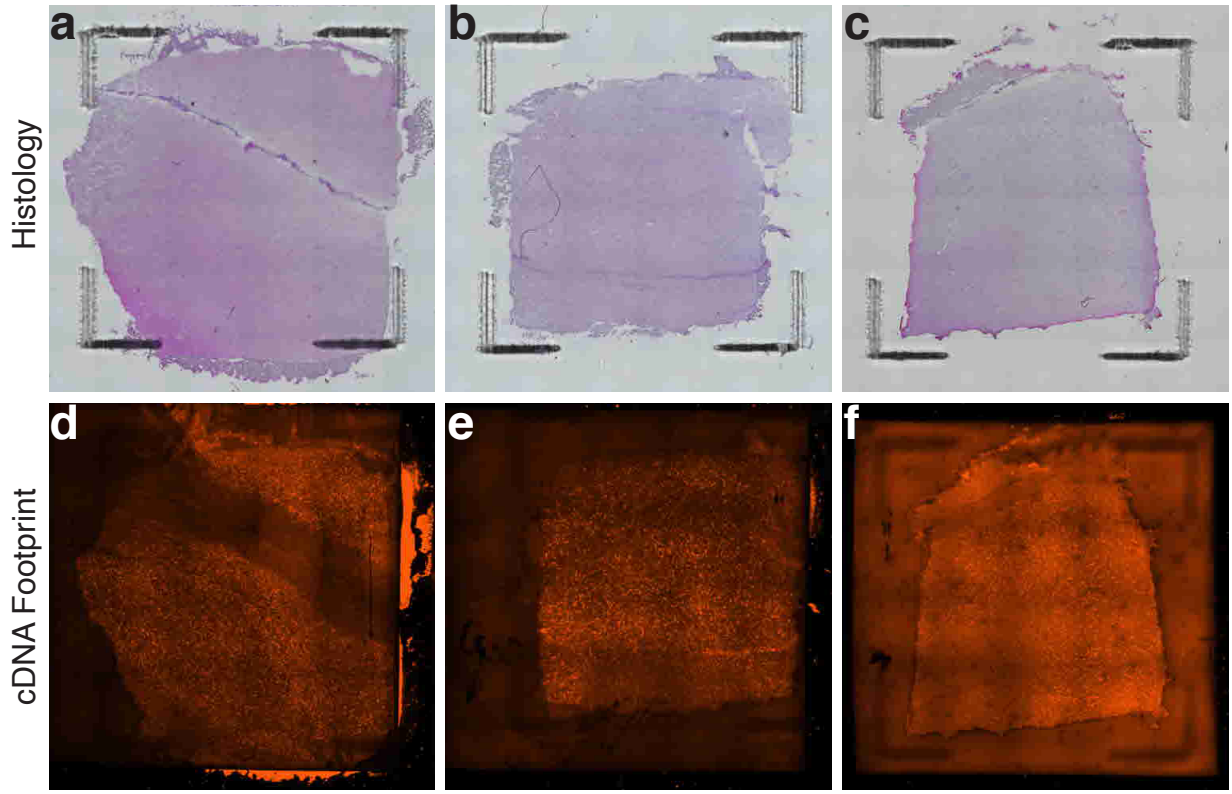
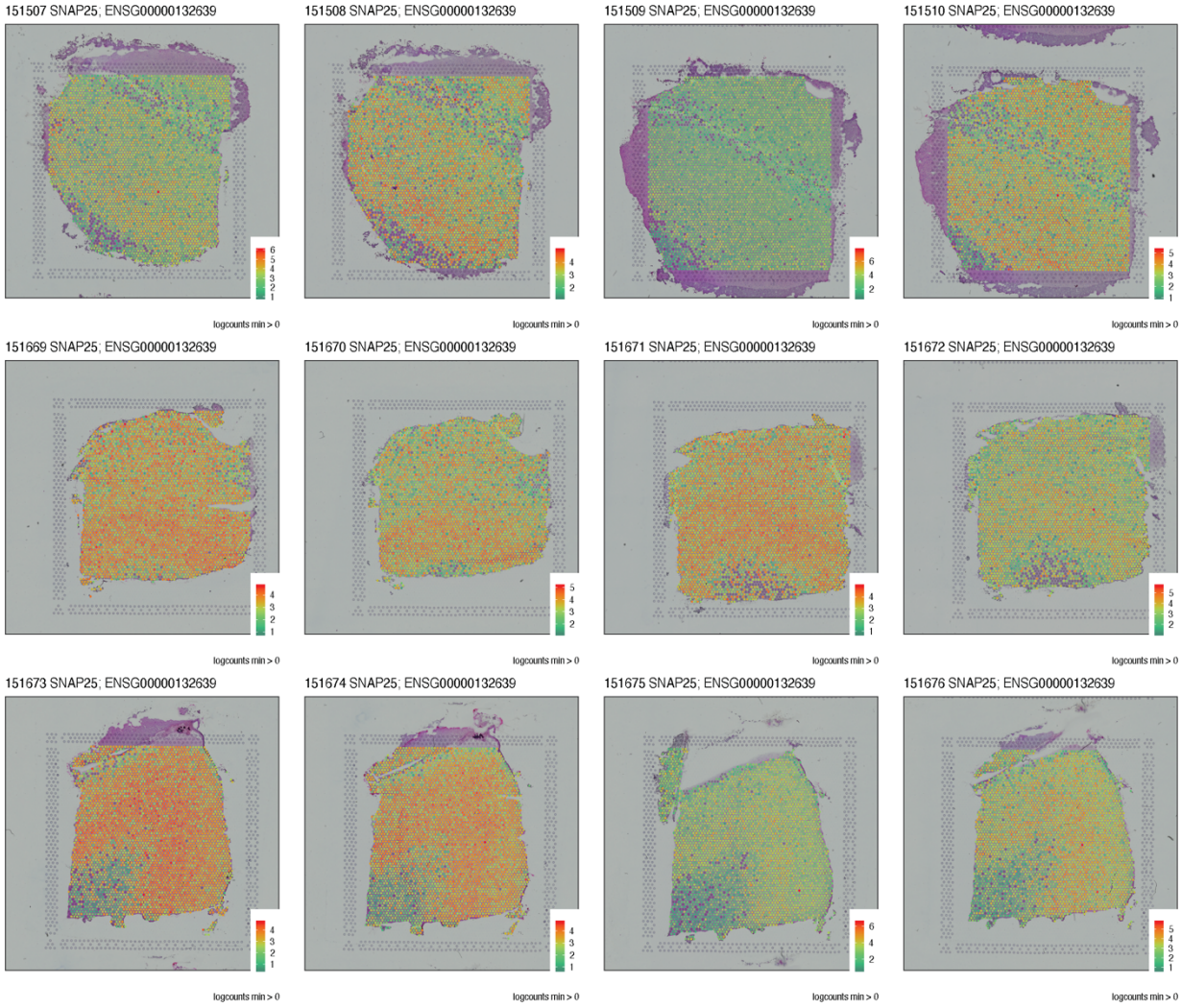
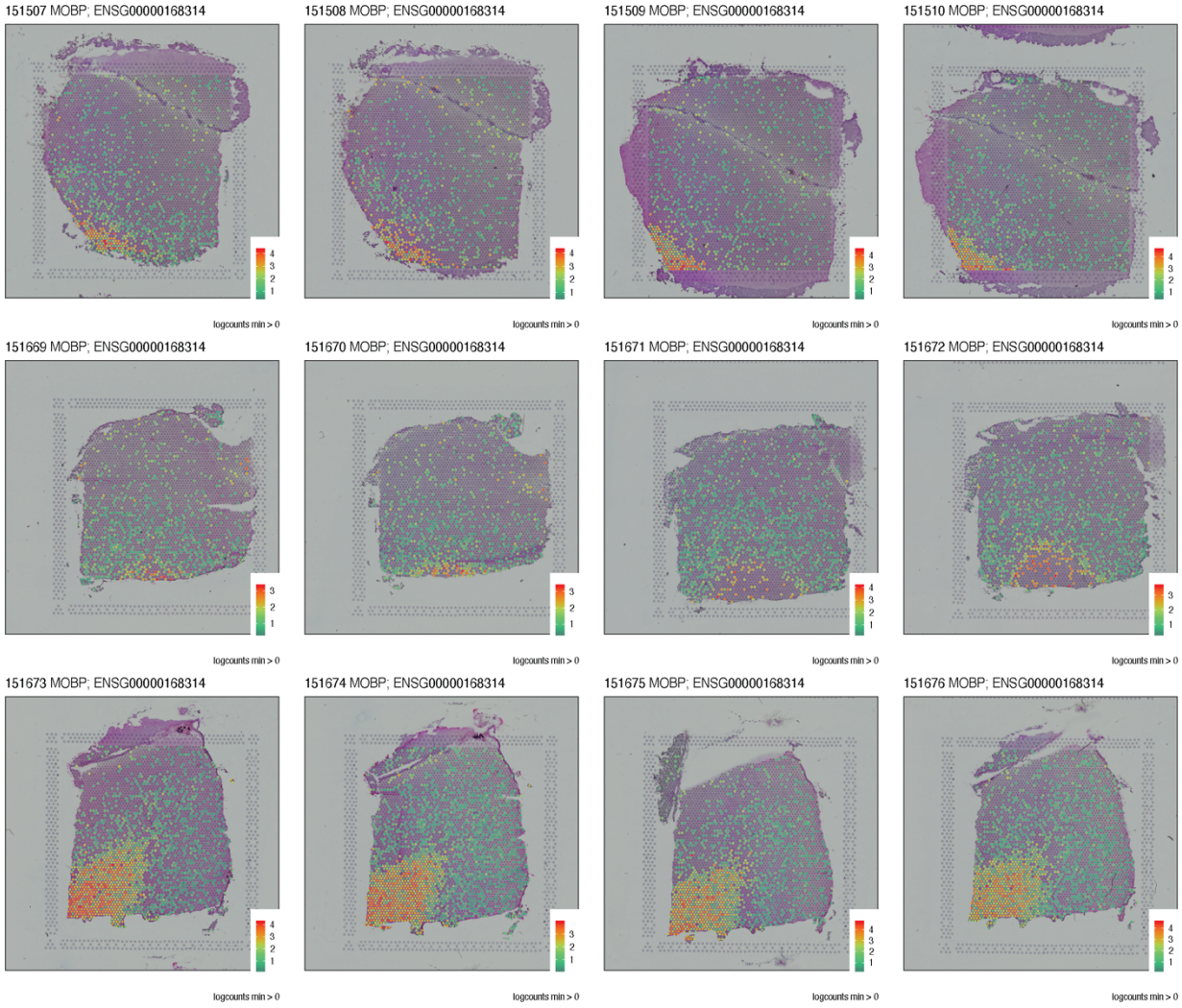
Supplemental Information



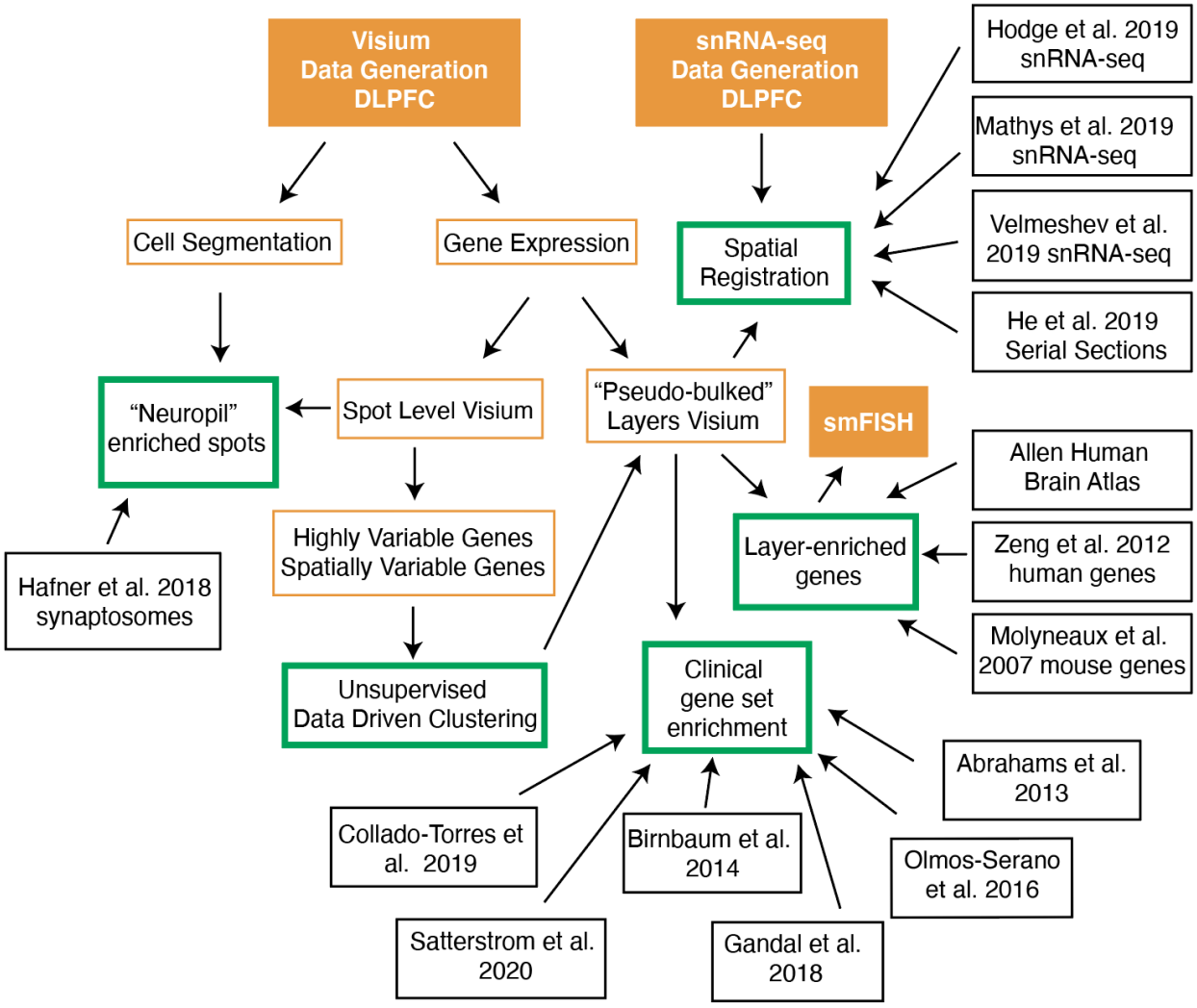
Supplementary Figure 1. Tissue optimization experiments, related to Figure 1. Brightfield histology images (A-C) and corresponding fluorescently labeled cDNA (D-F) for each subject after 18 minutes of tissue permeabilization. The size of the 10X Genomics capture frame is 6.5mm X 6.5mm as detailed at https://kb.10xgenomics.com/hc/en-us/articles/360035487572-What-is-the-spatial-resolution-and-configuration-of-the-capture-area-of-the-Visium-Gene-Expression-Slide-.



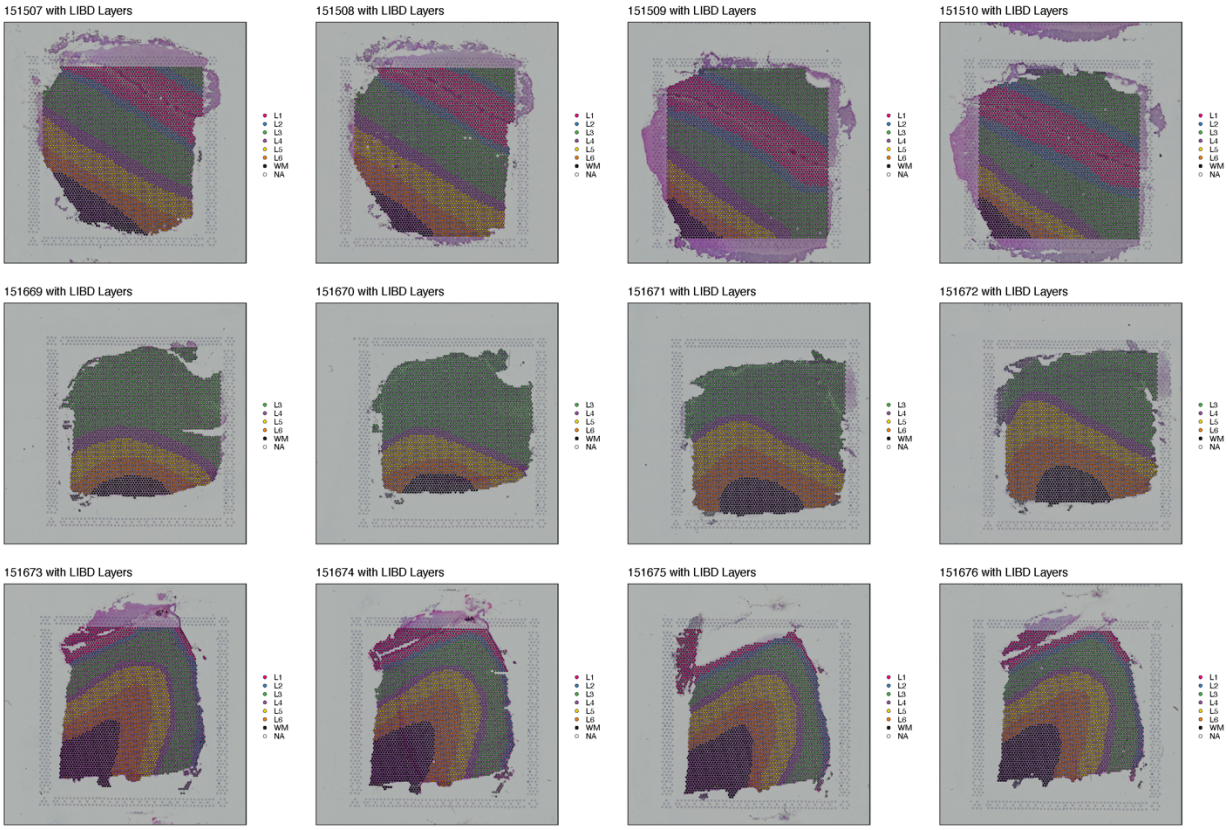
Supplementary Figure 2. *SNAP25* expression, related to Figure 1 D. Log-transformed normalized (logcounts) for *SNAP25* gene expression across all 12 samples arranged in rows by subject.



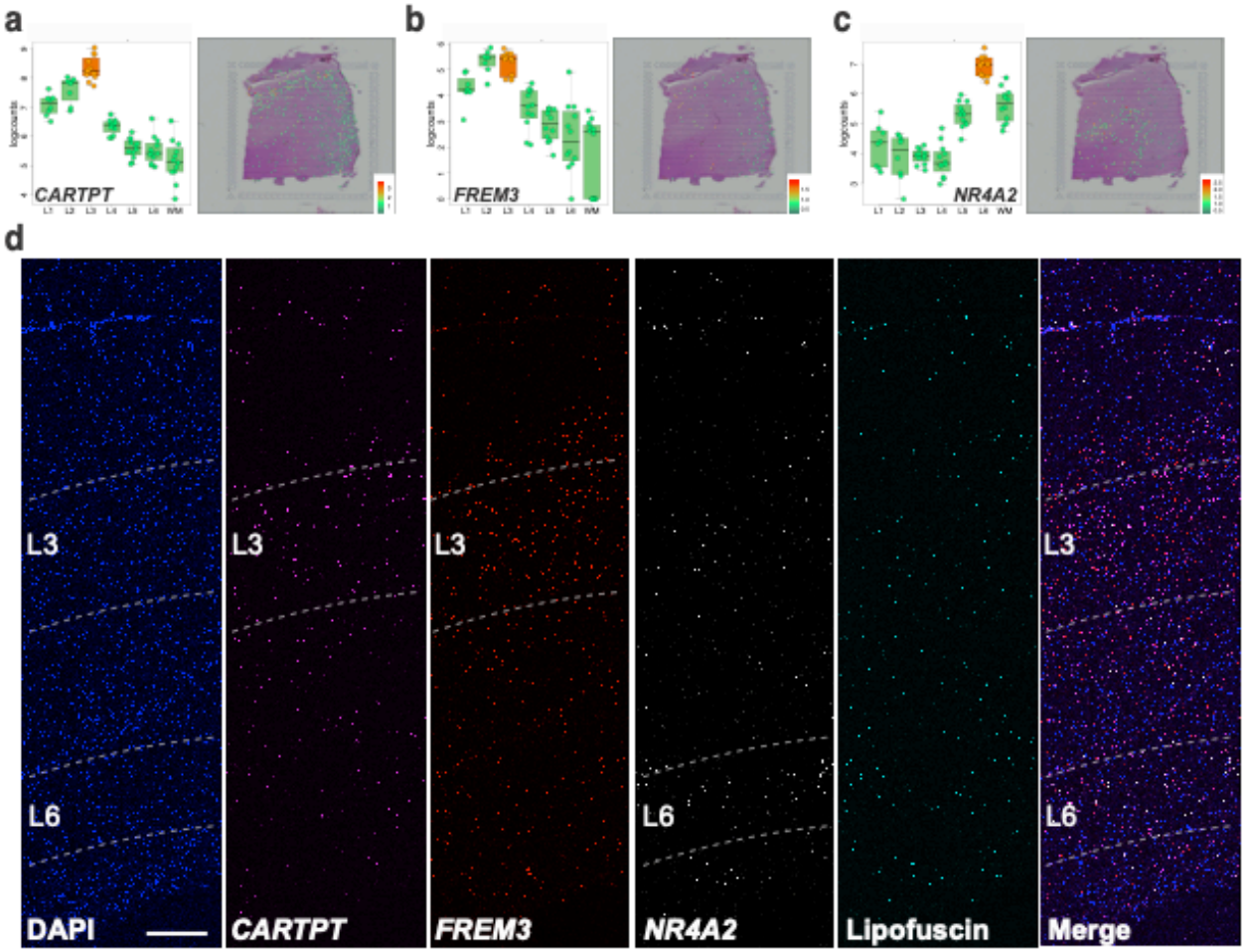
Supplementary Figure 3. *MOBP* expression, related to Figure 1 E. Log-transformed normalized (logcounts) for *MOBP* gene expression across all 12 samples arranged in rows by subject.



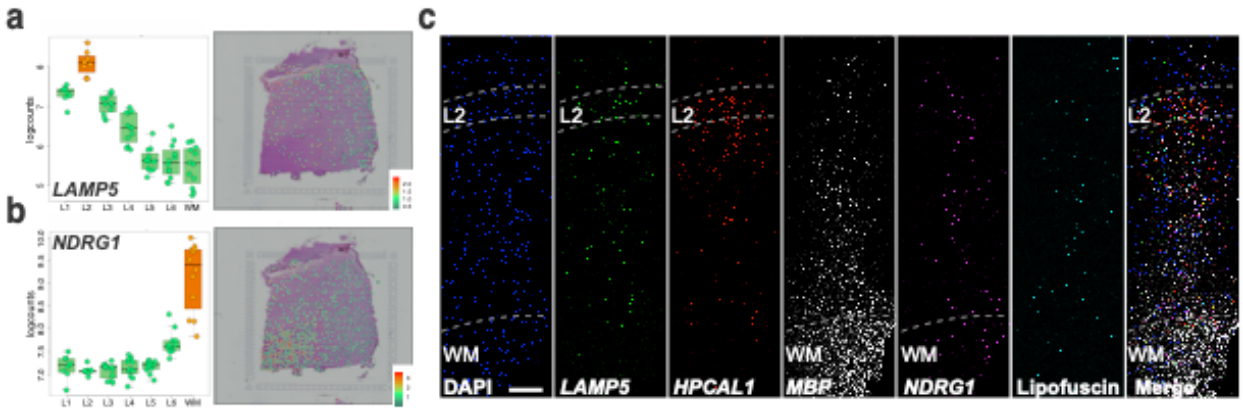
Supplementary Figure 4. Flowchart of data generation and analyses. Orange/thin line boxes highlight newly generated data. Green/thick line boxes highlight major analyses performed. Black boxes indicate publicly available datasets utilized in this study. Furthermore, spatial registration, layer-enriched genes, and clinical gene set enrichment can be applied to other datasets directly through our spatialLIBD web application available at http://spatial.libd.org/spatialLIBD/.



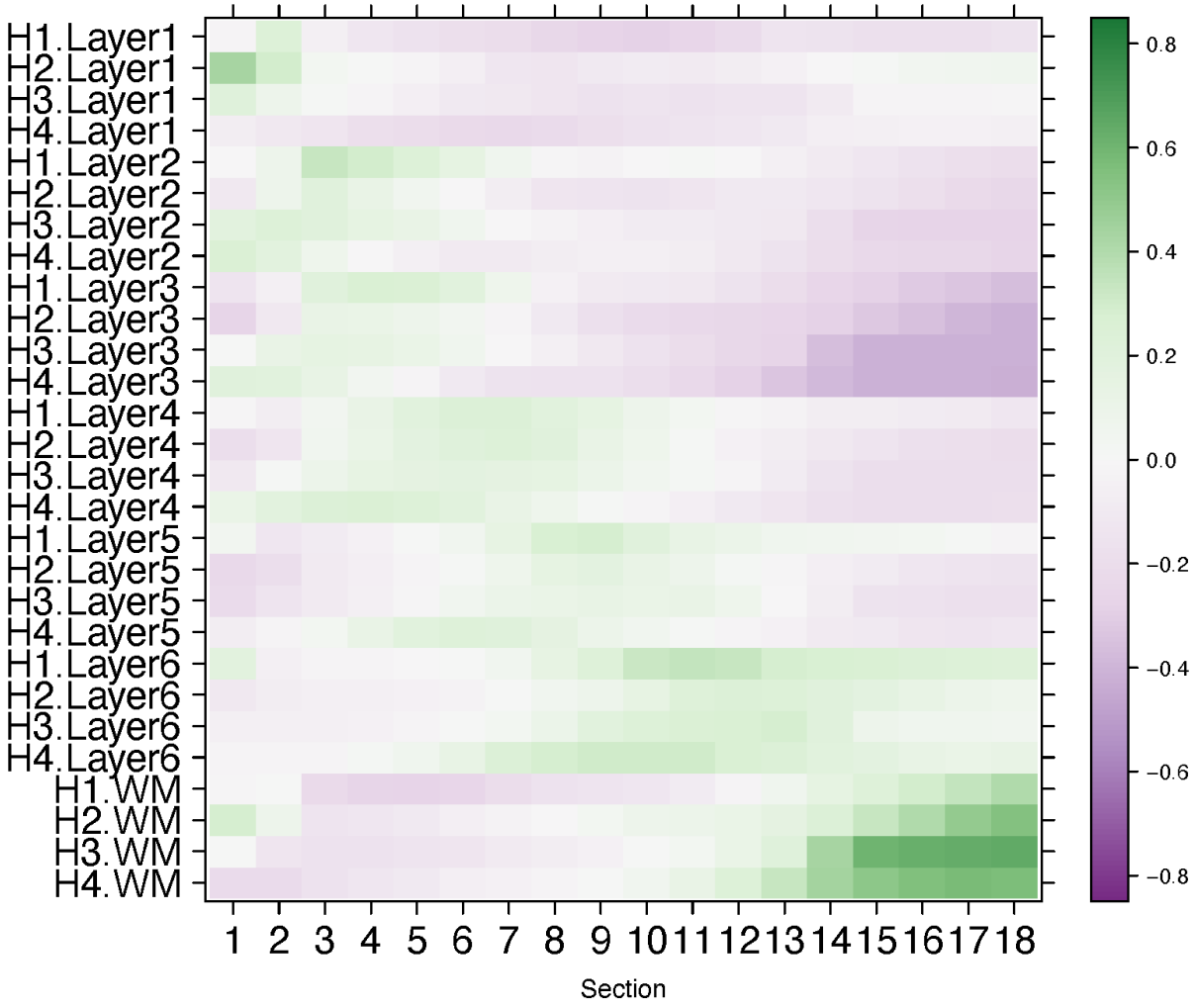
Supplementary Figure 5. Supervised annotation of layers based on cytoarchitecture and known marker gene expression, related to Figure 2. Manual annotation of cortical layers across all 12 samples arranged in rows by subject. See also **Extended Data 2**.



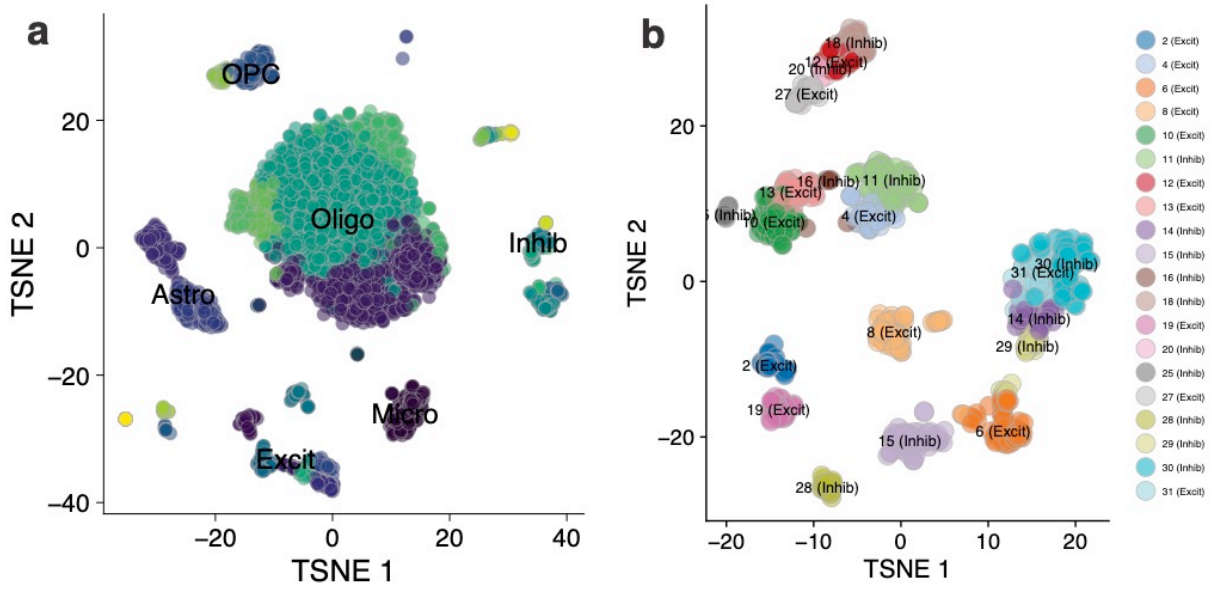
Supplementary Figure 6. **smFISH validation of L3- and L6-enriched genes, related to Figure 4. (A-C)** Left panels: Boxplots of log-transformed normalized expression (logcounts) for previously identified L3 and L6 marker genes *CARTPT* (**A**, L3>rest, *p*=2.07e-12) and *NR4A2* (**C**, L6>rest, *p*=1.15e-13), respectively, and Visium-identified gene L3 gene *FREM3* (**B**, L3>rest, *p*=8.16e-07). Right panels: Spotplots of log-transformed normalized expression (logcounts) for sample 151673 for corresponding genes. (**D**) Multiplex single molecule fluorescent in situ hybridization (smFISH) in a cortical strip of DLPFC. Maximum intensity confocal projections depicting expression of DAPI (nuclei), *CARTPT* (L3), *FREM3* (L3), *NR4A2* (L6) and lipofuscin autofluorescence. Merged image without lipofuscin autofluorescence. Scale bar=500 µm. 76 pseudo-bulked layers were used for computing the statistics in **A-C**.



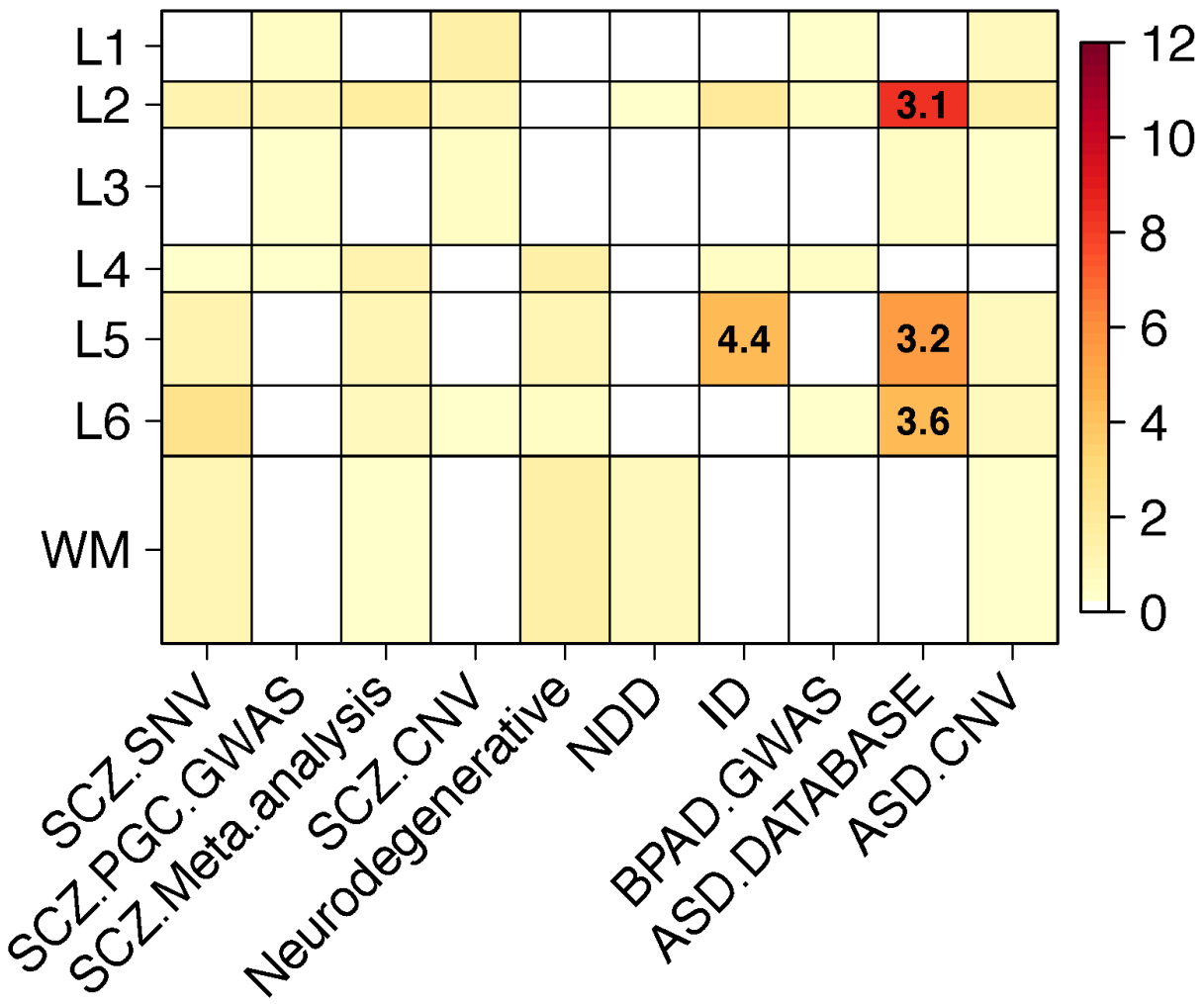
Supplementary Figure 7. **smFISH validation of L2- and WM-enriched genes, related to Figure 4. (A-B)** Left panels: Boxplots of log-transformed normalized expression (logcounts) for Visium-identified L2 and WM genes *LAMP5* (**A**, L2>rest, *p*=2.60e-09) and *NDRG1* (**B**, WM>rest, *p*=1.26e-26), respectively. Right panels: Spotplots of log-transformed normalized expression (logcounts) for sample 151673 for *LAMP5* (**A**) and *NDRG1* (**B**). Corresponding boxplots and spotplots for *HPCAL1* in **Figure 4** and *MBP* in **Figure S9.** (**C**) Multiplex single molecule fluorescent in situ hybridization (smFISH) in a cortical strip of DLPFC. Maximum intensity confocal projections depicting expression of DAPI (nuclei), *LAMP5* (L2), *HPCAL1* (L2), *MBP* (WM)*, NDRG1* (WM) and lipofuscin autofluorescence*.* Merged image without lipofuscin autofluorescence. Scale bar=500 µm. 76 pseudo-bulked layers were used for computing the statistics in **A-B**.



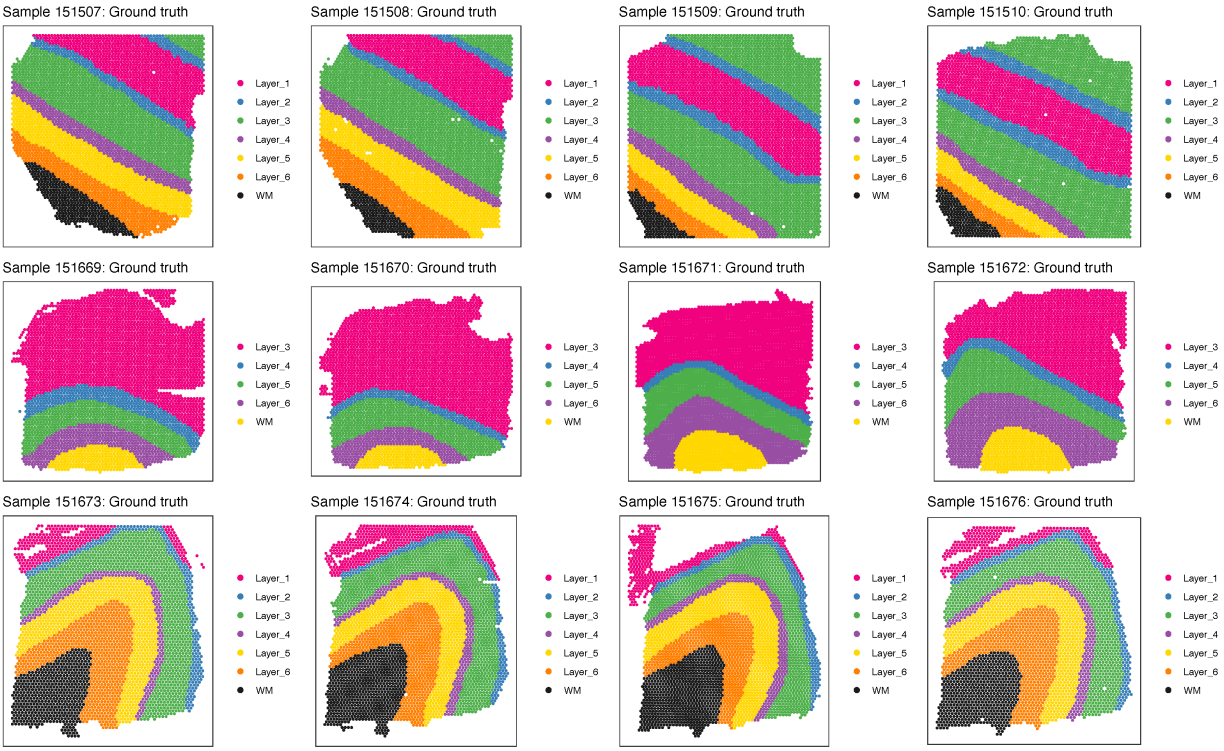
Supplementary Figure 8. Spatial registration of bulk RNA-seq data from serial sections from He *et al.*, related to Figure 5. Heatmaps of Pearson correlation values evaluating the relationship between our Visium-derived layer-enriched statistics across 700 genes for each of the four individuals from that study (y-axis) across the 18 serial sections for each donor.



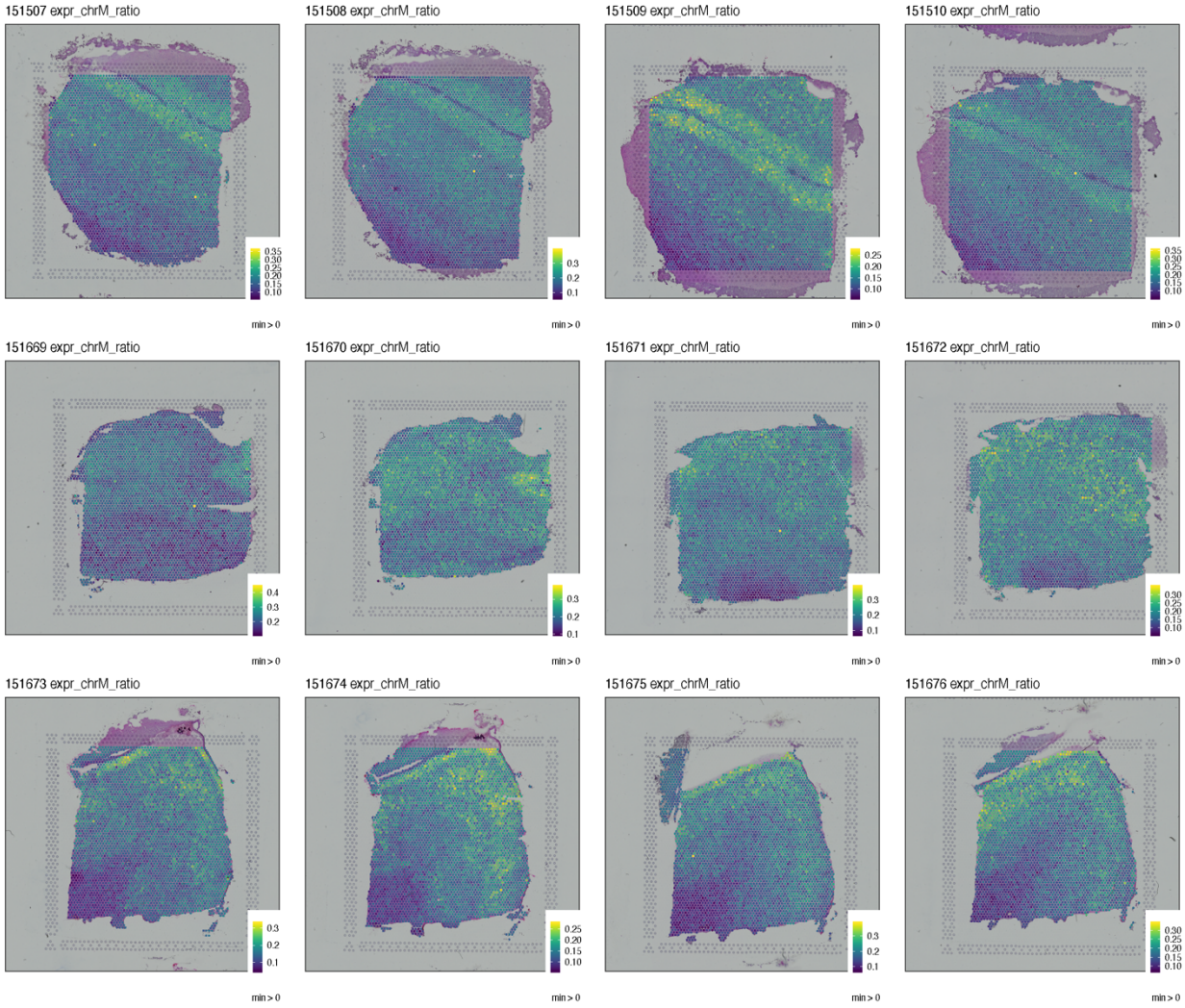
Supplementary Figure 9: t-SNE plots of snRNA-seq data from DLPFC, related to Figure 5. (**A**) tSNE plot of all nuclei, across 31 clusters. (**B**) tSNE plot of the subset of all neuronal nuclei.



Supplementary Figure 10. Enrichment of clinical gene sets for different neuropsychiatric and neurodevelopmental disorders, related to Figure 6. Shown are Fisher's exact test odds ratios and *p*-values for our Visium-derived layer-enriched statistics versus a series of predefined gene sets. Color scales indicate -log10(*p*-values), which were thresholded at $p=10^{-12}$, and numbers within significant heatmap cells indicate odds ratios (ORs) for the enrichments.



Supplementary Figure 11. Supervised annotation of DLPFC layers across all samples, related to Figure 7. These 'manually annotated' layers were used as the 'ground truth' for evaluating the data-driven clustering results for each sample. Colors represent the six DLPFC layers and white matter (WM), and are arranged in a consistent order across samples.



Supplementary Figure 12. Mitochondrial proportion of expression at the spot-level, related to Discussion. Visualization of the proportion of mitochondrial gene expression compared to the total gene expression at the spot-level. Each sample has its own color scale in order for the dynamic range to be visible for each sample.

Supplementary Tables

Note: the titles and legends are also included in the first sheet of the XLSX file.

Table S1. Sample metrics from Space Ranger and demographic information, related to Figure 1. A tab-separated table with the sequencing and alignment metrics produced by Space Ranger as well as the age, sex and LIBD brain ID for the samples sequenced in this project.

Table S2. Percent of spots with zero or one cell across layers, related to Results: Gene expression in the DLPFC across cortical laminae. This table shows the percentage of spots in each annotated layer with zero or one segmented cells.

Table S3. Spot cell count differential expression statistics, related to Results: Gene expression in the DLPFC across cortical laminae. Differential expression statistics comparing spots with 0 cells to >0 cells. Positive log2 fold changes indicate higher expression in spots without cells.

Table S4. Layer level differential expression statistics, related Figure 2. Differential expression statistics for the (**A**) ANOVA model (one model per gene), (**B**) Enrichment model (7 models per gene, 1 per layer), and (**C**) Pairwise model (21 models per gene, 1 per pair of layers).

Table S5. Optimal model results for known layer marker genes, related to Figure 3. Differential expression statistics for the optimal model for each known human or mouse brain marker gene as well as the top ranked gene using the layer-level data.

Table S6. Clinical gene sets layer enrichment statistics, related to Figure 6. Each row is a different gene set obtained from the literature. PE: psychENCODE, BS: BrainSeq, DS: Down Syndrome, DE: Differential Expression, TWAS: transcriptome-wide association study, OR: odds ratio, NumSig: number of significant layer-enriched genes in the gene set for that particular layer.

Table S7. Clinical gene set enrichment results with MAGMA, related to Figure 6. P-values for MAGMA gene set test for layer-enriched genes across four GWAS for SCZD, MDD, ASD and BPD. Bold indicates FDR < 0.05 significance and red indicates Bonferroni < 0.05 significance.

Table S8. LDSC results, related to Figure 6. Genomic enrichments of GWAS risk SNPs using partitioned heritability analysis. Prop = proportion, h2 = heritability, p = p-value, holm = Holm's adjusted p-values.

Table S9. Summary of *SpatialDE* genes, related to Figure 7. Number of statistically significant spatially variable genes (SVGs) identified per sample using *SpatialDE* [83], before and after additional filtering for lowly-expressed genes and mitochondrial genes.

Table S10. Description of clustering methods used for the data-driven layer-enriched clustering analyses, related to Figure 7. Summary of combinations of design choices that were

implemented for the clustering methods used for the data-driven spatial clustering analyses. Columns describe: (i) method names, (ii) the type of clustering method, (iii) the type of dimension reduction used to summarize gene expression, (iv) the source of gene sets used, and (v) whether spatial coordinates were included as additional features for clustering. The names of the clustering methods correspond to those shown in **Extended Data 9** and **Extended Data 10**.