Visium CytAssist Spatial Gene Expression for FFPE: Reagents, Workflow, & Data Comparison

Introduction

The Visium CytAssist Spatial Gene Expression for FFPE assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) tissue samples, using probes to target the whole transcriptome. Visium CytAssist is a compact instrument designed to simplify the Visium workflow by facilitating the transfer of transcriptomic probes from tissue slides to Visium slides. This enables spatial profiling insights to be gained from an expanded range of FFPE samples, including archived tissue blocks and slides.

This Technical Note compares reagents, workflow, and data generated from the Visium Spatial Gene Expression for FFPE assay ("Direct placement" data) and data generated using the Visium CytAssist Spatial Gene Expression assay with the CytAssist instrument ("CytAssist-enabled" data) with FFPE tissue samples. It highlights the performance improvements to spatial resolution clustering patterns, specificity, and gene sensitivity in multiple sample types.

Contents

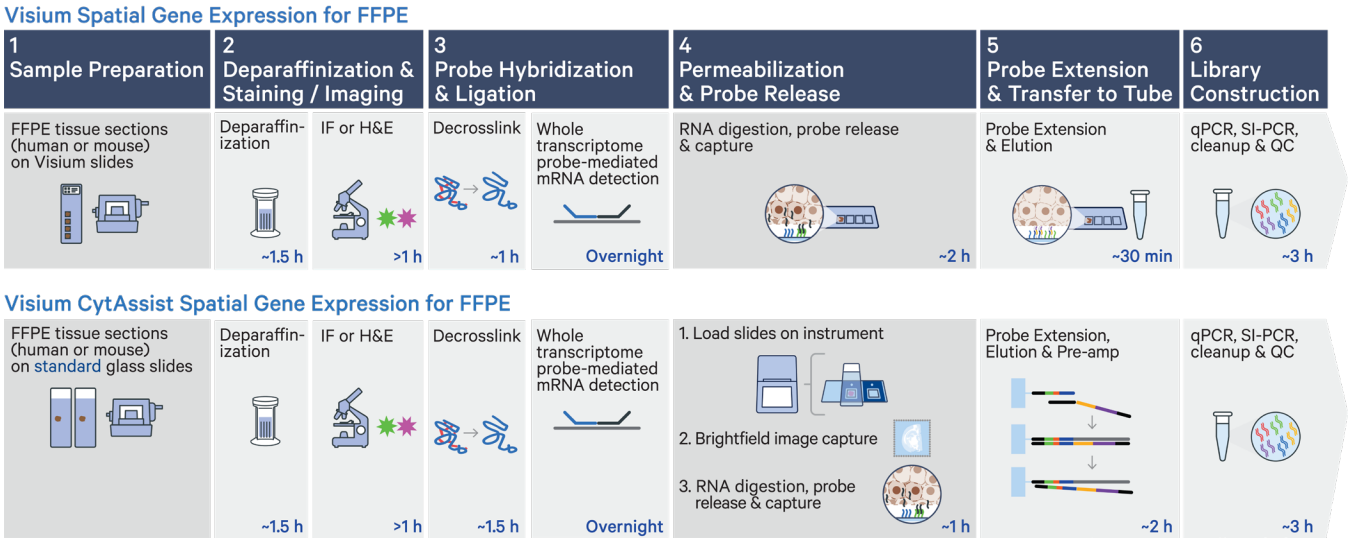

Figure 1. Workflow comparison between Visium Spatial Gene Expression for FFPE (Direct placement) and Visium CytAssist Spatial Gene Expression for FFPE (CytAssist-enabled) assays.

Assay Differences

Key differences between the Direct placement and the CytAssist-enabled assays performed in this document are presented in the table below and highlighted in Figure 1. For detailed information on protocol differences, refer to Appendix A1: Protocol Step Differences. Consult the relevant documentation for complete information.

	Direct placement	CytAssist-enabled
Instrument	N/A	Visium CytAssist
Tissue Types	Freshly placed FFPE sections	Freshly placed FFPE sections Archived hematoxylin & eosin (H&E) or immunofluorescence (IF) + DAPI stained and imaged sections
Slide Types	Visium Spatial Gene Expression Slides	Superfrost Plus Slides Visium CytAssist Spatial Gene Expression Slides v2
DV200 Recommendation	≥50%	≥30%
Reagent Kits		
10x Genomics Reagents	Visium Spatial Gene Expression for FFPE Kit, Human Transcriptome Visium Spatial Gene Expression for FFPE Kit, Mouse Transcriptome	Visium CytAssist Spatial Gene Expression for FFPE Kit, Human Transcriptome Visium CytAssist Spatial Gene Expression for FFPE Kit, Mouse Transcriptome
Software		
Software Analysis with Space Ranger	Requires an individual brightfield or fluorescence microscope image of each tissue Capture Area, including the fiducial frame, on the Visium Spatial Gene Expression Slide	Requires specifying the CytAssist instrument captured image containing the fiducial frame using --cytaimge with optional brightfield or fluorescence microscope image of the same tissue section on a blank slide

Sample Preparation Methods

To compare performance between the Direct placement and CytAssist-enabled assays, 5 µm FFPE tissue sections were generated from the following tissues:

Organism	Tissue	Disease State	Vendor
Mouse	Brain	Healthy	Charles River
	Spleen		
Human	Brain	Healthy	Avaden Biosciences
	Ovary	Serous Carcinoma	

All samples being compared between the assays were either derived from serial sections or proximal sections on the same tissue. Sections were placed on either Visium Spatial Gene Expression Slides (Direct placement) or blank slides (CytAssist-enabled). Samples were stained separately due to differences in the Direct placement and CytAssist-enabled staining protocols (refer to Appendix A1 for more information). Sections were then processed according to either the Direct placement or CytAssist-enabled workflows, as described in the table below (one representative replicate per condition). The CytAssist-enabled assay uses the same probe set as Direct placement for mouse samples (Visium Mouse Transcriptome Kit v1), but not for human samples (Visium Human Transcriptome Kit v1 and v2). While the genes covered in the Visium Human Transcriptome Kit v1 and v2 are not exactly the same, the analyses in this Technical Note focus only at the genes common to each panel.

Species	Assay	Probe Set Version
Mouse	Direct placement	Visium Mouse Transcriptome Kit v1
	CytAssist-enabled	
Human	Direct placement	Visium Human Transcriptome Kit v1
	CytAssist-enabled	Visium Human Transcriptome Kit v2

All samples were processed using the documentation below:

Direct placement	CytAssist-enabled
Visium Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000408)	Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000518)
Visium Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000409)	Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000520)
Visium Spatial Gene Expression Reagent Kits for FFPE User Guide (CG000407)	Visium CytAssist Spatial Gene Expression Reagent Kits for FFPE User Guide (CG000495)

Data Analysis Methods

To compare data between the Direct placement and CytAssist-enabled assays, all samples were downsampled to an equivalent read depth. To reduce batch effects between samples, analyses were conducted on the intersection of genes between the two probe sets, as the genes within each probe set are slightly different from one another. The union of variable genes was selected across samples to run a principal component analysis (PCA). The top twenty principal components were selected to run Harmony embeddings to cluster the data. The resolution hyperparameter is specific for each tissue, as biological diversity is tissue-specific. Figure 2 shows a summary of the analysis workflow.

To compare biological information between the two assays, genes expressed within the same cluster across samples were compared. The differential gene expression (DGE) list is filtered to adj.p.value<0.05 and abs(Log2FC)>0.5.

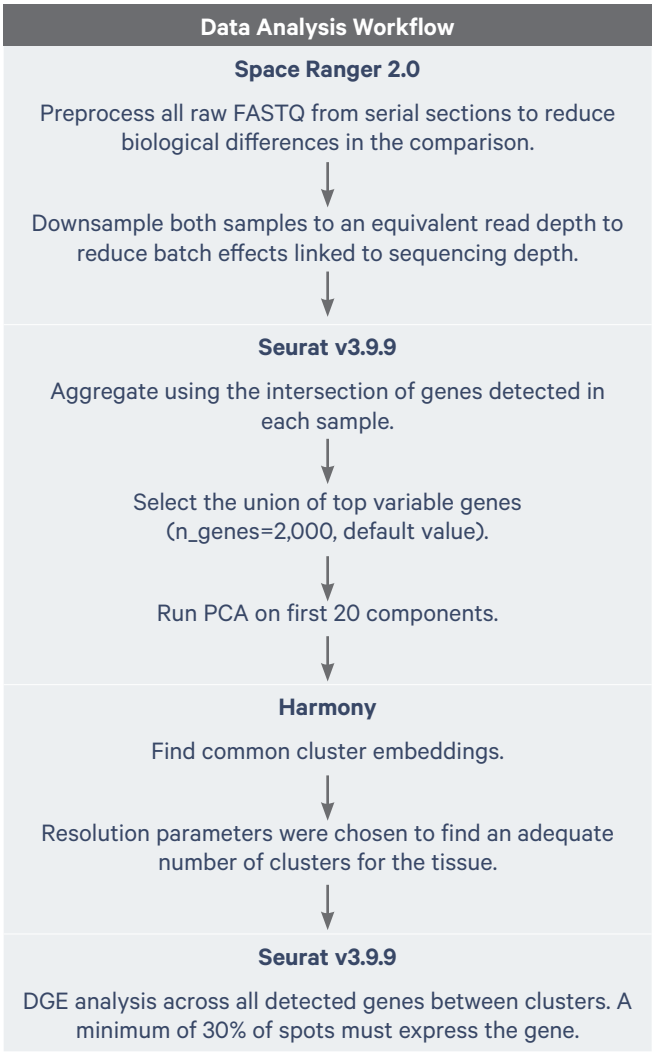

Figure 2. Guidelines for DGE analysis.

Results

Mouse Tissues

Table 1 summarizes key performance metrics from mouse tissue libraries generated with either the Direct placement or CytAssist-enabled assay. Both assays yield comparable data when working with healthy mouse tissue sections with similar morphology. The CytAssist-enabled assay leads to improvements in the fraction reads under tissue metric, suggesting that the CytAssist-enabled assay has less noise outside of tissue. Genes and UMIs were downsampled to equivalent read depths to allow for direct comparison.

Tissue	Assay	Reads Confidently Mapped to Probe Set	Mean Genes/Spot	Mean UMIs/Spot	Fraction Reads Under Tissue
Mouse Brain	Direct placement	98.0%	4,960	13,357	94.5%
	CytAssist-enabled	98.5%	4,805	13,537	97.0%
Mouse Spleen	Direct placement	97.9%	4,753	12,470	84.8%
	CytAssist-enabled	98.3%	5,326	13,356	94.4%

Table 1. A comparison of metrics for mouse tissues processed with the Direct placement or CytAssist-enabled assays.

Figure 3 shows that most of the genes found in the Direct placement assay are recovered with the CytAssist-enabled assay. Many more unique genes that were not found using Direct placement were found using the CytAssist-enabled assay when looking at differentially expressed genes in Harmony clusters. Saturation levels were lower in CytAssist-enabled samples (average of ~27%) as compared to Direct placement samples (average of ~48%); thus, deeper sequencing would likely reveal additional genes uniquely recovered in the CytAssist-enabled assay.

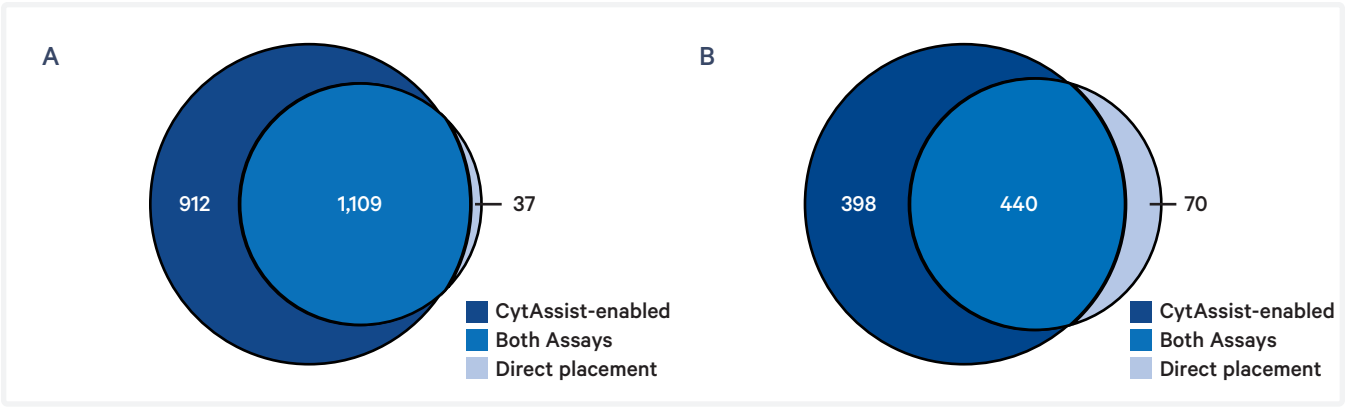

Figure 3. DGE analysis for mouse brain **(A)** and mouse spleen **(B)** showing the number of genes differentially expressed in either the CytAssist-enabled, Direct placement, or both assays (abs(Log2fc > 0.5), adj.p.value < 0.05) in the same clusters provided by Harmony.

Results

Mouse Tissues

Figures 4 (mouse brain) and 6 (mouse spleen) demonstrate that clustering through Harmony aligns well between the assays. Single *k*-means clustering of each assay with the same *k* as Harmony demonstrates that the CytAssist-enabled assay is capable of more distinction, as shown by the additional two clusters found in the dentate gyrus in the center of the tissue section.

Figure 5 shows individual gene expression within the mouse brain, while Figure 7 shows individual gene expression within the mouse spleen. Individual markers shown in Figure 5 show improved specificity to expected areas of the brain and lower background signal for CytAssist-enabled samples vs. Direct placement. In the spleen, follicular structures show more defined spatial clustering (Figure 6), with sharper distributions of individual markers (Figure 7).

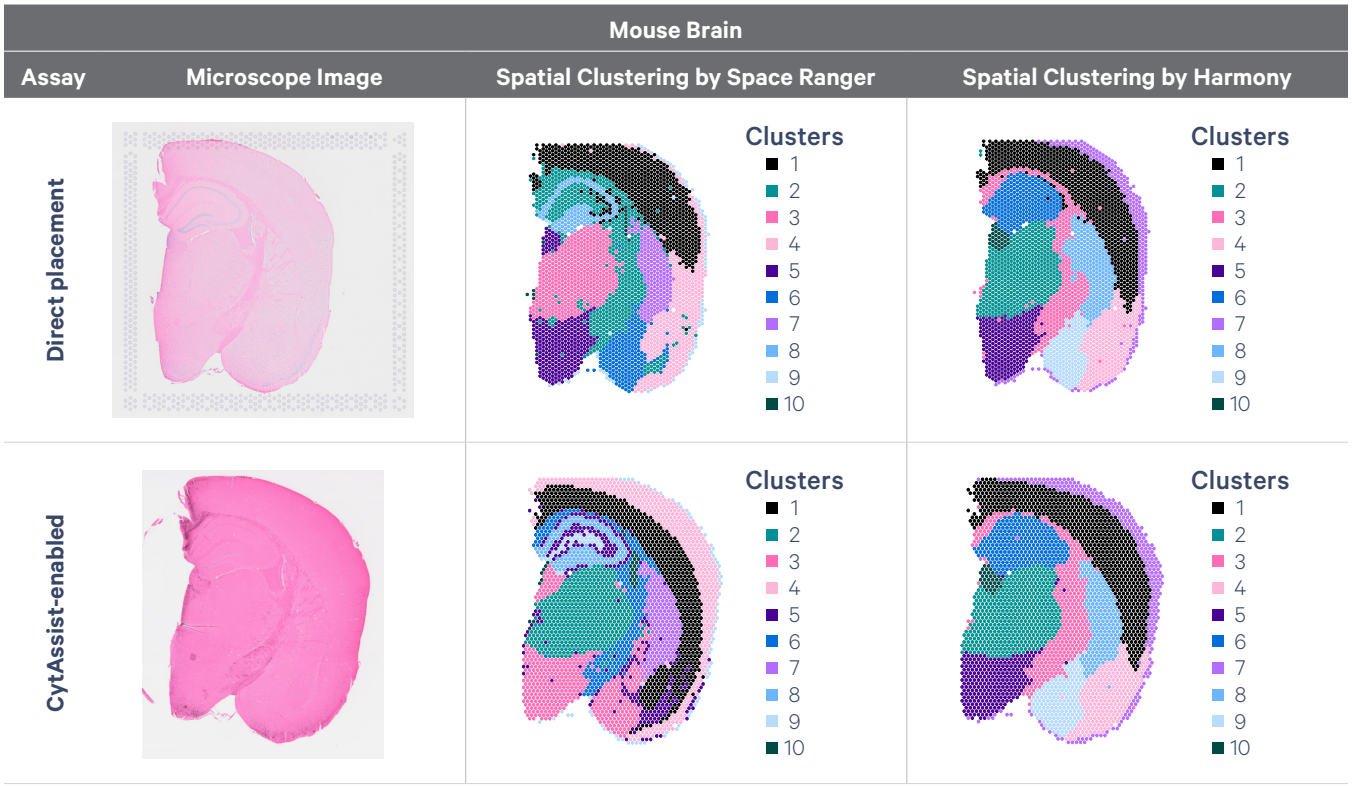

Figure 4. Microscope images (left) and spatial clustering of mouse brain tissue using both Direct placement (top) and CytAssist-enabled (bottom) assays produced with Space Ranger 2.0 (middle) or Harmony (right). Both Harmony clustering plots share the same cluster assignments, while each Space Ranger clustering plot is clustered independently.

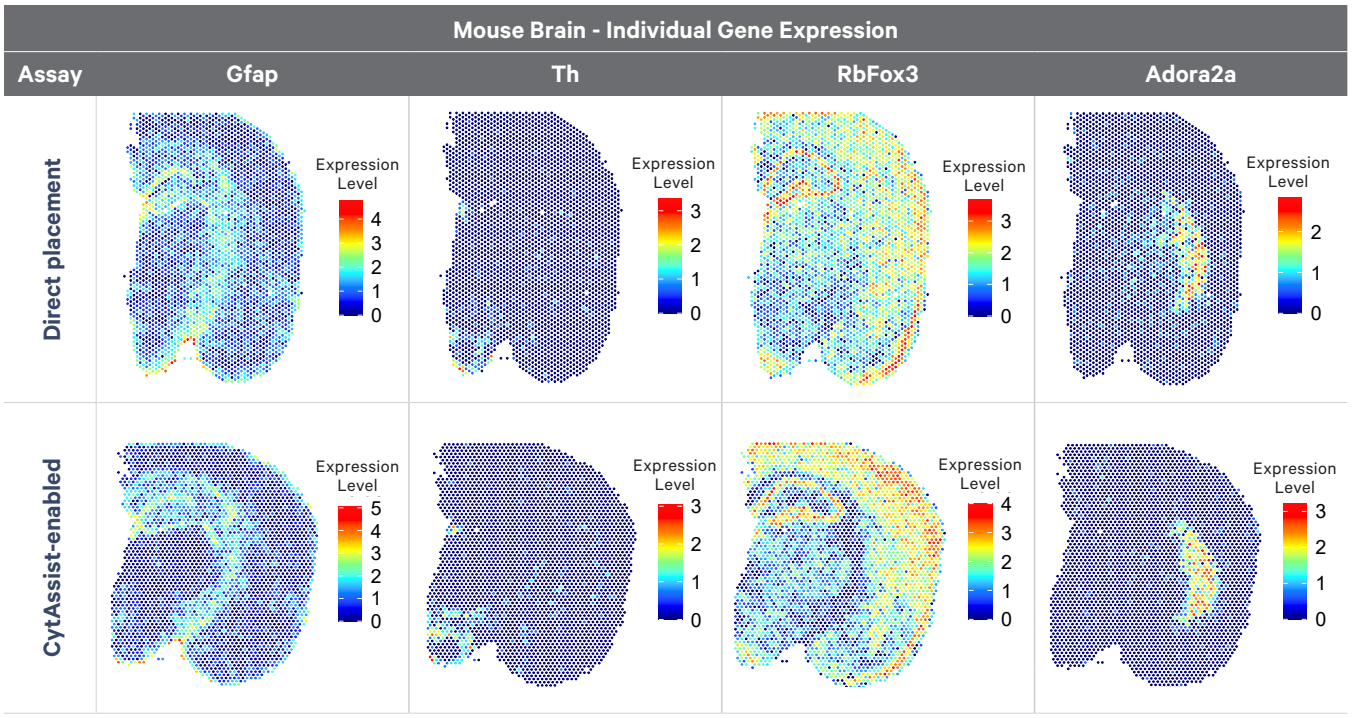

Figure 5. Individual gene expression in mouse brain tissue for both Direct placement (top) and CytAssist-enabled (bottom) assays. All expression levels are represented as log1p (natural log (X + 1). Gfap is an astrocyte marker found in white matter. Th encodes tyrosine hydroxylase, which helps convert tyrosine to dopamine in the frontal lobe. Rbfox3 produces NeuN, a marker for mature neurons that should be expressed throughout the section. Adora2a codes for an adenosine receptor expressed in the basal ganglia.

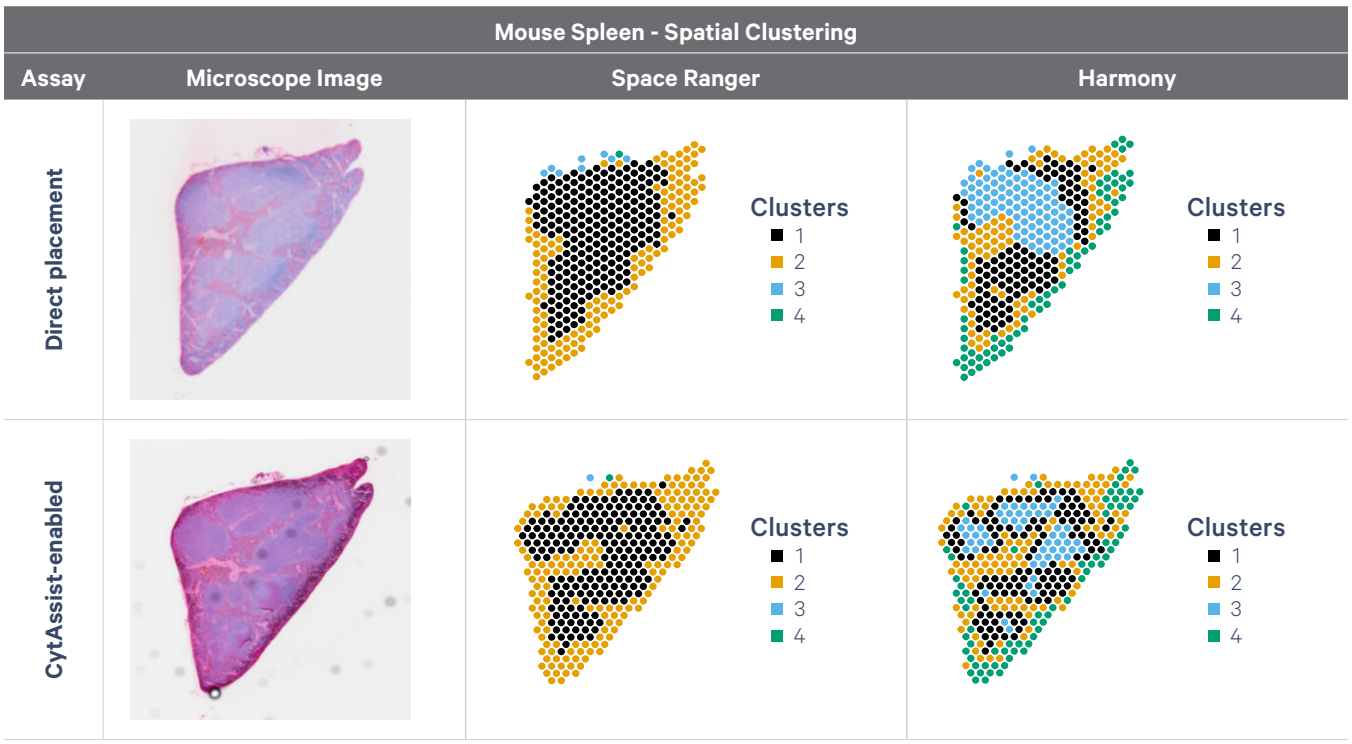

Figure 6. Microscope images (left) and spatial clustering of mouse spleen tissue using both CytAssist-enabled (top) and Direct placement (bottom) assays produced with Space Ranger 2.0 (middle) or Harmony (right). Small bubbles in the CytAssist-enabled high resolution image are from glycerol during coverslipping and do not affect assay performance.

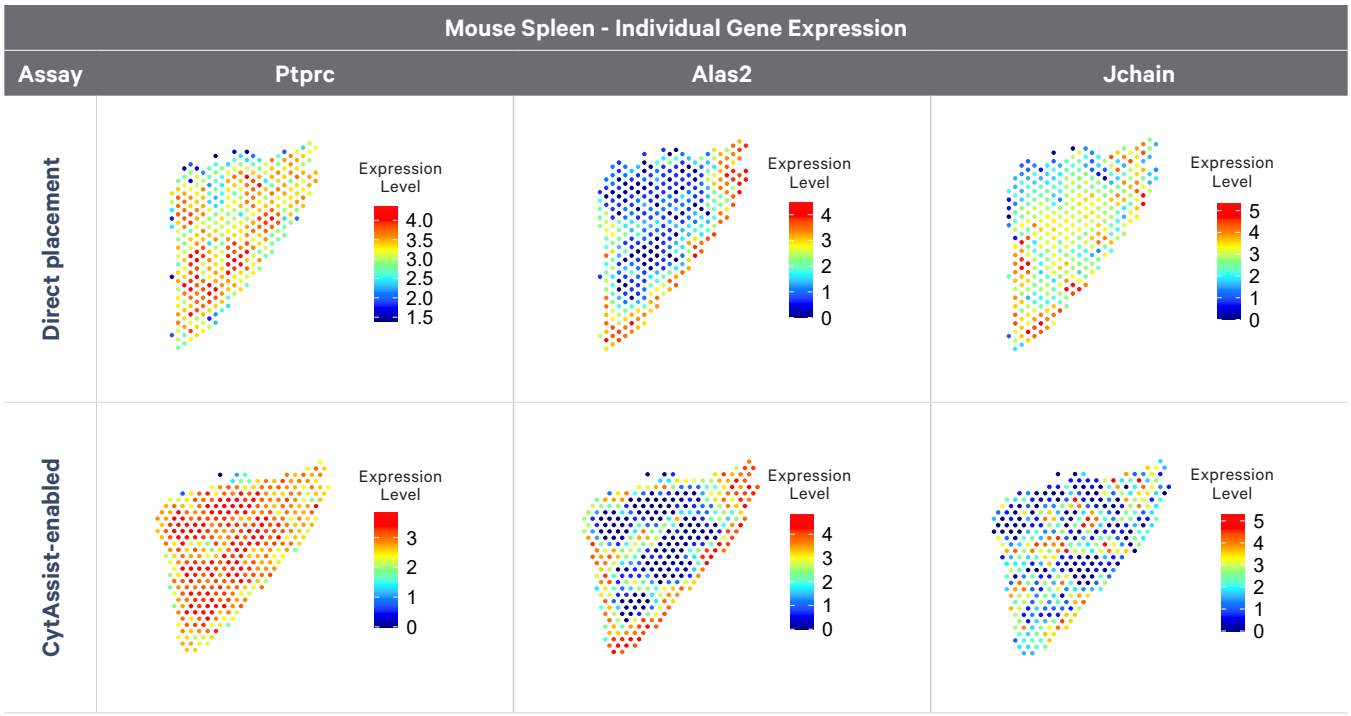

Figure 7. Individual gene expression in mouse spleen for both Direct placement (top) and CytAssist-enabled (bottom) assays. All expression levels are represented as log1p (natural log (X + 1). Ptprc encodes CD45, a regular of T and B cell antigen receptor signaling. Alas2 codes for an enzyme that catalyzes heme biosynthesis. Jchain plays a role in IgA and IgM binding and polymerization. Follicular structures show more defined spatial clustering, with sharper distributions of individual markers.

Human Tissues

Table 2 summarizes key performance metrics from human tissue libraries generated with either the Direct placement or CytAssist-enabled assay. Table 2 demonstrates that using the new human probe set v2 (Visium Human Transcriptome Kit v2) with the CytAssist-enabled assay results in more robust and sensitive data than v1, despite minor UMIs from Genomic DNA (gDNA). gDNA detection was not possible with probe set v1 (Visium Human Transcriptome Kit v1). For a deeper discussion on gDNA, consult the Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA Technical Note (CG000605). The human probe set also differs in gene coverage (~3 probe pairs per gene in v2 vs. ~1 probe pair per gene in v1). This difference contributes to an increase in UMIs observed per tissue-covered spot.

Tissue	Assay	Reads Confidently Mapped to Probe Set	Mean Genes/Spot	Mean UMIs/Spot	Genomic DNA Content
Human Ovarian Cancer	Direct placement	97.9%	3,703	9,904	-
	CytAssist-enabled	98.1%	5,695	14,988	2.5%
Human Healthy Brain	Direct placement	91.1%	2,275	3,704	-
	CytAssist-enabled	97.5%	5,095	13,119	1.8%

Table 2. A comparison of metrics for human tissues processed with the Direct placement or CytAssist-enabled assays.

Figure 8 shows that most of the genes found in the Direct placement assay are recovered with the CytAssist-enabled assay. Many more unique genes that were not found using Direct placement were found using the CytAssist-enabled assay.

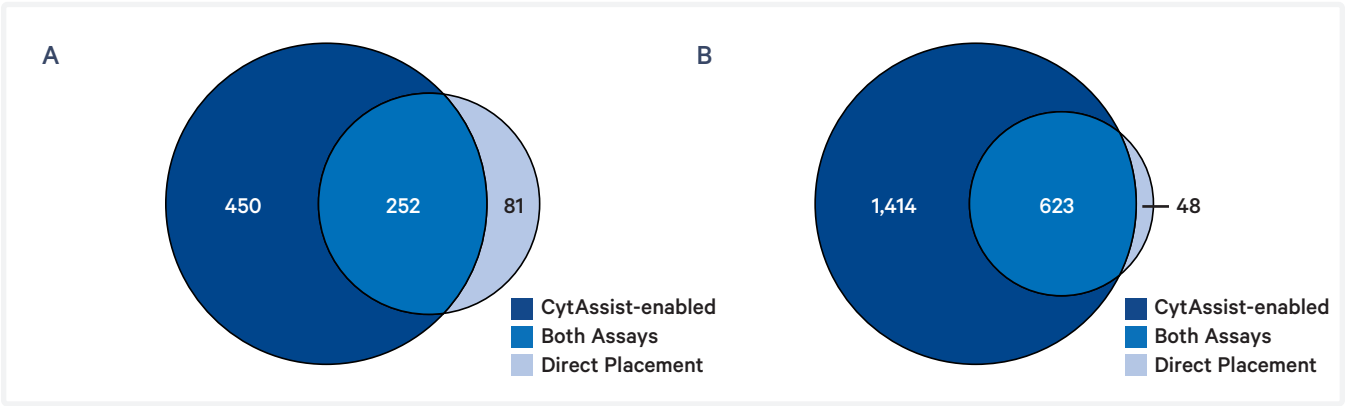

Figure 8. DGE analysis for human ovarian cancer **(A)** and human brain **(B)** showing the number of genes differentially expressed in either the CytAssist-enabled, Direct placement, or both assays (abs(log2fc > 0.5, adj.p.value < 0.05) in the same clusters provided by Harmony for both samples.

Human Tissues

Figures 9 (human ovarian cancer) and 11 (human brain) demonstrate that clustering through Harmony is comparable between the assays. CytAssist-enabled samples processed with the new human probe set show improved coverage (more of the transcriptome is covered by the probe set) and sensitivity.

Figure 10 shows individual gene expression for human ovarian cancer, while Figure 12 shows individual gene expression for human brain. For human ovarian cancer, CytAssist-enabled data demonstrate increased sensitivity and coverage while maintaining comparable overall spatial organization, as demonstrated by harmony clustering.

For human brain, the CytAssist-enabled data demonstrate successful spatial characterization from an FFPE block that performed suboptimally with the Direct placement assay. Spatial clustering in the CytAssist-enabled assay reveals layers of gene clusters forming around a capillary. Individual gene maps for the cell-specific markers AQP4, SNAP25, and MOBP reveal the differential expression of these cells across clusters. AQP4 and RELN (which encodes the protein Reelin) play a role in regulating the blood-brain barrier and can be found in the cluster near the capillary. Gene maps derived from the Direct placement assay show similar distribution patterns, but at lower intensity and with less distinction between signal and background.

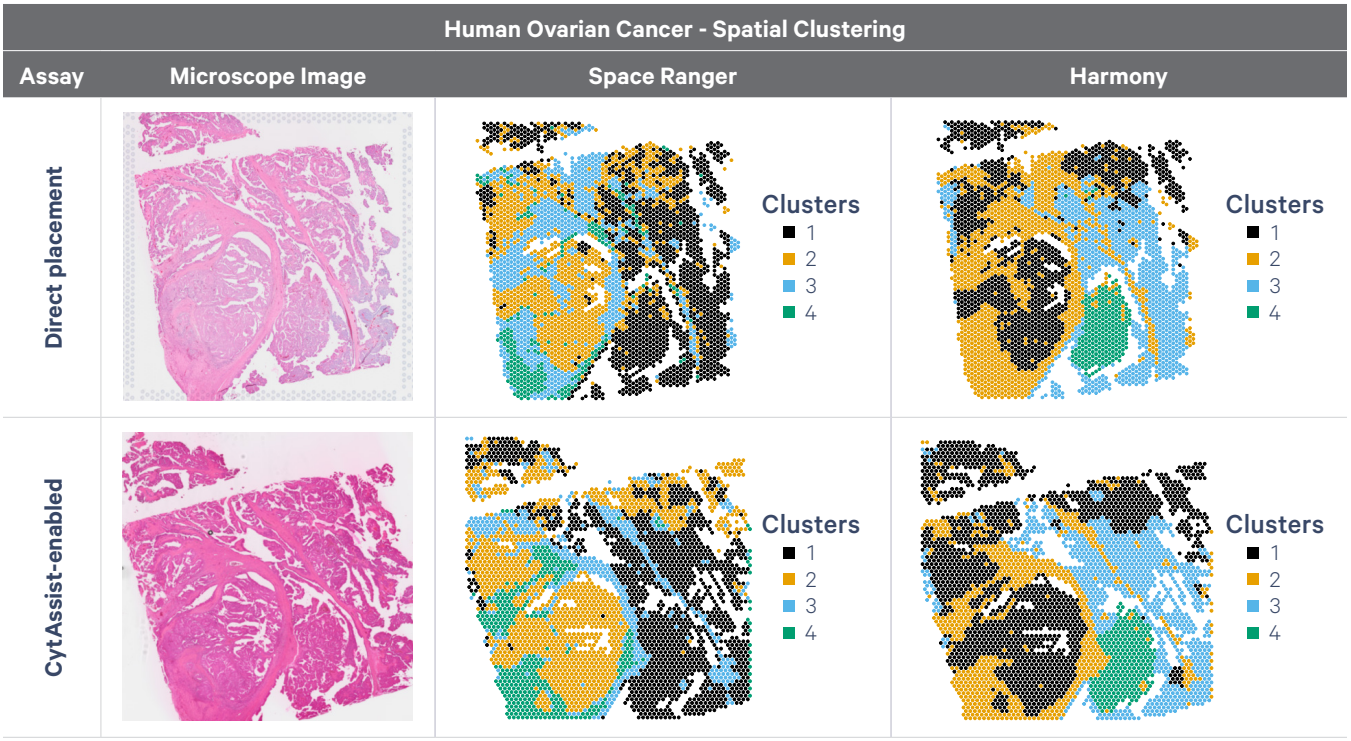

Figure 9. Microscope images (left) and spatial clustering of human ovarian cancer tissue using both Direct placement (top) and CytAssist-enabled (bottom) assays produced with Space Ranger 2.0 (middle) or Harmony (right). Both Harmony clustering plots share the same cluster assignments, while each Space Ranger clustering plot is clustered independently.

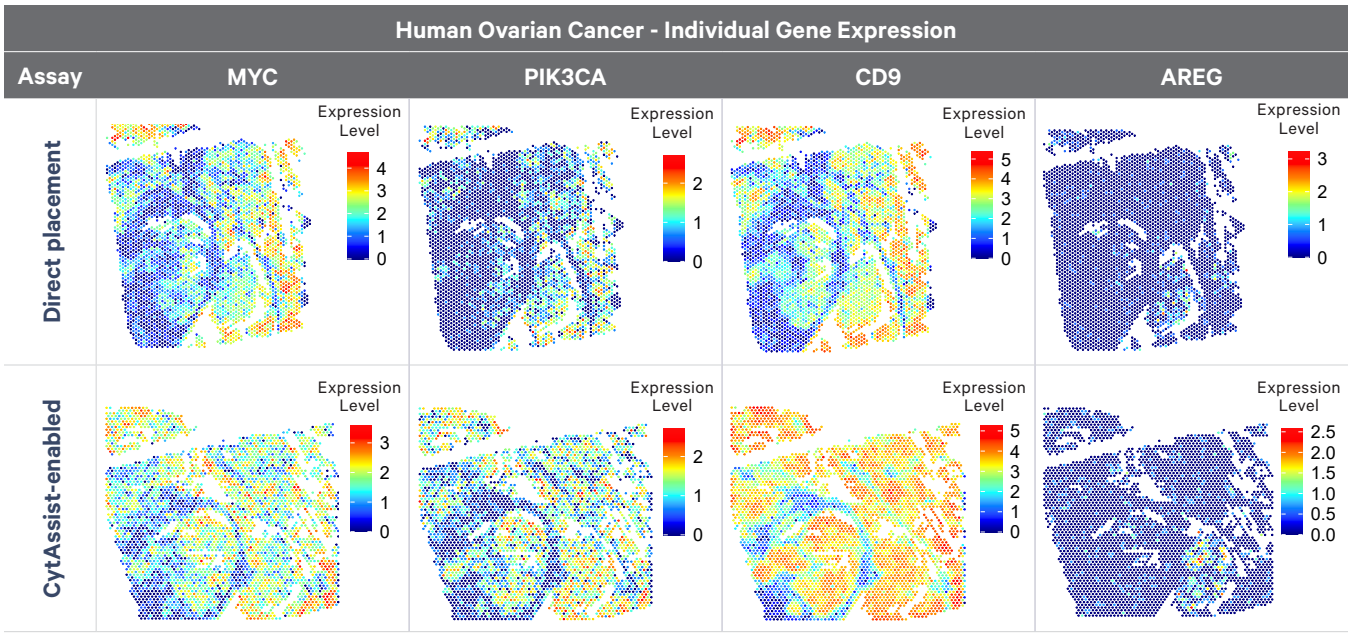

Figure 10. Individual gene expression in human ovarian cancer for both Direct placement (top) and CytAssist-enabled (bottom) assays. All expression levels are represented as log1p (natural log (X + 1). MYC is a common oncogene. PIK3CA is an ovarian cancer oncogene. CD9 is found on natural killer cells. REG encodes amphiregulin. Both CD9 and REG are associated with ovarian cancer cell proliferation and invasion.

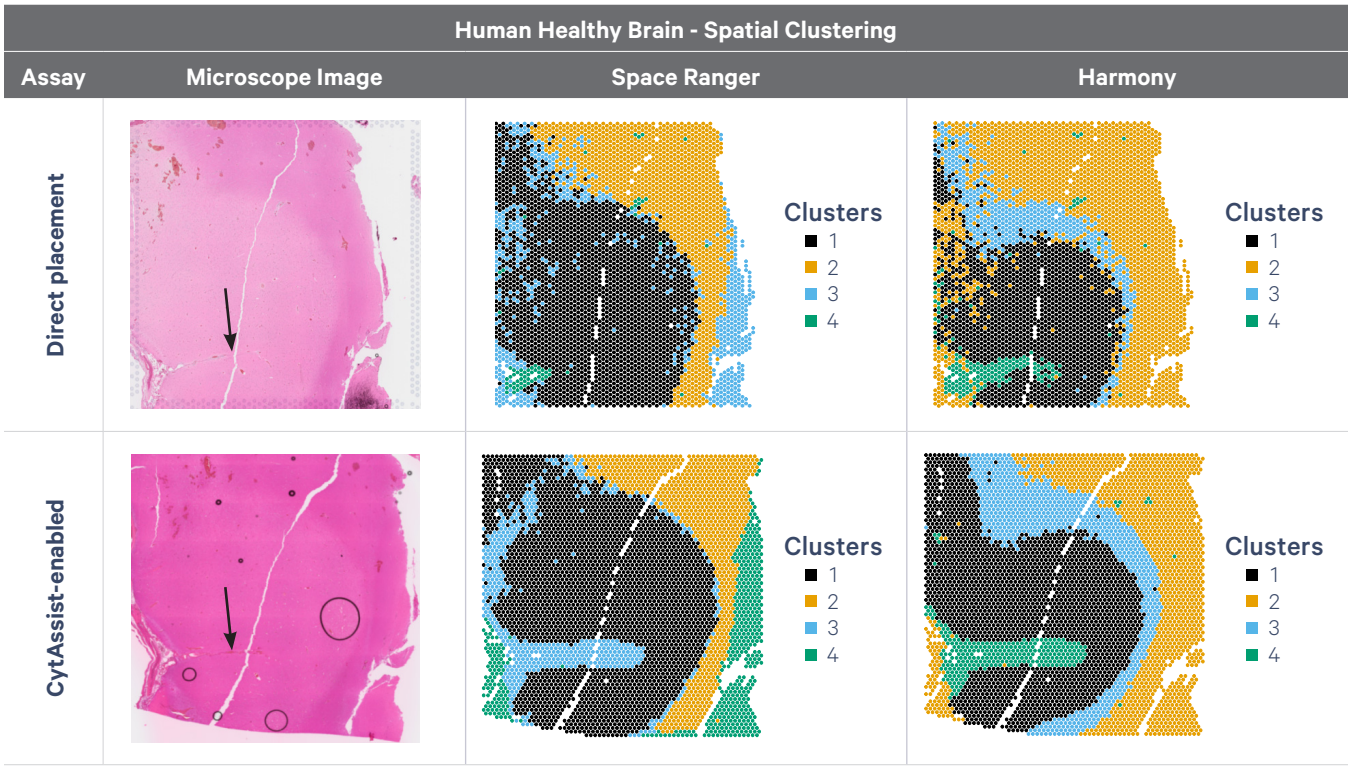

Figure 11. Microscope images (left) and spatial clustering of human brain tissue (frontal cortex) using both CytAssist-enabled (top) and Direct placement (bottom) assays produced with Space Ranger 2.0 (middle) or Harmony (right). The arrow in the microscope images point to a capillary. The bubbles in the CytAssist-enabled microscope image are from glycerol applied during coverslipping and do not affect assay performance.

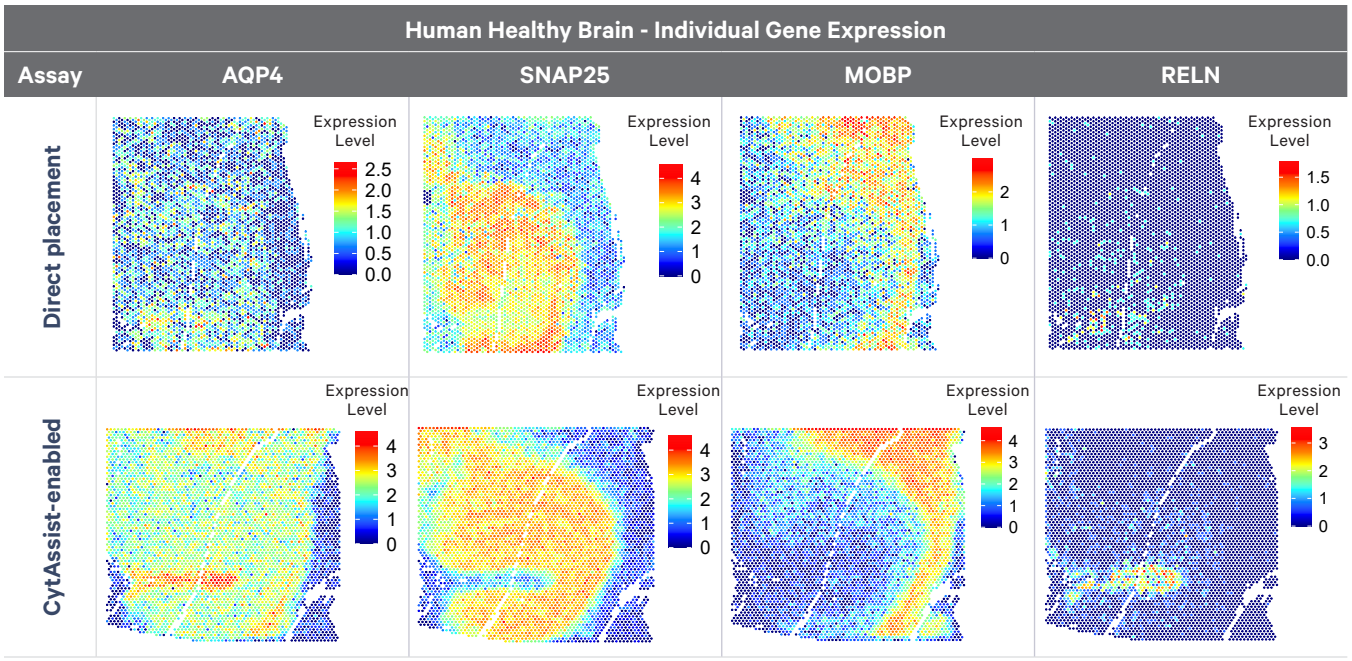

Figure 12. Individual gene expression in human brain for both Direct placement and CytAssist-enabled assays. All expression levels are represented as log1p (natural log (X + 1). AQP4 is an astrocyte marker. SNAP25 is a synapse marker. MOBP is an oligodendrocyte marker. RELN is an axon and dendrite marker.

Additional Comparisons

Figure 13 is a direct comparison of UMIs per tissue-covered spot and genes per tissue-covered spot between the two assays. These data show that while mouse metrics are similar between assays, human metrics show an increase with the CytAssist-enabled assay (Brain: +124% genes, +254.2 UMIs; Ovarian Cancer: +53.8% genes, +51.3% UMIs).

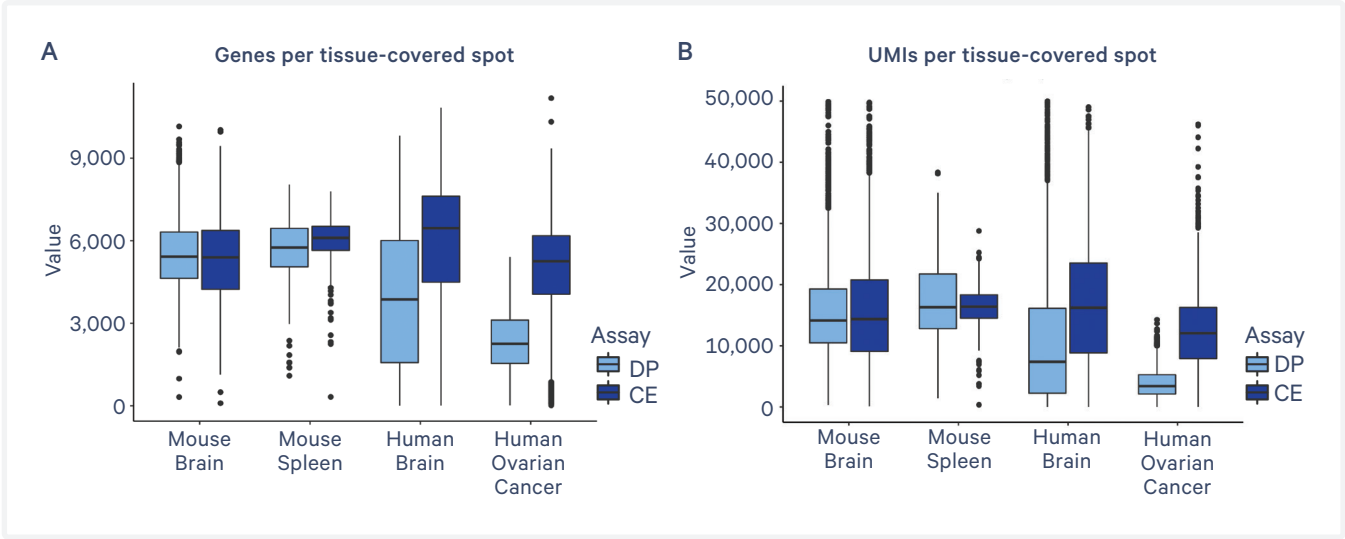

Figure 13. A comparison of genes per tissue-covered spot **(A)** and UMIs per tissue-covered spot **(B)** for Direct placement (DP) and CytAssist-enabled (CE) assays.

To verify that these increases in assay performance are specific, the co-clustering DGE analyses provided in previous sections were performed. For each comparison, differentially expressed genes in clusters provided by Harmony co-clustering were analyzed. These analyses should only find genes in both Direct placement and CytAssist-enabled assays that are differentially expressed in the same clusters and with the same sign (positively or negatively expressed). These results are shown in Figure 14. Differentially expressed genes found in a given cluster in the Direct placement assay are found in the same cluster in the CytAssist-enabled assay, as shown by the adherence of each gene to the diagonal line in the plot. The slight shift in gene expression is indicative that in the CytAssist-enabled assay, differentially expressed genes are more pronounced, which suggests improved specificity.

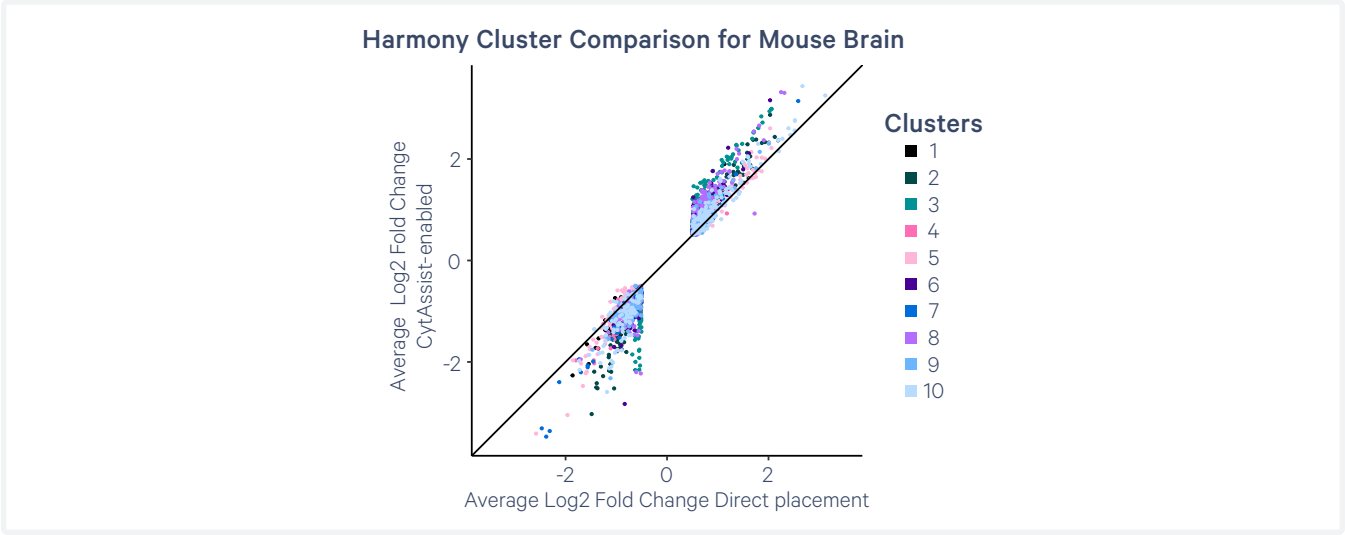

Figure 14. Harmony cluster comparison for mouse brain comparing fold change for Direct placement and CytAssist-enabled assays. All four tissue type comparisons in this Technical Note show similar trends.

Additional Comparisons

Figure 15 shows differentially expressed genes with positive expression. Positively expressed genes were filtered to avoid biasing the total number of differentially expressed genes, as a gene may be both positively or negatively differentially expressed. The portion of genes that are only found in the Direct placement assay is low across samples, with around 90% of Direct placement genes also detected by the CytAssist-enabled assay. Additionally, the CytAssist-enabled assay greatly expands on number of new genes that were not detectable in the Direct placement assay. Together, these results indicate that the Direct placement and CytAssist-enabled assays perform similarly but the CytAssist-enabled assay allows for further resolution.

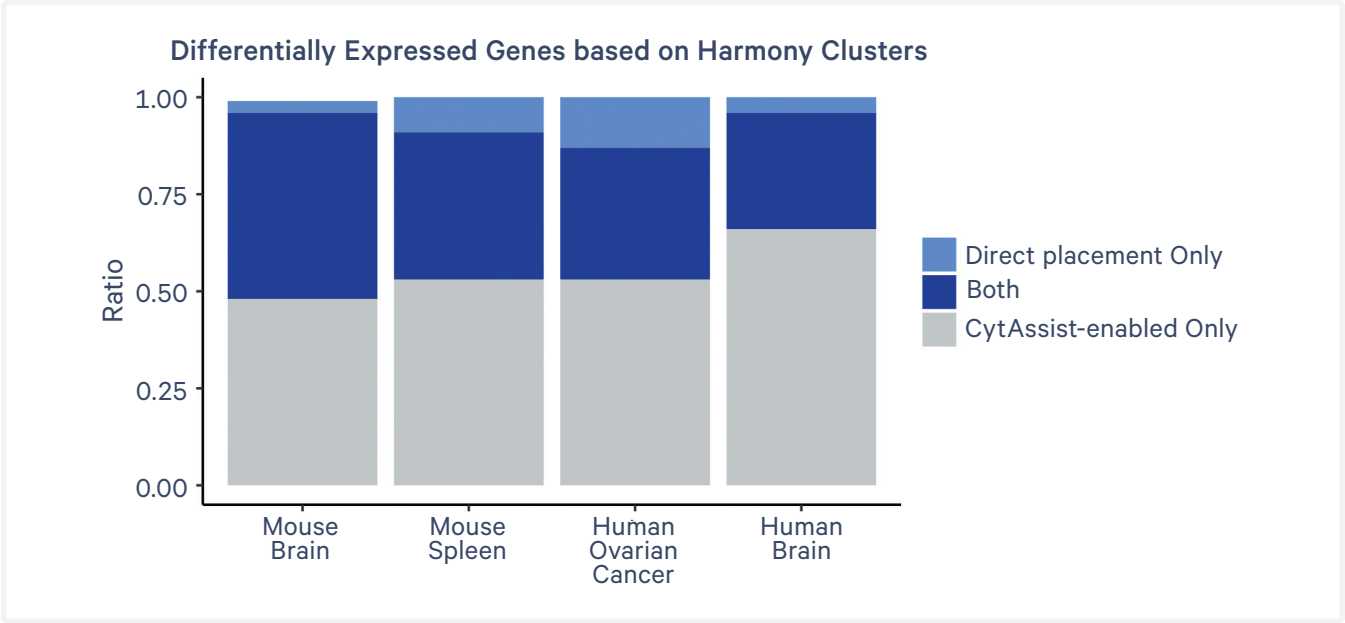

Figure 15. Differentially expressed genes that appear in the Direct placement assay, the CytAssist-enabled assay, or both.

Conclusions

In this Technical Note, reagents, workflow, and data from the Direct placement and CytAssist-enabled assays were compared.

Gene and UMIs per tissue-covered spot were used to compare the coverage and complexity of the data from both assays. Mapping metrics and fraction reads under tissue were used to evaluate the quality of that data. Data were downsampled to equivalent read depths.

DGE analysis allows one to establish whether the information derived from two assays is comparable. The data presented in this Technical Note demonstrate that most of the genes found in the Direct placement assay are recovered with the CytAssist-enabled assay, while unique genes that were not found using Direct placement were found using the CytAssist-enabled assay.

Spatial clustering and individual gene maps allow one to place information back in its original morphological context in the tissue. Spatial gene expression patterns and individual gene maps were used to compare spatiality and resolution of the Direct placement and CytAssist-enabled assays. The data presented in this Technical Note demonstrate that samples processed via the CytAssist instrument result in:

- Sharper spatial resolution
- Improved specificity
- Improved gene sensitivity

In addition to the above mentioned data improvements, the CytAssist-enabled assay has the following additional advantages over the Direct placement assay:

- Improved tissue retention as a result of enabling the use of glass slides.
- Ease of tissue section placement with larger allowable area.
- Compatibility with previously stained and hard-set coverslipped (archived) samples.
- Improved transcript coverage with human samples due to the use of Visium Human Probe Set v2.

References

1. Visium Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000408)
2. Visium Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000409)
3. Visium Spatial Gene Expression Reagent Kits for FFPE User Guide (CG000407)
4. Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000518)
5. Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000520)
6. Visium Spatial Gene Expression Reagent Kits for FFPE User Guide (CG000495)
7. Visium CytAssist Spatial Gene Expression for FFPE: Robust Data Analysis with Minimal Impact of Genomic DNA (CG000605)

Appendix

A1: Protocol Differences Between Direct placement and CytAssist-enabled Assays

	Direct placement	CytAssist-enabled
Protocol Steps		
Destaining	0.1N HCl Incubate at room temperature 3x for 1 min	0.1N HCl Incubate at 42°C 3x for 15 min
Decrosslinking	Tris-EDTA, pH 9 Incubate at 70°C for 1 h	Decrosslinking Buffer Incubate at 95°C for 1 h
Coverslip Removal	Wash 15x	Wash 30x
Pre-Hybridization	Nuclease-free Water 10X PBS, pH 7.4 Diluted Perm Enzyme B 10% Tween-20	Nuclease-free Water 10X PBS, pH 7.4 10% Tween-20
Hybridization	Nuclease-free Water FFPE Hyb Buffer Human/Mouse WT Probes - RHS Human/Mouse WT Probes - LHS	Nuclease-free Water FFPE Hyb Buffer Human v2/Mouse WT Probes - RHS Human v2/Mouse WT Probes - LHS
RNA Digestion	Nuclease-free Water 2X RNase Buffer RNase Enzyme Incubate at 37°C for 1 h	N/A
Non-CytAssist Probe Release	Perm Buffer B Perm Enzyme B Incubate at 37°C for 1 h	N/A
CytAssist Probe Release	N/A	Nuclease-free Water RNase Buffer B RNase Enzyme Tissue Removal Enzyme On-instrument incubation at 37°C for 30 min
Neutralization	Tris-HCl pH 7.0	Tris-HCl pH 8.0

	Direct placement	CytAssist-enabled
Pre-amplification	N/A	TS Primer Mix B Amp Mix B Nuclease-free Water Amplify 10x for 6.5 mm and 11 mm samples
Pre-amplification SPRIselect Cleanup	N/A	1.2X Cleanup
Cycle Number Determination (qPCR)	KAPA Sybr Fast qPCR Master Mix TS Primer Mix Nuclease-free Water Undiluted Sample	KAPA Sybr Fast qPCR Mix Diluted TS Primer Mix B Nuclease-free Water Diluted Sample (1:5)
Material Carried Forward	100% of barcoded ligation product	25% of barcoded ligation product
Sample Index PCR	Amp Mix Dual Index Plate TS Set A Amplify using Cq value +2 after rounding	Nuclease-free Water Amp Mix B Dual Index Plate TS Set A Amplify using Cq value +2 after rounding up
Library QC	Run diluted library on electrophoresis instrument	Run diluted library (1:50) on electrophoresis instrument

Document Revision Summary

Document Number	CG000618
Title	Visium CytAssist Spatial Gene Expression Data and Workflow Comparison
Revision	Rev B
Revision Date	March 2023
Specific Changes	• Corrected values shown in Figure 8. • Updated mouse tissue results section to include saturation levels on page 5.
General Changes	Updated for general minor consistency of language and terms throughout.

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