

An integrated single-nucleus and spatial transcriptomics atlas reveals the molecular landscape of the human hippocampus

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Supplementary Notes

Supplementary Note 1 | Spot-level deconvolution algorithm benchmarking

Given that individual Visium spots often contain multiple cell types (**Extended Data Fig. 4A**), we performed spot deconvolution to better understand the cellular composition of spots mapping to unsupervised spatial domains. While several algorithms have been developed to predict cell type proportions within individual Visium spots using single cell reference data (`cell2location` (1), RCTD (2), and `Tangram` (3)), these methods have not yet been comprehensively benchmarked across various brain regions. This study provides a great opportunity to perform such benchmarking based on the paired SRT and snRNA-seq data from the same donors and the presence of anatomical regions enriched for specific cell types. In order to provide the final aspect of a gold standard reference dataset, that is known cell type abundances in each gene expression spot, we performed Visium Spatial Proteogenomics assay (Visium-SPG) for 4 broad cell types, similar to our previous study in the dIPFC (4). We selected 2 out of the 10 brain samples, one male and one female, based on exceptional morphology and inclusion of all spatial domains (**Supplemental Fig. 14A**). Following multispectral fluorescence imaging, we proceeded with the standard Visium protocol to generate corresponding gene expression libraries from the same tissue section. This provided an orthogonal measure of broad cellular identity for each spot.

Visium-SPG data generation

Visium-SPG replaces H&E histology with immunofluorescence staining to label proteins of interest with fluorescent dyes. We opted to label cell type-specific proteins, including NEUN (marking neurons), OLIG2 (marking oligodendrocytes), GFAP (marking astrocytes), and TMEM119 (marking microglia). We performed combinatorial immunofluorescence staining paired with spatial gene expression using the Visium-SPG protocol (protocol CG000312, revision D, 10x Genomics), with some modifications. Briefly, gene expression slides were fixed in methanol, incubated in blocking buffer (3X SSC supplemented with 2% BSA, 0.1% Triton X100 and 2U/ul RNase inhibitor (RiboLock, Thermofisher, Cat# EO0384)), then incubated with primary antibodies for 30 minutes at room temperature. Primary antibodies used were mouse anti-NeuN antibody conjugated to Alexa 488 (Sigma Aldrich, Cat# MAB377X, 1:100), rabbit anti-TMEM119 antibody (Sigma Aldrich, Cat# HPA051870, 1:20), rat anti-GFAP antibody (Thermofisher, Cat# 13-0300, 1:100), and goat anti-OLIG2 antibody (R&D systems, Cat# AF2418, 1:20). Slides were washed with wash buffer (3X SSC with 2% BSA, 0.1% Triton X100, 2U/ul RNase inhibitor and supplemented with ribonucleoside vanadyl complex (Concentration, NEB S1402S)), then incubated for 30 minutes with secondary antibodies at RT. Secondary antibodies used were donkey anti-rabbit IgG conjugated to Alexa 555 (Thermofisher, Cat# A-31572, 1:300), donkey anti-rat IgG conjugated to Alexa 594 (Thermofisher, Cat# A-21209, 1:600), and donkey anti-goat IgG conjugated to Alexa 647 (Thermofisher, Cat# A-21447, 1:400). DAPI (Thermofisher, Cat# D1306, 1:3000, Final 1.67 µg/ml) was included as a nuclear counterstain. The slide was coverslipped with glycerol containing RNase Inhibitor (2 U/µl) and scanned on a Vectra Polaris slide scanner (Akoya Biosciences) at 20x magnification with the following exposure time per given channel: 2.70 msec for DAPI; 185 msec for Opal 520; 900 msec for Opal 570; 160 msec for Opal 620; 1300 msec for Opal 690; 100 msec for autofluorescence.

Following imaging, on-slide cDNA synthesis was performed, and sequencing libraries were generated for all Visium samples following the manufacturer's protocol (CG000239, revision C, 10x Genomics). Libraries were sequenced on a NovaSeq 6000 System (Illumina) at the Johns Hopkins Single Cell Transcriptomics core according to manufacturer's instructions at a minimum depth of 60000 reads per spot.

Visium-SPG quality control and domain annotation

FASTQ and image data were pre-processed with the 10x SpaceRanger pipeline and aligned to reference genome GRCh38 2020-A (version 2.0.0) (5). Quality control metrics were calculated with `scuttle` (6,7) and we applied a 3x median absolute deviation (MAD) threshold to discard spots with low library sizes and/or low numbers of detected genes (**Supplemental Fig. 14B**). Following QC, we retained 37,845 spots in $n=8$ capture areas for the Visium-SPG data (**Supplemental Fig. 14C**). Raw counts were then normalized based on library size and $\log_2$ -transformed.

In order to evaluate the performance of spot-level deconvolution in Visium-SPG across the different HPC spatial domains, we used `RcppML` to transfer spatial domain labels from the Visium-H&E SRT dataset (8). To achieve this, the Visium-H&E SRT dataset was pseudo-bulked to the level of spatial domain, summing the $\log_2$ CPM expression for each of the 16 spatial domains. Lowly expressed genes were removed with the `filterByExpr()` function from the `edgeR` package (9,10). We rescaled columns of the pseudo-bulked matrix such that each column summed to 1, akin to the scaling diagonal used in NMF calculations in `RcppML` (8). We then used the `project()` function from `RcppML` to estimate PRECAST spatial domain membership of each spot in the Visium-SPG samples. When implemented in this manner, this function predicts HPC spatial domain membership of each spot in the Visium-SPG samples. These predicted domain membership values were continuous. Therefore, for each spot, we assigned domain membership based on the highest predicted domain value (**Supplemental Fig. 14D**).

Immunofluorescence-based cell type classification

We leveraged the immunofluorescence (IF) images in the Visium-SPG assay to directly quantify cell type abundance and provide an imaging-based calculation of cell counts for comparison with outputs from spot deconvolution tools. Starting from the `cyto` model provided by `Cellpose 2.0` (11), nuclear segmentations of Br3942 and Br8325 were iteratively improved in the `Cellpose` GUI, and a new model was trained until DAPI segmentations visually satisfied our accuracy standards (examples **Supplemental Fig. 26A**). Segmentation masks were read into python 3.8.12, and mean fluorescence intensity for the NeuN, OLIG2, GFAP and TMEM119-marked channels was quantified using `skimage.measure.regionprops_table()` from the `scikit-image` (12) v0.19.2 library. For 2 capture areas (V12D07-332_D1 and V12D07-335_D1) which represented all HPC regions, an expert experimenter (co-author SCP) used `Samui Browser` (13) to randomly select and annotate a spatially diverse set of 200 cells of each immunolabeled cell type – e.g. neuron, oligodendrocyte, astrocyte, microglia, or other (i.e. presence of DAPI, but absence of other immunofluorescent signals). The resulting dataset included ~1000 cells with a cell type label, and the corresponding mean fluorescence intensities from the respective channels. A classification and regression tree (CART) was implemented using `scikit-learn` (14) to classify 80% of the expert-annotated nuclei into either one of the 4 immunolabeled cell types or “other” (**Supplemental Fig. 26B**). Following training (94% accuracy), the CART model was tested on the remaining expert-annotated nuclei and achieved 95% testing accuracy. The trained and tested model was then applied to the full Visium-SPG dataset, and CART-derived cell type labels were used to calculate cell counts per Visium spot for neurons, astrocytes, microglia, and oligodendrocytes (**Extended Data Fig. 4B**).

Input marker gene detection

All the tested deconvolution algorithms use a subset of genes in the snRNA-seq reference dataset to identify cell types before predicting the relative abundance of those learned cell types in the query SRT dataset. We selected marker genes for two resolutions of snRNA-seq clustering to probe the performance of deconvolution algorithms at multiple annotation depths (“fine” and “mid” cell type classifications, **Supplemental Fig. 27A**). We confirmed that at these resolutions there was no donor bias in the cell type classifications

(**Supplemental Fig. 27B-D**). Three snRNA-seq clusters from the fine grouping (HATA, Amy, and GABA.PENK) were excluded as these were not represented in any Visium-SPG samples and could occlude marker gene identification for other clusters. We retained the thalamus cluster because one of the Visium-SPG donors included a very small amount of thalamus (**Supplemental Fig. 18**) which was transcriptionally distinct from hippocampal cells.

To identify marker genes, we utilized a “mean ratio” method, implemented with `DeconvoBuddies` (version 0.99.0), where the ratio of expression between a target cell type and the next-highest-expressing cell type is used to rank candidate marker genes (15). Mitochondrial genes and genes not present in the spatial data were excluded from consideration. We evaluated a range of top n genes and determined that the top 25 provided optimal signal-to-noise ratio (**Supplemental Fig. 28**). The top-25-ranked markers by mean ratio were taken for each cell type and we confirmed that the mean ratio exceeded 1 for each selected marker gene (15). This resulted in a total of 200 mid-resolution markers and 525 fine-resolution markers used downstream for spot deconvolution (**Supplemental Table 7**). We confirmed the utility of the snRNA-seq-derived marker genes for spot deconvolution by evaluating the number of the top 25 marker genes with non-zero expression for each “mid” and “fine” cell type across spatial domains in the Visium-SPG data (**Extended Data Fig. 4C**, **Supplemental Fig. 29**).

Post-hoc, we found that `RCTD`, but not `cell2location` and `Tangram` (**Supplemental Fig. 30A**), dropped some spots when using the mean ratio marker genes (**Supplemental Fig. 30B**). We observed that `RCTD` failed for spots containing low sum UMI ($\sim <100$) calculated after filtering the genes to include only the user derived marker genes. Therefore, the default `RCTD` marker gene detection strategy was employed to run `RCTD` (**Supplemental Fig. 30C**).

Implementing spot-level deconvolution with Visium-SPG data

Having verified the selection of robust marker genes for each cell type, we ran `cell2location` (1), `RCTD` (2) and `Tangram` (3) and calculated the predicted cell type counts or proportions per spot. `Cell2location` v0.8a0, `RCTD` v2.2.0 and `Tangram` v1.0.2 were applied with default parameters following the respective tutorials provided by their authors, with a few exceptions. The mean ratio marker genes were used to train `cell2location` and `Tangram` and `RCTD` marker genes were used to train `RCTD`. `Tangram` requires an accurate cell count per spot, and thus was provided total cell counts per spot derived through nuclei segmentation with `Cellpose` v2.0 (Visium-SPG images) and through `VistoSeg` (Visium-H&E images). `Cell2location` and `RCTD` require an average count of nuclei per spot, which was calculated as 5 across all Visium-SPG capture areas. While `Tangram` and `Cell2location` predict cell type abundances in each spot, `RCTD` predicts the cell type proportion per spot. Therefore, to more directly compare across algorithms, we multiplied the cell type proportions obtained from `RCTD` by the nuclei counts to obtain spot-level abundance values.

Evaluating performance of spot deconvolution methods

The cell type counts estimated by each algorithm in the Visium-SPG data at fine and mid resolutions were “collapsed” down to the broad categories (neuron, astrocytes, microglia, oligodendrocytes, and other (comprised of OPCs, vasculature, and CSF-related cells)) to enable direct comparison with the CART-calculated counts (**Extended Data Fig. 4D**). We then visually compared the predicted cell type (of “mid” cell types collapsed down to broad cell types) proportion per spot from the 3 softwares to the CART-estimated proportions of immunolabeled cells (**Supplemental Fig. 31**). The collapsed counts from “mid” and “fine” resolutions were compared against each other which should theoretically match (**Supplemental Fig. 32**). To quantify the comparisons, the “other” cell type was excluded from the benchmarking analyses and counts for

each software tool and CART-quantified cell type were summed across each Visium-SPG section. Totals for each software method (gene expression) were compared against the CART-predicted (immunofluorescence) cell count totals using Pearson correlation and root mean squared error (RMSE) at “mid” (**Supplemental Fig. 32**) and “fine” (**Supplemental Fig. 32**) levels (**Supplemental Table 8**). Counts for each software tool and CART predictions were normalized to add to 1 across the four cell types which allowed the calculation of the Kulback-Liebr divergence (KL divergence) from each software tool’s predictions to the CART predictions. This treats the CART-predicted cell type composition as a ground-truth probability distribution that each software tool is attempting to estimate (**Supplemental Fig. 32**).

Given the challenge of deconvolving transcriptionally fine cell types, our Visium-SPG benchmark analyses confirmed RCTD showed the most consistent performance at both mid and fine resolution across all cell types and samples (**Supplemental Fig. 32**) closely aligning with biological expectations and revealing spatial variations in cell types (**Supplemental Fig. 33**). Tangram predicted a similar cellular composition in each domain and predicted the presence of all cell types in all spots. cell2location showed a similar pattern but with less pronounced consistency (**Extended Data Fig. 4D**). In summary, these analyses confirmed RCTD is most suitable for spot deconvolution of Visium HPC data because it accounts for the cellular heterogeneity seen across the HPC.

Applying spot deconvolution software to Visium-H&E data

Having evaluated the performance on the Visium-SPG data, we ran RCTD, the best performing method, on the whole SRT data for which orthogonal measures of spot-level cellular composition were not available (**Extended Data Fig. 4E, Figure 4, Supplemental Fig. 33**). The RCTD deconvolutions results for Visium-SPG and the SRT data are available on the Samui web application for visualization.

Supplemental Table Legends

Supplemental Table 1. Donor demographic information. Demographic information on brain donors, including Sample ID (BrNum), age at time of death, sex, psychiatric diagnosis, post-mortem interval (PMI), body mass index (BMI, calculated postmortem), Screening RNA Integrity Number (RIN, calculated at time of brain collection in the prefrontal cortex–PFC), and, for some samples, RIN measured in the tissue blocks included in this study. The assays performed on each tissue sample are included.

Supplemental Table 2. Abbreviations. Guide to abbreviations used in naming spatial domains and snRNA-seq cell types.

Supplemental Table 3. nnSVG gene ranks. Results from nnSVG analysis, used to identify spatially variable genes (SVGs) in the SRT data. Columns include ENSEMBL ID, gene name, overall gene rank, mean gene rank across all capture areas, number of capture areas for which each gene was highly ranked (within top 1000 most variable genes), and whether each gene was used for PRECAST clustering.

Supplemental Table 4. Differential expression results across pseudobulked spatial domains using the layer-enriched model. For each gene, a linear mixed-effects model was fit with counts aggregated (or pseudobulked) across spots within a spatial domain to identify differences in expression enriched in one domain compared to all other domains using Student's *t*-test statistics.

Supplemental Table 5. Differential expression results across four pseudobulked broad spatial domains using the layer-enriched model. Four broad domains: neuron-enriched domains (comprised of pyramidal, granule and GABAergic neuron clusters), neuropil-enriched domains (including ML, SL, SR, SLM.SGZ), WM, and vascular/cerebrospinal fluid (CSF)-related domains (comprised of the two domains mapping to vasculature and choroid plexus, respectively). For each gene, a linear mixed-effects model was fit with counts aggregated (or pseudobulked) across spots within a spatial domain to identify differences in expression enriched in one domain compared to all other domains using Student's *t*-test statistics.

Supplemental Table 6. Differential expression results across pseudobulked “superfine” cell types using the layer-enriched model. For each gene, a linear mixed-effects model was fit with counts aggregated (or pseudobulked) across nuclei within each superfine cell type to identify differences in expression enriched in one superfine cell type compared to all other superfine cell types using Student's *t*-test statistics.

Supplemental Table 7. Spot deconvolution cell type marker gene statistics. For each cell type at mid and fine resolution, statistics of top 25 marker genes (mean ratio>1) extracted with the *DeconvoBuddies* package using the filtered (genes that overlapped with Visium data only) snRNA-seq data.

Supplemental Table 8. RMSE and correlation stats. For each cell type at mid and fine resolution, Pearson Correlation and RMSE for spot-deconvolution software results compared with CART predictions were calculated for each Visum-SPG capture area.

Supplemental Table 9. Gene-level NMF weights. Table with gene-level weights for NMF patterns identified using RcppML. Each row is a gene and each column is an NMF pattern. Patterns were normalized such that each column sums to 1.

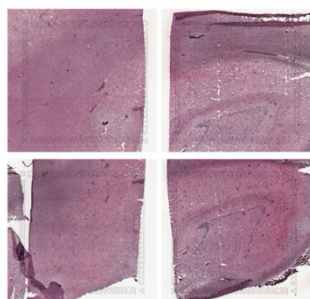
Supplemental Table 10. Top ten weighted genes for each NMF pattern. The top ten genes weighted to each pattern in Supplementary Table 9 are isolated and presented alongside the gene-level weights.

Supplemental Figures

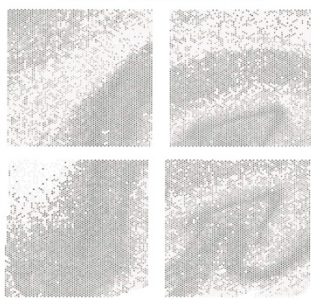
Supplemental Fig. 1. Expression of *SNAP25* and *MBP* reveal neuron-rich and oligodendrocyte-rich regions across hippocampus (HPC) samples.

H&E histology images (first column), $\log_2$ transformed CPM normalized counts for *SNAP25* (pan-neuronal marker, second column) for each donor.

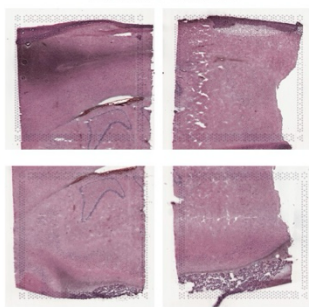
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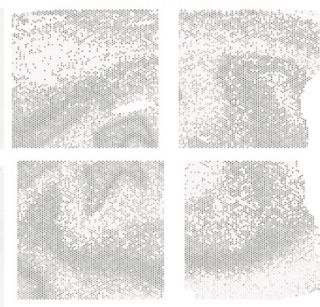
Br3942 SNAP25



Br6522 histo



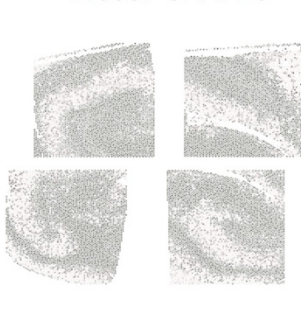
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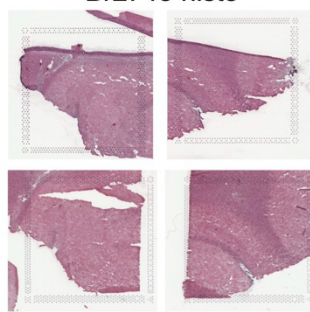
Br8667 histo



Br8667 SNAP25



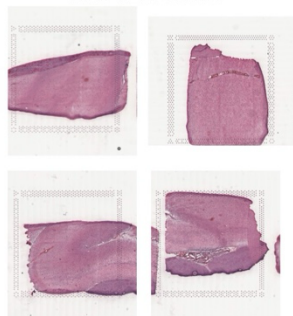
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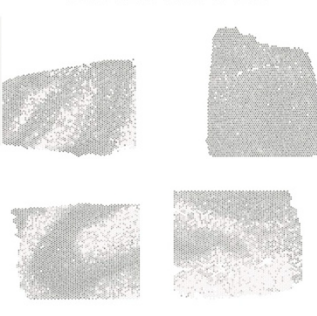
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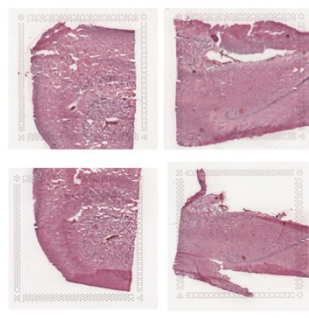
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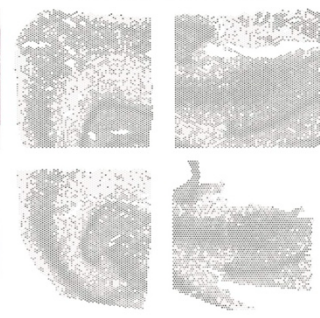
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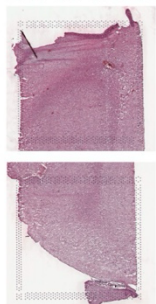
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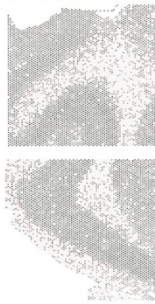
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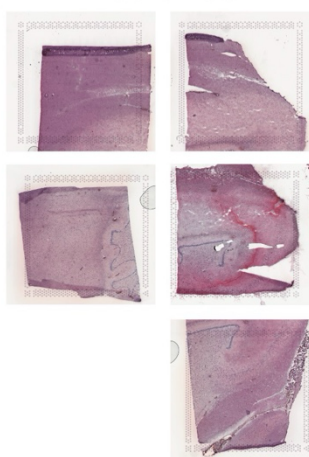
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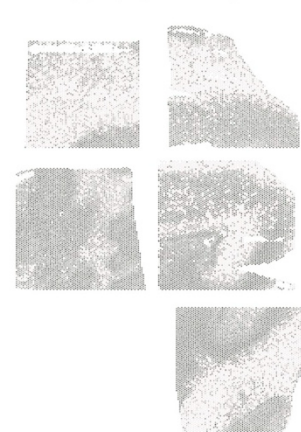
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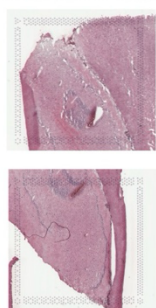
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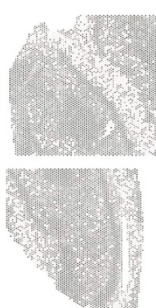
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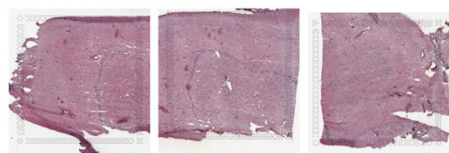
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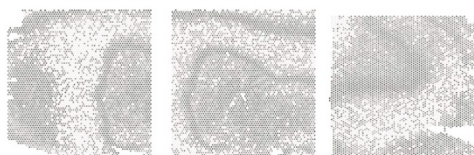
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Br6471 histo

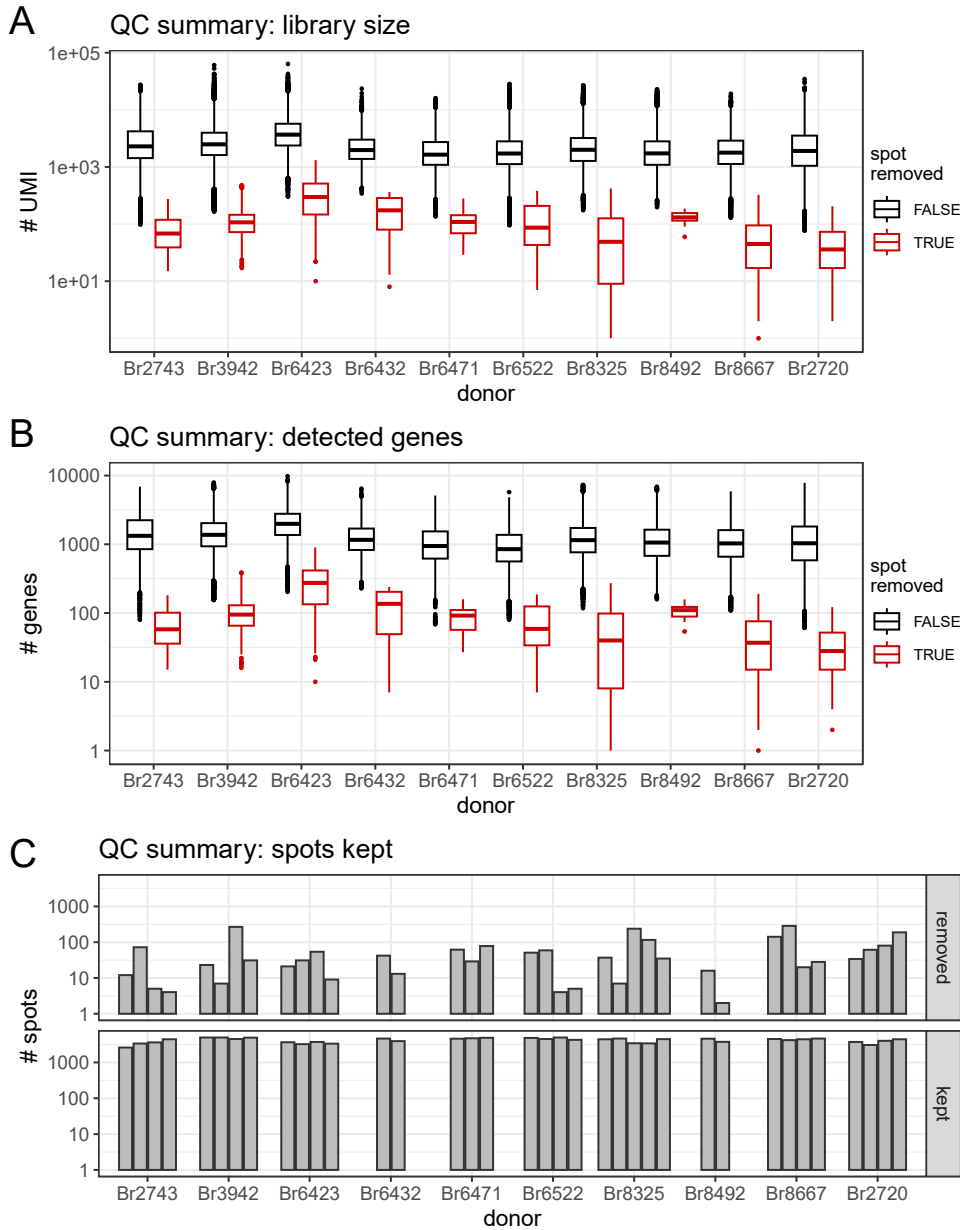


Br6471 SNAP25



Supplemental Fig. 2. Quality control (QC) metrics used to filter out low-quality spots.

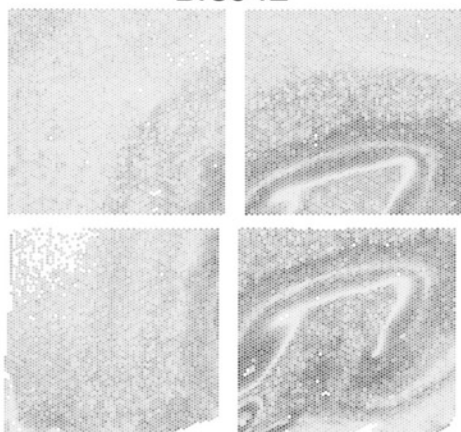
- (A) Boxplots of library size (y-axis, total number of reads in a single spot) based on three median absolute deviation (3MAD) threshold calculated for each capture area independently. The spots are grouped by donor (x-axis) and are stratified by whether the spots were kept (black) or removed (red).
- (B) Boxplots of number of detected genes (y-axis, total number of genes with at least one read in a single spot) based on three median absolute deviation (3MAD) threshold calculated for each capture area independently. The spots are grouped by donor (x-axis) and are stratified by whether the spots were kept (black) or removed (red).
- (C) Bar plots demonstrate the number of spots (y-axis, log scale) per sample (individual bar) for each donor (x-axis). The plot is faceted by whether the spots were removed based on QC filter (top) or kept in the final dataset (bottom).



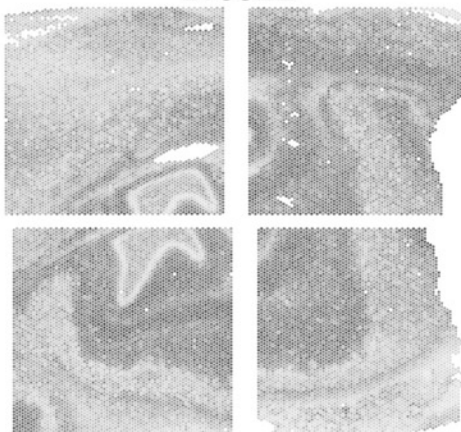
Supplemental Fig. 3. Spot plot of proportion of reads mapping to mitochondrial genes per spot for each capture area.

Within each capture area, each spot is colored by the proportion of reads mapping to mitochondrial genes (legend bottom left), which are enriched in hippocampal neuropil-enriched layers. Capture areas are grouped by donor number.

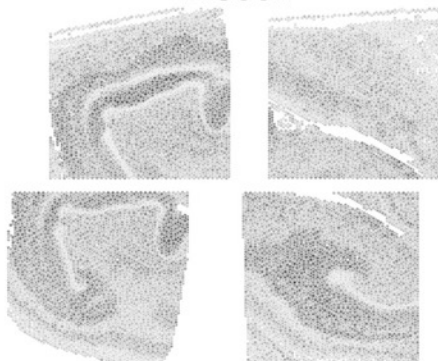
Br3942



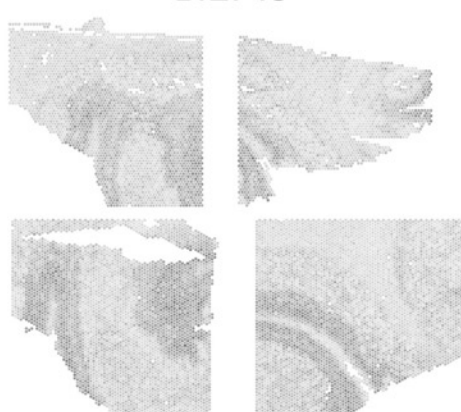
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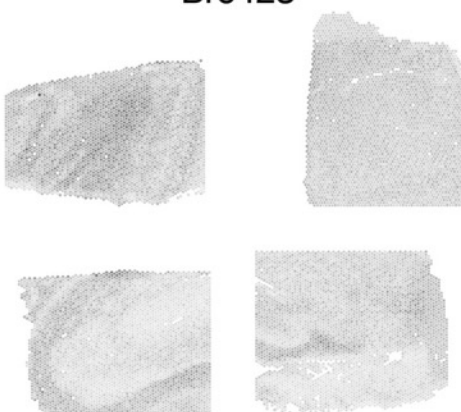
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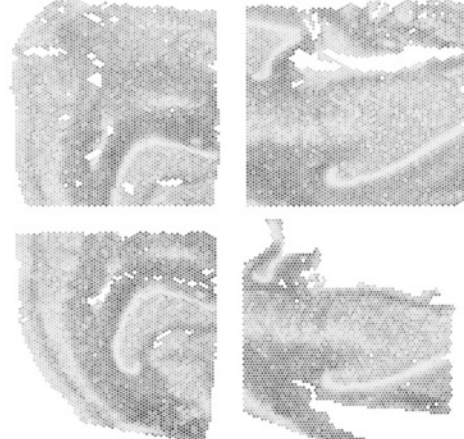
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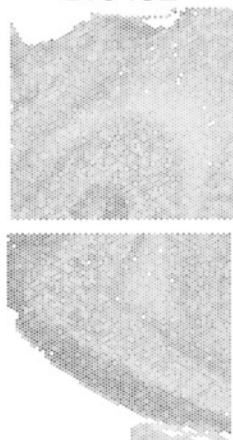
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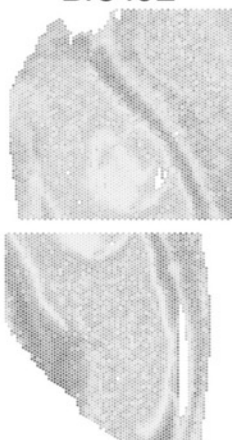
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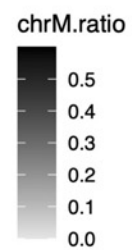
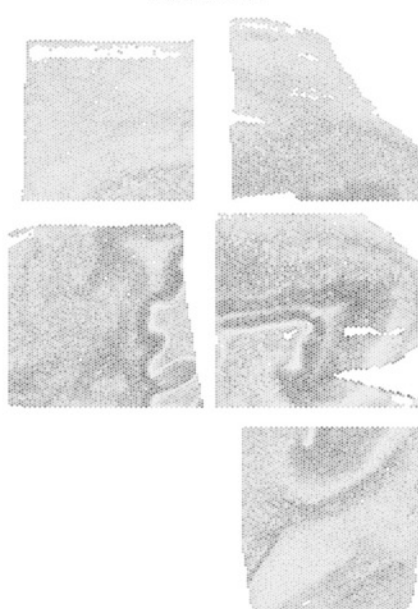
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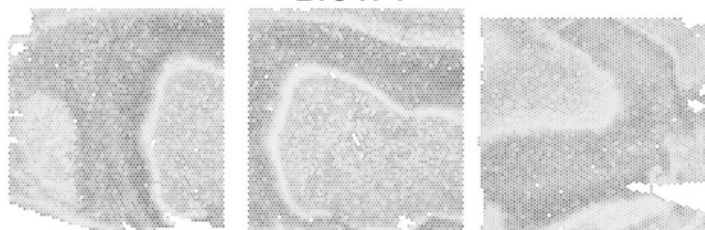
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Br8325



Br6471



Supplemental Fig. 4. Comparing PRECAST clustering results at $k=16$, $k=17$, and $k=18$.

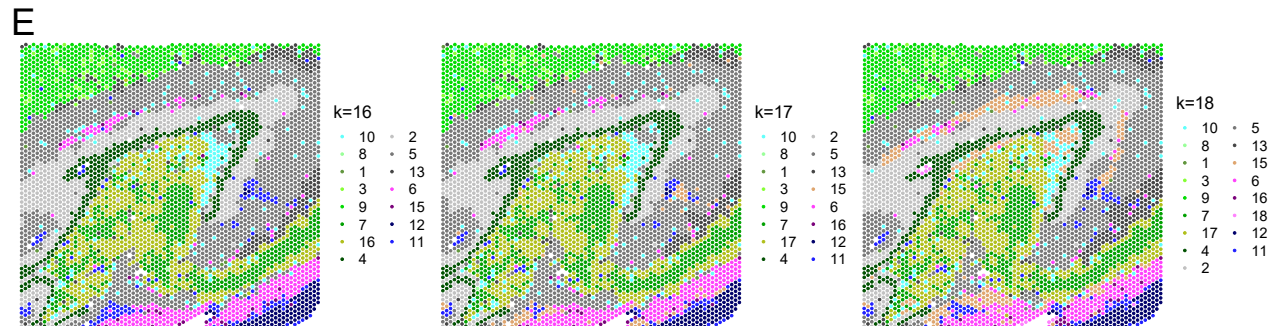
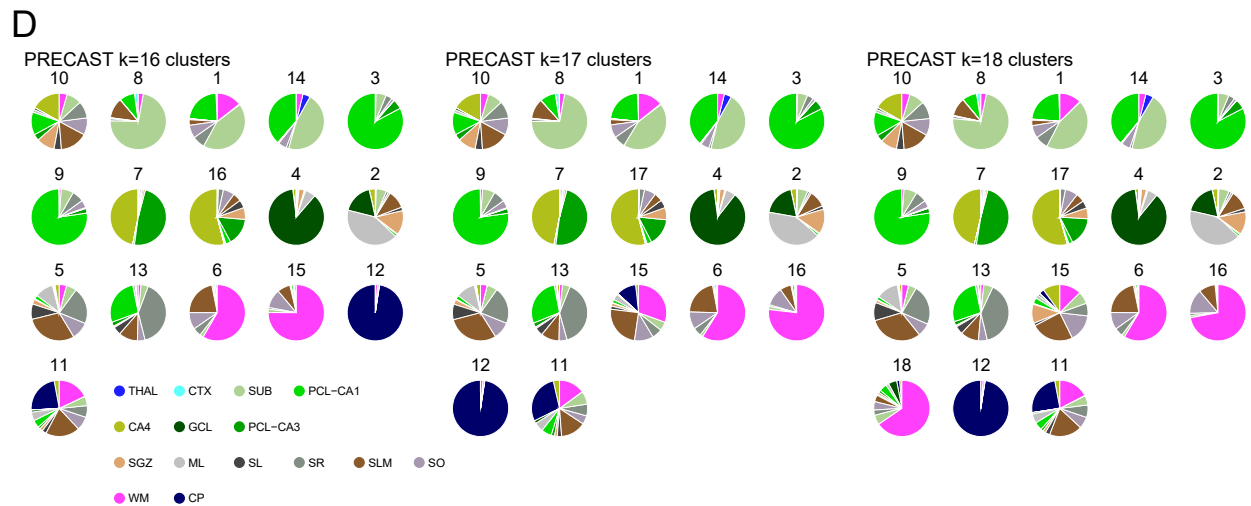
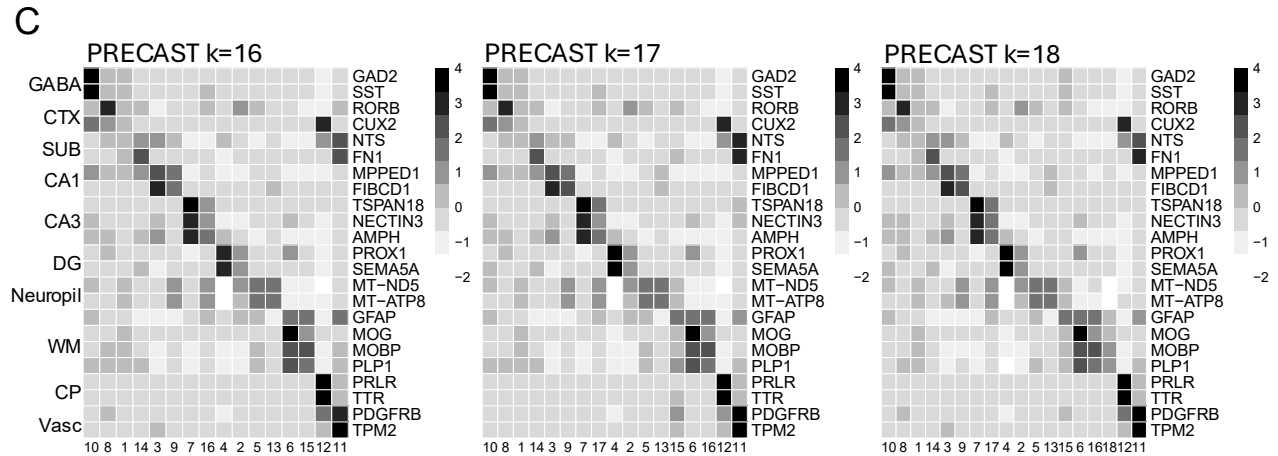
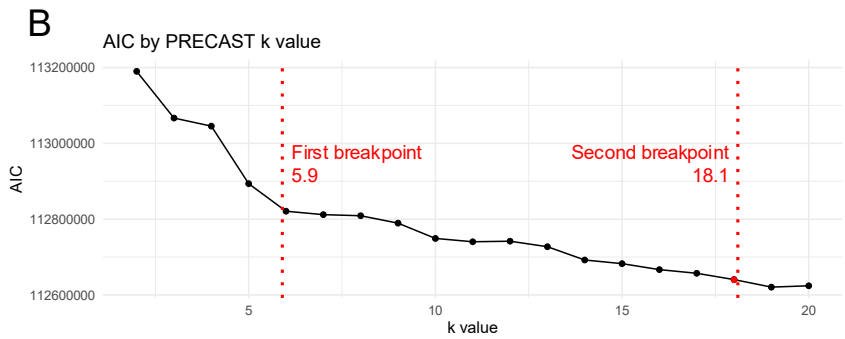
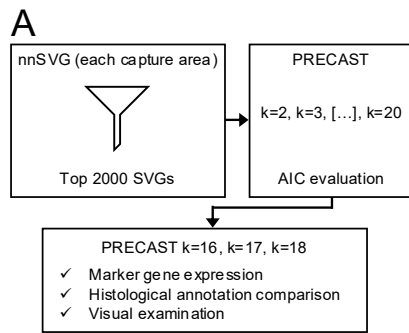
(A) Overview of feature selection with nnSVG and spatially-informed data-driven clustering with PRECAST., and the evaluation criteria of specific clustering results that led to the annotation of the final spatial domains used in this study.

(B) Plot showing Akaike Information Criterion (AIC, y-axis) by value of k (number of groups, x-axis) for $k=2$ through $k=20$. Dotted vertical lines mark breakpoints determined in segmented regression.

(C) Heatmaps for PRECAST clusters generated at (left) $k=16$, (middle) $k=17$, and (right) $k=18$ depict the expression ($\log_2$ normalized counts) for genes (rows) averaged across each cluster (columns). On the far left, rows are annotated by the HPC region for which they are a marker. The additional cluster gained at $k=17$ vs $k=16$ is outlined in orange and appears to be a neuropil cluster, which exhibits more refined marker gene expression at $k=18$ (dotted outline). The additional cluster gained at $k=18$ vs $k=17$ is outlined in pink and appears to be a WM cluster.

(D) Pie charts of PRECAST cluster (facets) colored by manually annotated HPC region for clusters generated at (left) $k=16$, (middle) $k=17$, and (right) $k=18$. The additional cluster gained at $k=17$ vs $k=16$ is outlined in orange and appears to be a neuropil cluster, which is maintained at $k=18$ (dotted outline). The additional cluster gained at $k=18$ vs $k=17$ is outlined in pink and appears to be a WM cluster. THAL: thalamus, CTX: cortex, SUB: subiculum, PCL: pyramidal cell layer, GCL: granule cell layer, SGZ: dentate gyrus, subgranular zone, ML: dentate gyrus molecular layer, SL: stratum lucidum, SR: stratum radiatum, SLM: stratum lacunosum-moleculare, SO: stratum oriens, WM: white matter, CP: choroid plexus. Manual annotation abbreviations are also presented in **Supplemental Table 2**.

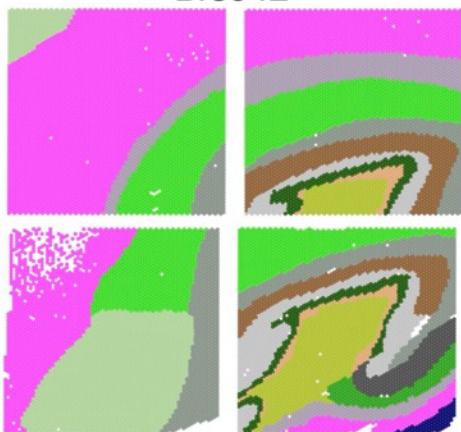
(E) Example spot plots (donor Br3942) of PRECAST clusters at (left) $k=16$, (middle) $k=17$, and (right) $k=18$ are colored by cluster. The new neuropil cluster at $k=17$ (tan) does not exhibit a spatial organization consistent with any known neuropil region, but the same cluster at $k=18$ exhibits a more stereotyped location to known neuropil-rich regions.



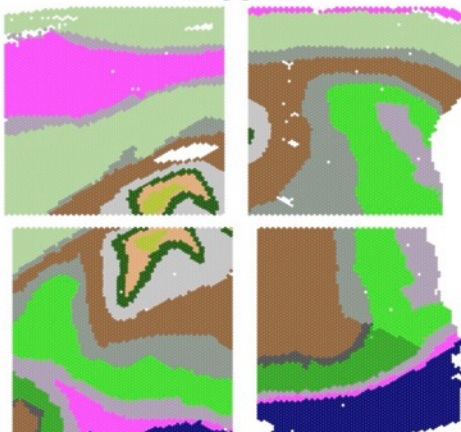
Supplemental Fig. 5. Histologically guided manual annotations of spatial domains.

Using the H&E histology and cytoarchitecture and canonical marker gene expression, each spot was manually annotated to a spatial domain across the $n=36$ Visium-H&E capture areas. Spot color indicates the manual annotation (legend in bottom right). Capture areas are grouped by donor number.

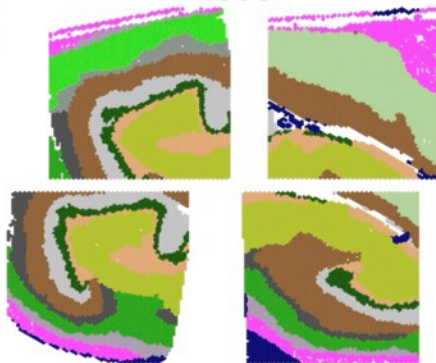
Br3942



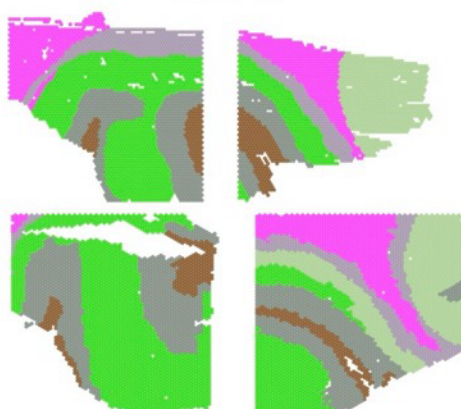
Br6522



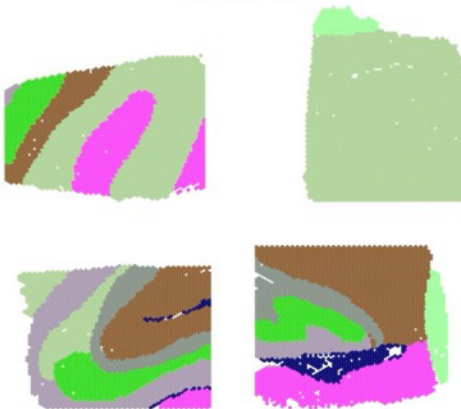
Br8667



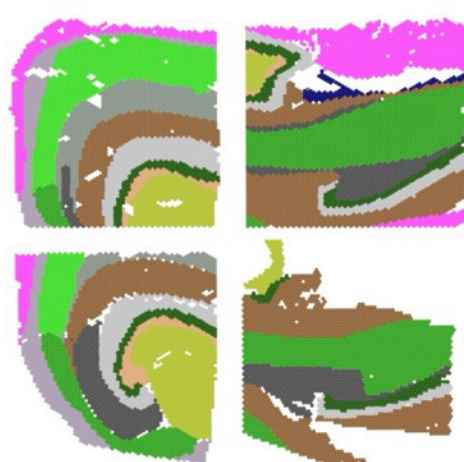
Br2743



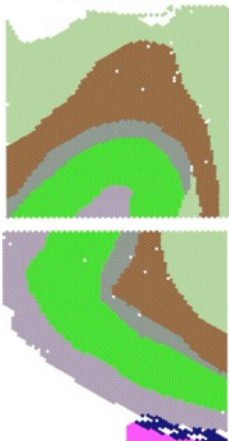
Br6423



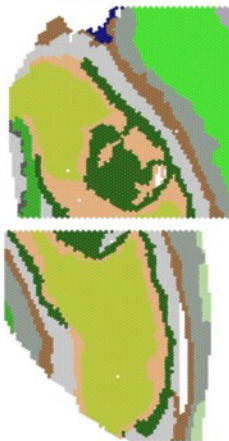
Br2720



Br6432



Br8492



Br8325



- GCL
- CA4
- PCL-CA3
- PCL-CA1
- SUB
- CTX
- THAL
- SL
- SO
- SR
- ML
- SLM
- SGZ
- WM
- CP

Br6471



Supplemental Fig. 6. PRECAST cluster annotation and spatial domain definitions.

(A) Heatmap showing expression of CA1 marker genes (*MPED1*, *FIBCD1*, *CMLP*), CA2 marker gene *RGS14*, CA3 marker genes (*TSPAN18*, *NECTIN3*, *AMPH*), and mossy cell/ hilus/ CA4 marker gene *CARTPT* across $k=18$ annotated PRECAST clusters. See **Supplemental Table 2** for abbreviations.

(B) Boxplots showing number of nuclei per spot and library size across CA2-4.1, CA2-4.2, CA1.1, and CA1.2. Outliers were removed prior to plotting.

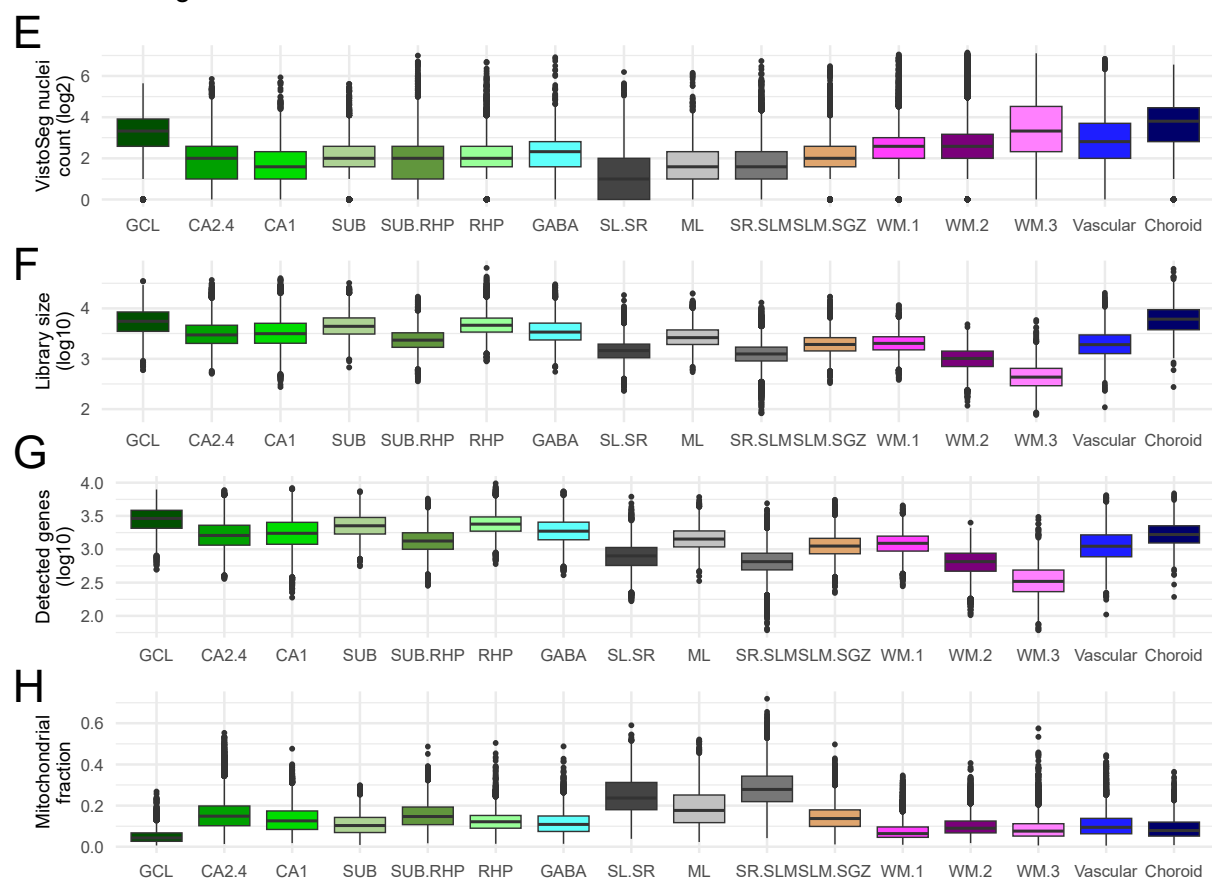
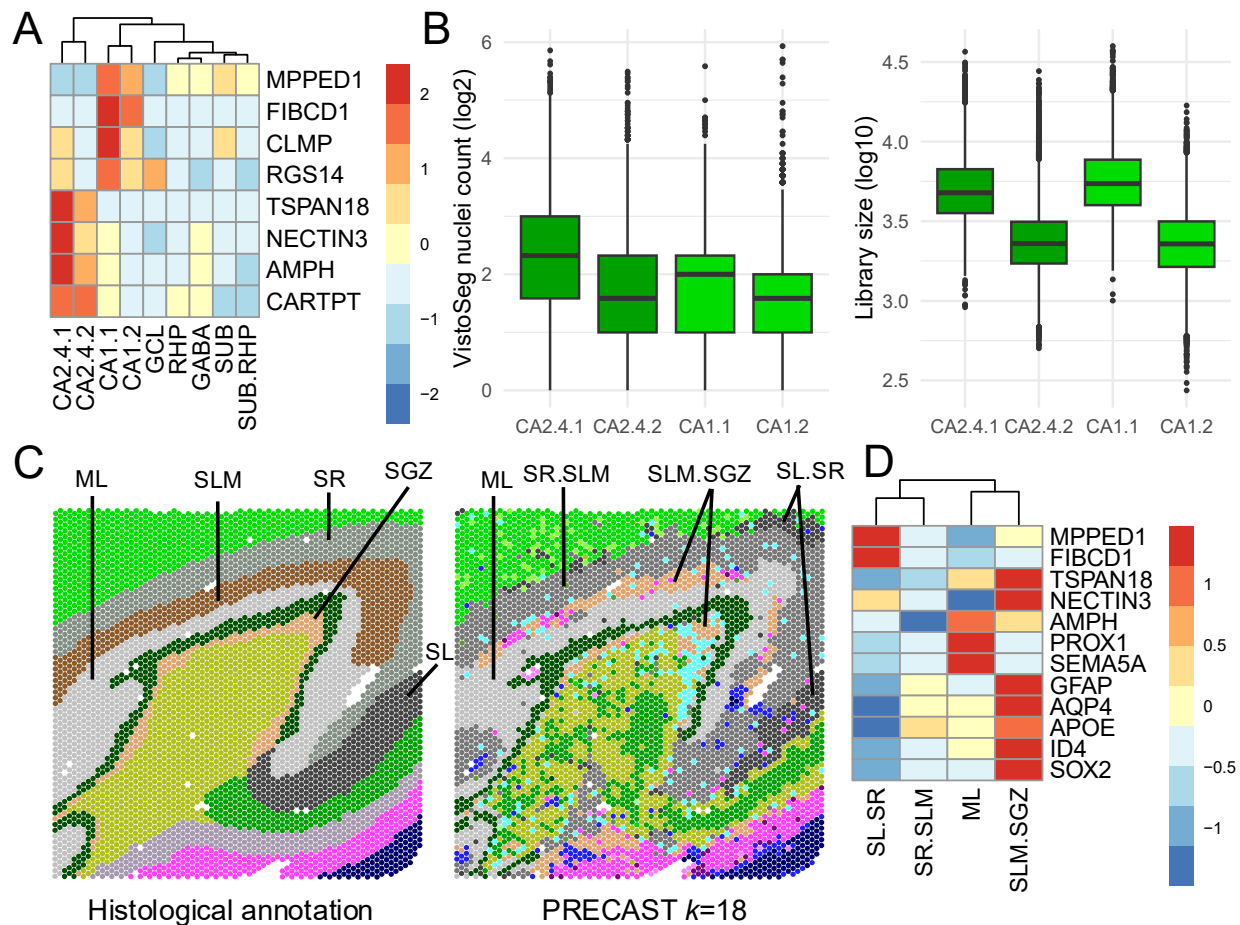
(C) Spot plot using example capture area from donor Br3942 highlighting manually annotated neuropil locations in comparison to annotated $k=18$ PRECAST clusters.

(D) Heatmap showing expression of HPC marker genes (CA1: *MPED1*, *FIBCD1*; CA3: *TSPAN18*, *NECTIN3*, *AMPH*; DG: *PROX1*, *SEMA5A*) and astrocyte-specific genes (*GFAP*, *AQP4*, *APOE*, *ID4*, *SOX2*) across neuropil spatial domains.

(E) Boxplots showing distribution of nuclei count per spot (y-axis) across final 16 spatial domains (x-axis).

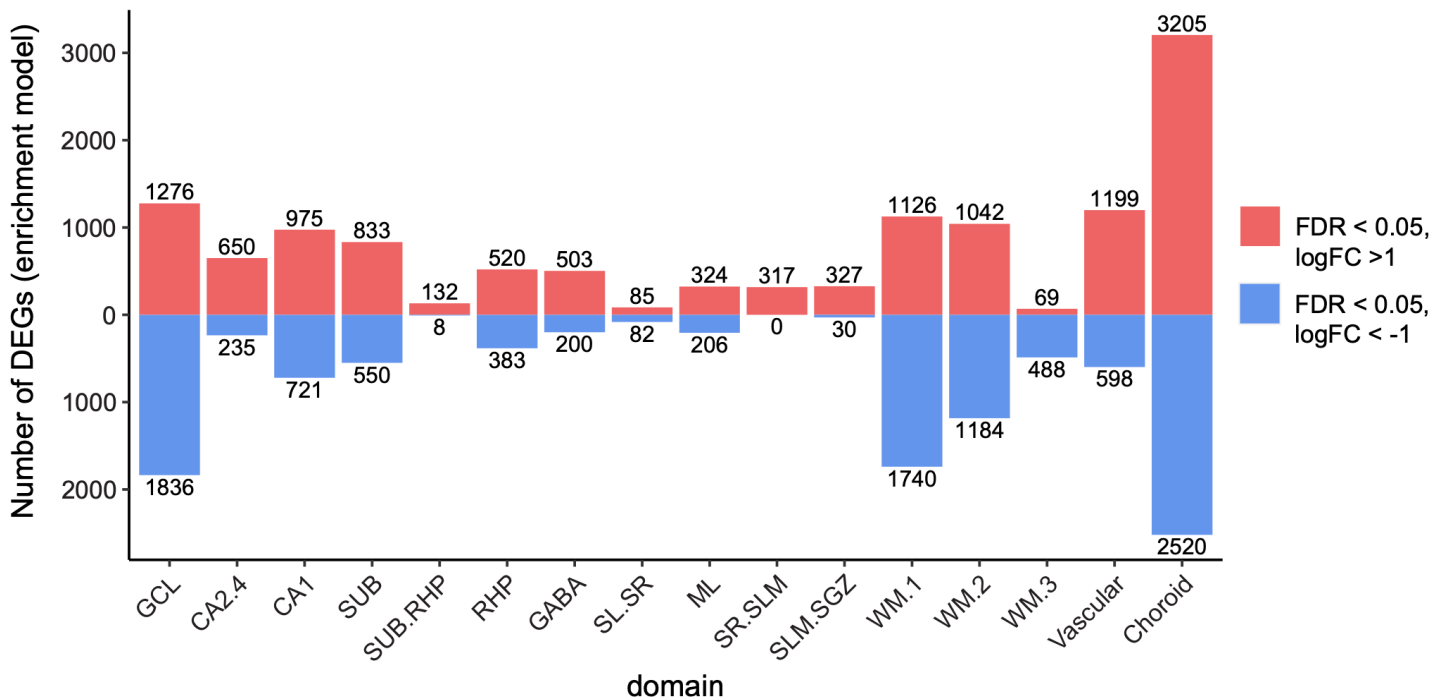
Violin plots across final spatial domains (x-axis) showing (F) library size (total number of UMI counts) per spot,

(G) number of detected genes per spot and (H) number of reads mapping to mitochondrial genes per spot.



Supplemental Fig. 7. Number of differentially expressed genes (DEGs) for each PRECAST spatial domain using enrichment model results.

Bar plot showing the number of DEGs (*y*-axis, FDR adjusted *p*-value < 0.01) in each domain (*x*-axis). Upregulated genes are shown in red (log₂ fold change > 1), while downregulated genes are shown in blue (log₂ fold change < -1). See **Supplemental Table 2** for abbreviations.



Supplemental Fig. 8. Quality control (QC) metrics used to filter out low-quality nuclei.

Boxplots of QC threshold (y-axis) calculated by three median absolute deviations (MAD) for the **(A)** maximum percent of reads originating from the mitochondrial genome (also known as mitochondrial fraction), **(B)** the minimum library size (total number of reads), and **(C)** the minimum number of genes with detected reads. Boxplots are grouped by sorting protocol (x-axis, propidium iodide (PI)+ or PI+NeuN+) and the individual values are the thresholds generated for each sample, colored by the round in which the sample was sequenced.

Boxplots of the number of nuclei excluded from each sample based on the raw 3MAD threshold for **(D)** maximum mitochondrial fraction, **(E)** minimum library size, and **(F)** minimum number of detected genes. Boxplots are grouped by sorting protocol (x-axis) and the individual values are the total number of nuclei removed from each sample based on the corresponding threshold in **A-C**, colored by the round in which the sample was sequenced.

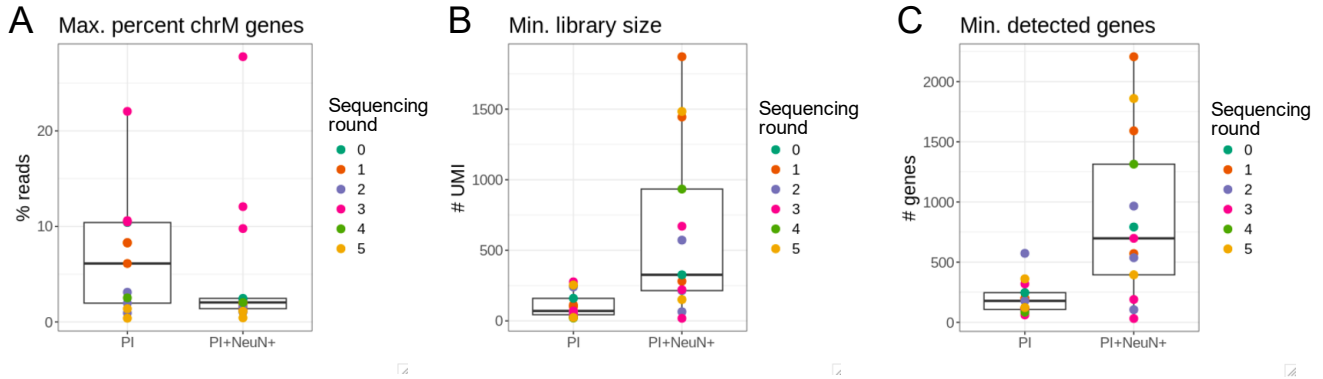
(G) Rather than calculating the 3MAD maximum mitochondrial fraction threshold on a per-sample basis, the threshold for mitochondrial fraction was re-calculated based on the 3MAD mitochondrial fraction of high quality samples that had an original mitochondrial fraction threshold of <5%. Boxplots depict the new maximum percentage of reads coming from the mitochondrial genome for each sample with the revised threshold derived from high quality samples. Boxplots are grouped by sorting protocol (x-axis) and the individual values are colored by the round in which the sample was sequenced.

(H) All samples with zero nuclei removed with the original 3MAD threshold for minimum library size were subject to a revised minimum library size threshold of 1000 reads. Boxplots depict the updated library size threshold for all samples. Boxplots are grouped by sorting protocol (x-axis) and the individual values are colored by the round in which the sample was sequenced.

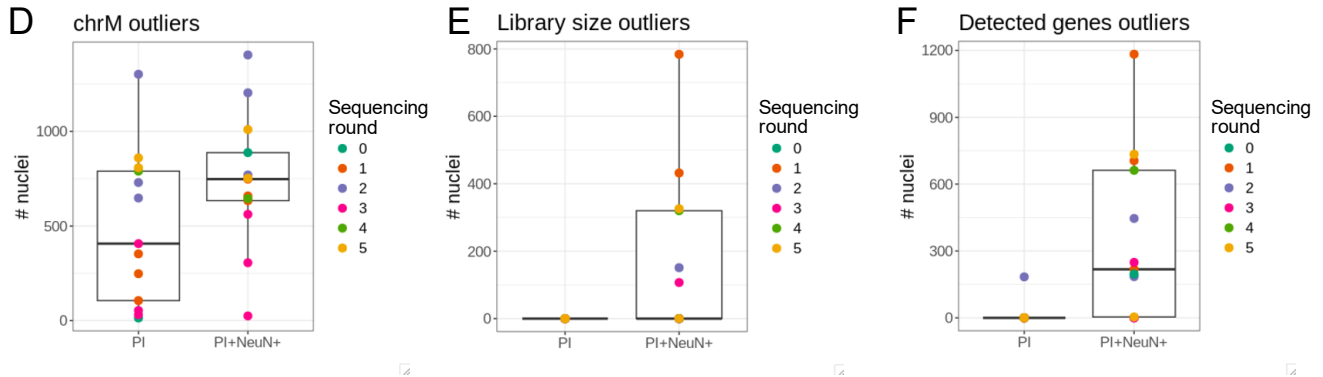
(I) All samples with zero nuclei removed with the original 3MAD threshold for minimum number of detected genes were subject to a revised minimum detected genes threshold of 1000 genes. Boxplots depict the updated detected genes threshold for all samples. Boxplots are grouped by sorting protocol (x-axis) and the individual values are colored by the round in which the sample was sequenced.

Boxplots of the number of nuclei excluded from each sample based on the revised 3MAD threshold for **(J)** maximum mitochondrial fraction, **(K)** minimum library size, and **(L)** minimum number of detected genes. Boxplots are grouped by sorting protocol (x-axis) and the individual values are the total number of nuclei removed from each sample based on the corresponding threshold in **G-I**, colored by the round in which the sample was sequenced.

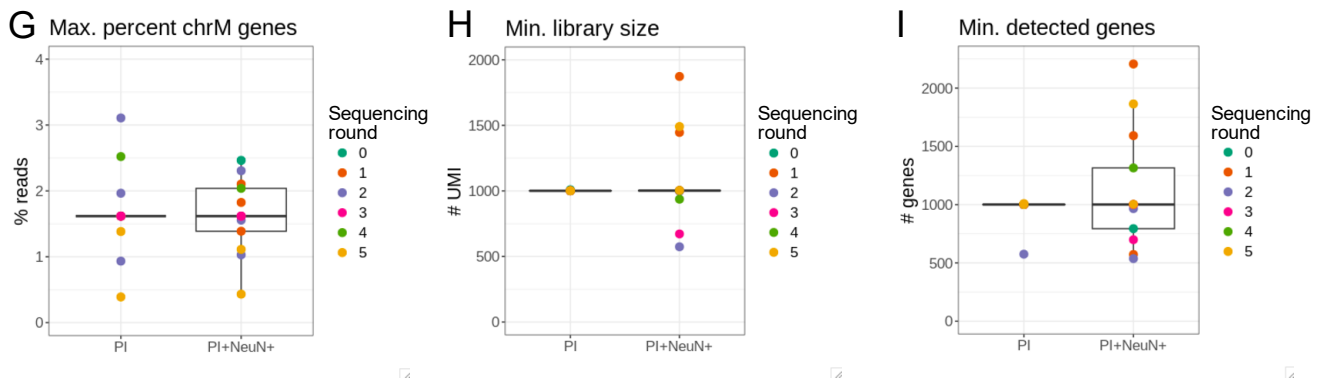
Original 3MAD QC thresholds (per-sample)



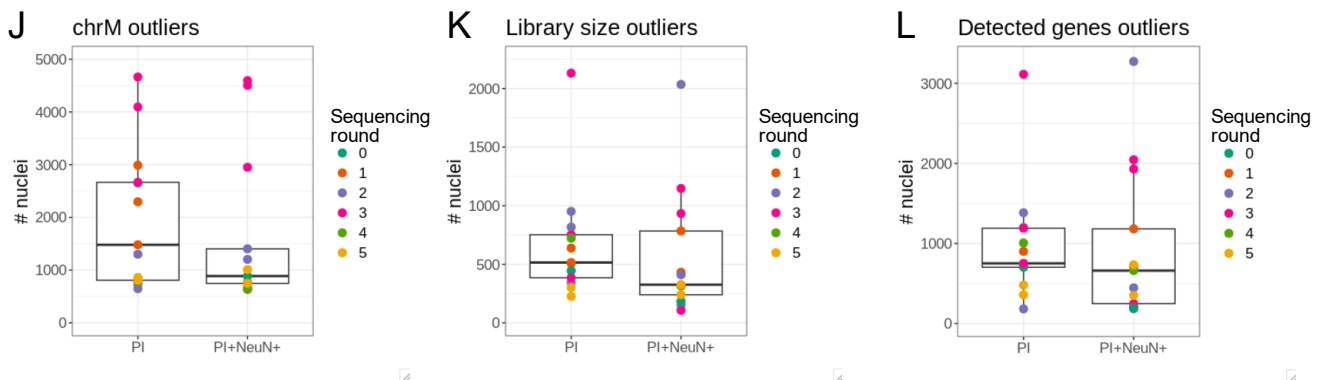
Nuclei excluded with original 3MAD thresholds



Revised 3MAD QC thresholds



Nuclei excluded with revised 3MAD thresholds



Supplemental Fig. 9. Summary of quality control (QC) metrics in discarded and retained nuclei.

(A) Violin plots show the distribution of mitochondrial fraction (y-axis; proportion of reads originating from mitochondrial genome) in populations of nuclei removed (left) or kept (right) based on QC thresholds, faceted by sorting protocol (propidium iodide (PI)+ samples (top) and PI+NeuN+ samples (bottom)). Each sample occupies a single position on the x-axis. The color of the violin plot indicates the round in which each sample was sequenced.

(B) Violin plots show the distribution of library size (y-axis; total number of reads) in populations of nuclei removed (left) or kept (right) based on QC thresholds, faceted by sorting protocol (PI+ samples (top) and PI+NeuN+ samples (bottom)). Each sample occupies a single position on the x-axis. The color of the violin plot indicates the round in which each sample was sequenced. The asterisk indicates sample 17c-scp for which the retained nuclei exhibit abnormally low library sizes.

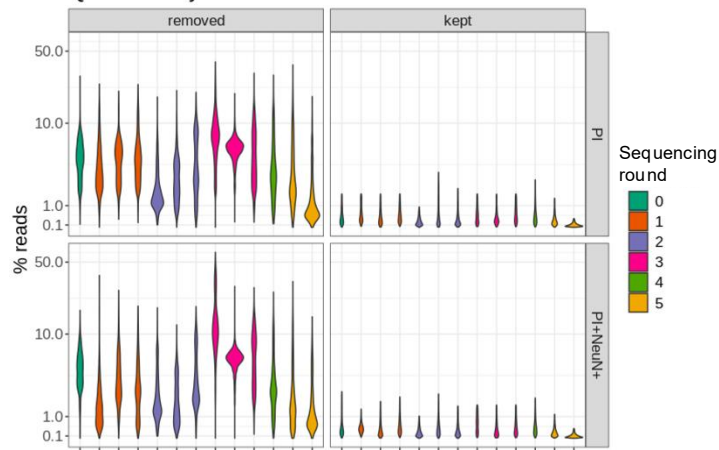
(C) Violin plots show the distribution of detected genes (y-axis) in populations of nuclei removed (left) or kept (right) based on QC thresholds, faceted by sorting protocol (PI+ samples (top) and PI+NeuN+ samples (bottom)). Each sample occupies a single position on the x-axis. The color of the violin plot indicates the round in which each sample was sequenced. The asterisk indicates sample 17c-scp for which the retained nuclei exhibit abnormally few detected genes.

(D) Temporary cell clusters were easily identified as neuronal or non-neuronal based on the proportion of nuclei within the cluster that possessed at least one read of *SYT1* gene (raw counts).

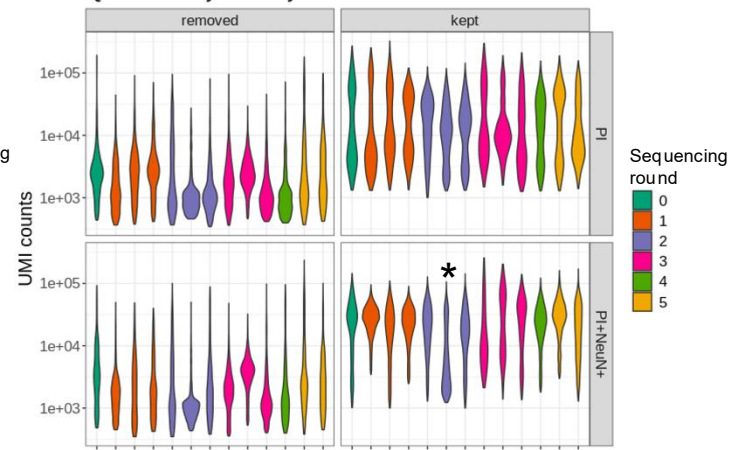
(E) Violin plots present the distribution of the number of detected reads in each temporary neuronal cell cluster (x-axis), colored and stratified by in the neuronal nuclei originated from sample 17c-scp (red) or any other sample (grey). The dotted line indicates 5000 detected genes, highlighting the disproportionate number of 17c-scp nuclei with fewer than 5000 detected genes across all temporary neuronal clusters.

(F) Bar plots show the number of nuclei (y-axis) that were removed (left) or kept (right) in the snRNA-seq dataset after all QC filters were applied. The plots are faceted by sorting protocol (PI+ samples (top) and PI+NeuN+ samples (bottom)) and the color of the bar indicated the round in which the sample was sequenced. Each sample occupies a single position on the x-axis, and the outlier purple bar is sample 17c-scp for which all nuclei with fewer than 5000 detected genes were removed.

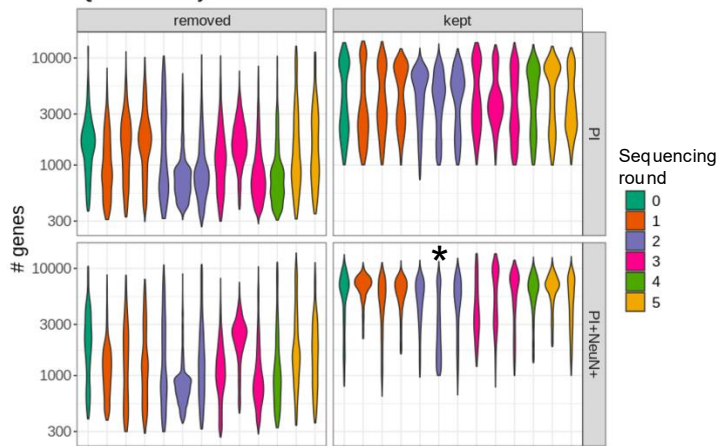
A QC summary: Mitochondrial fraction



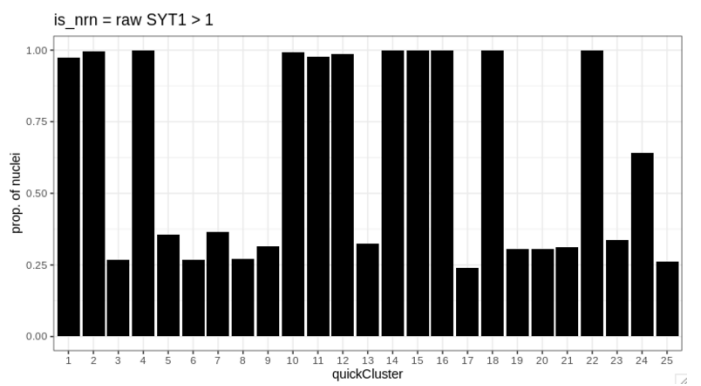
B QC summary: Library size



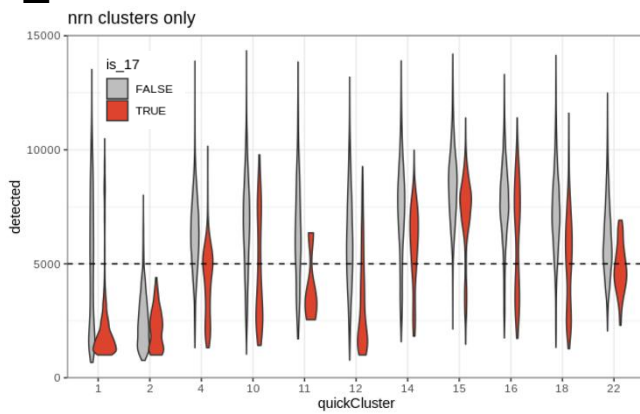
C QC summary: Genes detected



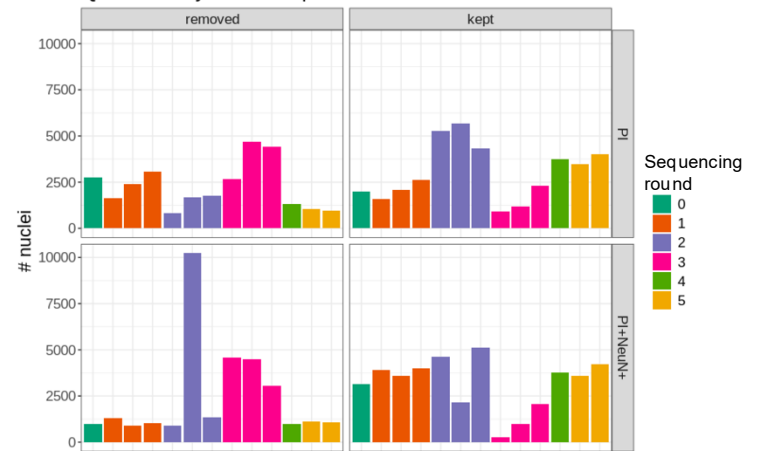
D



E



F QC summary: nuclei kept



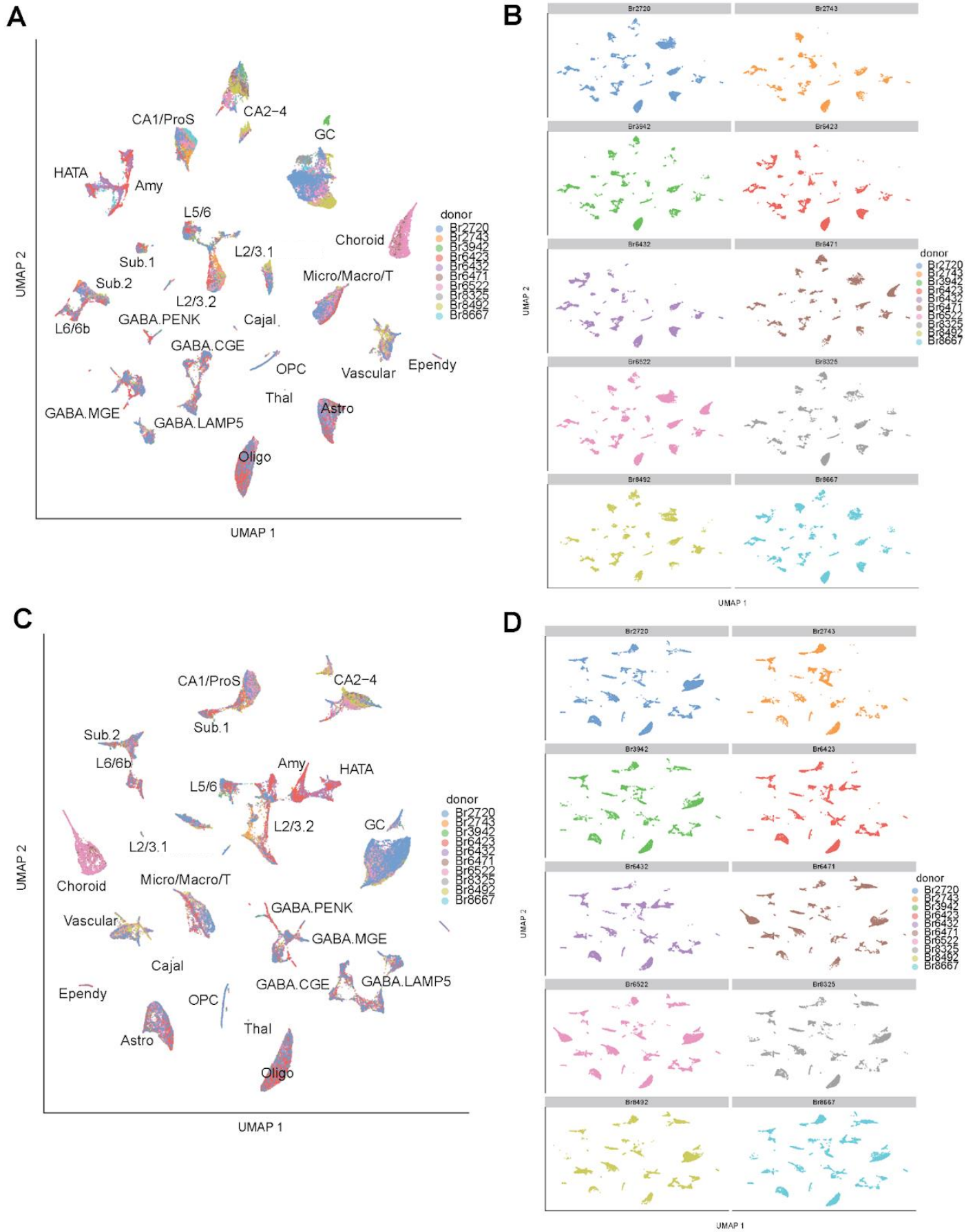
Supplemental Fig. 10. Batch correction for the hippocampus (HPC) single-nucleus RNA-sequencing (snRNA-seq) data.

(A) Uniform manifold approximation (UMAP) representation of snRNA-seq data before mutual nearest neighbors (MNN) batch correction. Points are colored by donor and labeled by cell types. See **Supplemental Table 2** for abbreviations.

(B) UMAP representation of snRNA-seq data before MNN batch correction. Each facet shows all nuclei from a given donor. Points are colored by donor.

(C) UMAP representation of snRNA-seq data after MNN batch correction. Points are colored by donor and labeled by cell types.

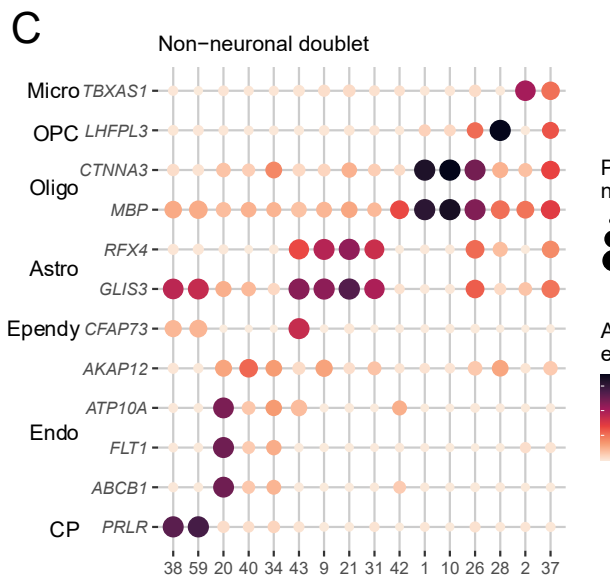
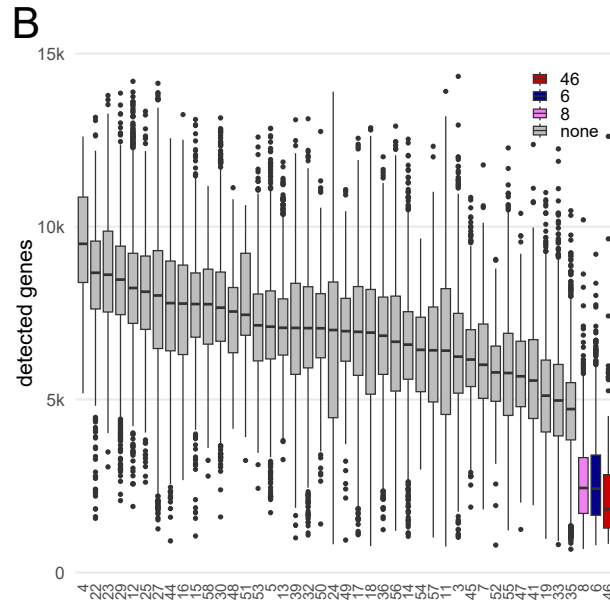
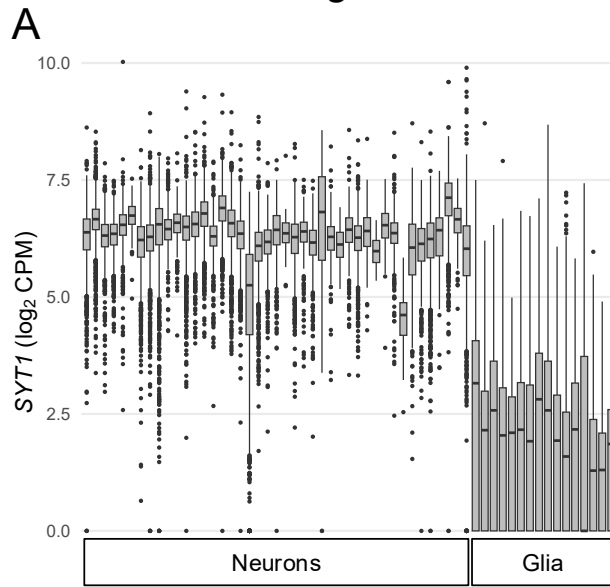
(D) UMAP representation of snRNA-seq data after MNN batch correction. Each facet shows all nuclei from a given donor. Points are colored by donor.



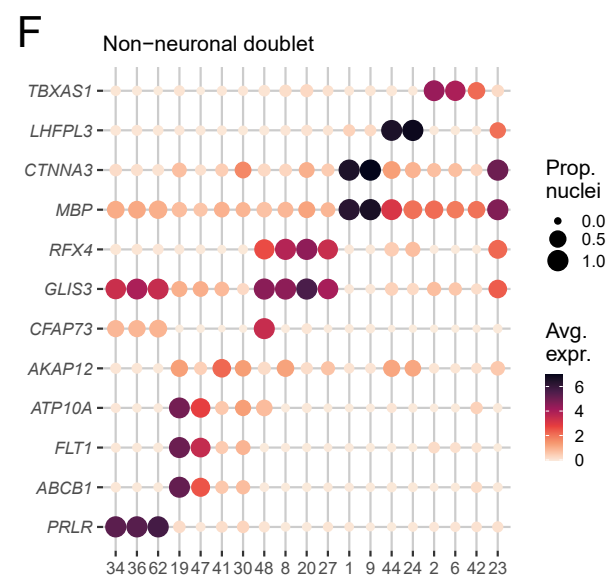
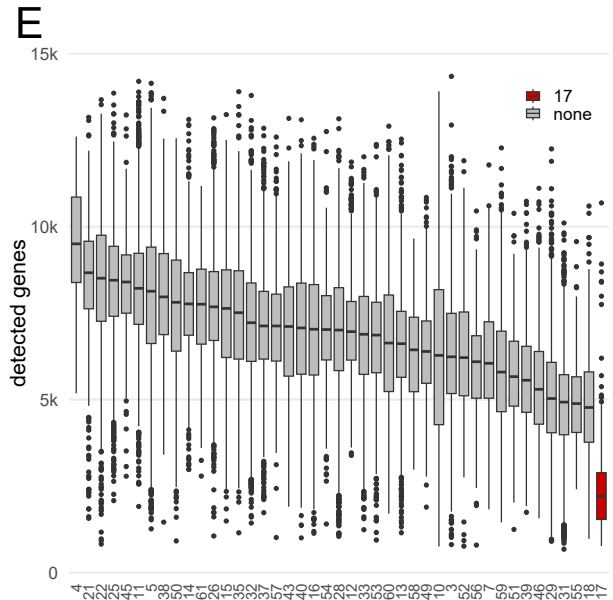
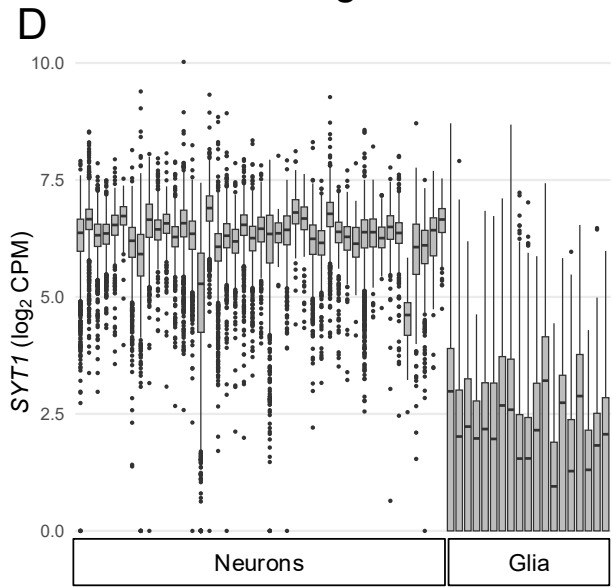
Supplemental Fig. 11. Determination of $n=60$ clusters used for snRNA-seq dataset

- (A) Boxplots depict the $\log_2$ normalized counts of *SYT1* (y-axis) in the initial Louvain clusters (x-axis) clearly segregating neuronal clusters from non-neuronal clusters.
- (B) Boxplots depict the number of detected genes (y-axis) in the initial Louvain neuronal clusters (x-axis), with boxplot color indicating three clusters that exhibited abnormally few detected genes and were therefore removed prior to re-clustering.
- (C) A dot plot displaying the proportion of nuclei (size) and the average $\log_2$ normalized counts within those nuclei (color) of select glial marker genes (y-axis) across the initial non-neuronal clusters (x-axis). The asterisk indicates a single cluster that equally expressed markers from distinct classes of glial cells and was therefore removed prior to re-clustering. See **Supplemental Table 2** for abbreviations.
- (D) Boxplots depict the $\log_2$ normalized counts of *SYT1* (y-axis) in the final Louvain clusters (x-axis) clearly segregating neuronal clusters from non-neuronal clusters.
- (E) Boxplots depict the number of detected genes (y-axis) in the final Louvain neuronal clusters (x-axis), with boxplot color indicating one cluster that exhibited abnormally few detected genes and was therefore removed from the final dataset.
- (F) A dot plot displaying the proportion of nuclei (size) and the average $\log_2$ normalized counts within those nuclei (color) of select glial marker genes (y-axis) across the final non-neuronal clusters (x-axis). The asterisk indicates a single cluster that equally expressed markers from distinct classes of glial cells and was therefore removed from the final dataset.

Initial clustering



Final clustering

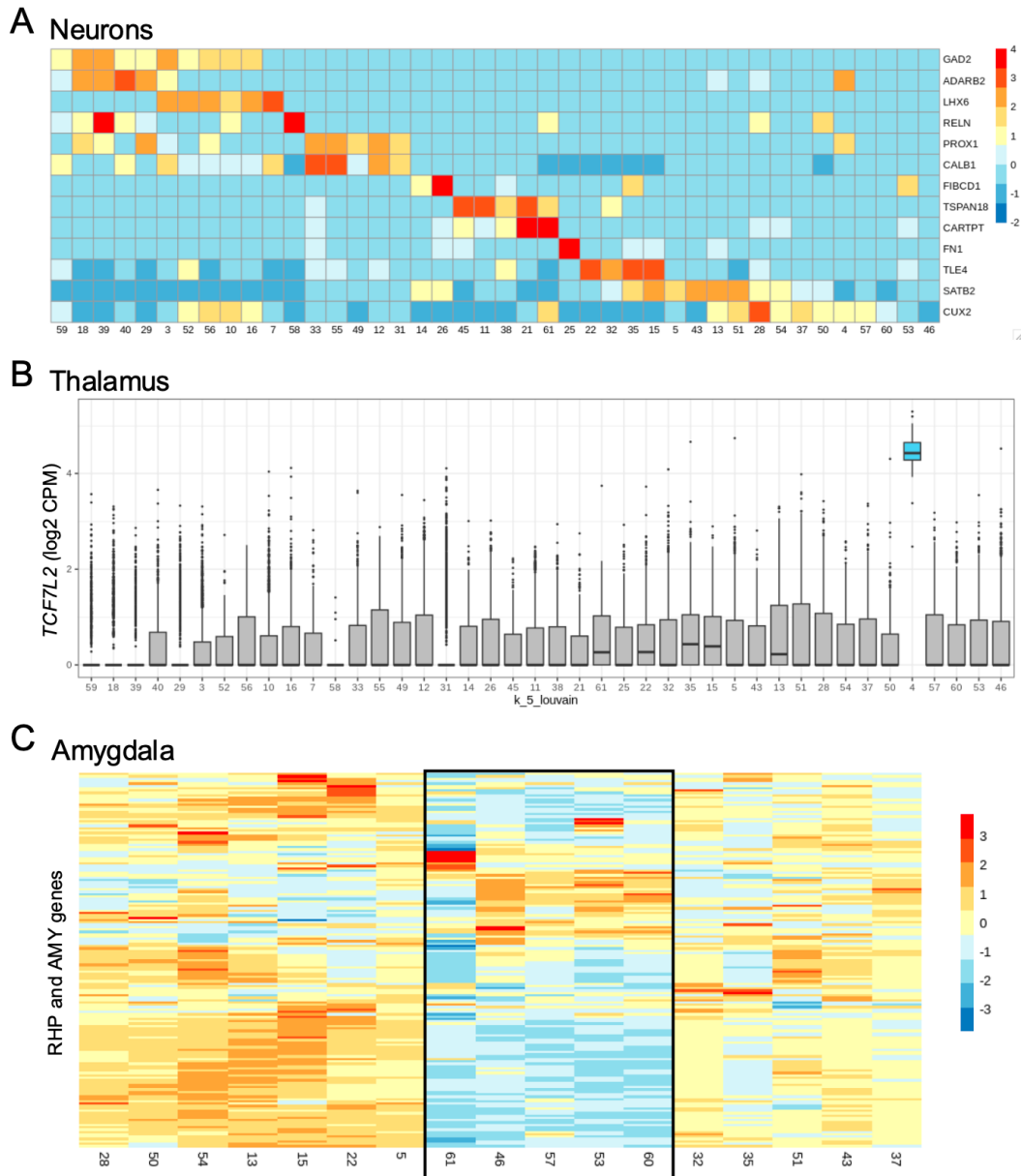


Supplemental Fig. 12. Preliminary annotation of neuronal nuclei.

(A) Heatmap depicting cluster average $\log_2$ normalized counts (color, scaled and centered) of known marker genes for inhibitory neurons (*GAD2*, *ADARB2*, *LHX6*) and Cajal Retzius cells (*RELN*) readily distinguish several clusters from the remaining excitatory neuron clusters. Excitatory neurons clusters were identified as hippocampal/ subiculum neurons (*PROX1*, *CALB1*, *FNDC1*, *TSPAN18*, *CARTPT*, *FN1*), with most of the remaining excitatory neuron clusters likely belong to laminar structures of the greater retrohippocampal formation based on the expression of cortical layer markers *TLE4*, *SATB2*, and *CUX2*. Few clusters did not express any of these markers in abundance.

(B) Boxplots showing average $\log_2$ normalized counts of *TCF7L2* (y-axis) clearly identifies cluster 4 as a cluster of thalamic neurons.

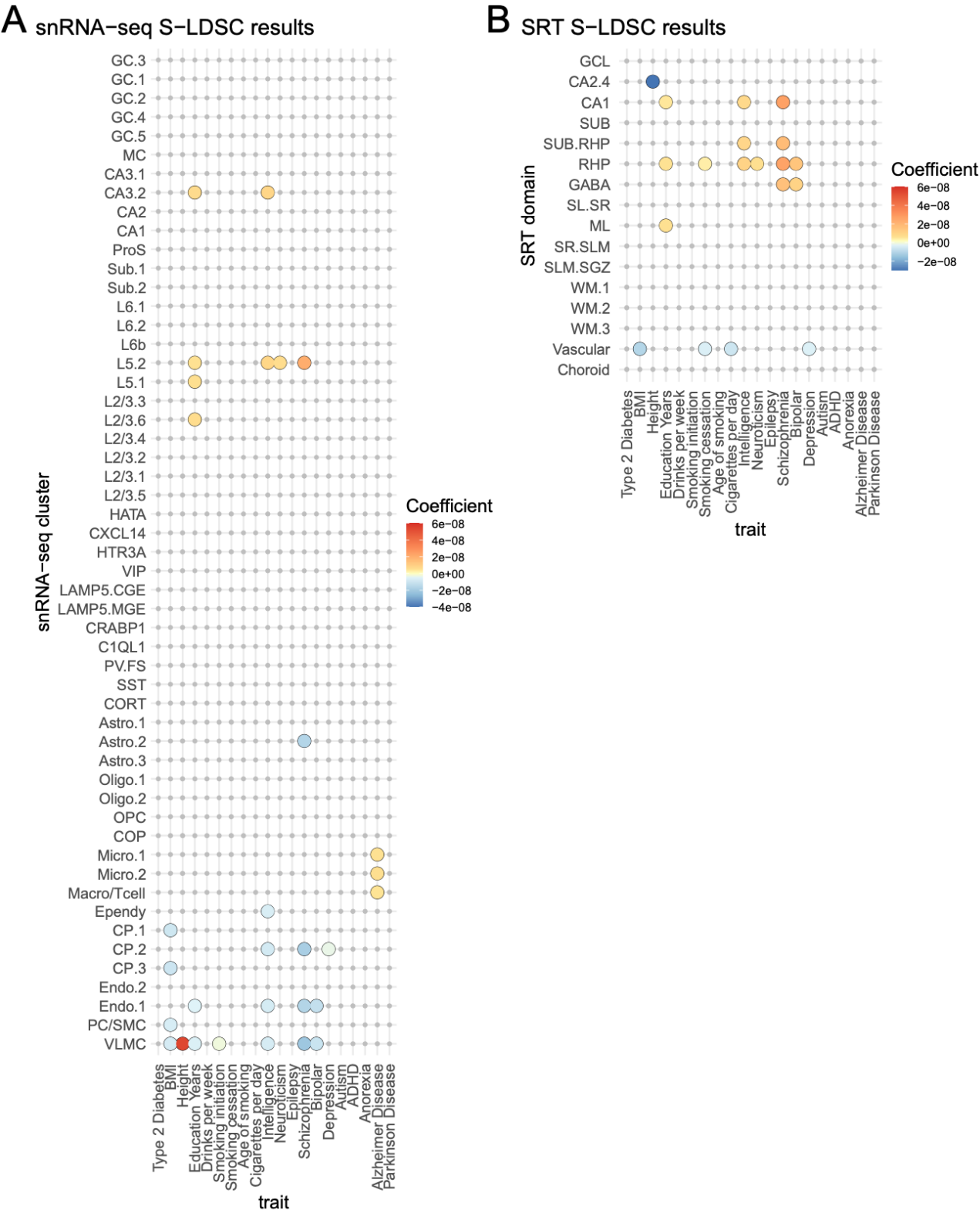
(C) Heatmap shows the average $\log_2$ normalized counts (color, scaled and centered) for genes (rows) found to be differentially expressed in amygdalar SRT spots compared to retrohippocampal (RHP) spots. Five of the excitatory neuron clusters in (A) exhibited increased expression of the amygdala genes and decreased expression of the RHP genes. These clusters (boxed) were subsequently annotated to the amygdala.



Supplemental Fig. 13. Linkage disequilibrium score regression identifies differential disease risk heritability across cell types and spatial domains.

(A) Dotplot showing significant (dot size, small= adj. $p>.05$, larger= adj. $p<.05$) linkage disequilibrium score regression (LDSC) coefficients (dot color, gray= adj. $p>.05$) for different polygenic traits (y-axis) across snRNA-seq cell types (x-axis).

(B) Same as **(A)**, but for SRT spatial domains (x-axis).



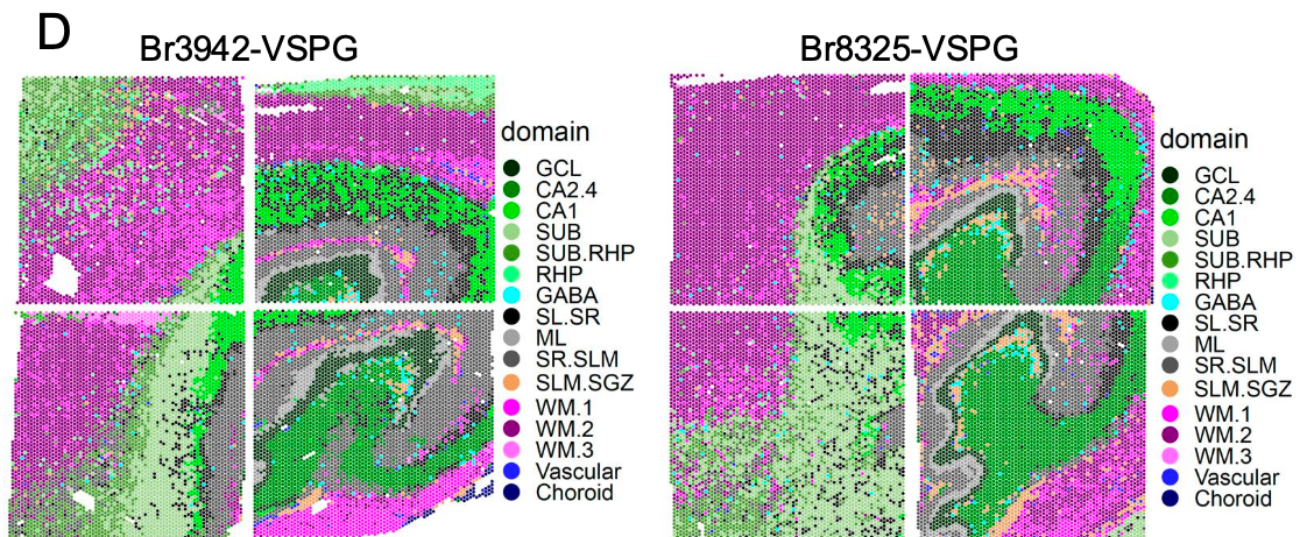
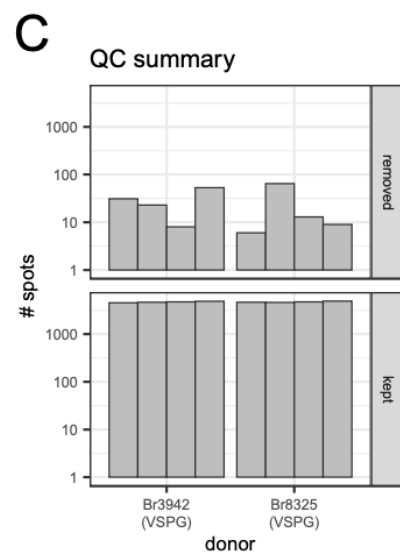
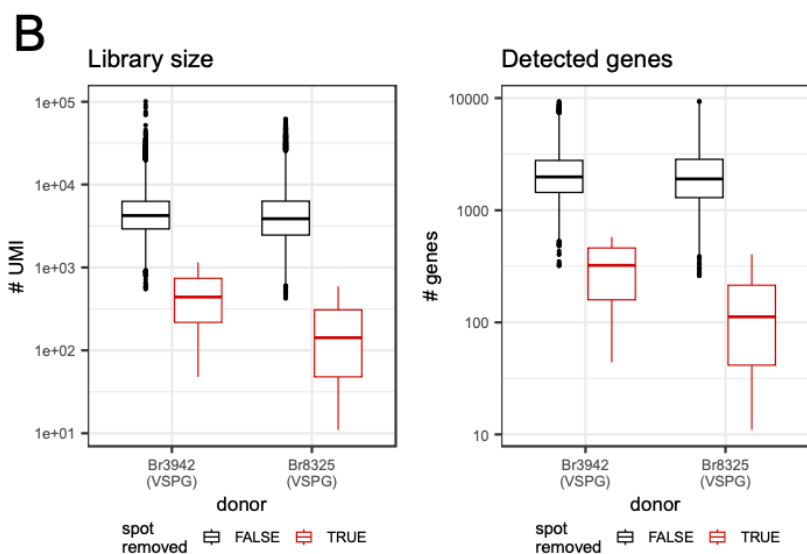
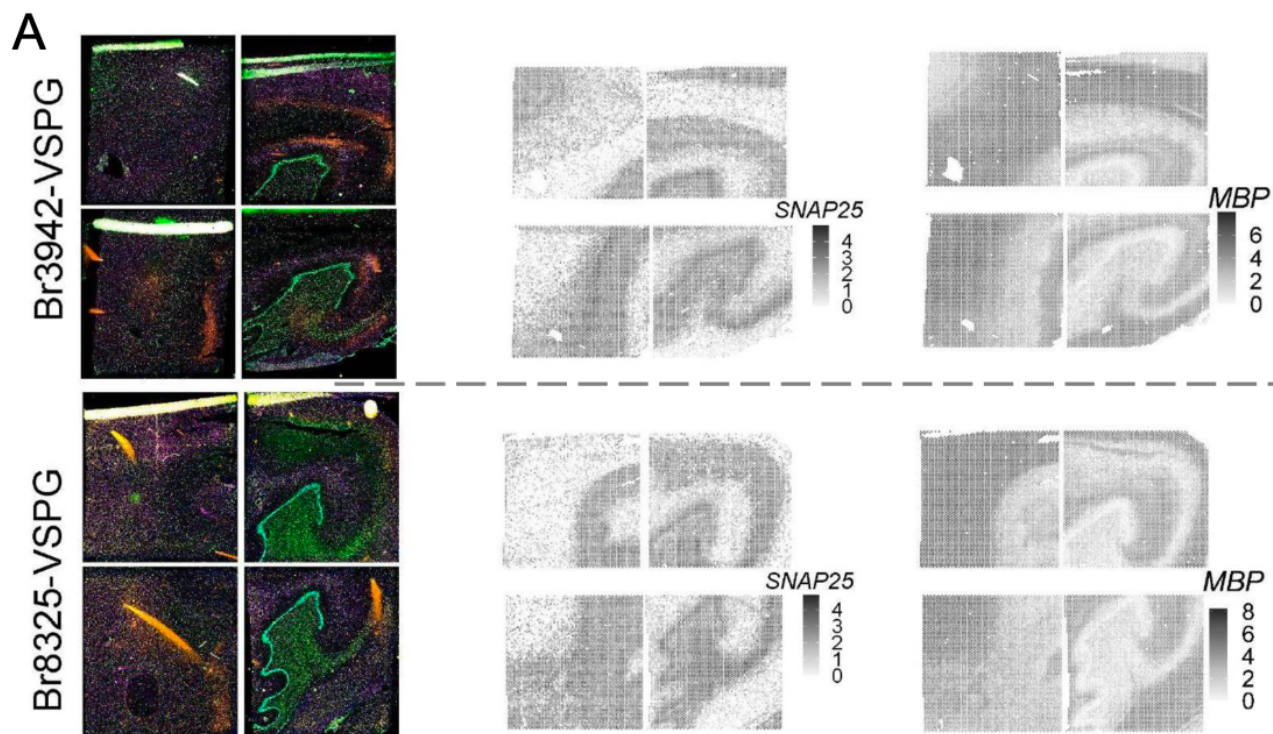
Supplemental Fig. 14. Visium Spatial Proteogenomics (Visium-SPG) quality control and spatial domain label transfer.

(A) Left: Merged immunofluorescence (IF) images from Visium Spatial Proteogenomics (SPG) data from donors Br3942 and Br8325. IF images represented DAPI (blue, all nuclei), NeuN (green, neuronal nuclei), TMEM119 (yellow, microglia), GFAP (red, astrocytes), and OLIG2 (magenta, oligodendrocytes). For each donor, spot plots of $\log_2$ transformed normalized counts for (middle) *SNAP25* and (right) *MBP*.

(B) Boxplots of library size (left: y -axis, total number of reads in a single spot) and detected genes (right: y -axis, total number of genes with at least one read in a single spot). The spots are grouped by donor (x -axis) and are stratified by whether the spots were kept (black) or removed (red) based on three median absolute deviation (3MAD) threshold calculated for each capture area independently.

(C) Bar plots demonstrate the number of spots (y -axis, log scale) per sample (individual bar) for each donor (x -axis). The plot is faceted by whether the spots were removed based on QC filter (top) or kept in the final dataset (bottom).

(D) Spot plots of Visium-SPG capture areas colored by spatial domain. Spatial domain labels annotated in the SRT dataset were projected to Visium-SPG dataset using `RcppML`.



Supplemental Fig. 15. Non-negative matrix factorization (NMF) identifies cell type-specific transcriptional patterns.

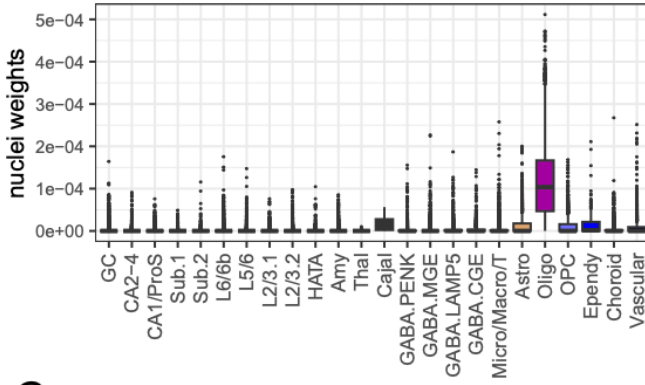
Boxplots of nuclei-level weights (y-axis) grouped by snRNA-seq fine cell classes (x-axis) for (A) oligodendrocyte pattern nmf77 and (B) by astrocyte pattern nmf81.

Boxplots of spot-level weights (y-axis) grouped by spatial domain (x-axis) for (C) oligodendrocyte pattern nmf77 and (D) by astrocyte pattern nmf81.

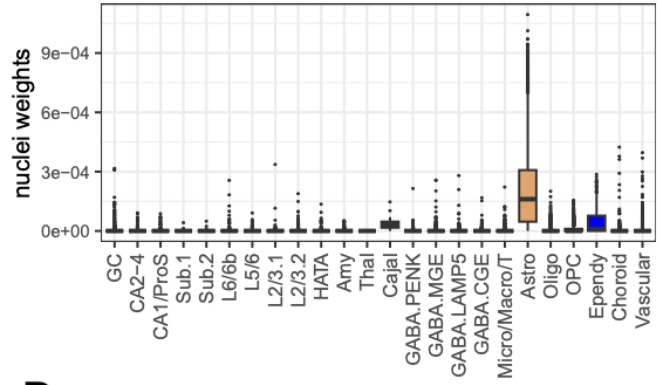
Boxplots of R_{CTD} -computed spot-level probability (y-axis) grouped by spatial domain (x-axis) for (E) oligodendrocyte cell identity and (F) by astrocyte cell identity.

Boxplots of spot-level weights (y-axis) grouped by manual annotation (x-axis) for (G) oligodendrocyte pattern nmf77 and (H) by astrocyte pattern nmf81.

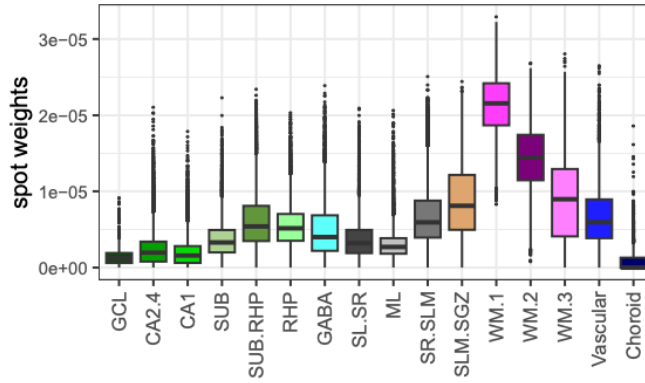
A Oligodendrocytes (nmf77)
snRNA-seq (source dataset)



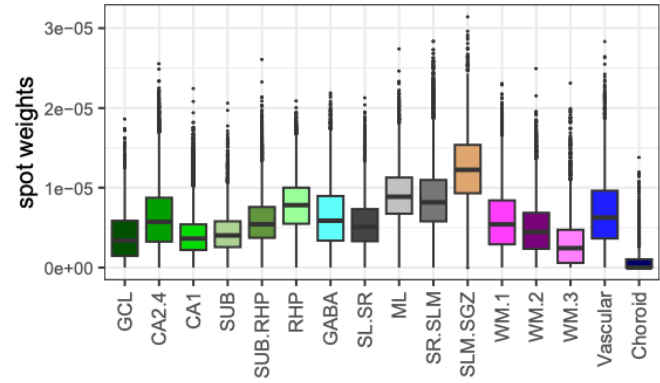
B Astrocytes (nmf81)
snRNA-seq (source dataset)



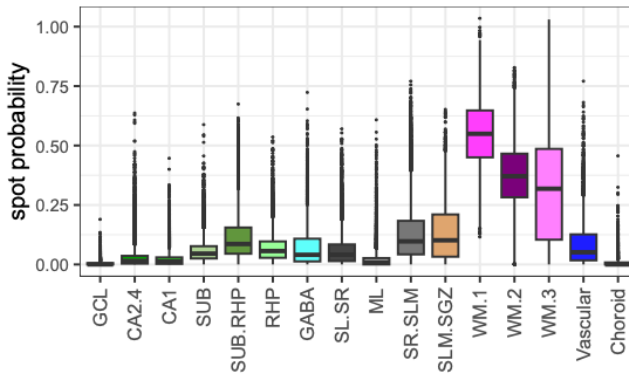
C Oligodendrocytes (nmf77)
Spatial domain (target dataset)



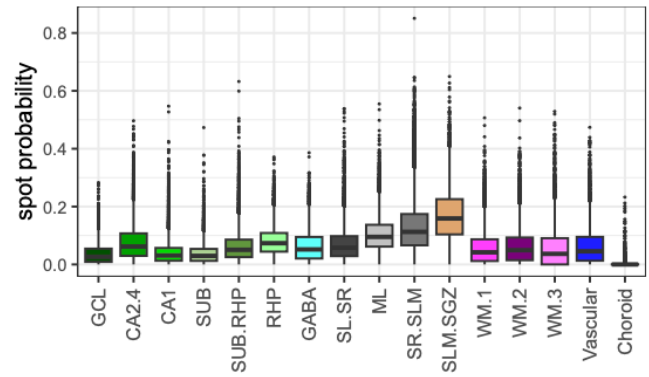
D Astrocytes (nmf81)
Spatial domain (target dataset)



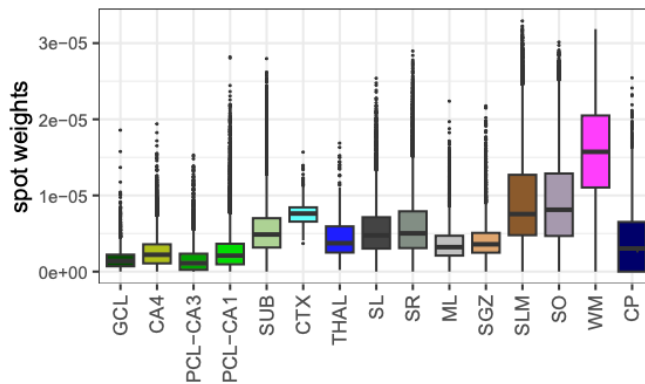
E Oligodendrocytes (RCTD)
Spatial domain



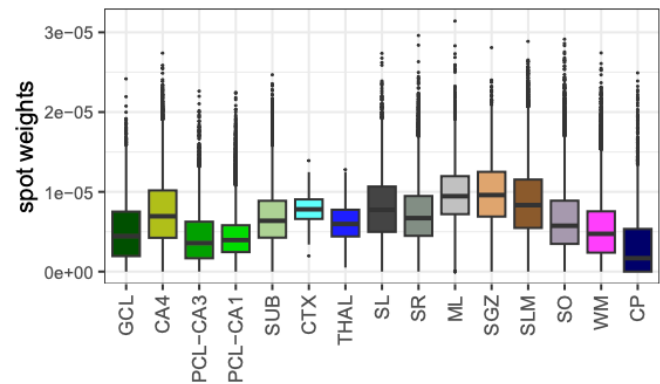
F Astrocytes (RCTD)
Spatial domain



G Oligodendrocytes (nmf77)
Manual annotation (target dataset)



H Astrocytes (nmf81)
Manual annotation (target dataset)



Supplemental Fig. 16. Non-negative matrix factorization (NMF) identifies trans-neuronal transcriptional patterns.

Boxplots of nuclei-level weights (*y*-axis) grouped by snRNA-seq fine cell classes (*x*-axis) for NMF patterns highlighting **(A)** excitatory postsynaptic specializations and **(B)** inhibitory postsynaptic specializations.

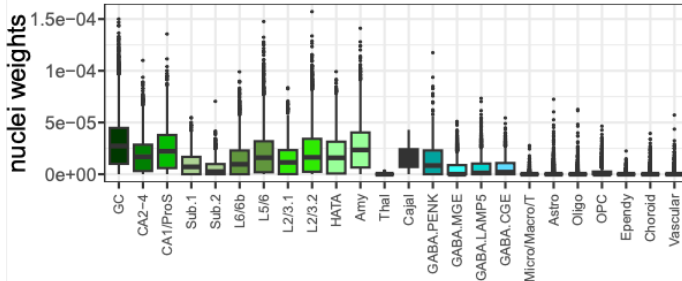
Boxplots of spot-level weights (*y*-axis) grouped by spatial domain (*x*-axis) for NMF patterns highlighting **(C)** excitatory postsynaptic specializations and **(D)** inhibitory postsynaptic specializations.

Boxplots of spot-level weights (*y*-axis) grouped by manual annotation (*x*-axis) for NMF patterns highlighting **(E)** excitatory postsynaptic specializations and **(F)** inhibitory postsynaptic specializations.

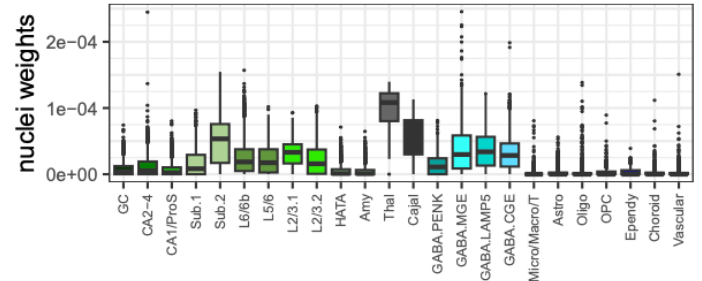
(G) Scatter plots of nuclei-level weights for nmf13 (*x*-axis) and nmf7 (*y*-axis) faceted by snRNA-seq fine cell classes.

(H) Scatter plots of spot-level weights for nmf13 (*x*-axis) and nmf7 (*y*-axis) faceted by SRT spatial domain.

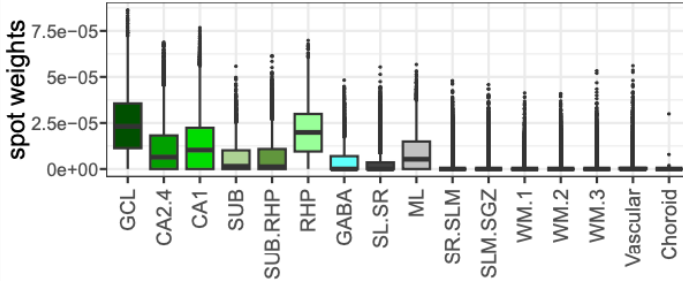
A Excitatory postsynaptic (nmf13)
snRNA-seq (source dataset)



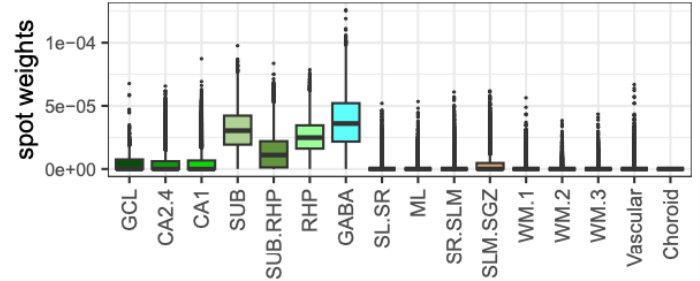
B Inhibitory postsynaptic (nmf7)
snRNA-seq (source dataset)



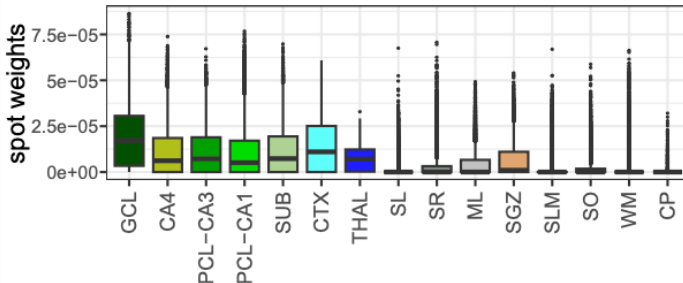
C Excitatory postsynaptic (nmf13)
Spatial domain (target dataset)



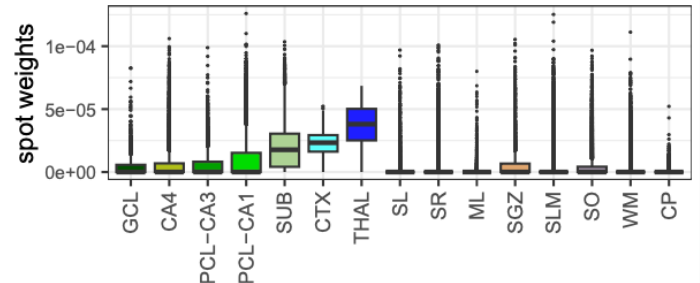
D Inhibitory postsynaptic (nmf7)
Spatial domain (target dataset)



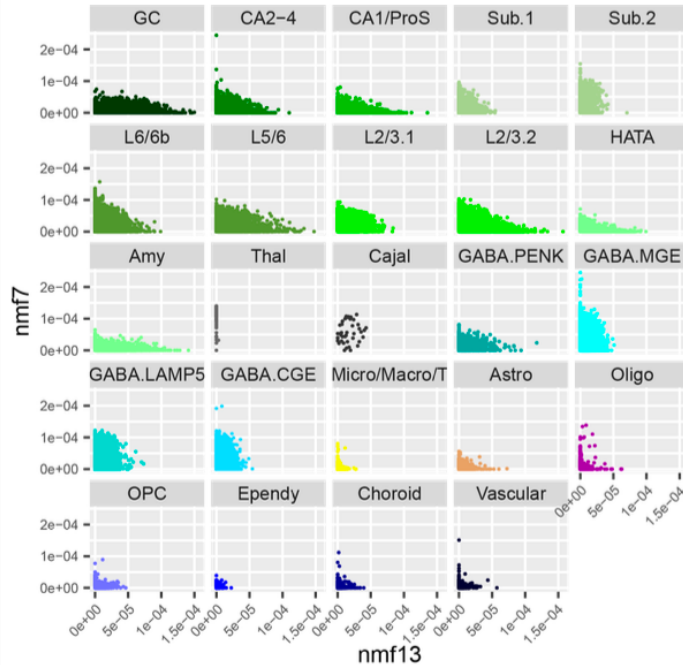
E Excitatory postsynaptic (nmf13)
Manual annotation (target dataset)



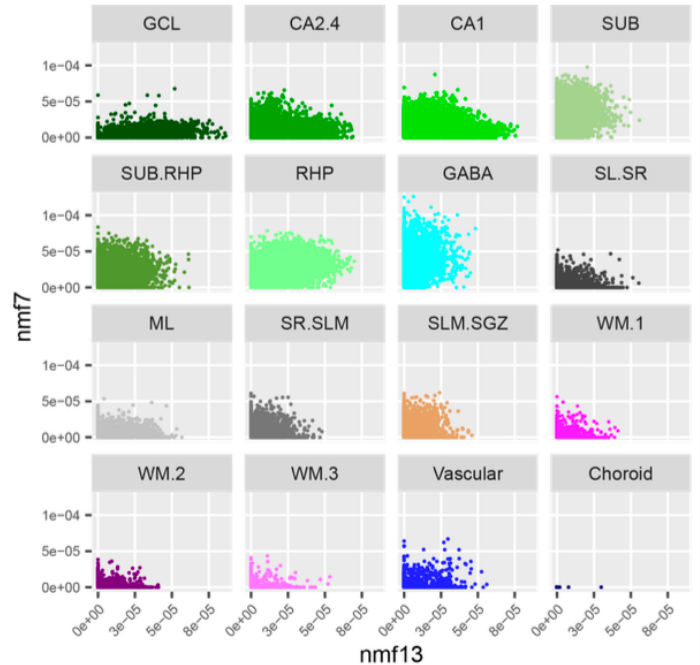
F Inhibitory postsynaptic (nmf7)
Manual annotation (target dataset)



G Co-occurrence of nmf13 and nmf7
snRNA-seq (source dataset)



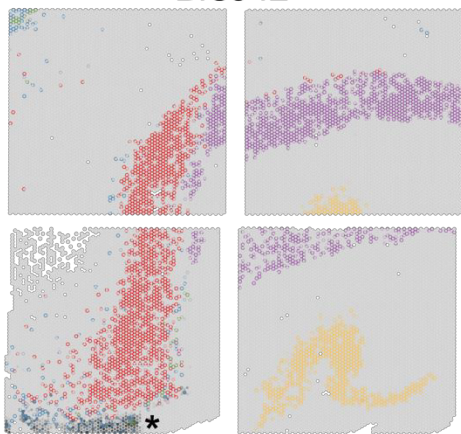
H Co-occurrence of nmf13 and nmf7
Spatial domain (target dataset)



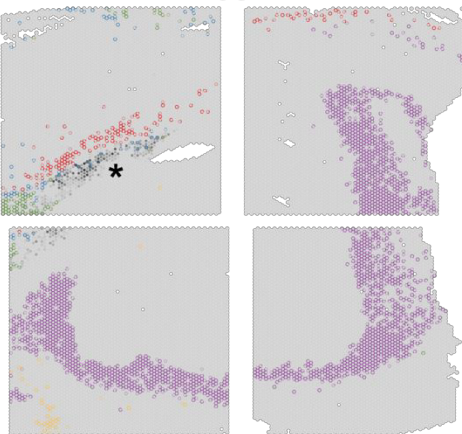
Supplemental Fig. 17. Spatial organization of nmf17 and identification of the presubiculum.

Spot outlines are colored by the combined annotation obtained by thresholding spatially-restricted and cell type-specific NMF patterns (legend lower right). Spot fill color indicates nmf17 weight (color bar lower right). The region believed to correspond to the presubiculum is indicated by an asterisk. Capture areas are grouped by donor number. ENT_L5: entorhinal cortex layer 5, ENT_sup: superficial entorhinal cortex.

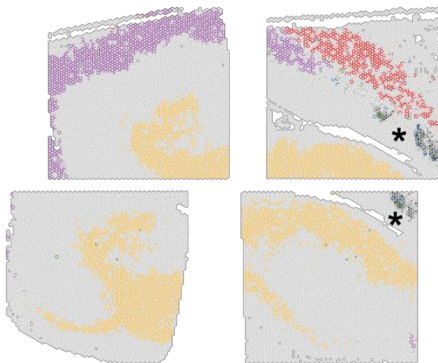
Br3942



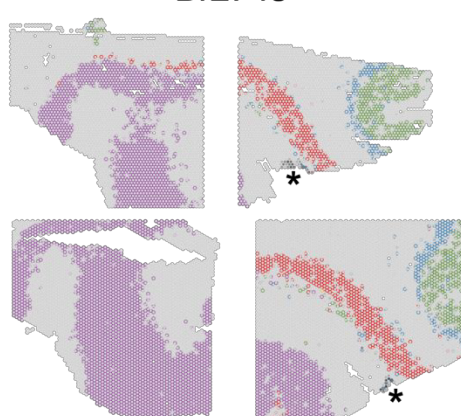
Br6522



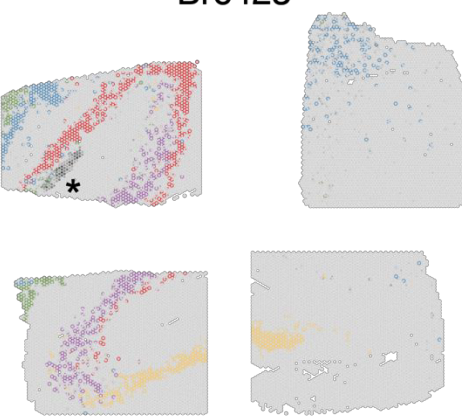
Br8667



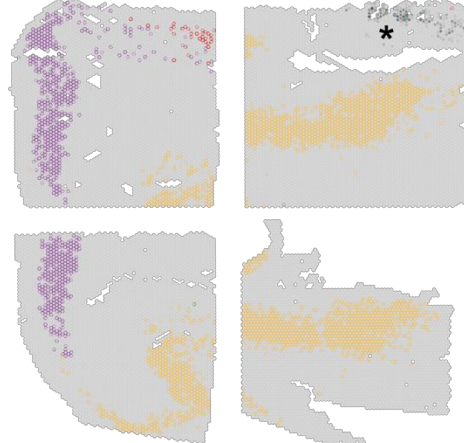
Br2743



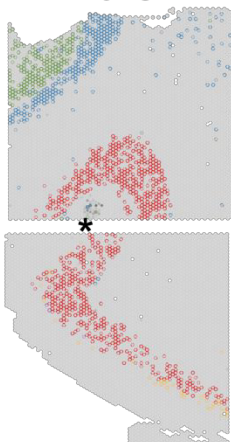
Br6423



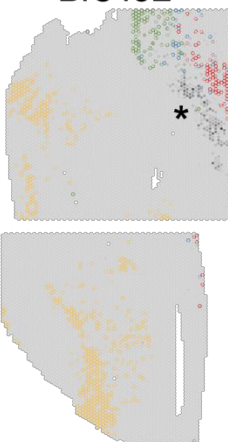
Br2720



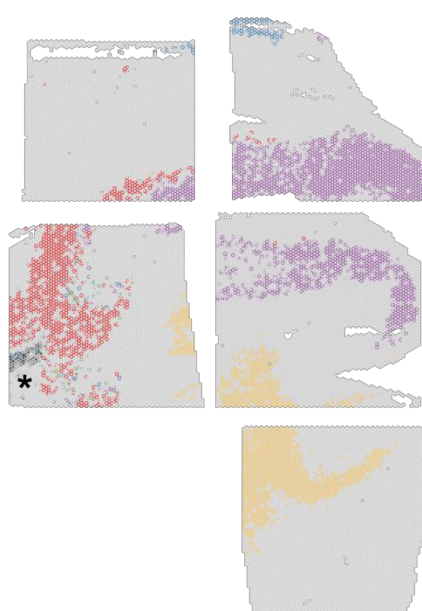
Br6432



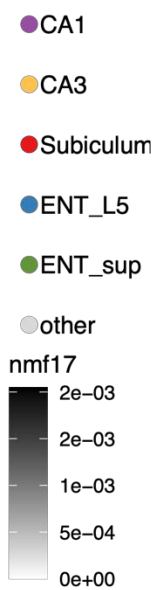
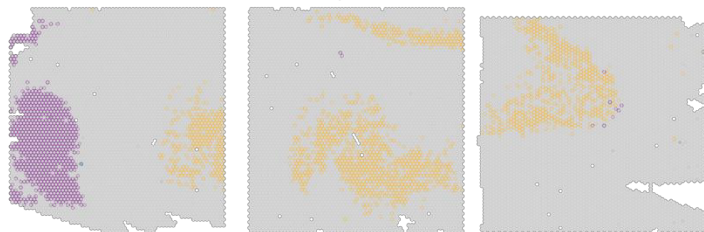
Br8492



Br8325



Br6471



Supplemental Fig. 18. Identification of thalamus and amygdala tissue in select SRT capture areas.

(A) H&E histology images of Br8325 capture areas. Putative thalamic tissue is circled.

(B) Spot plot showing expression of *TCF7L2*, a thalamus-specific transcription factor (21). Spots are filled by $\log_2$ normalized counts. Spot borders are colored by spatial domain.

(C) Spot plot showing expression of *SHOX2*, a thalamus-specific transcription factor (22). Spots are filled by $\log_2$ normalized counts. Spot borders are colored by spatial domain.

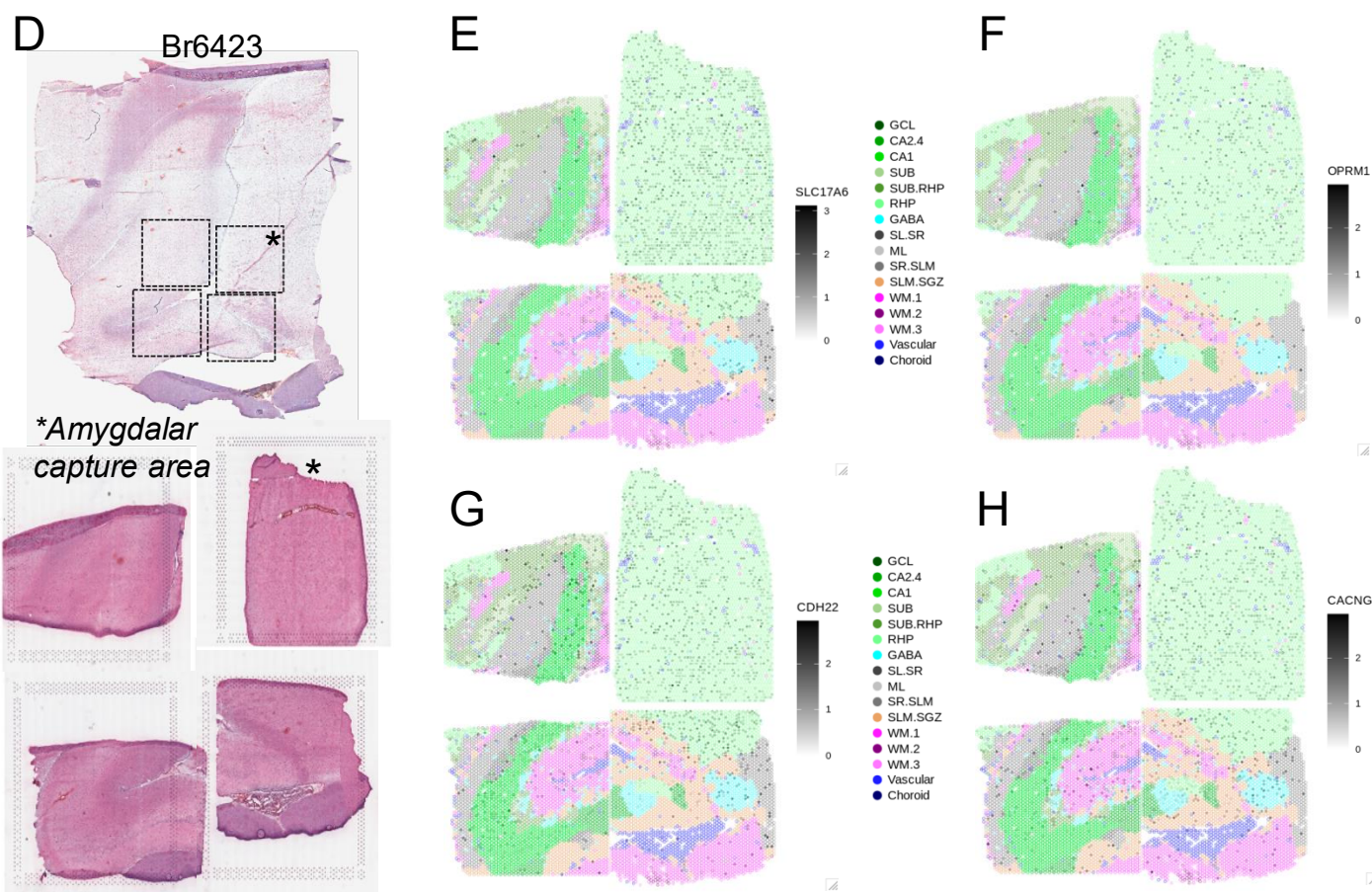
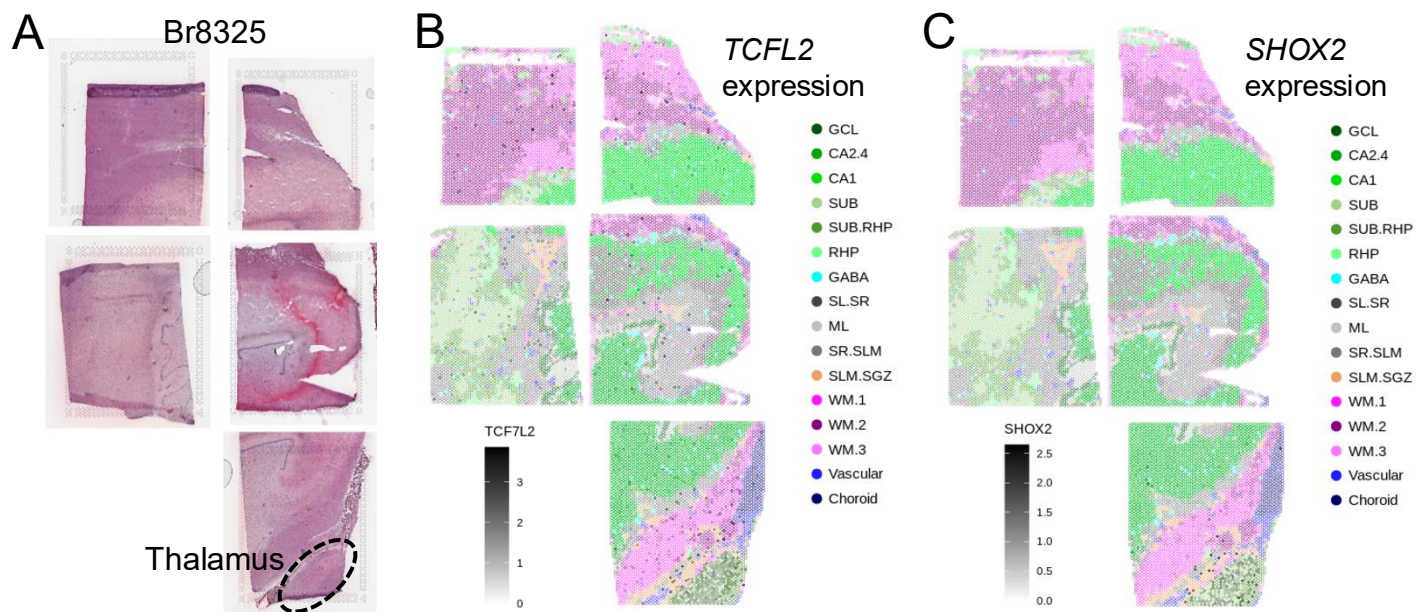
(D) Top: H&E histology image of example cryosection from Br6423 prior to scoring; approximate capture areas are outlined. Bottom: H&E histology images of Br6423 capture areas. Putative amygdala capture area (top right) is labeled.

(E) Spot plot showing expression of *SLC17A6*, which is enriched in human amygdala excitatory neurons (23). Spots are filled by $\log_2$ normalized counts. Spot borders are colored by spatial domain.

(F) Spot plot showing expression of *OPRM1*, which has been shown to be more highly enriched in amygdala than hippocampus (24). Spots are filled by $\log_2$ normalized counts. Spot borders are colored by spatial domain.

(G) Spot plot showing expression of *CDH22*, which is enriched in human amygdala excitatory neurons (23). Spots are filled by $\log_2$ normalized counts. Spot borders are colored by spatial domain.

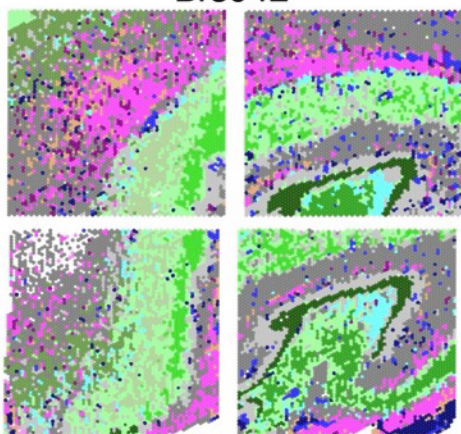
(H) Spot plot showing expression of *CACNG4*, which has been shown to be more highly enriched in amygdala than hippocampus (25). Spots are filled by $\log_2$ normalized counts. Spot borders are colored by spatial domain.



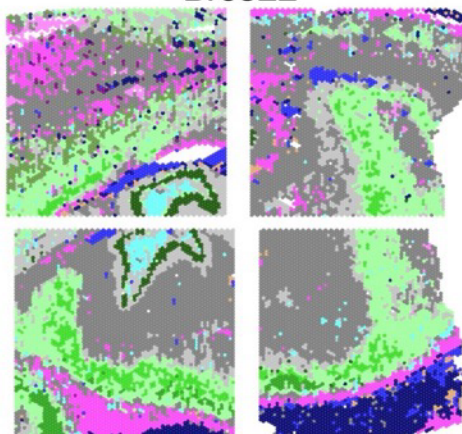
Supplemental Fig. 19. Spatial clustering results using GraphST for $k=16$.

GraphST clusters (color, legend in bottom right) are depicted across $n=36$ capture areas grouped by donor number.

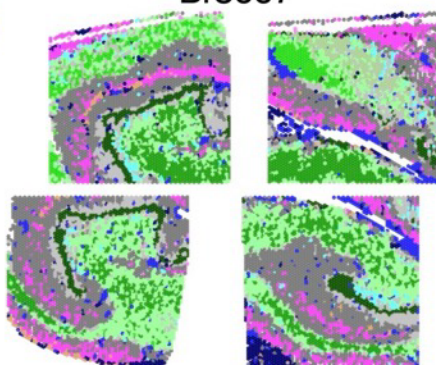
Br3942



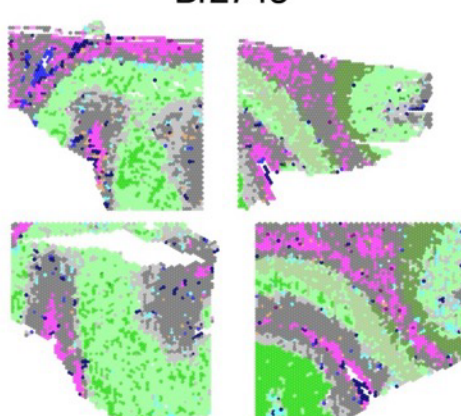
Br6522



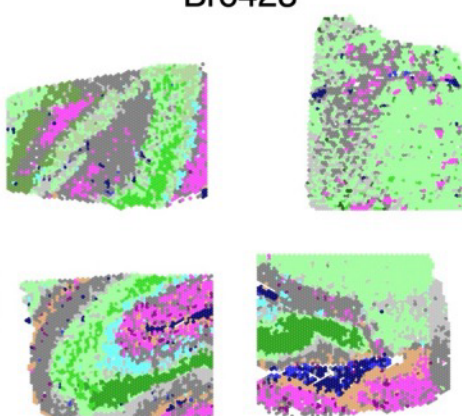
Br8667



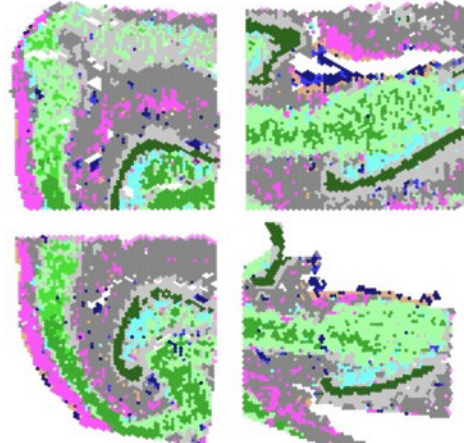
Br2743



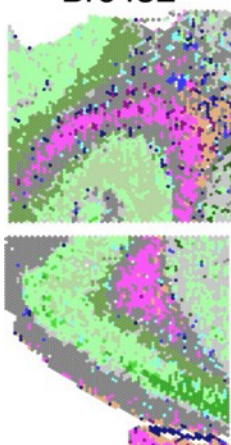
Br6423



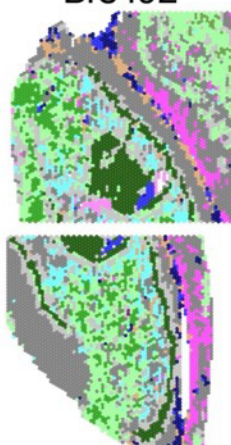
Br2720



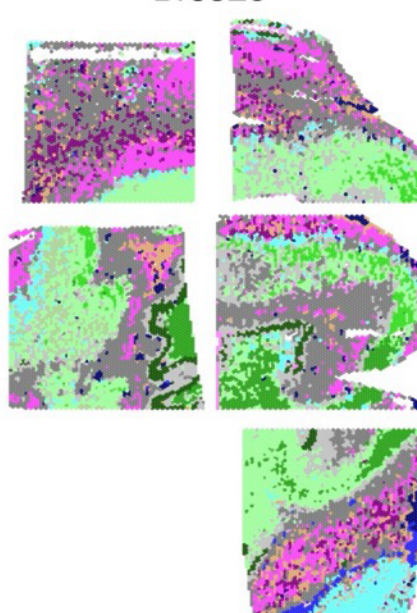
Br6432



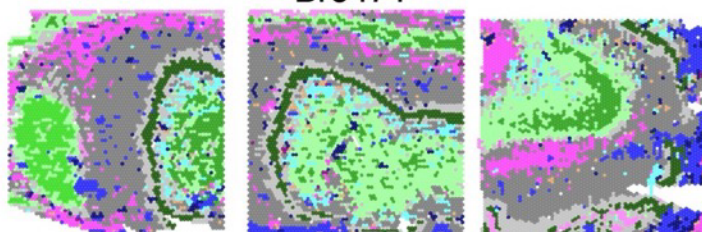
Br8492



Br8325



Br6471

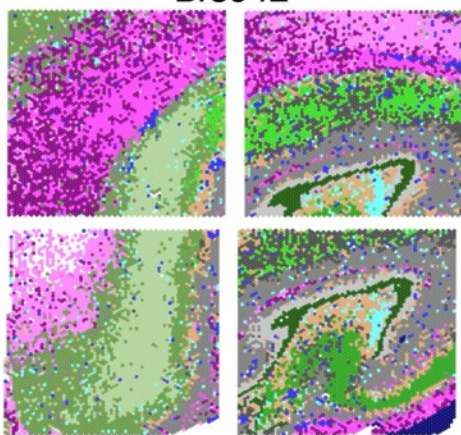


- 11
- 2
- 9
- 3
- 15
- 14
- 7
- 4
- 8
- 6
- 5
- 13
- 12
- 16
- 10
- 1

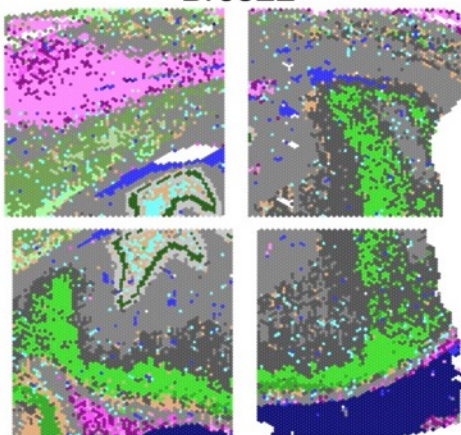
Supplemental Fig. 20. Spatial clustering results using `BayesSpace` for $k=18$.

`BayesSpace` clusters (color, legend in bottom right) are depicted across by $n=36$ capture areas grouped by donor number.

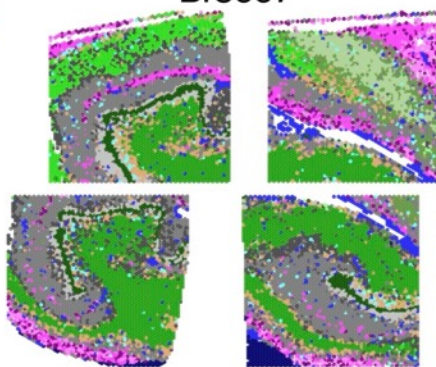
Br3942



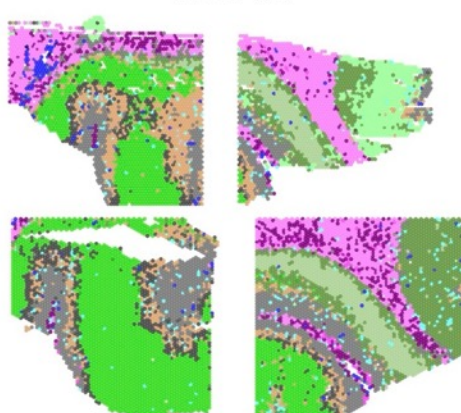
Br6522



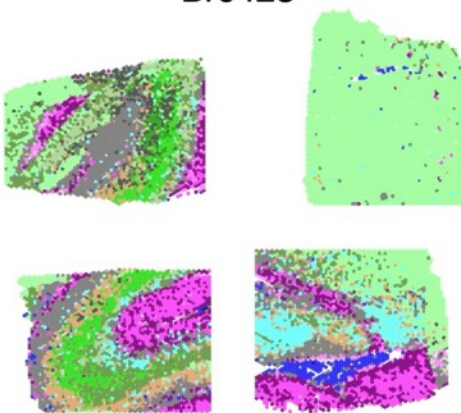
Br8667



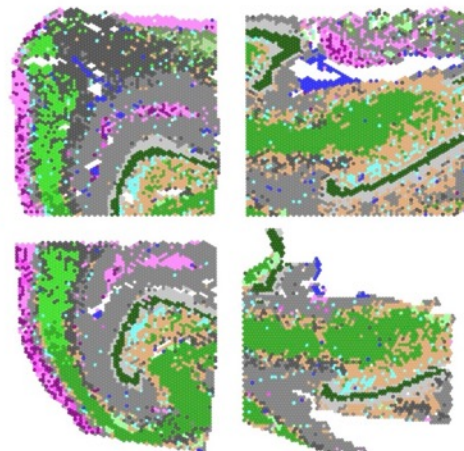
Br2743



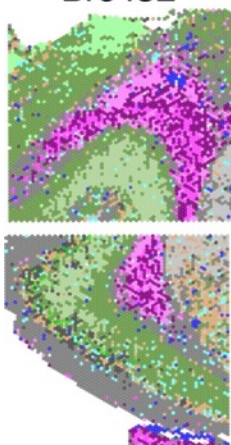
Br6423



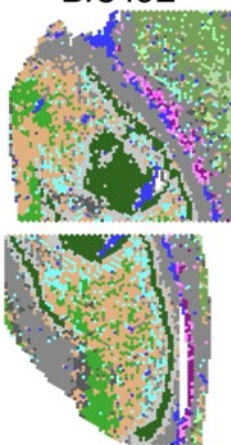
Br2720



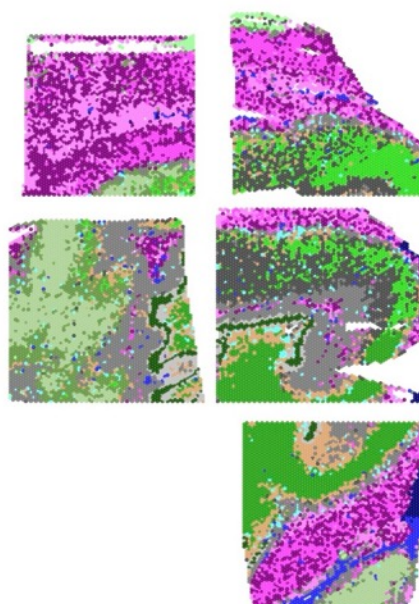
Br6432



Br8492



Br8325



Br6471



- 13
- 15
- 4
- 8
- 3
- 18
- 5
- 16
- 10
- 14
- 9
- 11
- 1
- 7
- 12
- 17
- 6
- 2

Supplemental Fig. 21. Comparison of spot-level assignment based on different annotation techniques.

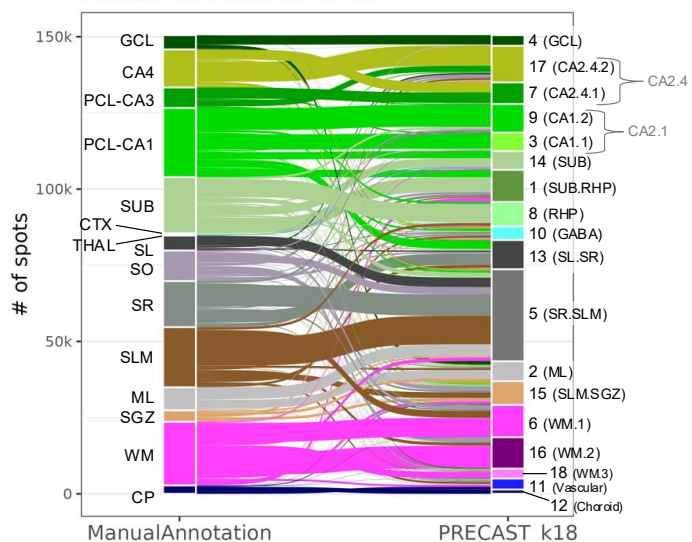
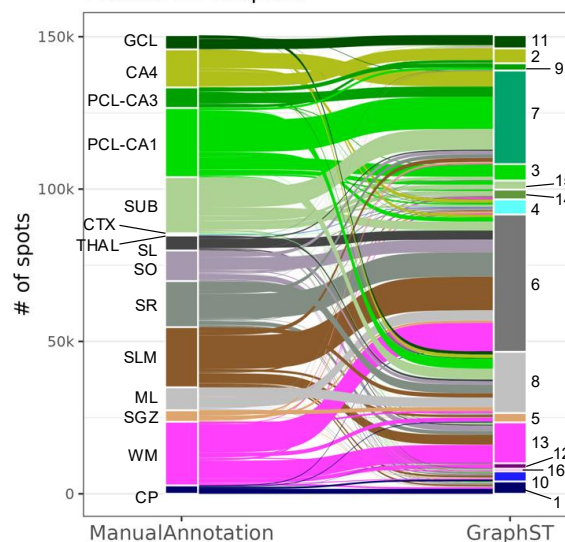
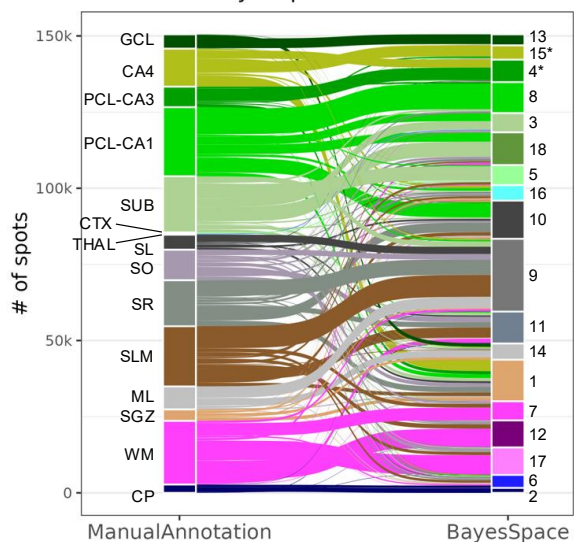
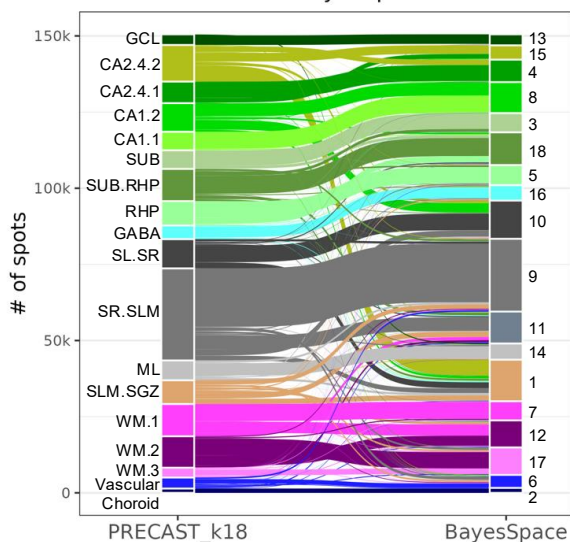
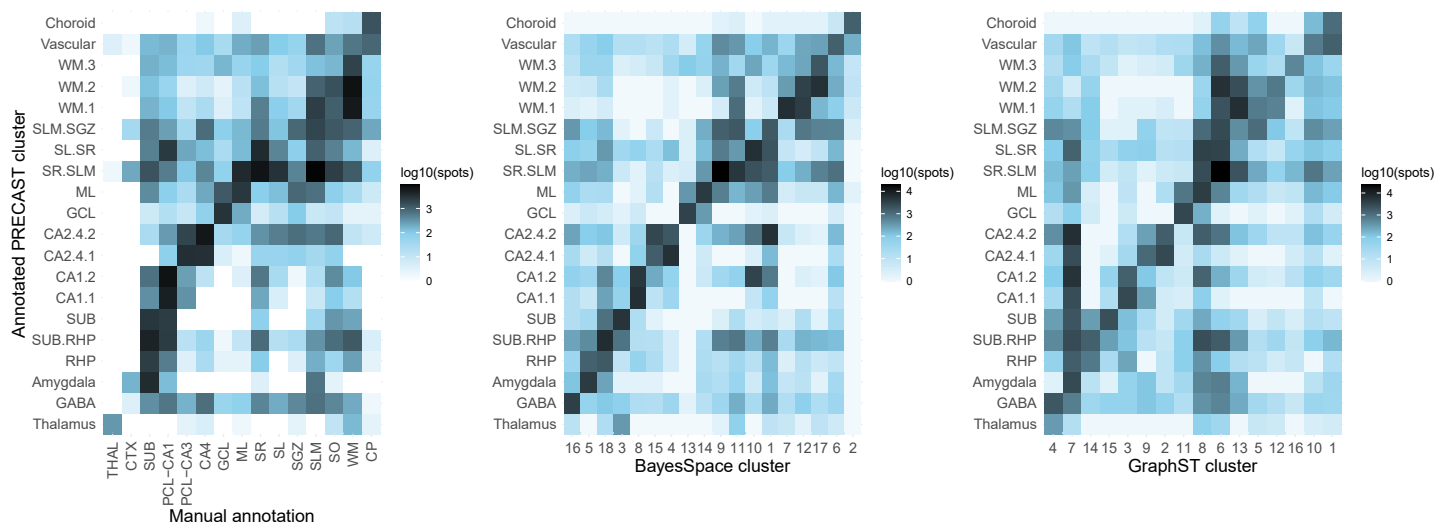
(A) Alluvial plot where flow groups indicate the number of spots (y-axis) annotated based on histologically-defined manual annotations and PRECAST k=18 clusters (x-axis). Color indicates group assignment. Text on the left labels the colors based on manual annotation groups. Text on the right labels the colors based on PRECAST k=18 cluster number, with cluster name in parentheses. The four total PRECAST clusters combined in the final spatial domain annotation are indicated with brackets.

(B) Alluvial plot where flow groups indicate the number of spots (y-axis) annotated based on histologically-defined manual annotations and GraphST k=16 clusters (x-axis). Color indicates group assignment. Text on the left labels the colors based on manual annotation groups. Text on the right labels the colors based on GraphST k=16 cluster number.

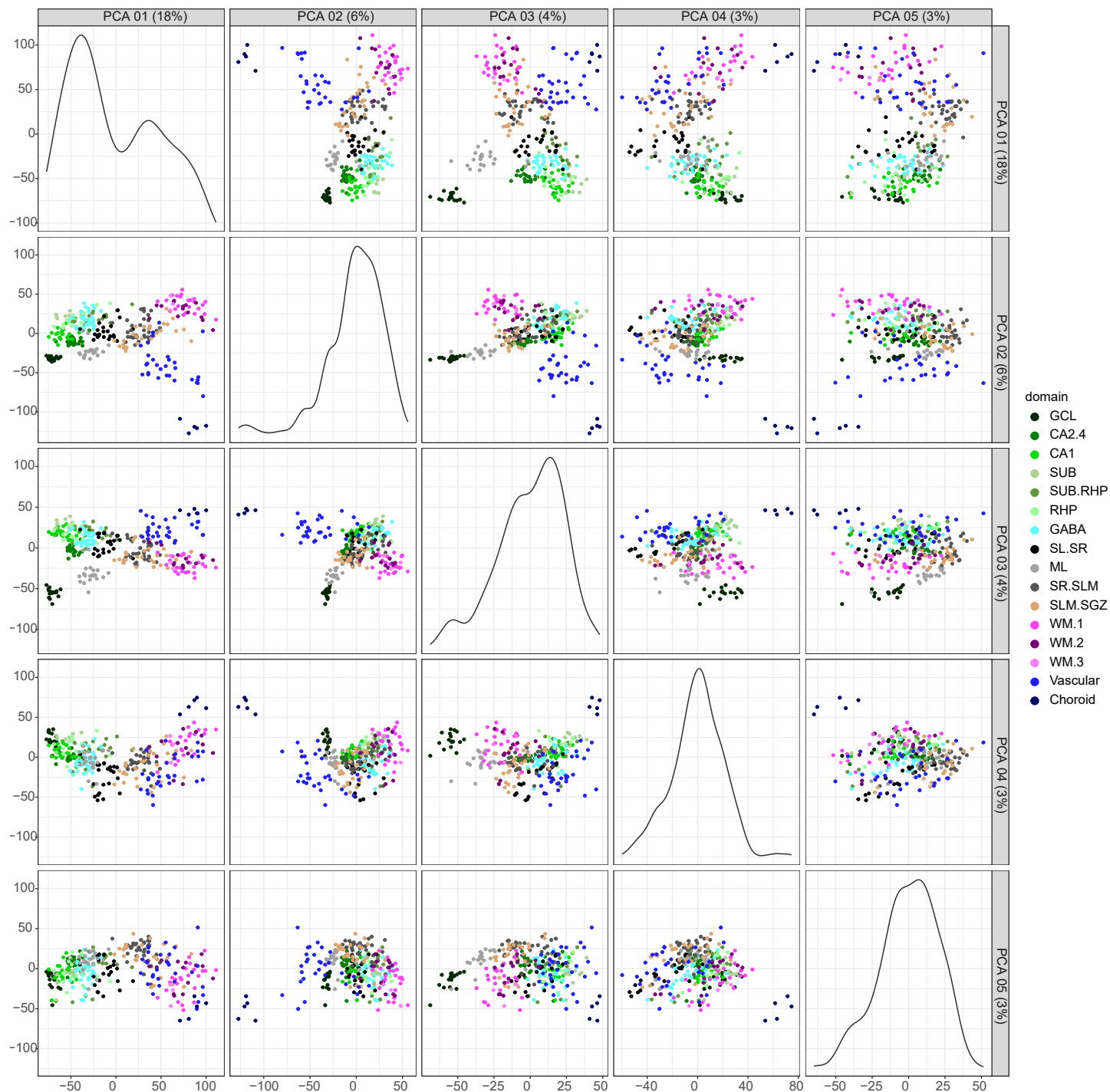
(C) Alluvial plot where flow groups indicate the number of spots (y-axis) annotated based on histologically-defined manual annotations and BayesSpace k=18 clusters (x-axis). Color indicates group assignment. Text on the left labels the colors based on manual annotation groups. Text on the right labels the colors based on BayesSpace k=18 cluster number.

(D) Alluvial plot where flow groups indicate the number of spots (y-axis) annotated based on PRECAST k=18 and BayesSpace k=18 clusters (x-axis). Color indicates group assignment. Text on the left labels the colors based on PRECAST k=18 cluster name. Text on the right labels the colors based on BayesSpace k=18 cluster number.

(E) Comparing PRECAST k=18 clusters (rows, amended to distinguish the limited amygdala and thalamus regions) to the manual annotations (left), BayesSpace clusters (middle), and GraphST clusters (right). In each case the color of the tile indicates the number of spots ($\log_{10}$ transformed) that were present in both the PRECAST-derived groups (rows) and the groups in the technique being compared (columns).

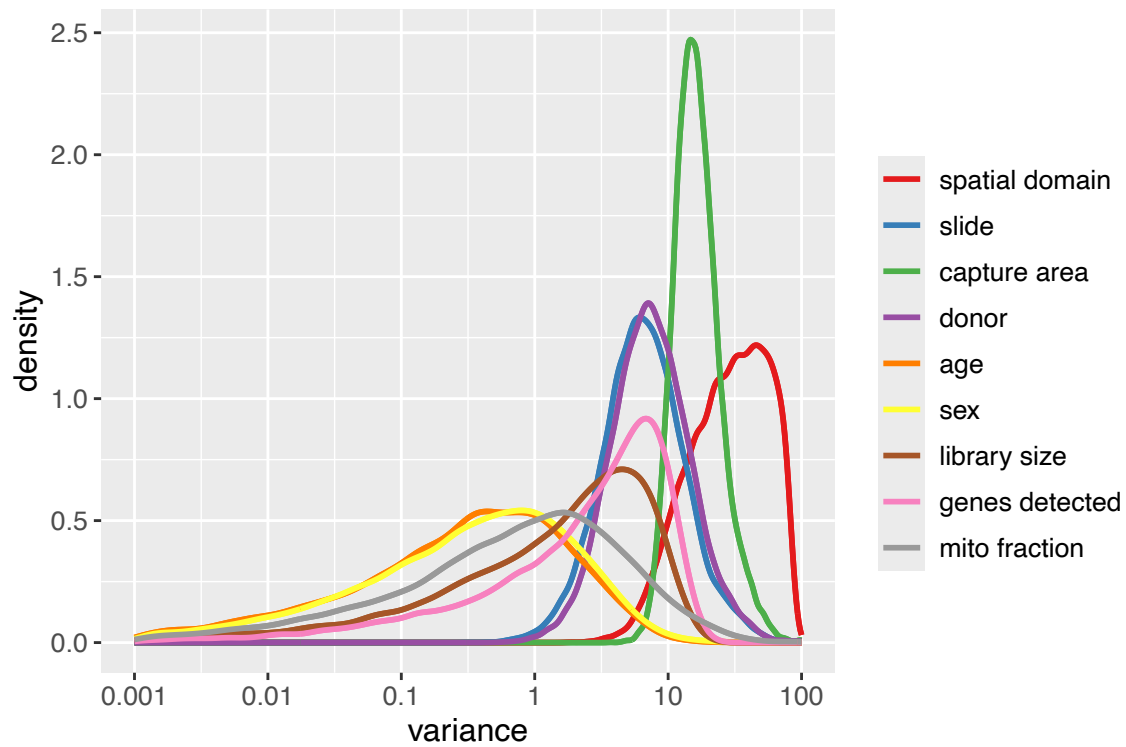
A Manual vs. PRECAST k=18**B** Manual vs. GraphST**C** Manual vs. BayesSpace**D** PRECAST k=18 vs. BayesSpace**E**

Principal component (PC) plots for the top 5 PCs (facets) after pseudo-bulking Visium spots across spatial domains (color) and 10 donors (total of 317 pseudo-bulked tissue samples). Facet labels include the percent variance explained for each PC.



Supplemental Fig. 23. Summary of gene-level variance explained by different variables.

Density plot for the percent of gene expression variance (x-axis) explained for several experimental variables in the pseudobulked spatial data across the $n=36$ SRT samples. The spatial domains (red line) contribute the largest percent of gene expression variance, importantly more than capture area (green) or donor (purple).



Supplemental Fig. 24. Preliminary annotation of neuronal nuclei in validation re-analysis.

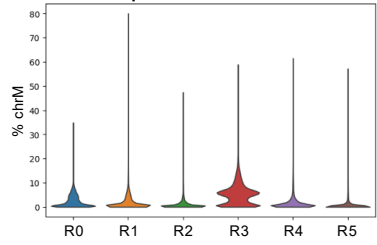
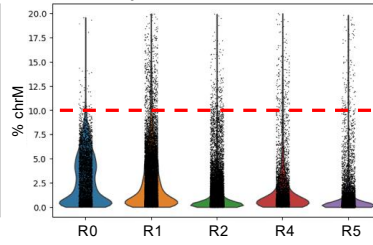
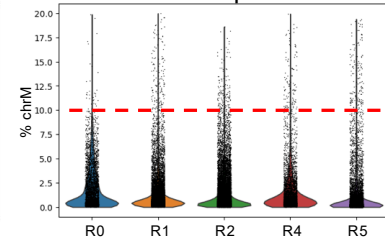
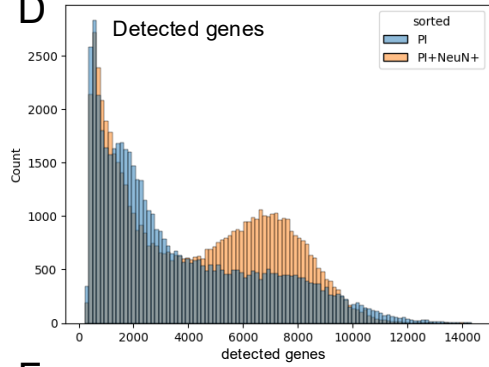
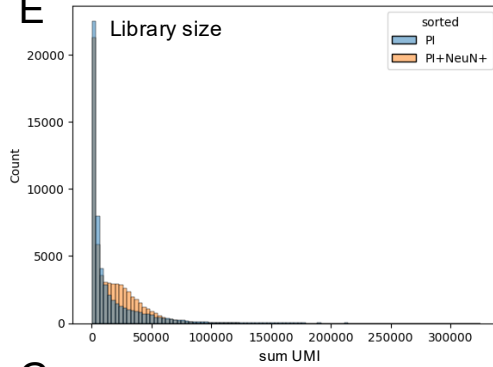
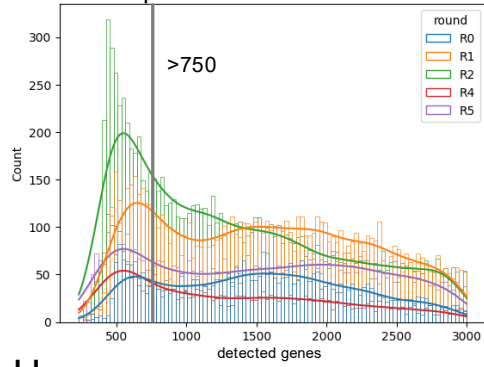
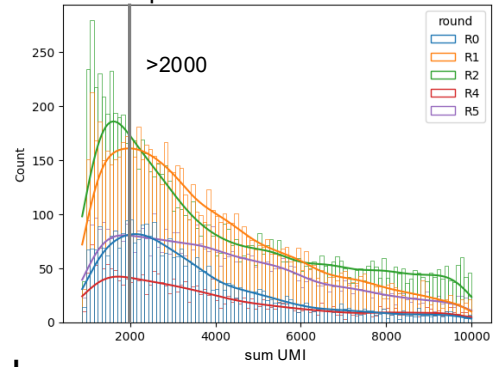
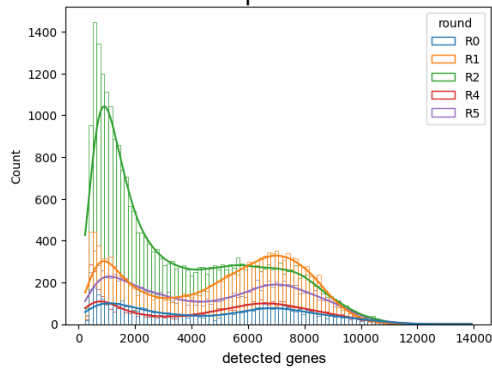
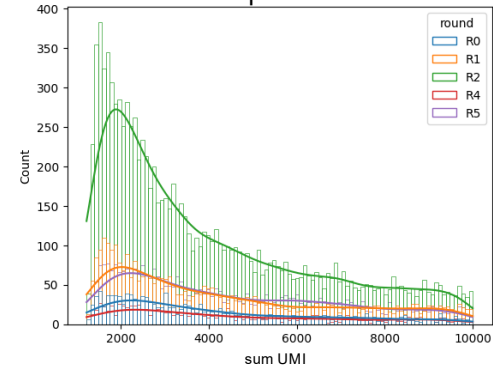
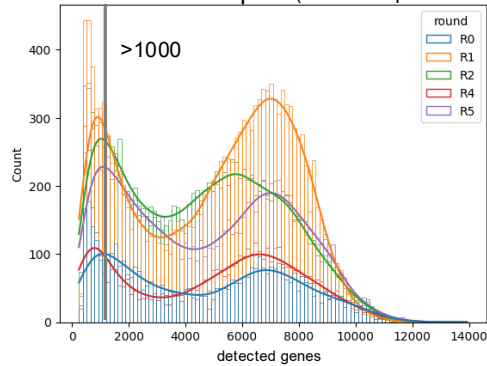
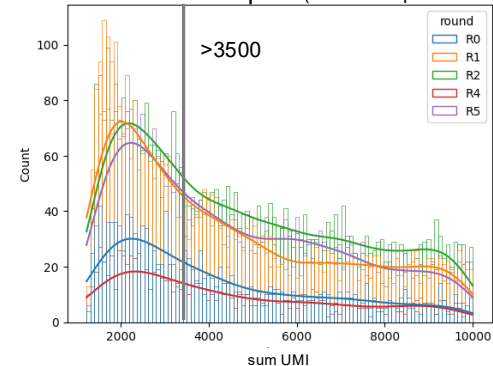
(**A**) Violin plots of nuclei mitochondrial fraction, separated by the sequencing round. An initial mitochondrial fraction threshold of 20% indicates that a stricter limit of 10% is suitable for (**B**) propidium iodide (PI)+ samples and (**C**) PI+NeuN+ samples.

Nuclei from PI+ and PI+NeuN+ samples exhibit distinct distributions of (**D**) the number of detected genes and (**E**) the number of reads per nucleus.

For PI+ samples, QC thresholds were set at a minimum of (**F**) 750 detected genes and (**G**) 2000 reads per nucleus.

For PI+NeuN+ samples, sequencing round 2 exhibited abnormal QC metrics (**H**, detected genes; **I**, library size), driven by one sample (17c-scp).

After removing the errant round 2 sample, QC thresholds for PI+NeuN+ were set at a minimum of (**J**) 1000 detected genes and (**K**) 3500 reads per nucleus.

A All samples**B PI samples****C PI+NeuN+ samples****D Detected genes****E Library size****F PI samples****G PI samples****H PI+NeuN+ samples****I PI+NeuN+ samples****J PI+NeuN+ samples (errant sample removed)****K PI+NeuN+ samples (errant sample removed)**

Supplemental Fig. 25. Clustering approach adopted in validation re-analysis.

(A) Uniform manifold approximation (UMAP) plot of initial Leiden clustering results based on *scVI* representations, annotated with cluster number.

(B) UMAP plot of initial Leiden clustering results based on *scVI* representations, colored based on the percent of reads originating from the mitochondrial genome (chrM) (also known as mitochondrial fraction).

(C) UMAP plot of initial Leiden clustering results based on *scVI* representations, colored based on the $\log_2$ normalized counts of *MALAT1*, a gene used to indicate nuclei quality.

(D) Violin plot depicts mitochondrial fraction (y-axis) across initial Leiden clusters (x-axis). Asterisks indicate clusters that were removed prior to re-clustering due to high mitochondrial fraction.

(E) Violin plot depicts *MALAT1* logcount expression (y-axis) across initial Leiden clusters (x-axis). Asterisks indicate clusters that were removed prior to re-clustering due to low *MALAT1* expression.

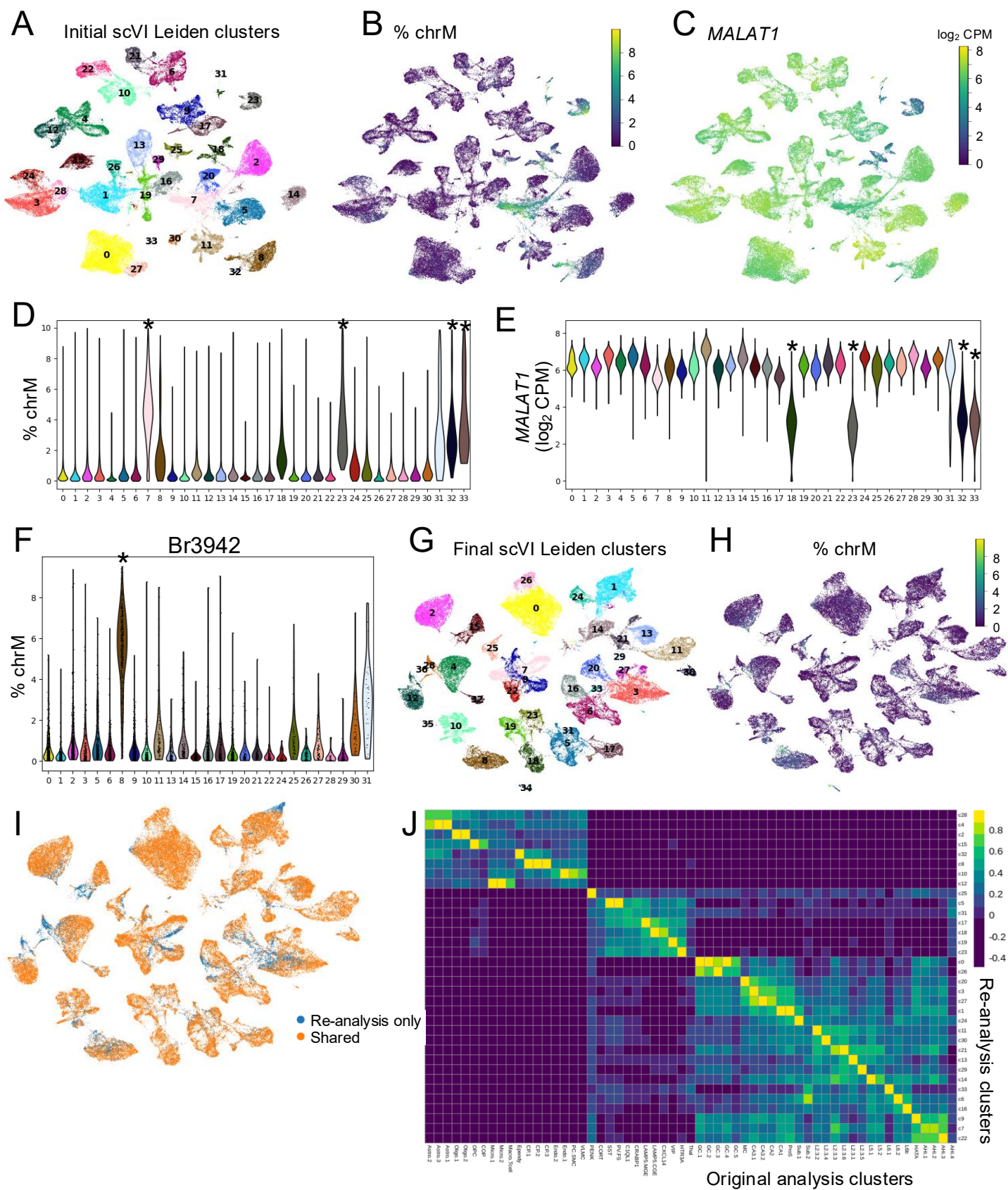
(F) Cluster 8 exhibited borderline exclusionary mitochondrial fraction that seemed to be due to nuclei from a single donor. This violin plot of mitochondrial fraction (y-axis) across all clusters from this sample only (x-axis) demonstrates that the increased mitochondrial fraction of cluster 8 is abnormal amongst this sample's nuclei. Nuclei belonging to the cluster labeled with an asterisk were removed prior to re-clustering.

(G) UMAP plot after re-clustering with $n=10$ neighbors following removal of low quality initial clusters. Color and number indicate final cluster annotations.

(H) UMAP plot demonstrating improved nuclei quality based on mitochondrial fraction (color) after removal of low quality initial clusters.

(I) The 74,216 nuclei obtained from the python-based re-analysis, with the 64,251 nuclei jointly present in the manuscript labeled in orange and the 9,965 nuclei unique to the re-analysis labeled in blue.

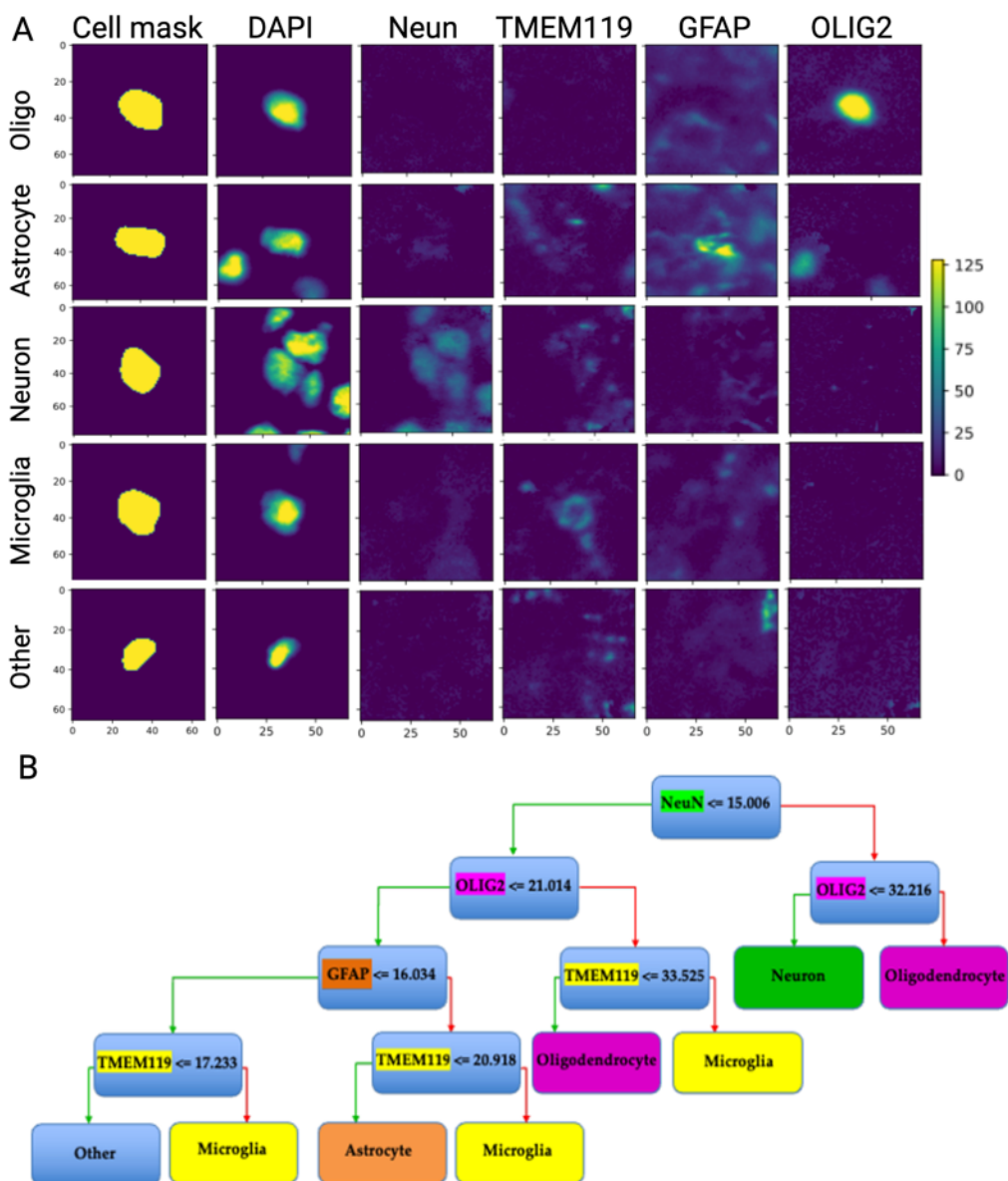
(J) Heatmap depicts the correlation coefficient (color) of the *t*-statistics generated from pseudobulked cluster enrichment differential expression analysis in the re-analyzed clusters (rows) and the original $n=60$ clusters (columns).



Supplemental Fig. 26. Decision-tree framework for classification and regression tree (CART) - cell types using immunofluorescence (IF) intensities.

(A) For each randomly chosen segmented nucleus, shown in the leftmost column (cell mask), fluorescence intensity for the DAPI channel and other channels marking each cell type is shown in the remaining columns. The decision tree uses the mean intensity values within the cell mask from each channel for classification.

(B) The decision-tree structure, which is traversed downwards. Each colored box represents a decision, made by comparing the mean fluorescence of a single channel for a given segmented cell against a learned threshold (indicated within each box). The red and green arrows indicate the direction of decision depending on the threshold indicated in the box, if mean intensity is below threshold tree follows red arrow otherwise it follows a green arrow. For example, for a cell to be classified as microglia, the mean pixel intensity of its cell mask should be less than 15 in the NeuN channel, less than 21 in the OLIG2 channel, less than 16 in the GFAP channel and exceeding 17 in the TMEM119 channel.



Supplemental Fig. 27. Hierarchy of snRNA-seq cluster resolutions.

(A) Dendrogram shows the hierarchical classification of cell type labels across cluster resolutions utilized for deconvolution.

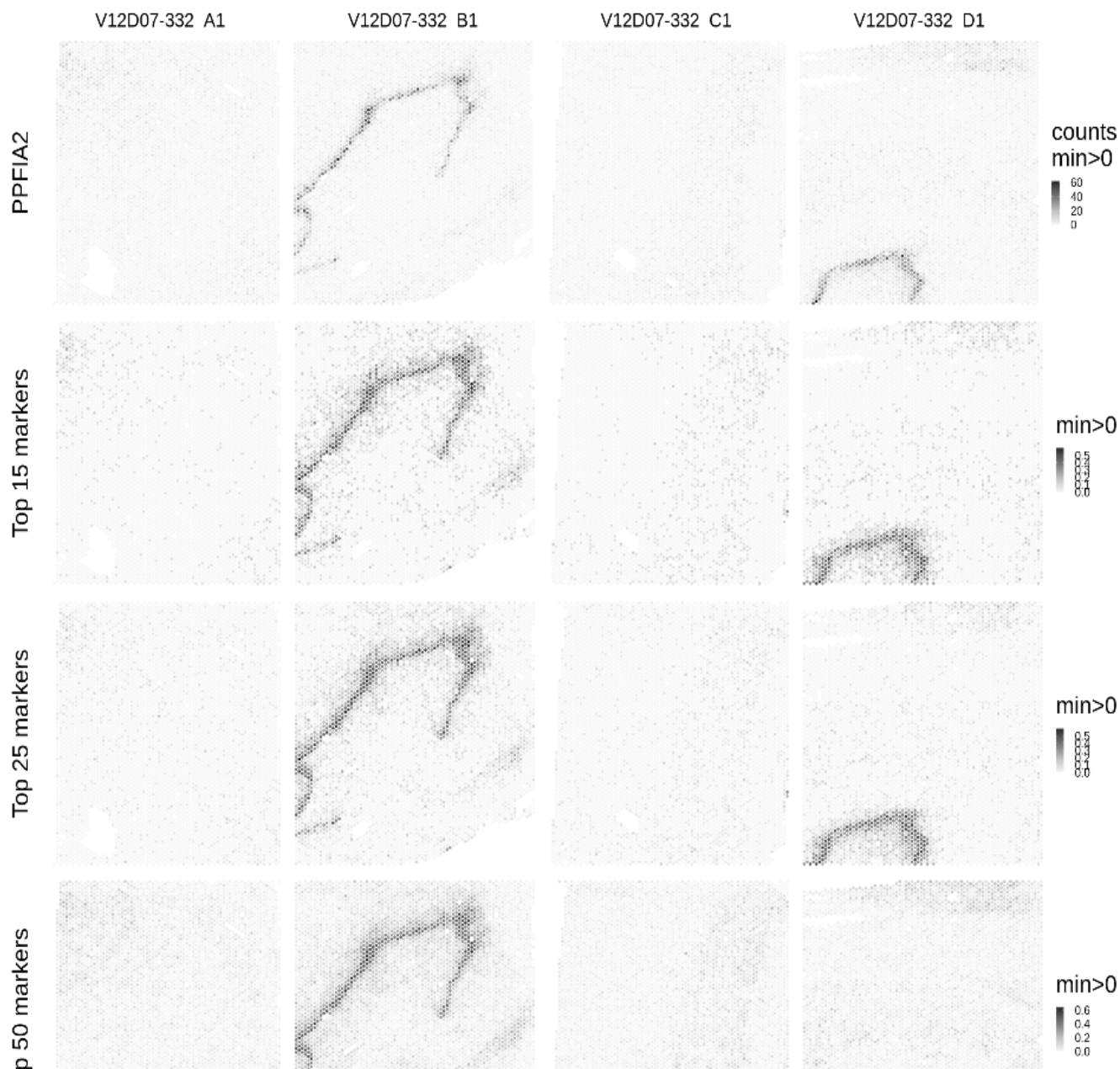
(B) Bar plots show the relative proportion of nuclei across cell types for each donor, each sorting strategy, and in total.

(C) Bar plots show the relative proportion of nuclei across cell types when excitatory neuron (ExcN) and inhibitory neuron (InhN) clusters are collapsed for each donor, each sorting strategy, and in total.

(D) Bar plots show the relative proportion of nuclei across cell types collapsed to basic annotations for each donor, each sorting strategy, and in total.

Supplemental Fig. 28. Determining the optimal number of gene markers for spot deconvolution.

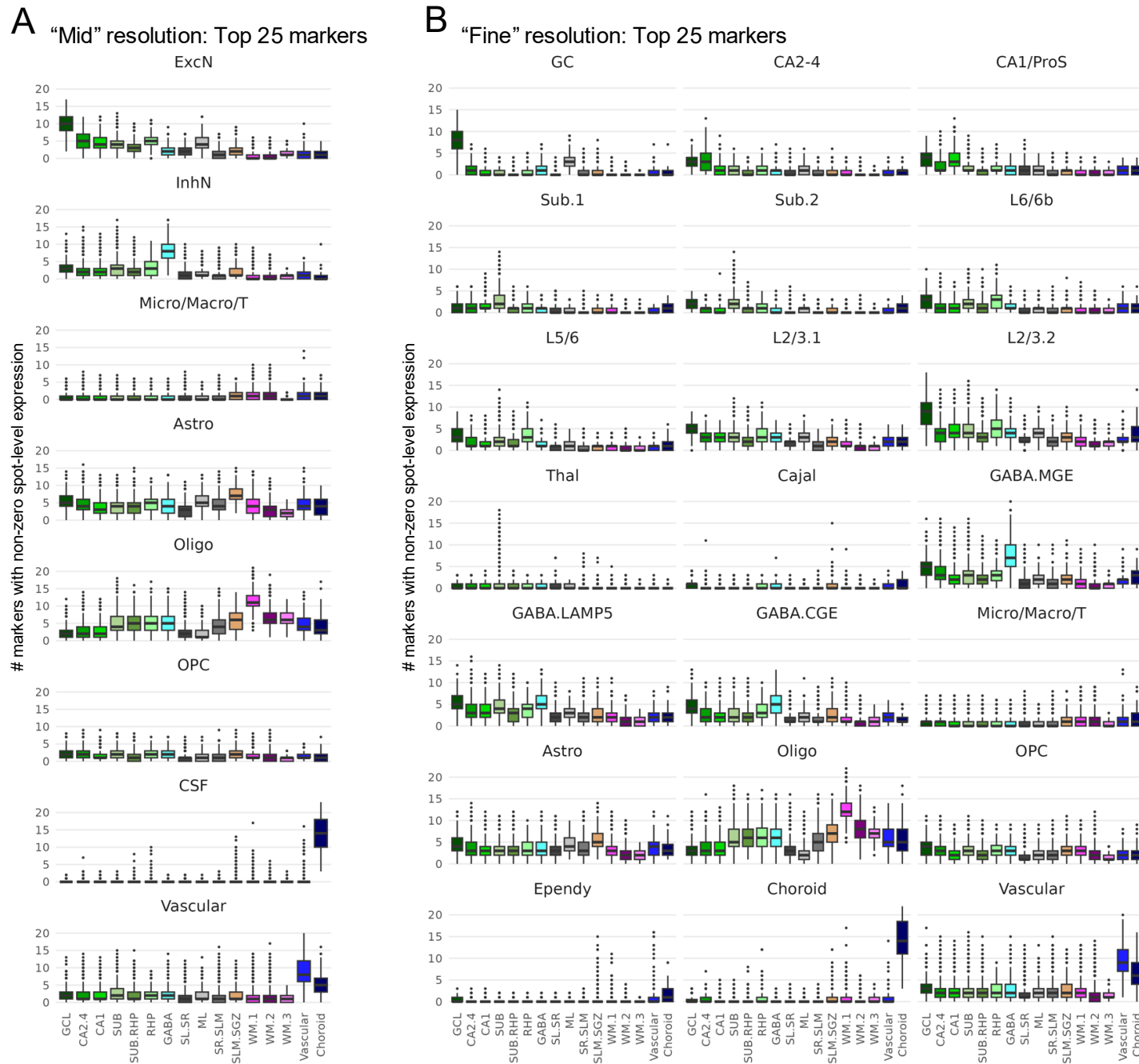
Comparison of *PPFIA2* counts to GC excitatory neuron marker gene expression with increasing number of marker genes. The top row shows spot plots for raw counts of *PPFIA2*, a marker for GCL, for all four Visium-SPG sections from donor Br3942. Remaining rows show spot plots depicting the proportion of GC marker genes with non-zero counts at each spot, when selecting the top 15, 25, or 50 marker genes. The choice of 25 marker genes per cell type results in an informative signal without significant expression outside of the expected layer.



Supplemental Fig. 29. Spatial validation of fine cell type marker gene (top 25) expression.

(A) Boxplots summarizing the number of top 25 marker genes (y-axis) expressed in each spot (individual data points) across Visium-SPG spatial domains (x-axis) for the “mid” resolution cell types (individual panels).

(B) Boxplots summarizing the number of top 25 marker genes (y-axis) expressed in each spot (individual data points) across Visium-SPG spatial domains (x-axis) for the “fine” resolution cell types (individual panels).

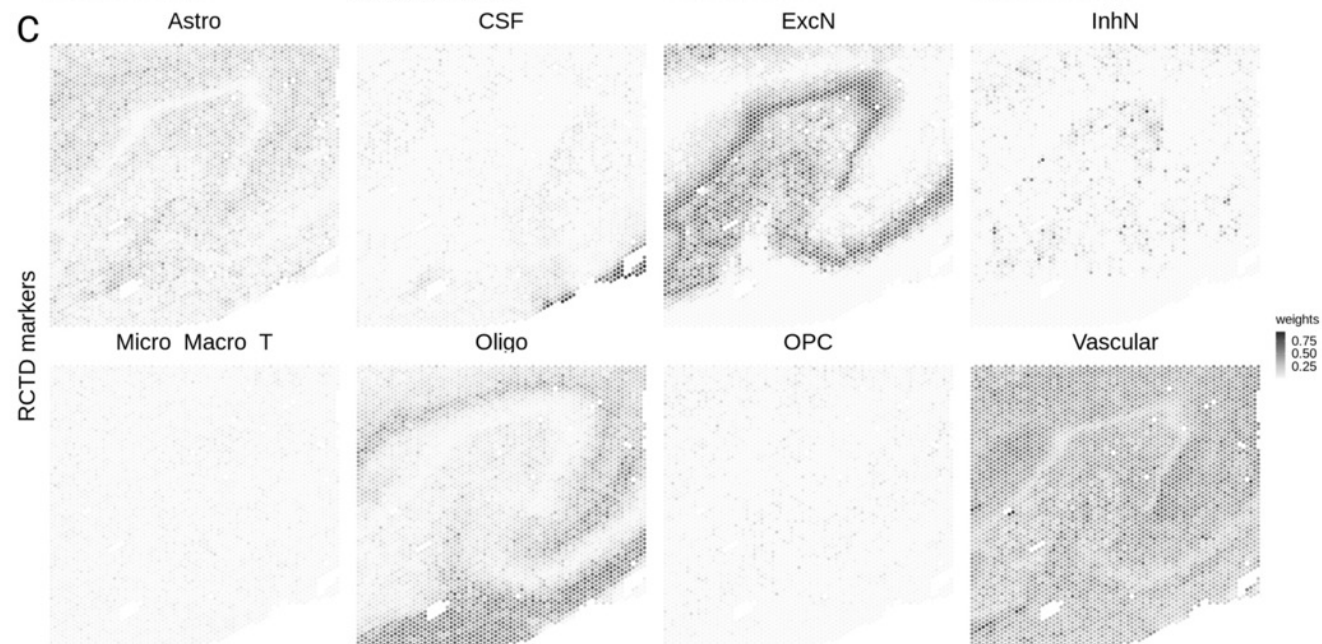
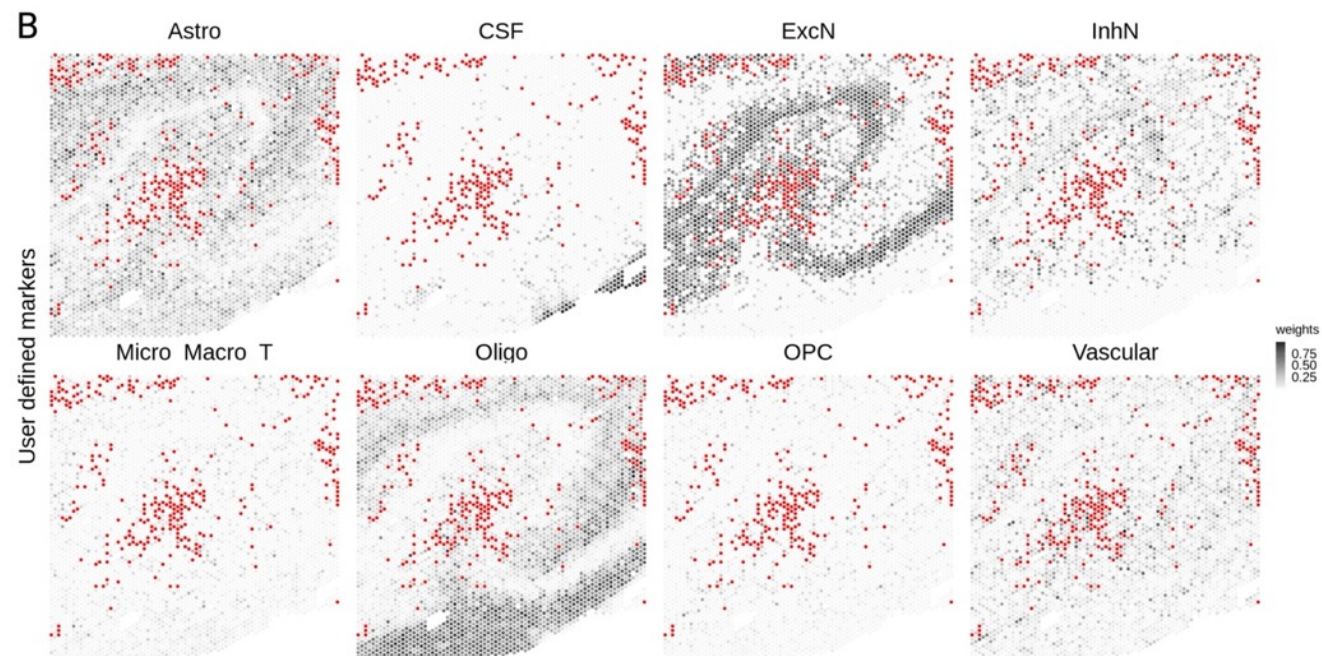
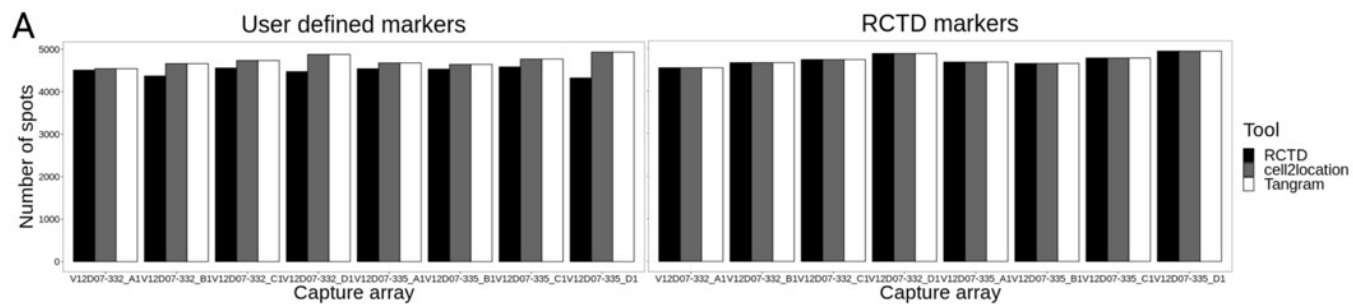


Supplemental Fig. 30. RCTD performance with user derived marker genes vs default marker genes.

(A) Bar plots show the number of spots retained (out of 5,000 per sample) across RCTD, cell2location, and Tangram. The x-axis represents eight Visium-SPG samples, and the y-axis indicates the number of retained spots. In the left panel, all tools use marker genes derived from DeconvoBuddies, where RCTD drops a notable number of spots compared to cell2location and Tangram. In the right panel, RCTD uses its default marker set, where it retains nearly all spots.

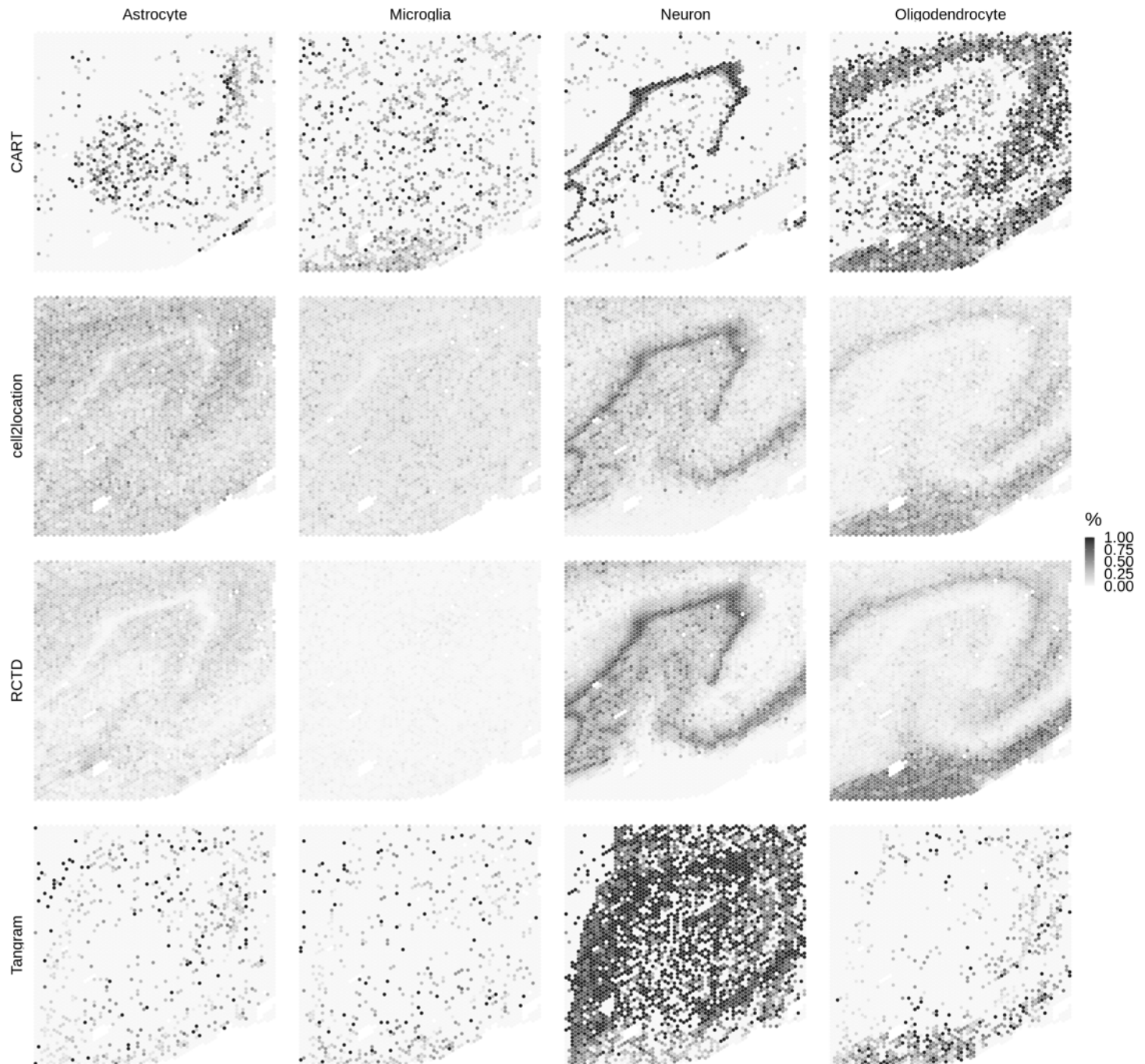
(B) RCTD predictions for mid cell type proportions with the user derived marker genes from DeconvoBuddies package (61). Red spots indicate the RCTD dropped spots when using user defined marker genes. See **Supplemental Table 2** for abbreviations.

(C) RCTD predictions for mid cell type proportions with default marker genes.



Supplemental Fig. 31. Spatial comparison of broad cell type proportions in classification and Regression Tree (CART) vs. spot deconvolution tool.

The top row shows spot plots of broad cell type counts from CART-predictions, for one Visium-SPG capture area V12D07-332_D1 from donor Br3942. The bottom three rows show collapsed mid cell type counts as estimated by cell2location, RCTD and Tangram.

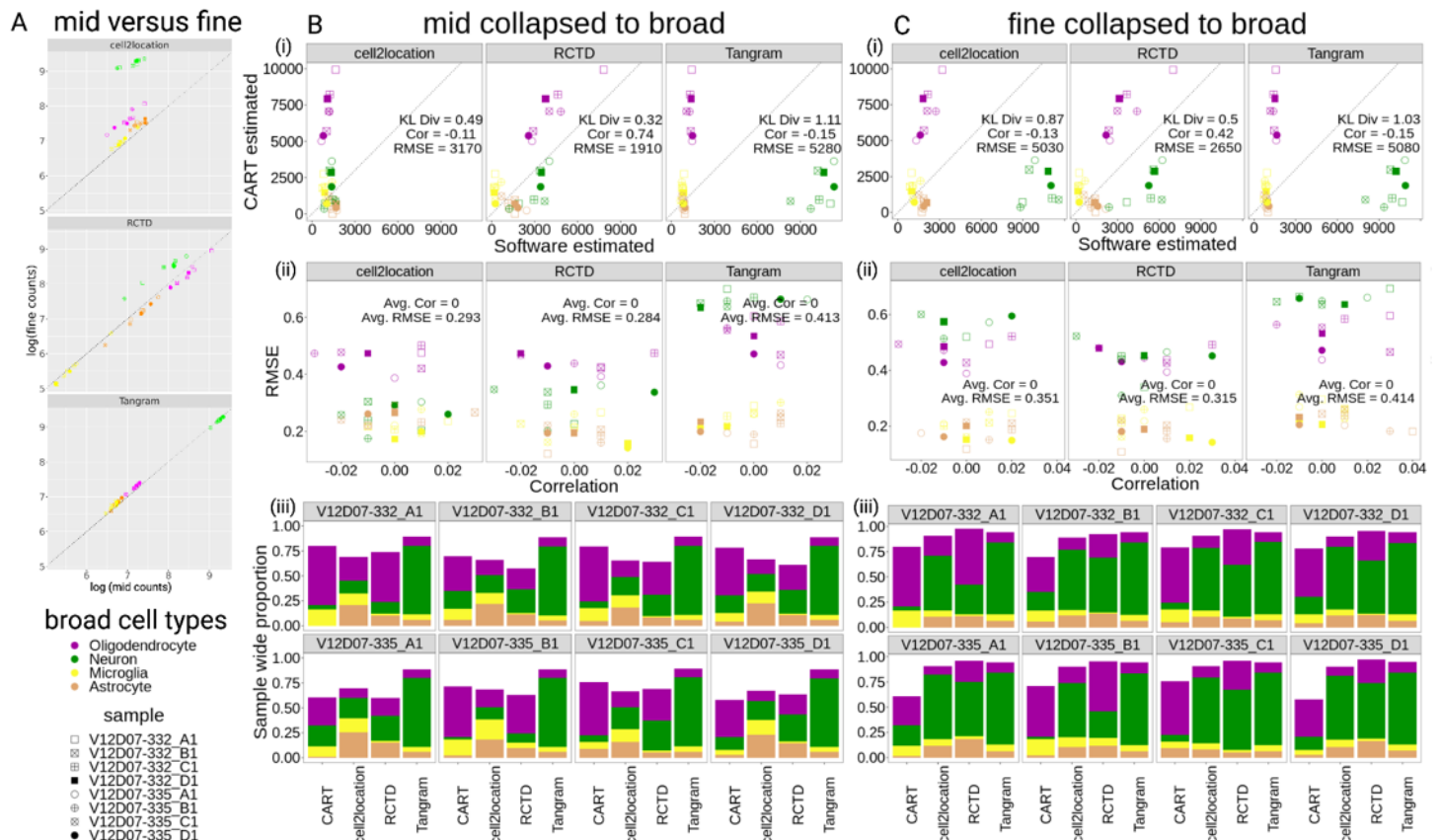


Supplemental Fig. 32. Benchmarking of spot deconvolution results.

(A) Comparison of cell type counts from mid (x-axis) and fine (y-axis) cell type classifications (both collapsed into broad cell types) from all three deconvolution tools.

(B) (i) Classification and Regression Tree (CART)-calculated vs. deconvolution-estimated counts for four immunolabeled cell types (Astro, Micro, Neuron, Oligo) across eight Visium-SPG sections. Each point represents the number of cells of the given cell type present in a tissue section, both as estimated by each deconvolution method (x-axis, facets) and as determined by the decision-tree classifier (y-axis). Since the CART framework can only predict the four cell types indicated by the immunofluorescence channels present in the Visium-SPG data, the deconvolution results that were predicted at mid cell type resolution are here collapsed down to broad cell types. Kulback-Leibler divergence (KL Div), Pearson correlation (Cor) and root mean squared error (RMSE) are the metrics computed to summarize the relationship between CART-calculated and deconvolution-estimated counts for a particular software method across four cell types and eight tissue sections. (ii) Predicted counts for a given method (facets), section (points), and mid cell type classification collapsed to broad (colors) are compared against the corresponding CART predictions by computing the Pearson correlation (x-axis) and RMSE (y-axis). Each of these values is then averaged to generate a single correlation (Avg. Cor) and RMSE (Avg. RMSE) value for each method (inset text). (iii) The predicted proportion (y-axis) of the broad cell types (fill color) by the four prediction methods (x-axis) for each sample (facets). Totals do not add to 1 because CART can classify cells as “other” (negative for Astro, Micro, Neuron, and Oligo), and the Vascular, OPC, CSF cell type predictions from the 3 softwares are grouped as others.

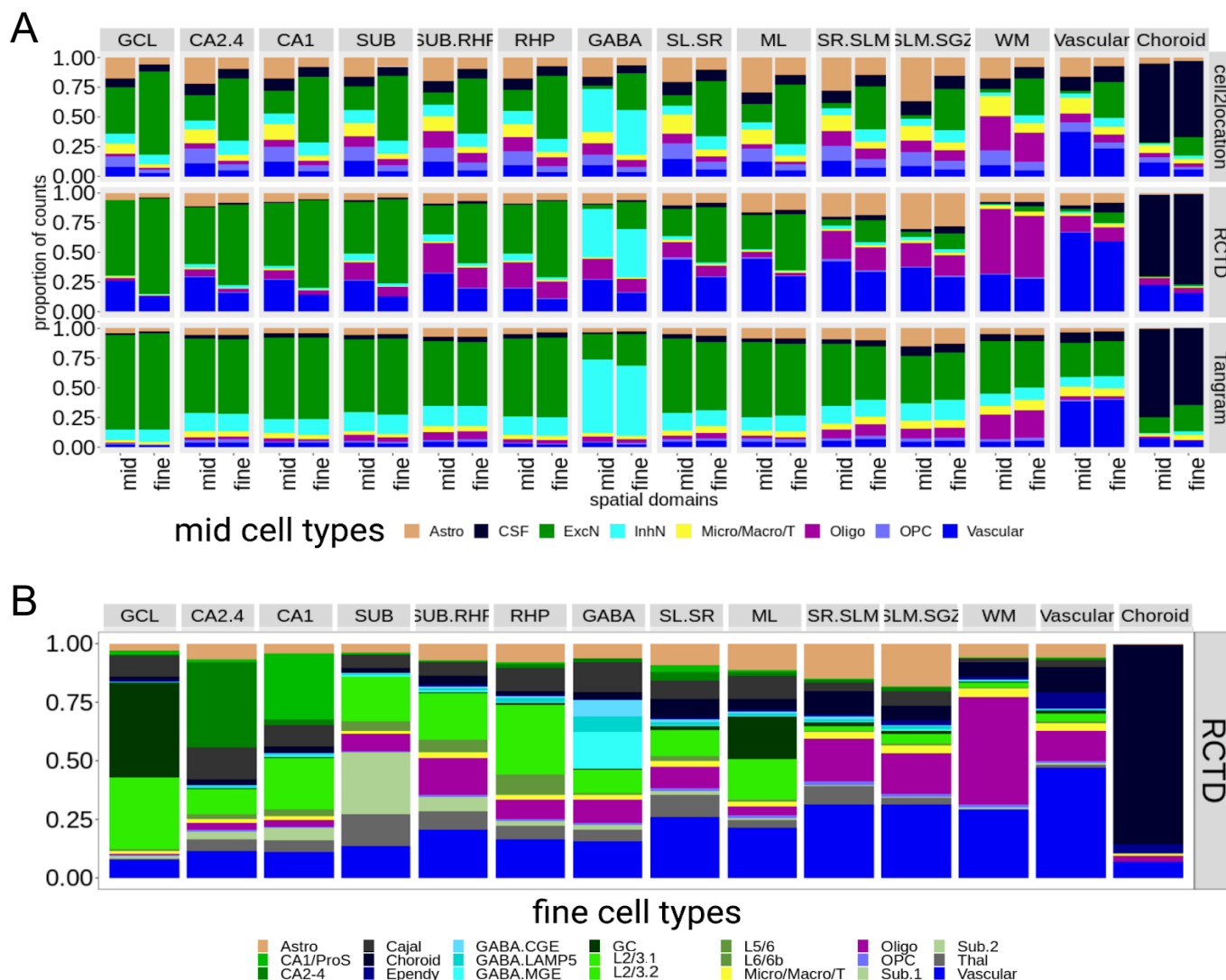
(C) The same metrics and plots from (B) were repeated for the fine cell types collapsed to broad cell type.



Supplemental Fig. 33. Distribution of fine versus mid cell type proportions.

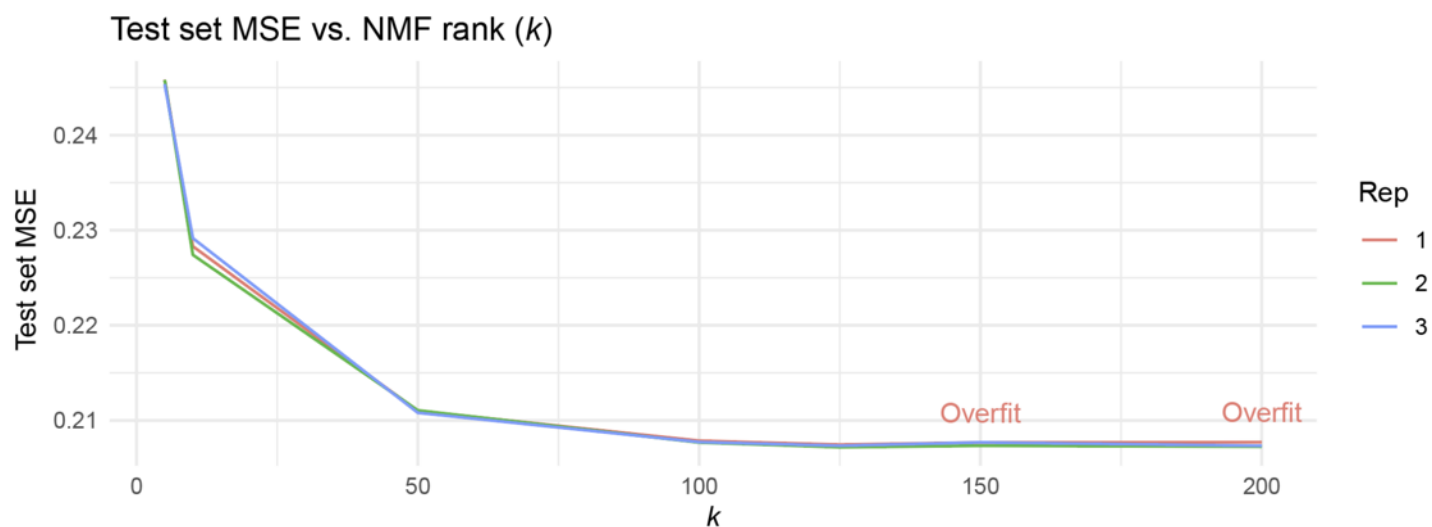
(A) Bar plots show the proportion of total cell type counts (y-axis) across PRECAST spatial domains (x-axis) in Visium-SPG data. Each bar represents the distribution of either 21 fine cell types (collapsed to 8 mid cell types) or 8 directly predicted mid cell types. Predictions from all three tools are included for comparison. This visualization highlights spatial patterns in cell type composition. See Supplemental Table 2 for spatial domain abbreviations.

(B) Cell type composition of 21 fine cell type predictions from RCTD in Visium-H&E data.



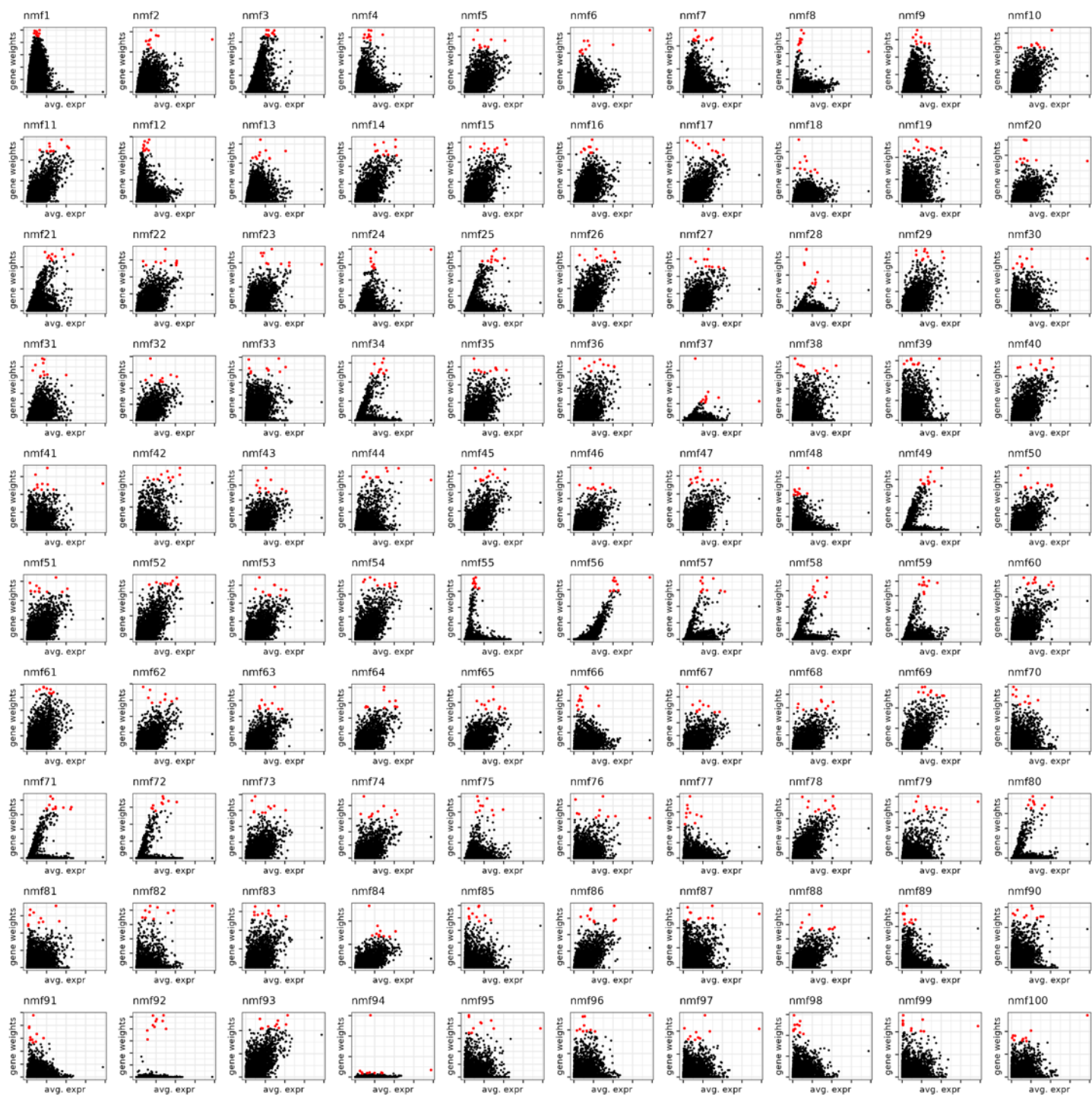
Supplemental Fig. 34. Results from non-negative matrix factorization (NMF) cross-validation using 3 replicates.

Line plot (lines= repeats) showing test set mean squared error (MSE) (y-axis) across different NMF ranks (x-axis).



Supplemental Fig. 35. Gene-level weights for all non-negative matrix factorization (NMF) patterns.

Scatter plots of gene-level weights (y -axis) stratified by the average $\log_2$ normalized expression across all nuclei in our snRNA-seq dataset (x -axis). The top 10 genes weighted to each NMF pattern are indicated in red and for ease of readability these points are 1.66x larger than the remaining genes in black.



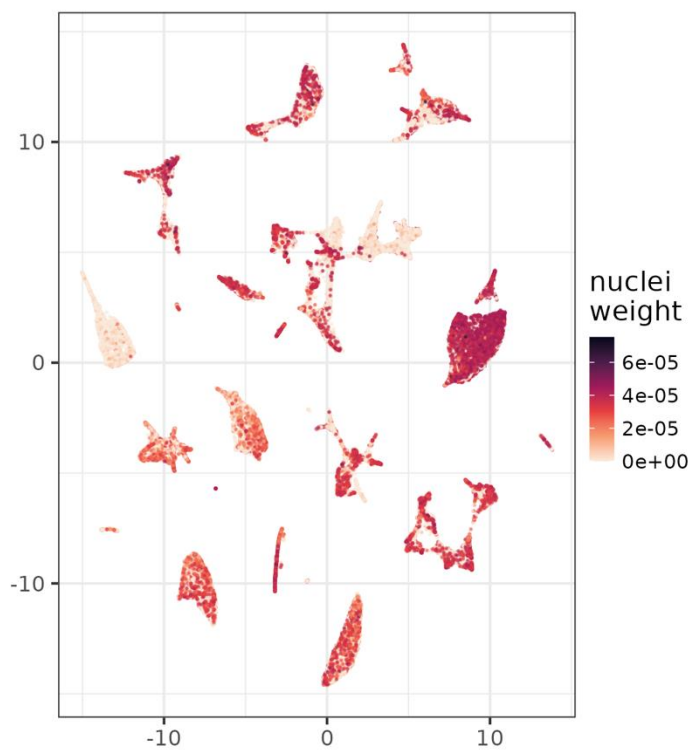
Supplemental Fig. 36. Non-negative matrix factorization (NMF) patterns capture sex-based gene expression programs.

(A) Uniform manifold approximation (UMAP) plot of snRNA-seq nuclei colored by nmf37 nuclei weights identified nuclei obtained from female donors and is almost identical to (B) the UMAP plot of $\log_2$ normalized expression of *XIST*, the top weighted gene for this pattern.

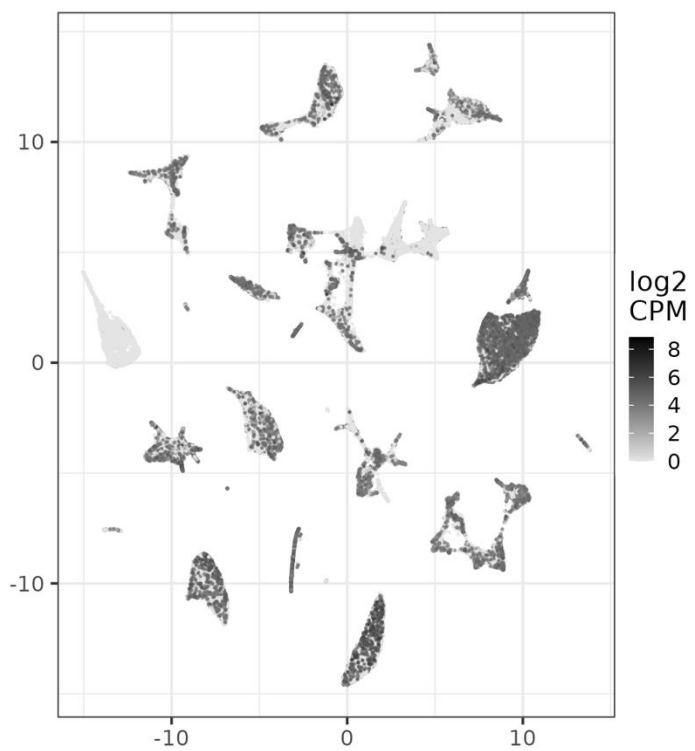
(C) UMAP plot of snRNA-seq nuclei colored by nmf28 nuclei weights identifies nuclei obtained male donors. The 4 genes most highly weighted to nmf28 originate from the Y chromosome: *TTY14*, *NLGN4Y*, *USP9Y*, and *UTY*.

(D) UMAP plot of snRNA-seq nuclei colored by $\log_2$ normalized expression of *USP9Y* is extremely similar to the pattern of nmf28 weights in (C).

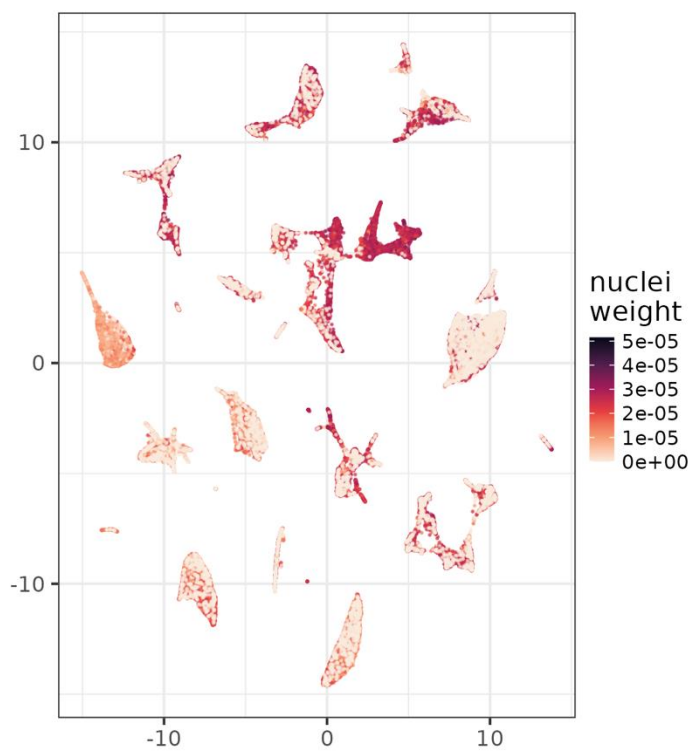
A nmf37



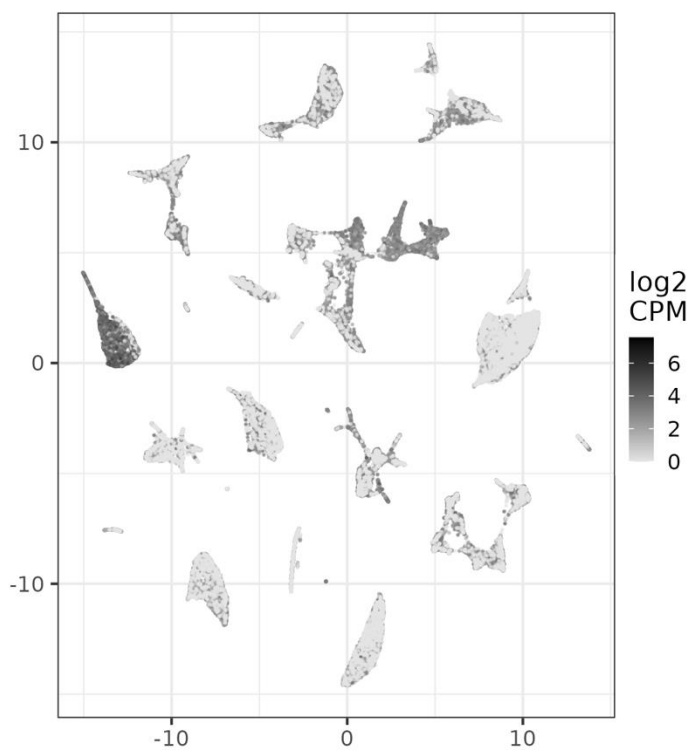
B XIST



C nmf28



D USP9Y



Supplemental Fig. 37. Non-negative matrix factorization (NMF), donor effect, and projection.

(A) The average nuclei (snRNA-seq, left) or spot (SRT, right) weights for nmf94 and nmf48 (x-axis), grouped by broad cell type (left) or domain (right) (y-axis). The size of the dots indicates the proportion of nuclei/spots in the y-axis group with non-zero weights for the given NMF pattern.

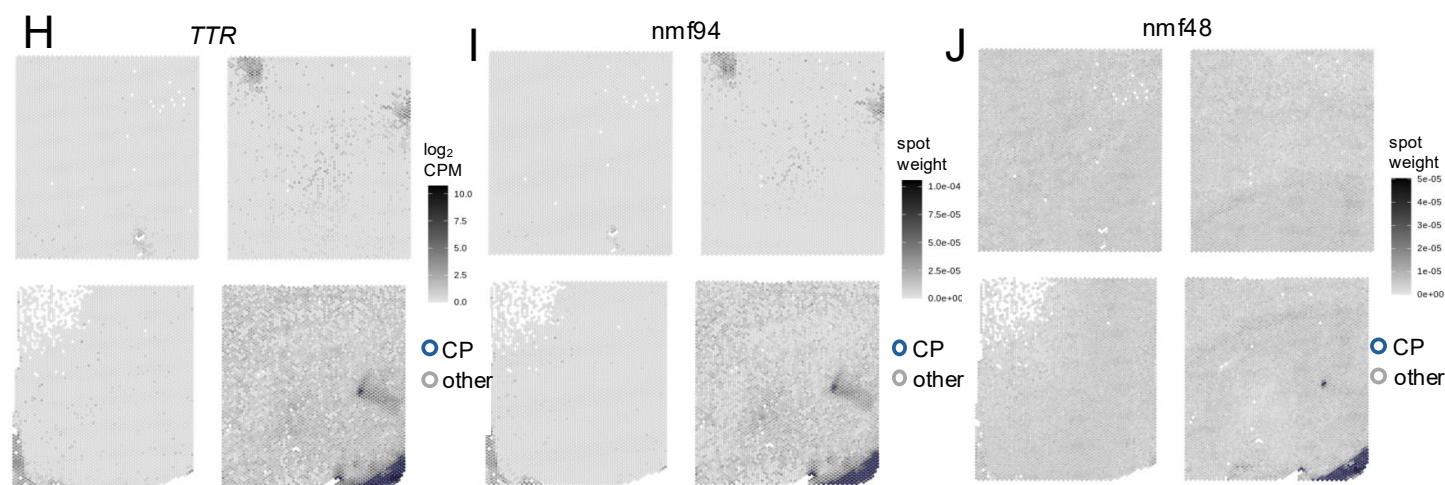
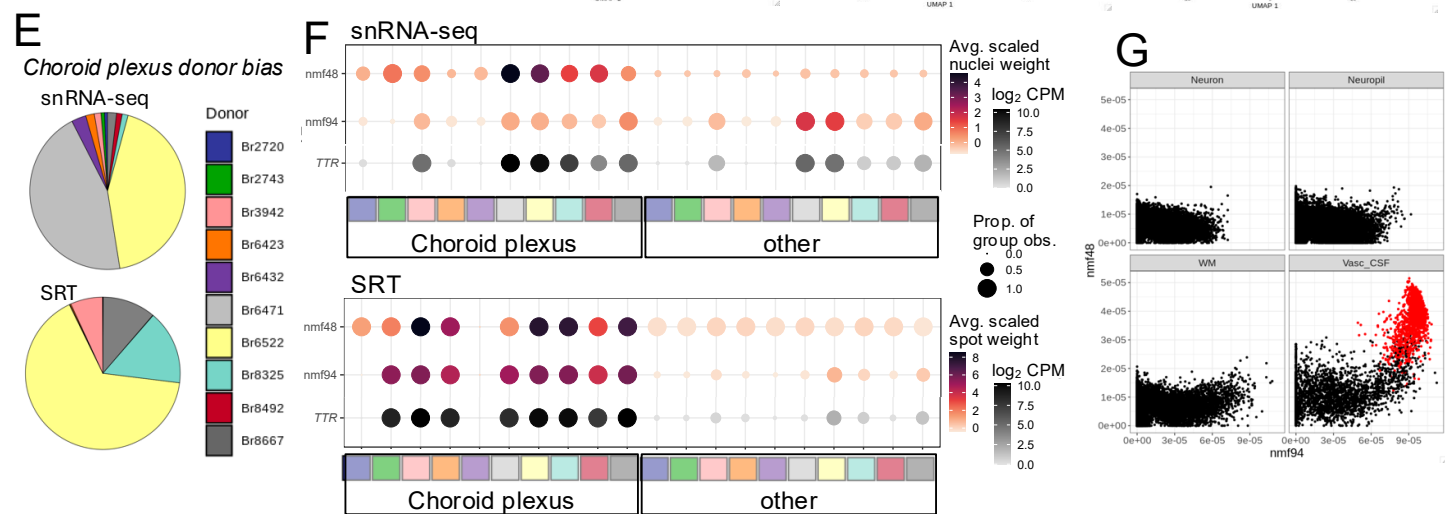
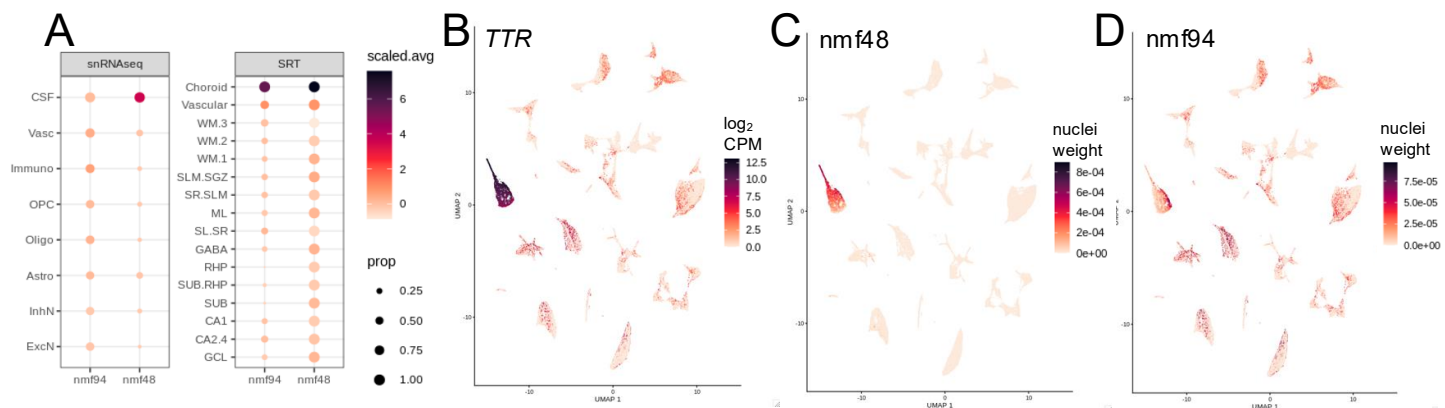
Uniform manifold approximation (UMAP) plot of snRNA-seq data colored by (B) $\log_2$ CPM *TTR* expression, (C) nmf48 nuclei weights, and (D) nmf94 nuclei weights.

(E) Choroid plexus (CP) nuclei (snRNA-seq, top) or spots (SRT, bottom) were isolated from a limited number of donors based on the scarcity of the tissue type in the HPC region. Pie charts depict the proportion of CP-annotated observations from either dataset originating from each donor (fill color). Top: Br6471 (light gray) n= 1613 nuclei; Br6522 (yellow) n= 1549 nuclei. Bottom: Br6522 (yellow) n= 942 spots; Br8325 (aqua) n= 225 spots; Br8667 (dark gray) n= 159 spots; Br3942 (pink) n= 96 spots.

(F) Dotplot indicating the observation-level measurements across donors (x-axis; colors correspond to legend in (E)) when nuclei or spots are grouped by CP or all other cell/tissue types. For the different metrics (y-axis), dot color indicates the scaled average nuclei-level (top) or spot-level (bottom) weight for nmf48 and nmf94 (peach to dark purple color gradient) and the average $\log_2$ CPM expression for *TTR* (grayscale gradient). For the different metrics (y-axis), dot size indicates the proportion of nuclei (top) or spots (bottom), grouped by donor and CP status, with non-zero weights or expression (depending on y-axis metric).

(G) Scatter plots of nmf94 spot-level weights (x-axis) and nmf48 spot-level weights (y-axis), faceted by the broad domain spot annotation.

Spot plots of spot-level (H) $\log_2$ CPM expression of *TTR*, (I) weights of nmf94, and (J) weights of nmf48 in example capture areas from donor Br3942.



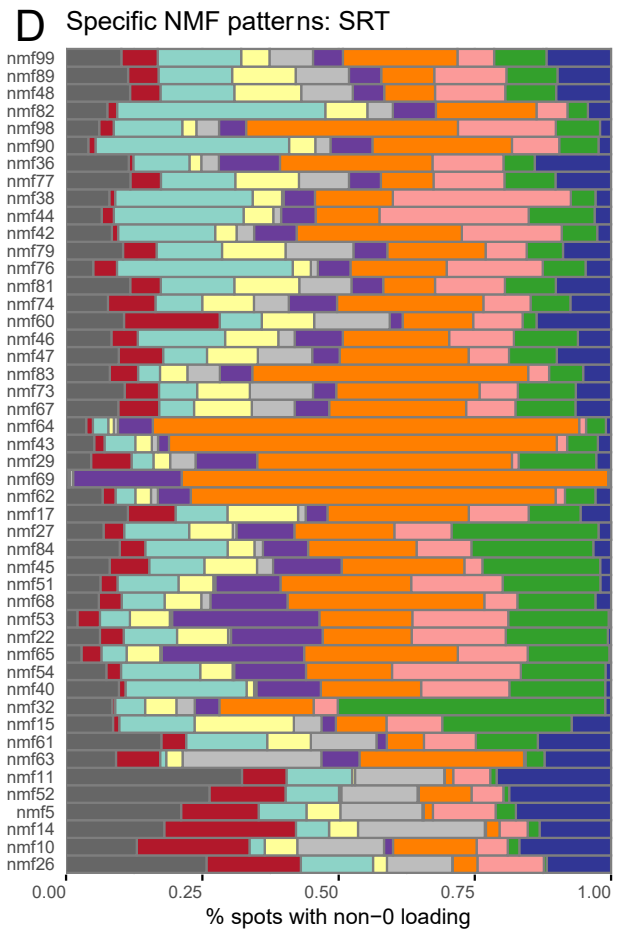
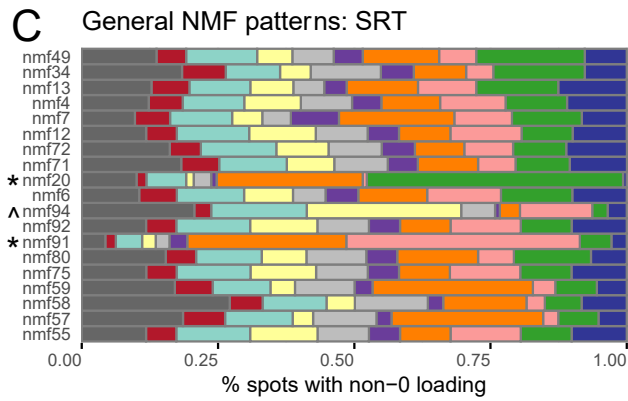
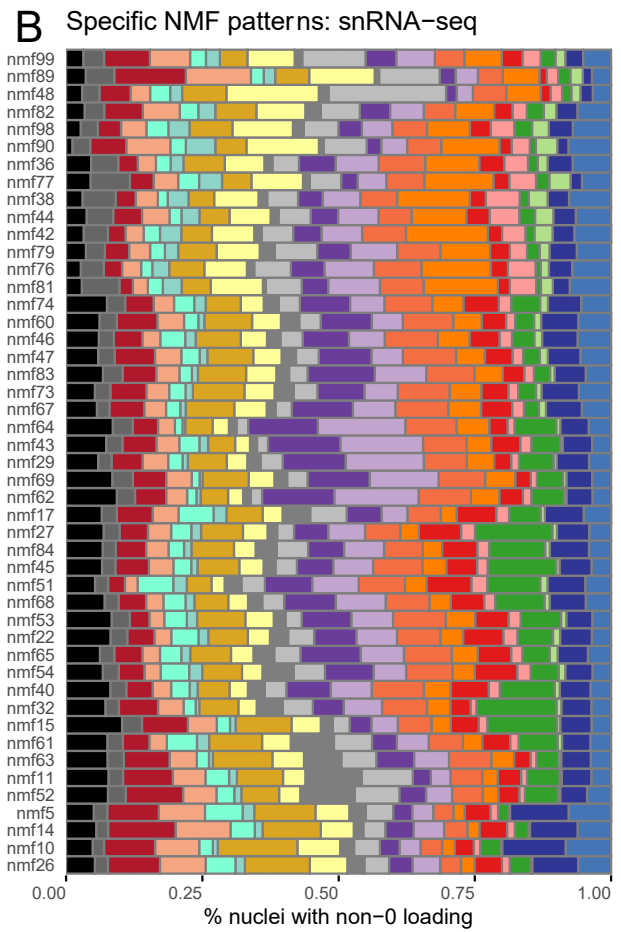
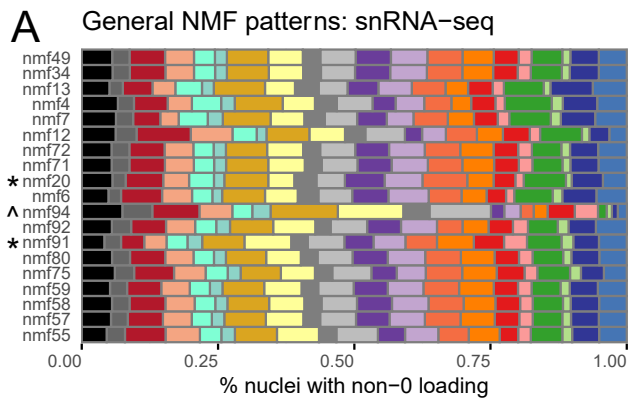
Supplemental Fig. 38. Non-negative matrix factorization (NMF) patterns by donor and sample.

(A) Bar plots show the proportion of snRNA-seq nuclei (x-axis) with non-zero weights to general NMF patterns (y-axis) originating from individual samples (fill color, labeled by donor and sorting protocol). Samples originating from the same donor are depicted in monochromatic palettes. nmf94 (carrot) exhibits enrichment in donors from which choroid plexus nuclei were collected (explored in **Supplemental Fig. 37**). nmf20 and nmf91 (asterisks) do not exhibit donor-specific enrichment in snRNA-seq nuclei.

(B) Bar plots show the proportion of snRNA-seq nuclei (x-axis) with non-zero weights to cell type-specific NMF patterns (y-axis) originating from individual samples (fill color, labeled by donor and sorting protocol) (see legend in (A)). Due to differences in tissue composition in the tissue block, samples from some donors, like Br2743 (greens), do not contain much dentate gyrus and therefore have fewer nuclei with non-zero weights in NMF patterns that specifically correspond to these regions (nmf26, nmf10, nmf14, nmf5). Other donors, like Br2720 (blues) have greater enrichment for dentate gyrus tissue and the corresponding NMF patterns.

(C) Bar plots show the proportion of SRT spots (x-axis) with non-zero weights to general NMF patterns (y-axis) originating from each donor (fill color). Other than nmf94 (carrot, discussed in **Supplemental Fig. 37**), nmf91 and nmf20 (asterisks) exhibit substantial donor-specific bias (explored in **Extended Data Fig. 7**)

(D) Bar plots show the proportion of SRT spots (x-axis) with non-zero weights to cell type- and/or domain-specific NMF patterns (y-axis) originating from each donor (fill color, see legend in (C)). As with the snRNA-seq data, differences in tissue composition are evident in certain NMF patterns that map to specific spatial domains that were enriched in specific donors (e.g. Br6423 (orange) and amygdala-associated NMF patterns nmf62, nmf69, nmf29, nmf43, nmf64).



Supplemental Fig. 39. Binarization of non-negative matrix factorization (NMF) patterns for simultaneous visualization on spot plots.

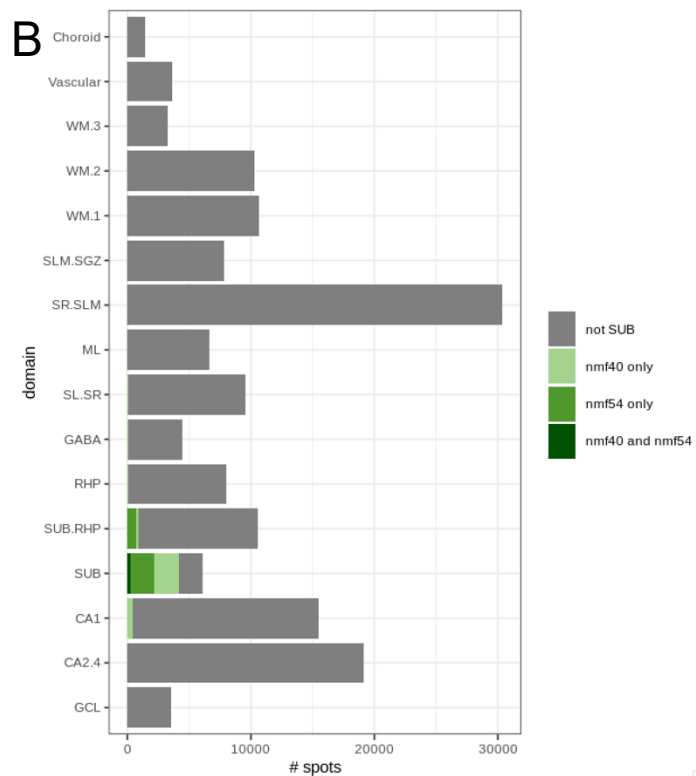
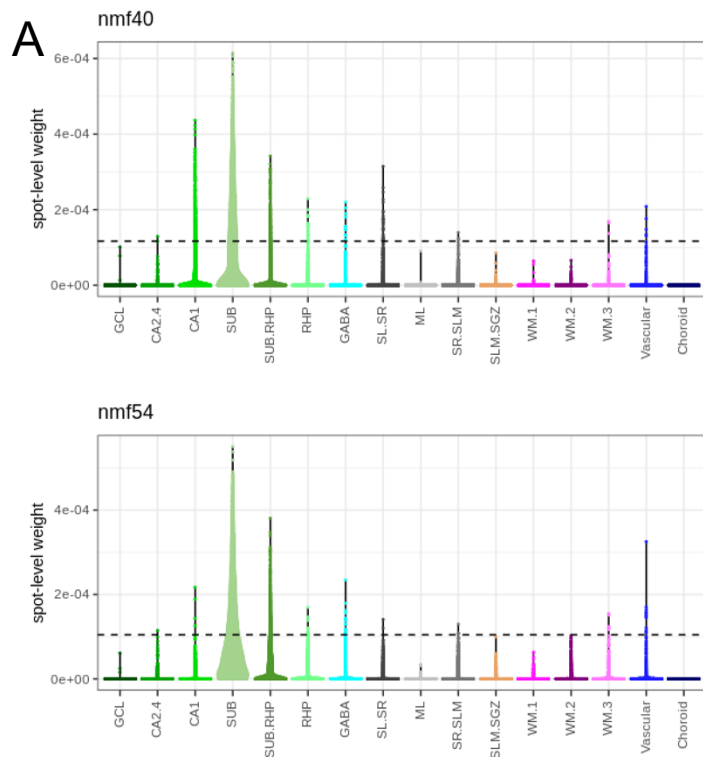
(A) To assist in determining whether RHP pyramidal NMF patterns overlapped with the subicular complex, we binarized the two patterns corresponding to the superficial (nmf40) and middle (nmf54) subiculum (SUB) (**Figure 6A**). Violin plots present the spot-level weights (y-axis) across spatial domains (x-axis). Thresholds are indicated by the dotted lines, determined by taking one fifth of the 95% max spot-level weight.

(B) Spots assigned to the SUB by binarizing NMF patterns were specific to the SUB spatial domain as annotated with PRECAST. Bar plots show the number of spots (x-axis) for each spatial domain (y-axis), colored by the new SUB identity as determined with binary thresholding. Spots not labeled as SUB are present in gray, spots newly labeled as subiculum are present in green, with different shades indicating the largely non-overlapping distribution of nmf40 and nmf54.

(C) Table indicating how many spots were classified to each individual threshold, with 1 indicating classification for the column and colored lightly. The total number of spots classified by each threshold are italicized at the bottom of the corresponding column. Spots that were classified to only one of the domains were included in the final combined annotation (bright colored cells). Final combined annotation sample sizes: 5263 subiculum spots, 12640 CA1 spots, 10430 CA3 spots, 1209 superficial entorhinal cortex (ENT_sup) spots, 1462 entorhinal cortex layer 5 (ENT_L5) spots.

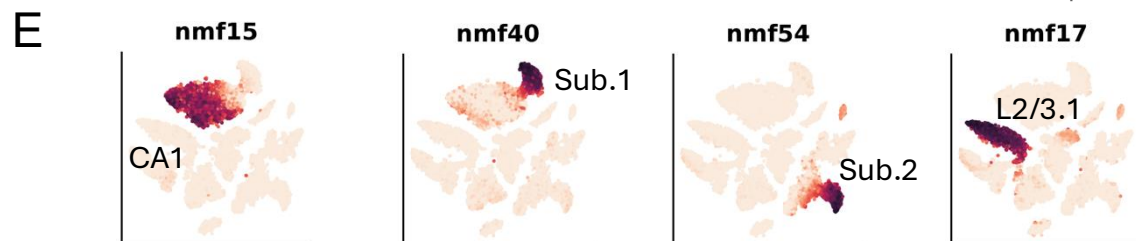
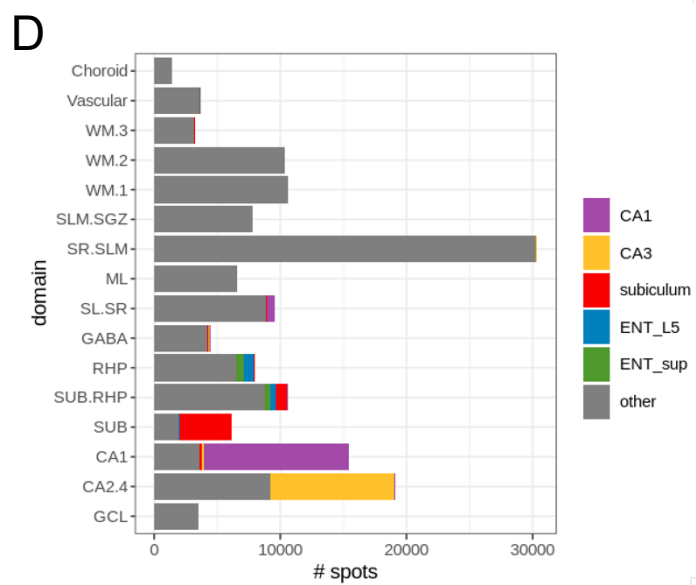
(D) The number of spots (x-axis) labeled by the final combined annotation (color) were distributed across the corresponding domains (y-axis).

(E) T-SNE embeddings of NMF patterns explored in **Figure 6** not otherwise presented.



C

subiculum	CA1	CA3	ENT_sup	ENT_L5	n spots
1	0	0	0	0	5263
1	0	0	0	1	115
1	0	0	1	0	13
1	0	0	1	1	1
1	0	1	0	0	5
1	1	0	0	0	266
1	1	1	0	0	9
1	1	1	0	1	1
0	1	0	0	0	12460
0	1	0	0	1	6
0	1	0	1	0	1
0	1	1	0	0	81
0	0	1	0	0	10430
0	0	0	1	0	1209
0	0	0	1	1	179
0	0	0	0	1	1462
0	0	0	0	0	119416
5673	12824	10526	1403	1764	



Supplement Bibliography

1. Kleshchevnikov V, Shmatko A, Dann E, Aivazidis A, King HW, Li T, et al. Cell2location maps fine-grained cell types in spatial transcriptomics. *Nat Biotechnol*. 2022 May;40(5):661–71.
2. Cable DM, Murray E, Zou LS, Goeva A, Macosko EZ, Chen F, et al. Robust decomposition of cell type mixtures in spatial transcriptomics. *Nat Biotechnol*. 2022 Apr;40(4):517–26.
3. Biancalani T, Scalia G, Buffoni L, Avasthi R, Lu Z, Sanger A, et al. Deep learning and alignment of spatially resolved single-cell transcriptomes with Tangram. *Nat Methods*. 2021 Nov;18(11):1352–62.
4. Huuki-Myers LA, Spangler A, Eagles NJ, Montgomery KD, Kwon SH, Guo B, et al. A data-driven single-cell and spatial transcriptomic map of the human prefrontal cortex. *Science*. 2024 May 24;384(6698):eadh1938.
5. 10x Genomics. spaceranger count, 10x Genomics [Internet]. [cited 2022 Apr 17]. Available from: <https://support.10xgenomics.com/spatial-gene-expression/software/pipelines/latest/using/count>
6. Lun ATL, McCarthy DJ, Marioni JC. A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor. [version 2; peer review: 3 approved, 2 approved with reservations]. *F1000Res*. 2016 Aug 31;5:2122.
7. McCarthy DJ, Campbell KR, Lun ATL, Wills QF. Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R. *Bioinformatics*. 2017 Apr 15;33(8):1179–86.
8. DeBruine ZJ, Melcher K, Triche TJ. Fast and robust non-negative matrix factorization for single-cell experiments. *BioRxiv*. 2021 Sep 1;
9. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010 Jan 1;26(1):139–40.
10. Lun ATL, Chen Y, Smyth GK. It's DE-licious: A Recipe for Differential Expression Analyses of RNA-seq Experiments Using Quasi-Likelihood Methods in edgeR. *Methods Mol Biol*. 2016;1418:391–416.
11. Pachitariu M, Stringer C. Cellpose 2.0: how to train your own model. *Nat Methods*. 2022 Dec;19(12):1634–41.
12. van der Walt S, Schönberger JL, Nunez-Iglesias J, Boulogne F, Warner JD, Yager N, et al. scikit-image: image processing in Python. *PeerJ*. 2014 Jun 19;2:e453.
13. Sriworarat C, Nguyen A, Eagles NJ, Collado-Torres L, Martinowich K, Maynard KR, et al. Performant web-based interactive visualization tool for spatially-resolved transcriptomics experiments. *Biol Imaging*. 2023 Jul 12;3:e15.
14. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: Machine Learning in Python. *Journal of Machine Learning Research*. 2011;12:2825–30.
15. Huuki-Myers LA, Montgomery KD, Kwon SH, Cinquemani S, Eagles NJ, Gonzalez-Padilla D, et al. Benchmark of cellular deconvolution methods using a multi-assay reference dataset from postmortem human prefrontal cortex. *BioRxiv*. 2024 Apr 7;
16. Lee M, Yoon J, Song H, Lee B, Lam DT, Yoon J, et al. Tcf7l2 plays crucial roles in forebrain development through regulation of thalamic and habenular neuron identity and connectivity. *Dev Biol*. 2017 Apr 1;424(1):62–76.
17. Nagalski A, Puelles L, Dabrowski M, Wegierski T, Kuznicki J, Wisniewska MB. Molecular anatomy of the thalamic complex and the underlying transcription factors. *Brain Struct Funct*. 2016 Jun;221(5):2493–

18. Tran MN, Maynard KR, Spangler A, Huuki LA, Montgomery KD, Sadashivaiah V, et al. Single-nucleus transcriptome analysis reveals cell-type-specific molecular signatures across reward circuitry in the human brain. *Neuron*. 2021 Oct 6;109(19):3088-3103.e5.
19. Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. *J Neurosci*. 1987 Aug;7(8):2445–64.
20. Letts VA, Mahaffey CL, Beyer B, Frankel WN. A targeted mutation in *Cacng4* exacerbates spike-wave seizures in stargazer (*Cacng2*) mice. *Proc Natl Acad Sci USA*. 2005 Feb 8;102(6):2123–8.