

1 Spatial Multi-Omics Workflow and Analytical Guidelines for Alzheimer's Neuropathology

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17 Abstract

18 Spatial biology technologies enable high-dimensional profiling within intact tissues,
19 revealing how molecular and cellular organization drives function and disease. As these
20 platforms gain broader adoption, standardized analytical frameworks are needed to ensure data
21 quality and reproducibility. Here, we present an end-to-end pipeline for the GeoMx Digital
22 Spatial Profiler that simultaneously generates whole-transcriptome and 637-protein
23 measurements from user-defined regions within the same tissue sections. The workflow
24 integrates morphology-guided region selection, quality control, normalization, and multi-modal
25 data interpretation. Applied to formalin-fixed cortical tissues from Alzheimer's disease, dementia
26 with Lewy bodies, amyotrophic lateral sclerosis, and controls, the framework resolves spatially
27 distinct molecular domains. Transcript and protein signals diverge across amyloid plaque cores
28 and surrounding glial-rich regions, with RNA–protein concordance varying by disease condition,
29 while single-neuron profiling with and without pathogenic tau deposition illustrates protein assay
30 sensitivity. This dataset provides a rigorously validated resource for spatial multi-omic analyses
31 and establishes broadly applicable guidelines for reliable, reproducible profiling of complex
32 tissues.

33 Introduction

34 Spatial organization underlies the function of biological systems, from single cells to
35 complex tissues. Spatial multi-omics technologies now enable high-plex measurement of RNA
36 and protein within intact tissues, linking molecular states to their architectural context. However,
37 standardized analytical frameworks for these datasets remain limited, particularly for integrated
38 transcriptomic-proteomic analyses. Neurodegenerative diseases exemplify the need for such
39 integrative approaches. Alzheimer's disease (AD)¹ and related disorders, including dementia
40 with Lewy bodies (DLB)² and amyotrophic lateral sclerosis (ALS)³, exhibit spatially
41 heterogeneous pathology that challenges conventional molecular profiling⁴⁻⁷. Amyloid plaques
42 are defining, but not exclusive hallmarks of AD: they are also observed in Parkinson's disease,
43 (DLB)^{8,9}, (ALS)¹⁰, and even in some clinically normal older adults¹¹. Although defined as
44 extracellular aggregates of amyloid β (Aβ), a cleavage product of amyloid precursor protein
45 (APP)¹², plaques contain dozens of associated proteins reflecting complex molecular
46 composition^{13,14}. Transcriptional changes in plaque-adjacent tissue are similarly context

47 dependent, varying by disease stage, brain region, and local cell composition^{5,15-17}. Because
48 plaque cores are largely acellular and RNA abundance is low, transcriptomic data alone cannot
49 fully resolve their biology. Post-transcriptional regulation and differences in assay sensitivity
50 further decouple RNA and protein signals, underscoring the need for integrated, multimodal,
51 spatially resolved measurements.

52 To address these challenges, we applied GeoMx Digital Spatial Profiling (DSP), a high-plex
53 platform that uses oligonucleotide-labeled antibodies and RNA probes to quantify molecular
54 targets within user-defined regions of interest in formalin-fixed, paraffin-embedded human brain
55 tissue¹⁸. We profiled 637 proteins and the whole transcriptome across amyloid plaque-centered
56 and matched control regions collected from primary motor and visual cortices of non-demented
57 controls (NDC), AD, DLB, and ALS cases. Recognizing that housekeeping genes and proteins
58 vary across disease states and pathologies, we systematically evaluated normalization strategies
59 and analytical approaches to ensure data robustness. Integrated RNA–protein analyses revealed
60 spatially distinct molecular domains and frequent RNA–protein discordance.

61 We further evaluated the protein assay sensitivity by profiling individual neurons with and
62 without pathogenic tau deposition as neurofibrillary tangles (NFTs), demonstrating single-cell
63 resolution and detection of neuronal heterogeneity. Finally, a subset of GeoMx protein targets
64 was validated using CellScape, a cyclic immunofluorescence platform that directly detects
65 proteins at subcellular resolution. This orthogonal validation reinforces the accuracy and
66 reproducibility of proteomic measurements.

67 Collectively, these studies establish a biology-informed framework for spatial multi-omics
68 that integrates RNA and protein landscapes with tissue architecture. The resulting dataset serves
69 as a validated resource and practical guide for rigorous, reproducible spatial multi-omic profiling
70 of complex tissues in neurodegeneration and beyond.

71

72 **Results**

73 **Study design and conventional baseline Quality Control (QC)**

74 We used four fluorescently labeled morphology markers to guide ROI selection: Syto83
75 for nuclei, a MAP2 and HuD antibody cocktail for neurons, AT8 (anti-pTauSer²⁰²/Thr²⁰⁵) for
76 neurofibrillary tangles (NFTs), and 6E10 (anti-Aβ N-terminal) for amyloid plaques. These
77 markers were chosen to visualize biologically relevant features without competing with protein

78 panel targets¹⁹. ROIs were selected from primary motor cortex (PMC) and visual cortex (VC)
79 across four neuropathological diagnoses: NDC, AD, DLB, and ALS (Fig. 1A; Table S1). For
80 plaque-associated ROIs, the Center area of illumination (AOI) encompassed the 6E10-positive
81 regions, Ring1 surrounded the Center, and Ring2 surrounded Ring1 (Figs. 1B–C). Adjacent
82 regions lacking 6E10 immunoreactivity were designated as within-tissue negative controls.

83 6E10-positive stains displayed diverse morphologies and sizes across disease conditions,
84 necessitating flexible AOI dimensions (Fig. 1D). We quantified protein and RNA expression
85 using a curated 637-protein panel (G_x), which combines the Immuno-oncology Proteome Atlas
86 with 63 spike-in proteins to interrogate neuropathology, immune states, stress responses, and
87 cellular senescence²⁰ and Whole Transcriptome Atlas (WTA) consisting of ~18,000 RNA targets
88 (Fig. 1E).

89 Proteomic data were processed first, using conventional baseline QC and normalization
90 in the GeoMx DSP Analysis Suite, followed by additional biology-informed QC. This included
91 evaluating amyloid plaque morphology with Cellscape, a cyclic immunofluorescence system
92 with high-dynamic-range microscopy, and confirming 6E10 panel expression; AOIs lacking
93 clear plaque signal were excluded (Fig. 1A).

94 Across all ROIs, comparable numbers of total cells and neurons were profiled (Figs.
95 S1D–E). Despite similar cell counts, AD and DLB cases exhibited lower protein and RNA
96 detection, including fewer raw reads and detected genes (Figs. 1E, S1A–C). While some group-
97 wise differences were statistically significant, overall variance was small, and cell numbers did
98 not correlate with raw read counts.

99

100 Considerations for proteomic data normalization

101 Raw protein counts correlated with AOI surface area (e.g., Center < Ring1 < Ring2; Fig.
102 S1A). To enable comparison across AOIs of differing sizes, we applied surface area-based
103 scaling: each AOI's surface area was divided by the minimum AOI area in the dataset. This
104 adjustment effectively scaled all counts relative to the smallest AOI.

105 We next evaluated five housekeeping proteins (RPS6, calreticulin, GAPDH, histone H3,
106 TOMM20) and negative control IgGs using the “Evaluate-Normalization-Options” script to
107 assess concordance and variance. Calreticulin, RPS6, and TOMM20 exhibited the highest
108 concordance and lowest variability (Figs. 2A, S2A). All housekeeping proteins displayed

109 disease-dependent variation (Fig. 2B). Since normalization to these proteins could obscure
110 biologically meaningful differences, we did not pursue this strategy in downstream analyses.
111 To assess background non-specific binding in the antibody-based GeoMx panels, we examined
112 negative control IgGs (rabbit, mouse IgG1/2b, hamster, and rat IgG2a; Figs. 2C-E). In GeoMx,
113 IgG controls can be used for normalization by scaling AOIs relative to the dataset-wide
114 geometric mean, or for background correction, which divides each protein's raw count by the
115 AOI-specific IgG geometric mean. Normalization balances variation across segments, whereas
116 background correction primarily reduces disproportionately high signals.

117 In our dataset, IgG controls showed high variability and poor concordance, especially at
118 low counts, and elevated IgG signals were limited to a subset of AOIs (Figs. 2C–E, S2B).
119 Comparison with two additional background measures, mean 6E10 fluorescence and negative
120 WTA probe counts, revealed low correlation (Fig. 2F). Notably, elevated IgG counts in the NDC
121 case did not correspond to these measures, indicating that IgGs did not reliably reflect non-
122 specific binding and were unsuitable for normalization.

123 To assess single-cell proteomics, we extended these methods to single-neuron profiling in
124 AD PMC tissue (25 μ m-diameter ROIs around AT8-positive and -negative neurons in cortical
125 layers II and V; Fig. S3E). Housekeeping protein expression varied between AT8-positive and -
126 negative neurons and across cortical layers (Fig. S3A). IgG background signals were inconsistent
127 across ROIs, particularly at low counts, and AT8-negative neurons often displayed more variable
128 IgG signals than AT8-positive neurons (Figs. S3B–F). Because all single-cell ROIs were
129 identical in size, area scaling was inapplicable. However, ROIs with elevated IgG levels also
130 exhibited disproportionately high total target counts, suggesting non-biological signal inflation
131 (Fig. S3F). Therefore, for single neurons, we applied signal-to-background correction, dividing
132 raw counts by the AOI-specific geometric mean of IgG controls.

133 Finally, we demonstrated that the choice of normalization strategy profoundly affects
134 downstream analyses. In the plaque dataset, proteins identified by differential expression (DE)
135 analysis between 6E10-positive and -negative AOIs varied substantially across three approaches:
136 housekeeping normalization, area scaling, and IgG-based background correction (Fig. 3). Each
137 method identified largely distinct sets of differentially expressed proteins (DEPs), with markedly
138 different distributions in volcano plots (Figs. 3A, 3B). Similar effects were observed in single-
139 neuron ROIs (Figs. 3C, 3D), with housekeeping normalization producing highly biased signals.

140 These results highlight that, in some cases, proceeding with minimally processed or raw data
141 may be the most appropriate approach. Based on these observations, we scaled plaque AOI
142 counts to the surface area of the smallest plaque without applying housekeeping or IgG-based
143 normalization, while choosing signal to background correction for single neurons.

144

145 **Biology-informed segment QC**

146 Non-specific staining of morphology antibodies can lead to inclusion of ROIs that do not
147 reflect relevant pathology. To ensure that selected AOIs captured authentic amyloid plaque
148 features, we applied additional “biology-informed” segment QC following conventional baseline
149 QC. We first quantified plaque load across all GeoMx-analyzed tissues. An auto-thresholding
150 algorithm with size-exclusion criteria was applied to the 6E10 channel within three 2 mm × 2
151 mm regions per brain section. Non-specific signals were identified and manually excluded from
152 final counts. Consistent with neuropathological diagnoses, no plaques were detected in NDC
153 cases, whereas plaque counts in other subjects correlated with Thal staging (Fig. 4A). While
154 6E10 recognizes the C-terminal region of A β (Fig. 4B), bright, speckled signals in NDC tissue
155 did not resemble true plaque morphology (Fig. 4C) and were excluded by automated
156 thresholding (Fig. 4A).

157 For the remaining cases, we validated that 6E10-positive regions corresponded to true
158 plaques by examining a subset of proteins from the G_x panel, including A β_{1-42} and known plaque
159 co-aggregate TDP43. Normalized counts confirmed that AOIs from AD and ALS cases exhibited
160 molecular features consistent with true plaques (Fig. 4D). Independent staining of serial ALS
161 sections using the orthogonal CellScape platform further confirmed plaque presence (Fig. 5).
162 Based on these evaluations, all 6E10-positive AOIs from AD and ALS cases were retained for
163 downstream analyses, whereas AOIs from NDC and DLB cases were excluded. These biology-
164 informed QC steps ensured that subsequent analyses focused on ROIs accurately representing
165 amyloid plaque pathology.

166

167 **Biology-informed target QC and validation of selective targets from the G_x panel with** 168 **CellScape**

169 G_x protein targets exhibited a broad dynamic range on GeoMx DSP (Fig. S4). To validate
170 selected targets across this range, we stained serial sections from the same FFPE blocks on

171 CellScape. Antibodies included MAP2, NeuN, 6E10, CD34, and A β 1-42, using the same clones
172 as in the G_x panel. MAP2, NeuN, and 6E10 were used for morphology, while CD34, NeuN, and
173 A β 1-42 represented low and high expressers on GeoMx, ensuring validation across differing signal
174 intensities (Fig. 5A).

175 Neuron staining with MAP2 and NeuN was robust on CellScape, with morphology
176 comparable to GeoMx readouts (Figs. 5A, C). A β 1-42 signals co-localized with 6E10 in the ALS
177 case, consistent with GeoMx measurements (Figs. 5C–E). CD34, a marker of vascular
178 endothelial cells²¹, exhibited generally low counts in GeoMx AOIs, which could reflect either
179 low antibody sensitivity or sparse vasculature (Figs. S4, 5A). On CellScape, CD34 staining
180 clearly delineates the vasculature (Figs. 5G, H). This comparison indicates that low GeoMx
181 CD34 counts correspond to regions with sparse CD34 \square cells, whereas high counts reflect dense
182 vascularization.

183 Based on these validations, we retained all G_x protein targets for downstream analysis
184 rather than pruning low-count targets. This approach preserves biologically meaningful variation
185 and ensures that both low- and high-abundance proteins inform spatially resolved tissue features.
186

187 **Proteomic data renormalization and transcriptomic data processing**

188 Proteomic data can require renormalization depending on AOI selection and the
189 normalization strategy employed. Following biology-informed segment and target QC, only
190 AOIs representing non-specific 6E10 staining were excluded. Counts had already been scaled to
191 the minimum AOI surface area (from an AD case), and no further renormalization was
192 performed. This approach preserved biologically meaningful variation while avoiding the
193 introduction of normalization artifacts that could distort downstream analyses.
194

195 **Biological insights: RNA and protein concordance**

196 With AOIs confirmed to reflect authentic plaque-associated regions, we processed WTA
197 data on the Rosalind.bio platform. Center AOIs were excluded due to low cell and transcript
198 counts, leaving 79 Ring1 and 83 Ring2 AOIs for downstream analysis. Because RNA and protein
199 were measured from the same ROIs, we directly compared abundance for QC-passed segments.
200 Antibodies recognizing post-translational modifications or targeting multiple proteins (e.g., pan-
201 lamin A/B/C) were excluded, resulting in 223 RNA/protein pairs. Pearson correlations across all

202 AOIs revealed positive, negative, and near-zero correlations (Figs. 6A–B). Positively correlated
203 pairs included *MAP2*, *MBP*, *UCHL1*, and *BACE1*, reflecting essential neuronal functions and
204 neuropathology. In contrast, *RPS6*, *FN1*, *MME*, and *FAS* were among the most negatively
205 correlated pairs (Fig. 6C), highlighting cases where protein abundance was largely decoupled
206 from transcript levels or subject to opposing regulatory mechanisms.

207 Pairs with some correlation strength (defined as above or below the mean ± 0.5 SD,
208 chosen to capture intermediate relationships) fell within a wide range of functional categories,
209 including neuropathology, metabolism and bioenergetics, and cell fate and survival. However, no
210 clear trend emerged linking strongly positive or negative correlations to any specific functional
211 group (Fig. 6D). Minimally correlated pairs (within mean ± 0.5 SD) spanned diverse functional
212 categories as well. Genes that encode key neurodegeneration-associated proteins such as *APP*,
213 *MAPT*, and *TARDBP* exhibited complete decoupling between transcript and protein abundance.
214 Important signaling molecules, such as *TP53*, *STING1*, *BCL2*, and *H2AX*, also showed minimal
215 RNA–protein correlation (Fig. S5).

216 Stratification by disease condition revealed similar overall distributions of correlation
217 coefficients (Fig. 6E), but individual targets often displayed marked shifts across conditions,
218 indicating that RNA/protein relationships may be disease dependent (Fig. 6F). For instance,
219 *NRGN* (neurogranin) exhibited strong RNA–protein correlation specifically in the DLB case but
220 showed minimal or negative correlation across other disease conditions. Conversely, *SOD2*
221 displayed strong positive correlation in the diseased individuals while displaying negative
222 correlation in the NDC case (Fig. 6G).

223

224 **Biological insights: G_x and WTA panels detected disease-state dependent changes**

225 We next evaluated whether this multi-omic approach captures molecular responses
226 associated with plaque pathology. DE analysis of G_x proteomics data, using a linear mixed model,
227 compared AOIs from plaque cores to matched control regions. As expected, A β_{1-42} was strongly
228 upregulated in plaque center AOIs, confirming that the assay accurately detected the core
229 pathological signature (Fig. 7A). Other DEPs included proteins involved in inflammation,
230 antigen presentation, and glial activation (Fig. 7A). In contrast, the surrounding
231 microenvironment exhibited relatively few changes (Fig. 7B), suggesting a localized molecular
232 signature in which immune cells are tightly associated with plaque cores. Comparing DE results

233 from Center AOIs before and after biology-informed QC revealed that this additional step not
234 only changed the number and identity of DEPs but also altered their statistical significance and
235 fold-change magnitudes, underscoring the importance of biology-informed ROI selection (Fig.
236 S6).

237 DE analysis of WTA data on the Rosalind.bio platform revealed only modest overlap in
238 differentially expressed genes (DEGs) between Ring1 and Ring2 AOIs (Fig. 7B). Gene ontology
239 analysis using g:Profiler indicated that Ring1 DEGs were enriched for pathways involved in
240 endothelial cell migration, consistent with the observed upregulation of α -SMA protein, a marker
241 of pericytes and endothelial cells within the plaque core, as well as response to ischemia and
242 stress fiber assembly. In contrast, Ring2 DEGs were enriched for pathways related to protein
243 export and plasma membrane localization, reflecting spatially distinct alterations in secretion and
244 membrane-associated signaling (Fig. 7C). Together, these findings demonstrate that integrated
245 proteomic and transcriptomic profiling captures a spatially resolved gradient of molecular
246 responses to plaque pathology, providing a multi-modal view of localized immune, vascular, and
247 cellular signaling changes in neurodegenerative disease.

248

249 **Discussion**

250 This study establishes a biology-informed framework for applying spatial multi-omics to
251 postmortem brain tissue across neurodegenerative diseases. By integrating high-plex proteomic
252 and transcriptomic profiling with rigorous normalization, quality control, and orthogonal
253 validation, we address a central limitation in current spatial biology workflows — the lack of
254 standardized, reproducible analytical approaches. This framework preserves biologically
255 meaningful variation, enables accurate RNA–protein integration, and supports robust
256 interpretation of complex tissue organization in both health and disease.

257 Protein normalization is a critical first step. Protein signals are sensitive to AOI/ROI-
258 specific factors, antibody performance, and background noise, where errors at this stage can
259 propagate into downstream RNA–protein integration. We evaluated commonly used
260 normalization strategies, including housekeeping proteins, IgG negative controls, and AOI/ROI
261 area scaling. Housekeeping proteins exhibited disease-dependent variation in both plaques and
262 tau tangles, underscoring that conventional internal references may not be stable in pathological
263 contexts. IgG signals were inconsistent across AOIs/ROIs and did not reliably reflect non-

264 specific background binding. For plaque AOIs, scaling to surface area produced the most
265 consistent results, whereas single-neurons were best corrected using ROI-specific signal-to-
266 background adjustment. Additionally, to enable cross-segment comparisons in downstream
267 analyses, we normalized all plaque AOIs together. Alternatively, AOIs can be separated into three
268 groups and normalized independently using signal-to-background correction, but only if cross-
269 segment comparison is not part of the analysis design. These findings emphasize the importance
270 of context-specific evaluation of protein normalization prior to RNA–protein integration,
271 preserving biologically meaningful variation while minimizing artifacts.

272 Beyond normalization, biology-informed QC was essential for robust analysis.
273 Conventional baseline QC alone can miss tissue artifacts, non-specific antibody binding, and
274 high endogenous autofluorescence, all of which may obscure true pathological signals. By
275 applying segment- and target-level QC guided by morphology, orthogonal validation (CellScape),
276 and pathological context, we ensured that downstream analyses were performed on biologically
277 meaningful, pathologically relevant features rather than technical or anatomical artifacts.

278 Integration of RNA and protein measurements revealed overall poor concordance. A
279 subset of protein targets exhibited minimal or even negative correlation with their corresponding
280 transcripts, and the strength of RNA–protein concordance varied by disease condition. This
281 highlights the risk of inferring protein-level changes from transcriptomic data alone and
282 reinforces the value of integrated multi-omic profiling for accurate biological interpretation.
283 Protein data provided robust detection in plaque cores, where RNA was sparse, revealing glial
284 and immune cell activation tightly associated with plaques. Transcriptomic profiling of
285 surrounding microenvironments captured region-specific gene expression changes across diverse
286 cell types, enabling complementary insights into spatially resolved molecular dynamics.

287 Importantly, this dataset itself represents a valuable resource for the community. It offers
288 high-plex, spatially resolved RNA and protein measurements across multiple neuropathological
289 features and brain regions, enabling further exploration of RNA–protein relationships, immune
290 and vascular microenvironments, and disease-specific molecular signatures. These data can serve
291 as a benchmark for validating analytical approaches and for future studies leveraging spatial
292 proteomics and integrated multi-omics to interrogate complex tissues.

293 Despite these advances, limitations should be acknowledged. The study included a
294 limited number of human cases and brain regions, and our observations should be validated in

295 independent cohorts. Our focus was on currently available normalization and QC strategies, and
296 new computational and experimental approaches will likely continue to emerge. The primary
297 goal was to provide broadly applicable, biology-informed guidelines for rigorous data processing
298 rather than to establish definitive biological conclusions.

299 Overall, this work demonstrates that rigorous, biology-informed QC, normalization, and
300 validation of spatial proteomic and transcriptomic data are important for reliable interpretation of
301 high-plex multi-omic assays. By establishing and validating these workflows, we provide a
302 framework that can be broadly applied across studies, enabling reproducible and accurate
303 exploration of complex tissue organization and molecular pathology in neurodegenerative
304 disease and beyond.

305 **Method**

306 **Human samples**

307 Brain tissues from the cases profiled were provided by the University of
308 Washington's Biorepository and Integrated Neuropathology Laboratory. The neuropathologic
309 diagnoses given at the time of original autopsy evaluation, hematoxylin and eosin (H&E), and
310 immunohistochemical (IHC) stains were reviewed by board-certified neuropathologist C.D.K to
311 ensure consistency in diagnoses.

312 **Tissue section preparation and ROI selection for GeoMx multi-omics profiling**

313 FFPE sections covering either the primary motor or visual cortices were deparaffinized and
314 processed according to the manufacturer's protocol, GeoMx DSP Spatial Proteogenomics Assay
315 manual (Bruker, MAN-10158-05-01). The WTA panel was used for transcript detection, and the
316 protein panel combined the Immuno-Oncology Proteome Atlas (IPA) with 63 spike-in proteins.
317 Because STING was represented in both the IPA and the custom spike-in's, the probe from the
318 IPA panel was excluded from all downstream analyses to prevent redundancy. In total, we
319 profiled 637 biological targets with 5 negative probes (IgGs). Tissue sections were incubated
320 with a panel of fluorescently labeled morphology markers to facilitate ROI selection: tau
321 phosphorylated at Ser202/Thr205 was used as a surrogate marker of neurofibrillary tangles (AT8
322 antibody, conjugated to Alexa Fluor 594 (AF594)²²; amyloid plaques were detected with the

323 6E10 antibody conjugated to AF488; neuronal markers HuD and MAP2 were co-labeled with
324 AF647; nuclei were visualized using SYTO 83 nucleic acid stain. For amyloid plaques, a circular
325 ROI was drawn around each plaque in cortical layer 5, with two concentric rings to capture the
326 surrounding microenvironment. For tau tangles, a 25 μm -diameter circle centered on the cell
327 body was drawn. The total number of ROIs/AOIs is reported in Figure 1.

328 Plaque ROI selection was verified by post-collection at the biology-informed QC step. Plaque
329 load was quantified using Fiji. Multichannel whole tissue scans were exported from the GeoMx
330 DSP platform, and plaque counts were performed on the 6E10 channel. For each sample, three
331 ROIs ($2 \text{ mm} \times 2 \text{ mm}$ each) were analyzed per tissue section, and the mean value was reported as
332 plaque count per 4 mm^2 . An appropriate auto global threshold and size exclusion criteria were
333 applied for each sample to isolate plaque-specific signals. Non-specific signals captured during
334 this process were manually reviewed and excluded from the final plaque count.

335 **GeoMx multi-omics data processing**

336 The G_x protein panel data were processed using the GeoMx Analysis Suite software, with quality
337 control and normalization details provided in the Results section. For the conventional baseline
338 QC, the following exclusions were applied: In the plaque dataset, one AOI was excluded due to
339 low sequencing saturation, one AOI was excluded due to low surface area, and three AOIs were
340 excluded due to uniformly low read counts (read value of 1 across all targets). In the single-
341 neuron dataset, three ROIs were excluded due to low raw read counts, two ROIs were excluded
342 due to a low percentage of aligned reads, and one ROI was excluded due to abnormally high raw
343 read counts across all targets. For the biology-informed QC, all plaque centers and associated
344 rings in DLB and NDC cases were excluded.

345 WTA data were processed using the GeoMx DCC (WTA) Methods workflow in Rosalind.bio
346 following default quality control (QC) metrics. Because non-template controls (NTCs) were not
347 included during sample collection, AOIs that failed sequencing were reassigned as NTCs to
348 ensure compatibility with the Rosalind analysis pipeline. Only AOIs that passed biology-
349 informed QC metrics were retained for downstream analysis. Grubbs' outlier test was applied to
350 identify and remove outlier negative probes. The Limit of Quantification (LOQ) was calculated

351 per AOI as two standard deviations above the geometric mean of the negative probes. Gene
352 detection threshold was set at 0% to select genes above the LOQ for AOI performance evaluation
353 and all center AOIs regardless of their detection level were excluded at this step. An AOI
354 detection rate threshold of 5% was applied to identify AOIs with sufficient gene detection for
355 assessing gene performance. Quantile normalization was used to normalize gene expression
356 values across AOIs.

357 Heatmaps were generated using area-scaled data normalized to a range of -4 to 4 for
358 visualization per ROI or AOI. Because background intensity was measured across the entire ROI,
359 IgG and negative WTA probe counts were calculated as the geometric mean of the three AOIs
360 within each ROI, representing the ROI-wide background values (Fig. 2F). Control and plaque
361 center AOIs were used to generate a heatmap of plaque-associated proteins with outlier AOIs
362 temporarily excluded during -4 to 4 scaling and reintroduced as a capped value of 4 for plotting
363 (Fig. 4D). Specifically, ALS PMC ($\text{A}\beta$ 1-42 and APOE), AD VC ($\text{A}\beta$ 1-40), and NDC PMC (APP)
364 plaque center AOIs were initially excluded to improve visualization of relative protein
365 expression across AOIs.

366 Concordance analysis was performed on the plaque dataset using AOIs that passed biology-
367 informed QC in both RNA and protein workflows. Antibodies recognizing post-translational
368 modifications or multiple proteins were excluded. Protein targets were matched to WTA
369 transcripts by HUGO gene symbol, and Pearson's r values were computed using the R "cor"
370 function.

371 **Differential expression analysis**

372 DE analysis of the G_x data was performed using the GeoMx Data Analysis Suite software.
373 Protein expression between plaque-positive and plaque-negative AOIs (Center, Ring1, and Ring2,
374 respectively) was compared using a linear mixed model (LMM), followed by Benjamini-
375 Hochberg correction to determine \log_2 fold changes and associated p-values. A random intercept
376 for Scan ID was included in the model to account for variability across tissue sections.
377 Differentially expressed proteins with p-value < 0.05 were reported.

378 DE analysis of the WTA data was performed using Rosalind.bio. A LMM was applied to estimate
379 differential gene expression, log \square fold changes, and associated p-values. The model
380 incorporated a random intercept for the assigned Tissue ID to account for variability between
381 tissue samples. To identify significantly differentially expressed genes, we applied the following
382 thresholds: absolute fold change > 1.25 and p-value < 0.05.

383 **Pathway analysis**

384 Pathway enrichment analysis was done using the gprofiler2 package in R. Genes with a p-value
385 < 0.05 from the differential expression analysis were included, and log \square fold change values were
386 used to rank the genes. Gene set enrichment was performed using the Gene Ontology Biological
387 Processes (GO: BP) database, with the default data source provided by g:Profiler. Multiple
388 testing correction was applied using the default g:SCS method. Enriched GO: BP terms were
389 visualized as bar plots, displaying the -log $\square\square$ (p-value) of each term.

390 **Tissue section preparation and image analysis for CellScape staining**

391 Serial sections covering the primary motor cortices from the same human subject were
392 deparaffinized and processed according to the manufacturer's protocol, CellScape Sample
393 Preparation and Instrument Operation manual (Bruker, MAN-10200-02). Tissue sections were
394 incubated with a panel of fluorescently labeled antibodies, using the same clones as those in
395 GeoMx profiling: MAP2, NeuN, CD34, 6E10, A β 1-42, and phosphorylated Tau (pTau S404).

396 ROIs centered on amyloid plaques and control regions, identified by 6E10 staining, were
397 annotated using QuPath (v0.6.0) and exported as multi-channel TIFF images for further analysis
398 in Fiji. Plaque masks were generated using the Moments auto-thresholding method, while control
399 region masks were defined as circular areas of comparable diameter. Mean pixel intensities of
400 6E10, A β 1-42, and CD34 were measured. The Pearson correlation coefficient between 6E10 and
401 A β 1-42 signals was calculated using the Coloc2 plugin in Fiji.

402 **Statistical analysis**

403 Except DE and concordance analyses, all other statistical analyses were performed using Prism
404 10 software (GraphPad). Specific tests used for each figure are indicated in the corresponding
405 figure legends.

406 **Data availability**

407 The pre-quality control and supporting data for all experiments are available from the authors on
408 reasonable request.

409 **Author contributions**

410 Conceptualization: M.E.O. and T.C.O.; data acquisition: T.C.O., A.R., M.I., and O.B.; data
411 analysis and interpretation: X.S., H.R.H., S.K., T.C.O, and M.E.O; writing—original draft: X.S.;
412 editing and final review of the manuscript: all authors; technical and material support: C.D.K.;
413 supervision: J.M.B. and M.E.O.; funding acquisition: M.E.O.

414 **Declaration of interests**

415 M.E.O. has a patent pending, ‘Detecting and Treating Conditions Associated with Neuronal
416 Senescence’ unrelated to this work. M.E.O. is the Director of a Bruker Spatial Biology Center of
417 Excellence.

418 A.R., M.I., O.B., and J.M.B. are or were employees of Bruker Spatial Biology, Inc.

419 The other authors declare no competing interests in relation to this work.

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428 stylistic refinement. The authors take full responsibility for the content of the manuscript.

429 **Figure Legends**

430 **Figure 1. Overview of study design, sample processing, and data analysis.**

- 431 A. Schematic of the study design and data analysis workflow. Primary motor cortices (PMC)
432 and visual cortices (VC) from 4 disease conditions (NDC, AD, DLB, and ALS) were
433 selected for GeoMx DSP multi-omics analysis. ROIs, including plaque and control areas,
434 were selected for both proteomic and transcriptomic profiling (probes used: G_x, immuno-
435 oncology proteome atlas plus customized 63 spike-in proteins; WTA, whole
436 transcriptome analysis). Each ROI was segmented into 3 AOIs (Center, Ring1, and Ring2)
437 based on 6E10 staining for downstream analysis.
- 438 B. Representative immunofluorescent staining of tissue sections. ROI selections were
439 highlighted by white circles.
- 440 C. Representative on-tissue control and 6E10 staining. Plaques were labeled using the 6E10
441 antibody (green, highlighted by white dashed circles), neurons were stained with
442 MAP2+HuD (yellow), tau phosphorylated at Ser²⁰²/Thr²⁰⁵ was stained with AT8
443 (magenta), and nuclei were stained with Syto83 (blue). In each condition, various 6E10
444 staining patterns were detected. The dashed circles in the lower right panel illustrated the
445 segmentation strategy (not to scale).
- 446 D. Surface area of collected AOIs.
- 447 E. Raw counts per AOI across disease conditions. Left: G_x probe counts; right: WTA probe
448 counts.

449 **Figure 2. Comparison of normalization strategies for GeoMx protein readout.**

- 450 A. Correlation between calreticulin and two additional housekeeping probes (RPS6 and
451 TOMM20). Each point represents a segmented AOI, with colors indicating disease
452 condition. Probe counts were scaled to the surface area of each AOI to account for size
453 differences. Since the data were not normally distributed, Spearman's correlation
454 coefficients (r) are reported.

- 455 B. Expression levels of housekeeping probes across disease conditions. Probe counts were
456 scaled to the surface area of each AOI to account for size differences. Significance was
457 determined using Kruskal-Wallis Anova tests with Dunn's correction.
458 C. Correlation between Rat IgG2a (used as a negative probe) and two additional negative
459 probes (mouse IgG1 and hamster IgG). Each point represents a segmented AOI, with
460 colors indicating disease condition. Probe counts were scaled to the surface area of each
461 AOI to account for size differences. Since the data were not normally distributed,
462 Spearman's correlation coefficients (r) are reported.
463 D. Count level of IgGs. Probe counts were scaled to the surface area of each AOI to account
464 for size differences.
465 E. Count level of IgGs across disease conditions. Probe counts were scaled to the surface
466 area of each AOI to account for size differences. Significance was determined using
467 Kruskal-Wallis Anova tests with Dunn's correction. To better visualize the data, one AOI
468 from the ALS case was excluded from the plot due to its high values (Ms IgG2b = 89.7,
469 Rb IgG = 88.7, Hmr IgG = 24.8, Rt IgG2a = 24.3, Ms IgG1 = 45.8).
470 F. Concordance between IgG counts, staining background, and WTA negative probe counts.
471 For each IgG and WTA negative probe, counts from individual AOIs were summed to
472 obtain values for each ROI. The geometric mean was then calculated across all IgGs or
473 WTA probes. Staining background was calculated by measuring the mean pixel intensity
474 in unstained areas of the 6E10 channel. The three metrics were scaled to a range of -4 to 4
475 and plotted on the heatmap (see Methods for details).

476 **Figure 3. Impact of normalization strategies on downstream analysis.**

- 477 A. Overlap of DEPs identified in Center AOIs under three normalization strategies. For the
478 plaque dataset, protein expression was normalized using one of three methods:
479 housekeeping normalization (yellow), scaling to area (green), and background correction
480 using IgGs (blue). Differential expression analysis compared Center AOIs from 6E10-
481 positive regions to those from 6E10-negative regions.
482 B. Overlay of volcano plots of DE analysis under three normalization strategies. DE analysis
483 was performed on center AOIs. Statistical significance and effect size (fold change)
484 varied depending on normalization strategies.

- 485 C. Overlap of DEPs identified in the NFT single-neuron dataset under three normalization
486 strategies. Protein expression was normalized using either housekeeping normalization
487 (yellow), background correction with IgGs (blue), or analyzed as raw data (red).
488 Differential expression analysis compared single neurons with and without NFT.
489 D. Overlay of volcano plots of DE analysis under three data processing strategies. DE
490 analysis was performed on NFT single-neuron dataset. Statistical significance and effect
491 size (fold change) varied depending on normalization strategies.

492 **Figure 4. Biology informed segment QC: validation of plaque ROI selection.**

- 493 A. Quantification of amyloid plaque load across all tissue sections analyzed on the GeoMx
494 DSP platform. Three 2 mm × 2 mm ROIs were drawn per section on 6E10-stained slides,
495 and the average number of semi-automatically detected plaques was reported.
496 B. Schematic illustrating APP processing and A β generation, including the epitope
497 recognized by the 6E10 antibody.
498 C. Representative images of 6E10 (green) staining from each case, highlighting differences
499 in plaque morphology and distribution. Hud/MAP2 counterstaining is in yellow,
500 D. Heatmap of selected G_x panel proteins associated with amyloid pathology. Expression
501 values were scaled to area, and AOIs with extreme outlier values were temporarily
502 excluded during scaling, then reintroduced and capped at a value of 4 for visualization
503 (see Methods for details).

504 **Figure 5. Biology informed target QC: Validation of selected G_x targets on CellScape.**

- 505 A. Signal strength of selected G_x targets from the GeoMx protein readout. Probe counts were
506 scaled to the surface area of each AOI and then log2 transformed
507 B. A β_{1-42} expression level measured by GeoMx. Each dot represents an AOI from either
508 control or plaque center in the ALS case. Significance was determined using unpaired
509 two-tailed t-test; error bar represents SEM.
510 C. Representative CellScape staining of a serial section from the ALS PMC. Plaques were
511 labeled using the 6E10 antibody (green). Neurons were labeled with MAP2 (yellow) and
512 NeuN (magenta).

- 513 D. Representative CellScape staining of a serial section from the ALS PMC. Plaques were
514 labeled using the 6E10 antibody (green) and an antibody against A β ₁₋₄₂ (magenta).
515 Neurons were labeled with MAP2 (yellow).
- 516 E. Quantification of 6E10 and β -Amyloid₁₋₄₂ staining. Plaque region was outlined by
517 automated thresholding algorithm in Fiji. Control regions were selected by drawing
518 circles of comparable size in plaque-free areas. Mean pixel intensity was measured for
519 each region. Significance was determined using unpaired two-tailed t-test; error bar
520 represents SEM.
- 521 F. Colocalization of 6E10 and β -Amyloid₁₋₄₂ staining. Pearson's correlation coefficient (r)
522 was calculated to describe the spatial colocalization between the two markers.
523 Colocalization analysis was performed using Fiji's Coloc2 plugin.
- 524 G. Representative CellScape staining of CD34 (magenta) on the serial section from the ALS
525 PMC. Plaques were labeled using the 6E10 antibody (green). Neurons were labeled with
526 MAP2 (yellow).
- 527 H. Quantification of CD34 signal within the plaque and control region as described in (B).

528 **Figure 6. Concordance between protein and transcript readouts in GeoMx DSP.**

- 529 A. Histogram showing the distribution of Pearson's r values for all matched RNA–protein
530 pairs. All AOIs passed biology-informed QC in both RNA and protein analysis workflow
531 were included in the analysis.
- 532 B. Scatter plots showing the concordance between protein and transcript of *MAP2*(left) and
533 *FNI*(right), respectively. Normalized RNA and protein counts were log₂-transformed
534 prior to plotting. The line represents a linear interpolation fit.
- 535 C. Pearson's r values for the top five most positively and most negatively correlated RNA–
536 protein pairs.
- 537 D. Sankey plot summarizing functional categories of RNA–protein pairs with some
538 correlation strength. Bruker annotations were consolidated into ten functional groups
539 (left), with targets sorted by Pearson's r values (right). Targets with more than one
540 functional annotations were shown in multiple links.

- 541 E. Violin plot showing the distribution of Pearson's r values for all matched RNA–protein
542 pairs. AOIs passed biology-informed quality control in both RNA and protein workflows
543 were stratified by disease condition.
544 F. Box-and-whisker plots showing the range (min to max) of Pearson's r values for each
545 RNA–protein pair across disease conditions. Each line represents one matched pair.
546 G. Heatmap illustrating protein/transcript pairs with the highest and lowest variance in
547 Pearson's r values across disease conditions.

548

549 **Figure 7. GeoMx multi-omics profiling revealed disease-state dependent molecular changes.**

- 550 A. DEPs between plaque and control AOIs. AOIs from all disease conditions were included
551 in the analysis. Dot size represents the $-\log_{10}(p\text{-value})$, and color intensity corresponds to
552 the $\log_2(\text{fold change})$. Differentially expressed proteins ($p < 0.05$) identified in each
553 spatial segment were also displayed in the other two segments. Probe counts were scaled
554 to the surface area of each AOI.
555 B. Number of DEPs and DEGs in Center, Ring1, and Ring2 AOIs. For DEPs, statistical
556 analysis was performed using the GeoMx Analysis Suite, including AOIs from all disease
557 conditions. DEPs were defined by a $p\text{-value} < 0.05$. No DEPs were detected in Ring2
558 AOIs. For DEGs, statistical analysis was performed using the Rosalind.bio platform,
559 including AOIs from all disease conditions. DEGs were defined by a $p\text{-value} < 0.05$ and
560 an absolute fold change > 1.25 .
561 C. Functional enrichment analysis of top DEGs in the immediate environment surrounding
562 plaques. DEGs were ranked by fold change, and analysis was performed using g:Profiler
563 with default settings. Pathway enrichment was conducted within Gene Ontology:
564 Biological Process. Adjusted p-values were corrected for multiple testing using the g:SCS
565 algorithm. Selective pathways were shown.

566 **Supplementary table 1 (related to figure 1). Information on human samples.**

567 **Supplementary figure 1 (related to figure 1). Detailed QC metrics and data processing.**

- 568 A. Raw counts from G_x analysis. Each dot represents an AOI.

- 569 B. Raw counts from WTA analysis. Each dot represents an AOI.
570 C. Number of detected genes from WTA analysis. Each dot represents an AOI.
571 D. Number of cells from each ROI. Syto83 nuclear staining was used for quantification.
572 Significance was determined using Brown-Forsythe and Welch Anova tests with Dunnett
573 T3 correction; error bar represents SEM.
574 E. Number of neurons from each ROI. MAP2+HuD staining was used for quantification.
575 Significance was determined using Brown-Forsythe and Welch Anova tests with Dunnett
576 T3 correction; error bar represents SEM.

577 **Supplementary figure 2 (related to figure 2). Results from the “Evaluate-Normalization-
578 Options” DSP DA Script.**

- 579 A. Overall agreement of the housekeeping proteins. Each dot represents an AOI and colored
580 by disease condition and brain region. The numbers on the plot refer to the correlation
581 variability.
582 B. Overall agreement of IgGs. Each dot represents an AOI and colored by disease condition
583 and brain region. The numbers on the plot refer to the correlation variability.

584 **Supplementary figure 3 (related to figure 2). Considerations of normalization for a single-
585 cell dataset.**

- 586 G. Expression levels of housekeeping probes across distinct neuronal populations. Neurons
587 harboring NFT and nearby tangle-negative neurons (Ctrl) were selected from cortical
588 layers 2 (L2) and 5 (L5) of the ADwNP PMC sample.
589 H. Count levels of IgGs.
590 I. Count levels of IgGs across different neuronal populations.
591 J. Correlation between mouse IgG2b (used as a negative control probe) and other IgG
592 probes. Each point represents an AOI. The zoomed-in panel (displaying AOIs with IgG
593 counts below 25) highlights a loss of linearity and concordance at low count levels.
594 K. A representative image showing varying IgG levels in adjacent neurons. Neurons were
595 stained with MAP2+HuD (yellow). Hyperphosphorylated tau species were stained with

596 AT8 (magenta). Profiled neurons were highlighted by white circles and labeled with
597 identifiers above. The corresponding raw IgG counts were provided in the table.
598 L. Correlation between IgG counts and total target counts for each ROI. The x-axis shows
599 the geometric mean of IgG probe counts for each region of interest (ROI), while the y-
600 axis represents the total raw counts of all protein probes in the corresponding ROI. Since
601 the data was not normally distributed, Spearman's correlation coefficient (R) and
602 associated p-value are reported.

603 **Supplementary figure 4 (related to figure 5). Log₂ signal-to-background ratio for all**
604 **targets.** This plot was automatically generated using the “Evaluate-Normalization-Options”
605 script in the DSP Data Analysis (DA) pipeline.

606 **Supplementary figure 5 (related to figure 6). Sankey plot summarizing functional**
607 **categories of minimally correlated RNA-protein pairs.** Bruker annotations were consolidated
608 into ten functional groups (left), with targets sorted by Pearson’s r values (right). Targets with
609 multiple functional annotations were shown in multiple links; key targets are highlighted.

610 **Supplementary figure 6 (related to figure 7). The impact of biology informed QC on**
611 **downstream analysis.**

- 612 A. Overlap of DEPs identified in Center AOIs before and after biology-informed QC.
613 Protein expression values were scaled to area prior to analysis. Differential expression
614 analysis compared Center AOIs before (green) and after (yellow) exclusion of segments
615 failing biology informed QC.
- 616 B. Overlay of volcano plots of DE analysis with Center AOIs before and after biology-
617 informed QC. Statistical significance and effect size (fold change) varied depending on
618 normalization strategies.
- 619 C. List of DEPs identified under both QC conditions. For each target, statistical significance
620 and effect size (fold change) are reported.

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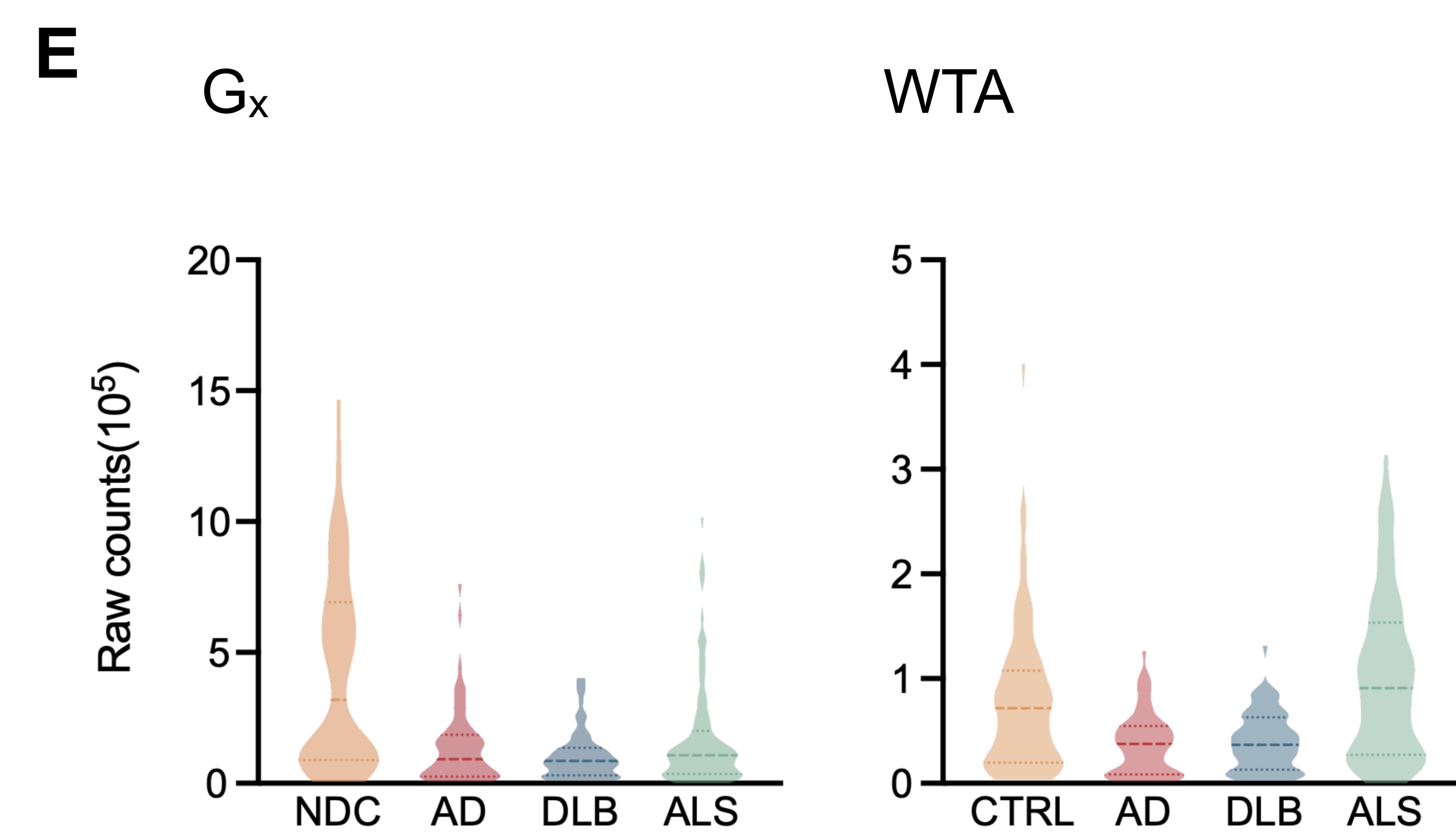
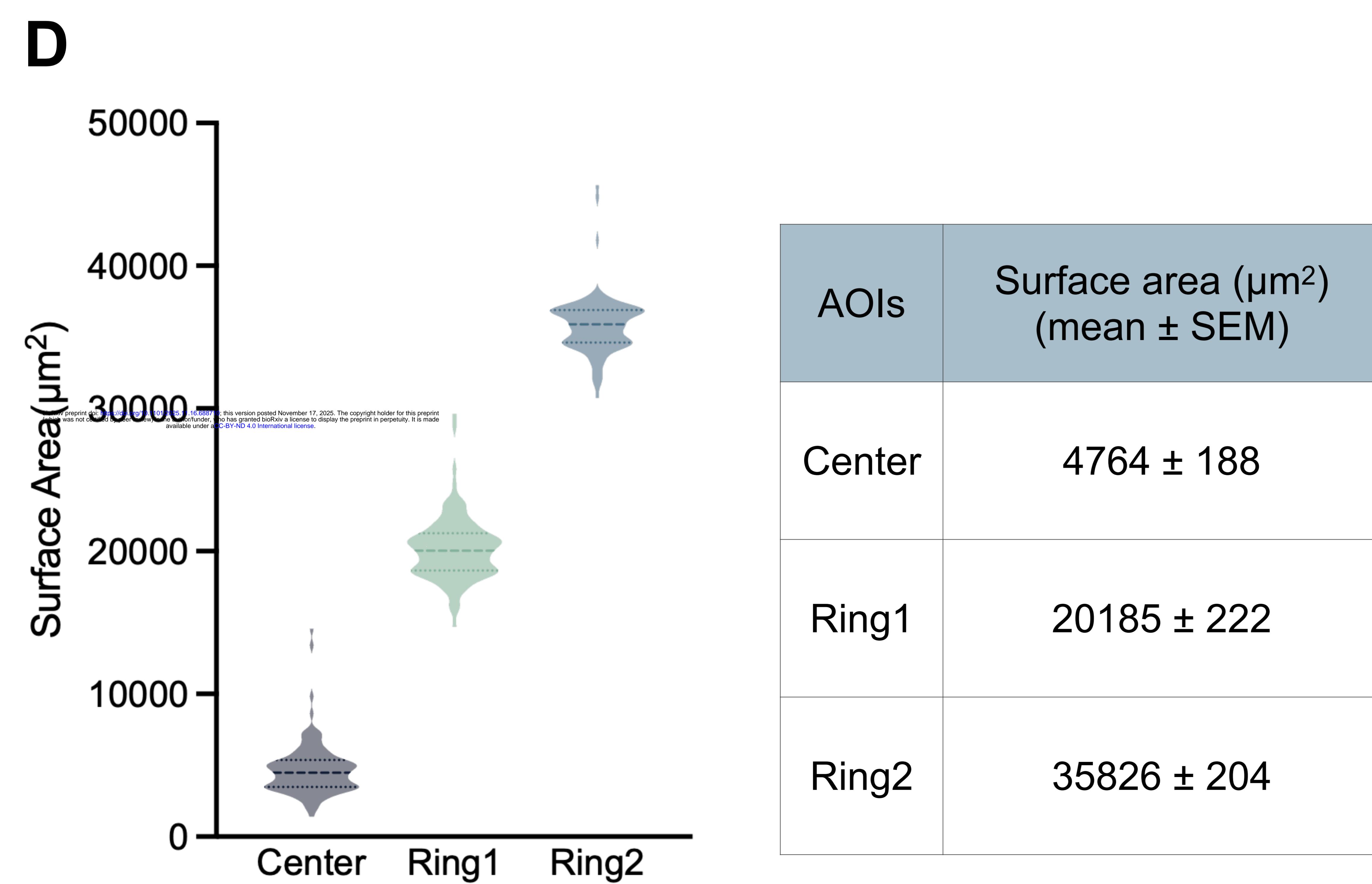
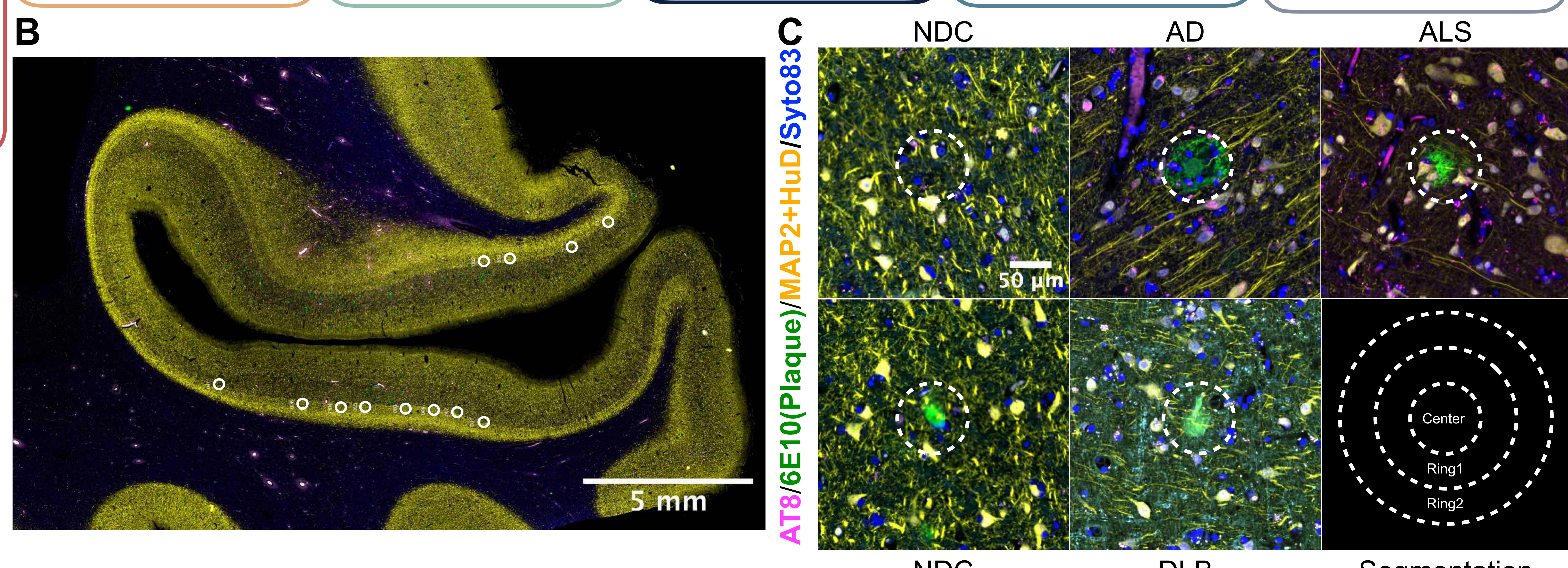
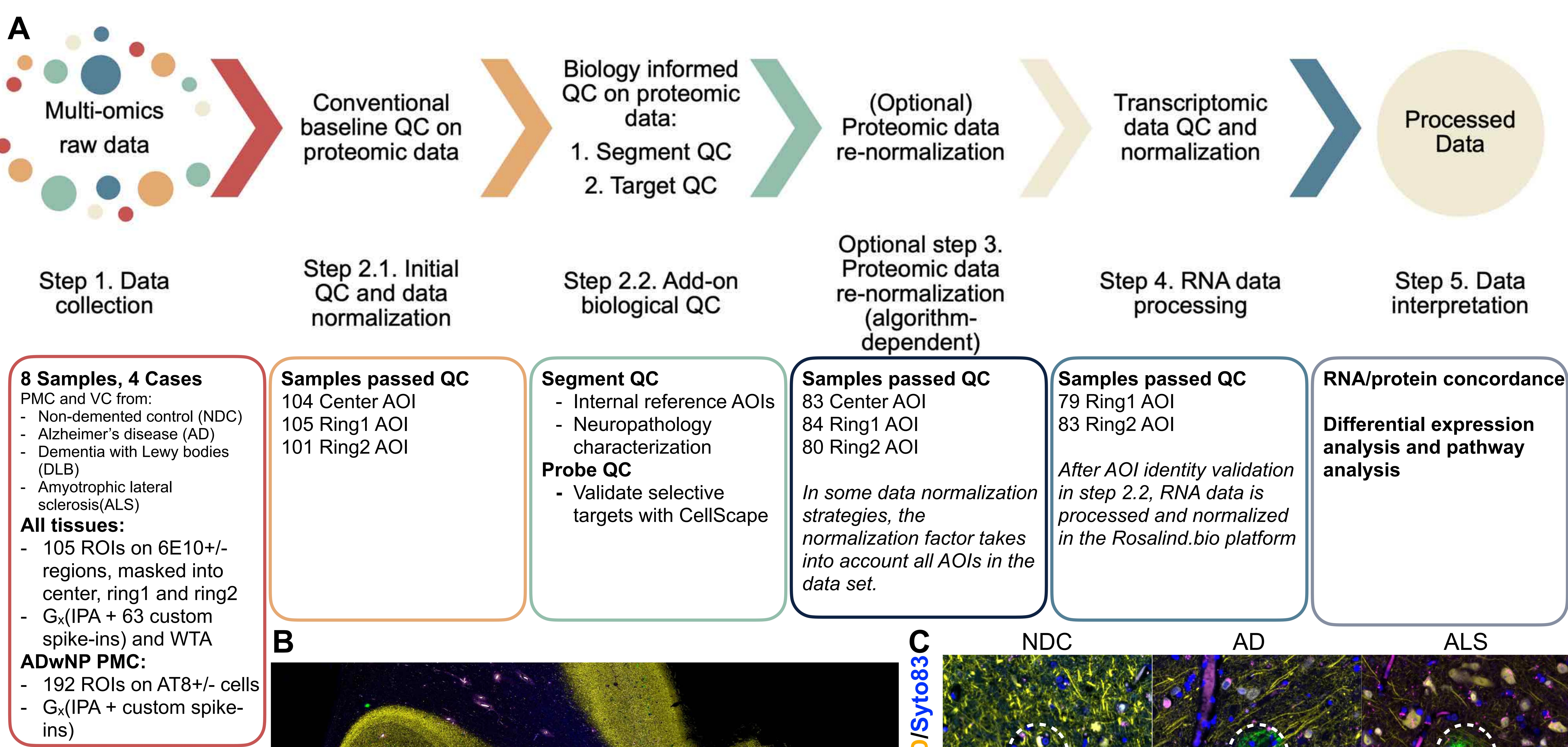


Figure 1

Step 2.1

Housekeeper

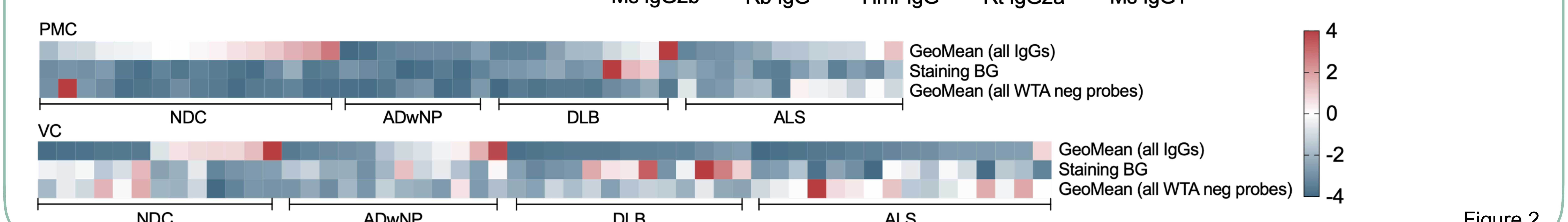
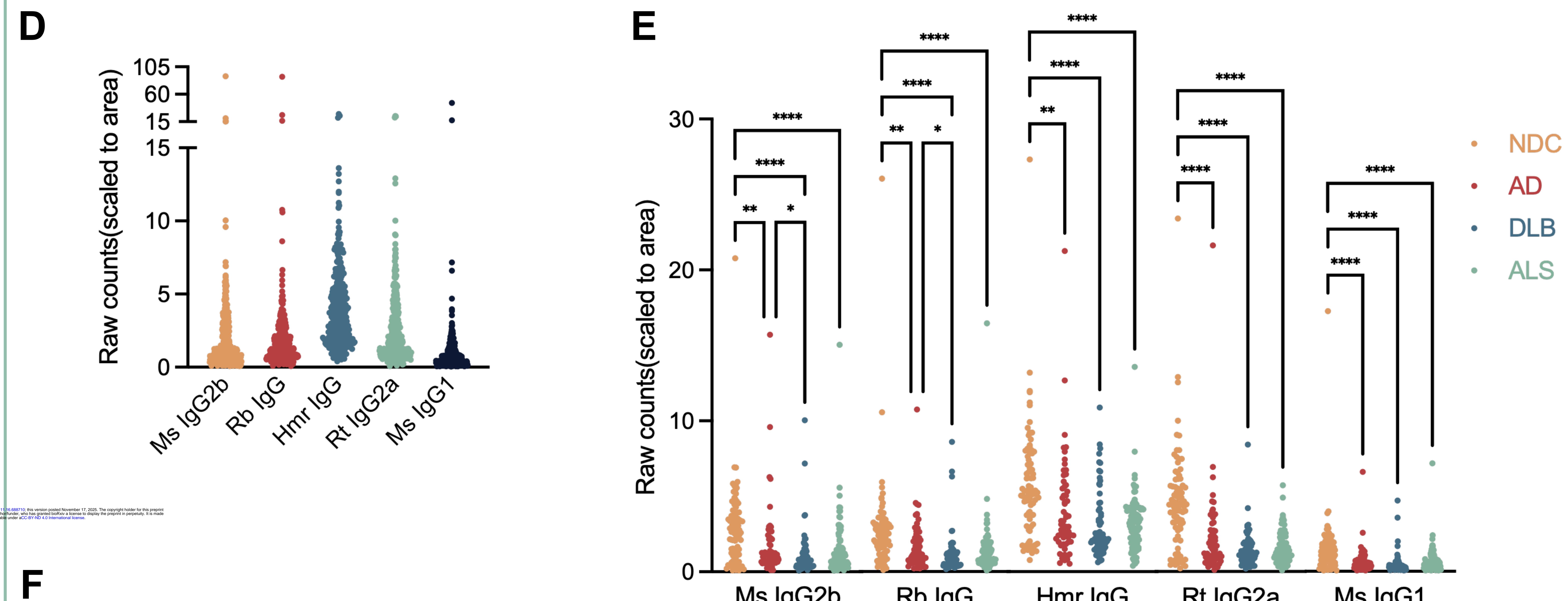
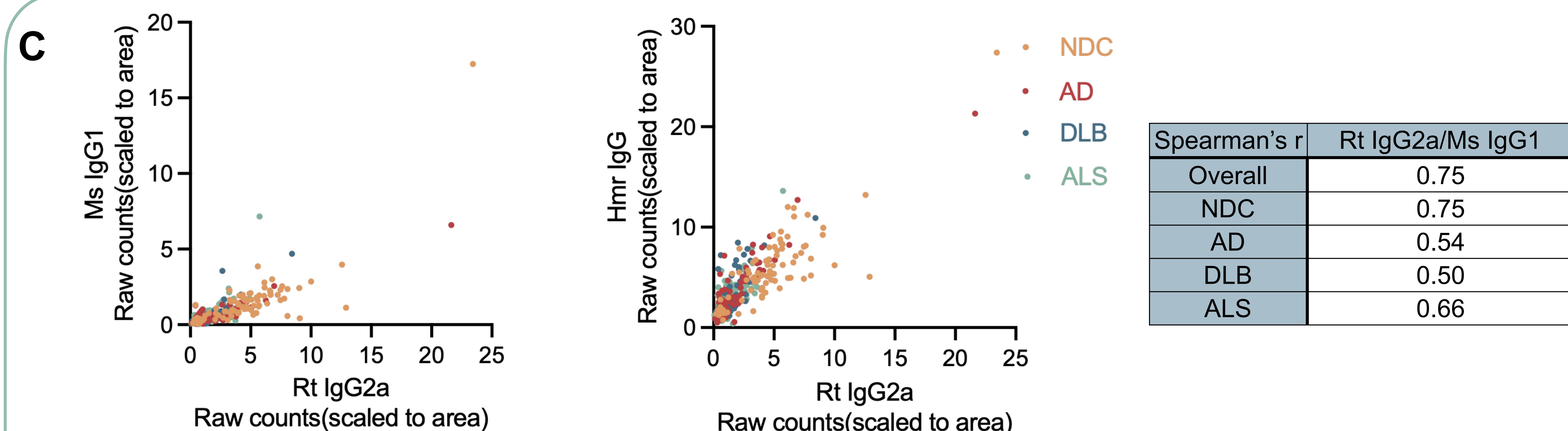
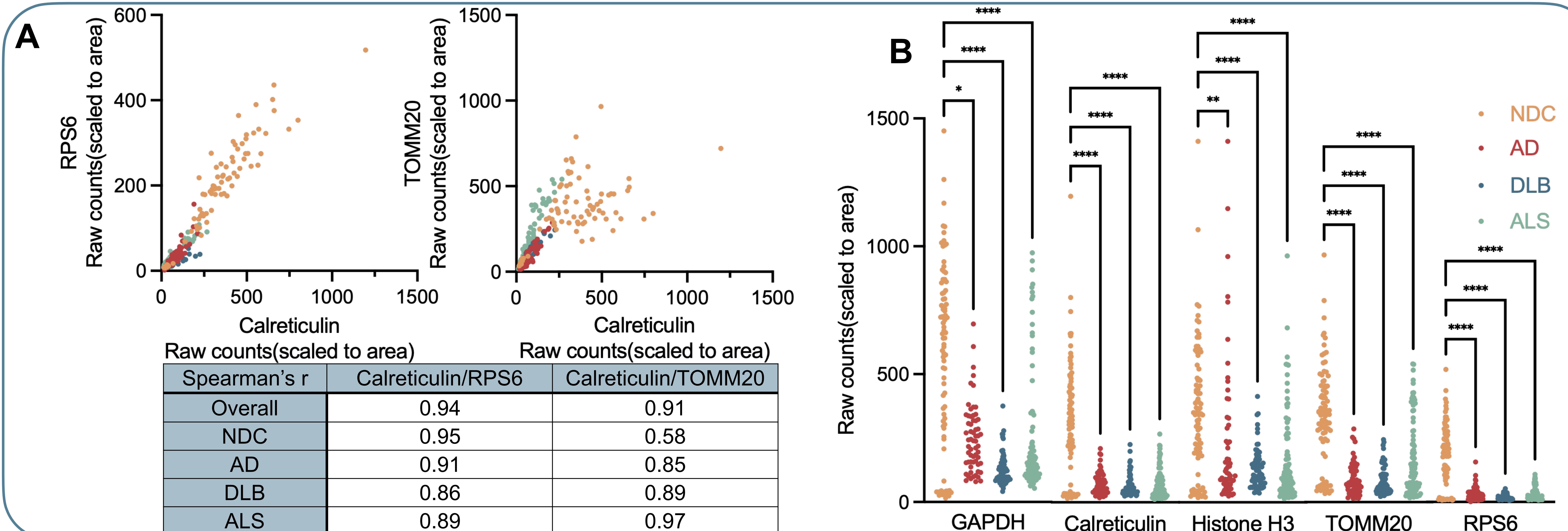
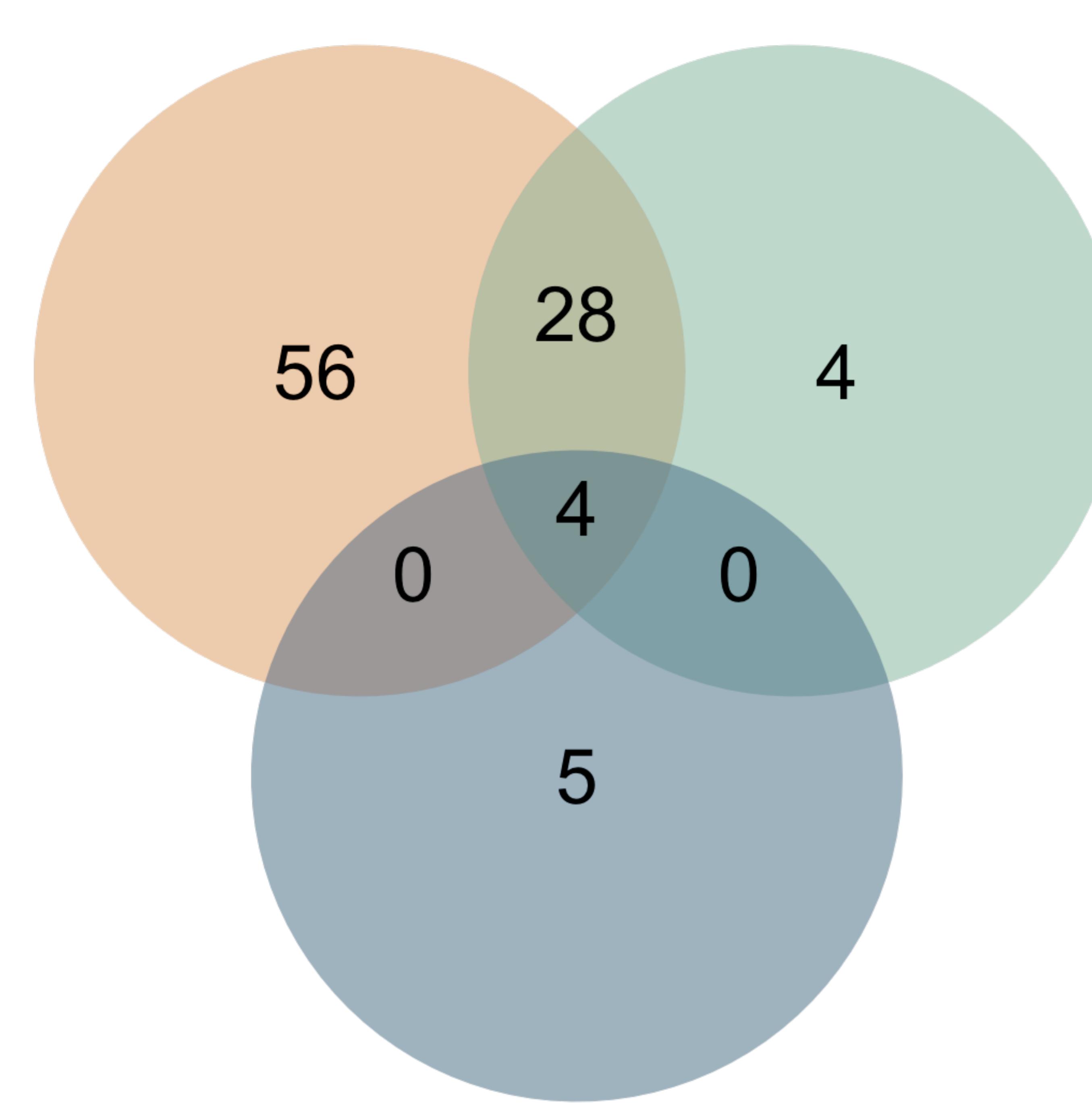


Figure 2

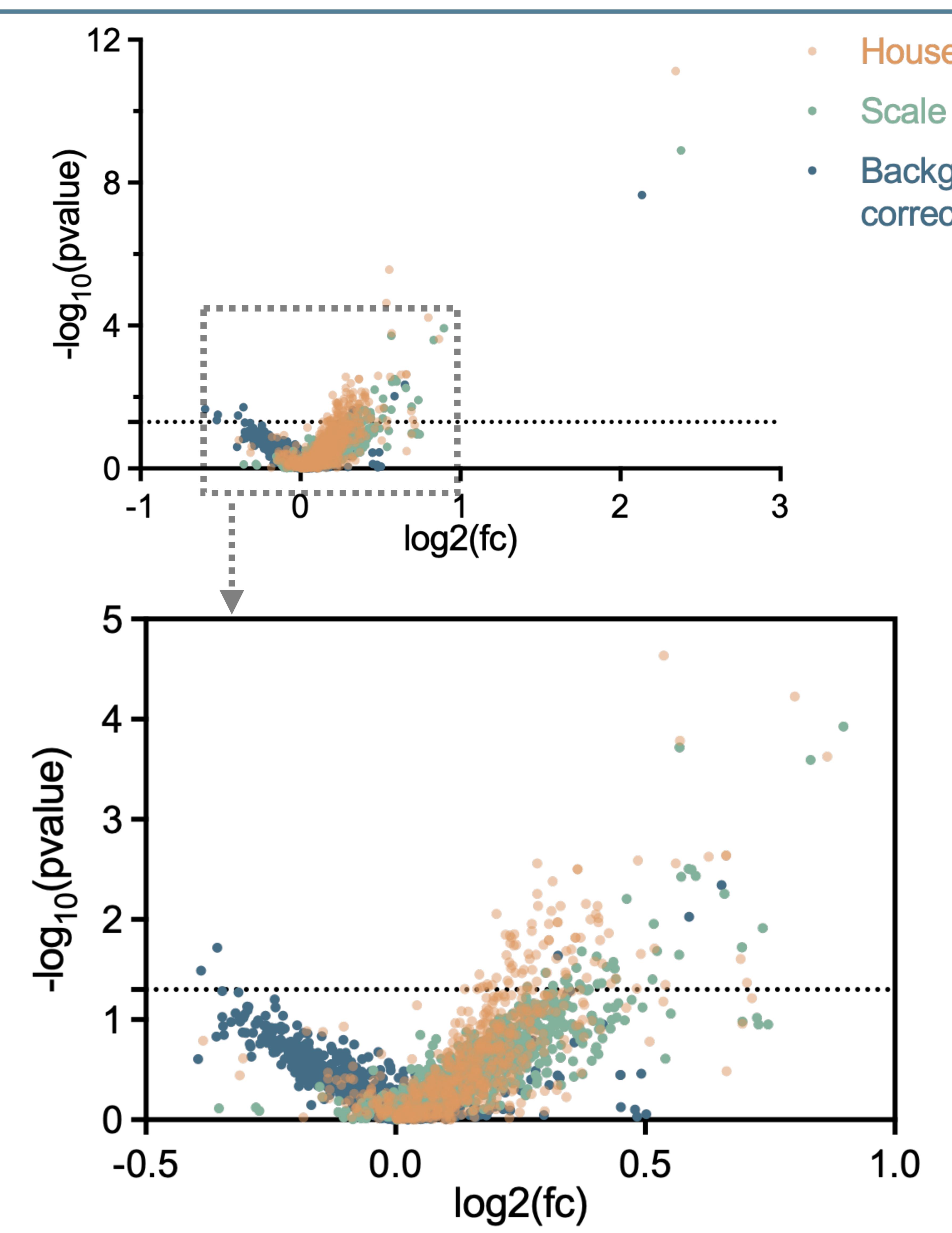
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2.1

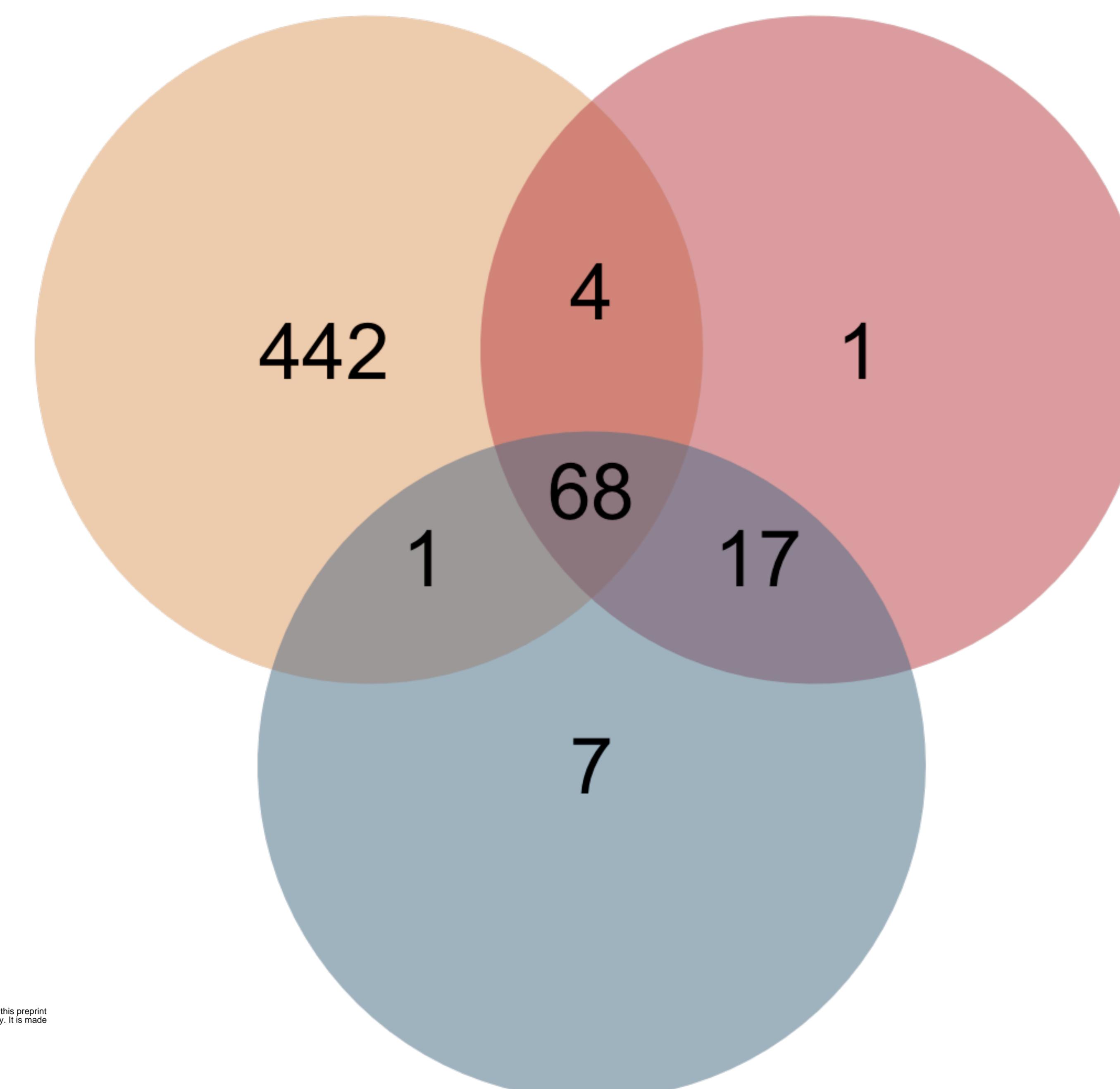
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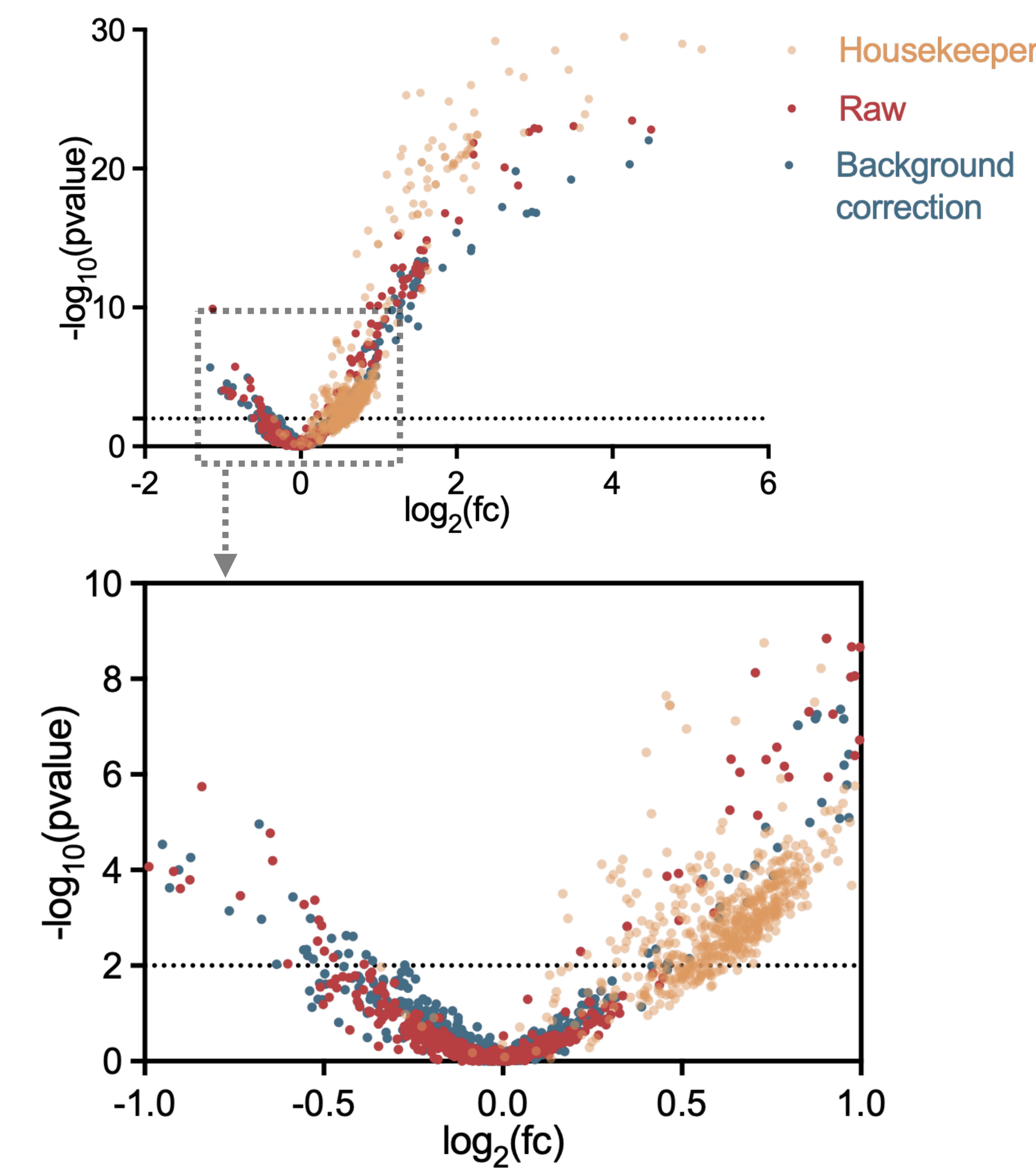
B



C



D



Amyloid data, plaque vs control centers

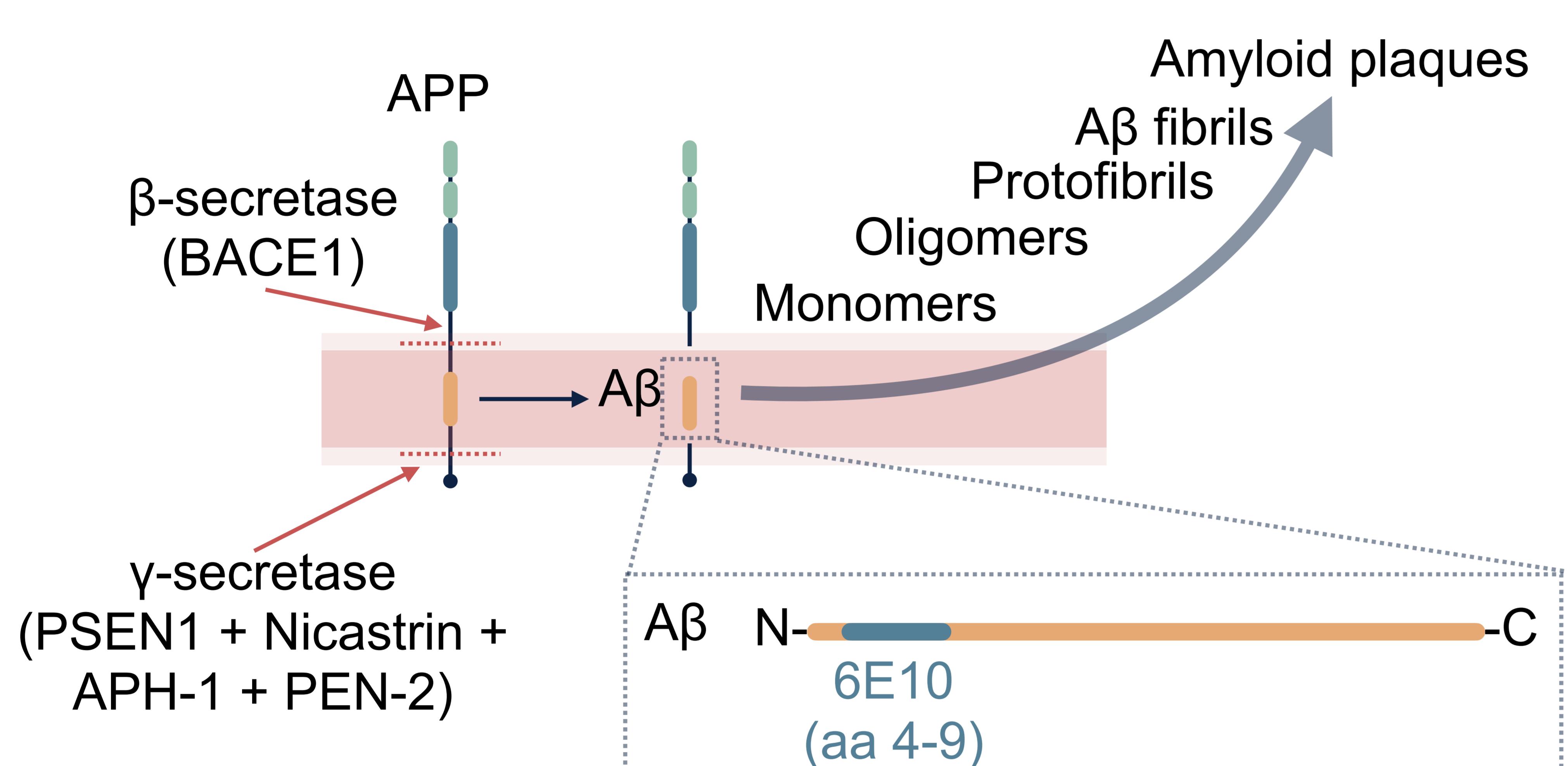
Tangle data

Figure 3

Step A
2.2.1

Disease	Thal	Brain region	Plaque count/4mm ²
NDC	0	PMC	Not detected
		VC	Not detected
AD	5	PMC	84.00
		VC	79.33
DLB	1	PMC	35.67
		VC	12.00
ALS	3	PMC	49.33
		VC	52.67

B



C

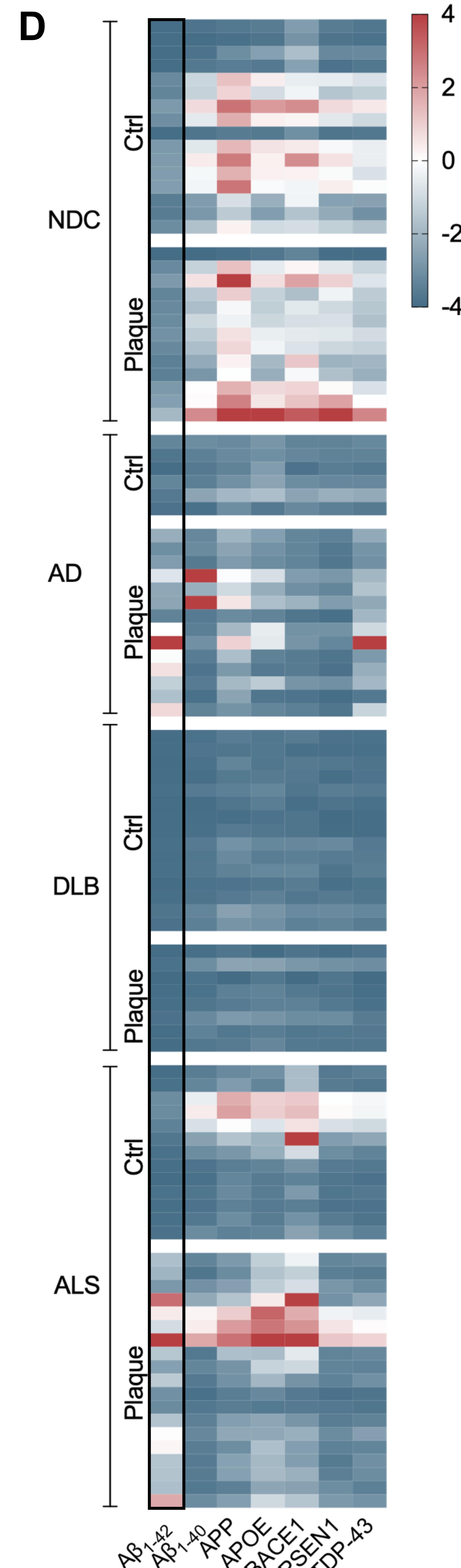
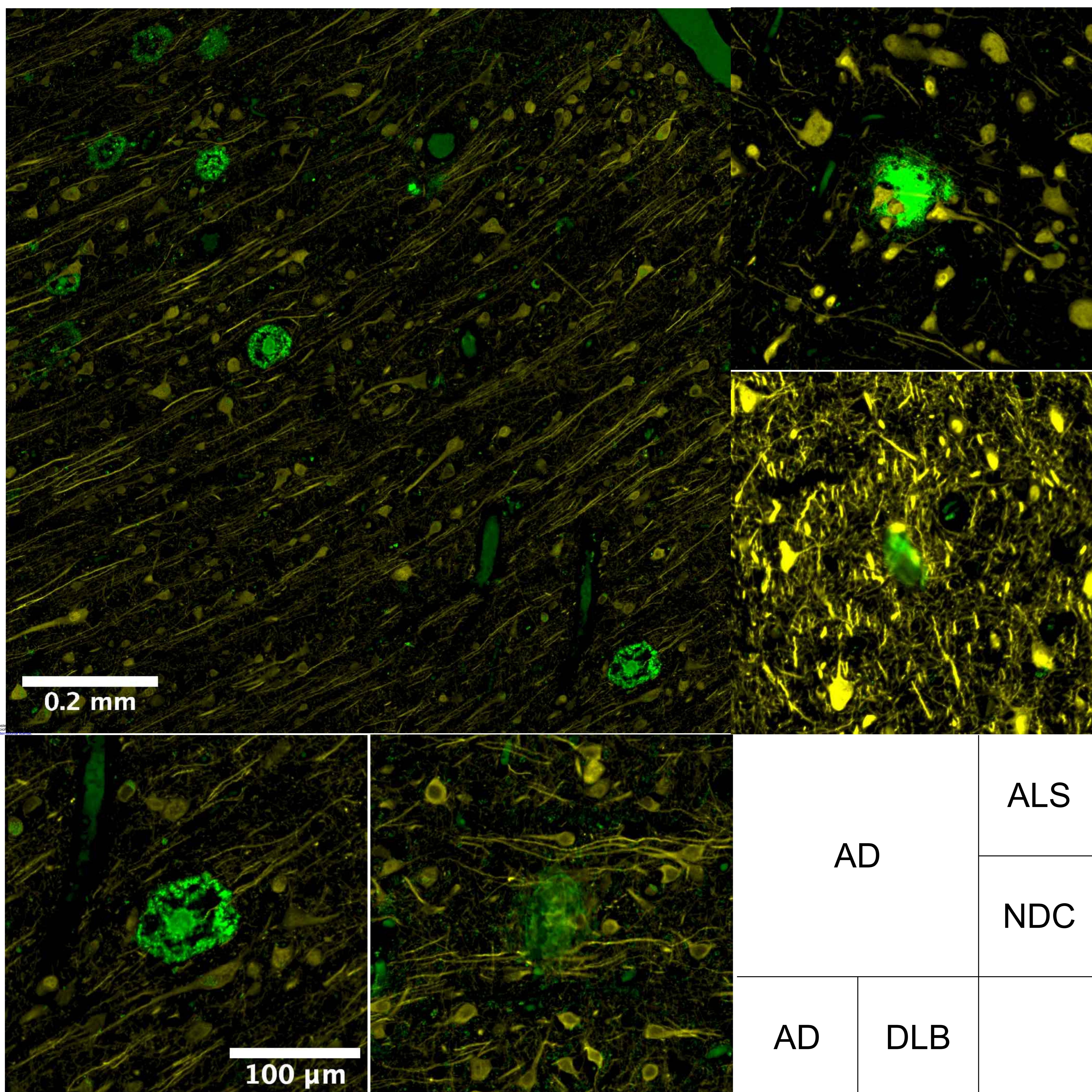
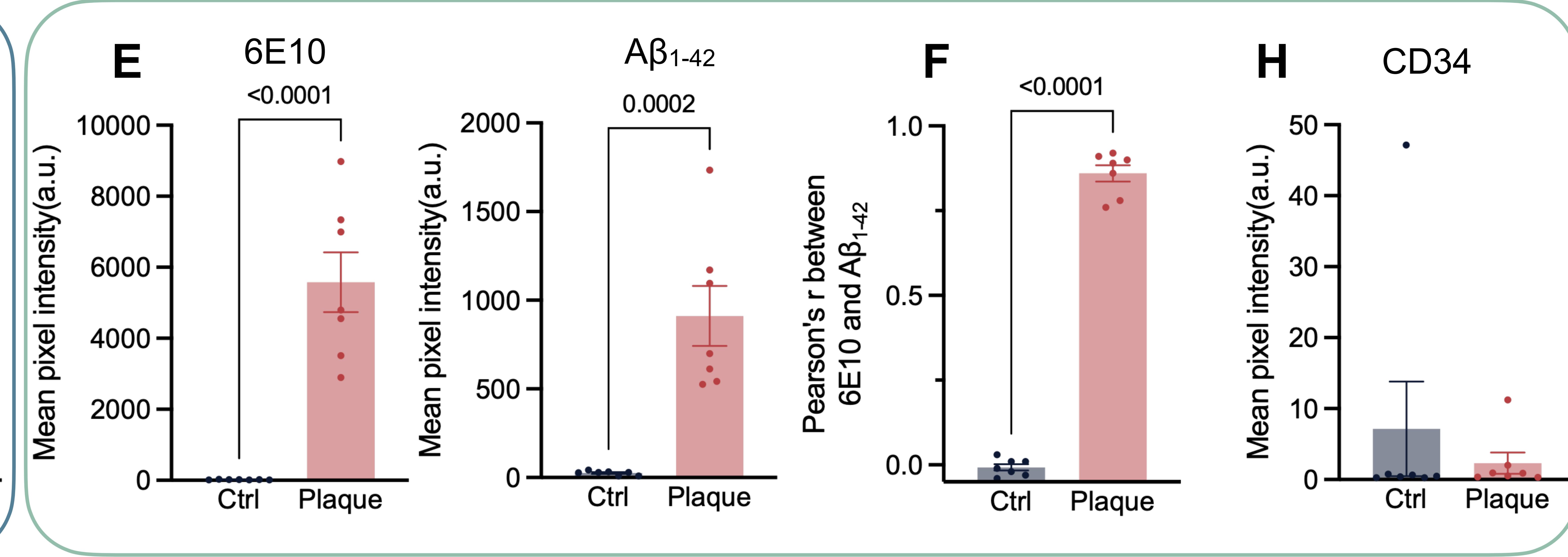
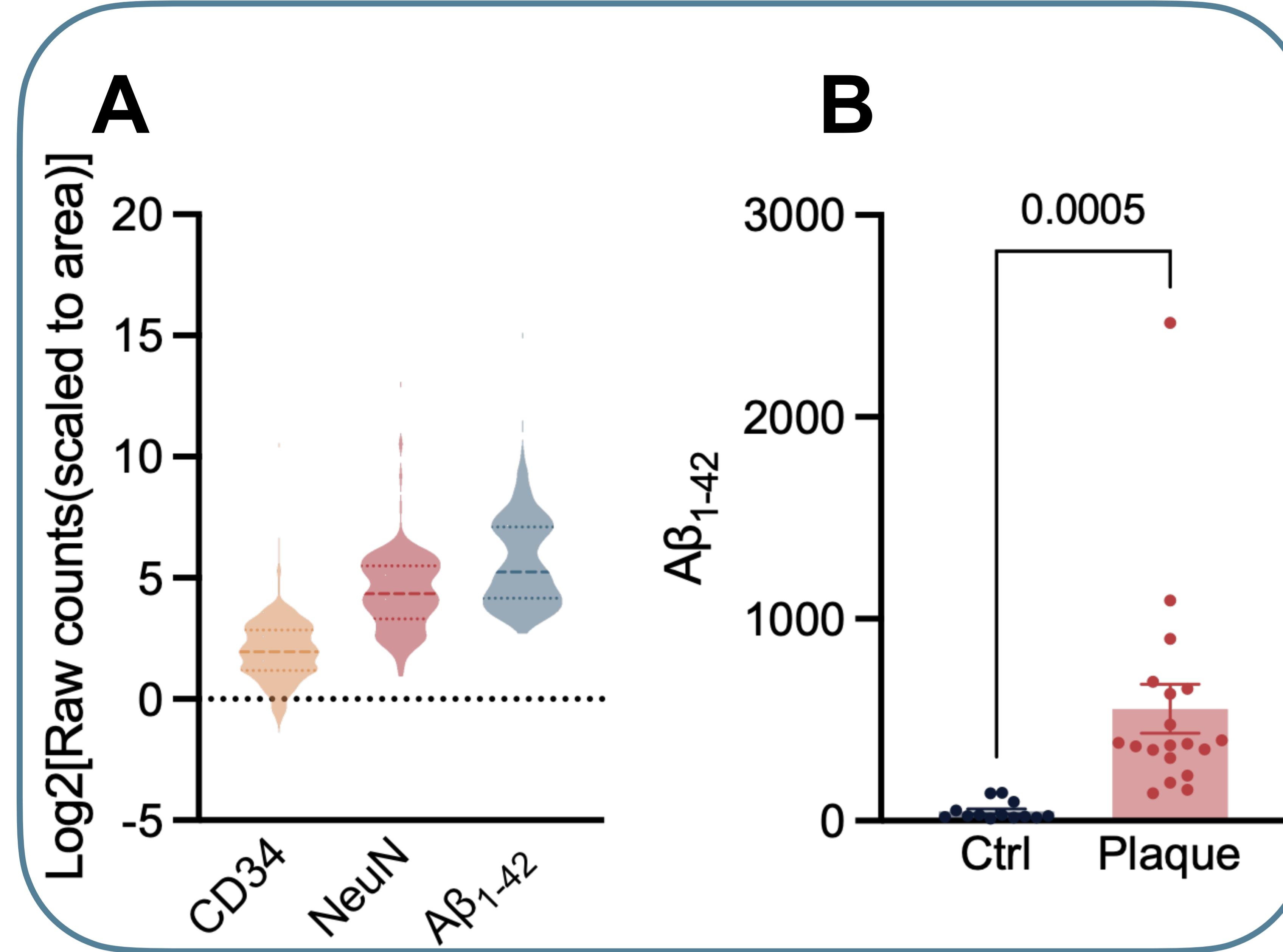


Figure 4

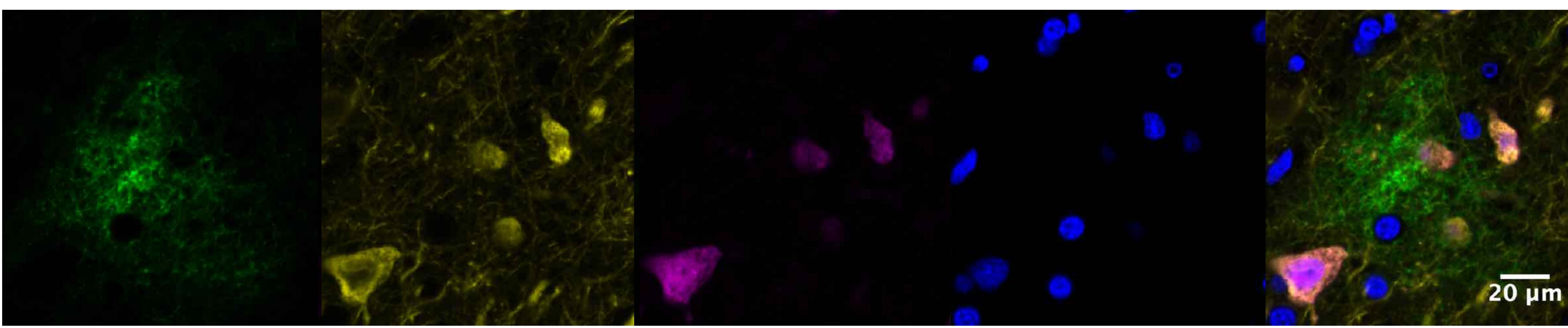
Step 2.2.2

GeoMx Count level



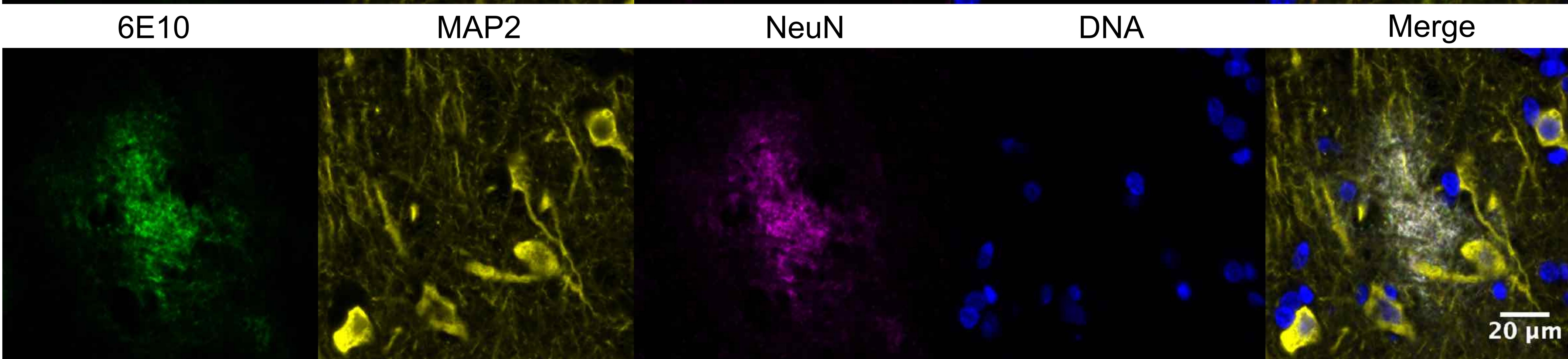
C

ALS



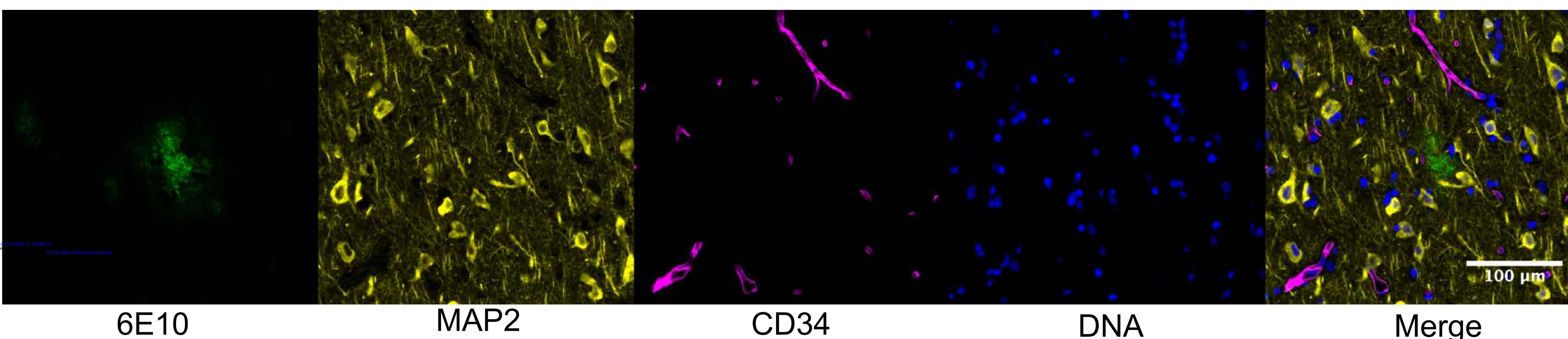
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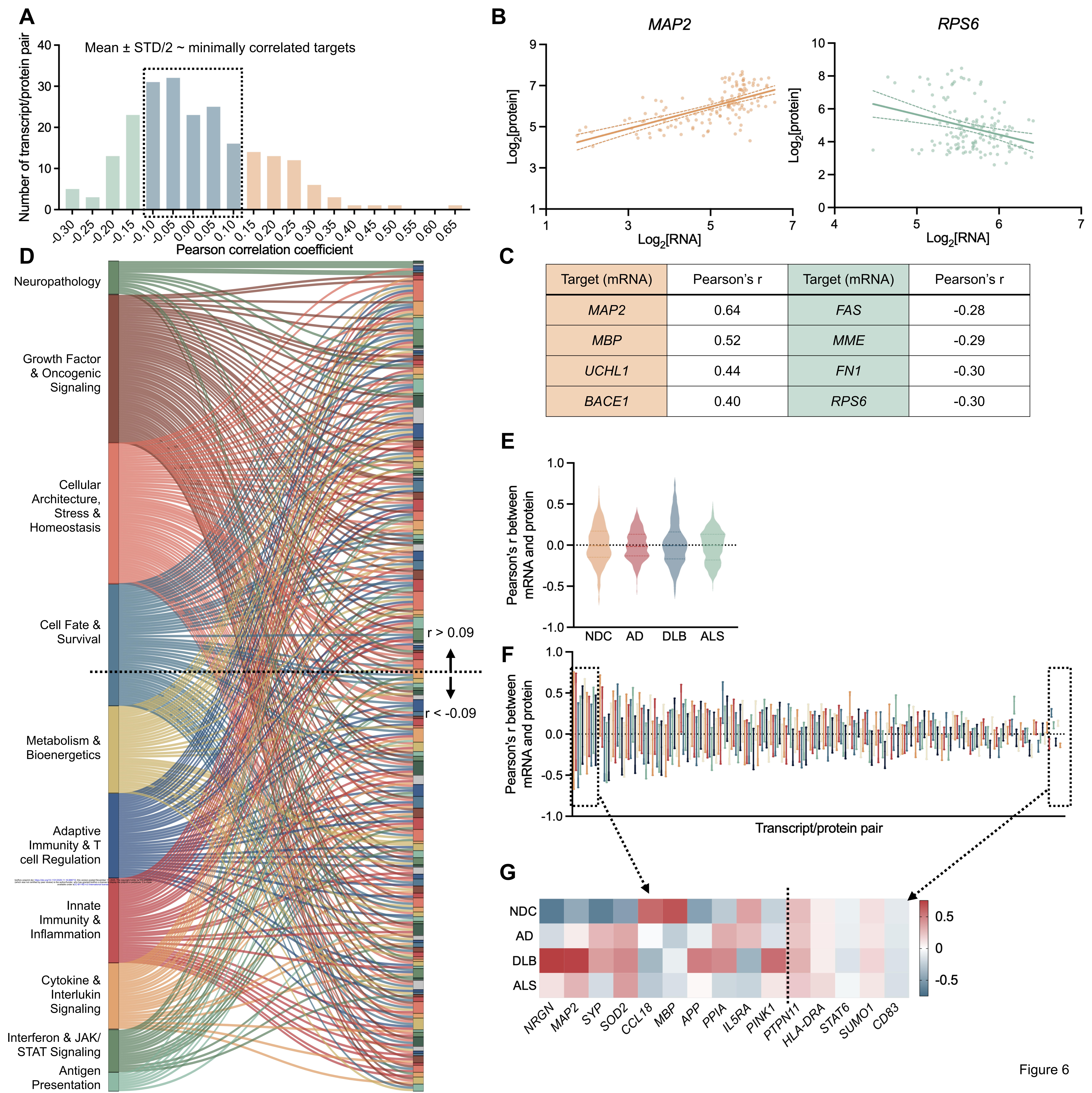
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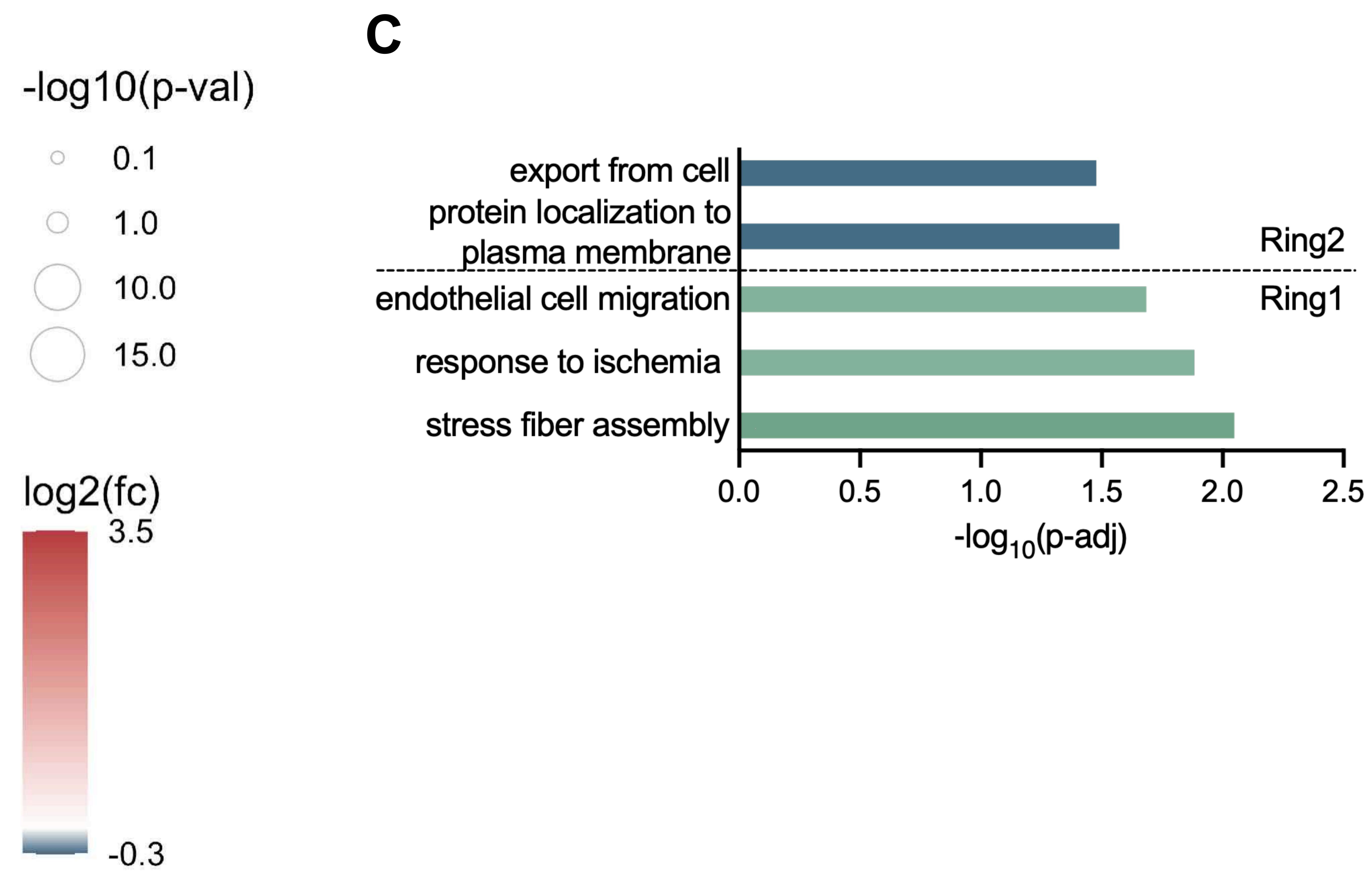
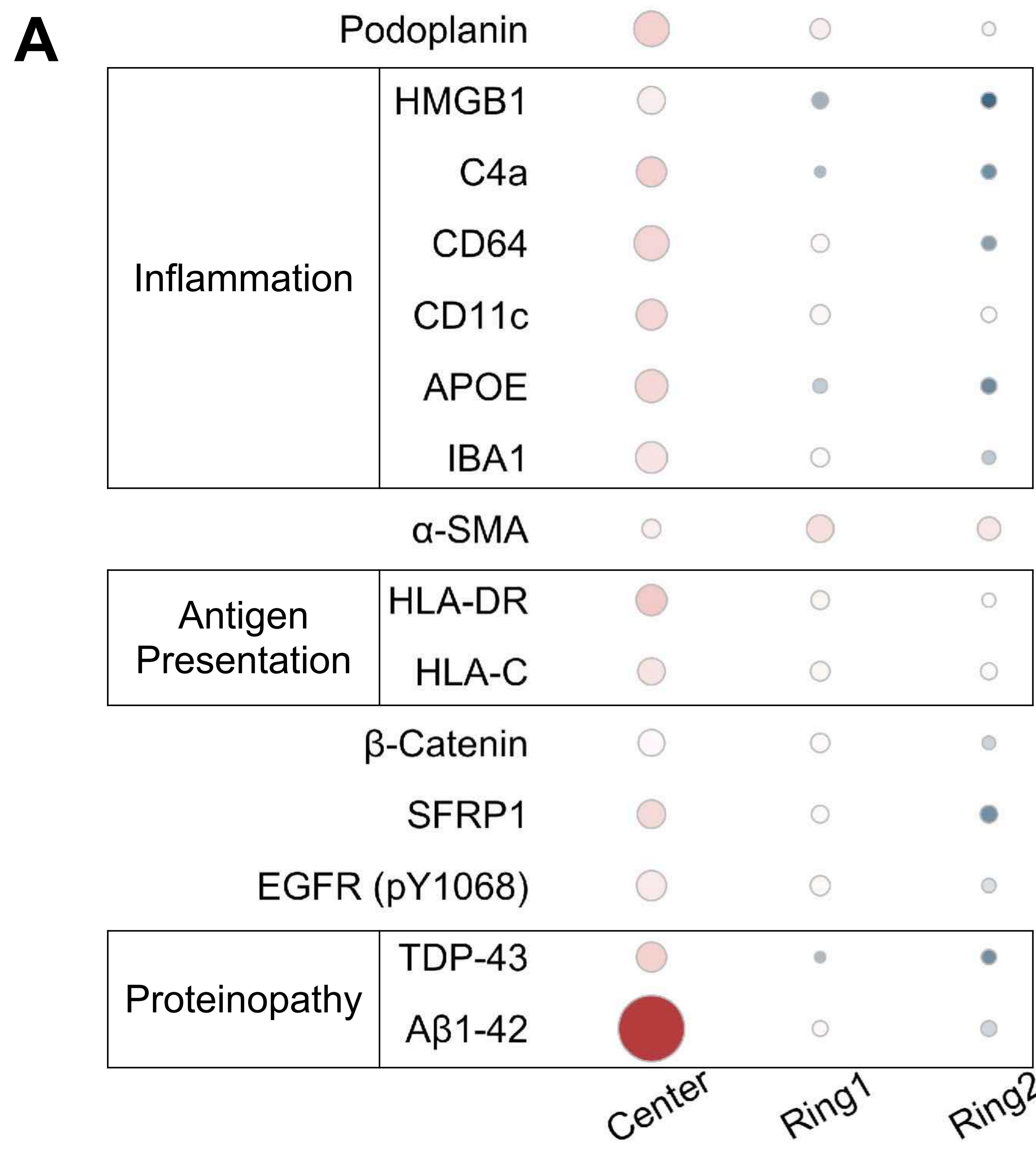


G

ALS







B Number of differentially expressed proteins/genes

