Visium HD Spatial Gene Expression Performance

Introduction

The Visium HD Spatial Gene Expression assay maps whole transcriptome mRNA expression from tissue sections at single cell scale. This Technical Note introduces key concepts and considerations specific to this technology and discusses the performance of the Visium HD assay in terms of resolution and sensitivity.

Key Terminology

Term	Abbreviation
Visium HD Spatial Gene Expression	Visium HD
Visium CytAssist Spatial Gene Expression	Visium v2

The Visium HD Assay

The Visium HD assay allows for whole-transcriptome spatial gene expression discovery in human or mouse tissue (Figure 1). The workflow begins with sectioning tissue onto glass slides, tissue staining, and imaging. Next, tissues are incubated with either human or mouse probes, which allows the probes to hybridize to target mRNAs. After probe ligation, tissue slides and Visium HD Slides are loaded onto the Visium CytAssist instrument. Single-stranded ligation products are released from the tissue and are captured by the spatially-barcoded oligonucleotides within the Visium HD slide Capture Area. After probe capture, the Visium HD Slide is removed from the Visium CytAssist for downstream library preparation. Gene expression libraries are then generated from each Capture Area and sequenced. After sequencing, the Space Ranger pipeline is used for downstream data processing (v3.0 or later).

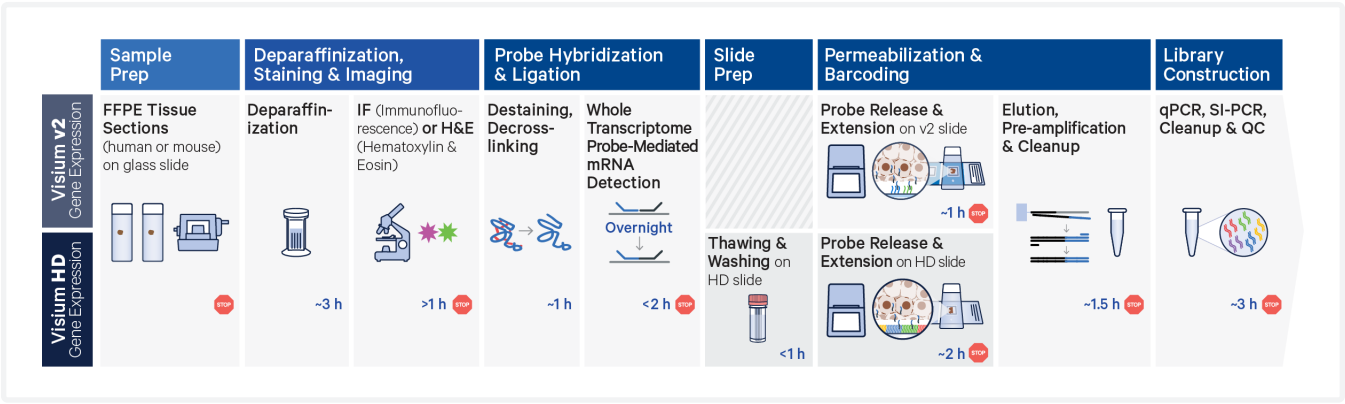

Figure 1. A workflow comparison between Visium HD and Visium v2 assays.

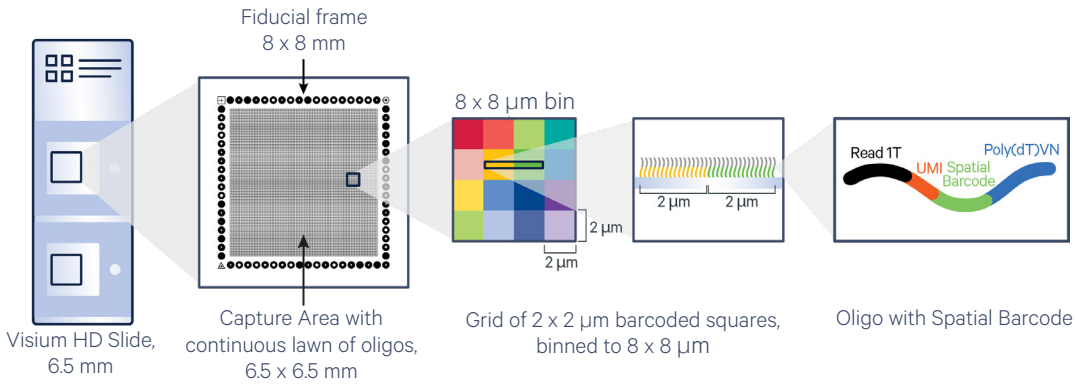

Figure 2. The Visium HD Gene Expression slide. The Visium HD slide Capture Area contains spatially barcoded oligos arranged in a grid of 2 x 2 µm barcoded squares.

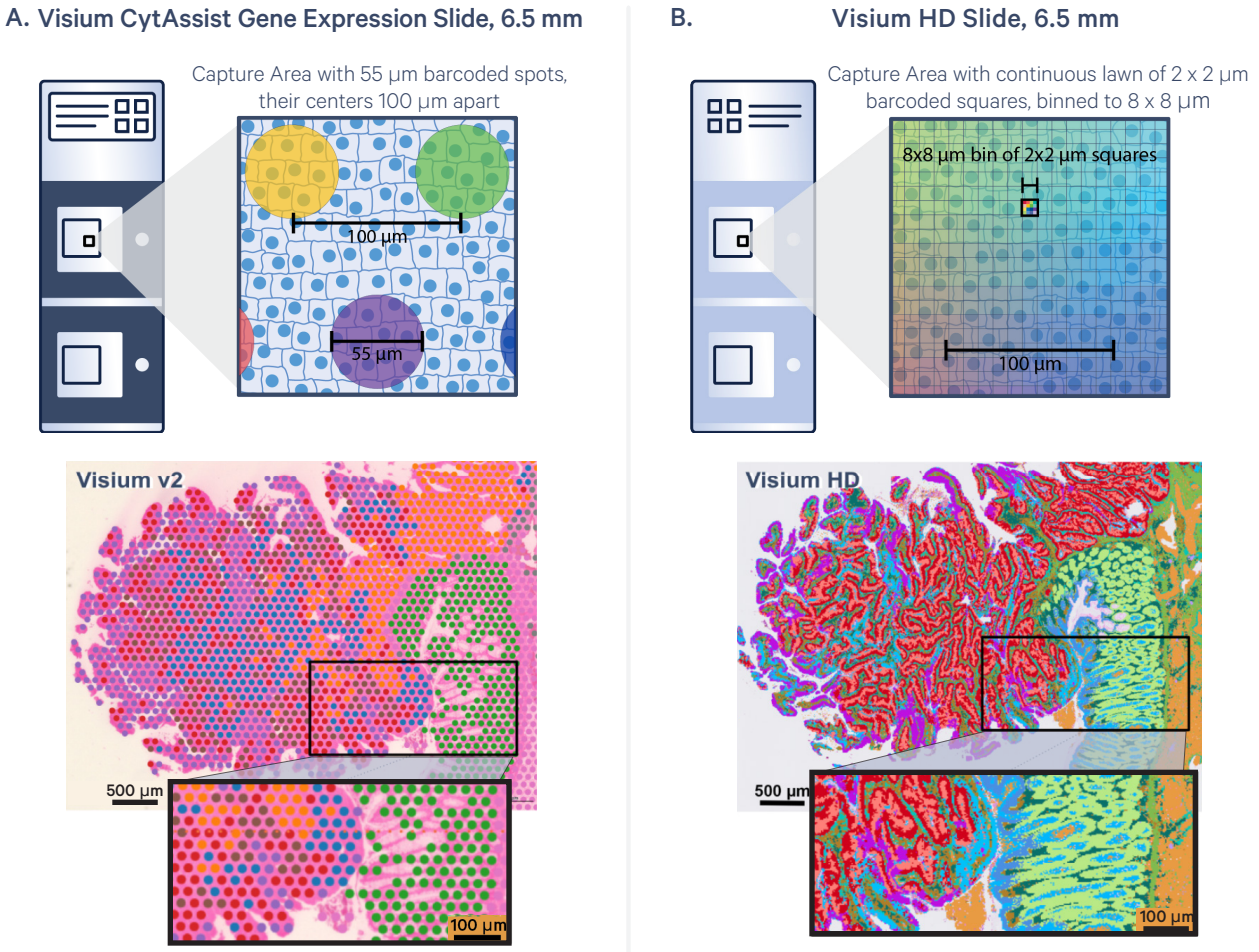

Figure 3. A comparison of sizes between Visium v2 barcoded spots **(A)** and Visium HD barcoded squares **(B)**, as well as unbiased clustering for adjacent or near-adjacent human colon cancer sections from the same tissue block. The blue cells shown in the zoomed in view of the Capture Areas are 15 µm wide and are scaled appropriately to the other items within the image.

The core technology driving the Visium HD assay are the barcoded oligonucleotides that link tissue location to gene expression (Figure 2). Visium v2 slides contain hexagonally-arranged barcoded spots that are 55 µm in diameter with a distance of 100 µm between the center of each spot. By contrast, Visium HD Spatial Gene Expression Slides contain 2 x 2 µm barcoded squares that are contiguous, forming a continuous lawn of oligos (Figure 3).

On the Visium HD slide, each 2 x 2 µm barcoded square is and contains a unique Spatial Barcode that allows for mapping gene expression data back to a specific square. This lawn of oligos on the Visium HD slide contains roughly 11 million barcoded squares. Due to the higher number of unique barcodes required, the length of the barcode and UMI region is 43 bases in Visium HD compared to 28 bases for Visium v2. The high-contrast fiducial frame on the Visium HD slide is imaged by the CytAssist instrument and used downstream by the Space Ranger pipeline to align gene expression data to a microscope image taken of the same tissue section.

The size and continuous nature of the spatially barcoded squares, along with the CytAssist instrument, facilitate mapping gene expression data at single cell scale. The Visium HD slide and assay are designed to optimize the assay resolution and sensitivity, while enabling alignment of the gene expression data to a high-resolution H&E or IF microscope image of the same tissue section that is taken before probe hybridization (Figure 1).

Methods Overview

Biomaterials and Reagents

The following tissue types were used for data generation in this Technical Note:

- Mouse FFPE tissues were obtained in block format from either AcePix Biosciences, BioIVT, or Charles Rivers Labs.
- Human FFPE tissues were obtained in block format from either AcePix Biosciences, Avaden Biosciences, BioIVT, Charles Rivers Labs, Cyence Biopathology, Discovery Life Sciences, or Precision For Medicine.

Tissues were cut into 5 µm sections and placed onto Fisherbrand Superfrost Plus slides. Tissue sections were then processed with either the Visium v2 assay or Visium HD assay. The Visium HD assay requires a Visium CytAssist instrument with firmware 2.0.0 or higher. The probe sets used for each assay are described in Table 1. The Visium HD assay uses a new version of the mouse probe set. Samples were prepared using documentation listed in the Appendix.

Species	Assay	Probe Set Version	Kit Part Number
Mouse	Visium v2	v1	1000365
	Visium HD	v2	1000667
Human	Visium v2	v2	1000466
	Visium HD	v2	

Table 1. Whole transcriptome probe sets used.

Data Analysis

The Space Ranger v3.0 pipeline was used to process FASTQ files, align reads to probe sets, produce primary output files including the gene-barcode matrices, and generate secondary analysis outputs including unbiased clustering and differential gene expression. Algorithm details can be found in Space Ranger documentation on the 10x Genomics support website. Loupe Browser v8.0 was used for data exploration and visualization of UMI counts, unbiased clustering, and differential gene expression comparisons between clusters. Input and output files are available on the 10x Genomics public datasets page.

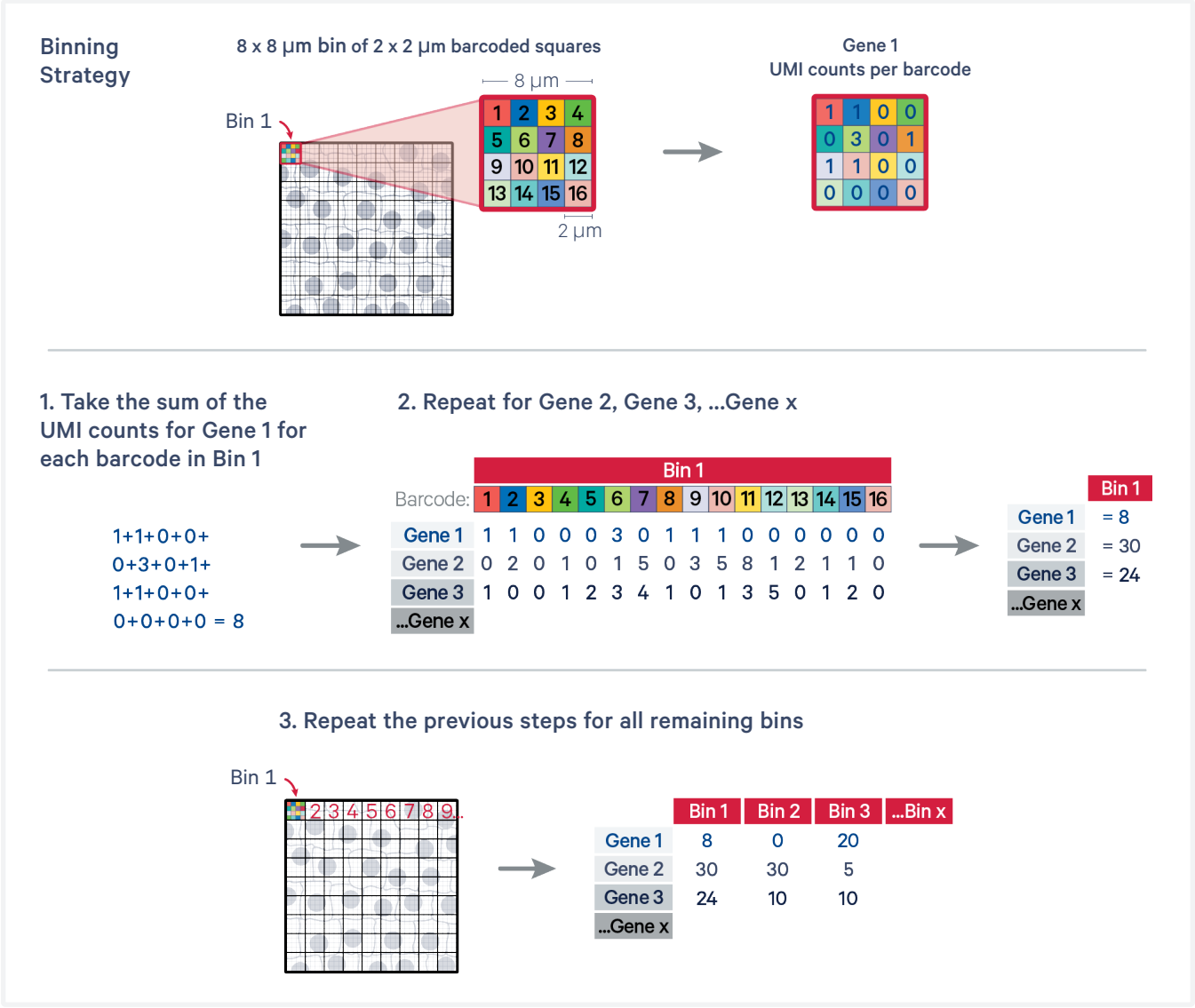

Figure 4. Visium HD binning overview. The UMI counts shown are illustrative and are not from an actual Visium HD sample.

Though each barcoded square is 2 x 2 µm, Space Ranger v3.0 outputs Visium HD data at three bin sizes, offering 8 µm and 16 µm bin sizes in addition to the native 2 µm feature size. Bins offer the advantage of including more mean UMI reads within the bin compared to a 2 x 2 µm barcoded square. For example, an 8 µm bin provides a 16-fold increase in the number of UMI reads on average within the bin compared to a 2 µm square. This increase improves the ability to resolve tissue structures via unbiased clustering or other analyses. A custom bin size (in microns at even integer values between 10 and 100) can be defined in Space Ranger or via third-party tools. Although optimal bin size may vary depending on the tissue type or other variables, the 8 µm bin size is an effective starting point for most researchers. It is the default binning size used to calculate some of the top-level metrics shown in the web summary and is what is shown in the Loupe Browser visualization. Figure 4 shows an overview of how Visium HD data are binned. The UMI counts shown in the example are illustrative and are not from an actual Visium HD sample.

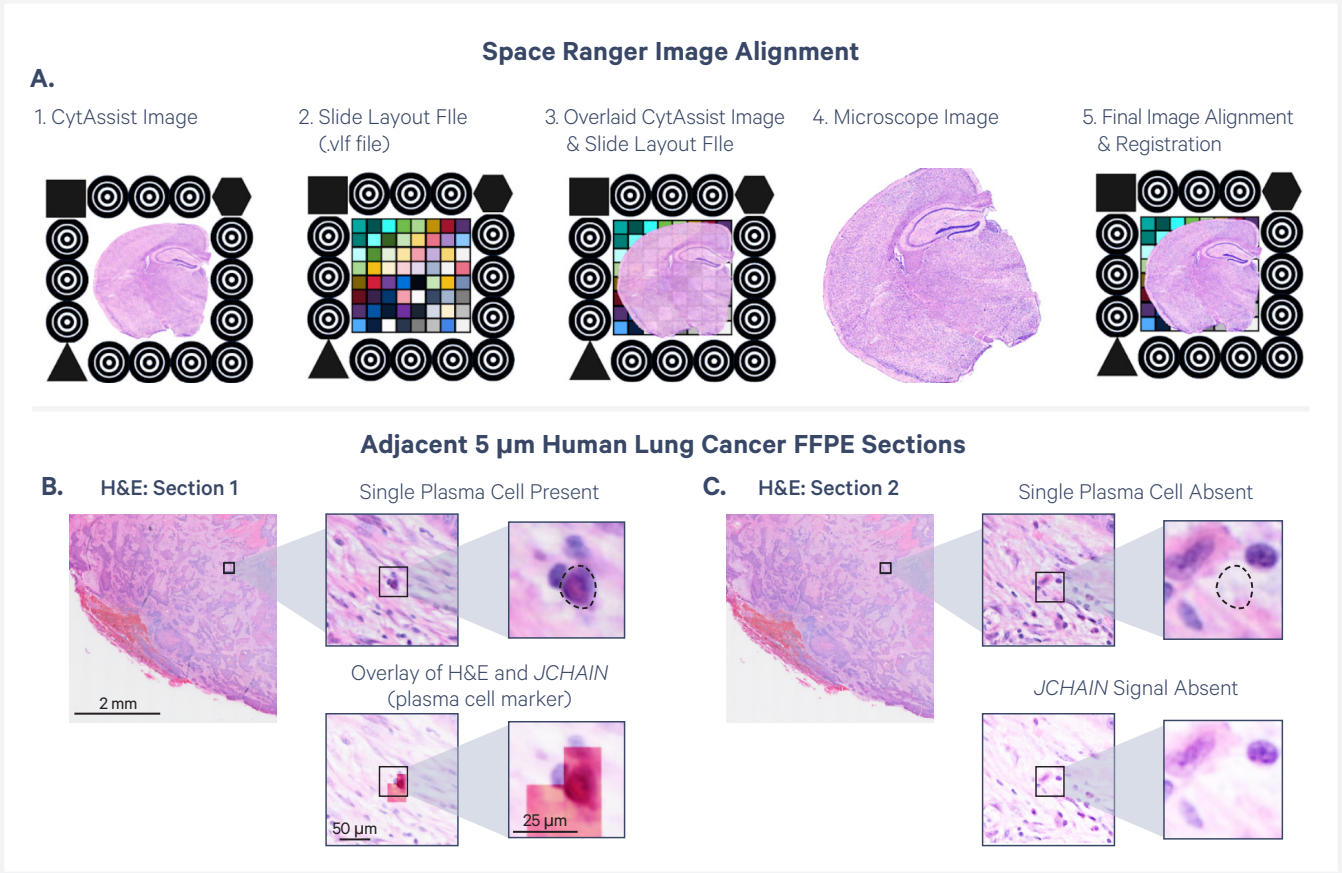

Figure 5. Space Ranger process to achieve accurate alignment between images and gene expression data **(A)**. First, the high-contrast circular fiducials surrounding the Capture Area in the CytAssist image are detected. Then, the slide-specific layout file (.vlf file) is used to determine the position and orientation of the barcoded squares relative to the fiducial frame. Finally, Space Ranger aligns the tissue in the CytAssist image to the tissue in the microscope image. By combining these steps, the barcoded squares can be localized both in the CytAssist and microscope images. The fiducial frame in this image is illustrative and is not to scale. In serial human lung cancer samples run simultaneously through the Visium HD assay, a plasma cell is present as marked by *JCHAIN* in one section **(B)** and absent in the adjacent section **(C)**.

Resolution

Effective spatial resolution is determined by a combination of Spatial Barcode dimensions (i.e. feature size and density), accuracy of transcript localization, and sensitivity. Visium HD optimizes for these dimensions of spatial resolution through a combination of Capture Area design, the CytAssist-enabled assay, and accurate image alignment.

As previously described, the Visium HD Capture Area has a continuous 2 x 2 µm lawn of oligos that enable the capture of transcripts at cellular resolution. The 2 µm square barcode size is the resolution limit of the assay, but effective spatial resolution also requires minimizing flow and diffusion that could localize transcripts to the wrong position. To address this, the Visium HD assay is performed on the CytAssist instrument, which provides an environment designed to minimize diffusion and flow during probe release and capture that would negatively impact accurate transcript localization.

Space Ranger aligns gene expression and imaging information using a three-step process to achieve accurate alignment (Figure 5A). Obtaining gene expression and imaging from the same tissue section versus an adjacent serial section is critical, as tissue section thickness is at the same length of a single cell, resulting in cell composition differences between tissue sections (Figure 5B-C).

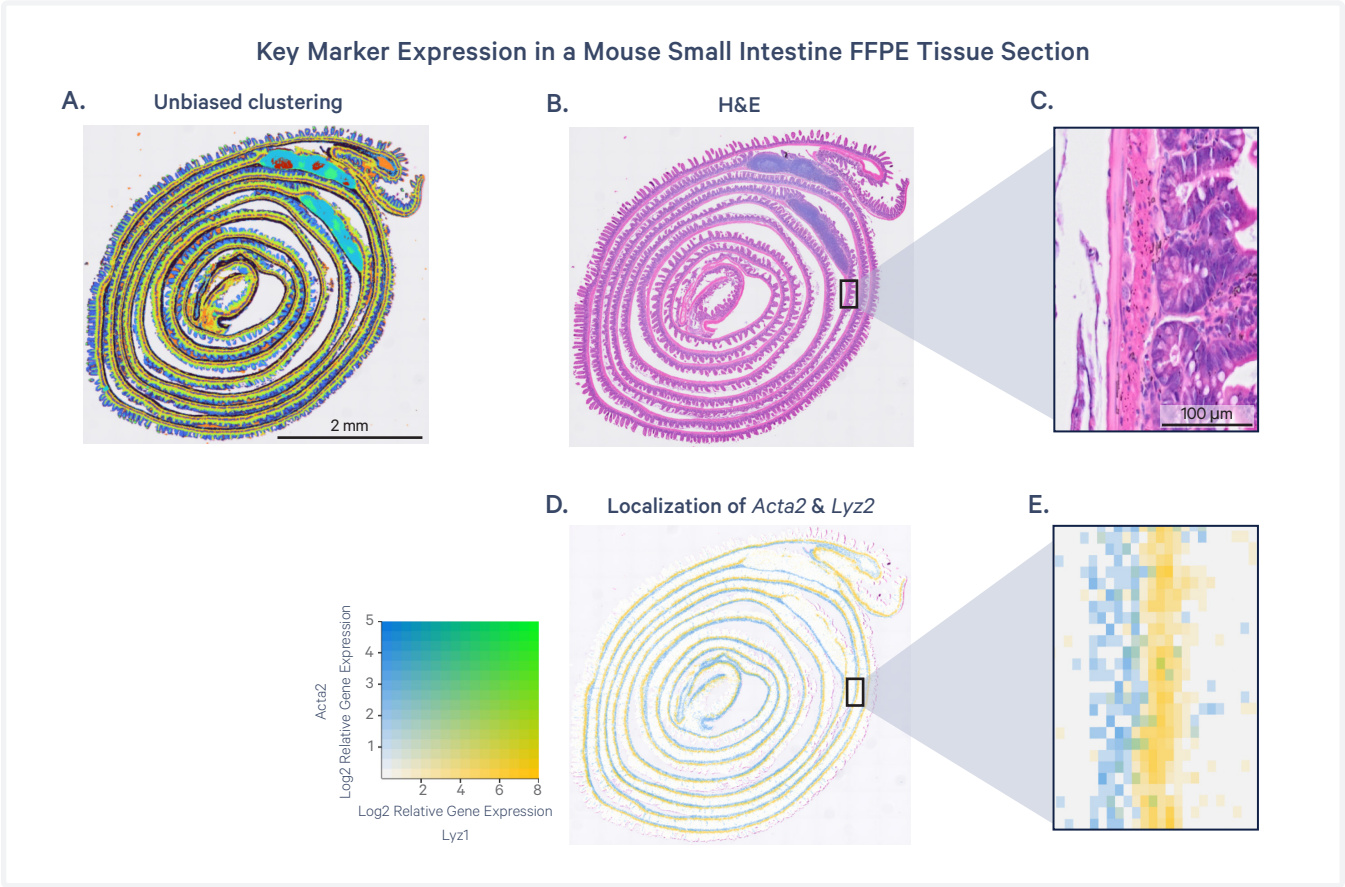

Figure 6. A mouse small intestine FFPE tissue section. Unbiased clustering **(A)** and H&E image **(B)** of the tissue section. A zoomed-in view of the region noted in the black box shows distinct crypts comprised of Paneth cells (dark purple) underlying smooth muscle **(C)**. As expected, the relative gene expression of Paneth cell marker *Lyz1* and the smooth muscle marker *Acta2* appear in distinct layers that correspond to the H&E image **(D-E)**. Colors in D-E correspond to relative gene expression of *Lyz1* and *Acta2* as shown in the heat map.

To demonstrate the accuracy of transcript localization in Visium HD data, qualitative and quantitative assessments were performed on mouse intestine FFPE sections based on marker gene expression. The mouse intestine has a known set of unique marker genes specific to adjacent cell types and a repetitive structure (Figure 6). When looking at the gene expression distribution of key markers in Figure 6, the gene expression pattern reveals clearly-defined separation between Paneth cells and underlying smooth muscle cells. Figure 7 shows that within manually annotated regions, 91% of Paneth cell markers are found in Paneth cells, while 86% of smooth muscle markers are found in smooth muscle. Taken together, these qualitative and quantitative assessments confirm that the Visium HD assay enables accurate transcript localization.

To further illustrate the ability of the Visium HD assay to achieve accurate transcript localization, mouse kidney FFPE tissue sections were analyzed (Figure 8). Structurally, the mouse kidney can be divided into three different regions enclosed by a fibrous outer layer called the renal capsule, the cortex, the outer medulla, and the inner medulla. As with the mouse intestine example, the expression of key marker genes shows clearly defined regions of the kidney, with defined borders between each respective layer and very little blurring, even between adjacent cells.

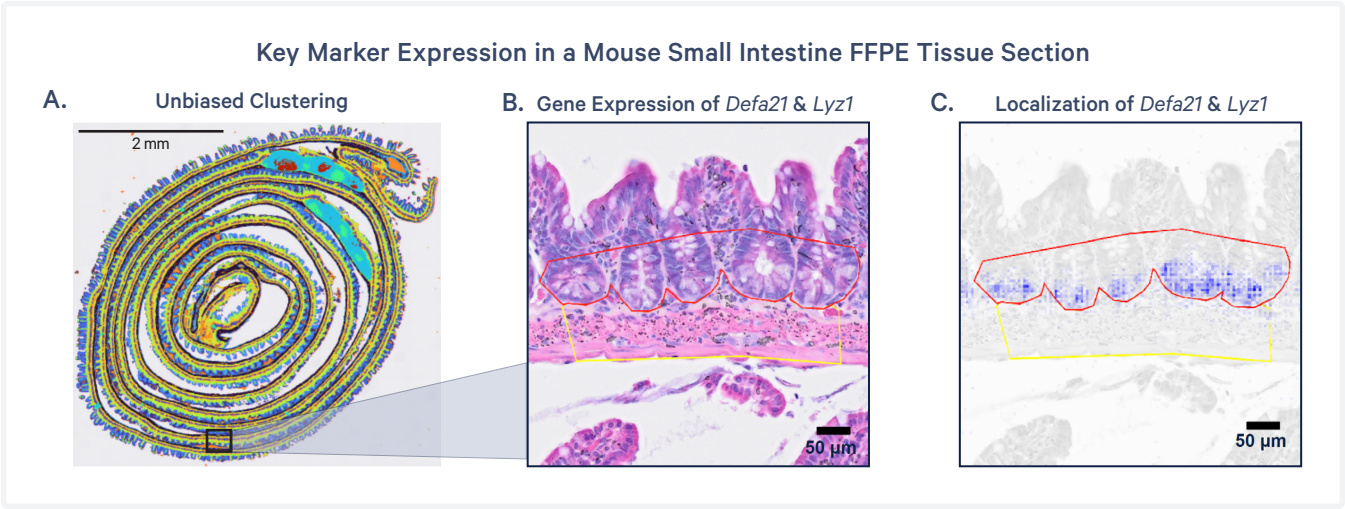

Figure 7. A mouse small intestine FFPE tissue section. **(A)**, H&E image of region marked in the black box **(B)**, and expression of the genes *Defa21* and *Lyz1* in the same region **(C)**. The red outline is a manual annotation of intestinal crypts, while the yellow outline is a manual annotation of the underlying smooth muscle. In this image, 91% of all *Lyz1* and *Defa21* expression exists within the red annotated region. When looking at five regions of interest, the fraction of *Defa21* and *Lyz1* transcripts localized to the appropriate crypt region was 86%, indicating that expected localization remained consistent throughout the tissue.

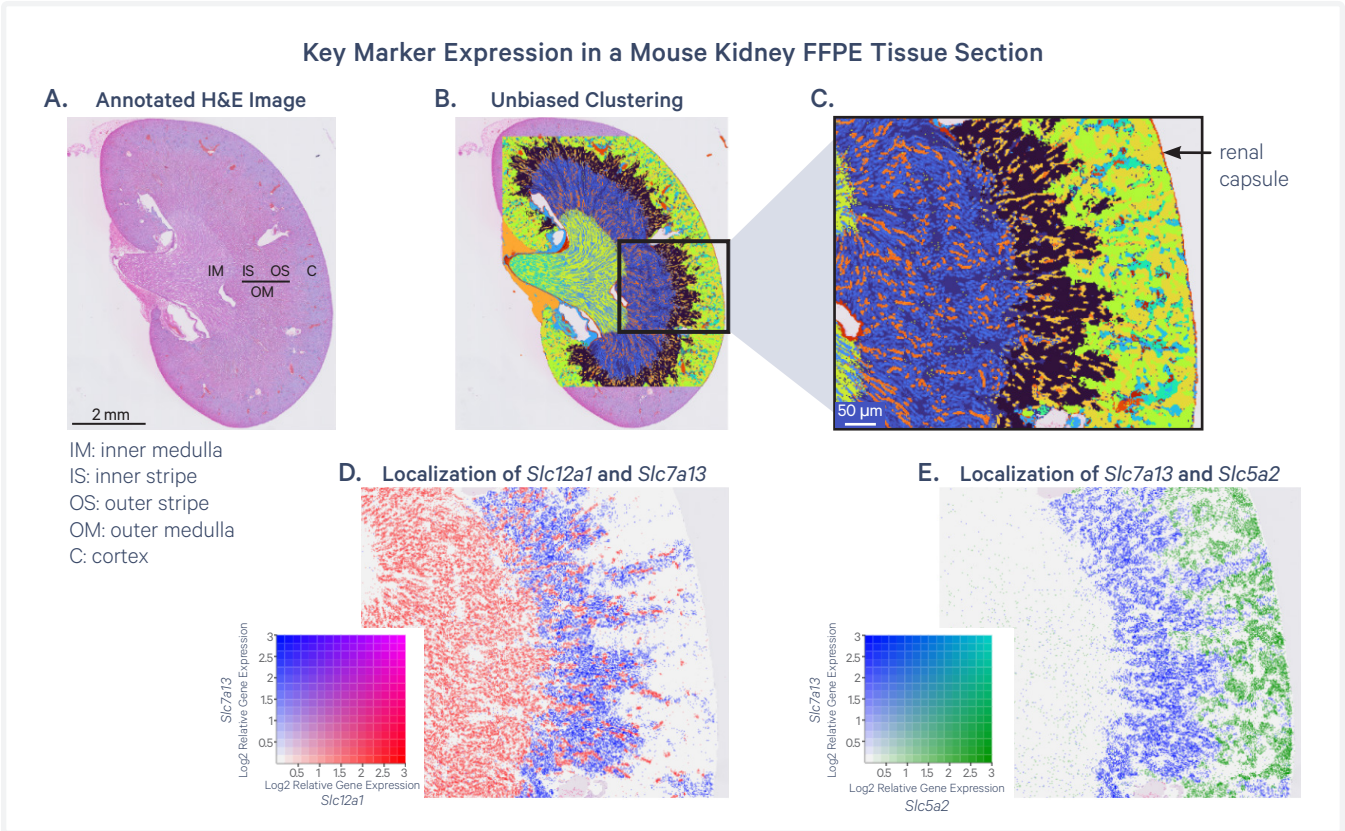

Figure 8. A mouse kidney FFPE tissue section processed with the Visium HD assay reveals expected localization of key cell markers. The mouse kidney is divided into three different regions enclosed by a layer called the renal capsule: the cortex (a peripheral layer), the outer medulla (composed of the outer and inner stripes), and the inner medulla as annotated in the H&E image **(A)**. Defined tissue structures matching known kidney regions is revealed by unbiased clustering **(B-C)**. Known gene markers were used to visualize the spatial localization of two regions: *Slc12a1*, which is expressed by the thick ascending limb of the inner stripe of the outer medulla and *Slc5a2*, which is expressed by proximal tubules within the outer stripe of the outer medulla **(D-E)**. Colors in D-E correspond to relative gene expression of *Slc7a13, Slc12a1, and Slc5a2* as shown in the heat maps.

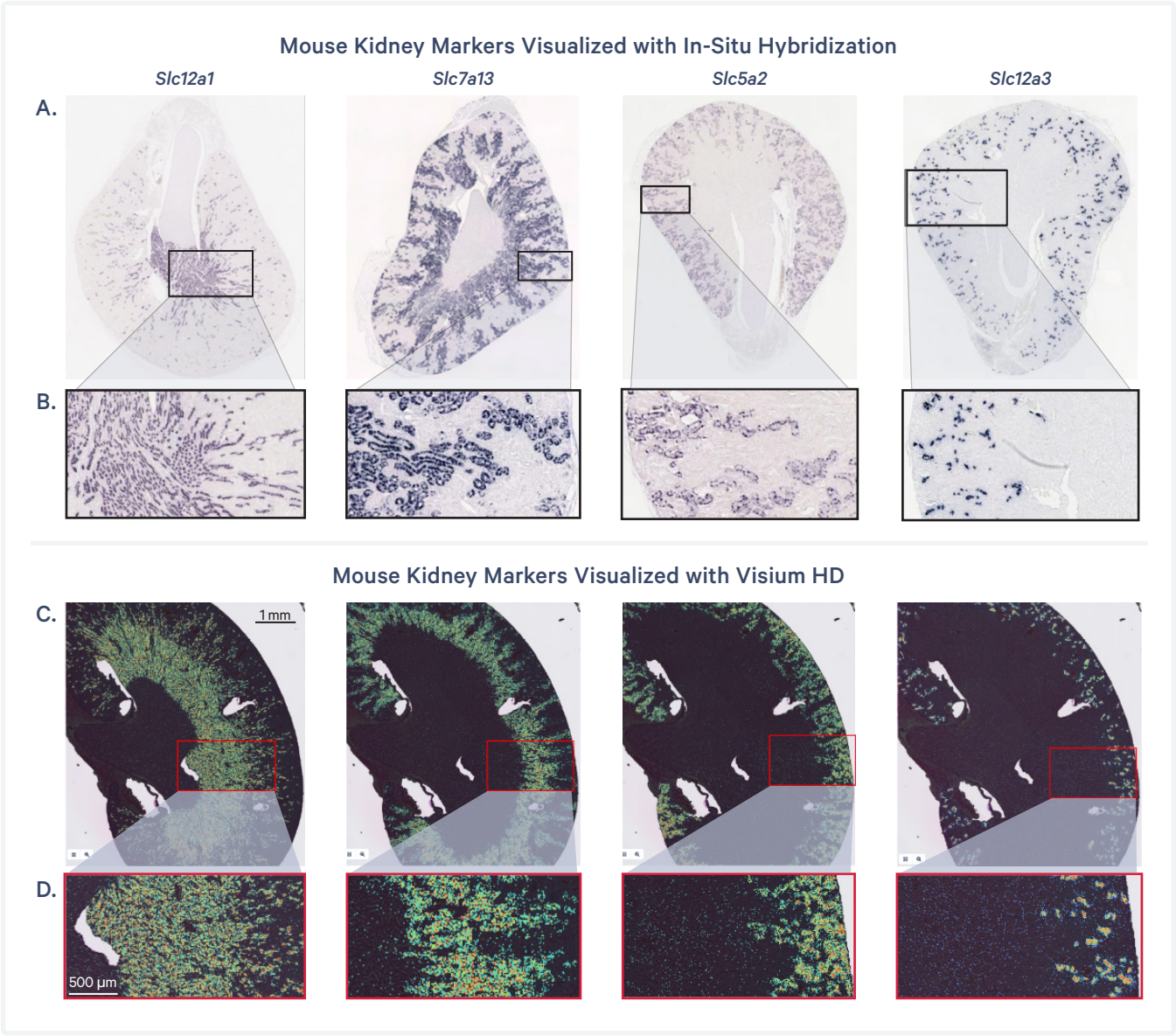

Figure 9. Comparison of Visium HD data with publicly available data for mouse kidney FFPE tissue. The spatial expression pattern of *Slc12a1, Slc7a13, Slc5a2,* and *Slc12a3* using in-situ hybridization (Mouse Genome Informatics, The Jackson Laboratories) **(A-B)** and gene expression from Visium HD using Loupe Browser **(C-D)** are comparably localized within the mouse kidney.

Finally, to confirm that the gene expression patterns detected by the assay match expected patterns based on publicly available data, Visium HD data from the mouse kidney was compared to a collection of in-situ hybridization images from the Jackson Laboratories Mouse Genome Informatics database (Figure 9). As expected, the expression of these key markers in the Visium HD data closely matches the in-situ hybridization results.

Assay Sensitivity

Assay sensitivity refers to the ability of the assay to detect transcripts of interest. To assess sensitivity, defined as the number of unique transcripts detected for the Visium HD assay, UMIs are counted on a per-tissue area basis. Due to differences in slide design between Visium v2 and Visium HD, making sensitivity comparisons on a per-tissue area is a more biologically relevant comparison than making per-active area (e.g. per-spot or per-square) comparisons.

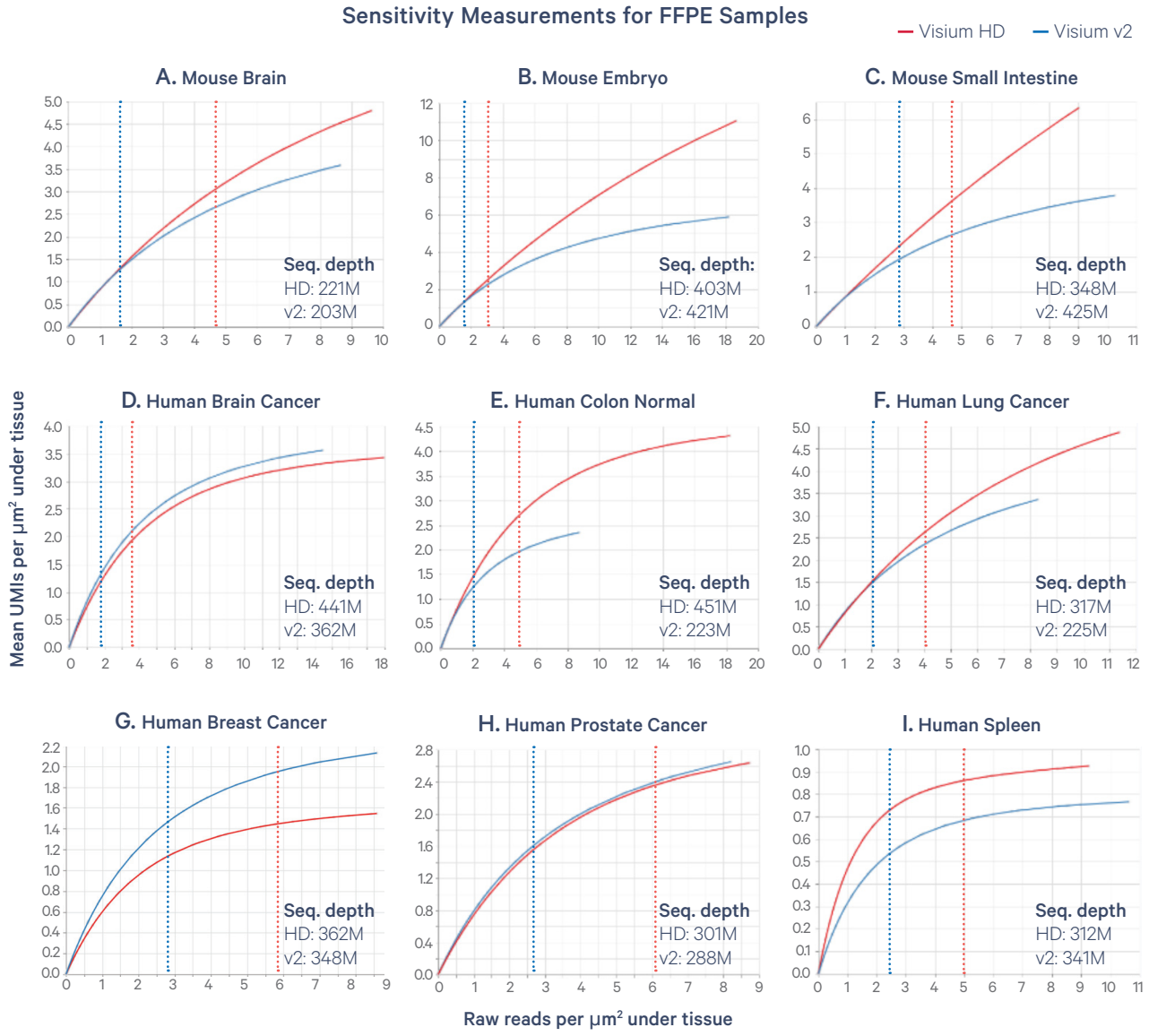

Figure 10. Sensitivity measurements at comparable sequencing depths for the following FFPE samples: **(A)** human embryo **(B)**, mouse small intestine **(C)**, human brain cancer **(D)**, human normal colon **(E)**, human lung cancer **(F)**, human breast cancer **(G)**, human prostate cancer **(H)**, and human spleen **(I)**. Sensitivity analysis is averaged across all sections/tissue types. Vertical dotted lines represent the minimum recommended sequencing depth for Visium HD (red) and Visium v2 (blue) based on recommended sequencing depth. For FFPE tissue processed with Visium HD, this is 275 million reads multiplied by the % Capture Area covered by tissue. For Visium v2, this is % Capture Area covered by tissue multiplied by 5,000 spots multiplied by 25,000 reads/spot. When compared to Visium v2, samples processed with the Visium HD demonstrate comparable or better performance, though this is largely tissue-dependent. Some tissues, such as human spleen, show sequencing saturation as sequencing depth increases. Other tissues, such as mouse intestine, do not show sequencing saturation as sequencing depth increases. While 10x Genomics provides a recommended sequencing depth, the ideal sequencing depth may be tissue-dependent.

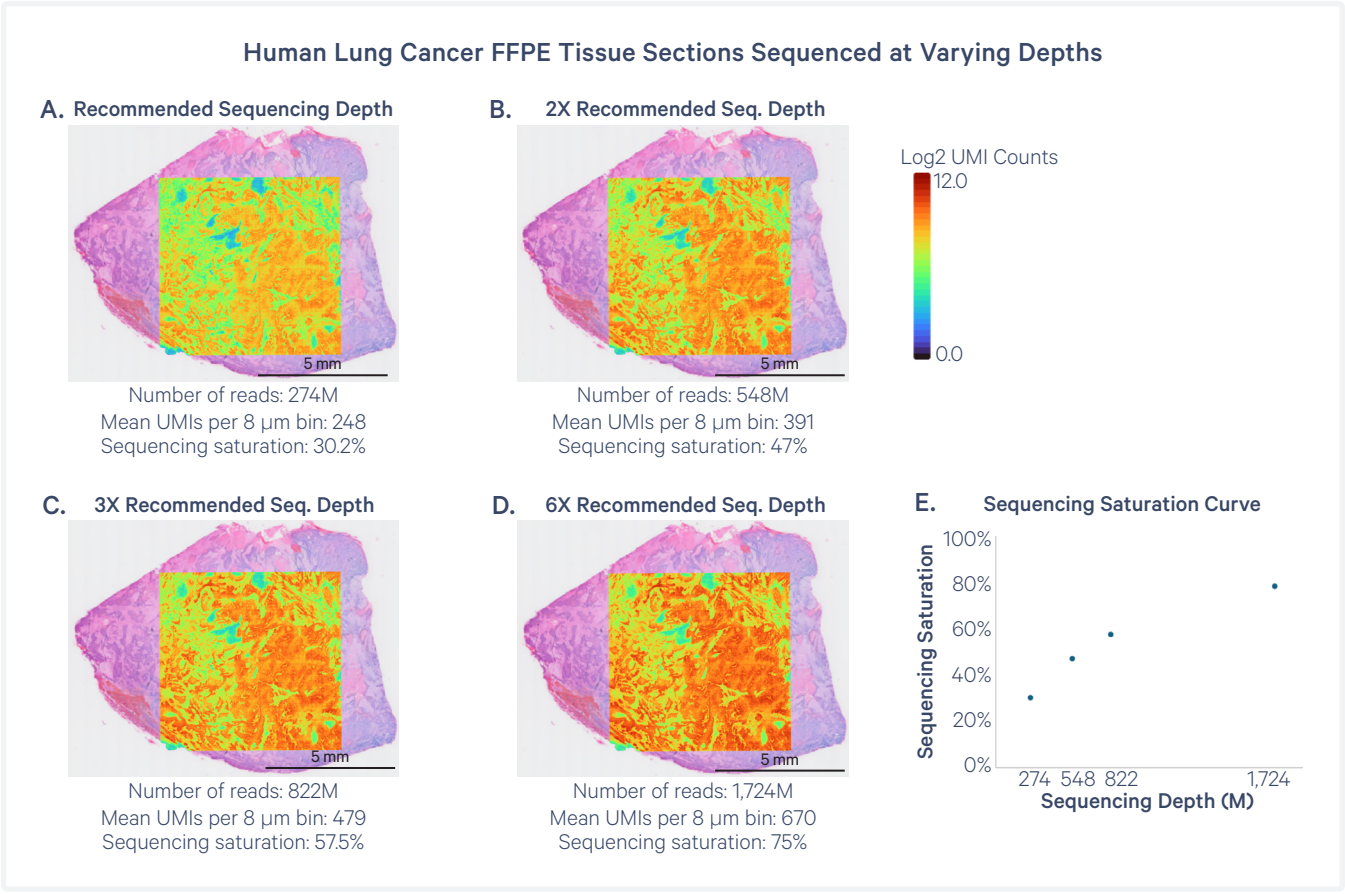

Figure 11. Human lung cancer FFPE samples sequenced at the recommended sequencing depth **(A)**, 2x the recommended sequencing depth **(B)**, 3x the recommended sequencing depth **(C)**, and 6x the recommended sequencing depth **(D)**. Binned UMI heat maps (log2) are overlaid onto the H&E image in each panel. Mean UMIs per 8 µm bin are 248 for (A), 391 for (B), 479 for (C), and 670 for (D). As sequencing depth increases, saturation increases up to 75% at 6x the recommended sequencing depth **(E)**.

The Visium HD slide enables the capture of target molecules under 100% of tissue versus Visium v2, where a portion of molecules can come from tissue located between barcoded spots (Figure 2). Thus, per-area comparisons describe the maximum amount of information that can be obtained from the sample. Figure 10 compares assay sensitivity between Visium v2 and Visium HD across various tissues. For most examples, Visium HD sensitivity is either similar to Visium v2 or exceeds it at matched sequencing depths. Tissue type, tissue composition, and tissue block quality will impact assay sensitivity. Note that the Visium HD assay uses a new version of the mouse probe set, contributing to gains in sensitivity in mouse tissues.

When evaluating sensitivity in the context of a specific sample, it is important to assess sequencing depth (i.e., the number of total reads per unit tissue area) and sequencing saturation (related to the number of reads sequenced per distinct UMI). In some tissue types, sequencing above the recommended sequencing depth (275 million read pairs multiplied by the percentage of the Capture Area covered by FFPE tissue) can result in much higher per-bin sensitivity (total UMIs per bin). An example of a human lung cancer sequenced at varying depths and reaching saturation at 6X the recommended sequencing depth is shown in Figure 11. However, there are diminishing returns for additional sequencing for samples with high sequencing saturation. Space Ranger provides web summary that contains a percent sequencing saturation metric together with sensitivity curves. These can be used as a guide to determine the value

in deeper sequencing. For more information on making sensitivity comparisons between Visium v2 and Visium HD, consult the "How do I Compare the Sensitivity of Visium HD to Visium v2" knowledge base article on the 10x Genomics support website.

Data Considerations

The Visium HD assay results in high-resolution, reproducible data with minimal contribution of genomic DNA. Figure 12 summarizes estimated UMI counts from gDNA across a variety of tissues processed with either the Visium HD or Visium v2 assay. The reduction in percentage of reads estimated to come from gDNA is the result of improvements made to the assay for Visium HD, including decreasing the decrosslinking temperature and updating the decrosslinking buffer. For more information on gDNA, consult the Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA Technical Note (Document CG000605). As shown in Figure 13, data are also reproducible across different lots of Visium HD slides, supported storage conditions, and supported thermal cyclers.

Due to design and manufacturing complexities of the Visium HD slides, some artifacts may manifest in Visium HD data that are not seen in Visium v2; however, these differences do not have significant effects on data interpretation. The reasons why these artifacts appear and additional context around data interpretation are discussed below.

When visualizing large numbers of UMIs at once, some rows and columns of bins or 2 µm squares may appear slightly darker or brighter than others. This effect is most apparent when viewing the total UMI map. These “line patterns” appear due to technical variations in the slide manufacturing process that result in a slight variance in the square size and appear as differences in sensitivity when visualized as idealized 2 µm squares. This affects all genes similarly and has minimal impact on spatiality. Since all genes are uniformly affected, secondary analysis such as

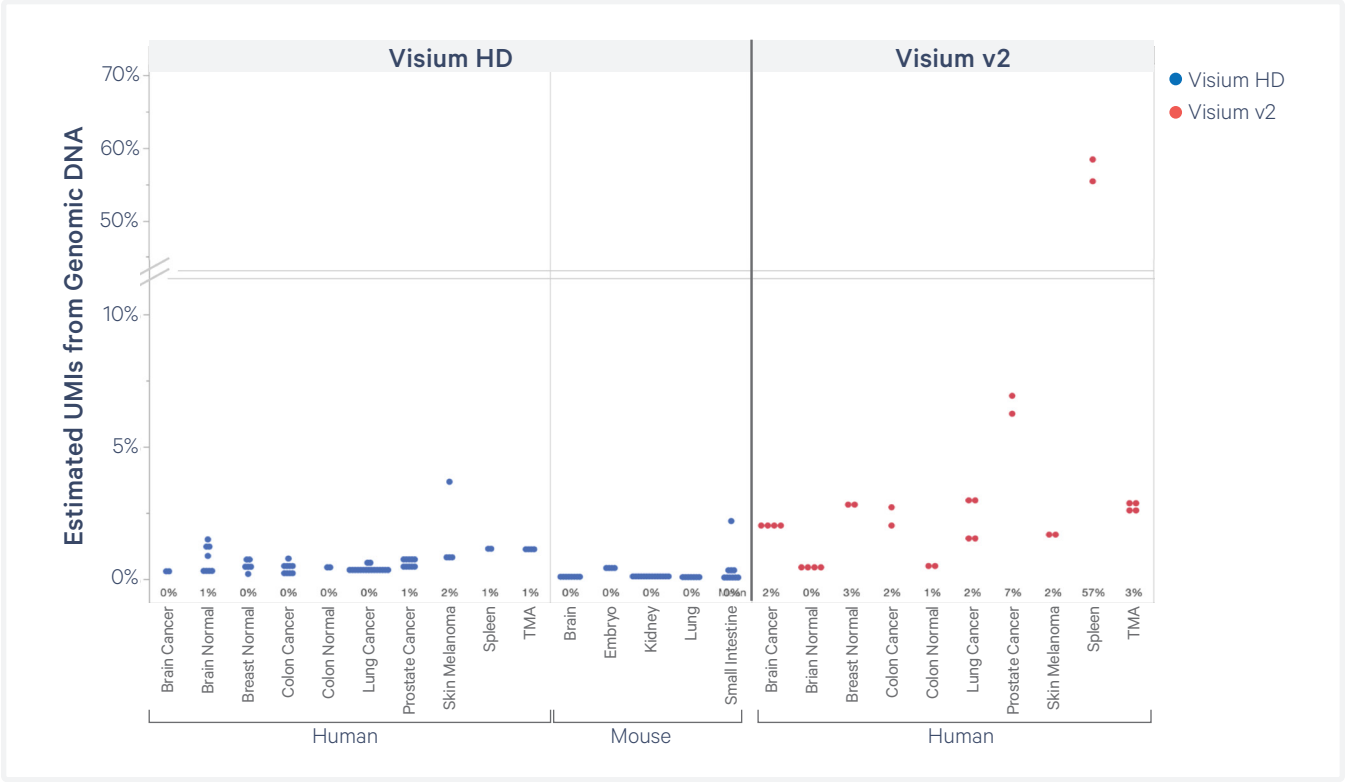

Figure 12. Estimated UMI counts from gDNA across various tissues. Mouse data from Visium v2 is not displayed because the Visium v2 assay uses v1 of the mouse probe set, which does not have the required gDNA probes for detection.

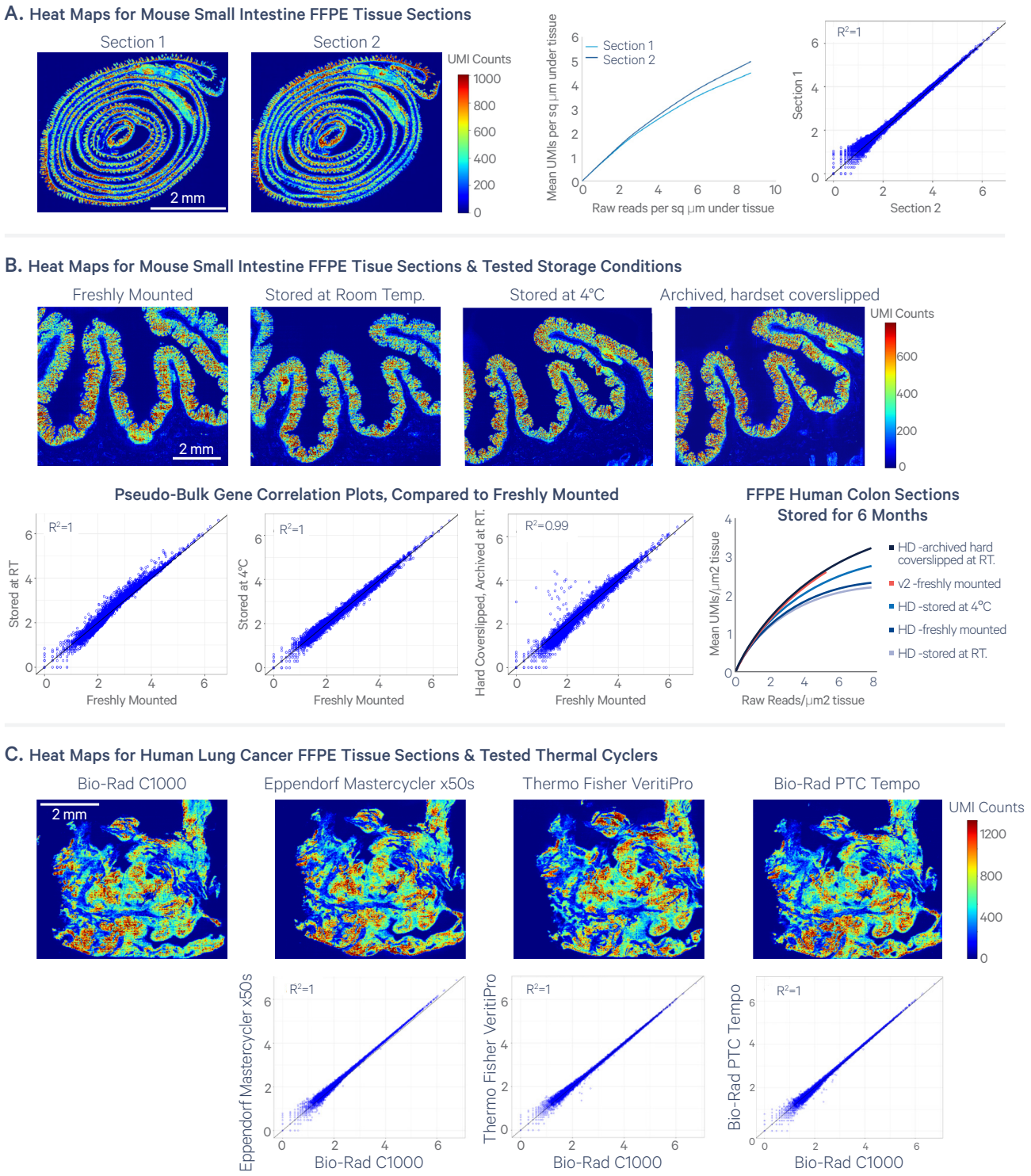

Figure 13. Assay reproducibility across slide lots, storage conditions, and thermal cyclers. **(A)** Two sections from the same mouse small intestine FFPE block have similar UMI counts, mean UMIs per square µm under tissue, and have strongly correlated gene expression profiles across slide lots. **(B)** Human colon FFPE tissue was processed under the indicated conditions. Unless otherwise noted, samples were not coverslipped. At 6 months, archived sections were processed using a freshly-sectioned/mounted tissue from the same block as control on different CytAssist instruments. UMI heat maps and pseudobulk gene correlation plots between replicates show high correlation. **(C)** Consecutive sections from a human lung cancer FFPE tissue block were used to test the indicated thermal cyclers. Each condition was run on a different CytAssist instrument. Replicates have consistent UMI heat maps as well as high gene expression correlation between the tested thermal cyclers.

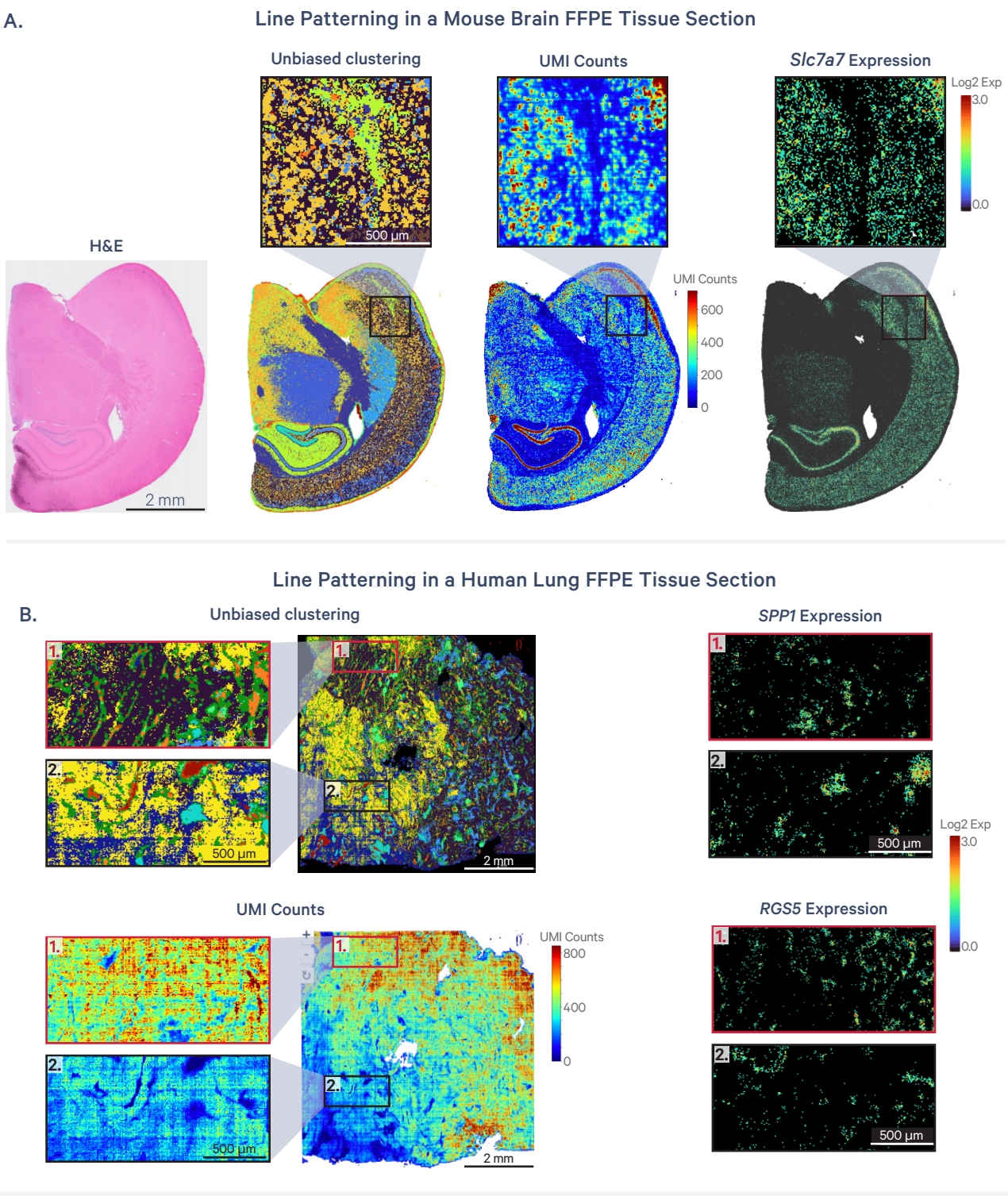

Figure 14. (A) A mouse brain FFPE tissue section that shows a line pattern when viewing unbiased clustering and total UMIs within the area marked with a black box. This line pattern has a negligible impact on the unbiased clustering or the expected localization of key marker genes, such as *Slc7a7*. **(B)** A human lung section, where line patterns are more apparent in areas of low expression than in areas of high expression as shown when viewing unbiased clustering or total UMI counts. As with the mouse brain FFPE tissue section, this line patterning has a negligible effect on the expected localization of key marker genes such as *SPP1* or *RGS5*.

differential expression or unbiased clustering – which normalize for differences in total UMI count per bin – are minimally impacted. Figure 14 shows the minimal impact of line patterns in areas of high gene expression and the minor impact to unbiased clustering in regions with very low UMI counts.

To illustrate the minimal impact of line patterns on unbiased clustering, the top differentially expressed genes between the dark blue and yellow clusters in the human lung sample were examined, as these form a line pattern (Figure 15). The most highly expressed genes in the dark blue cluster are also highly expressed in the cyan cluster, indicating that the dark blue cluster is composed of bins bridging the cells marked by cyan and the low transcript density yellow cluster. Additionally, there are very few genes that uniquely identify the yellow cluster, which is an area of low expression. There are no genes that distinguish the dark blue and yellow clusters in any meaningful way; thus, the appearance of the line between the clusters is considered inconsequential.

Visium HD slides undergo a rigorous manufacturing process that ensures that >99% of the Capture Area is covered with spatially-barcoded capture oligos; however, localized low UMI recovery can occur as a result of manufacturing defects within the Capture Area that result in missing oligos on the slide surface. Additionally, debris on the Visium HD slide or tissue slide or the presence of bubbles between the tissue and Capture Area may also result in low UMI recovery, as these may inhibit probe ligation products from being captured on the Visium HD

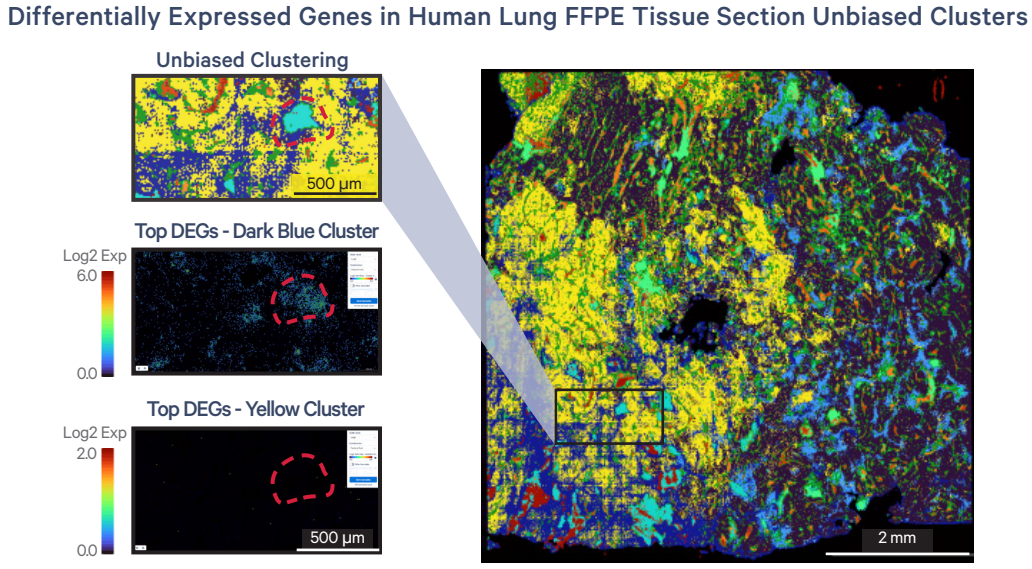

Figure 15. Examining the top differentially expressed genes (DEGs) between the yellow and dark blue clusters that form a line pattern in a human lung FFPE sample.

slide and assigned a UMI and Spatial Barcode. To assess the percentage of Spatial Barcodes that match the whitelist, a valid barcode rate is reported in the web summary produced by Space Ranger. For the Visium HD assay, this rate is typically above 85%.

There is a small chance (<1.5%) that errors in the Spatial Barcode oligo sequence result in the assignment of that barcode's sequence to an unexpected location (> 10 µm away). This will result in up to 1.5% of all transcripts being improperly located across the tissue. While this has minimal impact on clustering results, it creates low level noise in the data as well as a visual impact to individual gene localization analysis (Figure 16). While this is only noticeable for highly abundant genes, this mislocalization can occur for any transcript regardless of expression level.

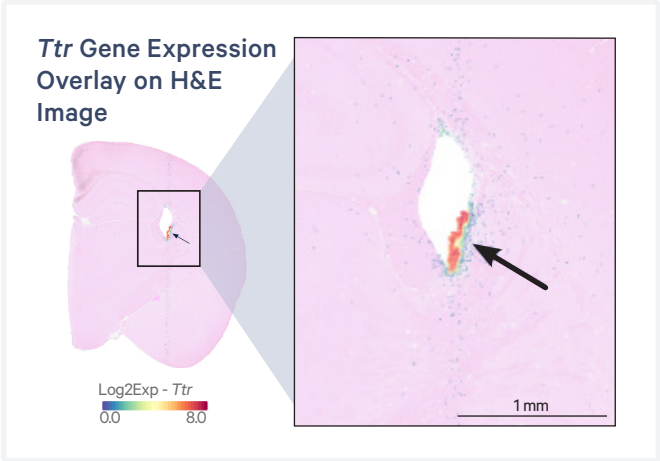

Figure 16. A mouse brain FFPE sample demonstrating mislocalization on the highly expressing and highly concentrated *Ttr* gene. *Ttr* is expected to only be expressed within the choroid plexus; however, mislocalization of *Ttr* signal is seen in both the X and Y axes.

Conclusion

The Visium HD Spatial Gene Expression assay enables single cell scale analysis of gene expression data. This Technical Note described the design of the Visium HD Slide, as well as the data binning strategy Space Ranger utilizes to optimize data interpretation. The data presented in this Technical Note shows that:

- Visium HD retains high spatial resolution through optimal transcript localization and feature dimension that allow whole-transcriptome gene expression data to be mapped clearly to relevant biological structures
- Sensitivity comparisons are tissue-dependent, but Visium HD typically has comparable sensitivity to Visium v2
- Visium HD is reproducible across different lots of Visium HD slides, CytAssist instruments, serial tissue sections, and thermal cyclers
- Visium HD optimizes data quality and minimizes the percentage of reads estimated to come from gDNA

References

- Visium CytAssist Tissue Preparation Guide (CG000518)
- Visium CytAssist H&E Staining Demonstrated Protocol (CG000520)
- Visium CytAssist Spatial Gene Expression Reagent Kits User Guide (CG000495)
- Visium HD FFPE Tissue Preparation Handbook (CG000698)
- Visium HD Spatial Applications Imaging Guidelines (CG000688)
- Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685)
- Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA (CG000605)

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