

1 **HyDrop: droplet-based scATAC-seq and scRNA-seq using dissolvable hydrogel beads**

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10 **Abstract**

11 Single-cell RNA-seq and single-cell ATAC-seq technologies are being used extensively to create
12 cell type atlases for a wide range of organisms, tissues, and disease processes. To increase the
13 scale of these atlases, lower the cost, and allow for more specialized multi-ome assays, custom
14 droplet microfluidics may provide complementary solutions to commercial setups. We developed
15 HyDrop, a flexible and generic droplet microfluidic platform encompassing three protocols. The
16 first protocol involves creating dissolvable hydrogel beads with custom oligos that can be released
17 in the droplets. In the second protocol, we demonstrate the use of these beads for HyDrop-ATAC,
18 a low-cost non-commercial scATAC-seq protocol in droplets. After validating HyDrop-ATAC, we
19 applied it to flash-frozen mouse cortex and generated 8,502 high-quality single-cell chromatin
20 accessibility profiles in a single run. In the third protocol, we adapt both the reaction chemistry
21 and the capture sequence of the barcoded hydrogel bead to capture mRNA, and demonstrate a
22 significant improvement in throughput and sensitivity compared to previous open-source droplet-
23 based scRNA-seq assays (Drop-seq and inDrop). Similarly, we applied HyDrop-RNA to flash-
24 frozen mouse cortex and generated 9,508 single-cell transcriptomes closely matching reference
25 single-cell gene expression data. Finally, we leveraged HyDrop-RNA's high capture rate to
26 analyse a small population of FAC-sorted neurons from the *Drosophila* brain, confirming the
27 protocol's applicability to low-input samples and small cells. HyDrop is currently capable of
28 generating single-cell data in high throughput and at a reduced cost compared to commercial
29 methods, and we envision that HyDrop can be further developed to be compatible with novel
30 (multi-) omics protocols.

31

32 Main

33 Droplet-microfluidic single-cell sequencing technologies have enabled the profiling of tens of
34 thousands^{1,2} - and recently millions^{3,4} - of single cells. Owing to their limited sensitivity (e.g. median
35 of 250-500 genes per cell in primary tissues^{1,5}), and relatively lengthy workflows compared to
36 commercial solutions such as 10x Genomics, generic protocols such as Drop-seq⁶ and InDrop⁷
37 have been used much less than the commercial alternatives^{5,8,9}. A second wave of droplet-based
38 assays has provided the ability to profile chromatin accessibility of single cells, particularly using
39 single-cell ATAC-seq¹⁰. To our knowledge, only one non-commercial droplet-based scATAC-seq
40 protocol has been published so far¹¹. Despite its elegant conceptual solution to droplet-based
41 scATAC/scRNA-seq, the SNARE-seq protocol is labour intensive compared to commercial
42 solutions such as 10x Genomics' Chromium¹² and Biorad's ddSEQ¹³, and the use of resin beads
43 in the SNARE-seq protocol leads to reduced cell capture and sensitivity. We developed HyDrop,
44 a new hydrogel-based droplet microfluidic platform to improve the sensitivity and usability of both
45 scRNA-seq and scATAC-seq in droplets, and to provide the first hydrogel-based solution for
46 droplet-based scATAC-seq. All HyDrop protocols and analysis code are freely available at
47 <https://www.protocols.io/workspaces/aertslab> and <https://hydrop.aertslab.org>.

48
49 In the first HyDrop protocol, we generate barcoded hydrogel beads that can dissolve and release
50 their embedded barcoded oligonucleotide. Polyacrylamide beads incorporating disulfide
51 crosslinkers and short oligonucleotide PCR handles are generated by droplet microfluidics similar
52 to a recently published method¹⁴. A custom droplet microfluidic chip (fig. S1) is employed to
53 produce beads of approximately 45 μm diameter, but flow rates can be tuned to change bead
54 diameter. These hydrogel beads are then barcoded using a modified three-round split-pool PCR
55 synthesis method^{7,15}, resulting in $96 \times 96 \times 96$ (884,736) barcode possibilities. The terminal
56 sequence used in the final round of barcoding can be varied depending on the assay the beads
57 will be used for (see Methods, fig. 1a). A sequence complementary to the Tn5 transposase
58 adapter is used to capture tagmented chromatin fragments in scATAC-seq and a unique
59 molecular identifiers (UMI)¹⁶ + poly(dT) sequence is used to capture and count poly(A)+ mRNA
60 in scRNA-seq (see further below under the HyDrop-RNA protocol). The barcoded beads are
61 stored in a glycerol-based freezing buffer at -80°C in order to prevent loss of primers over time
62 (fig. S2a).

63
64 We validated the purity and concentration of the hydrogel bead primers using fluorescent probes
65 complementary to the beads 3-prime terminal sequence¹⁵ (fig. 1b) or one of the 96 sub-barcode
66 possibilities (fig. 1c). These experiments show that there is no significant loss of primers or mixing
67 of barcodes throughout the barcoding process, and that the beads are uniform in size and primer
68 content. Additional testing revealed that our modified PCR barcoding method produced more
69 uniformly barcoded beads compared to the isothermal amplification protocol described in InDrop
70 (fig. S2b). Furthermore, the disulfide moieties incorporated in both the bead's polymer matrix and
71 oligonucleotide linker can be cleaved when exposed to reducing conditions, such as DTT. This
72 chemical method of release is more user-friendly compared to the UV-mediated^{7,15} primer release
73 as the beads do not have to be shielded from light. Furthermore, the entire barcoding reaction
74 can be executed in the reducing environment standard to many biochemical reactions. In addition

75 to improved primer release compared to non-dissolvable beads (fig. S2c), dissolvable beads also
76 do not disrupt the emulsion during the thermocycling needed for scATAC-seq (see methods) as
77 they dissolve within minutes in the droplet's reducing environment (fig. S3a, b). Finally, by varying
78 the concentration of the acrydite primer during bead synthesis, lower or higher amounts of
79 cleavable barcoded primers can be generated. When the acrydite primer concentration
80 incorporated in the bead is high (50 μ M, similar to InDrop¹⁵), excess unreacted barcodes cannot
81 be sufficiently filtered out in further downstream steps. These primers are carried over to
82 subsequent reactions, leading to random barcoding of free fragments after droplet merging, and
83 subsequently to cell-mixed expression or chromatin accessibility profiles. The bead primer
84 concentration with an optimal balance between sensitivity and library purity was found to be 6 to
85 12 μ M for both scATAC-seq and scRNA-seq (fig. S4a, b).

86
87 Our second HyDrop protocol provides a generic assay for droplet-based scATAC-seq. Here,
88 nuclei are co-encapsulated with HyDrop-ATAC beads after tagmentation in bulk. In order to co-
89 encapsulate beads and nuclei with a high capture rate and minimal microfluidic complexity, we
90 developed a custom microfluidic chip (fig. S1). The chip design features two inlets for beads and
91 cells or nuclei and one inlet for the emulsion carrier oil. Several layers of passive filters near the
92 inlet ports mitigate dust and debris buildup during droplet generation to prevent obstruction of the
93 channels. Beads and nuclei are loaded via a tip reservoir to reduce non-linear flow behaviour and
94 the potential accumulation of cells/nuclei and hydrogel beads associated with narrow tubes^{17,18}
95 (fig. 1d, e). Due to the stability of all flows and the deformable nature of the hydrogel beads, >
96 90% occupancy of hydrogel beads in droplets can be achieved¹⁹ (fig. 1f). After co-encapsulation
97 with the tagmented nuclei, the hydrogel beads dissolve in the presence of DTT in the nuclei/PCR
98 mix and release their uniquely barcoded primers inside the droplet as described above.
99 Subsequent thermocycling of the emulsion denatures the Tn5 protein complex and releases
100 accessible chromatin fragments within the droplet. These fragments are then linearly amplified
101 and cell-indexed by the bead's barcoded primers after which the emulsion is broken and the
102 indexed ATAC fragments are pooled, PCR amplified, and sequenced (fig. 1g). Pitstop, a selective
103 small molecule inhibitor of clathrin is supplemented during nuclei extraction and tagmentation to
104 increase nucleus permeability to Tn5²⁰.

105 To assess the purity of scATAC-seq libraries generated by HyDrop-ATAC, we performed two
106 mixed-species experiments. First, we generated single-nuclei ATAC-seq libraries from a 50:50
107 mixture of human breast cancer (MCF-7) and a mouse melanoma cell line generated previously
108 ²¹. For the pre-processing and mapping of HyDrop-ATAC data, we developed a custom
109 preprocessing pipeline²². After filtering the cell barcodes for a minimum TSS enrichment score of
110 7 and unique fragment count of 1,000, we recovered 1,353 cells from a target of 2,000, with a
111 median of 2,705 unique fragments per cell. We identified 98.4% of cells as either human or mouse
112 at a minimum purity of 95% fragments mapping to either species (fig. 2a). This implied doublet
113 rate of 3.1% is comparable to other droplet microfluidic protocols^{6,7,12}. Next, we generated libraries
114 from a mixture of MCF-7/PC-3/Mouse cortex (45:45:10) to evaluate whether two human cell types
115 can be distinguished. A spike-in of 10% mouse cells was used as an internal control. We
116 recovered 2,602 human cells, 466 mouse cells, and 93 species doublets after filtering for 95%
117 species purity. Clustering human cells (together with the MCF-7 cells from the first species mixing

118 experiment to evaluate batch effects) recovered two distinct populations, each exhibiting specific
119 ATAC-seq peaks near MCF-7 or PC-3 marker genes (fig. S5a). Aggregated reads per cluster
120 showed typical ATAC-seq profiles concordant with public bulk ATAC-seq data²³ (fig. 2b, fig. S5b).

121 To evaluate the performance of HyDrop-ATAC on primary tissue, we then generated single cell
122 libraries from snap-frozen, dissected adult mouse brain cortex. Libraries were sequenced to
123 approximately 75% duplication rate. After filtering for a minimum of 1,000 unique nuclear
124 fragments, a TSS enrichment score of 5, and removing 481 cells (5.4%) detected as doublets by
125 Scrublet²⁴, we recovered a total of 8,502 single nuclei. Cells passing the filters had a median of
126 4,148 fragments per cell, a median TSS enrichment score of 13, and a median of 53% of
127 fragments in peaks, reflecting high-quality cells and low levels of background signal (fig. 2c-f).
128 Even though the number of unique fragments per cell (~4K) is lower than that of commercial
129 methods (e.g., 17-20K per cell for 10x Genomics, see Methods), HyDrop-ATAC compares
130 favourably with these platforms in terms of TSS enrichment and FRIP scores, and due to the
131 possibility to profile large cell numbers (>8K cells in a single run), cell type clustering of mouse
132 brain achieved higher resolution compared to publicly available 10x Genomics data sets. We used
133 cisTopic²⁵ to reduce the dimensionality of the dataset and the Leiden algorithm²⁶ to cluster cells
134 (fig. 2g). Cell annotation using the aggregated ATAC signal around several neocortex markers^{27,28}
135 recovered 19 distinct cell types, similar to previously published scATAC-seq mouse cortex
136 data^{29,30} (fig. S6). For example, we identified oligodendrocyte precursors and mature
137 oligodendrocytes, marked by exclusive accessibility nearby *Sox10* and *Pdgfra2*, respectively.
138 Within ganglionic eminence-derived interneurons, we were able to further distinguish medial
139 ganglionic eminence-derived subtypes with specific ATAC-seq signal near either *Vip* or *Lamp5*,
140 and caudal ganglionic eminence-derived subtypes with accessibility near either *Sst* or *Pvalb*.
141 Finally, HyDrop-ATAC data revealed distinct cell-type specific differentially accessible regions (fig.
142 2h-i).

143 In the third HyDrop protocol, we implemented a new scRNA-seq assay using barcoded bead
144 primers carrying a 3-prime poly(dT) sequence. Single cells or nuclei are resuspended in a reverse
145 transcriptase mix and co-encapsulated into microdroplets with 3-prime poly(dT) HyDrop beads.
146 The same microfluidic chip design is used for both HyDrop-RNA and HyDrop-ATAC. Cells are
147 lysed inside the droplets upon contact with the lysis buffer in which the barcoded beads are
148 suspended. Simultaneously, barcoded primers are released from the hydrogel bead after
149 exposure to DTT present in the reverse transcriptase mix. Reverse transcription inside the
150 emulsion generates thousands of barcoded single-cell cDNA libraries in parallel. The emulsion is
151 then broken and the single-cell transcriptome libraries are processed further in a pooled manner
152 (fig. 1h), similarly to the InDrop protocol¹⁵. However, although both assays are based on hydrogel
153 beads, HyDrop-RNA differs significantly from InDrop. HyDrop-RNA employs a template switching
154 oligo (TSO) reverse transcription technique (similar to Drop-seq), rather than an *in vitro*
155 transcription/random hexamer priming workflow. This change simplifies and speeds up the
156 protocol significantly with no reduction in sensitivity. To optimize the sensitivity of the assay, we
157 compared several different reaction chemistries: (1) Exonuclease I treatment to remove excess
158 of unused barcode primers; (2) the use of a locked nucleic acid (LNA) TSO³¹; (3) the addition of
159 GTP/PEG into the reverse transcription step (similar to SMART-seq³²); and (4) the use of second
160 strand synthesis. For the latter, we tested both alkaline hydrolysis and enzymatic treatment

161 (RNase H) to remove the RNA strand from the first strand product, and we evaluated the
162 performance of both the Bst 2.0 DNA polymerase and the Klenow (exo-)^{33,34} fragment for second
163 strand synthesis. By comparing these variations on a 50:50 human-mouse (human melanoma,
164 mouse melanoma) mixture, we found that the GTP/PEG protocol with Exonuclease I treatment
165 performed best, yielding a median of 2,110 UMIs and 1,325 genes per cell with a species-purity
166 of 90.1% (fig. 3a, S7). Accordingly, the GTP/PEG method was used in all further HyDrop-RNA
167 experiments. Applying this protocol to a 50:50 human-mouse (MCF-7, mouse melanoma) mixture
168 recovered 1,235 human and 846 mouse cells with 169 species doublets at a cutoff of 95% species
169 purity, with a median of 1,439 UMIs and 904 genes per cell (fig. 3b).

170 We then used HyDrop-RNA to generate 9,508 single nuclei transcriptomes from snap-frozen
171 mouse cortex in a single experiment. At a saturation of approximately 60% duplicates and with >
172 55% of reads mapping to transcriptome, we achieve a median of 3,404 UMIs and 1,662 genes
173 per cell after filtering (fig. 2c), a significant improvement over the median of 1,521 UMIs and 1,097
174 genes reported by inDrop snRNA-seq on mouse auditory cortex neurons⁵ and the median of 1,389
175 UMIs and 922 genes reported by Drop-seq on mouse retina neurons⁶. 10x Chromium v2 gene
176 expression reference data reports a median genes of 775-2,679 and a median UMIs of 1,127-
177 6,570 on E18 and adult mouse brain nuclei (see methods). Comparing the top per-cluster
178 differentially expressed genes with markers from the Allen Brain Atlas SMART-seq data²⁷
179 revealed 30 distinct populations corresponding to previously identified cell types (fig. 2d-f, fig. S8).
180 In addition to the major neuronal and glial populations previously detected in our HyDrop-ATAC
181 experiment, we detect a small population of vascular leptomenigeal cells (VLMC) and layer 2
182 intratelencephalic neurons from the medial entorhinal area (L2 IT ENTm). We also detect both D1
183 and D2 medium spiny neurons (MSN) as a result of residual striatal tissue and layer 3 *Scnn1a*+
184 neurons from the retrosplenial and anterior cingulate area (L3 RSP ACA), concordant with Atlas
185 SMART-seq data²⁷.

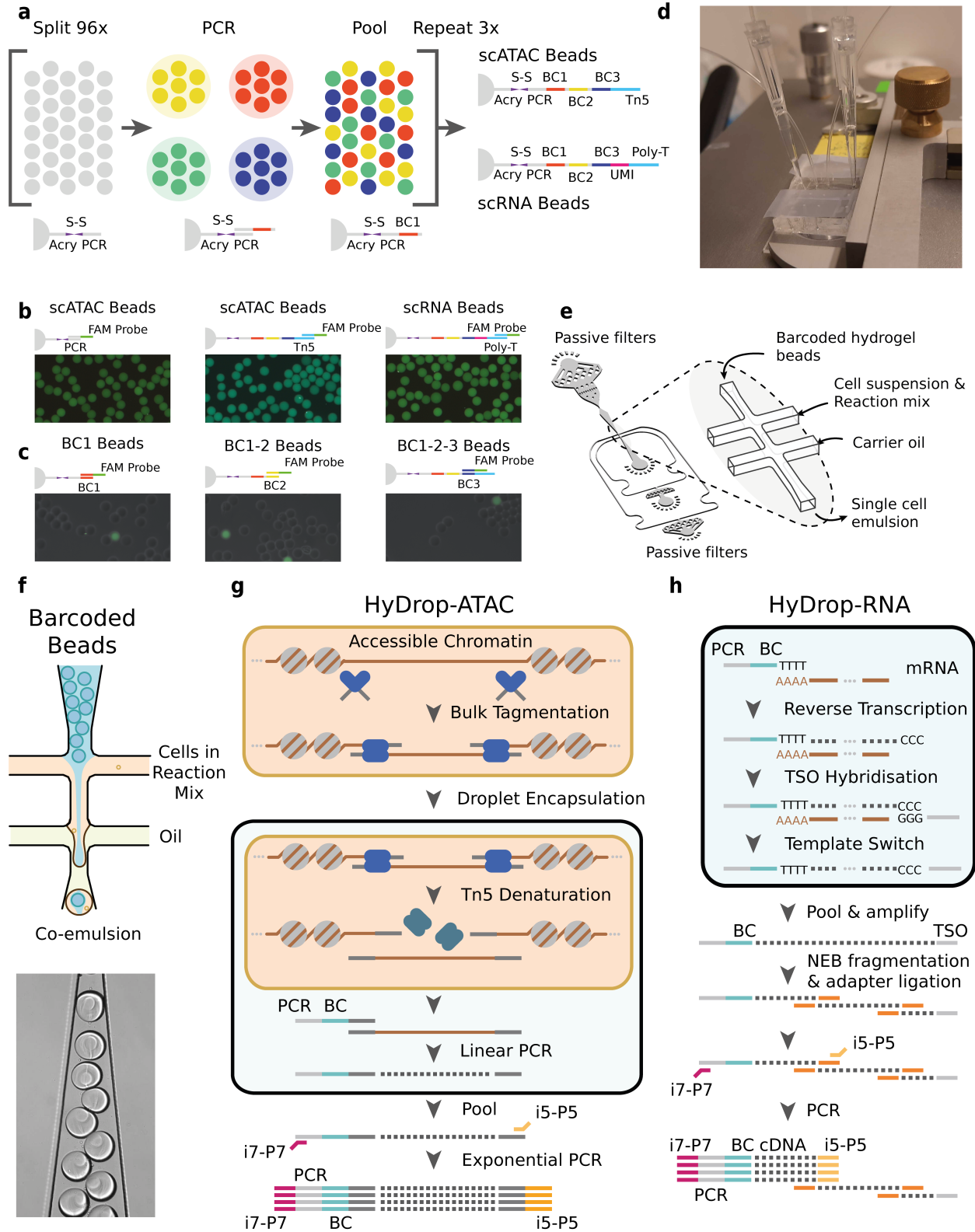
186 To assess HyDrop-RNA's performance on low cell input samples, we performed the protocol on
187 approximately 1500 FAC-sorted neurons from the *Drosophila* brain. We dissected brains in which
188 mCherry expression was driven in specific cell populations by a Gal4 driver line (R74G01-Gal4)
189 and used mCherry-positive cells as input for HyDrop-RNA (fig. 2h). Of the 1,500 cells obtained
190 after FACS sorting, we recovered 973 fly brain cells with a median of 1,307 UMIs and 640 genes
191 (fig. 2i). In-house Drop-seq performed on fly brain neurons recovered a median of 579 UMIs and
192 289 genes per cell at a deeper sequencing saturation¹. Annotation of the 973 single-cell
193 transcriptomes obtained by HyDrop confirmed the presence of all three Kenyon cell subtypes
194 alongside T1 and Tm1 neurons, as expected from our stainings and previous reports³⁵.
195 Surprisingly, we also detected a small population of Mi1 neurons (fig. j-l, fig. S9).

196 By applying HyDrop to generate thousands of mouse, human, and *Drosophila* single-cell gene
197 expression and chromatin accessibility profiles, we demonstrate the protocol's applicability to a
198 variety of different biological samples. Our experiments on mouse and fly tissues recapitulate
199 cellular heterogeneity in these complex samples and strongly agree with reference datasets from
200 both organisms. We show that HyDrop outperforms its open-source predecessors in terms of
201 sensitivity and user-friendliness. Moreover, at a per-cell library cost of < \$0.03 it does so at a
202 significantly lower cost compared to commercial droplet-microfluidic alternatives. We envision that

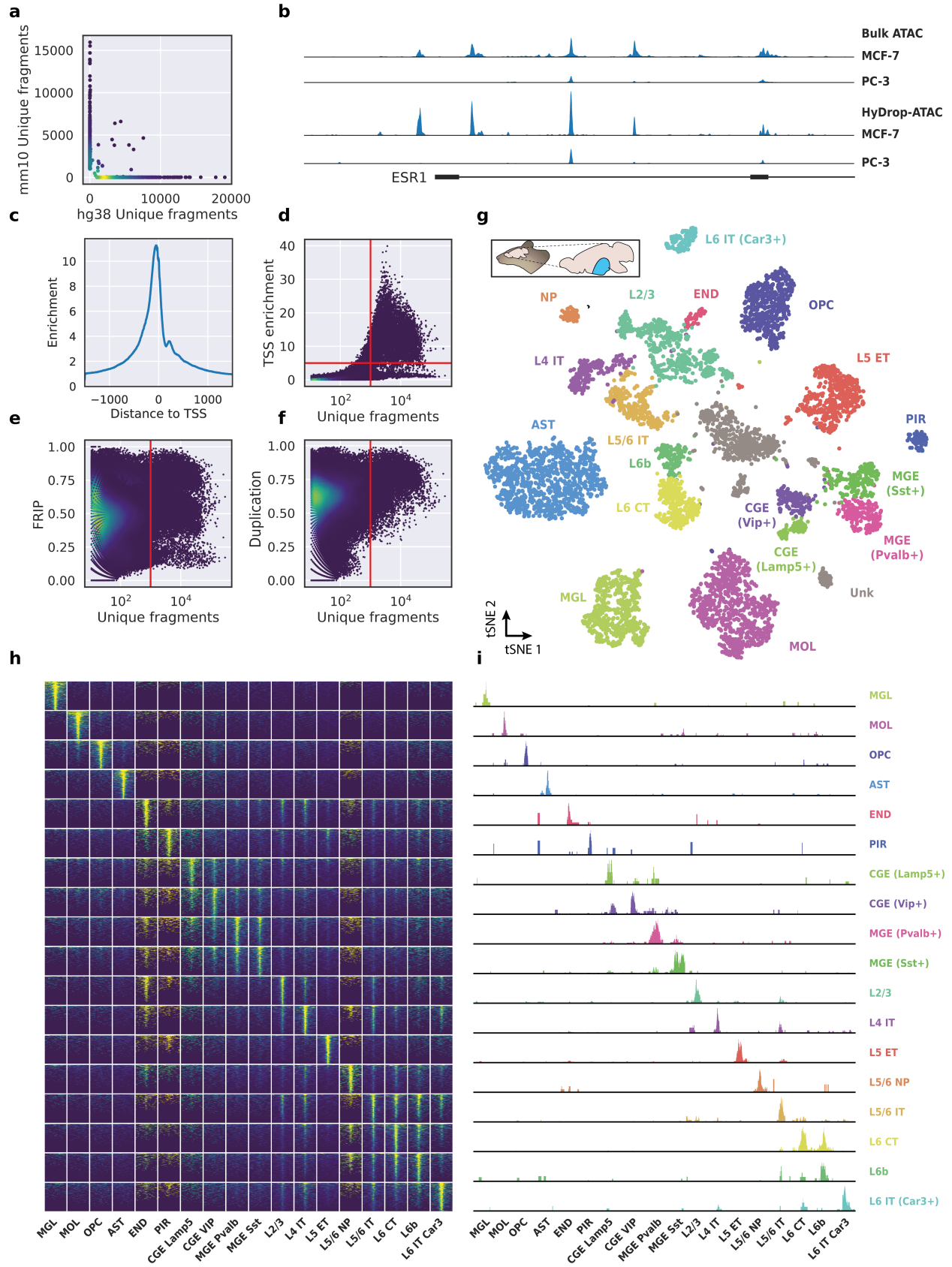
203 this reduction in both cost and labour will accelerate the scaling of large-scale atlasing efforts and
204 bring the benefits of single-cell sequencing to smaller projects. We believe that further
205 optimization and modification of the protocol's reaction chemistry and bead composition will lead
206 to improvements in sensitivity and stimulate the development of novel (multi-) omics droplet-
207 based assays. Additionally, HyDrop's flexible hydrogel bead synthesis toolkit may potentially be
208 exploited to design more complex single-cell (multi-) omics assays such as the capture of
209 accessible chromatin, (m)RNA, and proteins or antibodies from the same single cell.

210

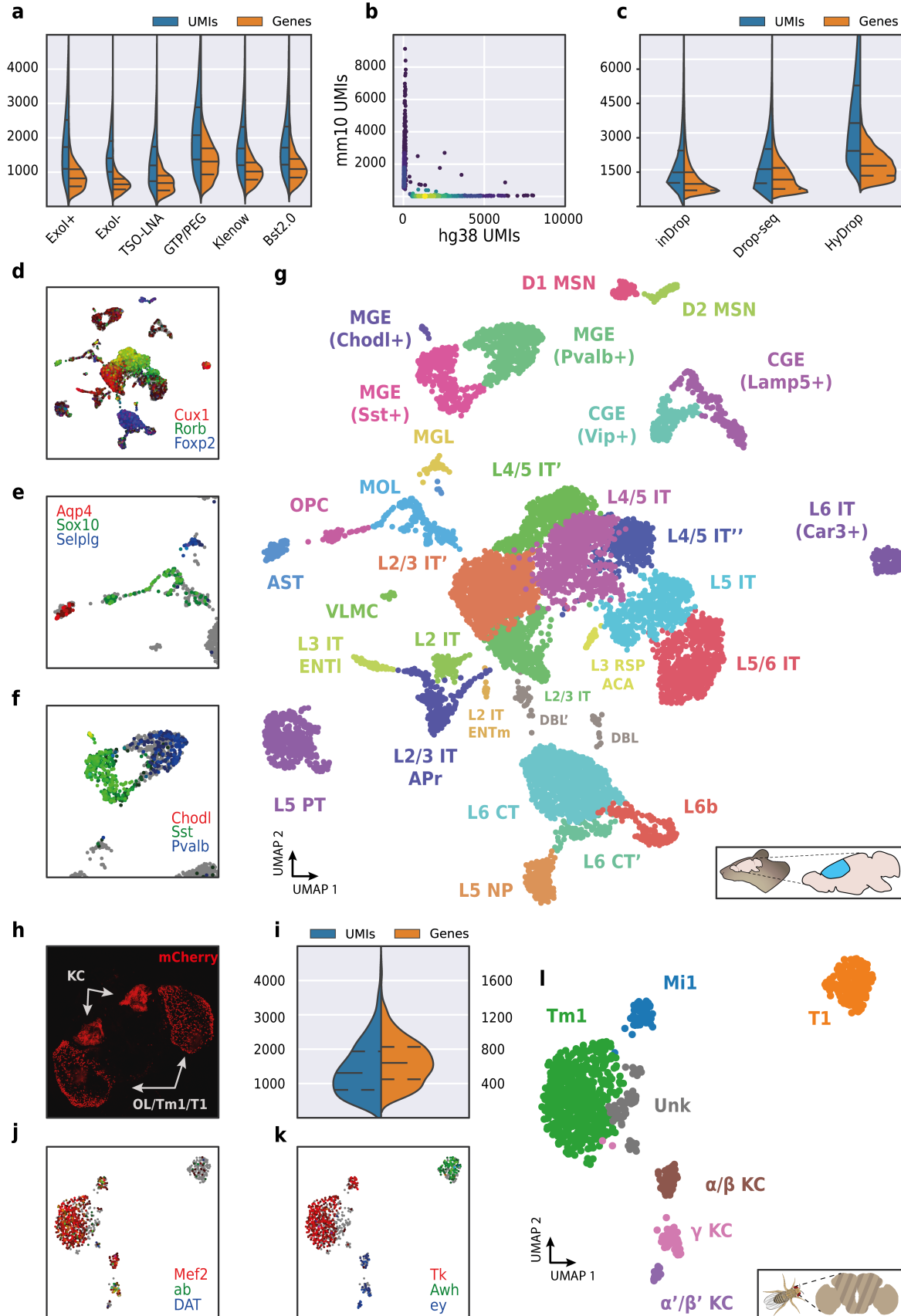
211 **Figures**



213 **Figure 1. a.** Split-pool process for barcoding of dissolvable hydrogel beads. Beads are
214 sequentially distributed over 96 wells, sub-barcoded, re-pooled, and distributed three times to
215 generate $96 \times 96 \times 96$ (884736) possible barcode combinations. Different 3'-terminal capture
216 sequences are possible depending on the oligonucleotide sequence appended in the last step.
217 **b.** Semi-quantitative assessment of bead primer incorporation by FISH after every sub-barcoding
218 step shows that bead fluorescence uniformity is retained throughout the barcoding process. **c.**
219 FISH with probes complementary to only one of 96 sub-barcode possibilities shows that
220 approximately 1/96 beads exhibit fluorescence for a selected sub-barcode probe. Fluorescence
221 signal is overlaid with a brightfield image at 50% transparency to indicate positions of non-
222 fluorescent beads. **d.** Microfluidic chip setup on the Onyx platform. Cells and beads are loaded
223 into pipette tips and plugged into a HyDrop Chip. Flow of oil and aqueous phases is achieved by
224 Onyx displacement syringe pumps. **e.** HyDrop chip design has three inlets: one each for carrier
225 oil, barcoded hydrogel beads and cell/reaction mix. Passive filters at each inlet prevent dust and
226 debris from entering the droplet generating junction. **f.** Diagram and snapshot of cell/bead droplet
227 encapsulation. Schematic overview of HyDrop-ATAC (**g**) and HyDrop-RNA (**h**) assay for single-
228 cell library generation. Nuclear membrane is visualised in salmon, water droplet is visualised in
229 blue.



231 **Figure 2. a.** Scatterplot of the number of unique fragments detected in a 50:50 mixture of human
232 MCF-7 and mouse melanoma cells coloured by local density estimation. **b.** RPGC-normalized
233 aggregate genome tracks comparing HyDrop-ATAC and bulk ATAC-seq profiles of human MCF-
234 7 and PC-3 cell lines around the Estrogen receptor 1 (ESR1) locus, scaled to maximum of all four
235 samples. Aggregate enrichment profile of reads around transcription start site (TSS) **(c)**, TSS
236 enrichment per barcode **(d)**, fraction of reads in peaks (FRIP) per barcode **(e)** and duplication rate
237 per barcode **(f)** in mouse cortex HyDrop-ATAC data. A minimum TSS enrichment of 5 and a
238 unique number of fragments of 1000 are used as cut-off values to separate cells from background
239 (red lines). Cells are colored by local density estimation. **g.** UMAP projection of 8502 mouse cortex
240 nuclei annotated with cell type inferred by accessibility near marker genes. Abbreviations:
241 microglia (MGL), mature oligodendrocytes (MOL), oligodendrocyte precursors (OPC), astrocytes
242 (AST), endothelial cells (END), piriform cortex neurons (PIR), caudal and medial ganglionic
243 eminence derived neurons (CGE, MGE), layers 2-6 intratelencephalic (IT), L5 extratelencephalic
244 (ET), L5/6 near projecting excitatory neurons (NP), L6 corticoencephalic (CT), and deep L6
245 excitatory neurons (L6b). **h.** Aggregate accessibility of top 1000 differentially accessible regions
246 per cluster. **i.** Row-scaled, CPM-normalized aggregate genome track covering the top 1
247 differentially accessible region (DAR) for each cluster.



249 **Figure 3. a.** Comparison of UMI and gene count of HyDrop-RNA with and without Exo I treatment
250 post-droplet merging, with the use of a locked nucleic acid (LNA) template switching oligo (TSO)
251 and with GTP/PEG, BST2.0 and Klenow fragment library preparation. Inner lines represent Q1,
252 median and Q3. **b.** Scatterplot of human and mouse UMIs detected in a 50:50 mixture of human
253 MCF-7 and mouse melanoma cells coloured by local density estimation. **c.** Comparison of UMI
254 and gene count of public inDrop mouse cortex data, public Drop-seq mouse retina data, and
255 HyDrop-RNA mouse cortex data. Inner lines represent Q1, median and Q3. Mouse cortex UMAP
256 is colored by log-scaled UMI counts of *Cux1*, *Rorb*, *Foxp2* (**d**), *Aqp4*, *Sox10*, *Selp1g* (**e**), *Chodl*,
257 *Sst* and *Pvalb* (**f**). Colors are scaled to minimum and maximum values. **g.** UMAP projection of
258 9507 mouse cortex nuclei annotated with cell type inferred by marker gene expression.
259 Abbreviations: microglia (MGL), mature oligodendrocytes (MOL), oligodendrocyte precursors
260 (OPC), astrocytes (AST), endothelial cells (END), piriform cortex neurons (PIR), caudal and
261 medial ganglionic eminence derived neurons (CGE, MGE), layers 2-6 intratelencephalic (IT),
262 pyramidal tract (PT), near projecting excitatory neurons (NP) and corticoencephalic (CT) neurons,
263 layer 2 intratelencephalic medial entorhinal area neurons (L2 IT ENTm), L2/3 intratelencephalic
264 area prostriata neurons (L2/3 IT APr), layer 3 intratelencephalic entorhinal neurons (L3 IT ENTl),
265 layer retrosplenial and anterior cingulate area neurons (L3 RSP ACA), deep L6 excitatory neurons
266 (L6b), D1 and D2 medium spiny neurons (MSN), and vascular leptomeningeal cells (VLMC). **h.**
267 Confocal maximum intensity projection of R74G01-Gal4>UAS-mCherry brain. **i.** Violin plot of
268 UMIs and genes detected in nuclei derived from FAC-sorted fly neurons. Inner lines represent
269 Q1, median and Q3. Fly neuron UMAP colored by log-scaled UMI counts of *Mef2*, *ab*, *DAT* (**j**)
270 and *Tk*, *Awh*, *ey* (**k**). Colors are scaled to minimum and maximum values. **l.** UMAP of 973 FAC-
271 sorted *Drosophila* neurons annotated with cell types inferred by marker gene expression (KC,
272 Kenyon cells; Tm1, transmedullary neuron; Mi1, medullary intrinsic neuron).

273 **Methods**

274 **Microfluidic droplet generator manufacturing**

275 Microfluidic droplet generators were produced using standard SU-8 lithography and PDMS
276 lithography according to well established protocols³⁶. Briefly, the design for droplet generators
277 were made in AutoCAD R2014 and the designs are printed onto a chrome mask using a laser
278 writer. The SU-8 lithography is performed on a 4 inch silicon wafer using SU 8-2050 (Microchem)
279 negative photoresist using UV aligner (EVG-620). As per manufacturers' recommendation, spin
280 coating of the wafer with SU8 was performed at 500 rpm (ramp 100rpm/s) - 10s and 2000 rpm
281 (ramp: 300rpm/s) - 30s, to achieve a feature height of 70-80 μm). For preparing the PDMS chip,
282 a mixture of PDMS monomer and crosslinker (Dow Corning SYLGARD 184) was prepared at a
283 ratio of 10:1 and mixed thoroughly. The mixture was degassed in vacuum for 45 minutes and
284 poured onto an SU-8 master template and baked at 80 °C for 4 hours. Inlet ports were cut using
285 a 1 mm biopsy needle after which the chips were exposed to high-voltage plasma for 30 s and
286 bonded onto a glass slide. 5 μL of 2% Trichloro(1H,1H,2H,2H-perfluorooctyl)silane in HFE was
287 injected into each channel, incubated for 10 mins at room temperature and excess oil was
288 removed by applying pressurized air. Chips were finally baked at 100 °C for 2 hours (more detailed
289 methods for photolithography and PDMS lithography in
290 <https://www.protocols.io/workspaces/aertslab>).

291 **Barcoded hydrogel bead manufacturing & storage**

292 Dissolvable hydrogel beads are synthesized similar to a previously published protocol¹⁴ and
293 barcoded according to a modified inDrop protocol¹⁵. For synthesizing 2-3 mL batch of beads, 2
294 mL of Bead Monomer Mix (6% acrylamide, 0.55% bisacryloylcystoamine, 10% TBSET (10 mM
295 Tris-HCl pH 8, 137 mM NaCl, 2.7 mM KCl, 10 mM EDTA, 0.1% Triton X-100), 12 μM acrydite
296 primer, 0.6% ammonium persulfate) was encapsulated into 50 μm diameter droplets in HFE-7500
297 Novac oil with EA-008 surfactant (RAN Biotech). 1 mL aliquots of the resulting emulsion was
298 layered with 400 μL of mineral oil and incubated at 65 °C for 14 hours. Excess mineral oil and the
299 emulsion oil was removed and 2-3 washes with 1 mL of droplet breaking solution (20% PFO in
300 HFE) was performed. Beads were pelleted at 5000 $\times g$, 4 °C for 30 seconds and washed twice in
301 1 mL of 1% SPAN-80 in hexane. Beads were sequentially washed in TBSET until all hexane
302 phase was removed.

303 Beads were washed twice in Bead Wash Buffer (10 mM Tris-HCl pH 8, 0.1% Tween-20), twice in
304 PCR Wash Buffer (10 mM Tris-HCl pH8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Tween-20). The
305 subsequent liquid handling in the 96-well plate is performed using Hamilton microlab STAR robot.
306 22.5 μL of beads were distributed to a 96 well plate. 2.5 μL of 100 μM sub-barcode primer and 25
307 μL of KAPA HiFi Hotstart master mix (Roche) was added to each well and the plate was
308 thermocycled (95 °C 3 min., 5 cycles of 98 °C 20s, 38 °C 4 min., 72 °C 2 min., 1 cycle of 98 °C 1
309 min., 38 °C 10 min., 72 °C 4 min., followed by a final hold on 4 °C) with intermittent vortexing
310 during every annealing step. 50 μL of STOP-25 (10 mM Tris-HCl pH 8, 25 mM EDTA, 0.1%
311 Tween-20, 100 mM KCl) was added to each well to deactivate the polymerase and its contents
312 were pooled. Remaining beads in wells were washed out with 100 μL of STOP-25 and the beads

313 were rotated at room temperature for 30 minutes. Beads were then washed with STOP-10 (10
314 mM Tris-HCl pH 8, 10 mM EDTA, 0.1% Tween-20, 100 mM KCl) and rotated for 10 minutes in
315 Denaturation Solution (150 mM NaOH, 85 mM Brij-35). Beads were washed twice in
316 Denaturation Solution and twice more in Neutralisation Solution (100 mM Tris-HCl pH 8, 10 mM
317 EDTA, 0.1% Tween-20, 100 mM NaCl). The sub-barcoding step was repeated twice more for a
318 total of 3 sub-barcodes.

319 Hydrogel beads were sequentially filtered using a 70 µm strainer (Falcon). For both the HyDrop-
320 ATAC and RNA beads were pelleted at 300 xg, 4 °C and resuspended in 5 mL of Bead Freezing
321 Buffer (150 mM NaCl, 125 mM Tris-HCl pH 7, 10 mM MgCl₂, 4% Tween-20, 0.75% Triton X-100,
322 30% glycerol, 0.3% BSA). Beads were pelleted at 300 xg, 4 °C and resuspended in 5 mL of Bead
323 Freezing Buffer a second time and incubated at 4 °C for at least 3 hours. Beads were pelleted at
324 1000 xg, 4 °C and the pellet was aliquoted into 35 µL aliquots and stored at -80 °C for long term
325 storage (further method details in <https://www.protocols.io/workspaces/aertslab>)

326 **Hydrogel bead fluorescence in-situ hybridisation quality control**

327 Bead QC was performed as described previously¹⁵. Briefly, 10 µL of hydrogel beads were
328 resuspended in 1 mL of hybridization buffer (5 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.05% Tween-
329 20, 1M KCl) and centrifuged for 1 min. at 1000 xg. The wash was repeated once more, and 960
330 µL of the supernatant was removed. 4 µL of 200 µM specific FAM probe was added depending
331 on which part of the barcode needed testing (see supplementary table 1). The beads were
332 incubated at room temperature in the dark for 30 min. Beads were washed thrice in QC buffer
333 and visualised under a Zeiss Axioplan 2 microscope (300 ms exposure time, 80% lamp intensity).

334 **Cell culture and cell dissociation**

335 MCF-7 cells were cultured in RPMI1640 (ThermoFisher 11875093) medium supplemented with
336 10% FBS (ThermoFisher 10270-106), 1% penicillin/streptomycin (Life Technologies 15140122),
337 and 10 ug/mL insulin (Sigma Aldrich I9278) and passaged twice per week. PC-3 cells were
338 cultured in RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and
339 passaged twice per week. Mouse melanoma cells were cultured in DMEM (ThermoFisher
340 13345364) supplemented with 10% FBS and 1% penicillin/streptomycin and passaged once per
341 week. MM087 melanoma cells were cultured in F-10 Nutrient mix supplemented with 10% FBS
342 and 1% penicillin/streptomycin and passaged once per week. Cells were washed in PBS and
343 dissociated into single cell suspensions by adding 1.5 mL of 0.05% Trypsin (Life Technologies
344 25300054) and waiting for 5 to 7 minutes. The single-cell suspension was centrifuged at 500 rcf
345 for 5 min at 4°C and the resulting pellet was resuspended in PBS. This PBS wash was repeated
346 once more and the single-cell suspension was processed further.

347 **Fly rearing and cell dissociation**

348 GMR74G01-Gal4 (BL#39868) and UAS-mCherry (BL#38425) flies were obtained from
349 Bloomington Drosophila Stock Center. The resulting cross strain was raised on standard
350 cornmeal-agar medium at 25°C at a 12h light/dark cycle. 50 adult brains were dissected in DPBS

351 and transferred to a tube containing 100 μ L of cold DPBS solution. Samples were centrifuged at
352 500 rcf for 1 min and the supernatant was replaced by 50 μ L of dispase (3 mg/mL, Sigma-Aldrich,
353 D4818, 2mg) and 75 μ L of collagenase I (100 mg/mL, Invitrogen, 17100-017). Brains were
354 dissociated in a Thermoshaker (Grant Bio PCMT) at 500 rpm for 2 h at 25°C, with pipette mixing
355 every 15 min. Cells were subsequently washed with 1000 μ L of cold DPBS solution and
356 centrifuged at 500 rcf for 5 min at 4°C and resuspended in 250 μ L of DPBS with 0.04% BSA. Cell
357 suspensions were passed through a 10 μ M pluriStrainer (ImTec Diagnostics, 435001050). Cells
358 were sorted based on viability and mCherry positivity using the Sony MA900 cell sorter. Sorted
359 cells were collected into Eppendorf tubes pre-coated with 1% BSA.

360 **Cell line nuclei extraction**

361 A pellet of 1 million dissociated cells or fewer was incubated on ice in 200 μ L of ATAC Lysis Buffer
362 (1% BSA, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.1% Tween-20, 0.1% NP-40, 3 mM MgCl₂, 70
363 μ M Pitstop, 0.01% Digitonin) for 5 to 7 minutes. 1 mL of ATAC Nuclei Wash Buffer (1% BSA, 10
364 mM Tris-HCl pH 7.5, 0.1% Tween-20, 10 mM NaCl, 3 mM MgCl₂) was added and the nuclei were
365 pelleted at 500 xg, 4 °C for 5 minutes. The resulting pellet was resuspended in 100 μ L of ice-cold
366 PBS and filtered with a 40 μ m strainer (Flowmi).

367 **Mouse cortex dissection**

368 All animal experiments were conducted according to the KU Leuven ethical guidelines and
369 approved by the KU Leuven Ethical Committee for Animal Experimentation (approved protocol
370 numbers ECD P183/2017). Mice were maintained in a specific pathogen-free facility under
371 standard housing conditions with continuous access to food and water. Mice used in the study
372 were 57 days old and were maintained on 14 h light, 10 h dark light cycle from 7 to 21 hours. In
373 this study, cortical brain tissue from female P57 BL/6Jax was used. Animals were anesthetized
374 with isoflurane, and decapitated. Cortices were collected, divided in four equal quadrants along
375 the dorso-ventral and anterior-posterior axis, and immediately snap-frozen in liquid nitrogen. For
376 HyDrop-ATAC, a ventral/posterior quadrant from the left hemisphere was used. For HyDrop-RNA,
377 a dorsal/anterior quadrant was used from the left hemisphere of a second mouse.

378 **Snap-frozen mouse cortex nuclei extraction**

379 For the preparation of nuclei for RNAseq, we used a modified protocol from the recently published
380 single nuclei preparation toolbox³⁷ to extract nuclei from snap-frozen mouse cortex samples.
381 Briefly, a ~1 cm³ frozen piece of mouse cortex tissue was transferred to 0.5 mL of ice-cold
382 homogenisation buffer (Salt-tris solution - 146 mM NaCl, 10 mM Tris 7.5, 1 mM CaCl₂, 21mM
383 MgCl₂, 250 mM Sucrose, 0.03% Tween-20, 0.01% BSA, 25 mM KCl, 1mM 2-Mercaptoethanol,
384 1X cOmplete protease inhibitor, 0.5U/ul of RNase In Plus (Promega)) in a Dounce homogenizer
385 mortar and thawed for 2minutes. The tissue was homogenised with 10 strokes of pestle A and 5
386 strokes of pestle B until a homogeneous nuclei suspension was achieved. The resulting
387 homogenate was filtered through a 70 μ m cell strainer (Corning). The homogenizer and the filter
388 is rinsed with an additional 500 ul of homogenization buffer. The tissue material was pelleted at
389 500xg and the supernatant was discarded. The tissue pellet was resuspended in a

390 homogenization buffer without Tween-20. An addition 1.65 ml of homogenization buffer was
391 topped up and mixed with 2.65 ml of Gradient Medium (75 mM sucrose, 1mM CaCl₂, 50%
392 Optiprep, 5mM MgCl₂, 10mM Tris 7.5, 1mM 2-Mercaptoethanol 1X cOmplete protease inhibitor,
393 0.5U/ul of RNase In Plus (Promega)). 4 mL of 29% iodoxanol cushion was prepared with a Diluent
394 medium (250 mM Sucrose, 150mM KCl, 30mM MgCl₂, 60mM Tris 8) and was loaded into an
395 ultracentrifuge tube. 5.3 mL of sample in homogenization buffer + gradient medium was gently
396 layered on top of the 29% iodoxanol cushion. Sample was centrifuged at 7700 xg, 4°C for 30
397 minutes and the supernatant was gently removed without disturbing the nuclei pellet. Nuclei were
398 resuspended in 100 µL of Nuclei buffer (1% BSA in PBS + 1U/ul of RNase Inhibitor).

399 For the preparation of nuclei for ATAC seq, we used a slightly modified protocol to extract nuclei
400 from snap-frozen mouse cortex samples. Briefly, a ~1 cm³ frozen piece of mouse cortex tissue
401 was transferred to 1 mL of ice-cold homogenisation buffer (320 mM Sucrose, 10 mM NaCl, 3mM
402 Mg(OAc), 10mM Tris 7.5, 0.1mM EDTA, 0.1% IGEPAL-CA360, 1X cOmplete protease inhibitor
403 and 1 mM DTT) in a Dounce homogenizer mortar and thawed for 2 minutes. The tissue was
404 homogenised with 10 strokes of pestle A and 5 strokes of pestle B until a homogeneous nuclei
405 suspension was achieved. The resulting homogenate was filtered through a 70 µm cell strainer
406 (Corning). 2.65 mL of ice-cold gradient medium was added to 2.65 mL of homogenate and mixed
407 well. 4 mL of 29% iodoxanol cushion (129.2 mM Sucrose, 77.5 mM KCl, 15.5 mM MgCl, 31 mM
408 Tris-HCl pH 7.5, 29% Iodoxanol) was loaded into ultracentrifuge tube. 5.3 mL of sample in
409 homogenization buffer + gradient medium was gently layered on top of the 29% iodoxanol
410 cushion. Sample was centrifuged at 7700 xg, 4°C for 30 minutes and the supernatant was gently
411 removed without disturbing the nuclei pellet. Nuclei were resuspended in 100 µL of Nuclei buffer
412 (1% BSA in PBS). For the HyDrop-ATAC experiment, quadrant X was used. For the HyDrop-RNA
413 experiment, quadrant X was used.

414 **HyDrop-ATAC library preparation**

415 50 000 nuclei were resuspended in 50 µL of ATAC Reaction Mix (10% DMF, 10% Tris-HCl pH
416 7.4, 5 mM MgCl₂, 5 ng/µL Tn5, 70 µM Pitstop, 0.1% Tween-20, 0.01% Digitonin) and incubated
417 at 37 °C for 1 hour. 100 µL of 0.1% BSA in PBS was added and the nuclei were pelleted at 500
418 xg, 4 °C for 5 minutes and resuspended in 40 µL of 0.1% BSA in PBS.

419 Tagmented nuclei were added to 100 µL of PCR mix (1.3X Phusion HF Buffer, 15% Optiprep, 1.3
420 mM dNTPs, 39 mM DTT, 0.065 U/µL Phusion HF Polymerase, 0.065 U/µL Deep Vent
421 Polymerase, 0.013 U/µL ET SSB). PCR mix was co-encapsulated with 35 µL of freshly thawed
422 HyDrop-ATAC beads in HFE-7500 Novac oil with EA-008 surfactant (RAN Biotech) on the Onyx
423 microfluidics platform (Droplet Genomics). The resulting emulsion was collected in aliquots of 50
424 µL total volume and thermocycled according to the PCR1 program (72 °C 15 min., 98 °C 3 min.,
425 13 PCR cycles of [98 °C 10 s, 63 °C 30 s, 72 °C 1 min.], followed by a final hold on 4 °C). 125 µL
426 of recovery agent (20% PFO in HFE), 55 µL of GITC Buffer (5 M GITC, 25 mM EDTA, 50 mM
427 Tris-HCl pH 7.4) and 5 µL of 1 M DTT was added to each separate aliquot of 50 µL thermocycled
428 emulsion and incubated on ice for 5 minutes. 5 µL of Dynabeads was added to the aqueous phase
429 and incubated for 10 minutes. Dynabeads were pelleted on a Nd magnet and washed twice with
430 80% EtOH. Elution was performed in 50 µL of EB-DTT-Tween (10 mM DTT, 0.1% Tween-20 in

431 EB (10 mM Tris-HCl, pH 8.5)). A 1x Ampure bead purification was performed according to
432 manufacturer's recommendations. Elution was performed in 30 μ L of EB-DTT (10 mM DTT in
433 EB). Eluted library was further amplified in 100 μ L of PCR2 mix (1x KAPA HiFi, 1 μ M index i7
434 primer, 1 μ M index i5 primer). Final library was purified in a 0.4x-1.2x double-sided Ampure
435 purification and eluted in 25 μ L of EB-DTT (10 mM DTT in EB).

436 **HyDrop-RNA single cell library preparation**

437 For a recovery of 2000 cells, 3795 cells were resuspended in 85 μ L of RT mix (1x Maxima RT
438 Buffer, 0.9 mM dNTPs, 25 mM DTT, 1.3 mM GTP, 15 % Optiprep, 1.3 U/ μ L RNase inhibitor, 15
439 U/ μ L Maxima hRT, 12.5 μ M TSO, 4.4% PEG-8000). RT mix was co-encapsulated with 35 μ L of
440 freshly thawed HyDrop-RNA beads in RAN oil on the Onyx microfluidics platform. The resulting
441 emulsion was collected in aliquots of 50 μ L total volume and thermocycled according to the RT
442 program (42 $^{\circ}$ C for 90 min., 11 cycles of [50 $^{\circ}$ C for 2 min., 42 $^{\circ}$ C for 2 min.], 85 $^{\circ}$ C for 5 min.,
443 followed by a final hold on 4 $^{\circ}$ C). 125 μ L of recovery agent (20% PFO in HFE), 55 μ L of GITC
444 Buffer (5 M GITC, 25 mM EDTA, 50 mM Tris-HCl pH 7.4) and 5 μ L of 1 M DTT was added to
445 each separate aliquot of 50 μ L thermocycled emulsion and incubated on ice for 5 minutes. 99 μ L
446 of Ampure XP beads was added to the aqueous phase and incubated for 10 minutes. Ampure
447 beads were pelleted on a Nd magnet and washed twice with 80% EtOH. Elution was performed
448 in 30 μ L of EB-DTT-Tween (10 mM DTT, 0.1% Tween-20 in EB). Exonuclease treatment was
449 performed by adding 4 μ L of 10x NEBuffer 3.1, 4 μ L of Exo I, and 2 μ L of dH₂O to 30 μ L of eluted
450 library. The Exo I reaction mix was incubated at 37 $^{\circ}$ C for 5 min., 80 $^{\circ}$ C for 1 min. for heat
451 inactivation. followed by a final hold at 4 $^{\circ}$ C. 2 μ L of 1 M DTT was added and a 0.8x Ampure XP
452 purification was performed according to manufacturer's recommendations. cDNA was eluted in
453 40.5 μ L of EB-DTT (10 mM DTT in EB) and added to ISPCR mix (40 μ L library, 50 μ L 2x KAPA
454 HiFi, 10 μ L 10 μ M TSO-P primer). PCR cycling was performed according to the ISPCR program
455 (95 $^{\circ}$ C for 3 min., 13 cycles of [98 $^{\circ}$ C for 20s, 63 $^{\circ}$ C for 20s, 72 $^{\circ}$ C for 3 min.], 72 $^{\circ}$ C for 5 min.
456 followed by a final hold at 4 $^{\circ}$ C. 2 μ L of 1M DTT was added and a 0.6x Ampure XP purification
457 was performed according to manufacturer's recommendations. cDNA was eluted in 28.5 μ L of
458 EB-DTT. Final sequencing library was prepared according to the following customised NEB Ultra
459 II FS protocol (NEB E7805S). 80 ng of amplified cDNA was fragmented in Ultra II fragmentation
460 mix (26 μ L of amplified cDNA, 7 μ L of NEBNext Ultra II FS Reaction Buffer, 2 μ L of NEBNext Ultra
461 II FS Enzyme Mix) on the following thermocycling program: 37 $^{\circ}$ C for 10 min., 65 $^{\circ}$ C for 30 min.
462 and a final hold at 4 $^{\circ}$ C. 15 μ L of EB was added and a 0.8x Ampure purification was performed
463 according to manufacturer's recommendation and eluted in 35 μ L. Fragmented library was
464 adapter-ligated in NEBNext Ultra II adapter ligation mix (35 μ L of fragmented library, 30 μ L of
465 NEBNext Ultra II Ligation Master Mix, 1 μ L of NEBNext Ligation Enhancer, 2.5 μ L of NEBNext
466 Adapter for Illumina) at 20 $^{\circ}$ C for 15 min., with 4 $^{\circ}$ C final hold. 28.5 μ L of EB was added and a
467 0.8X Ampure purification was performed according to manufacturer's recommendation and eluted
468 in 30 μ L. Eluted library was amplified in PCR master mix (50 μ L 2x KAPA HiFi, 10 μ L 10 μ M Hy-
469 i7 primer, 10 μ L 10 μ M Hy-i5 primer, 30 μ L eluted library) in the following thermocycling program:
470 95 $^{\circ}$ C for 3 min., 13 cycles of [98 $^{\circ}$ C for 20 s, 64 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 30 s], 72 $^{\circ}$ C for 5 min. and
471 a final hold at 4 $^{\circ}$ C. Sequencing-ready library was purified using a 0.8x Ampure purification and
472 eluted in 30 μ L of EB.

473 **HyDrop-RNA optimisation trials**

474 We performed 6 trials on a 50:50 mixture of human melanoma (MM087) and mouse melanoma
475 (MMel). Trials were performed as described in the general HyDrop-RNA protocol, but with the
476 following changes. All trials, except for the GTP/PEG trial, were performed using the following RT
477 reaction mix (1.6x Maxima h-RT buffer, 1.6 mM dNTPs, 47 mM DTT, 15% Optiprep, 1.6 U/ μ L
478 RNase Inhibitor, 15.7 U/ μ L Maxima hRT, 12.5 μ M TSO). For the Exo- condition, the Exonuclease
479 I treatment was skipped. For all other conditions the Exonuclease I treatment was performed as
480 described above. For the TSO-LNA trial, a locked nucleic acid TSO was used instead of the
481 regular TSO. For the GTP/PEG trial, all steps were performed as described in the main protocol.
482

483 For the Klenow fragment second strand synthesis trial, the purified first strand product was treated
484 with 1 μ L of E. coli RNase H (NEB M0297S). The mixture was incubated at 37 °C for 30 minutes
485 after which the enzyme was inactivated using 10 mM EDTA. The single stranded product was
486 purified using 1.2x Ampure XP bead purification (BD sciences) and eluted in 25 μ L of EB buffer.
487 dN-SMRT primer was added to the single strand product to a final concentration of 2.5 μ M and
488 the mixture was denatured by incubation at 95 °C for 5 minutes. The sample was then allowed to
489 cool to room temperature and incorporated in the Klenow enzyme mix (1x Maxima h-RT buffer,
490 1mM dNTP, 1U/ μ L of Klenow Exo-; NEB M0212L) was added to the single strand library. The
491 Klenow enzyme mix was incubated at 37 °C for 60 min. The second strand reaction was stopped
492 by heating the product at 85 °C for 5 min. The sample was purified using 1X Ampure XP and
493 eluted in 40 μ L of EB buffer. The purified second strand product was amplified with ISPCR primers
494 as described above.

495
496 For the BST 2.0 polymerase second strand synthesis trial, the purified first strand product was
497 treated with 1 μ L of E. coli RNase H (NEB M0297S). The mixture was incubated at 37 °C for 30
498 minutes after which the enzyme was inactivated using 10 mM EDTA. The single stranded product
499 was purified using 1.2X Ampure XP bead purification (BD sciences) and eluted in 25 μ L of EB
500 buffer. dN-SMRT primer was added to the single strand product to a final concentration of 2.5 μ M
501 and the mixture was denatured by incubation at 95 °C for 5 minutes. The sample was then allowed
502 to cool to room temperature and incorporated in the Bst 2.0 enzyme mix (1X Isothermal
503 amplification buffer, 1mM dNTP, 1U/ μ L of Bst 2.0 DNA polymerase; NEB M0537L) was added to
504 the denatured library and the mixture was incubated at 55 °C for 10 mins and 60 °C for 45 minutes.
505 The second strand reaction was stopped by heating the product at 85 °C for 5 minutes. The
506 sample was purified using 1X Ampure XP and eluted in 40 μ L of EB buffer. The purified second
507 strand product was amplified with ISPCR primers as described above.

508 **Sequencing**

509 HyDrop-ATAC libraries were sequenced on Illumina NextSeq500 or NextSeq2000 systems using
510 50 cycles for read 1 (ATAC paired-end mate 1), 52 cycles for index 1 (barcode), 10 cycles for
511 index 2 (sample index) and 50 cycles for read 2 (ATAC paired-end mate 2).

512 HyDrop-RNA libraries were sequenced on Illumina NextSeq2000 systems using 50 cycles for
513 read 1 (3' cDNA), 10 cycles for index 1 (sample index, custom i7 read primer), 10 cycles for index
514 2 (sample index) and 58 cycles for read 2 (barcode + UMI, custom read 2 primer).

515 **HyDrop-ATAC data processing**

516 Barcode reads were trimmed to exclude the intersub-barcode PCR adapters using a mawk script.
517 Next, the VSN scATAC-seq pre-processing pipeline²² was used to map the reads to the reference
518 genome and generate a fragments file for downstream analysis. Here, barcode reads were
519 compared to a whitelist (of 884736 valid barcodes), and corrected, allowing for a maximum 1 bp
520 mismatch. Uncorrected and corrected barcodes were appended to the fastq sequence identifier
521 of the paired-end ATAC-seq reads. Reads were mapped to the reference genome using bwa-
522 mem with default settings, and the barcode information was added as tags to each read in the
523 bam file. Duplicate-marking was performed using samtools markup. In the final step of the
524 pipeline, fragments files were generated using Sinto (<https://github.com/timoast/sinto>). For mixed-
525 species data, cells were filtered for a minimum of 1000 unique fragments and a minimum TSS
526 enrichment of 7. For mouse cortex data, higher level analysis such as clustering and differential
527 accessibility were performed using cisTopic²⁵. In brief, cells were filtered for a minimum of 1000
528 unique fragments and a minimum TSS enrichment of 5. Fragments overlapping mouse candidate
529 cis-regulatory regions³⁸ were counted, and the resulting matrix was filtered for potential cell
530 doublets using a Scrublet²⁴ threshold of 0.35. Cells were Leiden-clustered based on the cell-topic
531 probability matrix generated by an initial cisTopic LDA incorporating 51 topics, at a resolution of
532 0.9 with 10 neighbours. A consensus peak set was generated from per-cluster peaks and used
533 to recount fragments. Cells were filtered using the same filtering parameters and a new model
534 with 50 topics was trained. Cells were again Leiden-clustered based on the cell-topic probability
535 matrix generated by the second LDA, at a resolution of 0.9 with 10 neighbours. Region
536 accessibility was imputed based on binarised topic-region and cell-topic distributions. Gene
537 activity was imputed based on Gini index-weighted imputed accessibility in a 10 kb
538 up/downstream decaying window around each gene including promoters. Leiden clusters were
539 annotated based on imputed gene accessibility around marker genes^{27,28}. Differentially accessible
540 regions were called using one-versus-all Wilcoxon rank-sum tests for each cell type, with an
541 adjusted p-value of 0.05 and log2FC of 1.5. RPGC-normalized aggregate genome coverage
542 bigwigs were generated from BAM files using DeepTools³⁹. Per-cluster genome coverage tracks
543 were generated using pyBigWig.

544 **HyDrop-RNA data processing**

545 Barcode reads were trimmed to exclude the intersub-barcode PCR adapters using a mawk script.
546 Reads were then mapped and cell-demultiplexed using STARsolo⁴⁰ in CB_UMI_Complex mode.
547 The resulting STARsolo-filtered count matrices were further analysed using Scanpy⁴¹. In short,
548 cells were filtered on expression of a maximum of 4000 genes, and a maximum of 1% UMIs from
549 mitochondrial genes. Genes were filtered on expression in a minimum of 3 cells. Potential cell
550 doublets were filtered out using a Scrublet²⁴ threshold of 0.25. The filtered expression matrix was
551 scaled to total counts and log-normalized. Total counts and mitochondrial reads were regressed
552 out and UMAP embedding was performed after PCA. Cells were annotated and fine tuned based

553 on differential gene expression of marker genes sourced from either the Davie et al. Drosophila
554 brain atlas¹ or the Allen Brain RNA-seq Database²⁷.

555 Public inDrop and Drop-seq data^{5,6} were downloaded from their respective GEO repositories.
556 Both Drop-seq and inDrop cells were filtered for a minimum of 500 genes per cell as described in
557 their respective papers. Public reference 10x single-cell ATAC-seq data was sourced from
558 <https://support.10xgenomics.com/single-cell-atac/datasets> (“Flash frozen cortex, hippocampus,
559 and ventricular zone from embryonic mouse brain (E18)”, “Fresh cortex from adult mouse brain
560 (P50)”). Public reference 10x single-cell gene expression data was sourced from
561 <https://support.10xgenomics.com/single-cell-gene-expression/datasets> (“1k Brain Nuclei from an
562 E18 Mouse”, “2k Brain Nuclei from an Adult Mouse (>8 weeks)”). Public PC-3 and MCF-7 ATAC-
563 seq data was sourced from ENCODE (ENCFF772EFK, ENCFF024FNF).

564 Data was visualised using a combination of Seaborn⁴² and Matplotlib⁴³. A vector image
565 representing mouse head and cortex was sourced from SciDraw⁴⁴.

566

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581 **Ethical approval**

582 All animal experiments were conducted according to the KU Leuven ethical guidelines and
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584 numbers ECD P037/2016, P014/2017, and P062/2017). All use of cell lines was approved by the
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586 **Author contributions**

587 FDR, SP and SA wrote the manuscript. SP designed and fabricated the chips used for HyDrop
588 bead production and single cell encapsulation. SP, FDR, SA conceived the HyDrop protocols and
589 designed the experiments. SP conceived and developed the HyDrop bead barcoding strategy
590 and optimized the protocols with FDR, KT. FDR, SP, JI performed the HyDrop experiments. GM
591 performed mouse cortex microdissections. CF, GH, FDR developed the HyDrop pre-processing
592 and mapping pipeline. FDR, CBG, JJ performed the downstream data analysis.

593 **Competing interests**

594 The authors declare no competing interests or commercial affiliations.

595 **Data availability statement**

596 The datasets generated during and/or analysed during the current study are available on the
597 Gene Expression Omnibus (GEO) with the primary accession number GSE175684
598 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175684>), and on SCoPe
599 (https://scope.aertslab.org/#/HyDrop/*/welcome). Step-by-step user protocols are available on
600 Protocols.io (<https://www.protocols.io/workspaces/aertslab>). Data analysis tutorials for HyDrop
601 are available on GitHub (https://github.com/aertslab/hydrop_data_analysis).

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