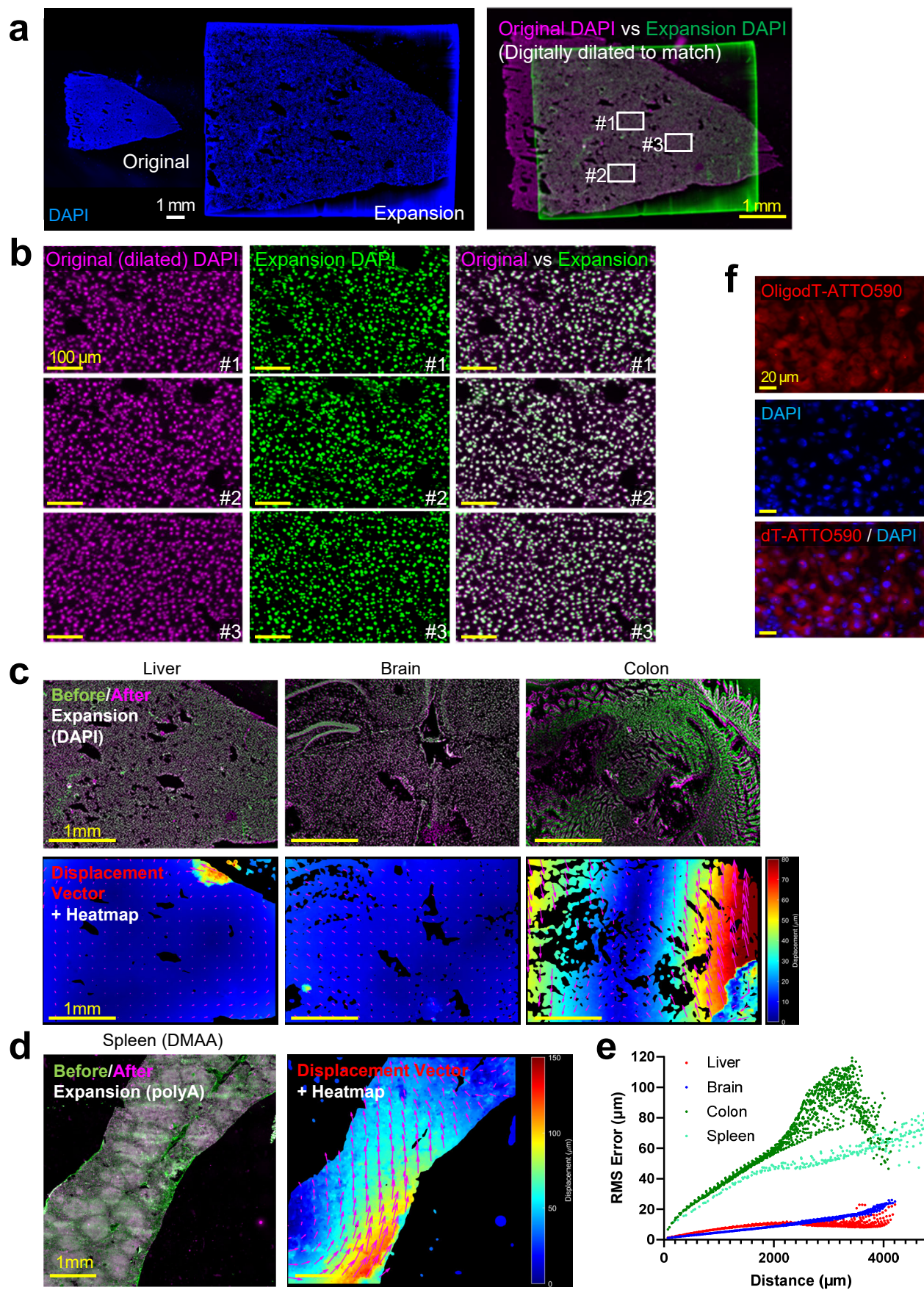




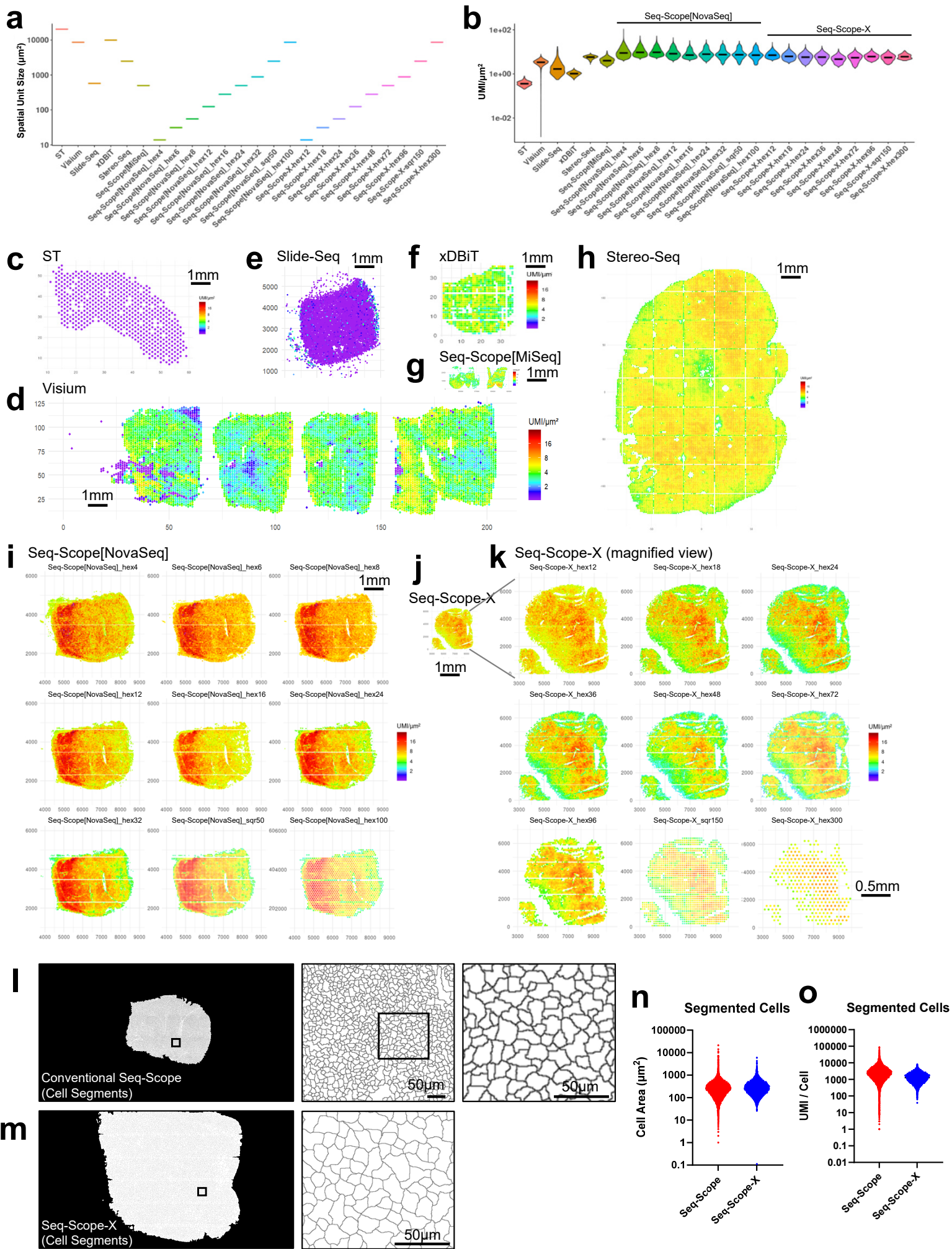
Supplementary Fig. 1. Assessment of Tissue Expansion Fidelity in Seq-Scope-X.

(a, b) Comparison of the liver tissue before and after tissue expansion. The liver section was imaged using conventional DAPI staining (Original). After hydrogel embedding and expansion ($2.7\times$ in the current data), the same tissue was imaged again (Expansion), and presented side-by-side to compare the scale (a, left). Each image was digitally colored into magenta (Original DAPI) and green (Expansion DAPI), and the original DAPI was digitally dilated to match the scale of expansion DAPI, and overlaid to each other. The overlaid image (a, right) demonstrates that the tissue expansion was uniformly applied to the whole tissue without any significant distortion or artifacts. Three regions (#1-#3 in A, right) were selected and further magnified (b) to show that microscopic structures were also well preserved before and after expansion. The experiment was repeated twice independently using separate liver sections, with similar results.

(c-e) Distortion analysis of expanded tissues. (c, d) DAPI staining was performed on liver (c, left), brain (c, center), and colon (c, right), and polyA detection was performed on spleen (d) before (green) and after (magenta) tissue expansion through standard (c) or DMAA (d) protocol. Corresponding displacement vectors (red arrows) and distortion heat maps are shown in a separate panel. Distortion analysis was performed on one tissue section per organ. (e) Distance-RMS error scatterplot demonstrating that the average distortion is $<5\%$ of distance across all samples and $<1\%$ for liver and brain tissues. In (e), $n=893$ matched feature pairs for standard expansion (c) and $n=336$ matched feature pairs for DMAA expansion (d) were analyzed from the same tissue section.

(f) Liver tissue was hybridized with ATTO590-labeled oligo-dT acrydite probe, embedded in a polyacrylate-containing expandable hydrogel, digested and expanded. The gel was imaged for red (OligodT-ATTO590) and DAPI (blue) fluorescence, which revealed both nuclear and cytoplasmic presence of captured mRNAs. The experiment was performed on one liver tissue section from 8-week-old *Alb-Cre/Tsc1^{F/F}/Depdc5^{F/F}* female mice, and the image shown is representative.

Scale bars represent the scale corresponding to the original tissue. Source data are provided as a Source Data file.



Supplementary Fig. 2. Benchmarking Transcriptome Capture Efficiency of Seq-Scope-X.

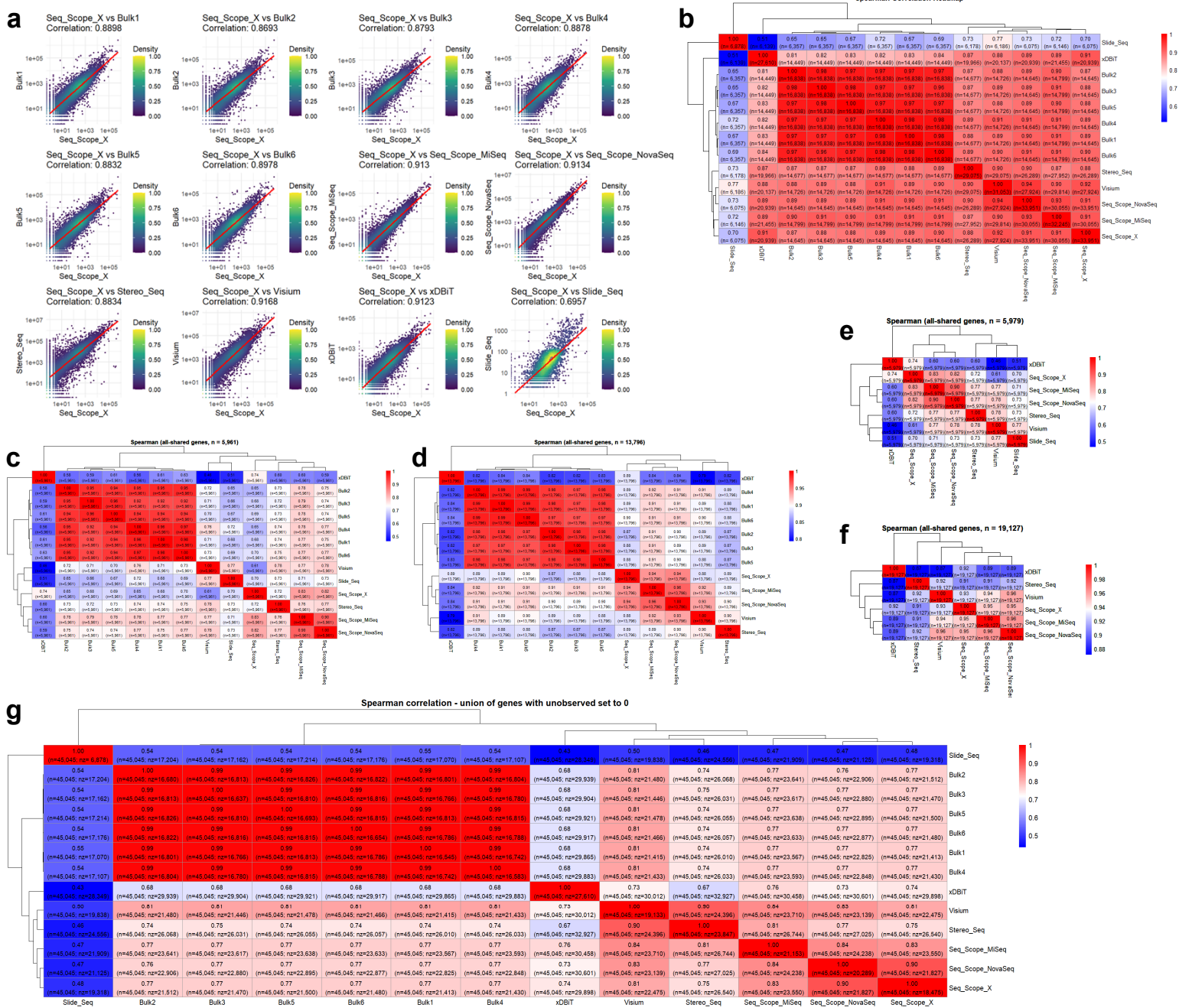
(a) Spatial unit sizes used to calculate UMI/ μm^2 for each of the technologies. Seq-Scope[NovaSeq]_hex100 (binned with 100 μm flat-to-flat height hexagons) and Seq-Scope-X_hex300 (binned with 300 μm flat-to-flat height hexagons) have spatial unit sizes comparable to Visium or xDBiT. Seq-Scope[NovaSeq]_sqr50 (binned with 50 μm -sided squares) and Seq-Scope-X_sqr150 (binned with 150 μm -sided squares) have spatial unit sizes comparable to Stereo-Seq, which was also binned with 50 μm -sided squares. Seq-Scope[MiSeq] (binned with 24 μm flat-to-flat height hexagons), Seq-Scope[NovaSeq]_hex24, and Seq-Scope-X_hex72 have spatial unit sizes comparable to Slide-Seq.

(b) UMI/ μm^2 calculated for each dataset. Number of data points: ST (n=598); Visium (n=7,625); Slide-Seq (n=459); xDBiT (n=836); Stereo-Seq (n=34,201); Seq-Scope[MiSeq] (n=3,619); Seq-Scope[NovaSeq]_hex4 (n=598,114); Seq-Scope[NovaSeq]_hex6 (n=241,288); Seq-Scope[NovaSeq]_hex8 (n=133,669); Seq-Scope[NovaSeq]_hex12 (n=75,568); Seq-Scope[NovaSeq]_hex16 (n=35,125); Seq-Scope[NovaSeq]_hex24 (n=20,530); Seq-Scope[NovaSeq]_hex32 (n=12,482); Seq-Scope[NovaSeq]_sqr50 (n=4,493); Seq-Scope[NovaSeq]_hex100 (n=1,355); Seq-Scope-X_hex12 (n=155,475); Seq-Scope-X_hex18 (n=89,910); Seq-Scope-X_hex24 (n=60,060); Seq-Scope-X_hex36 (n=26,987); Seq-Scope-X_hex48 (n=14,558); Seq-Scope-X_hex72 (n=7,522); Seq-Scope-X_hex96 (n=3,263); Seq-Scope-X_sqr150 (n=1,421); Seq-Scope-X_hex300 (n=314).

(c-k) Comparison of transcriptome capture efficiency (UMI/ μm^2) in liver tissue across various spatial transcriptomics (ST) technologies. Seven publicly available ST datasets (c-i) and the current Seq-Scope-X dataset (j, k) are shown to illustrate the spatial distribution of transcriptome capture efficiency (UMI/ μm^2) for the datasets shown in (a, b), with the comparable magnification scale. The datasets include: Original ST (c), 10x Visium (d), Slide-Seq (v1; e), xDBiT-Seq (f), Seq-Scope[MiSeq] (g; binned by 10 μm -sided squares), Stereo-Seq (h; binned by 50 μm -sided squares), Seq-Scope[NovaSeq] (i; binned as indicated), and the Seq-Scope-X dataset (j, k; binned as indicated). The total distribution of transcriptome capture performance is summarized in Fig. 1q.

(l-o) UMI/cell analysis of Seq-Scope and Seq-Scope-X using cell segmentation methods. (l) Cell segmentation information for Conventional Seq-Scope data is from the previous paper¹⁵, where H&E histology was used to estimate the cell boundary. (m) Seq-Scope-X data was segmented using RNA density images. (n,o) The cell area (n; n=35,515 for Seq-Scope, n=12,381 for Seq-Scope-X) and UMI per cell (o; n=24,820 for Seq-Scope, n=11,721 for Seq-Scope-X) are slightly lower in Seq-Scope-X (two-tailed unpaired Student's t-test; $P < 2.2 \times 10^{-16}$ for both comparisons).

Scale bars represent the scale corresponding to the original tissue. Source data are provided as a Source Data file.



Supplementary Fig. 3. Transcriptomic Characteristics of Seq-Scope-X Mouse Liver Data.

(a-g) Comparison of mouse liver transcriptomes from different spatial transcriptomics (ST) technologies and bulk RNA-seq. Datasets include six batches of bulk RNA-seq (Bulk1-6)²⁹, 10x Visium²⁶, Slide-Seq (v1)⁸, xDBiT-Seq²⁷, Stereo-Seq²⁸, Seq-Scope^{MISEQ}¹², Seq-Scope^{NOVASEQ}¹⁵, and the Seq-Scope-X datasets. All data were produced from young (2 to 4-month-old) C57BL/6 male mice, except Slide-Seq (v1) data, whose animal source was not identified. To facilitate comparisons, all ST datasets were aggregated into pseudo-bulk gene expression count tables.

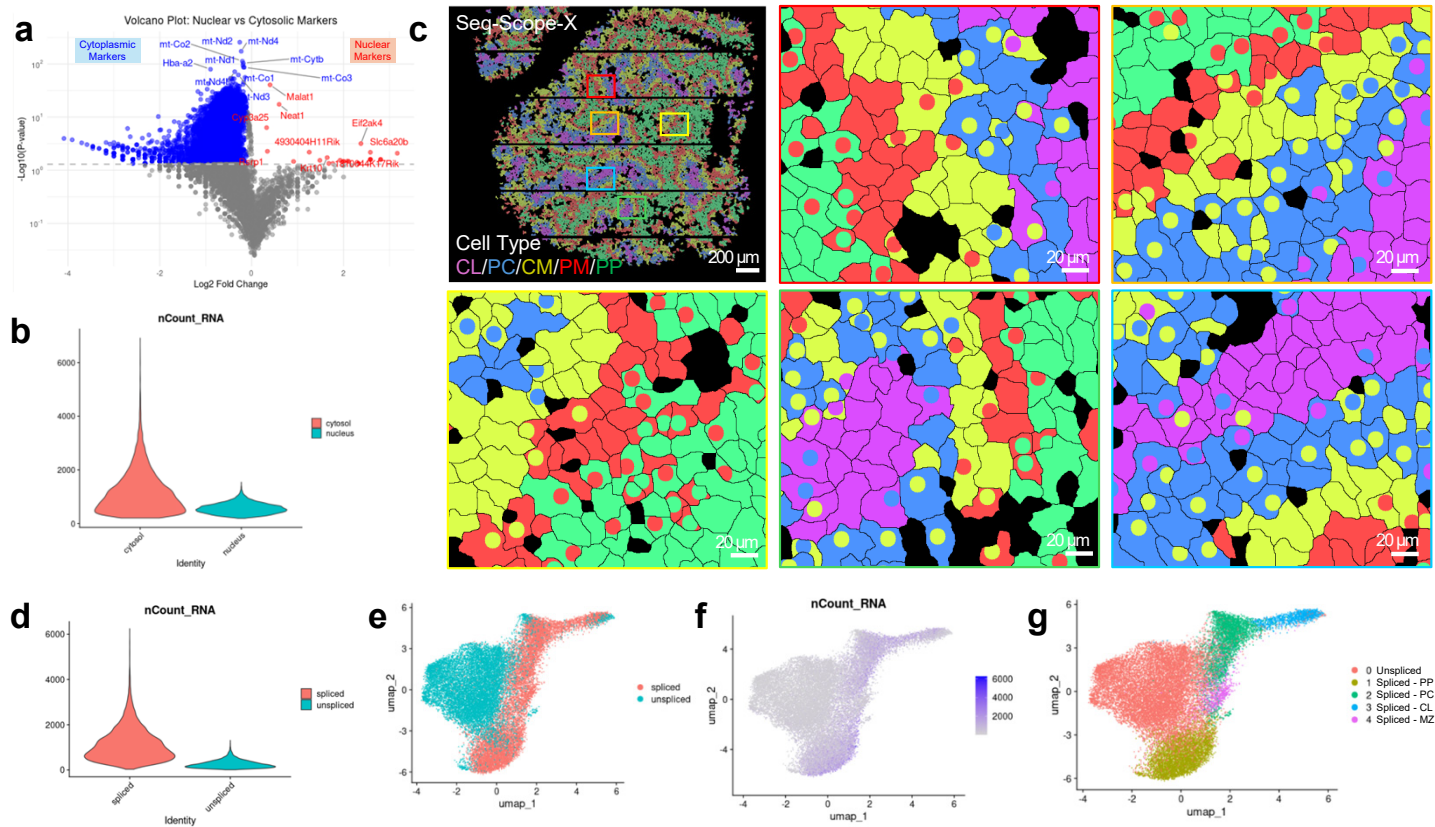
(a) Scatterplots show correlations between datasets, with each dot representing the expression of a shared gene in two datasets. The red trendline indicates the correlation, and Spearman correlation coefficients (r) are displayed.

(b) Spearman correlation heatmap table with dendrogram indicates the strong correlation ($r > 0.8$) among all liver bulk RNA-seq and ST datasets except Slide-Seq (v1). The number of genes used for comparison in every pair was indicated in parentheses.

(c-f) Additional Spearman correlation heatmap tables using the globally shared gene set. (c) All datasets.

(d) All datasets except Slide-Seq. (e) All ST datasets. (f) ST datasets except Slide-Seq. All data are consistent with our previous conclusion, indicating that Seq-Scope-X produces transcriptome data similar to Bulk RNA-Seq and other ST technologies.

(g) Spearman correlation heatmap computed using the full union of genes, with all unobserved values treated as zero. The correlation pattern remains consistent with results obtained using pairwise shared or globally shared gene sets, confirming the robustness of dataset relationships. n , total number of genes considered in each comparison (union size); nz , number of genes with non-zero expression in at least one dataset of the pair.



Supplementary Fig. 4. Subcellular Transcriptome Heterogeneity in Seq-Scope-X Data.

(a) A volcano plot shows genes differentially expressed between nuclear (red) and cytoplasmic (blue) compartments. The gray dots represent transcripts below the classification threshold and, therefore, are not assigned as either nuclear or cytoplasmic markers. As expected, nuclear-enriched non-coding RNAs, such as *Malat1* and *Neat1*, are enriched in nuclear segments, while mitochondrial genes are highly enriched in cytoplasmic segments.

(b) A violin plot compares the number of transcripts in nuclear (red) and cytoplasmic (green) segments, showing slightly fewer transcripts in nuclear segments (two-tailed Wilcoxon rank-sum test; $P < 2.2 \times 10^{-16}$).

(c) Segments are color-coded according to the cell type mapping results, using the same color scheme as Fig. 2c and 2d. Regions not shown in Fig. 2c are included here to provide additional histological context. Colored boxes in the upper-left panel mark areas that are magnified in the other panels, with each zoomed image outlined in the corresponding box color. The magnified regions illustrate the layered organization of hepatocytes reflecting metabolic zonation, as well as the presence of nuclear–cytoplasmic transcriptomic disparities.

(d-g) Spliced and unspliced transcriptome analysis based on whole-cell segments of Seq-Scope-X data.

(d) A violin plot shows the number of spliced (red) and unspliced (green) transcripts in different whole cell segments, showing significantly fewer unspliced transcripts in whole cell segments (two-tailed Wilcoxon rank-sum test; $P < 2.2 \times 10^{-16}$).

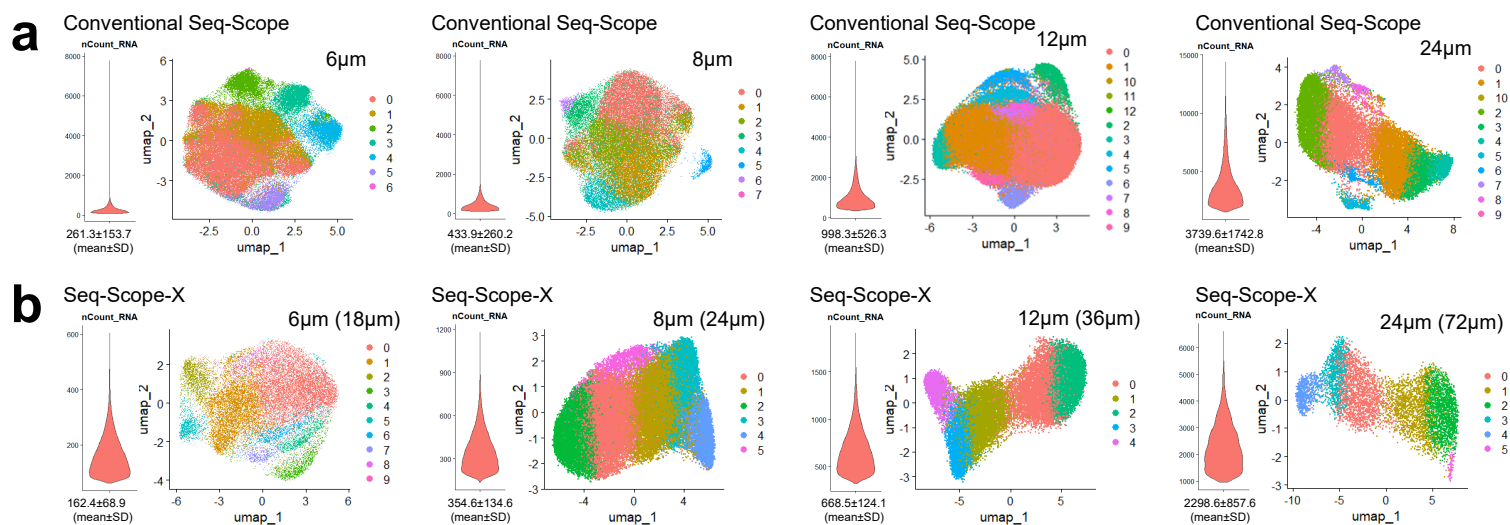
(e-g) UMAP visualizations of cell type clustering based on spliced and unspliced transcriptomes.

(e) Data points are color-coded to distinguish spliced (red) and unspliced (green) data.

(f) Data points are colored by the number of unique transcripts identified in each data.

(g) Cell type clustering analysis highlights hepatocellular zonation clusters in the spliced dataset. Clusters 1-4 represent periportal (PP), pericentral (PC), centrilobular (CL), and midzone (MZ) hepatocytes, all corresponding to spliced data. In contrast, the unspliced data forms a single globular cluster (cluster 0), likely due to its sparse and biased transcriptome content.

Number of data points analyzed: $n=18,448$ (a-c), $23,512$ (d-g). Scale bars represent the scale corresponding to the original tissue.



Supplementary Fig. 5. Comparative Spatial Performance of Seq-Scope-X and conventional Seq-Scope (a, b) UMAP manifolds display cell type clustering using hexagons with specified flat-to-flat height (in μm) for conventional Seq-Scope (a) and Seq-Scope-X (b) liver datasets. Heights in parentheses (b) represent raw flat-to-flat dimensions, unadjusted for the expansion factor. Hexagons with a 24 μm height (unadjusted scale) correspond to the 14 μm -sided hexagons used in Fig. 3a and 3d. While the overall cluster geometry is similar between datasets, Seq-Scope-X demonstrates improved spatial precision, as shown in Fig 3. Number of hexagons analyzed: n=372,564, 227,723, 94,367, 23,564 (a), 102,031, 55,191, 22,723, 7,013 (b).

Supplementary Fig. 6. Cross-Platform Integration of Liver Spatial Transcriptomes from Seq-Scope-X and Conventional Seq-Scope.

(a-l) Integrated analysis of liver spatial transcriptomes profiled using Seq-Scope-X and conventional Seq-Scope. The datasets were analyzed using hexagonal grids with flat-to-flat width of 24 μm (a–d; n=7,522 hexagons for Seq-Scope-X, 20,529 hexagons for conventional Seq-Scope), 18 μm (e–h; n=14,558, 35,125), and 12 μm (i–l; n=26,986, 75,564), corresponding to the original tissue scale (adjusted for the expansion factor in Seq-Scope-X data).

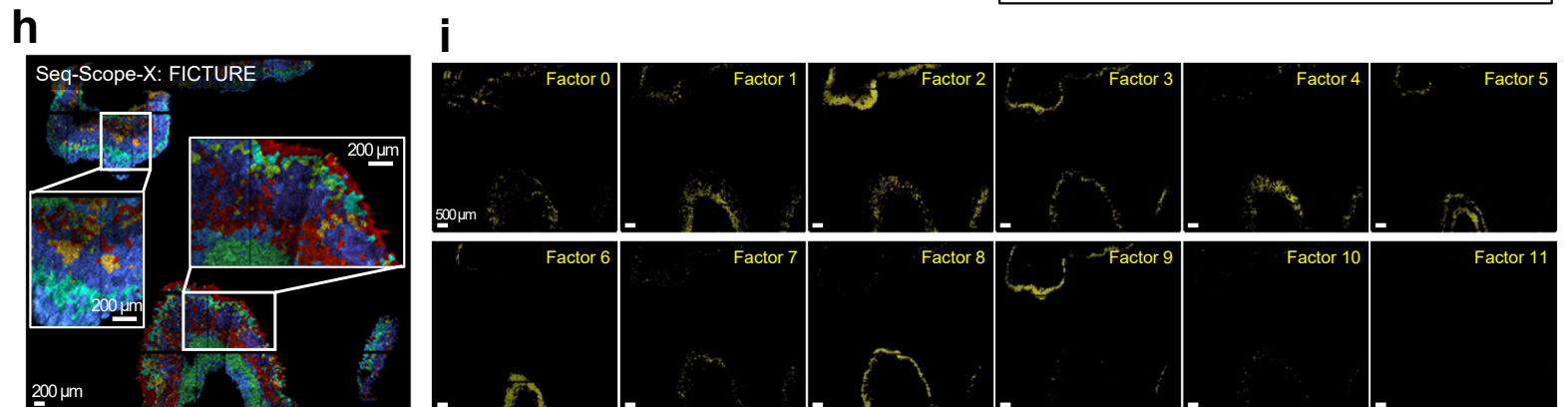
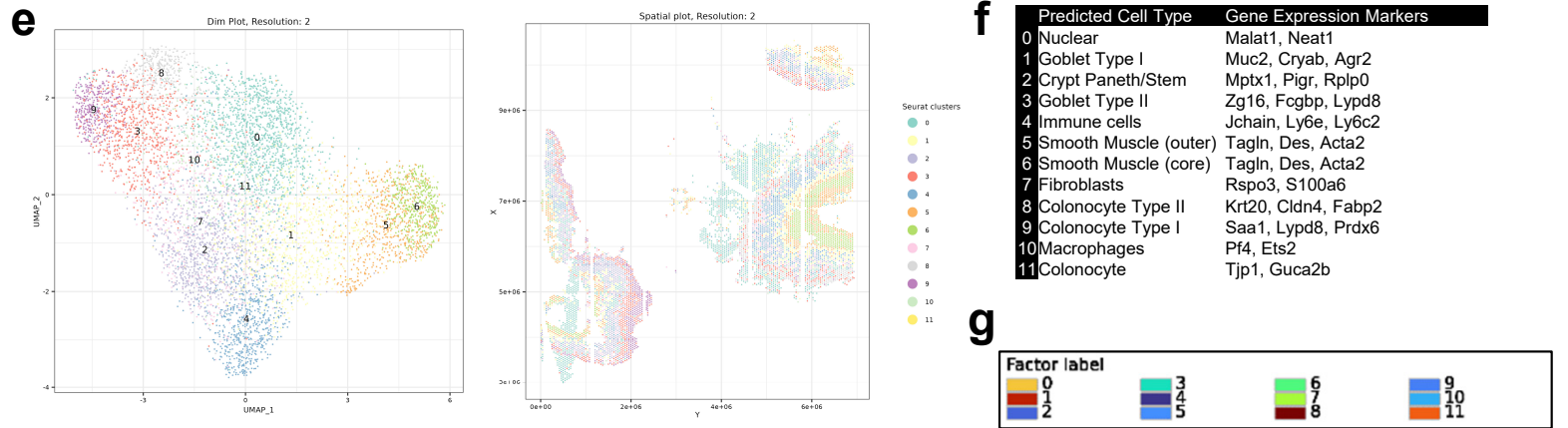
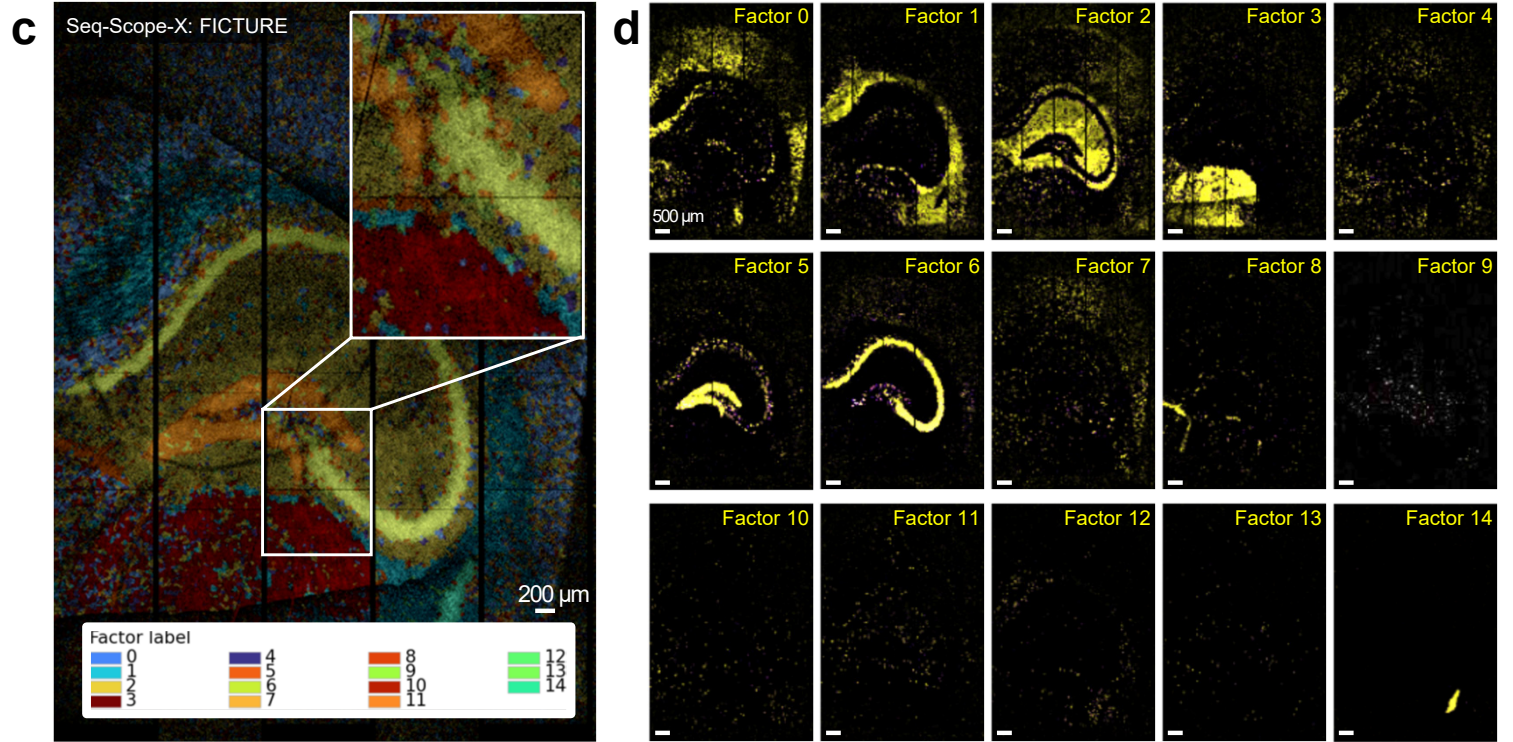
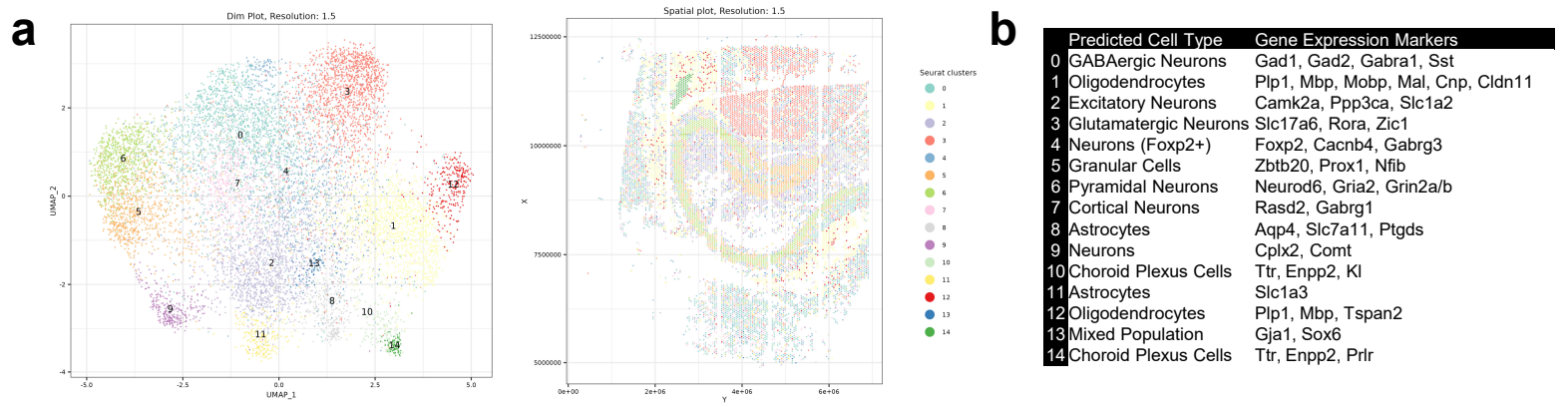
(a, e, i) UMAP plots showing effective data integration between conventional Seq-Scope (blue) and Seq-Scope-X (red) datasets.

(b, f, j) UMAP plots illustrating distinct hepatocellular and non-parenchymal cell types identified by multidimensional clustering.

(c, g, k) Spatial maps of hexagonal grids color-coded by cell type. Boxed areas were magnified in insets.

(d, h, l) Proportions of different cell types derived from each analysis, demonstrating consistent cell type composition between Seq-Scope-X and conventional Seq-Scope.

PP, periportal hepatocytes; PM, portal-side midzone hepatocytes; CM, central-side midzone hepatocytes; PC, pericentral hepatocytes; CL, centrilobular hepatocytes; NP, nonparenchymal; HSC-N, normal hepatic stellate cells; Hep_injury, injured hepatocytes; RBCs, red blood cells.



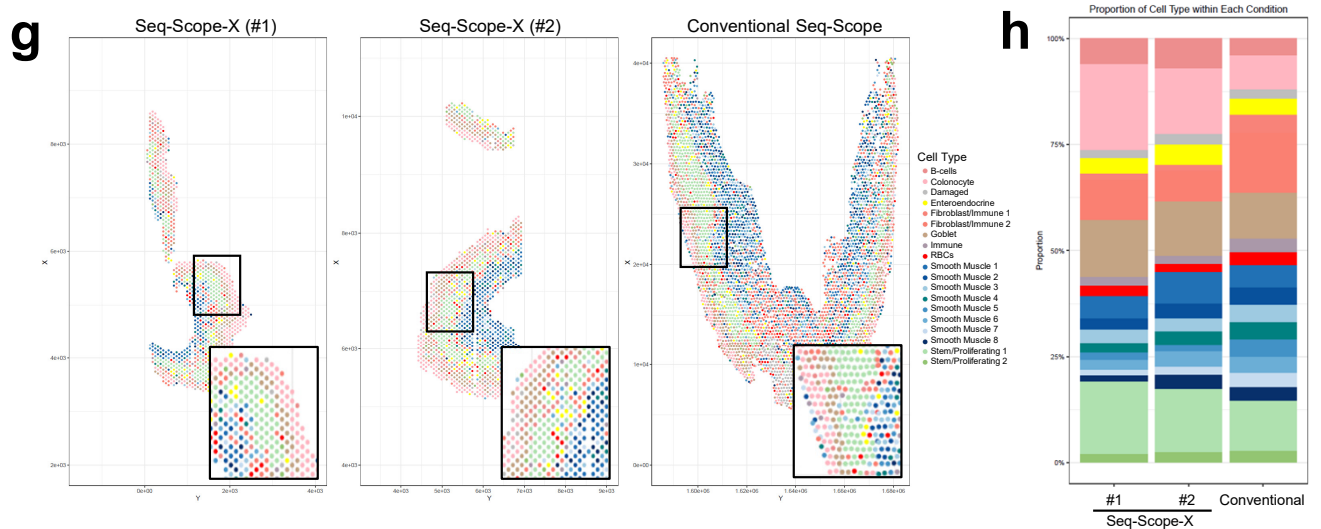
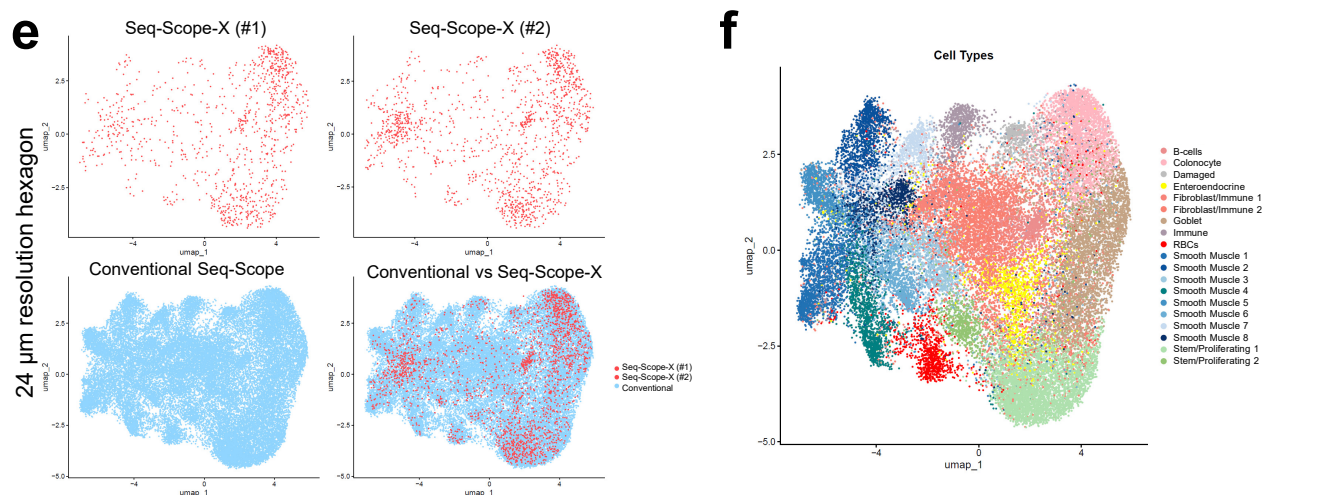
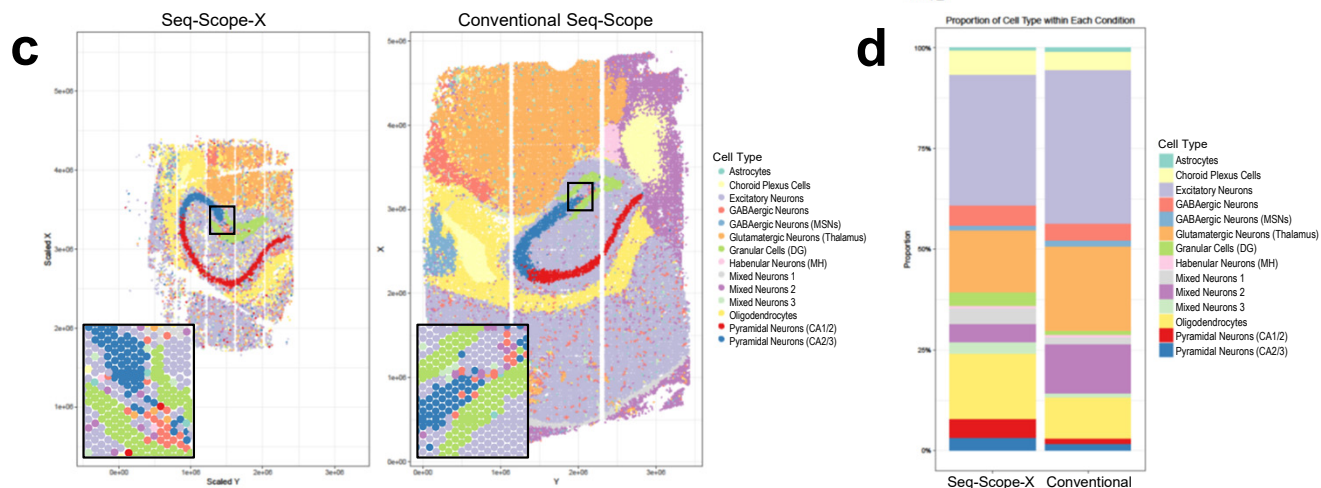
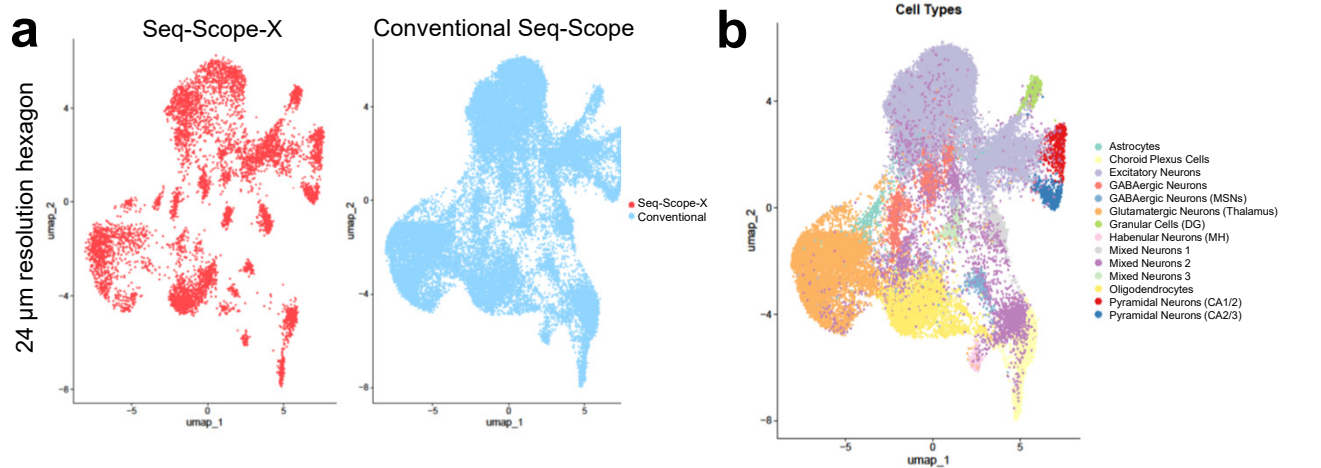
Supplementary Fig. 7. Detailed Tissue and Cell Type Insights from Seq-Scope-X Analysis

(a-i) Cell type clustering and spatial projection results of mouse brain (a-d; n=11,699 hexagons) and colon (e-i; n=7,224 hexagons) Seq-Scope-X datasets, described in Fig. 4.

(a, b, e, f) Cell type clustering results are shown as UMAP manifolds (a, e; left) and spatial maps (a, e; right) using 10 μm -sided hexagons (16 μm flat-to-flat height, reflecting 3X tissue expansion) from the Seq-Scope-X dataset. The same cell type-specific color code is applied for both UMAP and spatial plots. Cluster annotations and representative marker genes are summarized in a table for each dataset (b, f). The same clustering results were used for FICTURE projections (c, d, g-i).

(c, d, g-i) FICTURE projections of the cell type clustering results in (a, b, e, f) are presented in composite figures (c, h) with cluster-specific color codes (c, inset; g) or as individual panels highlighting each factor separately. For the colon results (h, i), FICTURE output was spatially filtered using a mask to visualize only the expressions within the tissue boundary.

Scale bars represent the scale corresponding to the original tissue.



Supplementary Fig. 8. Integrated Analysis of Brain and Colon Spatial Transcriptomes from Seq-Scope-X and Conventional Seq-Scope.

(a-h) Integrated analysis of brain (a-d; n=7,416 hexagons for Seq-Scope-X, 29,254 hexagons for conventional Seq-Scope) and colon (e-h; n=19,129 hexagons for Seq-Scope-X, 32,665 hexagons for conventional Seq-Scope) spatial transcriptomes profiled using conventional Seq-Scope and Seq-Scope-X. The datasets were analyzed using hexagonal grids with flat-to-flat width of 24 μ m, corresponding to the original tissue scale (adjusted for the expansion factor).

(a, e) UMAP plots showing effective data integration between conventional Seq-Scope (blue) and Seq-Scope-X (red) datasets. Two batches of tissues were analyzed in Seq-Scope-X colon datasets (#1 and #2).

(b, f) UMAP plots illustrating distinct cell types identified by multidimensional clustering.

(c, g) Spatial maps of hexagonal grids color-coded by cell type. Boxed areas were magnified in insets.

(d, h) Proportions of different cell types derived from each analysis, demonstrating consistent cell type composition between conventional Seq-Scope and Seq-Scope-X.

DG, Dentate Gyrus; MSNs, Medium Spiny Neurons; MH, Medial Habenula; CA, Cornu Ammonis

Supplementary Fig. 9. Seq-Scope-X Analysis of Spatial Proteome in Mouse Spleen.

(a) Spatial expression plots of all 128 AbTags used for profiling the mouse spleen spatial proteome, as shown in Fig. 5b-5f.

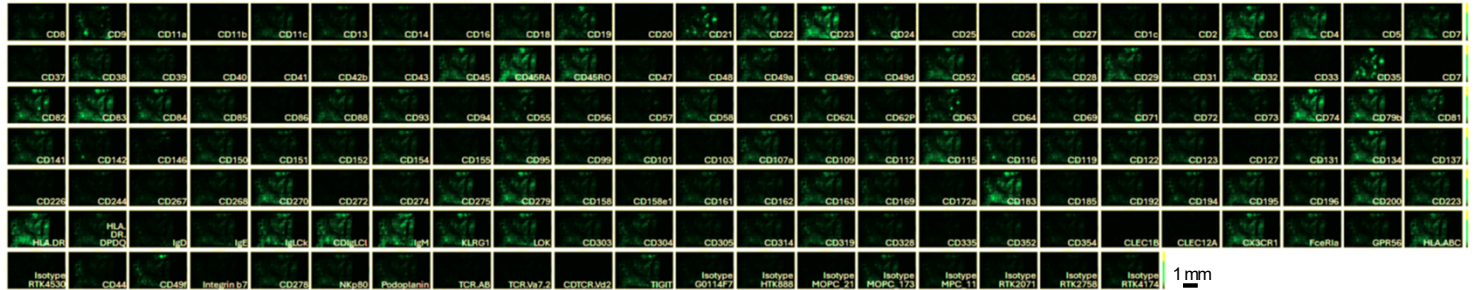
(b) Individual maps of all 12 LDA factors described in Fig. 5e.

(c) Volcano plot of proteins highly expressed in pixels corresponding to each factor. Black dots represent proteins consistent with the annotated factor identity, while gray dots represent proteins that may not align with the annotated cell type but could reflect regional context.

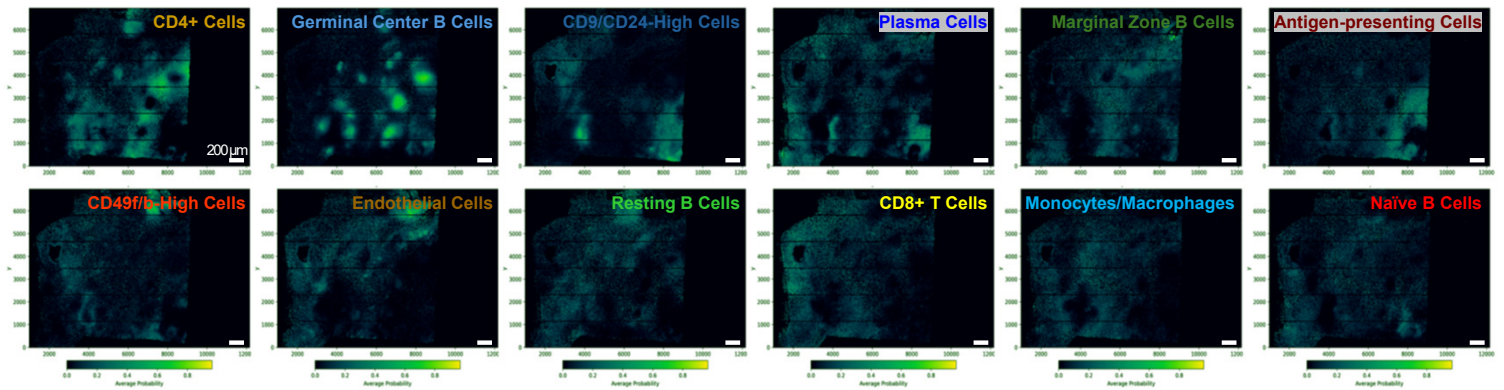
(d, e) Side-by-side comparison of CD4, CD8b, and CD21-35 immunodetection using immunofluorescence confocal microscopy (IF-Confocal) and Seq-Scope-X. Nearby sections from the same mouse spleen were processed by either IF-Confocal or Seq-Scope-X to visualize CD4 (magenta, d), CD8b (green, d), and CD21-35 (green, e). The spatial patterns show strong overall concordance between the two methods, with minor differences expected from the use of neighboring, rather than identical, sections. Yellow arrows mark salient structures present in both images. Boxed regions are shown at higher magnification in the lower panels.

Scale bars represent the scale corresponding to the original tissue.

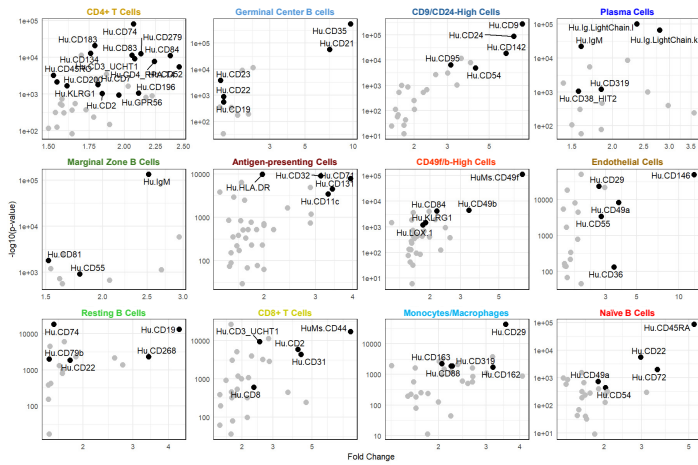
a



b



c



Supplementary Fig. 10. Seq-Scope-X Analysis of Spatial Proteome in Human Tonsil.

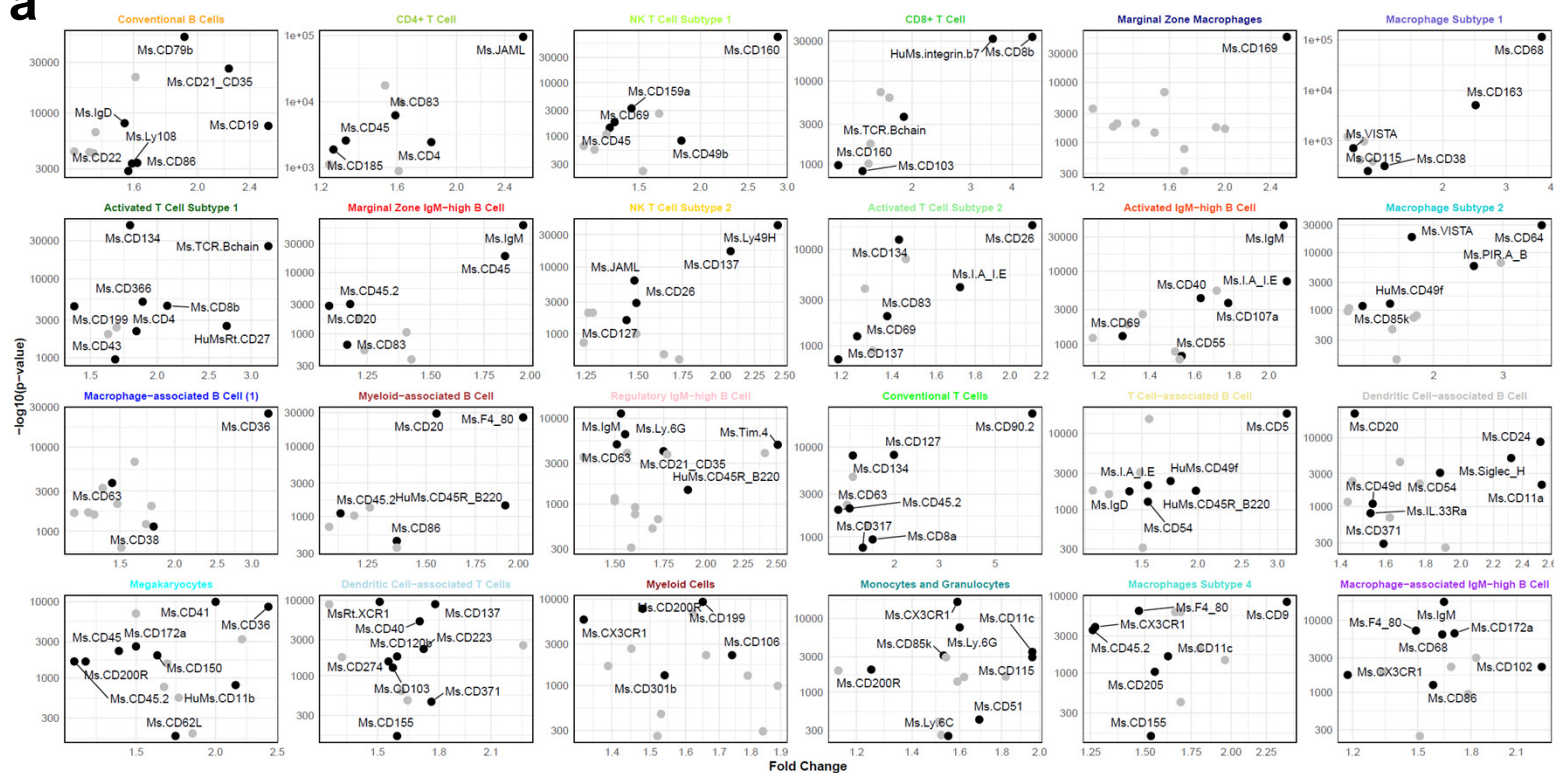
(a) Spatial expression plots of all 163 antibody tags used for profiling the human tonsil spatial proteome, as shown in Fig. 5g, 5h.

(b) Individual maps of all 12 LDA factors described in Fig. 5g, illustrating the positional probability of each factor.

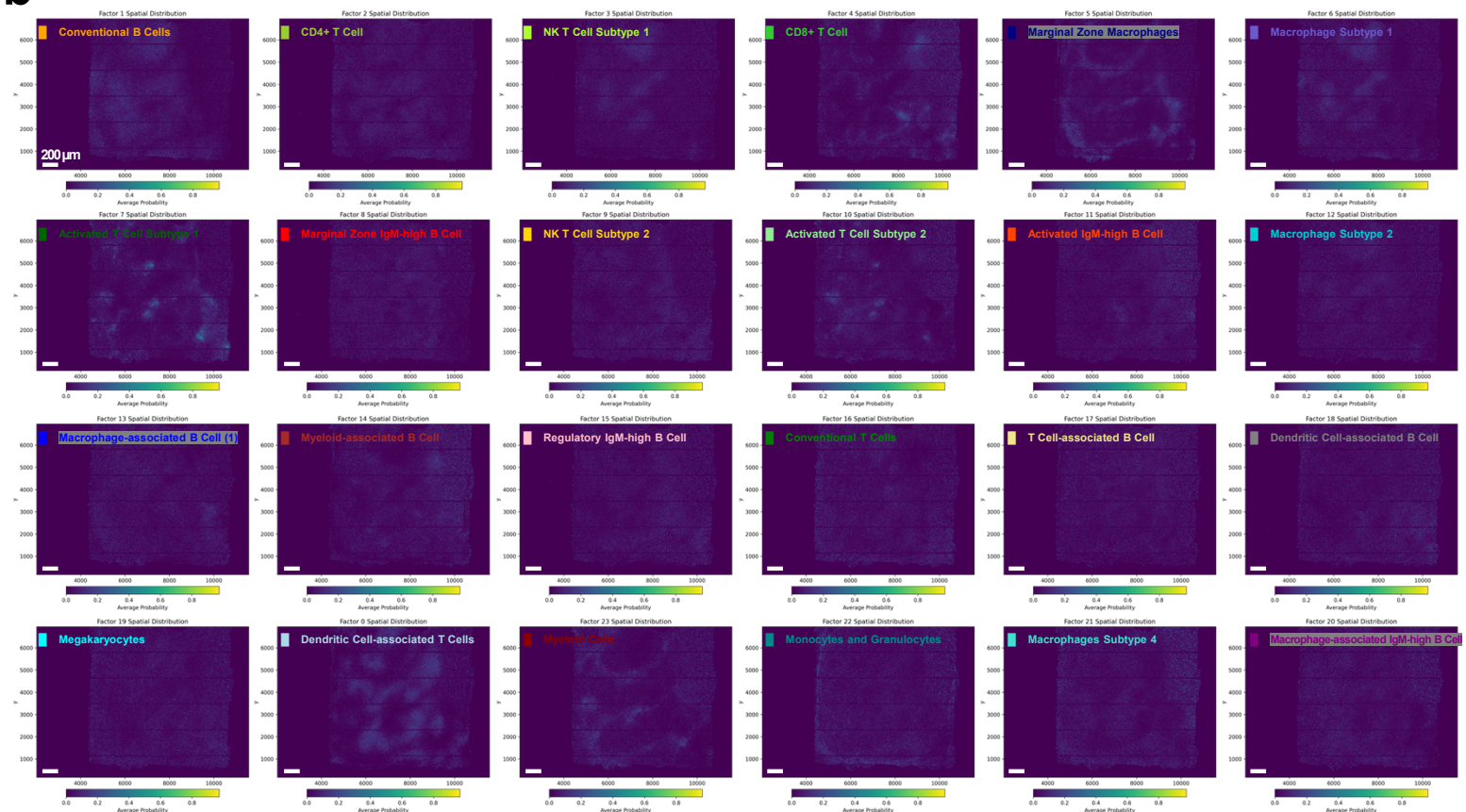
(c) Volcano plot of proteins highly expressed in pixels corresponding to each factor. Black dots represent proteins consistent with the annotated factor identity, while gray dots represent proteins that may not align with the annotated cell type but could reflect regional context.

Scale bars represent the scale corresponding to the original tissue.

a



b



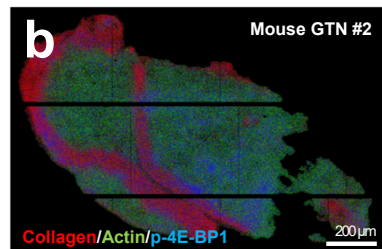
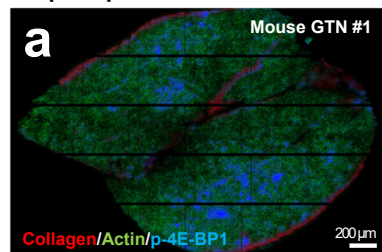
Supplementary Fig. 11. Analysis of the Mouse Spleen Spatial Proteome Profiled by DMAA-Enhanced Seq-Scope-X

(a) Volcano plot showing proteins highly expressed in pixels corresponding to each LDA factor described in Fig. 6. Black dots represent proteins consistent with the annotated factor identity, while gray dots indicate proteins that may not align with the annotated cell type but could reflect regional context.

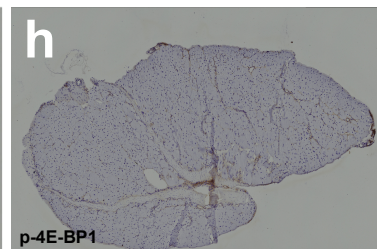
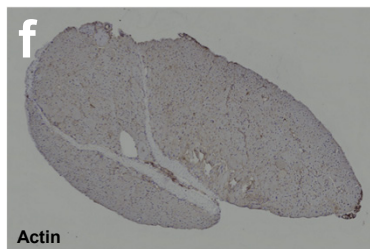
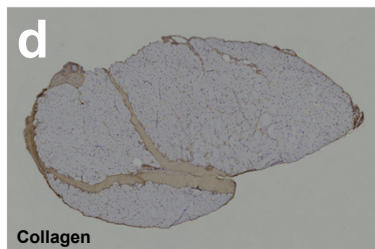
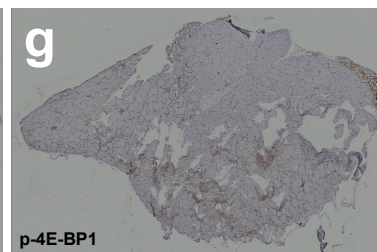
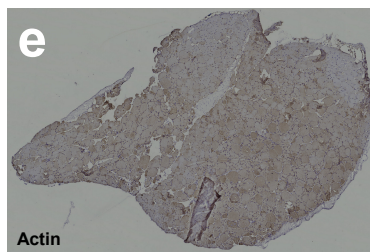
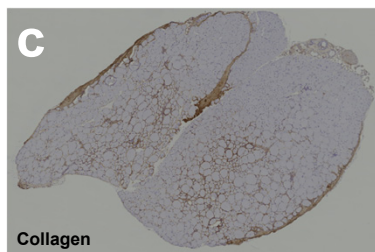
(b) Individual maps of all 24 LDA factors described in Fig. 6, demonstrating the positional probability of each factor within the histological space.

Scale bars represent the scale corresponding to the original tissue.

Seq-Scope-X: Protein Detection



IHC: Serial Sections from the Same Tissue



Supplementary Fig. 12. Detection of Extracellular, Intracellular, and Post-Translationally Modified Targets by Seq-Scope-X.

(a, b) COL1A1 (red), ACTA1 (green), and p-4E-BP1 (blue) antibodies were labeled with oligonucleotide tags, and used for Seq-Scope-X analysis of gastrocnemius (GTN) muscle from *CKM-Cre/Tsc1^{F/F}/Depdc5^{F/F}* (mTORC1-activated) mice ⁴³. Spatially detected antibody tags were visualized through spatial plotting.

(c-h) Expression patterns of each of these proteins were examined through traditional immunohistochemistry using the sections from the same tissue, showing similar patterns of expression.

Two independent GTN tissues were analyzed, and images from both tissues are shown. Scale bars represent the scale corresponding to the original tissue.