

1 Scalable spatial single-cell transcriptomics and translomics in 3D thick tissue blocks

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14

15 Abstract

16 Characterizing the transcriptional and translational gene expression patterns at the
17 single-cell level within their three-dimensional (3D) tissue context is essential for revealing how
18 genes shape tissue structure and function in health and disease. However, most existing spatial
19 profiling techniques are limited to 5-20 μm thin tissue sections. Here, we developed Deep-
20 STARmap and Deep-RIBOmap, which enable 3D *in situ* quantification of thousands of gene
21 transcripts and their corresponding translation activities, respectively, within 200- μm thick tissue
22 blocks. This is achieved through scalable probe synthesis, hydrogel embedding with efficient
23 probe anchoring, and robust cDNA crosslinking. We first utilized Deep-STARmap in
24 combination with multicolor fluorescent protein imaging for simultaneous molecular cell typing
25 and 3D neuron morphology tracing in the mouse brain. We also demonstrate that 3D spatial
26 profiling facilitates comprehensive and quantitative analysis of tumor-immune interactions in
27 human skin cancer.

28

29 Introduction

30 The spatial regulation of gene expression and translation is critical for tissue function¹⁻⁶.
31 *In situ* profiling technologies enable the study of both the transcriptome and translome within
32 their original spatial contexts⁷⁻¹⁰. However, most spatial omics techniques are confined to
33 analyzing thin tissue sections (5-20 μm). Many functional and anatomical studies in tissue
34 biology require 3D profiling in tissue blocks across multiple cellular layers^{11,12}. For instance, in
35 neuroscience, 3D morphological profiling and long-range projection mapping¹³⁻¹⁶, *in situ*
36 electrophysiology¹⁷⁻²², and *in vivo* neural activity imaging²³⁻²⁵ in the brain require direct
37 measurements in 3D brain volumes (100-300 μm) where thin tissue sections are inadequate. In
38 cancer pathology, 3D samples offer a more accurate representation of tumor architecture,
39 microenvironment, and cell-cell interactions in patient samples²⁶.

40 Although reconstructing 3D volumes using serial thin sections is feasible, this method
41 faces three significant challenges. Firstly, tissue sectioning often fragments cells, resulting in
42 partial RNA readouts and an increased risk of RNA content loss during handling, complicating
43 the accurate analysis of the transcriptome. Secondly, tissue deformation during sectioning
44 presents a persistent challenge for computational reconstruction methods. Thirdly, this

45 approach requires substantial manual labor. Therefore, developing effective spatial omics
46 methods for 3D profiling from thick tissue blocks is imperative.

47 Current thick-tissue spatial profiling approaches achieved quantitative *in situ*
48 measurements of transcriptome in thick samples using single or multi-round Fluorescence *In*
49 *Situ* Hybridization (FISH)²⁷⁻⁴⁰, but are limited in several aspects. The number of genes they can
50 analyze, typically fewer than 300, and the size of the imaging areas, often restricted to a single
51 brain region. These limitations arise primarily because these methods often employ linear
52 coding instead of exponential coding, and rely on RNA integrity to preserve the imaging signal,
53 significantly reducing their efficiency of RNA detection, scalability of gene numbers and tissue
54 volume, and flexibility of sample handling and imaging time²⁸⁻⁴⁰. Additionally, the displacement
55 of RNA molecules between imaging rounds further restricts the number of imaging cycles that
56 can be performed²⁷. Moreover, current thick-tissue spatial profiling methods are limited to
57 mapping spatial transcriptomics and lack the capability to map the translome, thus hindering
58 multiplexed characterization of gene translation at single-cell resolution.

59 Here, we have developed Deep-STARmap and Deep-RIBOmap to address the
60 aforementioned limitations by introducing a novel and scalable strategy for probe synthesis and
61 embedding as well as robust cDNA amplicons crosslinking, enabling scalable *in situ*
62 quantification of thousands of RNA transcripts and their respective translational activities within
63 large intact thick tissue samples. Utilizing Deep-STARmap and Deep-RIBOmap, we profiled the
64 transcription and translation of 1,017 genes in intact mouse brain tissue at 300 nm voxel size
65 within a thick hydrogel-tissue scaffold, revealing heterogeneity in protein translation across cell
66 types. Additionally, by combining our method with multicolor fluorescence labeling (Tetbow)¹⁴,
67 we simultaneously profiled neuronal morphology and molecular signatures in single cells,
68 achieving multimodal mapping of the adult mouse brain in a scalable manner. Lastly, we
69 demonstrated the applicability of our method on human cutaneous squamous cell carcinomas
70 (cSCC) samples, uncovering tumor-immune interactions with more accurate and quantitative
71 spatial distributions compared to thin tissue analyses. We anticipate that Deep-STARmap and
72 Deep-RIBOmap will also yield important biological insights into the pathophysiology of cancers
73 and other diseases.

74

75 **Results**

76 **Deep-STARmap and Deep-RIBOmap workflow**

77 We designed the workflow of Deep-STARmap and Deep-RIBOmap (**Fig. 1a**) as follows:
78 it begins with the hybridization of pre-designed oligonucleotide probe sets to target either all
79 RNA molecules of a gene or ribosome-bound RNAs, respectively, in PFA-fixed tissues followed
80 by hydrogel matrix embedding; the samples are then subjected to protein digestion and lipid
81 removal to enhance enzyme penetration, ensuring sufficient depth coverage in thick tissue
82 samples; subsequently, *in situ* cDNA amplicons are synthesized through enzymatic ligation and
83 rolling circle amplification (RCA); each cDNA amplicon contains a pre-designed gene-specific
84 identifier, which is finally decoded through cyclic sequencing, imaging, and stripping steps
85 (SEDAL sequencing³⁰). In comparison with previously published thick-tissue STARmap protocol
86 (linear encoding, 28 genes), the new developments of Deep-STARmap and Deep-RIBOmap
87 solved the issues of scalable probe preparation, cDNA amplicon anchoring, signal decay, and
88 translation mapping capability as detailed below.

89 STARmap employs a padlock probe, designed to target specific mRNA species of
90 interest, along with a primer that binds to the same mRNA transcript adjacent to the padlock
91 probe binding site. In the previous publication of the STARmap protocol adapted for thick
92 tissue³⁰, each primer contains a 5' Acrydite modification to anchor the probe into the hydrogel.
93 However, this modification is expensive to synthesize and not scalable for large gene numbers.
94 In this report, Deep-STARmap incorporates a common “flanking linker sequence” at the 5' end
95 of all primers. This addition enables an Acrydite-modified adapter to hybridize and covalently
96 crosslink with the flanking linker, allowing the whole probe set of primers to be conjugated to
97 polyacrylamide hydrogels during polymerization (**Fig. 1b**). Covalent crosslinking is achieved
98 with a nucleoside analog, 3-cyanovinylcarbazole nucleoside (^{CNV}K)⁴¹, incorporated into the
99 adapter. Upon 366 nm UV irradiation, the ^{CNV}K-containing adapter undergoes rapid
100 photocrosslinking to the complementary strand via an adjacent pyrimidine base, a process
101 shown to be non-damaging to DNA^{42,43}. Experimental optimization revealed that an adapter-to-
102 primer ratio of 5:1 is sufficient for complete conversion of primer, and higher ratios do not
103 increase the number of amplicons in mouse brain samples (**Extended Data Fig. 1a-c**). Notably,
104 probes with a photocrosslinked 5' Acrydite adapter performed equivalently to those with 5'
105 Acrydite modifications incorporated during solid phase synthesis (**Fig. 1c**). It is worth noting that
106 the photocrosslinked adapter approach is markedly more efficient and scalable, as it employs a
107 universal flanking linker sequence and corresponding adapter for all primers to allow pooled
108 synthesis. In contrast, attachment of 5' Acrydite for each individual probe during solid phase
109 synthesis is extremely costly, especially in the setting of >5,000 probes with up to 70 bases in
110 length. Due to its high multiplexing capability, our method enables the embedding of a large
111 number of probe sets into the hydrogel, expanding the number of targetable RNA species from
112 dozens to thousands. Our findings demonstrate that UV crosslinking significantly enhances
113 probe incorporation efficiency, resulting in a higher detection yield of cDNA amplicons compared
114 to mere adapter-primer hybridization. This method substantially outperforms the approach of
115 relying solely on hydrogel physical retention (**Fig. 1c**). We also demonstrated that anchoring
116 probe sets into the hydrogel is more efficient than the previously reported strategies^{40,44} of
117 anchoring RNA molecules into the hydrogel in our experimental setting. (**Extended Data Fig. 1f,**
118 **g**).

119 After hydrogel polymerization using a mixture of redox initiator and thermal initiator to
120 embed the tissue and polymerizable primers (**Extended Data Fig. 1d, e**), we performed protein
121 digestion, enzymatic ligation, and rolling circle amplification (RCA) to construct *in situ* cDNA
122 amplicons. We observed that primer polymerization alone could not efficiently retain cDNA
123 amplicons as puncta for more than 4 imaging cycles, potentially because they are prone to
124 displacement, disassemble, and even fragmentation caused by buffer-dependent hydrogel
125 expansion and contraction between imaging cycles, resulting in progressively lower SNR (**Fig.**
126 **1d**). To maintain the position and integrity of the amplicons through multiple detection cycles, a
127 second round of hydrogel embedding was introduced following RCA, which outperformed
128 several alternative re-embedding strategies (**Extended Data Fig. 2a-d**). Collectively, the
129 implementation of these strategies devised explicitly for Deep-STARmap significantly enhances
130 its robustness and scalability, enabling consistent spatial transcriptomics readouts across 200-
131 μm thick sections of the mouse brain (**Fig. 1e**).

132 Next, we leveraged the insights gained from developing Deep-STARmap to establish
133 Deep-RIBOmap for investigating spatial translomics in thick tissue samples. RIBOmap utilizes
134 a tri-probe design strategy to selectively detect and amplify ribosome-bound mRNAs: in addition
135 to the padlock and primer, an additional splint DNA probe hybridizes to ribosomal RNAs
136 (rRNAs)⁴⁵. Building upon this design, Deep-RIBOmap incorporates a “flanking linker sequence”
137 at the 5' end of both the primer and the splint DNA probe (**Fig. 1f**). An Acrydite-modified adapter
138 covalently crosslinks to these flanking linkers, enabling the integration of the entire tri-probe set
139 into polyacrylamide hydrogels during polymerization. Using the same workflow as Deep-
140 STARmap, Deep-RIBOmap achieves spatial translomic profiling in thick tissue blocks.

141

142 **Deep-STARmap and Deep-RIBOmap in mouse brain with 1,017 genes**

143 To evaluate the scalability of Deep-STARmap and Deep-RIBOmap for high-throughput
144 3D intact tissue transcriptomic and translomic sequencing, we applied these techniques to
145 thick mouse brain sections (Methods), targeting a curated list of 1,017 genes. This gene list was
146 compiled from reported cell-type marker genes in adult mouse CNS single-cell RNA sequencing
147 (scRNA-seq) datasets and spatial transcriptomic mouse brain atlases^{46–49}. Gene identities
148 encoded by five-nucleotide sequences on the padlock probes were read out through six rounds
149 of sequencing by ligation with error rejection (SEDAL).

150 We performed pairwise Deep-STARmap and Deep-RIBOmap mapping on adjacent 150-
151 μm -thick coronal sections of the mouse hemisphere, encompassing multiple brain regions
152 (198,675 cells for Deep-STARmap and 164,029 cells for Deep-RIBOmap). To annotate cell
153 types and align them with established nomenclature, we integrated our Deep-STARmap and
154 Deep-RIBOmap with a published spatial brain atlas with curated cell typing annotations, using
155 two different approaches independently. In the first approach, we used FuseMap⁵⁰, a recently
156 developed integration method that transfers cell type annotations leveraging both spatial and
157 cellular information (**Figure 2a**). We also benchmarked the results using a second approach,
158 where an established method, Harmony⁵¹, is used solely relying on single-cell gene expression
159 information (**Figure 2b**). Both methods were applied independently to the same datasets and
160 yielded consistent results: the confusion matrix of major cell type assignments showed that
161 FuseMap's cell types were highly concordant (82.4% matched labels) with those identified by
162 the traditional single-cell sequencing integration method (**Fig. 2c**). Since FuseMap is a pre-
163 trained model that integrates multiple large-scale spatial transcriptomic datasets and cell-type
164 annotations of the mouse brain⁵⁰ and has demonstrated higher accuracy in sublevel transferred
165 annotations, we proceeded with FuseMap for downstream analyses.

166 FuseMap integration, followed by nearest-neighbor label transfer, identified 19 main cell
167 types, including 9 neuronal, 5 glial, 1 immune, and 4 vascular cell clusters, all of which exhibited
168 canonical marker genes and expected spatial distributions. Further hierarchical clustering within
169 each main cluster resulted in 137 subclusters (**Fig. 2d**). These major and subcluster annotations
170 were consistent with previously published brain atlas datasets (**Fig. 2d**)^{49,52,53}. Our spatial
171 transcription and translation patterns of canonical cell-type marker genes and neurotransmitter
172 genes aligned well with previously published spatial transcriptomic and translomic sequencing
173 results (**Extended Data Fig. 3a, b**). Based on these cell typing results, we generated spatial cell
174 maps of the imaged hemibrain region. Our analysis demonstrated consistent cell typing

175 between Deep-STARmap and Deep-RIBOmap in terms of gene expression patterns, cell-type
176 composition, and spatial distribution of cell types (**Fig. 2d and Extended Data Fig. 3c**).

177 By exploiting the single-cell and spatial resolution of paired Deep-STARmap and Deep-
178 RIBOmap datasets, we probed the heterogeneity in translational regulation across various cell
179 types and brain regions. To investigate translationally regulated genes across different cell
180 types, we first performed gene clustering using Deep-STARmap and Deep-RIBOmap profiles,
181 identifying 18 gene modules (**Extended Data Fig. 4a**) with distinct functions and expression
182 patterns (**Extended Data Fig. 4b**). Prior research has shown that non-neuronal cells,
183 particularly oligodendrocytes, exhibit significant translational regulation⁴⁵. We analyzed a gene
184 module comprising 74 genes predominantly expressed across the oligodendrocyte lineage, from
185 oligodendrocyte progenitor cells (OPCs) to mature subtypes (OLG1 and OLG2). Our findings
186 recapitulate previous observations⁴⁵, demonstrating that genes with higher translation efficiency
187 in OPCs are linked to oligodendrocyte differentiation, while those with elevated translation
188 efficiency in mature oligodendrocytes are associated with myelination (**Extended Data Fig. 4c**).

189 Beyond our extensive transcriptome analysis of the brain, we focused on investigating
190 translational control at the subcellular level. Translation localized to the soma and processes in
191 brain tissue plays a pivotal role in the organization and plasticity of neuronal and glial networks
192 in response to physiological stimuli during neurodevelopment and memory formation (**Extended**
193 **Data Fig. 5a**). To dissect this localized translation, we categorized Deep-RIBOmap reads in 3D
194 thick tissue blocks into somata-localized reads (within the cell body, identified using Watershed
195 3D⁵⁴) and processes-localized reads (the rest of the reads). We then identified the top 10% of
196 genes with the highest and lowest processes-to-somata ratios, designating them as enriched in
197 processes and somata, respectively (**Extended Data Fig. 5b, d**). Gene ontology (GO) analysis
198 indicated that genes enriched in processes are involved in cell projection, cell junction, and cell-
199 cell signaling, while those enriched in somata are associated with the extracellular matrix and
200 various receptors (**Extended Data Fig. 5c**).

201 Given the ability of Deep-STARmap and Deep-RIBOmap to measure multiple layers of
202 cells, we next tested whether our methods could resolve volumetric patterns of cell organization
203 in 3D. We performed a detailed analysis of the nearest-neighbor distances among various
204 interneuron subtypes. Prior studies have demonstrated that interneurons of identical subtypes
205 frequently form juxtaposed pairs in the mouse visual cortex. Our result substantiates these
206 findings, indicating that an inhibitory neuron is predominantly adjacent to another of the same
207 subtype (Lamp5, Vip, Sst, or Pvalb) rather than other inhibitory subtypes (**Extended Data Fig.**
208 **5e, f**). This close spatial proximity might be related to the formation of gap junctions, which are
209 crucial for synchronized firing patterns and may enhance visual responses in the cortex⁵⁵⁻⁵⁷.

210 **Single-cell morphology analysis of molecular cell types with Tetbow**

211 Understanding the brain function necessitates a detailed mapping of its neuroanatomy.
212 Electron microscopy (EM) remains the gold standard for neuroanatomical studies, offering
213 nanometer-scale resolution^{58,59}. However, EM reconstructions are largely incompatible with
214 molecular cell-typing, resulting in a trade-off between spatial resolution and molecular
215 information. Additionally, the current analytical throughput of EM is inadequate for studying the
216 long-range spatial organization of mouse and mammalian neurons. The integration of stochastic
217 multicolor labeling techniques^{13,14,60} with spatial transcriptomic mapping offers a promising
218

219 solution. This combined approach enables the generation of comprehensive, co-profiling of
220 transcriptome and morphology of individual neurons within densely labeled neural circuits.

221 To simultaneously interrogate transcriptomic readouts and morphology within single cells
222 by exploiting the unique advantages of thick tissue mapping, we integrated the stochastic
223 multicolor genetic labeling tool, Tetbow¹⁴, into our workflow (**Fig. 3a**). Tetbow enables bright and
224 high-resolution mapping of intermingled neurons *in situ* by tagging individual neurons with
225 stochastic combinations of three cytoplasmically-localized fluorescent proteins. It has also been
226 demonstrated that systemically delivered AAVs allow a more uniform distribution of labeled cells
227 and color diversity⁶¹. Thus, we utilized the AAV-PHP.eB⁶¹ variant to co-administer three
228 separate vectors encoding three fluorescent proteins, along with a tTA expression vector to
229 activate combinatorial fluorescent protein expression across the entire brain. (**Fig. 3b**,
230 **Extended Data Fig. 6a**).

231 After tissue sectioning and embedding the probe sets into the hydrogel through
232 polymerization, we performed imaging for the three Tetbow fluorescent proteins (FPs) along
233 with DAPI, and observed bright, high-quality labeling of diverse neuronal cell types across all
234 regions of the brain (**Fig. 3a**). Following morphology imaging, we digested the FPs from the
235 sample using tissue clearing to enable subsequent transcriptome profiling of 1,017 genes.
236 cDNA amplicons were constructed and sequenced as previously described. We additionally
237 used DAPI as a fiducial marker for image registration between the two imaging modalities to
238 correspond each FP-labeled neuron to its molecular subtype identity resolved by Deep-
239 STARmap (**Extended Data Fig. 6b, c**).

240 To visualize the morphological diversity of labeled neurons, we established a semi-
241 automated morphological reconstruction pipeline in Bitplane Imaris. In total, we reconstructed
242 the dendritic arbors of 40 principal cells and interneurons within the imaged volume, spanning
243 across 34 molecular subtypes (**Fig. 3c**). We focused our study on the dendritic arbor to
244 maximize the accuracy of our traces, as it has been demonstrated that fine axonal morphologies
245 cannot be consistently and faithfully recapitulate without a membrane-localized marker⁶⁰. In
246 agreement with past findings^{62,63}, we were able to resolve the characteristic dendritic trees of
247 principal pyramidal neurons in different brain regions, including CA1 hippocampal
248 (TEGLU_3,7,8,9,10,11,12,14,15,18,21,22,26,35,37,39,41) and layer V neocortical pyramidal
249 neurons (TEGLU_4,5,6). As expected, we resolved the most prominent dendritic structure of
250 pyramidal neurons: the apical dendrite, which in CA1 hippocampal neurons extends towards the
251 stratum lacunosum-moleculare (SLM), while in layer V neocortical neurons extend towards the
252 cortical surface, both branching to form tree-like structures. Similarly, although cortical
253 GABAergic inhibitory interneurons constitute a minority of the total neocortical neuronal
254 population, we additionally confirmed that their morphology was resolvable with our approach,
255 and that they exhibit a wide diversity of dendritic morphologies. In this study, we also elucidate
256 the morphological diversity of several major subclasses of GABAergic neurons⁶⁴, classified by
257 their transcriptomic profiles, within the mouse cerebral cortex. In conclusion, we simultaneously
258 profiled molecular cell types and morphologies at single-cell resolution in the adult mammalian
259 brain in a scalable way.

260

261 **Deep-STARmap in human cutaneous squamous cell carcinoma**

262 Deep-STARmap's potential extends beyond neuroscience. One particularly promising
263 application lies in the field of oncology, where a comprehensive understanding of the spatial
264 organization of tumors, their microenvironments and immune interactions is crucial. Skin
265 cancers account for ~90% of all human malignancies. The second-most prevalent skin cancer is
266 cutaneous squamous cell carcinoma (cSCC), which arises from keratinocytes (the major cell
267 type of the epidermis). Over 1 million new cSCC cases are diagnosed annually in the United
268 States⁶⁵, with an estimated 3.7% of cSCCs leading to metastatic disease and 1.5% of cases
269 resulting in death from disease⁶⁵. The leading risk factor for cSCC is chronic ultraviolet radiation
270 (UVR) exposure, which has mutagenic effects on the skin. UVR-induced somatic mutations
271 translate to a large burden of tumor neoantigens that are thought to be responsible for the high
272 immunogenicity of cSCCs⁶⁶. Of note, immunosuppressed patients are at a 65-100 fold higher
273 risk of developing cSCC and are significantly more likely to be diagnosed with multiple and
274 metastatic cSCCs due to a failure of cancer immunosurveillance^{67,68}. Immunotherapies such as
275 immune checkpoint inhibitors have shown promise in the treatment of advanced cSCC⁶⁹,
276 however many patients fail to respond and the biomarkers, precise cell subpopulations, and
277 mechanisms underlying response versus resistance are not well understood. There is great
278 interest in assessing the spatial organization and signaling between tumor, immune, and
279 stromal cells in the native tumor microenvironment. Prior spatial studies of cSCC have been
280 limited to thin or 2D tissue samples that do not capture the full complexity of tumor architecture,
281 as human skin's barrier function makes it resistant to enzymatic digestion and macromolecule
282 penetration. Thus, we applied Deep-STARmap to more comprehensively assess tumor
283 organization and tumor-immune cell interactions in cSCC.

284 We curated a targeted list of 254 genes from previously published scRNA-seq studies of
285 normal skin and skin cancers, including markers for common skin and immune cell types⁷⁰⁻⁷³.
286 Deep-STARmap was performed on a 60- μ m-thick section of human cSCC obtained from Mohs
287 micrographic surgery (MMS), a sample that included both cSCC tumor and adjacent normal skin
288 (**Extended Data Fig. 7a**). Following cell segmentation in 3D, data processing, and integration
289 with a published cSCC scRNA-seq dataset⁷¹, we conducted cell typing and visualized cell
290 clusters on the UMAP space based on single-cell RNA expression (**Fig. 4a**). 9 cell types were
291 identified using known marker genes: keratinocytes, tumor-specific keratinocytes (TSKs),
292 fibroblasts, endothelial cells, B cells, Langerhans cells, macrophages/dendritic cells (DCs),
293 cytotoxic T cells, and regulatory T cells/exhausted T cells (**Fig. 4a, b**). Deep-STARmap enabled
294 dissection of tumor spatial organization at single-cell resolution. Consistent with histologic tumor
295 spatial patterns noted at the time of MMS, tumor-specific keratinocytes in this sample were
296 primarily localized to the center of the tissue while non-tumor keratinocytes were localized to
297 normal skin at the sample periphery (**Fig. 4c**).

298 299 **Mapping cell-cell interactions in human cSCC**

300 To characterize cell-cell interactions, we generated a mesh graph via Delaunay
301 triangulation of cells and computed a near-range cell-cell adjacency matrix from spatial
302 connectivity as previously described^{74,75}. This allowed us to identify the nearest neighbors of
303 each cell and to quantify the number of edges between cells of each type with cells of the same
304 or other cell types. A heat map of cell type frequencies among first-tier neighbors revealed clear
305 patterns of cell type-specific cell-cell communication (**Fig. 4d**). The same analysis was

306 performed on a thin 15- μ m section of the cSCC sample taken within the same 3D volume
307 (**Extended Data Fig. 7b, c**). As expected, more cell-cell contacts were detected in thick tissue
308 (mean of 14.3 connected cells) compared to thin tissue (mean of 6.0 connected cells)
309 (**Extended Data Fig. 7d, e**).

310 Across the cSCC sample, strong interactions were detected among cells of the same
311 type, with more same cell type interactions identified in thick tissue compared to pseudo-thin
312 tissue (**Extended Data Fig. 7b, c**). Similarly, immune cell interactions with other immune cell
313 types such as macrophages/DCs with T cells were more strongly detected in thick compared to
314 pseudo-thin tissue (**Extended Data Fig. 7b, c**). Interestingly, tumor-specific keratinocytes only
315 interacted strongly with two cell types: other tumor-specific keratinocytes or Langerhans cells
316 (**Fig. 4d, e**). This was again more evident in thick tissue than thin tissue, demonstrating that the
317 additional 3D morphological information provided by Deep-STARmap increases the sensitivity
318 and robustness for quantifying cell-cell contacts.

319 Langerhans cells (LCs) are the major resident antigen-presenting cells of the skin and
320 are known to interact with keratinocytes via E-cadherin. LCs have been reported to encounter
321 cSCC cells prior to other DC subtypes⁷⁶ and stimulate cytotoxic CD8 T cells and NK cells more
322 efficiently than other DC subsets⁷⁷. In our cSCC sample, LCs interacted with T cells and tumor-
323 specific keratinocytes, but not normal keratinocytes outside the tumor, indicating tumor-specific
324 immune responses (**Fig. 4e**). Taken together, our Deep-STARmap cSCC data identified a
325 disease-relevant interaction between tumor-specific keratinocytes and Langerhans with more
326 accurate and quantitative spatial distribution compared to thin tissue analyses.

327

328 Discussion

329 In this study, we present Deep-STARmap and Deep-RIBOmap as novel imaging
330 platforms for *in situ* transcriptomic and translomic sequencing within intact tissue blocks. To
331 enable robust performance and scalability over existing approaches, we introduced new
332 strategies for thick tissue RNA imaging, including scalable probe synthesis, efficient probe
333 anchoring, and robust cDNA crosslinking. These technological developments are pivotal for
334 scaling up 3D *in situ* transcriptomic and translomic profiling to encompass thousands of genes
335 and across larger tissue regions. This scalability facilitates the integration of molecular
336 characterizations with morphology mapping in neuroscience within thick tissue blocks. We
337 demonstrated that Deep-STARmap and Deep-RIBOmap could profile the transcription and
338 translation of over 1,000 genes within intact thick mouse brain tissue sections, significantly
339 expanding the readouts from larger cell populations. Incorporating combinatorial fluorescence
340 labeling using the Tetbow system allowed high-throughput *in situ* co-profiling of spatial
341 transcriptomics and single-neuron morphology in thick tissue blocks, enabling multimodal
342 mapping on a volumetric scale previously unattainable. For example, our platform is potentially
343 compatible with MAPseq¹⁵ and BARseq¹⁶ to uncover the organizing principles of neuronal
344 circuitry in thick tissue blocks. Moreover, our platform can be further applied to decode the
345 spatial transcriptomics and translomics of specific neurons with activity dynamics being
346 collected by live imaging.

347 Our platform can also be generalized to study various heterogeneous cell populations in
348 diverse tissues. We demonstrated that our 3D *in situ* profiling platform is adaptable for profiling
349 difficult-to-digest human skin cancer samples, providing more accurate and quantitative

350 measurements of tumor-immune spatial patterns. Furthermore, we anticipate that our 3D *in situ*
351 profiling platform will be highly useful for studying human organoid cultures, which are
352 extensively used to replicate *in vivo* 3D organ development from 2D embryonic germ layers
353 during organogenesis⁷⁸. These organoids, typically measuring hundreds of micrometers,
354 necessitate *in situ* profiling in both healthy and diseased states to advance our understanding of
355 human tissue development, pathology, and therapeutic responses.

356 In summary, 3D *in-situ* spatial transcriptomics and translomics, exemplified by Deep-
357 STARmap and Deep-RIBOmap, offer a robust methodology for integrating molecular data with
358 high-resolution cellular imaging. This comprehensive approach allows for detailed analysis of
359 anatomical and functional dynamics within tissues. Such techniques are poised to substantially
360 enhance our understanding of the underlying mechanisms of tissue functionality and pathology,
361 thereby facilitating deeper scientific exploration and potential therapeutic innovations.

362

363 **Author contributions**

364 X.S. and X.W. conceived the idea and developed Deep-STARmap and Deep-RIBOmap
365 for the study. X.S. carried out experimental work, performed *in situ* sequencing, and conducted
366 computational and data analyses. J.A.L. designed the gene lists for human cSCC, acquired
367 samples, and made significant contributions to the analysis of human cSCC. S.L., Y.H., and Z.T.
368 performed data analysis. Z.L. and J.L. helped with method optimization. Y.Z. helped with AAV
369 packaging and conducted animal work. W.X.W helped with morphology analysis. X.S., J.A.L.,
370 and X.W. wrote the manuscript with input from all authors. X.W. supervised the study.

371

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384

385 **Competing interests**

386 X.W. and X.S. are inventors on pending patent applications related to Deep-STARmap
387 and Deep-RIBOmap. X.W. is a scientific co-founder and consultant of Stellaromics. Other
388 authors declare no competing interests.

389

390 **Methods**

391 Mouse lines

392 All animal procedures adhered to the care guidelines approved by the Institutional
393 Animal Care and Use Committee (IACUC) of the Broad Institute of MIT and Harvard, under

394 animal protocol #0255-08-19. For the experiments, C57/BL6 mice aged between 6 to 10 weeks
395 were procured from The Jackson Laboratory (JAX). Mice were housed with 4~5 animals per
396 cage with arbitrary food and water in a room with 18~23 °C temperature and 40~60% humidity
397 under a 12-h light-dark cycle.

398 Human cutaneous squamous cell carcinoma samples

399 Human cutaneous squamous cell carcinoma tissue was obtained from deidentified
400 discarded hospital specimens approved under the Massachusetts General Hospital Research
401 Committee/IRB protocol #2013P000093.

402 Tetbow AAV injections

403 The AAV plasmids utilized in this study include pAAV-TRE-mTurquoise2-WPRE
404 (Addgene #104110), pAAV-TRE-EYFP-WPRE (Addgene #104111), pAAV-TRE-tdTomato-
405 WPRE (Addgene #104112), and pAAV-ihSyn1-tTA (Addgene #99120). Tetbow components
406 were packaged into AAV.PHP.eB as previously described⁶¹. In brief, for each capsid, HEK 293T
407 cells (ATCC CRL-3216) were transfected with a combination of pAAV plasmid and two AAV
408 packaging plasmids (kiCAP-AAV-PHP.eB and pHelper) in a 1:4:2 weight ratio, using
409 polyethylenimine, with a total of 40 µg of DNA per 150-mm culture dish. Fluorescence
410 expression, when applicable, was evaluated via microscopy, and the media was refreshed 20-
411 24 hours post-transfection. Viral particles were collected 72 hours post-transfection from both
412 the cells and the medium by centrifugation, forming cell pellets. These cell pellets were then
413 resuspended in a buffer containing 500 mM NaCl, 40 mM Tris, 10 mM MgCl₂, pH ~10 and 100
414 U/mL of salt-activated nuclease (SAN, 25 U/µL, Arcticzymes, 70910-202) and incubated at 37°C
415 for 1.5 hour. Following incubation, the cell lysates were subjected to centrifugation at 2,000g to
416 remove cellular debris. The viral particles were then isolated through a series of iodixanol
417 gradient steps (15%, 25%, 40%, and 60%). Viruses were collected from both the 40/60%
418 interface and the 40% iodixanol layer. The concentration of the viral particles and buffer change
419 were achieved using Pierce™ Protein Concentrators (Thermo Scientific, 88528), and they were
420 subsequently suspended in sterile phosphate-buffered saline (PBS). To quantify viral titers, viral
421 genomes were measured using quantitative PCR (qPCR). The procedure included treating
422 samples with DNase I (Roche Diagnostics, 4716728001) to eliminate non-packaged DNA and
423 subsequently with proteinase K (Roche Diagnostics, 03115828001) to digest the viral capsid,
424 thereby exposing the viral genomes for qPCR quantification. A linearized genome plasmid
425 served as the reference standard. The viral titers for tTA, tdTomato, EYFP, and mTurquoise2
426 were 2.15×10^{13} , 2.31×10^{13} , 3.04×10^{13} , and 2.63×10^{13} vg/ml, respectively.

427 Intravenous administration of AAV.PHP.eB mixture (1×10^{11} vg tTA, 3.33×10^{11} vg
428 tdTomato, 3.33×10^{11} vg EYFP, 3.33×10^{11} vg mTurquoise2) was performed via injection into
429 the retro-orbital sinus of adult female C57BL/6 mice (8-10 weeks of age). Twenty-eight days
430 post-injection, the mice were anesthetized with isoflurane. Transcardial perfusion was carried
431 out, initially with 50 mL of cold PBS, followed by 50 mL of 4% PFA. The entire brain was then
432 post-fixed in 4% PFA at 4°C for 3 hours. Subsequently, the brain was washed multiple times
433 with PBS and placed in a 30% sucrose solution (in PBS) at 4°C overnight or until it had sunk.
434 Finally, the brain was embedded in O.C.T. (Fisher, 23-730-571) and frozen in liquid nitrogen
435 and stored at -80 °C. Thick tissue sections were prepared and carefully transferred into
436 pretreated glass-bottom plates.

437 Chemicals and enzymes

438 Chemicals and enzymes listed as name (supplier, catalog number): 12-Well Plate, No.
439 1.5 Coverslip, 14 mm Glass Diameter, Uncoated (MatTek, P12G-1.5-14-F); PlusOne Bind-
440 Silane (Sigma, 17-1330-01); 16% PFA, EM grade (Electron Microscope Sciences, 15710-S);
441 Methanol (Sigma-Aldrich, 34860-1L-R); Tween-20, 10% solution (Teknova, T0710); Triton-X100,
442 10% solution (Sigma-Aldrich, 93443-100ML); 10X PBS (Thermo Fisher, 70011044); 1X PBS
443 (Thermo Fisher, 10010049); 20X SSC buffer (Thermo Fisher, 15557044); Methacrylic acid N-
444 hydroxysuccinimide ester, 98% (Sigma-Aldrich, 730300-1G); Acrylamide solution, 40% (Bio-Rad,
445 161-0140); Bis Solution, 2% (Bio-Rad, 161-0142); Ammonium persulfate (Sigma-Aldrich,
446 A3678-100G); N,N,N',N'-Tetramethylethylenediamine (Sigma-Aldrich, T9281-50ML); OmniPur
447 SDS, 20% (Calbiochem/Sigma, 7990-200ML); NeuroTrace Fluorescent Nissl Stains, yellow
448 (Molecular Probes/Fisher Scientific, N21480); COVER GLASS CIRCLE, 12mm, #2, 1oz/BX
449 (Electron Microscopy Sciences, 72226-01); Gel Slick Solution (Lonza, 50640); Formamide,
450 Deionized (Sigma aldrich, 4650-500ML); Antarctic Phosphatase Reaction Buffer (New England
451 Biolabs, B0289S); Antarctic Phosphatase (New England Biolabs, M0289L); BSA (New England
452 Biolabs, B9200S); Glycine (Sigma aldrich, 50046-250G); Ribonucleoside Vanadyl Complex
453 (New England Biolabs, S1402S); DMSO, anhydrous (Invitrogen/Thermo Fisher, D12345);
454 DNase/RNase-Free Distilled Water (Invitrogen/Thermo Fisher, 10977023); 4',6-diamidino-2-
455 phenylindole (DAPI) (Thermo Fisher, 62248); Acetic acid (Sigma-Aldrich, A6283-100ML); Poly-
456 D-Lysine (Thermo Fisher, A3890401); dNTP mix (thermofisher, 18427089); 5-(3-aminoallyl)-
457 dUTP (Invitrogen, AM8439); BSPEG9 (thermofisher, 21582); Proteinase K Solution (Invitrogen,
458 25530049); SUPERase•In RNase Inhibitor (Thermo Fisher, AM2696); T4 DNA Ligase (Thermo
459 Fisher, EL0012); Phi29 DNA Polymerase (Thermo Fisher, EP0094); Yeast tRNA (Thermo
460 Fisher, AM7119)

461 Deep-STARmap and Deep-RIBOmap probe design

462 The Deep-STARmap and Deep-RIBOmap padlock and primer probes were developed
463 based on the methodologies outlined in Wang et al. and Zeng et al., with specific
464 modifications^{30,45}. Each Deep-STARmap and Deep-RIBOmap primer incorporated a “flanking
465 linker sequence” (CCTACCAGTACGACGTATTTAGCAA) at the 5' end to enable hybridization
466 with an Acrydite-modified oligonucleotide. The Deep-RIBOmap additionally required a splint
467 probe, composed of three segments: a 25-nucleotide sequence at the 5' end complementary to
468 the 18S ribosomal RNA (rRNA), a stretch of 50 deoxyadenosine nucleotides (dA), and a 12-
469 nucleotide padlock template at the 3' end. To prevent the 3' terminus of the splint probes from
470 serving as an RCA primer, a 3' Inverted dT modification was included. Additionally, each splint
471 probe incorporated a “flanking linker sequence” (CCTACCAGTACGACGTATTTAGCAA) at the
472 5' end to facilitate the hybridization process with the Acrydite-modified oligonucleotide.

473 Adapter and primer pre-treatment

474 The ^{CNV}K-containing adapter ([5Acryd]GCTA^{[CNV]K}ATACGTCGTACTGGTAGG[Inv-dT],
475 ordered from Gene Link with PAGE purification) undergoes rapid photo cross-linking to the
476 complementary strand through an adjacent pyrimidine base upon UV irradiation. The irradiation
477 process was conducted using the Boekel UV Crosslinker (234100) equipped with 368 nm-
478 wavelength bulbs (Boekel Part Number 920-0307). The adapter to primer was maintained at a
479 molar ratio of 5:1.

480 Deep-STARmap and Deep-RIBOmap protocol

481 Glass-bottom 12-well plates (Mattek, P12G-1.5-14-F) were treated with oxygen plasma
482 using the Anatech Barrel Plasma System at 100W and 40% O₂ for 5 min. Following this, the
483 plates were immersed in a 1% methacryloxypropyltrimethoxysilane (Bind-Silane) solution for 60
484 min at room temperature. The plates then underwent three consecutive ethanol washes and
485 were allowed to air dry. Subsequently, a 0.1 mg/mL Poly-D-lysine solution was applied to the
486 plates for 1 hour, followed by three rinses with distilled water.

487 Tissue slices were transferred and adhered to pre-treated glass-bottom 12-well plates.
488 The samples were permeabilized using 1 mL of pre-chilled methanol at -20°C for one hour.
489 During this period, PBST solution, comprising 0.1% Triton-X 100 in PBS, was prepared. The
490 samples were then washed with 500 µL of PBSTR (0.1 U/mL SUPERase-In in PBST) for 30 min.
491 This was followed by a quenching step with 500 µL of quenching solution (1 mg/mL Yeast tRNA,
492 100 mM Glycine in PBSTR) at room temperature for 30 minutes, followed by another 30-min
493 wash with PBSTR. Subsequently, hybridization buffers were prepared. The base composition of
494 the hybridization buffer included 2x SSC, 10% formamide, 1% Triton-X 100, 20 mM RVC, 0.1
495 mg/mL yeast tRNA, 0.1 U/µL SUPERase-In, and 0.2% SDS. For the Deep-STARmap samples,
496 this buffer was supplemented with pooled Deep-STARmap padlock and pre-treated primer at a
497 concentration of 5 nM per oligo. For the Deep-RIBOmap samples, the hybridization buffer
498 additionally contained 100 nM of pre-treated splint probe for RIBOmap. The samples were
499 incubated in 300 µL of hybridization buffer in a 40°C humidified oven with gentle shaking for 36
500 hours. After incubation, the samples were washed for 30 min with PBSTR, followed by a 30-min
501 wash in high salt buffer (4x SSC in PBSTR) at 37°C. Finally, the samples were washed once
502 more with PBSTR at 37°C.

503 To cast the tissue-hydrogel hybrid, the samples were first incubated with monomer
504 buffer (4% acrylamide, 0.2% bis-acrylamide, 2x SSC) supplemented with 0.2% TEMED and
505 0.25% VA-044 at 4°C for 60 min. Following incubation, the buffer was aspirated, and 55 µL of a
506 polymerization mixture (0.2% TEMED, 0.2% ammonium persulfate, and 0.25% VA-044 in
507 monomer buffer) was added to the center of the sample and immediately covered with a Gel
508 Slick-coated coverslip. The polymerization process was conducted in a 40°C N₂ oven for 90 min.
509 Subsequently, the sample was washed with PBSTR three times for 15 min each. For Tetbow
510 samples, the tissue was stained with DAPI for 3 hours and then immersed in a washing and
511 imaging buffer (10% formamide in 2x SSC buffer) containing 0.1 U/µL SUPERase-In RNase
512 inhibitor. Confocal images of Tetbow fluorescent proteins (tdTomato, EYFP, and mTurquoise2)
513 and DAPI were acquired using an inverted confocal microscope, Leica TCS SP8 (version
514 3.5.5.19976), equipped with a 405 nm and 442 nm diode, a white light laser, HyD detectors, and
515 a 25x water-immersion objective (NA 0.95). The voxel size for imaging was 0.32 µm × 0.32 µm
516 × 0.70 µm. The following wavelengths were used for imaging: 405 nm for DAPI, 442 nm for
517 mTurquoise2, 506 nm for EYFP, and 550 nm for tdTomato.

518 The tissue-gel hybrids were then digested with 1 mL Proteinase K mixture (0.4 mg/mL
519 Proteinase K in 2x SSC and 1% SDS) at 37°C for overnight, then washed by PBSTR 3 times for
520 30 min each. The sample was then incubated in ligation mixture (0.25 U/µL T4 DNA ligase,
521 1:100 BSA, 0.2 U/µL SUPERase-In RNase inhibitor) at room temperature overnight with gentle
522 shaking and then washed with PBSTR three times for 30 mins each. Then the sample was
523 incubated with 400 µl rolling-circle amplification mixture (0.5 U/µL Phi29 DNA polymerase, 250
524 µM dNTP, 20 µM 5-(3-aminoallyl)-dUTP, 1:100 BSA and 0.2U/µL of SUPERase-In RNase

525 inhibitor in 1X Phi29 buffer) at 4°C for 60 min for equilibrium before incubating at 30 °C for 8-14
526 hours for amplification and then washed with PBST 3 times for 30 mins each. The samples were
527 then treated with 20 mM methacrylic acid N-hydroxysuccinimide ester in 100 mM sodium
528 bicarbonate buffer for 4 hours to overnight at room temperature. Following the exact same
529 procedures casting tissue-hydrogel hybrid, cDNA amplicons were re-embedded with 2%
530 acrylamide, 0.05% bis-acrylamide to enable cDNA amplicon crosslinking in the tissue-hydrogel
531 setting, and such cross-linking is essential to maintain the position and integrity of the amplicons
532 through many cycles of detection. Samples were stored in PBST or wash and imaging buffer at
533 4°C until imaging and sequencing.

534 Before SEDAL, the samples were treated with the dephosphorylation mixture (0.25 U/ μ L
535 Antarctic Phosphatase, 1x BSA, in 1x Antarctic Phosphatase buffer) at 37 °C for 4 hours and
536 washed by PBST three times for 30 min each. Each sequencing cycle began with treating the
537 sample three times, 15 min each, with the stripping buffer (60% formamide and 0.1% Triton X-
538 100 in water) at room temperature, followed by washing with PBST three times for 15 min each.
539 Then the samples were incubated with a at minimal 300 μ L sequencing-by-ligation mixture (0.2
540 U/ μ L T4 DNA ligase, 1x BSA, 10 μ M reading probe, and 5 μ M fluorescent decoding
541 oligonucleotides in 1x T4 DNA ligase buffer) at room temperature for overnight, followed by
542 rinsing with washing and imaging buffer three times for 10 min each before imaging. Images
543 were acquired using the same Leica TCS SP8 with a 25x water-immersion objective (NA 0.95).
544 The voxel size for imaging was 0.32 μ m \times 0.32 μ m \times 0.70 μ m. For each round, images were
545 acquired with Alexa 488, 546, 594, and 647 illumination. DAPI was dissolved in wash and
546 imaging buffer and used for nuclei staining for 3 hours before the first round. The DAPI signal
547 was collected at the first cycle of imaging with an additional 405 nm wavelength. Six cycles of
548 imaging were performed to detect 1017 genes.

549 Data processing for Deep-STARmap and Deep-RIBOmap.

550 *Deconvolution:* Image deconvolution was achieved with Huygens Essential version
551 23.4.0 (Scientific Volume Imaging, The Netherlands, <http://svi.nl>). We applied the classic
552 maximum likelihood estimation method with a signal-to-noise ratio of 10 and 10 iterations.

553 *Image registration, spot calling, and barcode filtering:* For image registration, spot
554 calling, and barcode filtering, we utilized our custom software package, Starfinder
555 (<https://github.com/wanglab-broad/starfinder>). This software corrects chromatic aberrations,
556 enhances signals, registers images, and extracts positive reads (amplicons). Adjustments were
557 made to accommodate the large datasets generated by thick tissue profiling. In short, image
558 clarity is enhanced by intensity normalization and histogram equalization where images in the
559 first sequencing round are used as reference. To ensure accurate and reliable identification of
560 each cDNA amplicon's barcode, we utilized a two-step registration process. First, we conducted a
561 global registration using 3D fast Fourier transform. Next, we applied a non-rigid registration using
562 MATLAB v.2023b's 'imregdemons' function. This method adjusts for any shifts and distortions
563 between imaging sessions, ensuring precise alignment of the same amplicon's positions across
564 different sequencing rounds. Since the amplicon size is larger than amplicons in thin tissue, we
565 applied a medium filter with 'medfilt2' function in a 3-by-3 or 2-by-2 (depending on the average
566 amplicon size) neighborhood around the corresponding pixel in the input image. Dots with
567 intensity at their centroids less than the threshold were removed. The process of identifying
568 individual amplicons in 3D was carried out using the 'imregionalmax' function in MATLAB to find

569 local maxima within the images from the first sequencing round. The dominant color for each
570 amplicon across all rounds of sequencing was then determined by estimating the amplicon size
571 and integrating the voxel volume intensity in each channel. Each dot's color composition was
572 represented by an L2-normalized vector with four elements, and dots showing multiple
573 maximum values within this vector were excluded. Initial filtering of dots was based on quality
574 scores, which were computed as the average of $-\log(\text{color vector value in the dominant channel})$
575 across all sequencing rounds. This metric quantified the degree to which each dot in each
576 sequencing round was derived from a single color rather than a blend of colors. Subsequently,
577 the barcode codebook was translated into color space, following the expected color sequence of
578 the two-base encoded barcode DNA sequence. Only dots that met the quality threshold and had
579 a matching barcode sequence in the codebook were retained, with all others being discarded.
580 The 3D physical locations and gene identities of these filtered dots were then preserved for
581 subsequent analysis.

582 *3D segmentation:* 3D image segmentation was performed based on the DAPI staining
583 image and the composite image containing amplicon channels to create reference
584 segmentations as previously described with minor adjustments^{45,74,79}. Unlike thin tissue analysis,
585 where images are stitched before segmentation, this approach is impractical for thick tissue
586 profiling because the stitched files are too large for effective segmentation. Therefore,
587 segmentation was performed on each field of view (FOV) individually, and the identified
588 amplicons were stitched afterward. For each FOV, images targeting different cellular
589 compartments were first processed using a median filter and then binarized with an
590 automatically determined threshold in FIJI. Distance Transformed Watershed 3D was
591 subsequently applied to generate a 3D segmentation mask for each cellular region. Connected
592 components (objects) with fewer than 500 voxels were removed from the binary image. Finally,
593 the images were dilated using a disk structure element with a radius of 10.

594 *Reads assignment and stitching:* Filtered amplicons overlapping each segmented cell
595 region in 3D were assigned to their respective regions to compute a per-cell gene expression
596 matrix. The TileConfiguration file generated from FIJI grid stitching was then used to merge
597 detected amplicon signals from each FOV, ensuring the removal of duplicated cells and
598 associated reads. Further strategies to exclude low-quality cells were applied as previously
599 described in thin tissue analysis^{30,45,74}.

600 *Cell type classification via FuseMap:* Cell type classification was performed using
601 transfer learning with a pretrained FuseMap model, as previously described⁵⁰. This model maps
602 and annotates new query data with cell-type labels based on cell embeddings. In this study, a
603 previously published brain spatial atlas served as the reference for training the FuseMap model,
604 while thick tissue sections were used as the query datasets for annotation.

605 *Harmony integration:* To benchmark FuseMap performance, Harmony integration was
606 employed. First, Deep-STARmap data were combined with Deep-RIBOmap data after
607 preprocessing, followed by batch correction using the `pp.combat` function. Harmony integration
608 was then applied to the combined dataset to create a joint PCA embedding⁸⁰. A k-nearest
609 neighbor (KNN) classifier was trained on the integrated PC space using cosine distance as the
610 metric. This classifier was used in a label transfer process to annotate each cell based on its
611 neighboring reference cells in the KNN graph. The label transfer was performed for the
612 annotation at the "Rank4_Refine" level.

613 Gene Ontology (GO) enrichment analysis

614 GO enrichment analysis was conducted using the DAVID database
615 (<https://david.ncifcrf.gov/>)^{81,82}. gProfiler (<https://biit.cs.ut.ee/gprofiler/gost>) was utilized for GO
616 analysis. Enriched GO terms were selected from biological processes (BP) and cellular
617 components (CC) with FDR < 0.05 for both cell-type-resolved Deep-STARmap and Deep-
618 RIBOmap profiles, as well as for somata-enriched translation genes and processes-enriched
619 translation genes.

620 Gene Clustering

621 The gene expression (*log₂_norm1e4*) of the 4 samples were first averaged across the
622 cell types within each sample, respectively. Subsequently, the average expression values were
623 standardized by calculating the Z-score within each sample. The standardized vectors were
624 merged and clustered with the Leiden algorithm from Scanpy⁸³ (Version 1.9.3).

625 Near-range cell-cell adjacency analysis

626 Near-range cell-cell adjacency analysis was performed to quantify the number of edges
627 between cells of each main cell type and cells of other main cell types, as previously
628 described^{75,84}. The adjacency value between cell types A and B was defined as the number of
629 A-B edges within a 1-hop neighborhood on the Delaunay tissue graph, calculated using
630 `scipy.spatial`. Raw counts were normalized against a null distribution created by 1,000 random
631 spatial shifts of cells.

632 Morphological reconstructions

633 3D reconstructions of single-neuron morphologies were generated from 3D image stacks
634 using Imaris (Oxford Instruments; v. 9.7.2-10.1.1). Dendritic arbors of Tetbow-labeled neurons
635 were initially reconstructed semi-automatically with the filament tracer in autodepth mode.
636 These reconstructions were then extensively manually corrected and curated using the filament
637 tracer in manual mode. A fully connected neuronal structure was reconstructed wherever
638 possible while remaining faithful to the image data. Any processes that could not be definitively
639 linked to the main structure were left unconnected.

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649 **Reference**

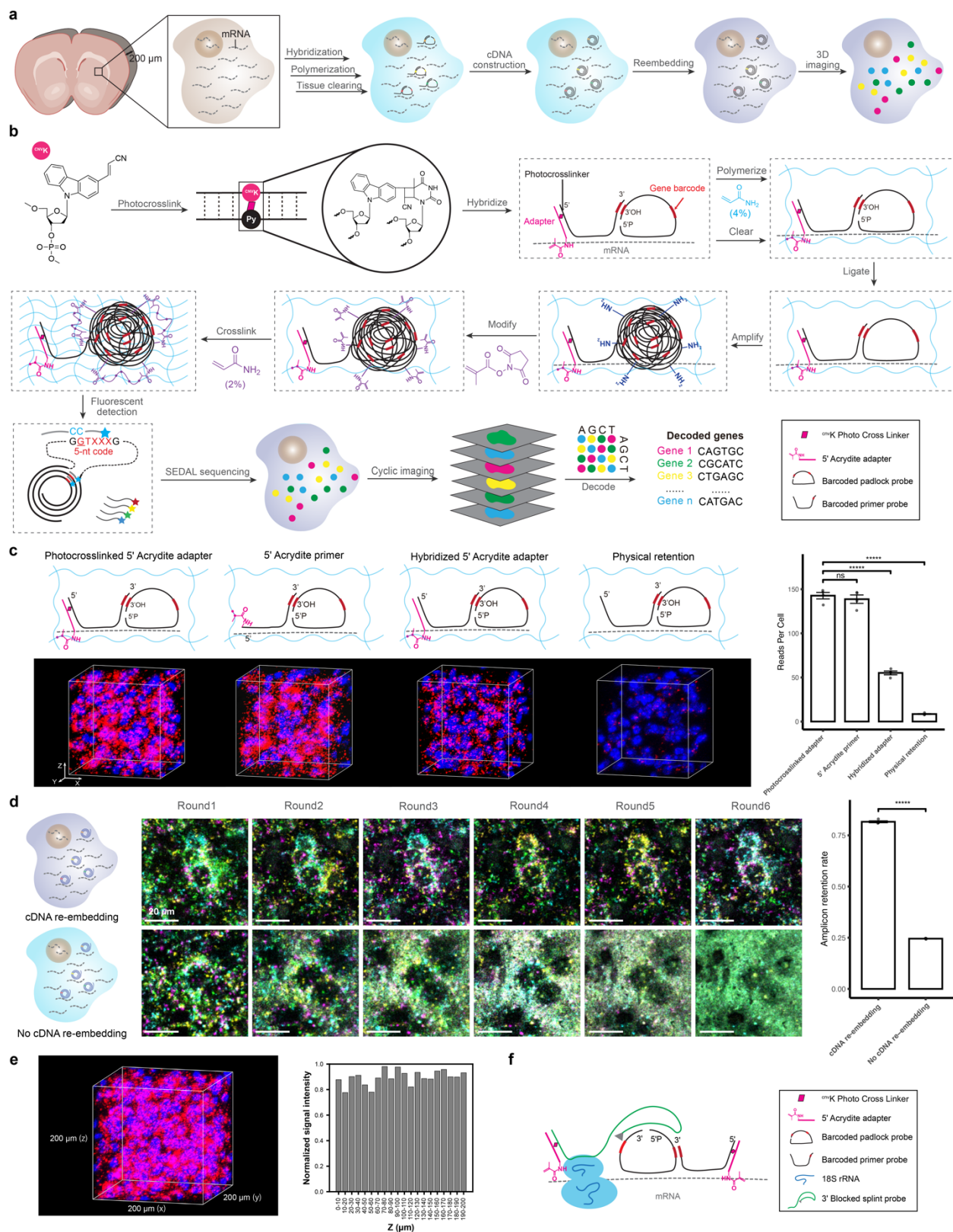
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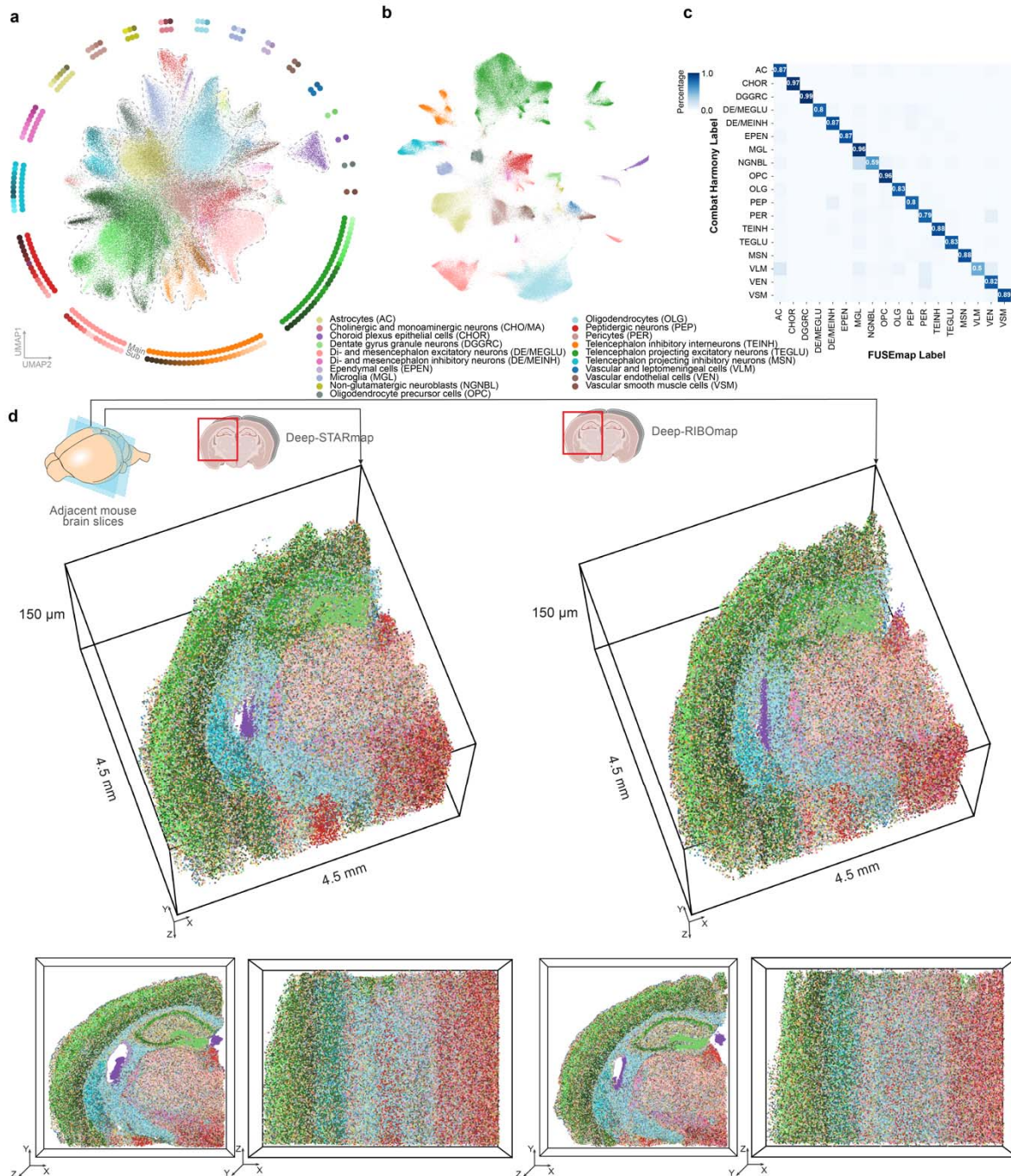
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Fig. 1 | Deep-STARmap and Deep-RIBomap enable spatiotemporally resolved transcriptomics and translomics in 200 μm thick tissue blocks. a, Schematic summary of

829 Deep-STARmap and Deep-RIBOmap workflow. **b**, *In situ* sequencing of transcriptional states in
830 thick tissue blocks: The primer, featuring a flanking linker sequence at its 5' end, is covalently
831 crosslinked (pink rhombus) to an Acrydite-modified oligonucleotide adapter (pink). This
832 crosslinking occurs through a photo-crosslinking reaction between ^{CNV}K and pyrimidines via a
833 [2+2] cycloaddition upon UV-A irradiation (366 nm). Following the preparation of thick tissue
834 slices (see Methods), the adapter (pink)-primer (black) complex and padlock (black) probes with
835 unique gene identifiers (red) hybridize to intracellular mRNAs (gray dashed line) within the intact
836 tissue. The probe set is copolymerized with acrylamide, forming a DNA-gel hybrid (blue wavy
837 lines) through the adapter's functionalized acrylic group, followed by the removal of unbound
838 lipids and proteins. Subsequently, enzymatic ligation and rolling circle amplification (RCA)
839 construct *in situ* cDNA amplicons. These cDNA amplicons are further anchored into the
840 hydrogel network via hydrogel re-embedding. Barcodes on the unique gene identifiers are read
841 out via cyclic *in situ* sequencing with error reduction by dynamic annealing and ligation (SEDAL).
842 This comprehensive quantification of RNA enables the elucidation of gene expression patterns
843 and the identification of cell types within the native 3D tissue context. **c**, Left: Schematics and
844 representative fluorescent images of negative and positive control experiments in 100 μm tissue
845 sections of the mouse cerebral cortex. Using a 5' Acrydite adapter photocrosslinked with the
846 primer produces equivalent results to direct 5' Acrydite modification of the primer, both
847 surpassing the performance of adapter-primer hybridization alone or hydrogel physical retention.
848 Right: Quantification of cell images showing the average amplicon reads per cell (n=4 images
849 per condition). Red: DNA amplicons from 4 cell type markers. Blue: DAPI. Scale bar: 20 μm .
850 Two-sided independent *t*-test, ****P < 0.0001. Data shown as mean \pm standard deviation. **d**, Left:
851 Schematics and representative fluorescent tissue images of 6 rounds of sequencing with and
852 without cDNA re-embedding. In the absence of cDNA re-embedding, PEGylated
853 bis(sulfosuccinimidyl)suberate (BSPEG) is used to crosslink cDNA. This results in background
854 accumulation and reduced cDNA detection efficiency. Fluorescent images show Ch1 to Ch4
855 (color-coded channels for barcode decoding) and cell nuclei (blue) in mouse brain slices. Right:
856 Quantification of cell images showing the average amplicon retention rate after 6 rounds of
857 sequencing (n=4 images per condition). Two-sided independent *t*-test, ****P < 0.0001. Data
858 shown as mean \pm standard deviation. **e**, Representative raw fluorescent tissue images across
859 200 μm and quantification of DNA amplicon signal intensity at different tissue depths. **f**, Deep-
860 RIBOmap probe design: Primer (black) and padlock (black) probes with unique gene identifiers
861 (red) hybridize to intracellular mRNAs (gray dashed line), while splint probes (green) bind to the
862 18S rRNA of ribosomes. Splint probes serve as splints for proximity ligation and circularization
863 of padlock probes. Both the primer and splint probe feature a flanking linker sequence at their 5'
864 ends and are covalently crosslinked (pink rhombus) to an acrydite-modified oligonucleotide
865 adapter (pink).
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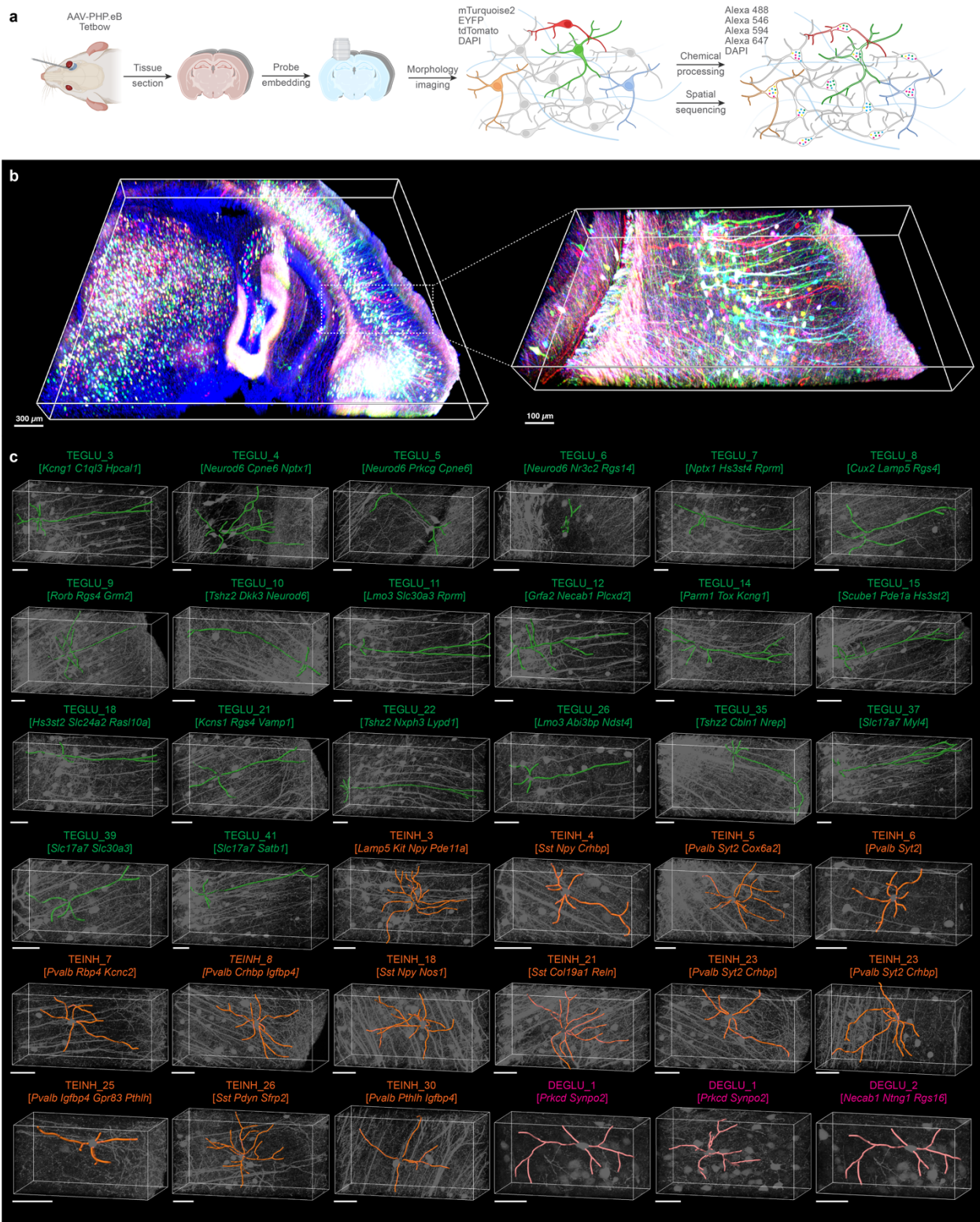


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Fig. 2 | Spatial single-cell transcriptomic and translational profiling of 1,017 genes in thick mouse brain slices: **a-b**, Uniform Manifold Approximation and Projection (UMAP) plot visualizations of transcriptional and translational profiles of 362,704 cells collected from mouse coronal hemibrains using FuseMap (**a**) and integration using Harmony (**b**). Surrounding diagrams display 137 subclusters derived from 19 main clusters. **c**, Confusion matrix of cell type labels obtained from FuseMap and Harmony integration, visualizing cell types with more than 100 cells in the sample. **d**, 3D molecular cell-type maps derived from Deep-STARmap (left) and Deep-RIBOmap (right) across adjacent 150- μ m thick sections from the mouse hemisphere.

877 Each dot represents one cell, colored by its subcluster identity, using the same color code as in
878 (a).

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Fig. 3 | Characterizing the morphological features of transcriptomic types. a, Deep-STARmap combined with Tetbow enables simultaneous profiling of gene expression and

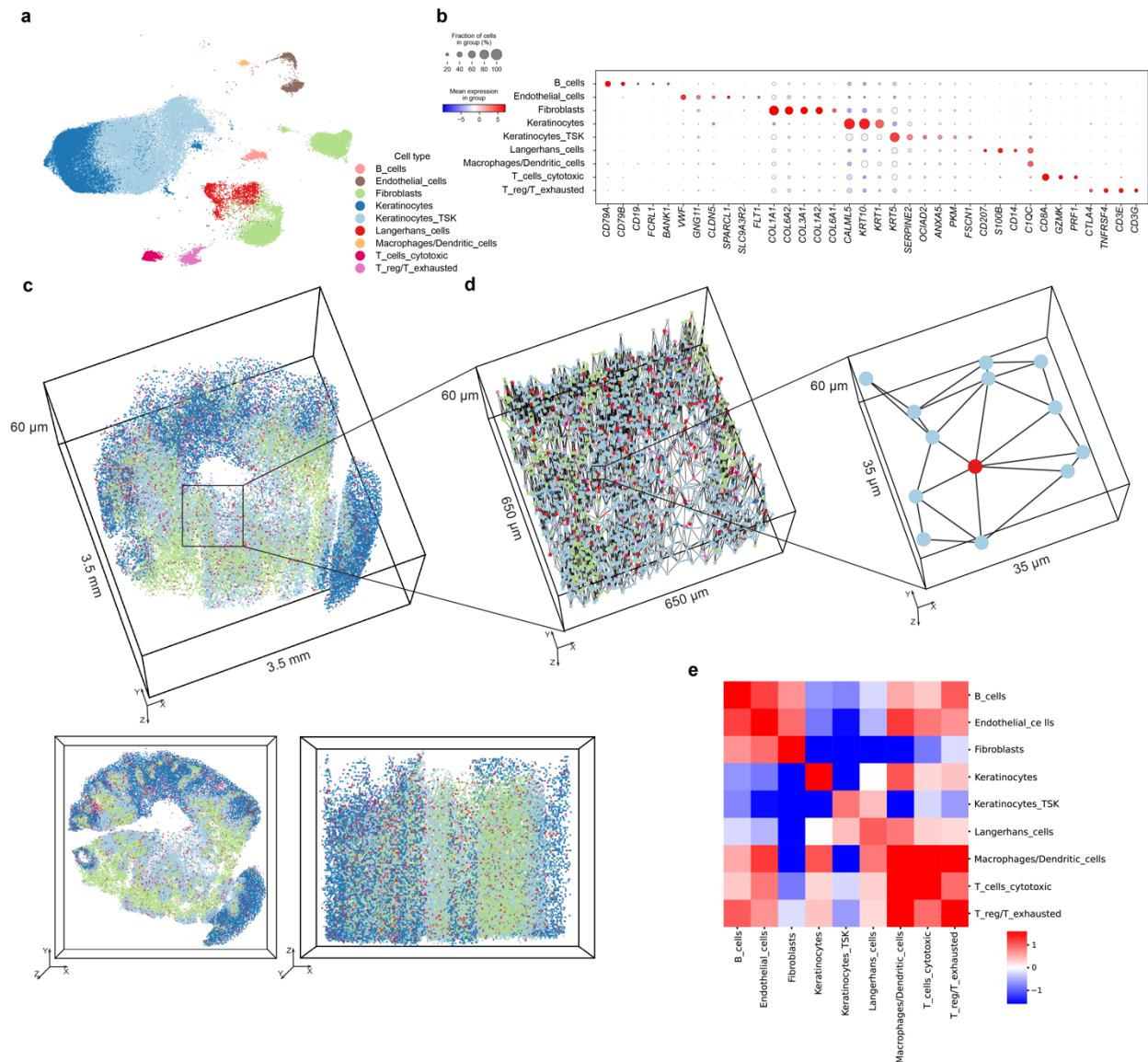
884 neuron morphologies. AAV-PHP.eB delivers vectors encoding fluorescent proteins and the tTA
885 expression vector. Following tissue sectioning and embedding probe sets into hydrogel, Tetbow
886 fluorescent proteins and DAPI are imaged. After protein digestion, cDNA amplicons are
887 constructed and sequenced. DAPI co-staining serves as a fiducial marker for image registration
888 between FP images and *in situ* sequencing images, enabling the identification of Tetbow-
889 labeled neurons by molecular subtype. **b**, Left: Volume rendering of neurons in the
890 hippocampus and thalamus labeled with Tetbow. Neurons exhibit unique colors generated by
891 the stochastic and combinatorial expression of three fluorescent proteins (tdTomato, EYFP, and
892 mTurquoise2), enabling high-resolution identification and differentiation of individual neurons.
893 Right: Zoom-in view of volume rendering of mouse cortical pyramidal neurons labeled with
894 Tetbow. **c**, Representative individual morphological reconstructions of 30 transcriptome-defined
895 subtypes of excitatory and inhibitory neurons. These reconstructions illustrate the distinct
896 morphologies associated with each neuronal subtype, providing insights into the structural
897 diversity within the neural network. Scale bar: 50 μm .

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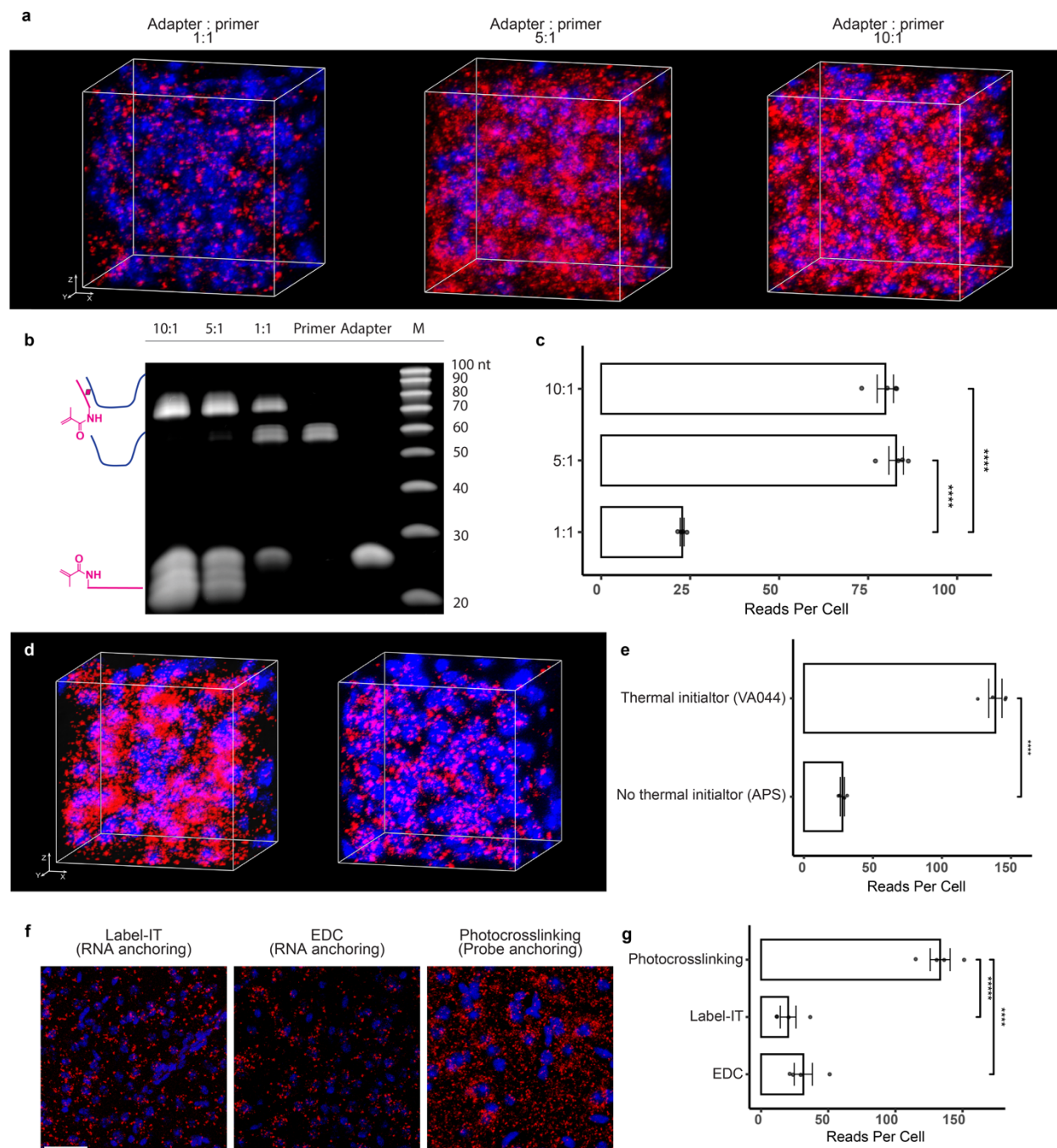
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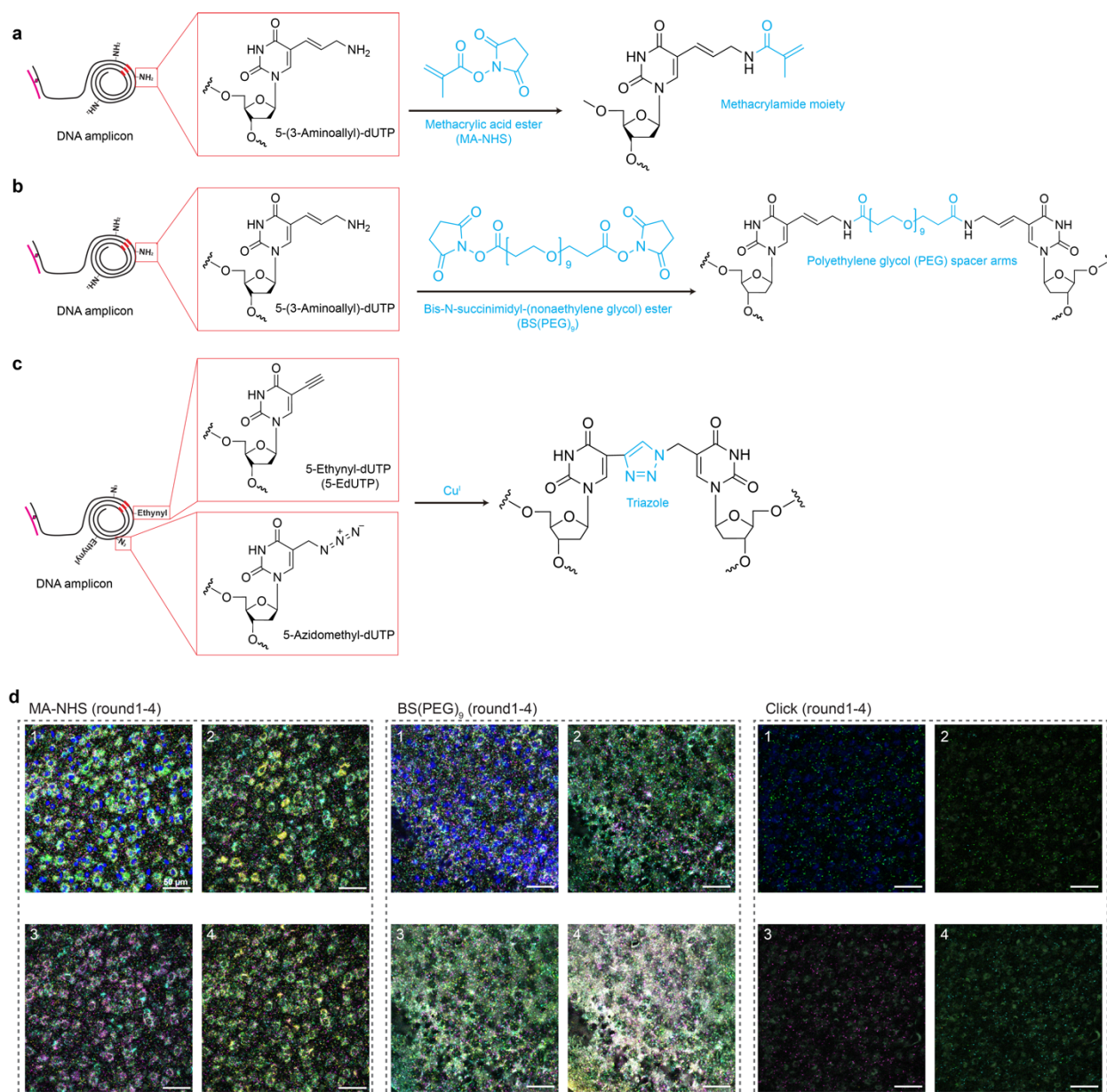
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Fig. 4 | Mapping cell-cell interactions in human cSCC. **a**, UMAP plot visualization of transcriptional profiles of 51,471 cells, integrated using Harmony with a published cSCC scRNA-seq dataset. Cells are color-coded according to their cell-type identity. **b**, Dot plot illustrating the top differentially expressed marker genes for each major cluster. The color scale represents the \log_2 fold change in gene expression compared to the mean gene expression values across all cells. The dot size indicates the percentage of cells expressing the genes within each major cell type. **c**, 3D molecular cell-type maps generated from Deep-STARmap, using the same color coding as in (a). **d**, Zoomed-in view of the interaction between Langerhans cells and tumor-specific keratinocytes within a mesh graph of physically neighboring cells. Each cell is depicted as a spot colored according to its main cell type, with physically neighboring cells connected by edges. **e**, 3D cell-cell adjacency quantified by the normalized number of edges between pairs of cell types.



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916 **Extended Data Fig. 1 | Optimization of probe crosslinking.** **a**, Representative fluorescent
917 imaging illustrating probe anchoring efficiency in a hydrogel matrix with various adapter-primer
918 ratios. Red: DNA amplicons from 4 cell type markers. Blue: DAPI. Scale bar: 10 μ m. **b**, 15%
919 TBE-Urea gels demonstrating UV crosslinking efficiency with varying adapter-primer molar
920 ratios. ^{CNV}K- and Acrydite-containing adapter used for UV crosslinking is
921 [5Acryd]GCTA[cnvK]ATACGTCGTA CTACTGGTAGG[Inv-dT] (24 nt). Primer used is 58 bp ssDNA
922 with a 24 nt flanking liner at the 5' end. M, Marker: IDT ssDNA 20/100 Ladder. **c**, Quantification
923 of cell images showing the average amplicon reads per cell ($n=4$ images per condition). Two-
924 sided independent t -test, **** $P < 0.0001$. Data presented as mean \pm standard deviation. **d**,

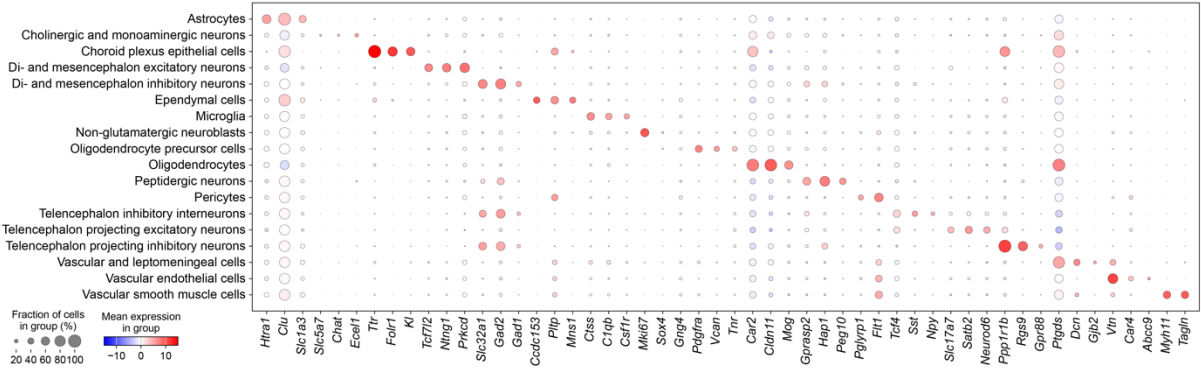
925 Representative fluorescent imaging demonstrating probe anchoring efficiency with and without
926 the use of the VA-044 thermal initiator in the first round of polymerization. Red: DNA amplicons
927 from 4 cell type markers. Blue: DAPI. Scale bar: 10 μm . **e**, Quantification of cell images showing
928 the average amplicon reads per cell ($n=4$ images per condition). Two-sided independent t -test,
929 **** $P < 0.0001$. Data presented as mean \pm standard deviation. **f**. Representative fluorescent
930 imaging demonstrating detection efficiency of covalently anchored RNA molecules or probes
931 within the hydrogel in the Deep-STARmap setting. Red: DNA amplicons from 4 cell type
932 markers. Blue: DAPI. Scale bar: 50 μm . **g**, Quantification of cell images showing the average
933 amplicon reads per cell ($n=4$ images per condition). Two-sided independent t -test, **** $P <$
934 0.0001. Data presented as mean \pm standard deviation.
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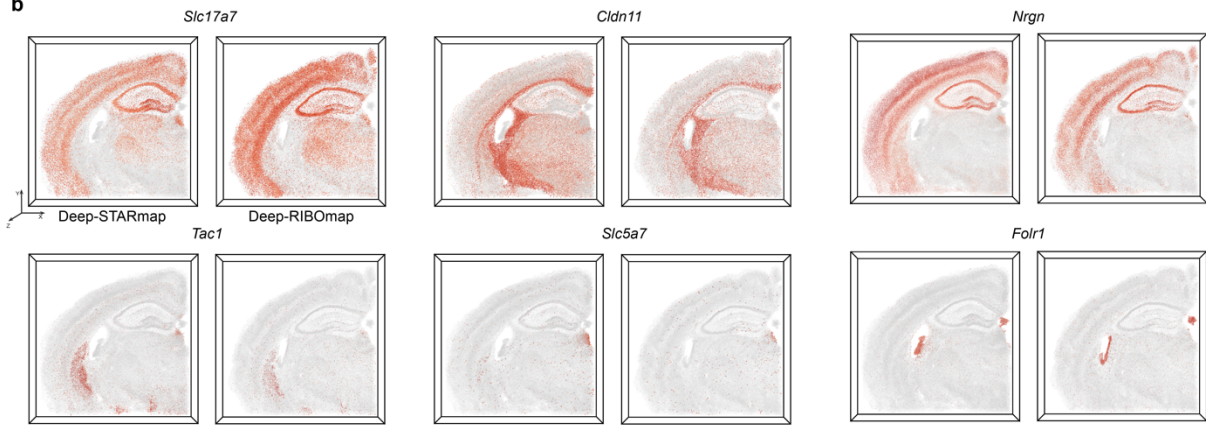
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 938 **Extended Data Fig. 2 | Optimization of re-embedding strategy.** **a**, Mechanism of cDNA
 939 crosslinking using hydrogel re-embedding. Amine-modified nucleotides were incorporated into
 940 the RCA reaction. MA-NHS enables rapid conjugation to nucleophilic groups on the amplicons
 941 via its NHS ester under mild conditions. These functionalized methacrylamide moieties are then
 942 integrated into the hydrogel, effectively immobilizing the cDNA amplicons. **b**, Mechanism of
 943 cDNA crosslinking using BSPEG. Amine-modified nucleotides were incorporated into the RCA
 944 reaction followed by BSPEG crosslinking, where the NHS esters of BSPEG react with the amino
 945 groups on the amplicons. **c**, Mechanism of cDNA crosslinking using Click chemistry. Azide and
 946 alkyne groups were incorporated during the RCA process, followed by the addition of copper to
 947 catalyze the azide-alkyne cycloaddition, forming a stable triazole ring as a crosslinking method.
 948 **d**, Representative fluorescent imaging demonstrating sequencing signal-to-noise ratio using
 949 different cDNA crosslinking strategies. BSPEG and Click chemistry crosslinking result in higher
 950 background noise compared to hydrogel re-embedding after several rounds of sequencing.

951 Additionally, the incorporation of azide and alkyne moieties during RCA significantly reduced
 952 amplification efficiency, leading to fewer amplicons.
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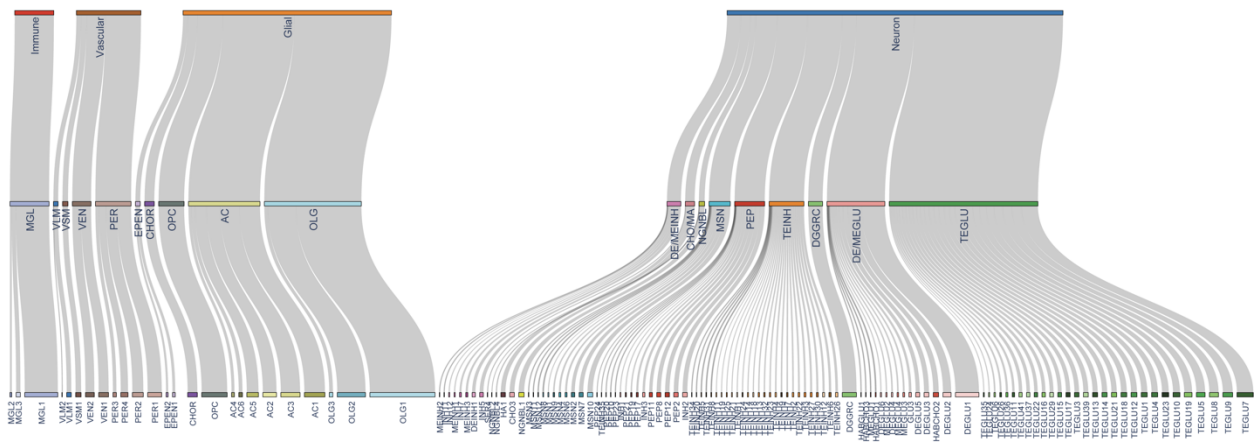
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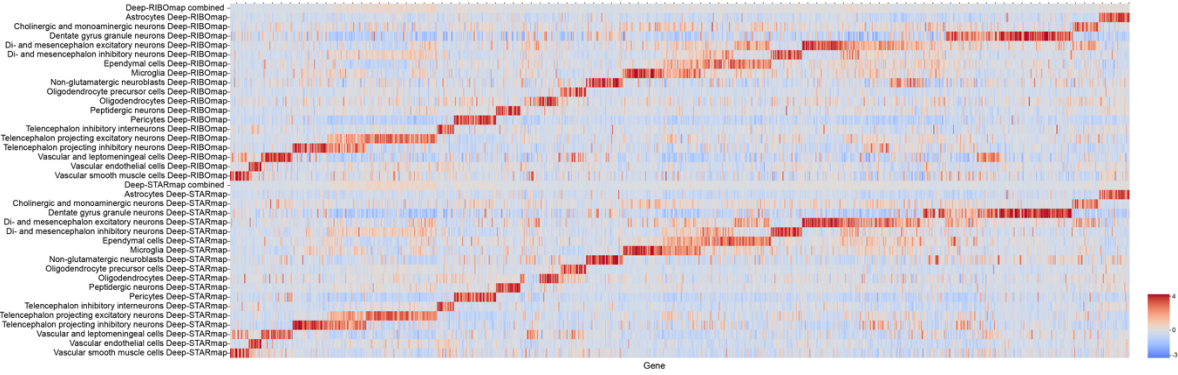


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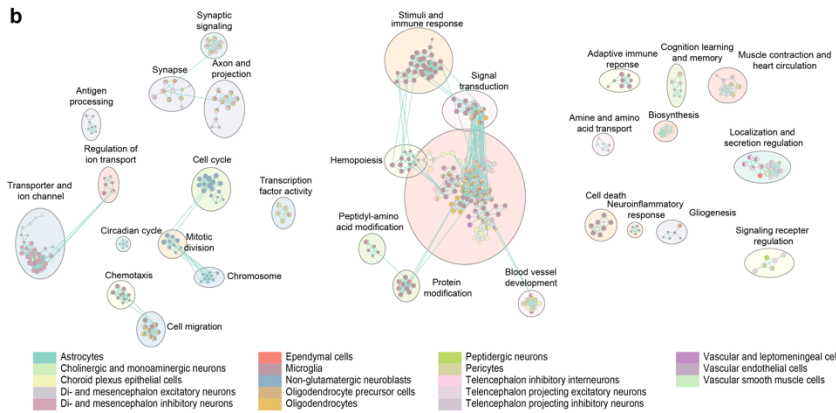
955 **Extended Data Fig. 3 | Spatially single-cell transcriptomic and translomic profiling of**
956 **1017 genes in the mouse brain. a**, Dot plot illustrating the expression levels of representative
957 markers across various major cell types using Deep-STARmap and Deep-RIBOmap. The color
958 scale represents the \log_2 fold change in gene expression compared to the mean gene
959 expression values across all cells. The dot size indicates the percentage of cells expressing the
960 genes within each major cell type. xyz size: 4.5 mm, 4.5 mm, 150 μm . **b**, Deep-STARmap (left)
961 and Deep-RIBOmap (right) images of example cell marker genes and neurotransmitter genes. **c**,
962 Hierarchical taxonomy of cell types showing the main level and subtype level cell-type
963 identification and annotations.

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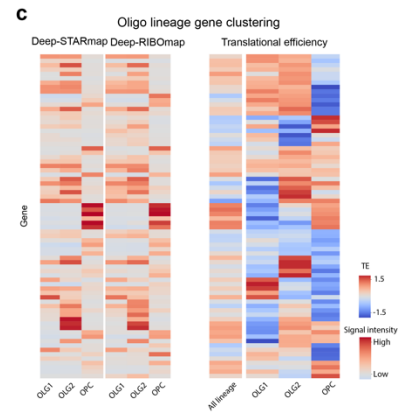
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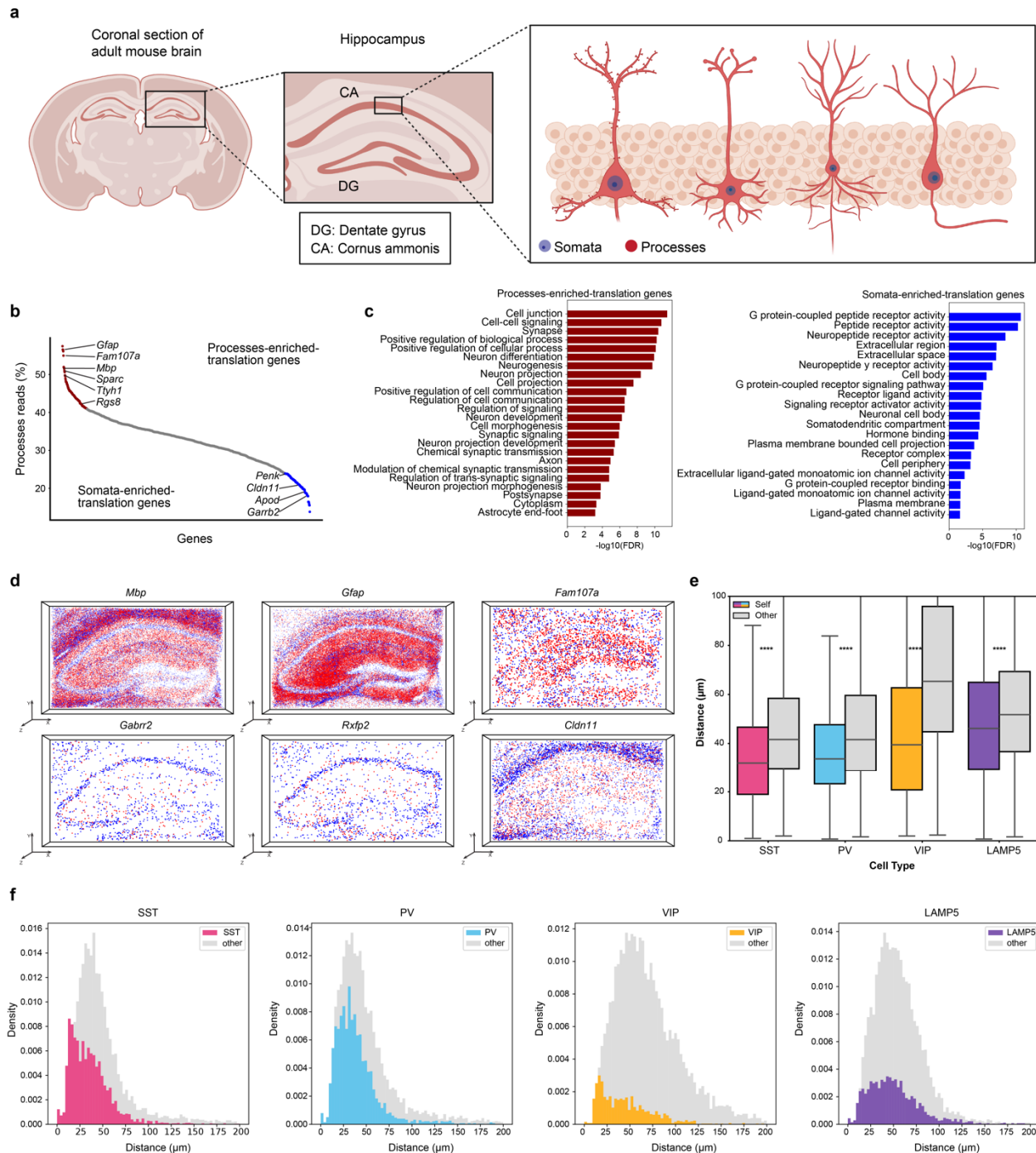
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Extended Data Fig. 4 | Comparison of spatial translome and transcriptome in the mouse brain and cell-cell adjacency analysis. **a**, Heatmap showing the gene clustering using the RIBOmap and STARmap results by cell type (Z-score expression). **b**, Visualization of enriched GO terms within each gene module, categorized and color-coded by module. In the enrichment map, nodes represent enriched GO terms, with the size of each node reflecting the number of genes associated with that term. Edges between nodes indicate shared genes among the GO terms. **c**, Heatmap displaying gene clustering based on Deep-STARmap and Deep-RIBOmap results across the three oligodendrocyte lineage cell types (left). The right panel shows the translational efficiency (TE) of these genes within each oligodendrocyte lineage cell type (Z-score expression).

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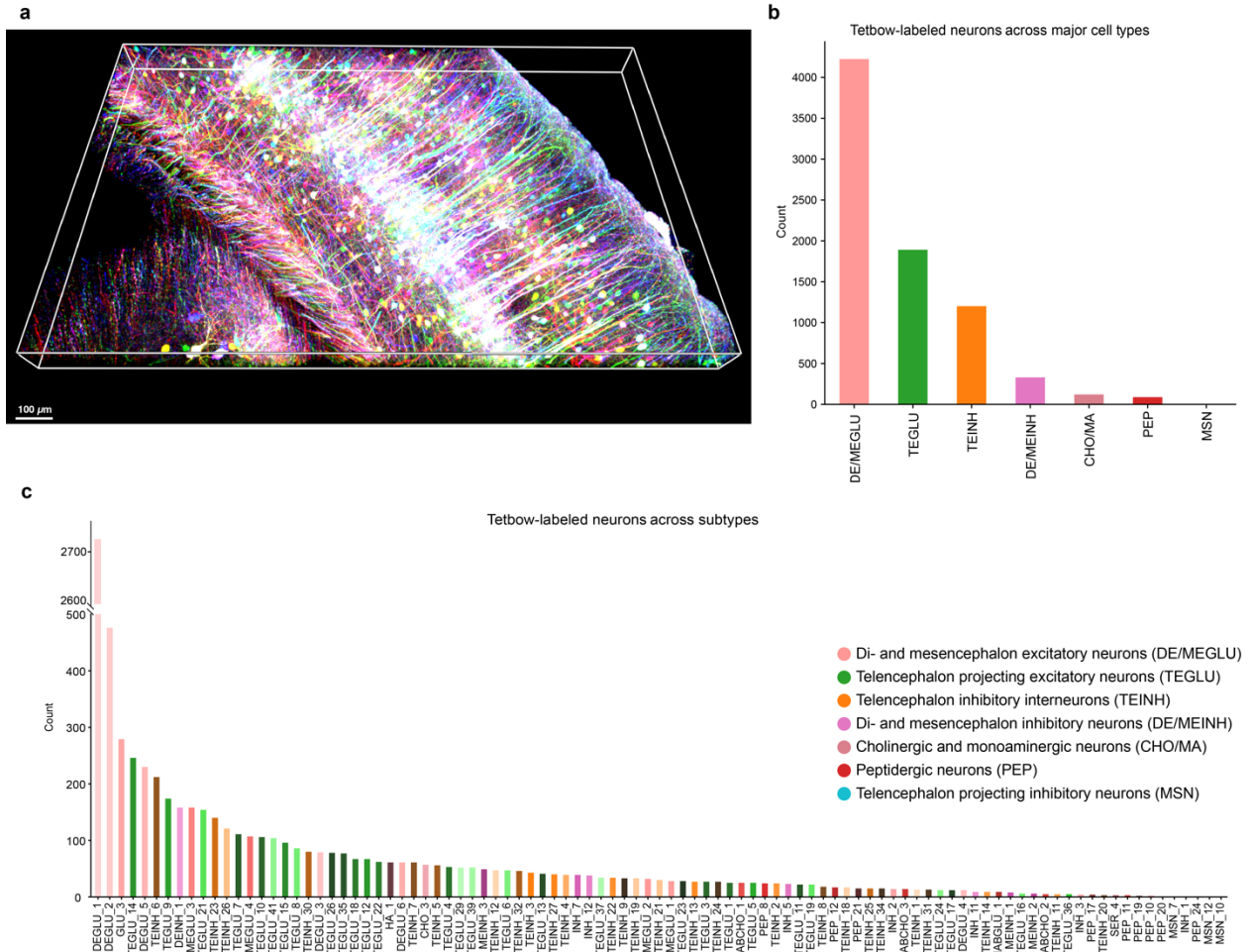
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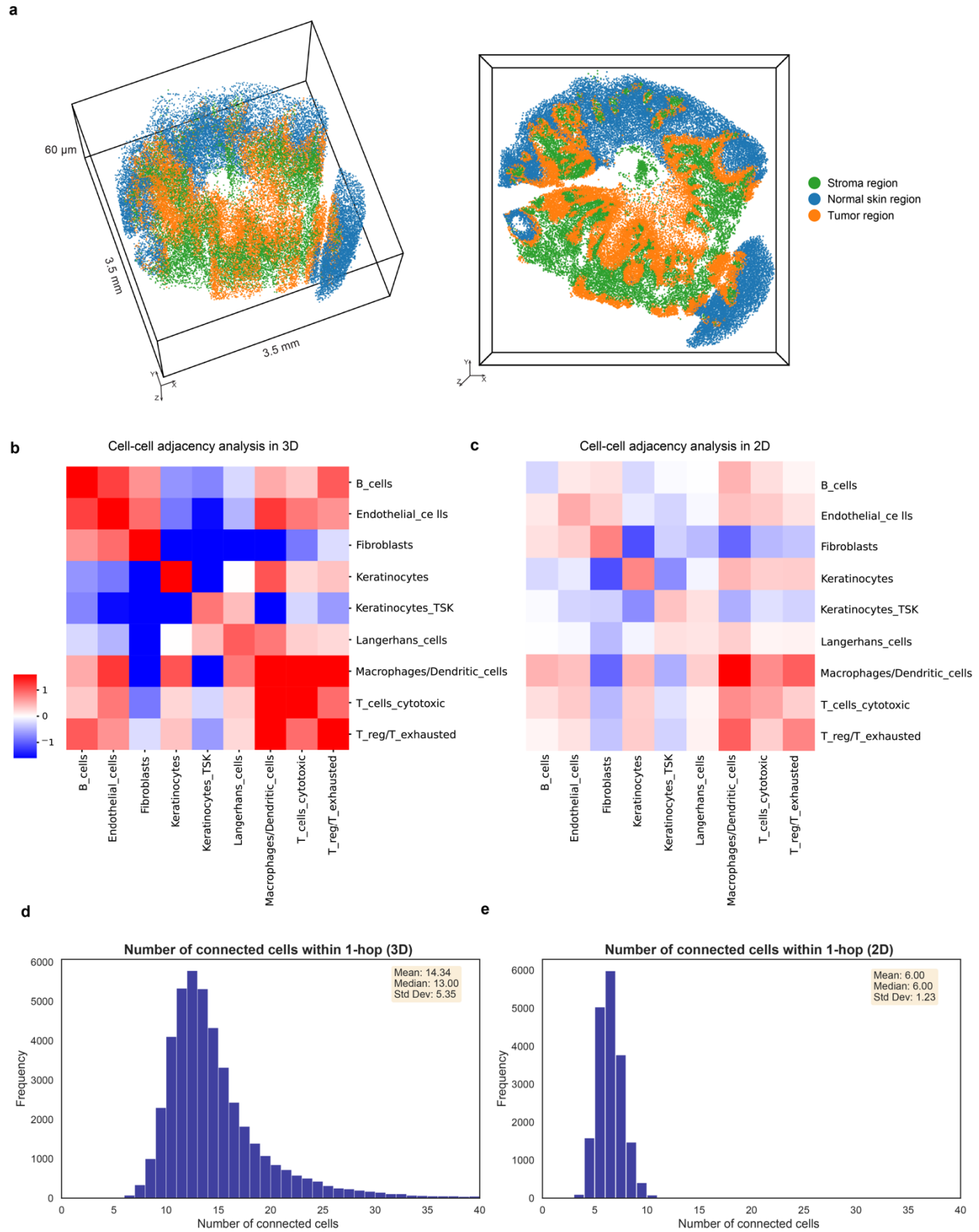
Extended Data Fig. 5 | Localized translation in the somata and processes of neuronal and glial cells in the mouse brain. **a**, Schematic illustration of a hippocampal slice highlighting the somata and processes of hippocampal neurons. **b**, Processes read percentages of individual translating genes with genes rank-ordered based on their processes reads percentage. **c**, Significantly enriched GO terms for processes-enriched and somata-enriched translating genes. **d**, Spatial translation map of representative genes with enriched translation in processes (top) and somata (bottom) within the hippocampus, depicting somata reads in blue and process

986 reads in red. **e-f**, Nearest-neighbor distance distributions in Deep-STARmap sample, comparing
987 distances from cells in specific inhibitory neuronal subclasses to cells within the same subclass
988 (“to self”) and to cells in different subclasses (“to other”).
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 992 **Extended Data Fig. 6 | Quantification of Tebow-labeled neurons. (a)** Another zoom-in view
 993 of volume rendering of mouse cortical pyramidal neurons labeled with Tebow. **(b,c)** Cell count
 994 quantification of Tebow-labeled neurons across major cell types (b) and subtypes (c).
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Extended Data Fig. 7 | Cell-cell adjacency analysis in 2D and 3D. **a**, Molecular tissue region maps visualized in 3D. Each dot represents a cell. Three molecular regions can be identified:

1000 tumor region, fibroblast region, and normal skin region. These regions were identified by
1001 analyzing low-frequency, large-scale patterns within the spatial neighbors graph. **b-c**,
1002 Quantification of cell-cell adjacency in 3D (b) and 2D (c) by the normalized number of edges
1003 between pairs of cell types. The 2D analysis is performed by projecting 15 μm (~1 cell layer)
1004 slices along the z-axis, taken within the same 3D volume as shown in Fig. 4. The 3D analysis
1005 reveals stronger cell-cell adjacency enrichment. **d-e**, The 3D analysis detects stronger cell-cell
1006 interactions because the number of connected cells (edges of a given cell in the mesh graph via
1007 Delaunay triangulation) is greater than in 2D. The 2D nearest-neighbor distances cannot
1008 accurately represent the 3D cellular environment.