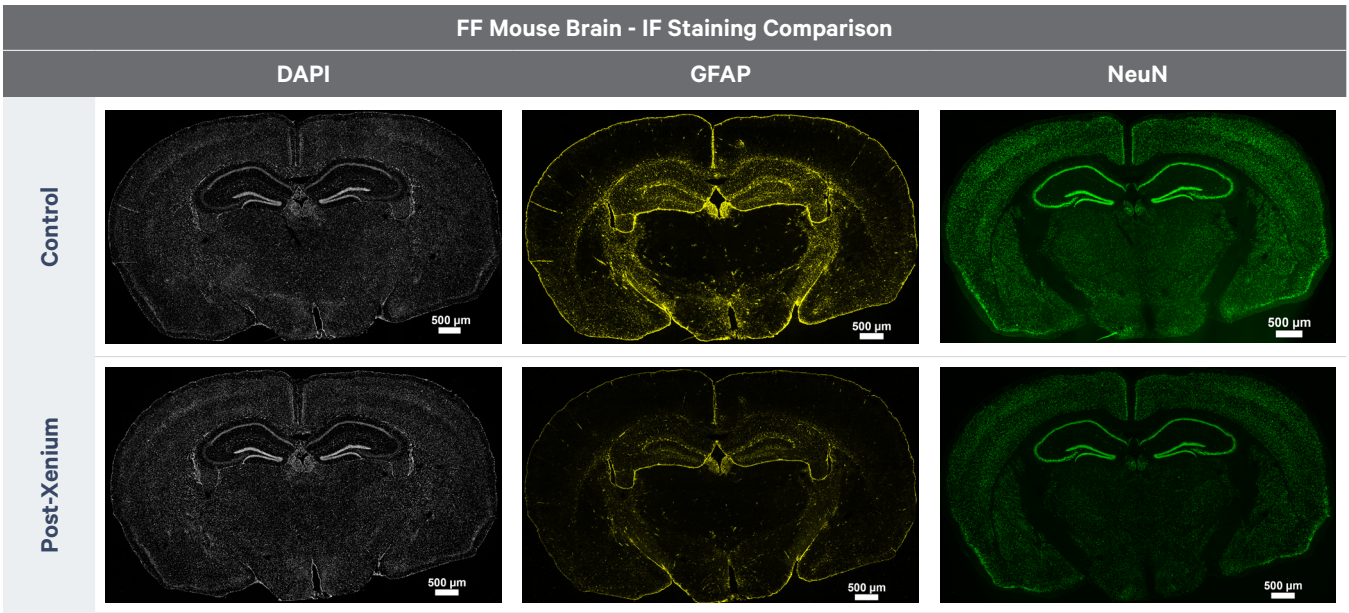

Figure 3. Side-by-side comparison of IF-stained (DAPI, GFAP, and NeuN) control sections not processed through the Xenium workflow prior to IF staining (top, representative replicate) and post-Xenium sections (bottom, representative replicate). DAPI is a marker for nuclei. GFAP is a marker for astrocytes. NeuN is a marker for post-mitotic neurons.

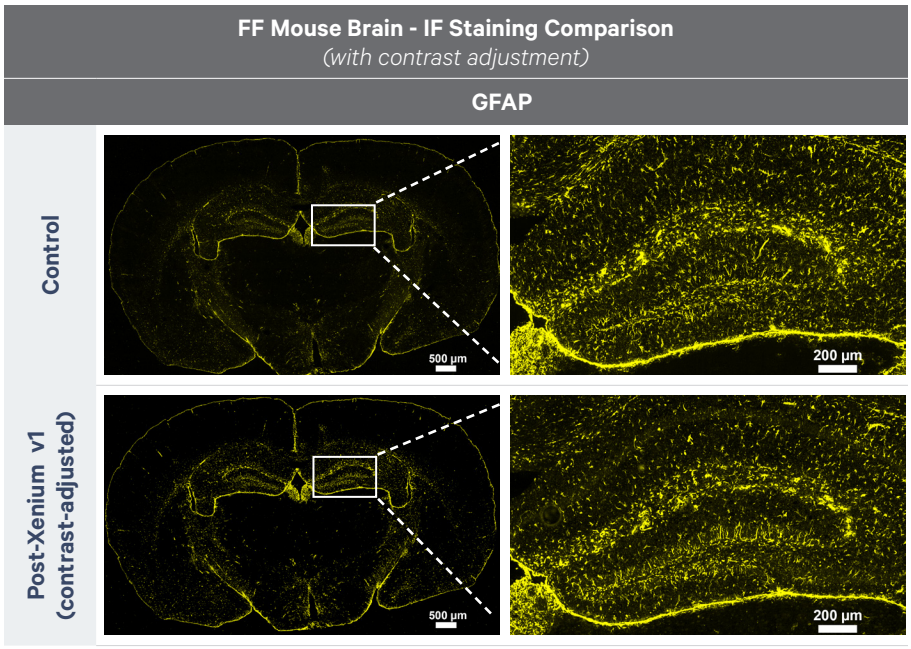

Figure 4. Comparison of a representative control section not processed through the Xenium workflow prior to IF staining with original image settings (top) and a representative IF-stained (GFAP) post-Xenium section in which the contrast was manually adjusted following image acquisition (bottom). Box highlights detailed images showing GFAP staining.

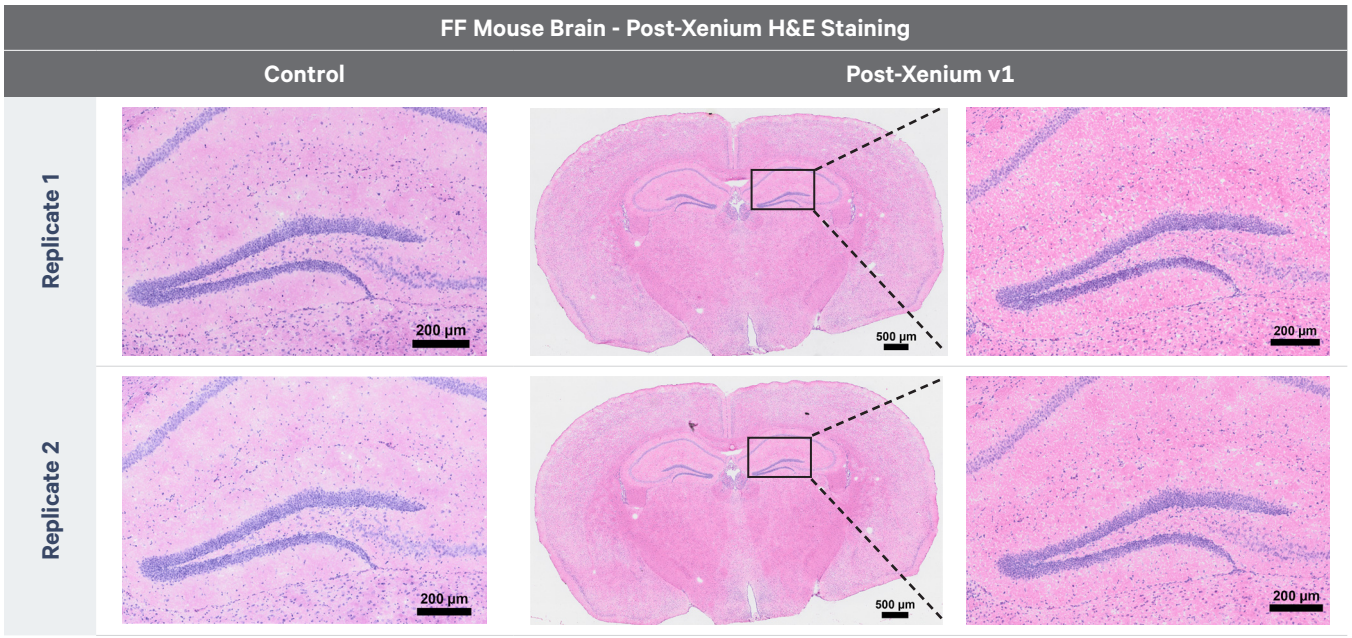

Figure 5. H&E staining on control sections not processed through the Xenium workflow prior to H&E staining (left panel) and post-Xenium sections (right panel). H&E staining was performed on sections following IF staining.

FFPE Human Breast

Protein detection via IF staining was performed on post-Xenium and control FFPE human breast tissue sections using DAPI and fluorescently-labeled antibodies for vimentin (VIM) and α-smooth muscle actin (α-SMA). Protein staining for VIM and α-SMA on post-Xenium FFPE tissue sections was reproducible across replicate samples (Figure 6). Although similar, IF staining in post-Xenium FFPE human breast tissue sections showed dimmer signal compared to controls not processed through the Xenium workflow using the same microscope imaging settings (Olympus VS 200 slide scanner, 20X/NA0.8 objective, 100% LED power; VIM, channel 532, exposure 1 ms; α-SMA, channel 590, exposure 1.25 ms; Figure 8). As observed in the FF mouse brain experiment, the FFPE human breast data demonstrates that protein detection post-Xenium is feasible, although some variation in staining intensity may be expected between staining-only controls and post-Xenium samples.

When the contrast was adjusted following microscope acquisition, IF staining in a post-Xenium section was comparable to the staining-only control (Figure 8), supporting that biological interpretations are retained for the tested antibodies. Additionally, H&E staining in the post-Xenium replicate samples was highly preserved and comparable to the control staining-only samples (Figure 9).

These results highlight the minimal impact of the Xenium workflow on tissue integrity in FFPE samples, enabling detection of protein and morphology analysis on the same sections.

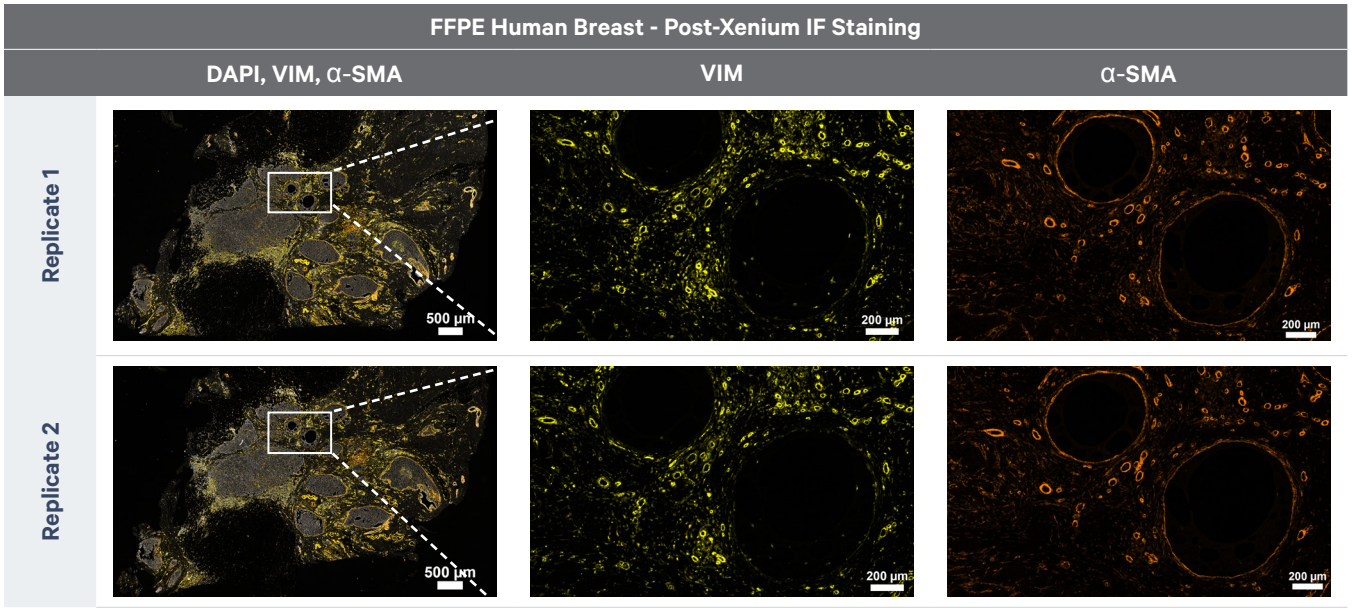

Figure 6. IF staining using DAPI (white) and fluorescently-labeled conjugated antibodies for VIM (yellow) and α-SMA (orange) on post-Xenium sections in two replicates (top and bottom). Box highlights detailed images showing VIM and α-SMA staining. DAPI is a marker for nuclei. VIM is a marker for the cytoskeleton. α-SMA is a marker for smooth muscle.

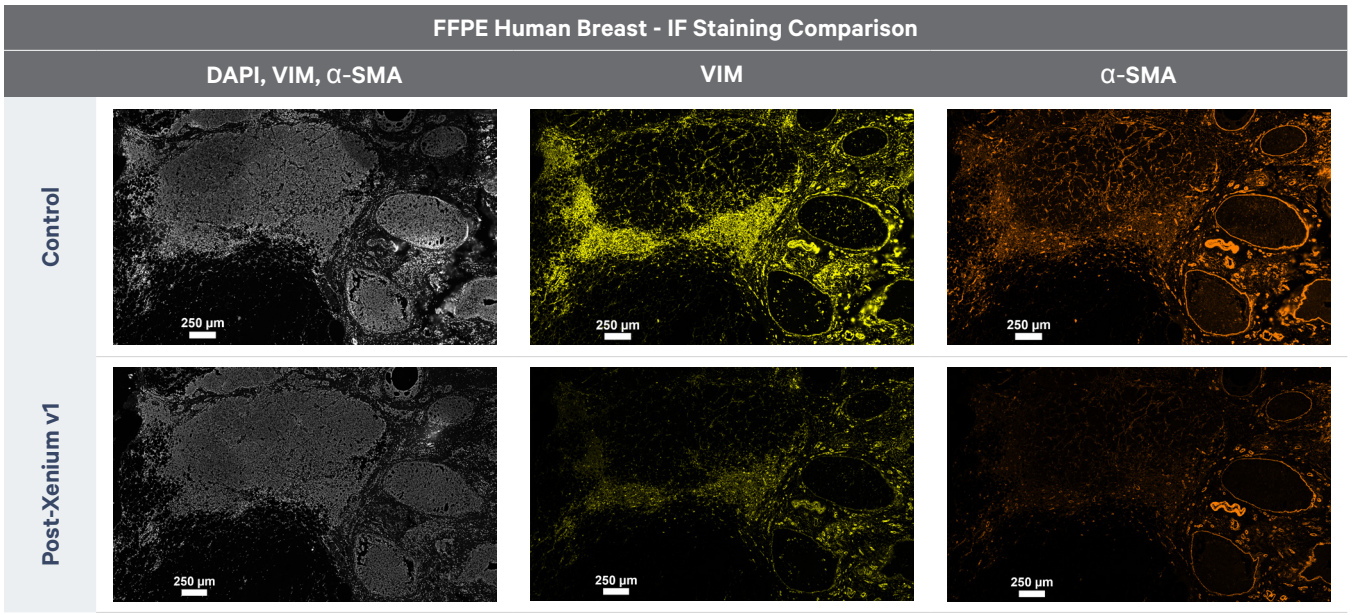

Figure 7. Side-by-side comparison of IF-stained (DAPI, VIM and α-SMA) control sections not processed through the Xenium workflow prior to IF staining (top, representative replicate) and post-Xenium sections (bottom, representative replicate). DAPI is a marker for nuclei. VIM is a marker for the cytoskeleton. α-SMA is a marker for smooth muscle.

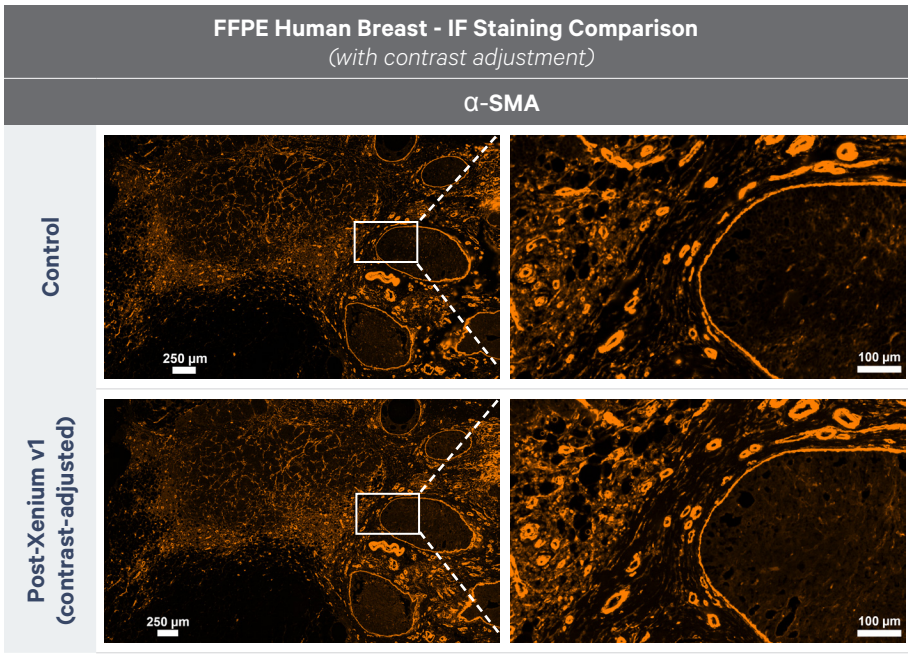

Figure 8. Comparison of a representative control section not processed through the Xenium workflow prior to IF staining with original image settings (top) and a representative IF-stained (α-SMA) post-Xenium section in which the contrast was manually adjusted following image acquisition (bottom). Box highlights detailed images showing α-SMA staining.

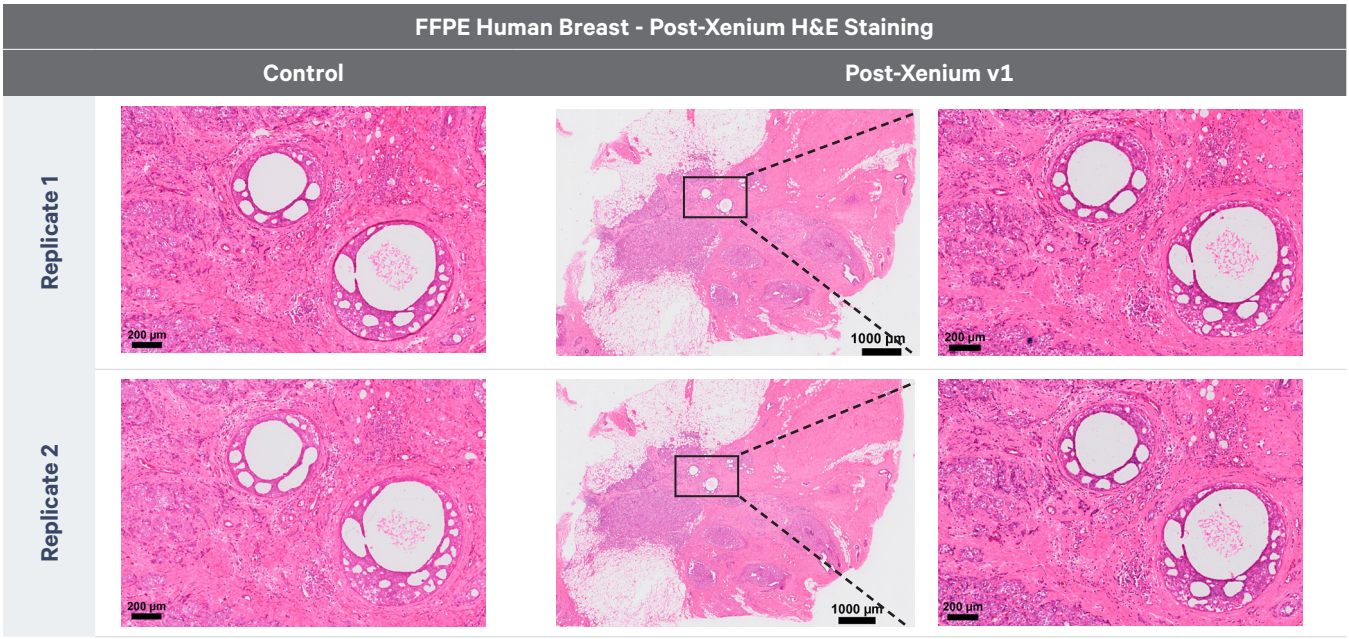

Figure 9. H&E staining on control sections not processed through the Xenium workflow prior to H&E staining (left panel) and post-Xenium sections (right panel). H&E staining was performed on sections following IF staining.

Visium v2 with H&E Staining Post-Xenium v1

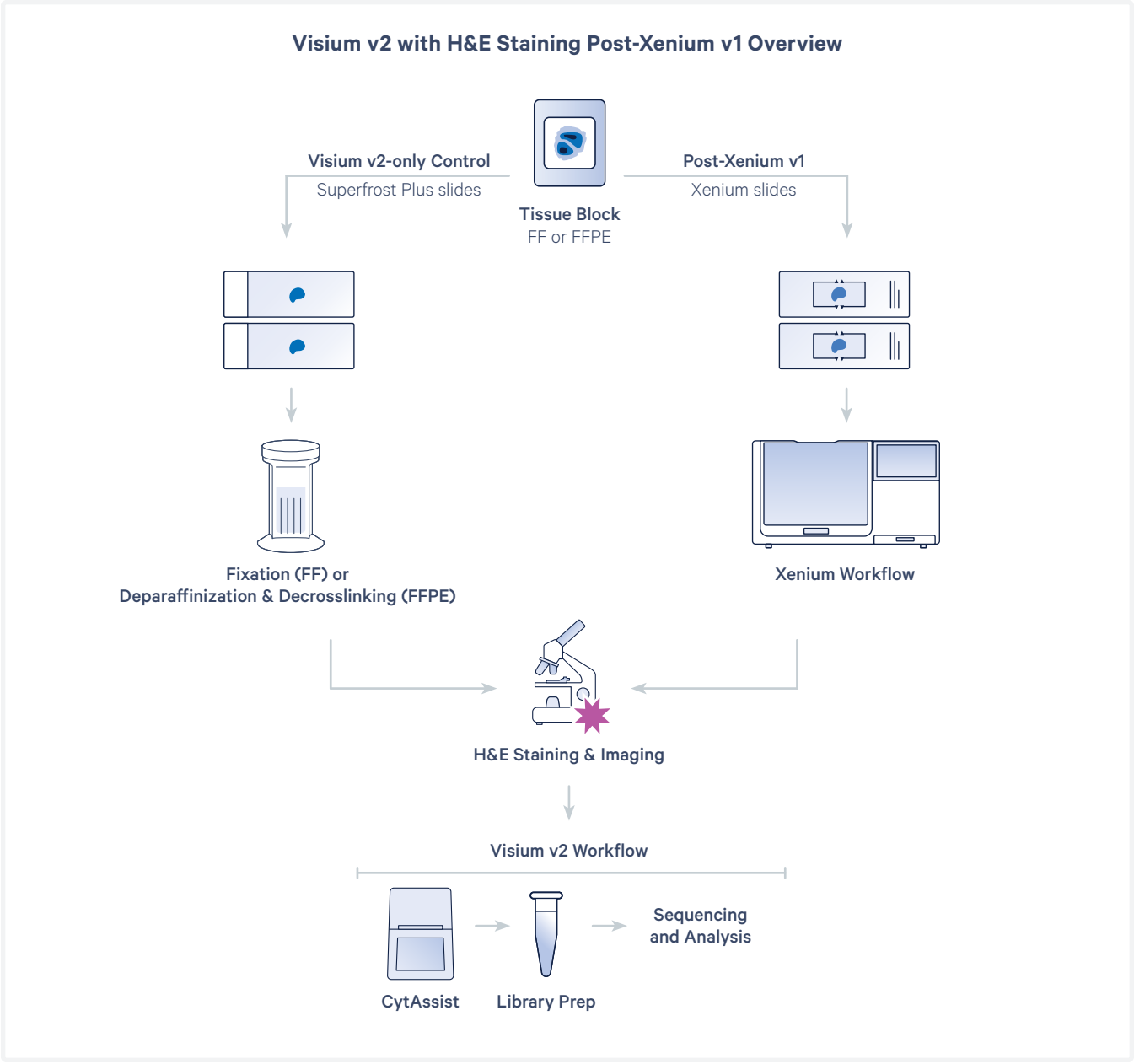

Figure 10. Post-Xenium v1 Visium CytAssist Spatial Gene Expression (Visium v2) is feasible with both FF (in OCT compound) and FFPE-embedded tissue samples. Serial sections from FF and FFPE tissue blocks were prepared. Sections 1 and 4 were placed onto Superfrost Plus slides and processed with the Visium v2 workflow (Visium v2-only controls) while sections 2 and 3 were placed onto Xenium slides and processed with the Xenium workflow on one Xenium Analyzer instrument run. See Methods section for more details about analysis in Visium v2-only control slides and post-Xenium slides.

Methods: *Visium v2 with H&E Staining Post-Xenium v1*

FF mouse brain and FFPE human colon samples were used to compare Visium v2 with H&E and post-Xenium Visium v2. The Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 11 mm, 2 rxns kit (PN-1000523) and the Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 11 mm, 2 rxns kit (PN-1000522) were used in the following control CytAssist-only and post-Xenium assays.

The post-Xenium Visium v2 workflow requires a tissue area to be selected that will fit either 6.5 x 6.5 mm or 11 x 11 mm (Capture Area sizes on Visium CytAssist slides). Tissue outside the selected region and/or additional sections on the Xenium slide will be lost during the Visium CytAssist workflow.

Post-Xenium Slides

Post-Xenium slides were immediately used or stored in 1,000 µl PBS-T (0.05%) for up to three days at 4°C. Prior to starting the Visium CytAssist Spatial Gene Expression workflow, PBS-T was removed, the Xenium Cassette was disassembled, and Autofluorescence Quenching Solution added during the Xenium workflow according to Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582) was removed according to the Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613), steps 1.1-1.3.

H&E staining, imaging, and coverslip addition were performed following Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking Demonstrated Protocol (CG000520), steps 1.3-2.3. Decoverslipped slides were placed in a Visium CytAssist Tissue Slide Cassette following Visium CytAssist Tissue Slide Cassette Assembly from the Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495), Rev C.

FF tissues were destained with 0.1N HCl according to Visium CytAssist for Fresh Frozen - Fixation, H&E Staining, Imaging & Destaining Demonstrated Protocol (CG000614), step 3.2.

FFPE tissues were destained with 0.1N HCl according to Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking Demonstrated Protocol (CG000520), step 3.2, skipping the decrosslinking step (3.3).

Post-Xenium slides were then immediately processed following the Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495) from step 1.1 Probe Hybridization. Pre-Hybridization Mix (PBS-T) was prepared and added to wells according to step 1.1 followed by a two minute incubation (instead of a fifteen minute incubation) prior to addition of freshly prepared Hybridization Mix to the wells. A shorter incubation time was used because the slide already went through a similar step in the Xenium workflow; here, the Pre-Hybridization Mix was used primarily to wash off additional HCl from destaining. It is recommended that the thermal cycler be prepared during the destaining step and Hybridization Mix be prepared shortly before use. Hybridization Mix was added following step 1.1, and the remaining workflow was performed until the libraries were ready for sequencing.

Control Slides

FF mouse brain control slides: FF mouse brain control slides were H&E stained according to Visium CytAssist Spatial Gene Expression for Fresh Frozen – Methanol Fixation, H&E Staining, Imaging & Destaining (CG000614). Libraries were then constructed following Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495).

FFPE human colon control slides: FFPE human colon control slides were H&E stained according to Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000520). Libraries were then constructed following Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495).

Results: *Visium v2 with H&E Staining Post-Xenium v1*

The following post-Xenium v1 Visium v2 and control Visium v2-only analysis results using the Visium v2 workflow are for two tissue types. Replicate samples indicate two serial sections placed on separate slides so that each post-Xenium slide has a comparable control slide (sections 2 and 3 were placed on Xenium slides and sections 1 and 4 were placed on Superfrost Plus slides). Figure 10 provides an overview of the post-Xenium Visium v2 workflow. FF mouse brain results are displayed in Figures 11-15 and FFPE human colon results are displayed in Figures 16-20.

Fresh Frozen Mouse Brain

Whole transcriptome spatial RNA sequencing data using Visium v2 from the same tissue sections processed in the Xenium workflow yielded comparable median distribution of UMIs and genes detected per spot compared to serial control sections not processed in the Xenium workflow (Figure 12). There was a strong correlation in UMI counts between the FF mouse brain post-Xenium Visium v2 and Visium v2-only control data (Figure 13), supporting the minimally destructive nature of the Xenium workflow at the RNA level. It is possible that a subset of shared gene targets between the Xenium and CytAssist probe panels may suffer from an expression bias, by either displaying increased or decreased expression on Visium v2 data (highlighted as colored triangles in Figure 13). One potential underlying factor could be that Rolling Circle Amplification Products (RCPs) generated during the Xenium workflow may remain in the tissue post-Xenium, and could potentially interfere with proper annealing of shared probe targets from the Visium v2 whole transcriptome probe set. However, the fraction of probe sequence overlap in shared targets was not correlated with observed gene expression levels among this subset of target genes.

Next, unsupervised clustering analysis of post-Xenium Visium v2 data generated similar spatial patterning (Figure 14), as well as spatial gene localization comparable to Visium v2-only controls, as shown by a set of genes selected for their known distinct spatial gene expression pattern (Figure 15), supporting that post-Xenium Visium v2 data yields comparable biological interpretations.

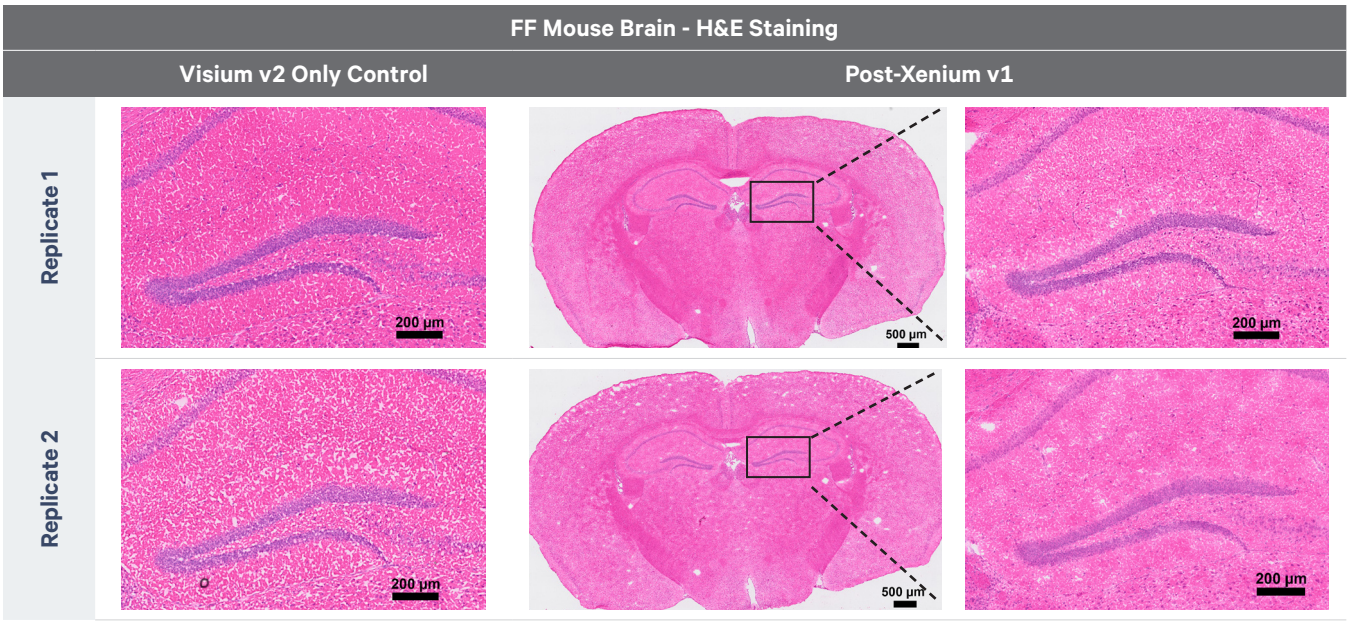

Figure 11. H&E staining on control sections not processed through the Xenium v1 workflow prior to H&E staining (left panel) and post-Xenium sections (right panel).

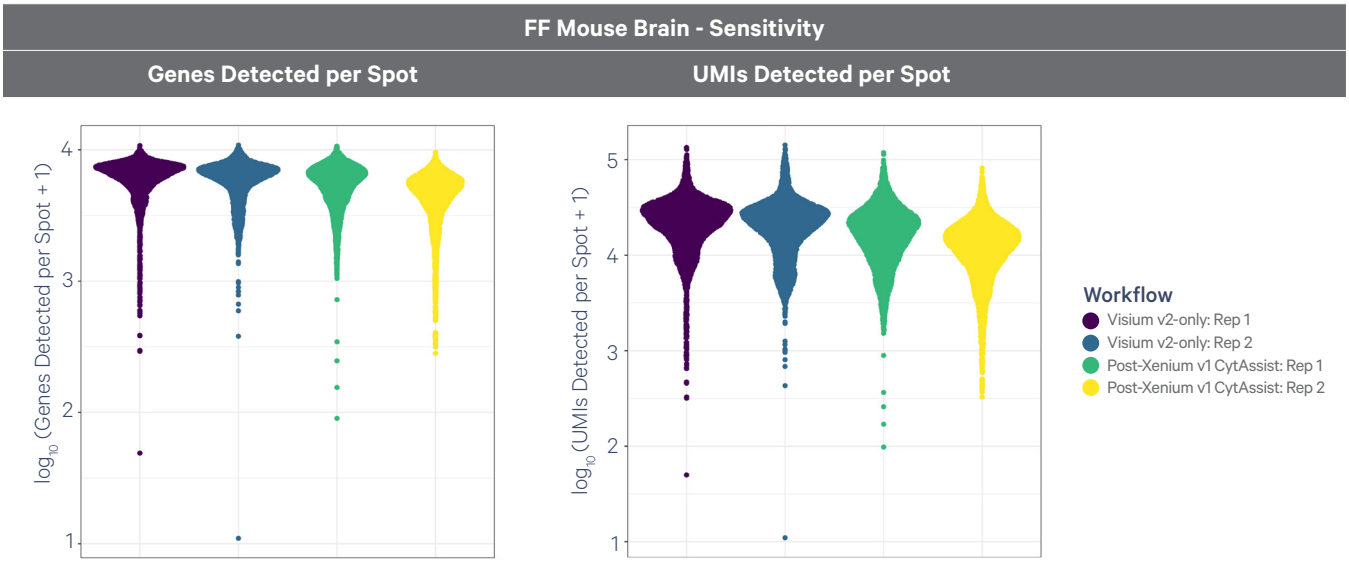

Figure 12. Log_{10} genes (left panel) and UMIs (right panel) detected per tissue covered spot across serial sections (2 Visium v2-only and 2 post-Xenium v1).

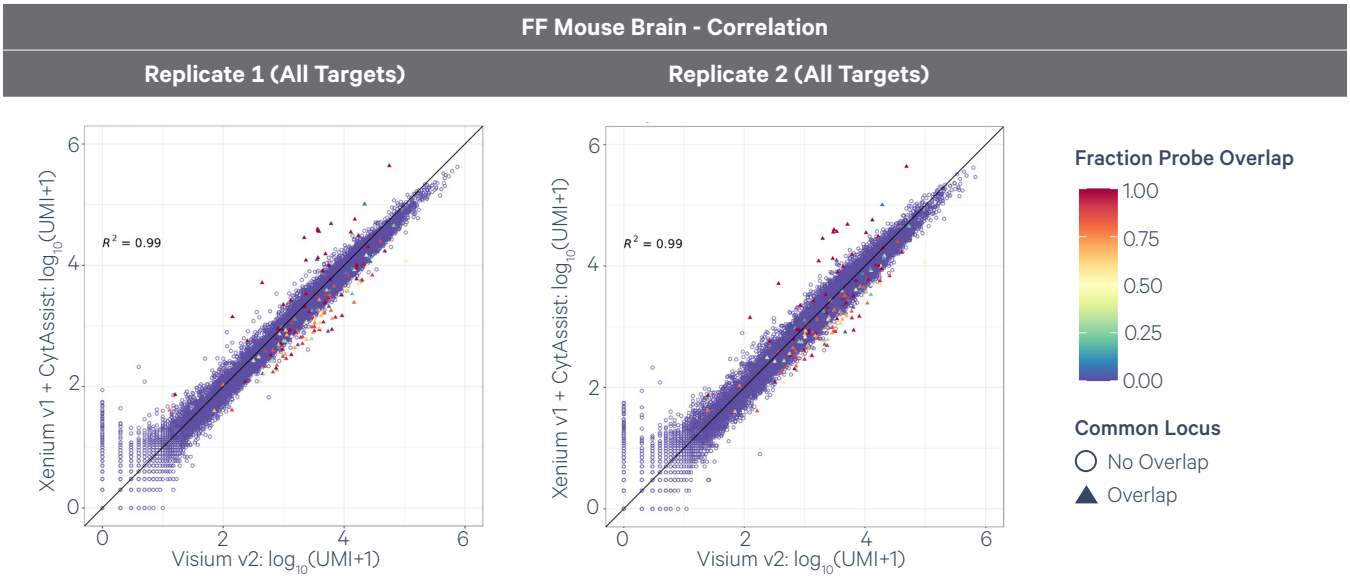

Figure 13. Correlation plots of UMI counts obtained from Visium v2 collected post-Xenium v1 and Visium v2-only controls. Open circles represent loci that are not shared targets between Visium mouse whole transcriptome probes v1 and the Xenium probe sets, while triangles represent genes that are shared targets between both probe sets (248 genes). The color bar shows fraction probe sequence overlap for the shared targets.

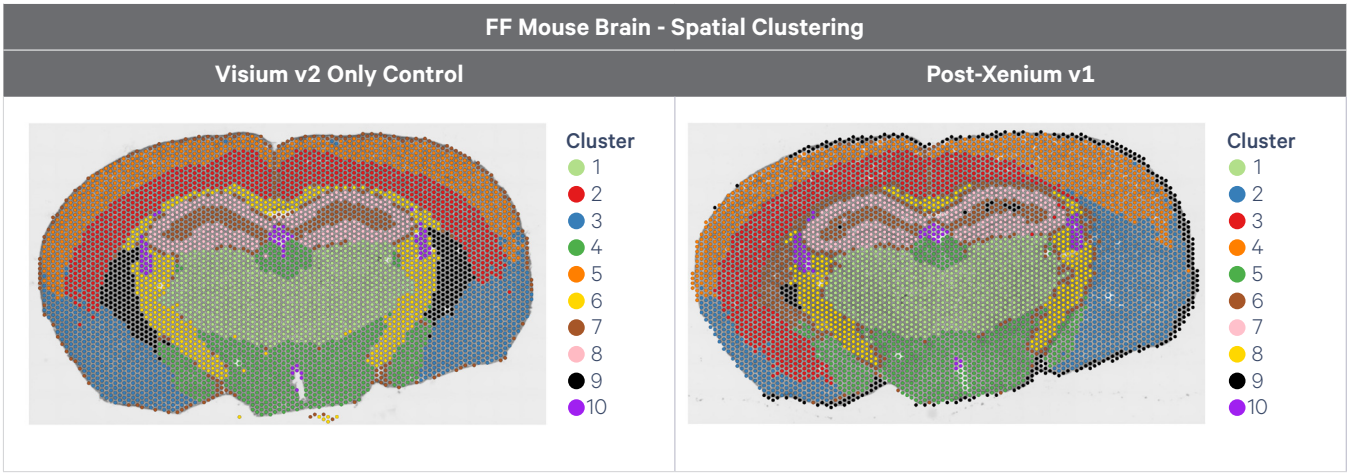

Figure 14. K-means 10 clustering for serial sections (Visium v2-only and post-Xenium v1), showing similar clusters were identified across Visium v2-only and post-Xenium sections.

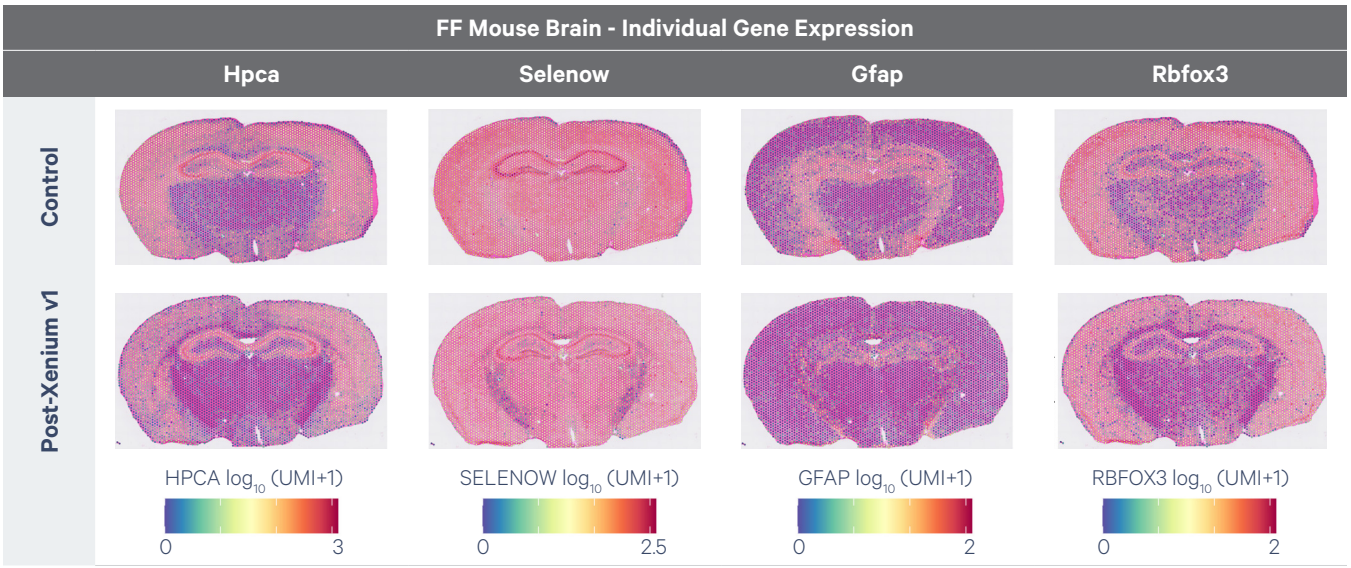

Figure 15. Selected gene markers across Visium v2-only (top) and post-Xenium v1 (bottom) serial sections demonstrating analysis of Visium v2 data after the Xenium workflow was consistent with serial sections run through the Visium v2-only workflow. The Hippocalcin (Hpca) gene in mice encodes for a calcium-binding protein that plays a role in regulation of voltage-dependent calcium channels, Selenoprotein W (Selenow) encodes a selenoprotein involved in muscle growth and differentiation, Glial fibrillary acidic protein (Gfap) gene encodes for a major intermediate filament protein of mature astrocytes, and RNA binding protein, fox-1 homolog (C. elegans) 3 (Rbfox3) encodes a protein involved in regulation of alternative splicing of pre-mRNA.

FFPE Human Colon

Visium v2 assay performance was also compared in FFPE human colon samples run through either the post-Xenium Visium v2 workflow or in Visium v2-only controls. H&E staining was consistent between post-Xenium replicates and was comparable to staining-only controls (Figure 16). Whole transcriptome spatial RNA sequencing data using Visium v2 in FFPE human colon yielded comparable median distribution of UMIs and genes detected per tissue covered spot between post-Xenium sections and Visium v2-only controls. Differences in the detection among lowly expressing targets did not skew the strong correlation in UMI counts observed between post-Xenium Visium v2 and Visium v2-only data (Figure 17). This difference in detection among lowly expressed genes that appear to be more highly expressed in Visium v2-only data as compared to post-Xenium Visium v2 data could be attributed to the reminiscent presence of genomic DNA (gDNA) in data derived from the the Visium v2-only control samples. It is possible that the difference in decrosslinking conditions between the Xenium and Visium v2 workflows accounts for this difference in gDNA. For more information about interpreting results impacted by gDNA, consult the Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA Technical Note (CG000605).

It is also possible that a subset of shared gene targets between the Xenium and Visium probe panels may suffer from an expression bias in post-Xenium FFPE human colon sections (Figure 18), as discussed above for the FF mouse brain data, that also does not seem to be associated with the fraction of probe sequence overlap between shared targets.

Unsupervised clustering analysis of post-Xenium Visium v2 data generated similar clustering data (Figure 19), as well as spatial gene localization comparable to Visium v2-only controls, as shown by a set of genes selected for their known distinct spatial expression pattern (Figure 20).

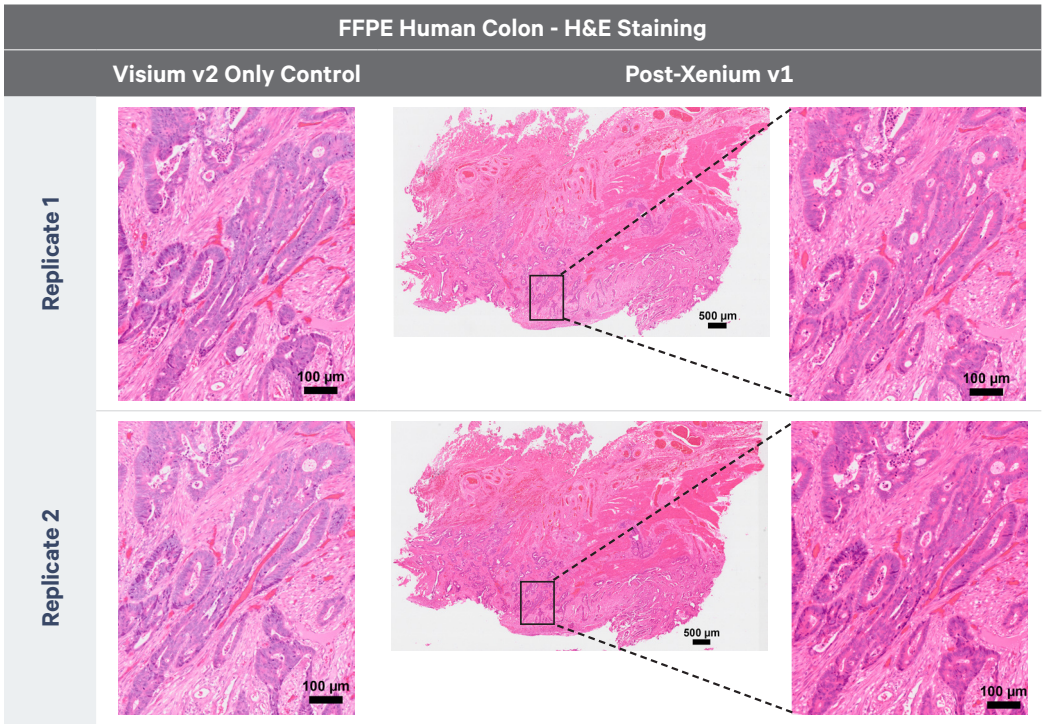

Figure 16. H&E staining on control sections not processed through the Xenium v1 workflow prior to H&E staining (left panel) and post-Xenium sections (right panel).

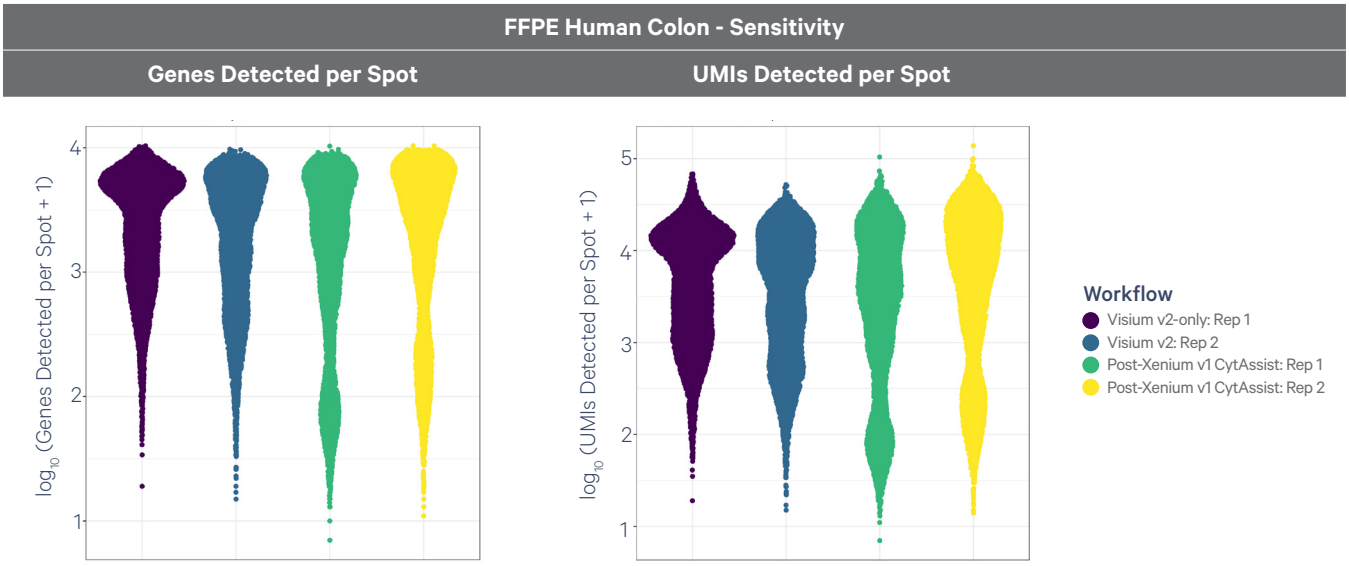

Figure 17. Log_{10} genes (left panel) and UMIs (right panel) detected per tissue covered spot across serial sections (2 visium v2-only and 2 post-Xenium v1).

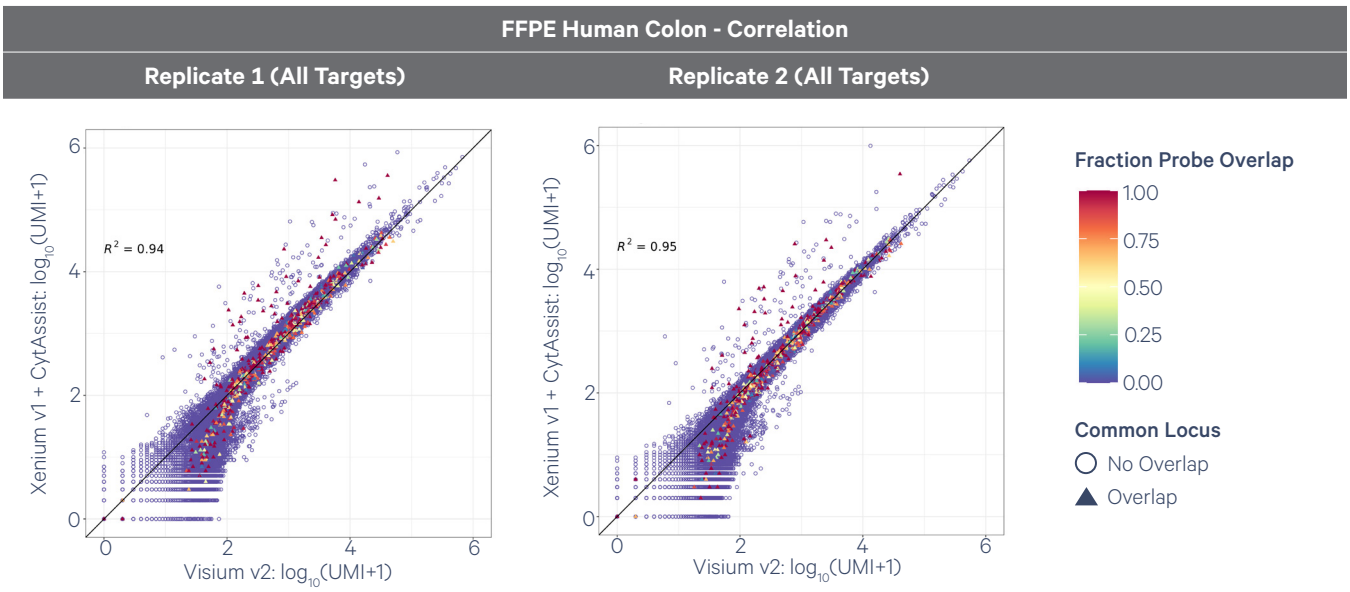

Figure 18. Correlation plots of UMI counts obtained from Visium v2 collected post-Xenium v1 and Visium v2-only controls. Open circles represent loci that are not shared targets between Visium human whole transcriptome probes v2 and the Xenium probe sets, while triangles represent genes that are shared targets between both probe sets (248 genes). The color bar shows fraction probe sequence overlap for the shared targets.

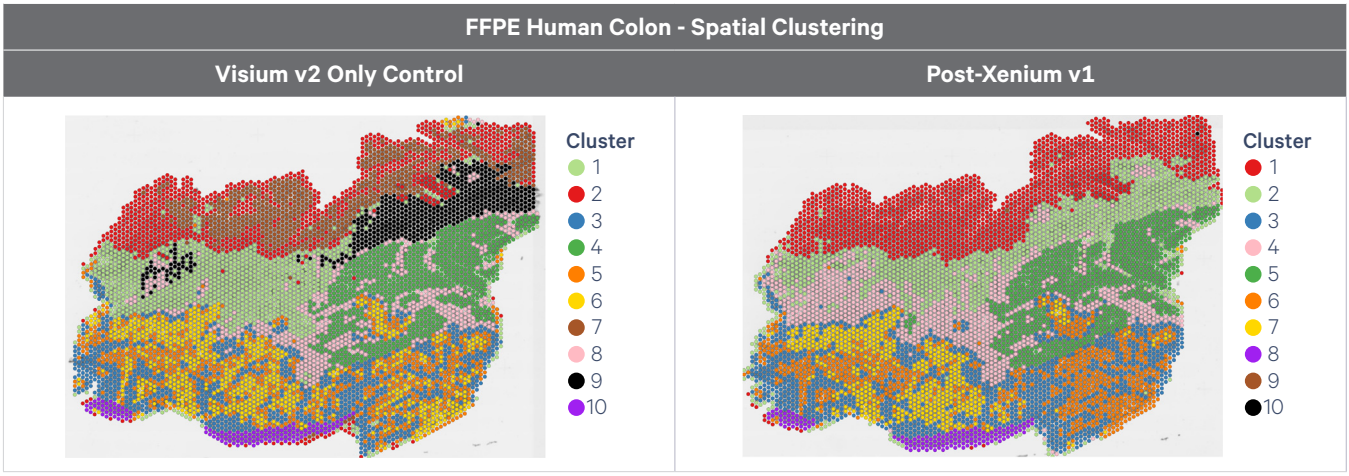

Figure 19. K-means 10 clustering for serial sections (Visium v2-only and post-Xenium v1), showing similar clusters were identified across Visium v2-only and post-Xenium sections.

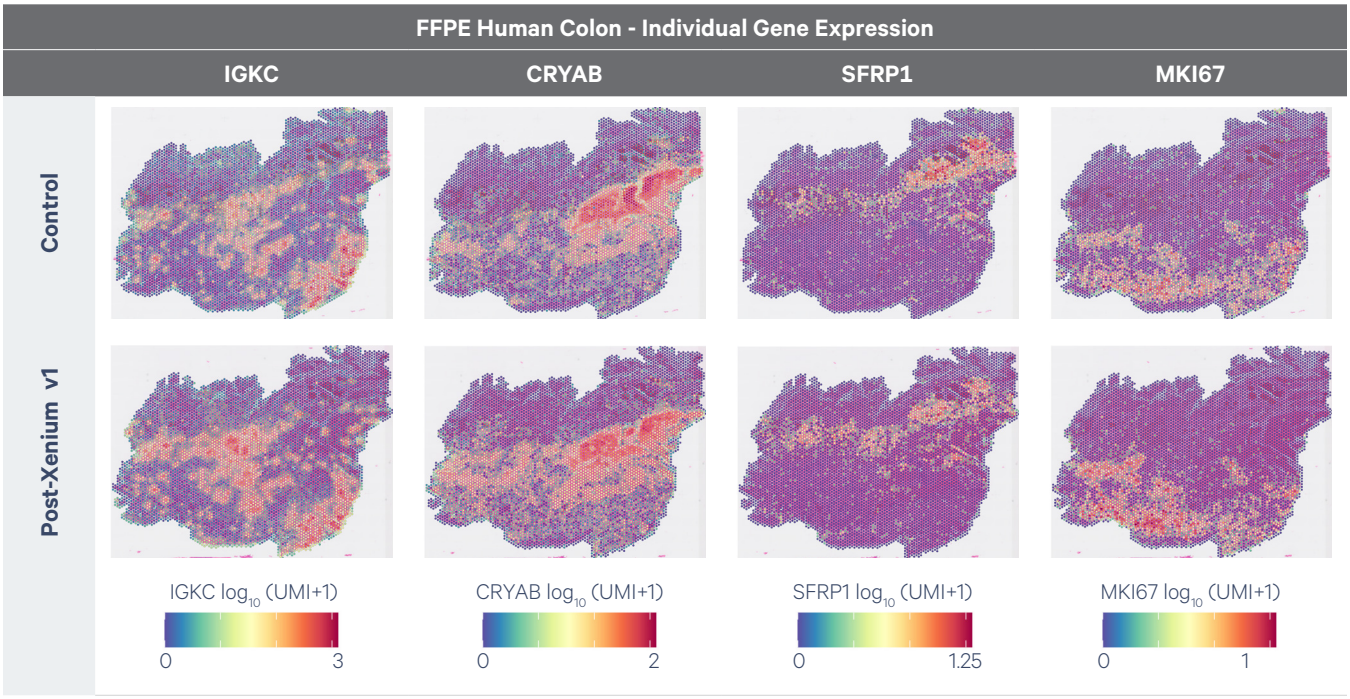

Figure 20. Selected gene markers across Visium v2-only (top) and post-Xenium v1 (bottom) serial sections demonstrating CytAssist analysis after the Xenium workflow was consistent with serial sections run through the Visium v2-only workflow. Immunoglobulin Kappa Constant (IGKC) gene encodes the constant domain of kappa-type light chains of antibodies, Alpha-Crystallin B Chain (CRYAB) gene encodes for a chaperone protein that blocks aggregation of denatured proteins, Secreted Frizzled Related Protein 1 (SFRP1) gene encodes for a protein that acts as an extracellular ligand of WNT signaling, and Marker of Proliferation Ki-67 (MKI67) gene encodes for a protein involved in regulation of chromosome segregation and mitotic nuclear division.

Visium HD Post-Xenium v1 or Xenium Prime 5K

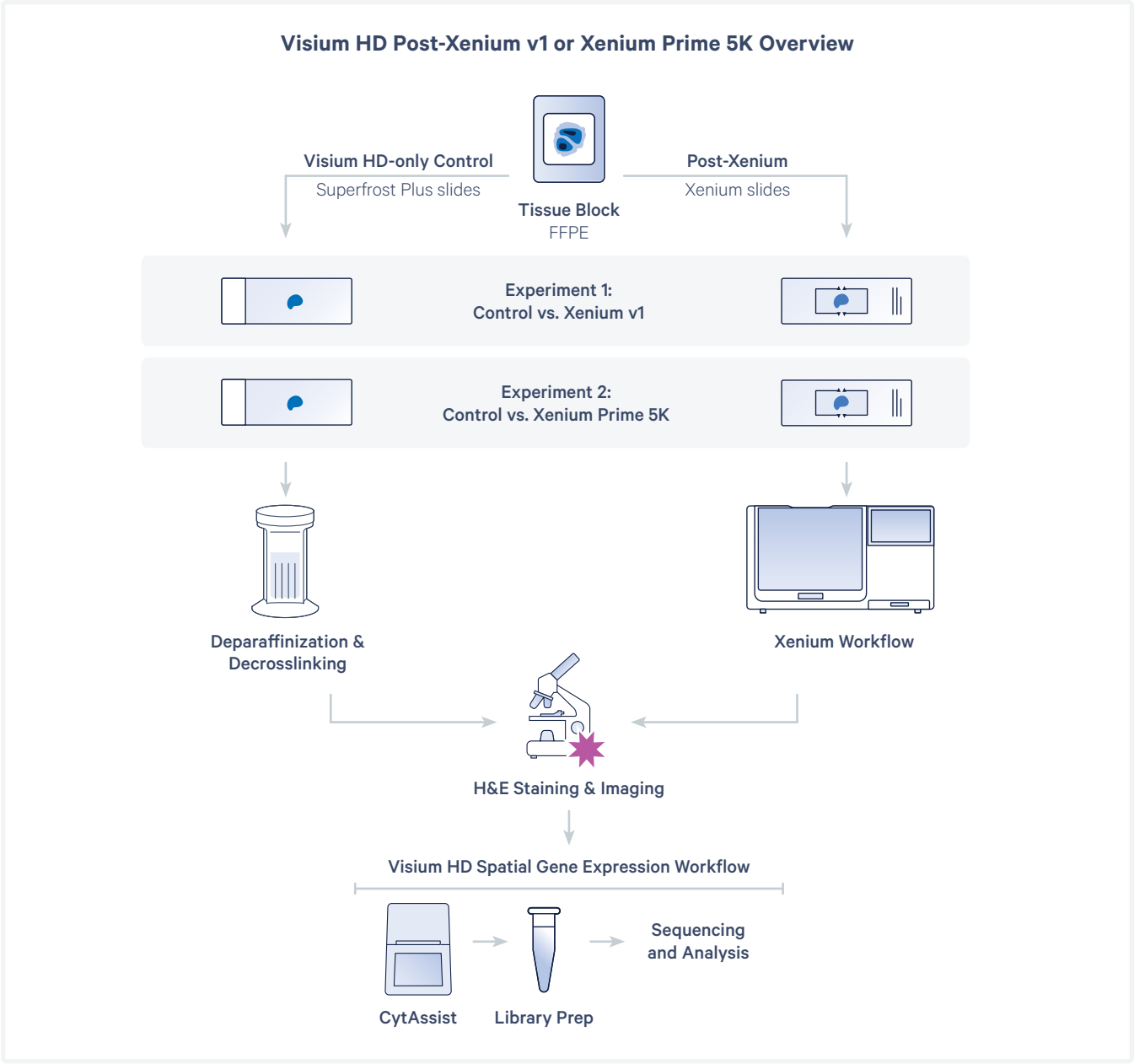

Figure 21. Post-Xenium Visium HD Spatial Gene Expression is feasible with FFPE-embedded tissue samples. Serial sections were prepared for both experiments. Experiment 1: Tissue section was processed with Visium HD workflow (Visium HD-only control) and the adjacent section on Xenium slide was processed with the Xenium v1 workflow. Experiment 2: Tissue section was processed with Visium HD workflow (Visium HD-only control) and the adjacent section on Xenium slide was processed with the Xenium Prime 5K workflow. See Methods section for more details about CytAssist analysis in Visium HD-only control slides and post-Xenium slides.

Methods: *Visium HD Post-Xenium v1 or Xenium Prime 5K*

FFPE human lung tissue was used to compare Visium HD and post-Xenium Visium HD. The Visium HD, Human Transcriptome, 6.5 mm, 2 rxns kit (PN-1000675) was used in the following control Visium HD-only and post-Xenium assays.

The post-Xenium Visium HD Spatial Gene Expression workflow requires a tissue area to be selected that will fit in a 6.5 x 6.5 mm Capture Area. Tumor tissue areas were captured for both control and post-Xenium samples. Tissue outside the selected region and/or additional sections on the Xenium slide were lost during the Visium HD workflow.

Post-Xenium v1 and Post-Xenium Prime 5K Slides

Post-Xenium slides were immediately used or stored in 1,000 µl PBS-T (0.05%) for up to three days at 4°C. Prior to starting the Visium HD workflow, PBS-T was removed, the Xenium Cassette was disassembled, and Autofluorescence Quenching Solution added during the Xenium workflow according to Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582) or Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining (CG000760) was removed according to the Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613), steps 1.1-1.3.

H&E staining, imaging, and coverslip addition were performed following the Visium HD FFPE Tissue Preparation Handbook (CG000684).

Decoverslipped slides were placed in a Visium Tissue Slide Cassette S3 following Visium Cassette S3 for Assembly & Disassembly Quick Reference Card (CG000730).

FFPE tissues were destained with 0.1N HCl according to Visium HD FFPE Tissue Preparation Handbook (CG000684), step 3.6, skipping the decrosslinking step (3.7).

Post-Xenium slides were then immediately processed following the Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685) from step 1.1 Probe Hybridization. Pre-Hybridization Mix was prepared and added to wells according to step 1.1 prior to addition of freshly prepared Probe Hybridization Mix to the wells. It is recommended that the thermal cycler be prepared during the destaining step and Probe Hybridization Mix be prepared shortly before use. Probe Hybridization Mix was added following step 1.1, and the remaining workflow was performed until the libraries were ready for sequencing.

Control Slides

FFPE human lung control slides were H&E stained according to the Visium HD FFPE Tissue Preparation Handbook (CG000684). Libraries were then constructed following Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685).

Results: *Visium HD Post-Xenium v1 or Xenium Prime 5K*

The following post-Xenium (Xenium v1 and Xenium Prime 5K) Visium HD and control Visium HD-only analysis results using the Visium HD workflow are FFPE human lung. Figure 21 provides an overview of the post-Xenium Visium HD workflow.

H&E staining was consistent between two post-Xenium experiments and was comparable to staining-only controls (Figure 22).

Whole transcriptome spatial RNA sequencing data using the Visium HD CytAssist-enabled workflow from the same tissue sections processed with either Xenium workflow revealed lower median distribution of UMIs and genes detected either per feature or per bin compared to Visium HD-only tissue sections (Figure 23).

There was a strong correlation in UMI counts between the FFPE human lung post-Xenium Visium HD and Visium HD-only control data (Figure 24), supporting the minimally destructive nature of the Xenium workflow.

It is possible that a subset of shared gene targets between the Xenium v1 and Visium HD probe panels may suffer from an expression bias, by either displaying increased or decreased expression on Visium HD (highlighted as colored triangles in Figure 24). Similar to the Visium v2 data, it is possible that Rolling Circle Amplification Products (RCPs) generated during the Xenium workflow may remain in the tissue post-Xenium and could potentially interfere with proper annealing of shared probe targets from the Visium HD whole transcriptome probe set. Expression biases were not seen when comparing post-Xenium Prime 5K to Visium HD only controls.

Next, unsupervised clustering analysis of post-Xenium Visium HD data generated similar spatial patterning, as well as spatial gene localization comparable to Visium HD-only controls, as shown by a set of genes selected for their known distinct spatial gene expression pattern (Figure 25-26), supporting that post-Xenium Visium HD data yields comparable biological interpretations.

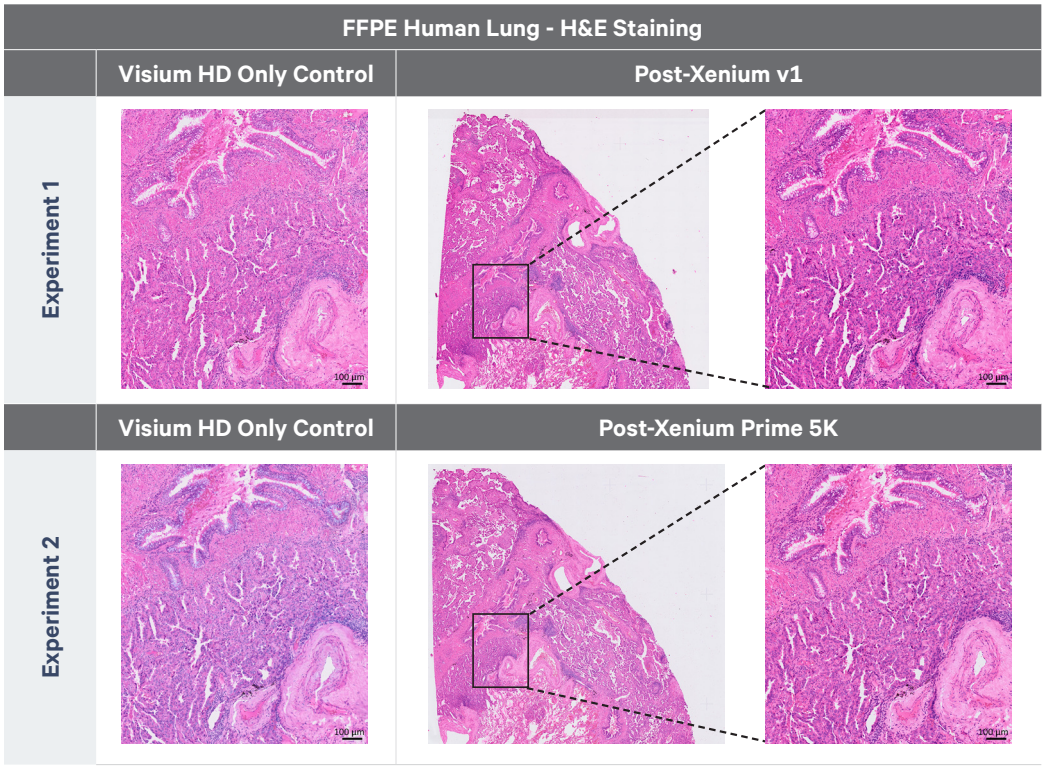

Figure 22. H&E staining on control sections not processed through the Xenium workflow prior to H&E staining (Visium HD only control) and H&E staining on sections processed with Visium HD after either the Xenium v1 or Xenium Prime 5K workflows.

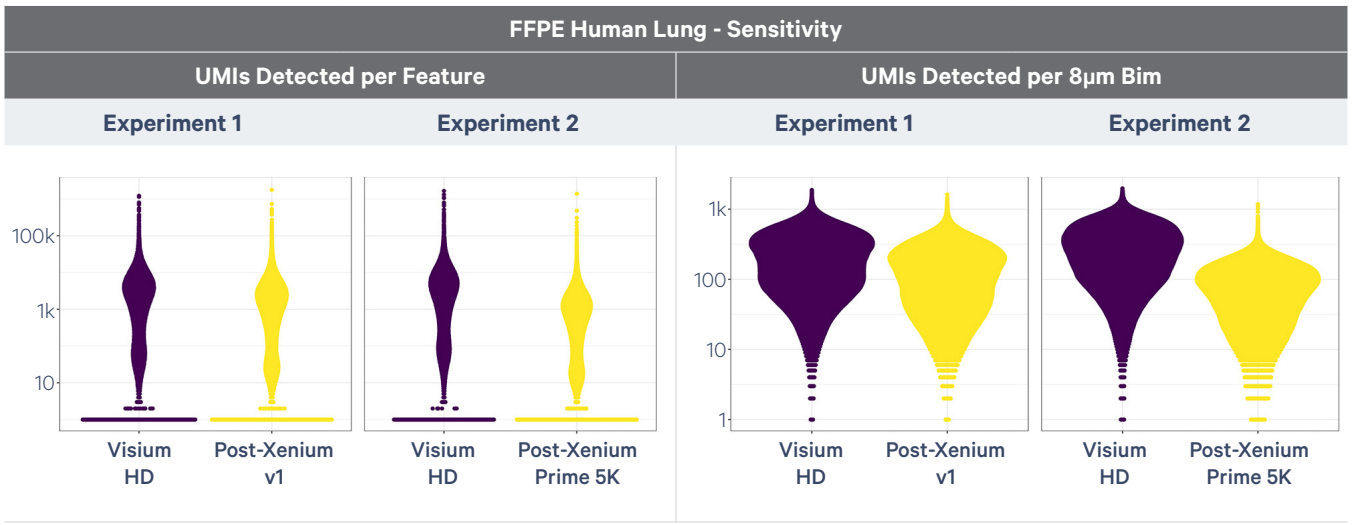

Figure 23. UMIs detected per feature (left panel) or per 8 micron bin (right panel) across serial sections (Visium HD-only and vs serial section processed with either Xenium v1 or Xenium Prime 5K).

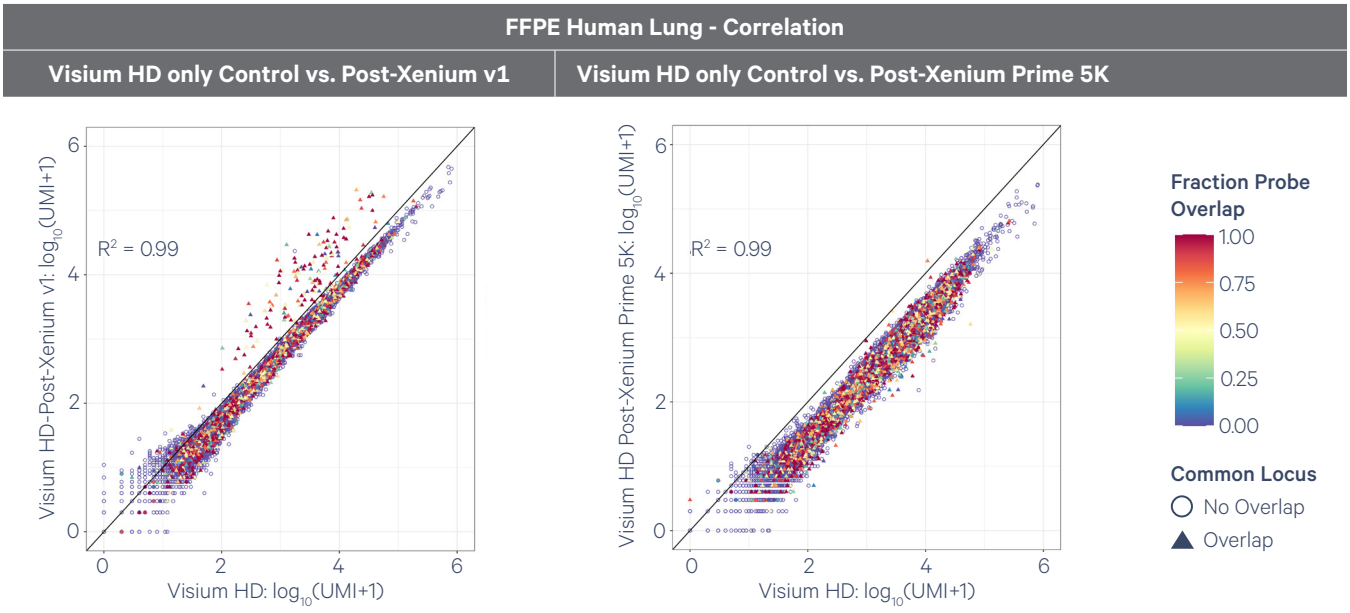

Figure 24. Correlation plots of UMI counts obtained from Visium HD-only and post-Xenium samples. Open circles represent loci that are not shared targets between Visum HD human whole transcriptome probes v2 and Xenium probe sets, while triangle represent loci that are shared targets between both probe sets. Geometric mean sensitivity difference between Visium HD-only and post-Xenium v1 = 1.7. Geometric mean sensitivity difference between Visium HD-only and post-Xenium Prime 5K = 4.0. The color bar shows fraction probe sequence overlap for the shared targets.

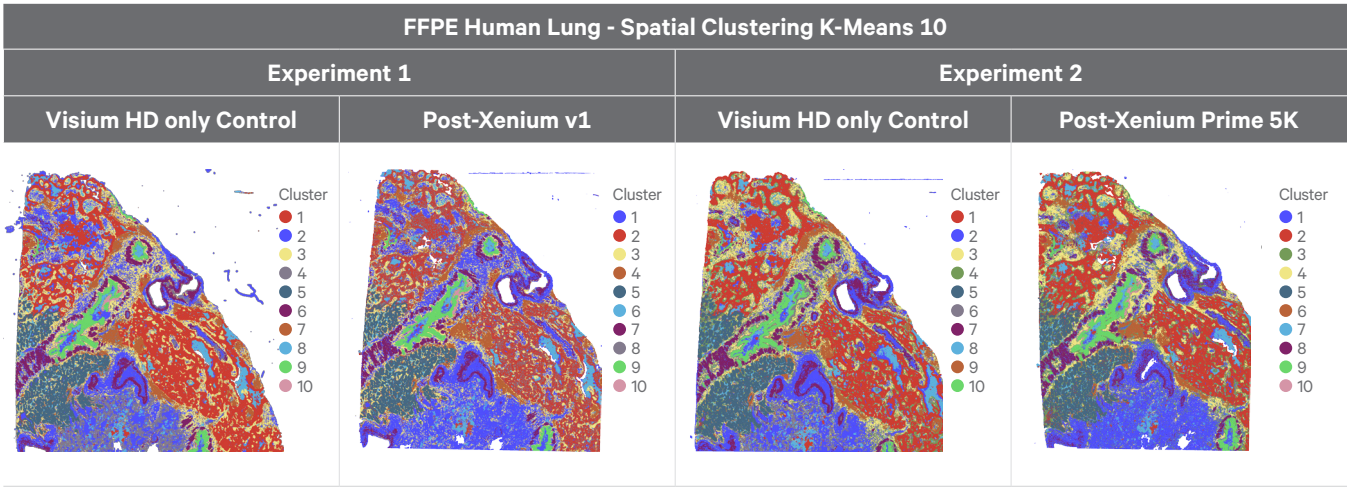

Figure 25. K-means 10 clustering for serial sections (Visium HD-only vs. post-Xenium), showing similar clusters were identified across Visium HD-only and post-Xenium sections.

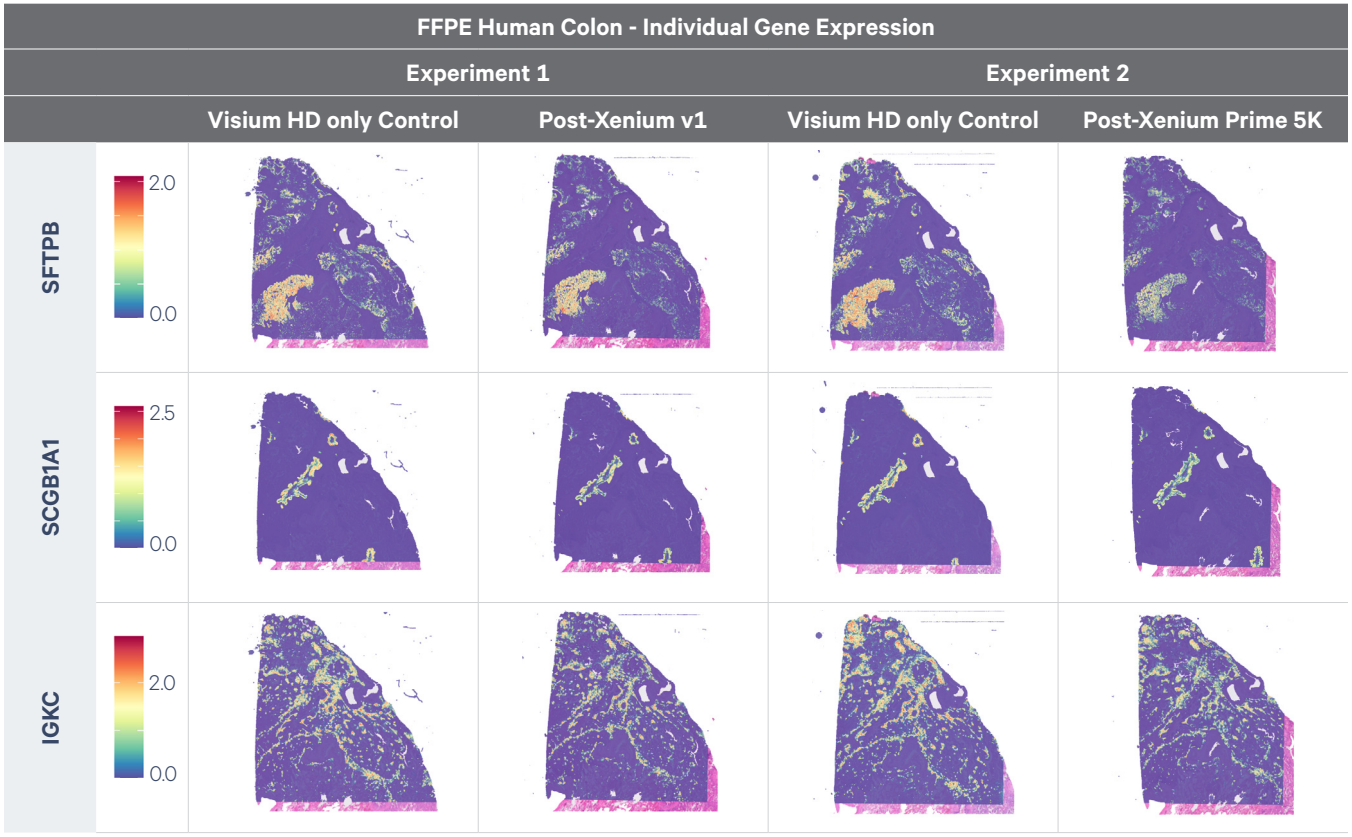

Figure 26. Selected gene markers across serial sections demonstrate that gene marker distribution remains consistent for Visium HD-only and post-Xenium tissue sections. Surfactant protein B (SFTPB) gene encodes an amphipathic surfactant protein essential for lung function and is primarily expressed in alveolar type II pneumocytes. Secretoglobin family 1A member 1 (SCGB1A1) gene encodes a protein essential for maintaining the airway epithelium and is primarily expressed in club cells. Immunoglobulin Kappa Constant (IGKC) gene encodes the constant domain of kappa-type light chains of antibodies. The legend represents gene expression levels, shown as log10 (UMI+1).

Conclusions

In this Technical Note, the feasibility of performing multiple applications post-Xenium including staining (IF and H&E) and Visium (Visium v2 and Visium HD) was demonstrated. Data were obtained in post-Xenium FF (Visium v2 only) and FFPE tissue sections and compared to controls (staining-only and Visium-only) not processed through the Xenium assay. Protein detection was obtained by antibody staining, while morphology of the tissues was analyzed by H&E staining. Visium v2 and Visium HD data were used to compare the sensitivity between workflows. Post-Xenium Prime 5K was only assessed with Visium HD.

Here, IF and H&E staining were performed on the same sections post-Xenium, generating highly comparable staining patterns to control sections. IF staining yielded slightly reduced signal intensities as compared to IF staining-only controls. This effect may be due to multiple differences between the workflows, including the presence of Autofluorescence Quenching Solution in the post-Xenium sections and differences in the sample preparation (i.e. decrosslinking conditions) between post-Xenium and controls slides. For detection of new proteins, users may want to screen for antibodies using serial sections of the same blocks prior to staining post-Xenium sections. Additionally, IF staining may be impacted by potential inaccessibility of certain epitopes, thus performance is not guaranteed for all antibodies in the post-Xenium workflow.

Visium v2 obtained post-Xenium v1 was highly comparable to the Visium-only data, with strong correlation for UMIs and gene counts per tissue covered spots, as well as similar spatial clustering and gene spatial distribution between workflows, with minimal impact on the overall sensitivity in the post-Xenium slides. A larger impact on sensitivity was seen when comparing post-Xenium data vs Visium HD-only, particularly with Xenium Prime 5K. There was a difference in sensitivity of a subset of shared gene targets between Visium and Xenium v1 probe sets. This reduced sensitivity did not affect biological interpretation of the data.

Together, these data highlight the minimally destructive nature of the Xenium workflow, allowing for downstream IF and H&E staining, and Visium v2 and Visium HD data collection on the same tissue sections. Though data are not shown in this Technical Note, IF and H&E staining is also compatible with post-Xenium Prime 5K samples.

Factors that may impact sensitivity include tissue type, sample input quality, and storage time of post-Xenium slides prior to starting staining and CytAssist workflows. High quality tissue should be used for optimal results. Post-Xenium slides should be used within three days for post-Xenium applications, as longer storage times may compromise performance.

In conclusion, the ability to obtain additional data post-Xenium on the same tissue section can be used as a powerful tool for understanding biology at multiple levels in a variety of tissue types.

Appendix

Item	Clone	Dilution	Fluorophore	Imaging Channel	Marker for	Supplier	Part Number (US)
4, 6-diamidino-2-phenylindole (DAPI)	N/A	5 µg/ml *(in PBS)*	Blue	385	Nuclei	Millipore Sigma	D9542
Glial fibrillary acidic protein (GFAP)	GA5	1:1000	Cy3 (Orange)	532	Astrocytes	Millipore Sigma	MAB3402C3
Neuronal nuclear antigen (NeuN)	EPR12763	1:10,000	488 (Green)	488	Post-mitotic neurons	Abcam	ab190195
Vimentin (VIM)	EPR3776	1:100	555 (Orange)	532	Cytoskeleton	Abcam	ab203428
alpha-Smooth Muscle Actin (α-SMA)	EPR5368	1:100	594 (Red)	590	Actin	Abcam	ab202510

Table 1. Staining Reagents and Conditions

Item	Description	Supplier	Part Number (US)
Nuclease-free Water	Nuclease-free Water (not DEPC treated)	Thermo Fisher Scientific	AM9937
PBS	PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
10% Tween	Tween 20 Surfact-Amps Detergent Solution (10% solution) *(not 100% Tween diluted to 10%)*	Thermo Fisher Scientific	28320
	10% Tween 20	Bio-Rad	1662404
FBS	Fetal Bovine Serum, qualified, heat inactivated	Thermo Fisher Scientific	16140-071
Dextran Sulfate	Dextran Sulfate Sodium Salt	Millipore Sigma	42867
Citrate Buffer	Citrate Buffer, pH 6.0, 10X, Antigen Retriever *(dilute 10-fold with water for 1X working solution)*	Millipore Sigma	C9999

Consult the Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582) for PBS-T preparation instructions.

Table 2. Additional Reagents

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. Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613).
5. Xenium In Situ for Fresh Frozen Tissues - Fixation & Permeabilization Demonstrated Protocol (CG000581).
6. Xenium In Situ for FFPE - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580).
7. Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000520).
8. Visium CytAssist Spatial Gene Expression for Fresh Frozen – Methanol Fixation, H&E Staining, Imaging & Destaining(CG000614).
9. Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495).
10. Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, Immunofluorescence Staining & Imaging (CG000519).
11. Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000518).
12. Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA (CG000605).
13. Xenium In Situ Gene Expression with Cell Segmentation Staining User Guide (CG000749).
14. Xenium In Situ Cell Segmentation: Workflow and Data Highlights Technical Note (CG000750).
15. Visium HD Spatial Gene Expression User Guide (CG000685).
16. Visium HD FFPE Tissue Preparation Handbook (CG000684).
17. Xenium Prime In Situ Gene Expression with Cell Segmentation Staining User Guide (CG000760).

Datasets

https://www.10xgenomics.com/datasets

Document Revision Summary

Document Number	CG000709
Title	Post-Xenium In Situ Applications: Immunofluorescence, H&E, and Visium CytAssist Spatial Gene Expression
Revision	Rev B to Rev C
Revision Date	September 2024

Description of Changes:

- Updated title to include Visium HD (p. 1)
- Added information on Visium HD to Experimental Design Overview section (p. 2)
- Added Post-Xenium Visium HD Spatial Gene Expression with H&E Staining section (p. 21-27)
- Updated for general minor consistency and language and terms throughout

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