Supplementary Materials for

Imaging cell lineage with a synthetic digital recording system

Ke-Huan K. Chow†, Mark W. Budde†, Alejandro A. Granados, Maria Cabrera, Shinae Yoon, Soomin Cho, Ting-hao Huang, Noushin Koulena, Kirsten L. Frieda, Long Cai, Carlos Lois*, Michael B. Elowitz*

†These authors contributed equally to this work.
*Corresponding author. Email: melowitz@caltech.edu (M.B.E.); clois@caltech.edu (C.L.)

Published 9 April 2021, Science 372, eabb3099 (2021)
DOI: 10.1126/science.abb3099

This PDF file includes:

Materials and Methods
Figs. S1 to S17
Captions for Tables S1 to S4
Captions for Movies S1 to S3
References

Other Supplementary Materials for this manuscript include the following:
(available at science.sciencemag.org/content/372/6538/eabb3099/suppl/DC1)

MDAR Reproducibility Checklist
Tables S1 to S4 (.xlsx)
Movies S1 to S3 (.mp4)
Materials and Methods

Plasmids preparation
Constructs were cloned using standard methods. Due to the repetitive sequence, inverted attPs were difficult to amplify in vitro, therefore PCR-based cloning methods were avoided for these regions. Mammalian constructs involving serine integrases Bxb1, phiC31, R4, and TP901 were cloned from, or used directly as, plasmid gifts from Mitsuo Oshimura (66). All constructs reported in this manuscript are listed in table S1, and sequence maps for constructs generated for the intMEMOIR system are available at (63). The Bxb1 and intMEMOIR array constructs are available on Addgene:
R26-pCAG-Ceru-10unit-BGHpA: https://www.addgene.org/158387/
PRExpress-Bxb1-hsp70pA: https://www.addgene.org/158391/
UAS-Ceru-10unit: https://www.addgene.org/158389/

Tissue culture
All tissue culture experiments were done with E14 mouse embryonic stem (mES) cell line (ATCC catalog number CRL-1821). Cells were cultured in humidified chambers at 37°C and 5% CO2, with filtered media composed of GMEM (Sigma), 15% FBS, PSG (100 units/mL penicillin, 100 µM/mL streptomycin, 2 mM L-glutamine) (ThermoFisher), 1mM sodium pyruvate (ThermoFisher), 1X Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA, ThermoFisher), and 100µM 2-Mercaptoethanol (ThermoFisher), with 1,000 units/mL Leukemia Inhibitory Factor (LIF, Millipore) added after filtering. Cells were maintained on polystyrene plates coated with 0.1% gelatin.

Flow cytometry
For flow cytometry experiments shown in Figures 2, C to E and S2, D and E, mES cells were plated on 24 well plates at approximately 70% confluency. Cells in each well were then cotransfected with 200 ng mTagBFP2, 400 ng integrase, and 400 ng of the sample’s corresponding prototype trit reporter. The transfections were performed with Lipofectamine LTX and PLUS reagent overnight (ThermoFisher).

Flow cytometry was performed two days after transfection on CytoFlex (Beckman Coulter). Cells were lifted from the plate with StemPro Accutase (ThermoFisher) and resuspended in buffer made of Hank’s Balanced Salt Solution (HBSS), 2.5mg/mL Bovine Serum Albumin (BSA), and 1mM EDTA. They were then filtered through a 40 µm cell strainer prior to flow cytometry. These experiments, including their respective transfections, were conducted in triplicate (Fig. 2C to E).

Flow cytometry data were analyzed using the EasyFlow Matlab program developed by Yaron Antebi, the version used for this manuscript available at (63), and the latest version available at
We gated for single cells using forward and side scatter (FSC and SSC), then gated for cells expressing high levels of the cotransfection marker mTagBFP2 to enrich for the transfected population in downstream analysis. For figure S2, D and E, we plotted the resulting distributions of Citrine and mCherry fluorescence for the relevant triplicates. For Figure 2C, we determined the median Citrine and mCherry fluorescence, background subtracted the fluorescence detected in the no integrase negative control, and calculated the mCherry/Citrine ratio for each replicate. We then plotted the average ratio of the experimental triplicates, with error bars representing the standard error of the mean (SEM). For Figure 2, D and E, we determined the median Citrine and mCherry fluorescence for each sample, background subtracted the fluorescence detected in the no integrase negative control, and averaged the values over the experimental triplicates. The resulting values were then normalized to the values for matching GT att sites and plotted for comparison, with the error bars representing normalized SEM.

Characterization of additional members of the serine integrase family

To characterize the ability of additional, non-Bxb1 serine integrases to function in mES cells, we constructed stable reporter cell lines containing either an integrase-specific reporter construct (fig. S2, A and B), or a 4 unit array with palindromic att sites (fig. S2C). These cell lines were then transiently transfected with their respective integrases, and the results evaluated through hybridization chain reaction FISH (described in section below).

To construct the stable reporter mES cell lines, the reporter constructs were site specifically inserted into the Rosa26 locus through Cas9-mediated homologous recombination by cotransfection of 600 ng of the reporter plasmid with 200 ng of pX330 Cas9 (gRNA sequence: CAGGACAACGCCCACACACC), followed by 500 µg/mL geneticin selection. Polyclonal stable cell lines were used to generate figure S2, A and B. Monoclonal cell lines were selected for figure S2C. The cells were then transiently co-transfected with 600 ng of their corresponding integrase and 200 ng of puromycin resistance plasmid. After one day, 1 µg/mL puromycin was added for two days to enrich for transfected cells in downstream experiments. After ending selection, cells recovered in regular media for one day, and were then plated on glass bottom 96-well plates (Cellvis) coated with 20 µg/mL of Laminin-511 (BioLamina).

For figure S2, A and B, cells were fixed one day after plating for HCR-FISH. Integrase activities were calculated as the percentage of manually counted cells with inverted reporters, out of all cells with the invariable barcode (fig. S2A). For figure S2C, cells were fixed approximately 4 hours after plating for HCR-FISH. The results were analyzed by a custom Matlab pipeline available at (63). Briefly, the program automatically segments the cells and counts HCR-FISH dots above manually determined thresholds. To account for different array expression levels, dot counts were normalized to nuclear CFP intensities before using them to call a unit state (i.e. unedited, inverted, and deleted), while the unnormalized dot counts were used to resolve rare conflicts between
unedited and inverted calls. The 4 unit array constructs contain a barcode without att sites located in the middle of the array, and only cells with unedited middle barcodes detected were used for analysis.

All transfections were performed with Lipofectamine LTX and PLUS reagent (ThermoFisher) overnight, on mES cells plated at approximately 70% confluency on a 24-well plate. All monoclonal selections involving site-specific integrations were screened with PCR.

**intMEM1 cell line construction**

To construct intMEM1, we began by integrating a landing pad containing FRT sites into the TIGRE locus using Cas9-mediated homologous recombination (construct modified from (39)). This was achieved through cotransfection of 600 ng of TIGRE-LandingPad-FRT-partialHygro-SV40pA and 200 ng of pX330 Cas9 (gRNA sequence: CTGCCATAACACCTAATT), followed by selection with 10 µg/mL blasticidin. After selecting a clone with correct integration, we introduced constitutive pEF1α-Tet3G through PiggyBac transposition (System Biosciences), transfecting with 600 ng of the Tet3G plasmid and 200 ng of the transposase, followed by 1 µg/mL puromycin treatment, and again selected for a single clone. We then inserted TRE-Bxb1-ecDHFR into the TIGRE landing pad with FlpE recombinase through cotransfection of 600 ng of TIGRE-TRE-(poorKozak)Bxb1-ecDHFR-BGHpA and 200 ng of FlpE, followed by 100 µg/mL hygromycin selection. The resulting polyclonal line was transfected with the 10-unit intMEMOIR array targeted to the Rosa26 locus through Cas9-mediated homologous recombination, by cotransfection of 600 ng of R26-pCAG-Ceru-10unit-BGHpA and 200 ng of pX330 Cas9 (gRNA sequence: CAGGACAACGCCCACACACC), 500 µg/mL geneticin selection, and monoclonal selection. Finally, we increased the fluorescence of these cells for time-lapse movie tracking by integrating PGK-mTurquoise2-Blast by PiggyBac, using 600 ng of the marker plasmid and 200 ng of transposase, followed by blasticidin and a second round of hygromycin selection. A final round of monoclonal selection resulted in the intMEM1 cell line. All transfections were performed with Lipofectamine LTX and PLUS reagent (ThermoFisher) overnight, on mES cells plated at approximately 70% confluency on a 24-well plate. All monoclonal selections involving site-specific integrations were screened with PCR.

**Time-lapse imaging for ground truth lineage**

Cells were plated on glass bottom 24-well plates (Eppendorf) coated with 20 µg/mL of Laminin-511 (BioLamina) overnight. Approximately 6,000 intMEM1 cells were seeded onto the coated wells, along with 18,000 parental E14 cells to increase cell density to support growth and survival. Media was changed prior to the start of the movie to remove any unattached cells. Imaging was done with an Olympus IX81 inverted epi-fluorescence microscope with Photometrics Prime 95b sCMOS camera, 20x air objective (0.75 numerical aperture), and equipped with an environmental chamber. intMEMOIR recording was initiated by adding 10 µM TMP (to block the DHFR degron) and 100 ng/mL doxycycline (to activate the TRE3G promoter). Inducers were omitted in negative
control samples. For each position, images were acquired every 15 minutes in both the visible light (DIC) and fluorescent (CFP) channels. 36 hours after the start of the movie we halted induction by washing off the induction media and replacing it with regular culture media. 54 hours after the start, we terminated time-lapse imaging and promptly fixed the sample at room temperature with 4% formaldehyde in PBS for 5 minutes, followed by HCR-FISH protocol (below).

**Constructing ground truth lineage**
Ground truth lineage trees were constructed by manually tracking the cells in the time-lapse images using a modified version of the EasyTrack software developed by Yaron Antebi (freely available at (63) and [https://github.com/AntebiLab/EasyTrack/tree/Memoir](https://github.com/AntebiLab/EasyTrack/tree/Memoir)). Cells were primarily tracked by their CFP fluorescence. Ground truth trees could begin at either the one or two cell stage depending on the colony’s cell cycle at the start of image acquisition, and were rooted at the two cell stage if the parent cells in question were likely sisters based on proximity, cell morphology, CFP intensity, as well as their cell movements and cycles in the subsequent frames. Ground truth trees for all colonies were outputted as Newick strings (table S2 for colonies used in lineage reconstruction).

**Hybridization Chain Reaction (HCR) FISH and imaging**

*Overview of imaging workflow:*
The imaging protocol consists of fixation and permeabilization steps, followed by multiple rounds of primary probe binding and signal amplification by HCR (fig. S3A). Below, we describe each of these steps in more detail.

*Fixation and permeabilization:*
HCR-FISH in tissue culture began after fixing the samples at room temperature with 4% formaldehyde in PBS for 5 minutes (as described above). Fixed cells were washed with PBS, followed by permeabilization in 70% RNase-free ethanol at -20°C overnight, and stored in 70% ethanol for up to 3 days at -20°C. Permeabilized cells were washed with 20% formamide wash buffer in 2X SSCT at room temperature for 5 minutes and pre-hybridized in 30% probe hybridization buffer at 37°C for 30 minutes.

*Primary probe hybridization:*
Primary probe hybridizations and hairpin amplifications were then carried out as previously described for HCR v3.0 (Molecular Instruments) (42). Primary probes for each round of hybridization were prepared in probe hybridization buffer (warmed to 37°C) at 4 nM per probe. The pre-hybridization solution was then replaced with the probe solution with an overnight incubation at 37°C. The samples were then washed 4 times with warm 30% probe wash buffer at 37°C, with 15 minutes incubation accompanying each wash. Finally, samples were washed once with 5X SSCT at room temperature for 5 minutes.
**HCR amplification:**
Samples were incubated in amplification buffer at room temperature for 30 minutes. Hairpins for amplification were prepared by snap cooling each at the stock concentration of 3 μM. This was done by heating the individual hairpins to 95°C for 90 seconds, then cooling them to room temperature in the dark for 30 minutes. The cooled hairpins were then mixed and prepared in amplification buffer at 60 nM final concentration for each hairpin. The pre-amplification solution on the sample was then replaced with the hairpin mix and incubated at room temperature from 4 hours to overnight. During incubation and for all subsequent steps, the sample plate was protected from light by covering it with aluminum foil except during pipetting and/or imaging.

Amplification was ended with two 5 minute washes, two 30 minute washes, and one 5 minute wash of 5X SSCT at room temperature. Finally, the cells were imaged in 5X SSCT.

**Materials:**
30% probe hybridization buffer, 30% probe wash buffer, amplification buffer, and HCR amplification hairpins were purchased from Molecular Instruments. Hairpins used for the intMEM1 experiments were (in the format of HCR initiator-fluorophore): B1-Alexa594, B2-Alexa647, B3-Alexa546, and B4-Alexa488. The probe binding regions for each intMEMOIR unit, along with their corresponding initiators, are listed in table S3, and the probes can be purchased from Molecular Instruments with order IDs 3049 and 3092.

**Rehybridization:**
Between hybridization rounds, probes were removed via DNase I treatment (Roche). Briefly, cells were washed with 1X DNase buffer, followed by incubation with 1 Kunitz unit/µL DNase I in 1X buffer for 2 to 4 hours at 37°C. Digestion was ended by washing the cells 3 times with 30% probe wash buffer, incubating the final wash for 15 minutes at 37°C. Finally, cells were washed once with 5X SSCT before the pre-hybridization step for the next round of HCR-FISH.

**Imaging:**
Cells were imaged using a Nikon Eclipse Ti inverted fluorescence microscope, with an Andor Zyla 4.2 sCMOS camera and a 60x oil objective (1.4 numerical aperture). For all HCR-FISH channels, each field of view was acquired with 0.5 μm z-steps for 20 z-slices. Maximum intensity projections from the in-focus slices were then used for downstream analysis.

**Antibody staining**
Upon completion of all rounds of HCR-FISH readout and a final round of DNase I treatment to remove any HCR-FISH signals, cells underwent antibody staining for membrane markers E-cadherin and β-catenin to facilitate segmentation. Immunostaining was performed following standard protocols, with most incubation and washing steps carried out on a gentle rocker. Briefly, samples were blocked with blocking buffer made in PBS (5% BSA, 1% DMSO, and 0.2% Triton...
X-100) for 1 hour at room temperature. They were then incubated with primary antibodies E-cadherin (R&D Systems, AF648, 1:20) and β-catenin (Abcam ab6301clone15B8, 1:750) overnight at 4°C. The following day, they were washed 5 times with PBST for 5 minutes each, then incubated with secondary antibodies (donkey anti-goat IgG 647 A21447, and donkey anti-mouse IgG 488 A21202, respectively) diluted 1:1000 in blocking buffer for 3 hours at room temperature. Finally, samples were washed 5 times with PBS for 5 minutes each, followed by imaging in fresh PBS.

Analysis of HCR-FISH readout in mES cells

To segment individual cells and identify their array edit states, we used a custom analysis pipeline in Matlab (fig. S4, available at (63)). For cell segmentation, immunofluorescence images of E-cadherin were first preprocessed with Ilastik (64) to generate a membrane probability map. The positions of cells in the final frame of the ground truth lineage analysis were used as watershed seeds overlaid on the membrane probability maps. The resulting segmented images were visually examined and manually curated. The four channels used for HCR-FISH analysis did not show significant fluorescent crosstalk (fig. S5).

For array state determination, the centers of the mRNA dots were determined using a Laplacian of Gaussian filter in Matlab. Barcode mRNA locations were called when multiple units localized to the same spot. Each barcode mRNA state was determined by looking at the binary state of each of the twenty unit HCR-FISH images. All of the barcodes located in each cell were used to generate a consensus barcode state. Cells with fewer than 50 detected units were excluded from analysis.

Calculation of mutual information between recording units

We pooled the observed states from all 1,453 cells and built a frequency matrix \( \Gamma_{3 \times 10} \) representing each of the 10 recording units and the observed frequency of each one of the three possible intMEMOIR states which define the distribution \( P(\mathbf{x}) \) per site. For each pair of sites, we then computed the joint distribution \( P(\mathbf{x}, \mathbf{y}) \) from the observed frequencies of pairs of states e.g. \([\{1, 1\}, \{1, 0\}, \{1, 2\}].\). We then combined the probabilities in \( \Gamma \) with the joint distribution to build a matrix of pairwise Mutual Information using Shannon’s formula, using \( \log_3 \) to normalize the maximum entropy of a single unit to 1 trit.

Lineage analysis of large mES cell colony

Cells were plated on glass bottom 24-well plates (Eppendorf) coated with 20 μg/mL of Laminin-511 overnight. Approximately 1,000 intMEM1 cells were seeded onto the coated wells and induced with 10 μM TMP and 100 ng/mL doxycycline, along with 9,000 parental E14 cells to increase cell density to support growth and survival. Induction lasted 36 hours, followed by approximately 70 hours of growth with no induction. Media was changed daily, and the cells were fixed at the end of the experiment with 4% formaldehyde in PBS for 5 minutes, followed by HCR-FISH protocol described above. Clone boundaries and barcode analysis for this colony were analyzed by hand.
D. memoiphila fly line generation

Fly lines containing UAS-Ceru-10unit and PRExpress-Bxb1-hsp70pA were site-specifically integrated into the attp2 and VK27 sites, respectively, using phiC31 (Bestgene Inc.). Flies with the 10-unit array were first crossed with an nSyb-Gal4 line (R57C10-Gal4, attp40, Bloomington Drosophila stock center) for pan-neuronal expression of the intMEMOIR array. The offspring were then crossed with the PRExpress-Bxb1-hsp70pA to generate the line capable of autonomous recording for downstream experiments. The generated fly lines are available from Bloomington Drosophila stock center with the following RRID:
PRExpress-Bxb1-hsp70pA: BDSC_90853
nSyb-Gal4; UAS-Ceru-10unit: BDSC_90854

D. memoiphila characterization

To determine if we could tune the edits in D. memoiphila embryo and read out the results in adult fly brains, we placed parents of the PRExpress-Bxb1-hsp70pA x nSyb-Gal4; UAS-Ceru-10unit cross in fresh vials overnight at 25°C to collect eggs. 3 to 4 hours after removing the parents, the embryos were heat shocked in 37°C water bath for 30 minutes, 1 hour, and 3 hours for the respective samples. Negative control samples were always kept at 25°C and not heat shocked. The resulting adult flies were sacrificed, and their brains were dissected in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 20 minutes. Samples were then washed 3 times with PBS for 10 minutes, transferred to Optimal Cutting Temperature (OCT) compound, and frozen on dry ice. Samples were cut into 20 µm-thick sections on a cryostat and transferred onto coverslips that had been pre-treated with 3-aminopropyltriethoxysilane (Sigma A3648, diluted to 2% v/v in Acetone), followed by post-fix with 4% PFA in PBS for approximately 25 minutes, 3 rinses with PBS, 1 rinse with 70% ethanol, and permeabilized in 70% ethanol overnight at 4°C. Tissues were then cleared with 8% SDS for 5 minutes at room temperature, rinsed once with PBS, then rinsed 3 times with 70% ethanol. After air drying the samples, we installed SecureSeal Hybridization Chambers (Grace Bio-Labs) onto the coverslips. intMEMOIR array states were then read out with HCR v3.0 as described above (Molecular Instruments) (42). Imaging for these samples were done on a Nikon Eclipse Ti inverted microscope, with a spinning disc unit Yokogawa CSU-W1, an electron-multiplying charge-coupled device camera Andor iXon Ultra, and a 40x oil objective (1.3 numerical aperture).

intMEMOIR activity was evaluated by calculating the ratio of inverted-unit-5 to unedited-unit-9. The mES cell HCR-FISH data demonstrated that these two units are efficiently edited (Fig. 2I), and the two states were both probed in the same round of HCR-FISH, enabling comparison of the exact same fields of view and eliminating errors or bias that might result from image alignment. Each data point in Fig. 5B corresponds to one imaging position.

intMEMOIR labeling of early neuroblast lineages
To label neuroblast lineages at early embryonic stages, parents of the PRExpress-Bxb1-hsp70pA x nSyb-Gal4; UAS-Ceru-10unit cross were placed in fresh vials at 25°C to collect freshly laid eggs. The vials were inspected every hour and, upon observing egg laying, the parents were removed, and 4 hours later the embryos were heat shocked at 37°C for 1 hour. The resulting adult flies, up to 1 week old, were incubated at 29°C overnight to enhance activity of the Gal4 transcription factor on the UAS promoter prior to brain collection and cryosection, followed by smFISH readout (fig. S3B and below).

smFISH readout in D. melanogaster brain section
To simultaneously examine spatial organization, cell state, and lineage information in the same tissue (Fig. 5D to M), the dissected fly brains were cryosectioned and attached onto coverslips treated with 1% bind-silane (GE 17-1330-0) and poly-D-lysine (Sigma P6407), and the resulting samples prepared and analyzed with sequential, automated rounds of smFISH in a manner similar to previously described (65). Briefly, sections were post-fixed with 4% PFA at room temperature for 15 minutes, followed by three PBS washes. They were then permeabilized in 70% ethanol (either at 4°C overnight or at room temperature for 2 hours), cleared with 8% SDS in 1X PBS for 20 minutes at room temperature, then washed with 70% ethanol prior to two rounds of overnight primary probe hybridizations at 37°C (in order to separate the hybridizations for unedited and inverted units). After each hybridization, samples were washed with 2X SSC for 3 times, incubated in 40% formamide in 2X SSC for 30 minutes at 37°C, followed by 3 additional rounds of 2X SSC wash. They were then stained with 100 µg/mL Concanavalin A-488 (ThermoFisher) in PBS with 0.1% BSA and 0.1% Triton X-100 for more than 5 hours at room temperature to facilitate segmentation in downstream analysis. After staining, the sample was washed three times with PBS plus 0.5% Triton X-100 (with an extended 5 minute incubation for the final wash), and stained with 10 µg/ml DAPI in 4X SSC for 15 seconds. Next, an anti-bleaching buffer solution made of 10% (w/v) glucose, 1:100 diluted catalase, 0.5 mg/ml glucose oxidase and 50 mM pH 8 Tris-HCL in 4X SSC was flowed through the samples. We used an automated imaging and fluidics delivery system described in (65) to image mCerulean transcripts, the intMEMOIR units, and 8 endogenous genes. Two fluorophores, 647 and Cy3B, were used to read out the 29 targets in 15 rounds of hybridizations. The probe sequences and their corresponding readout channels are listed in table S4. The microscope used in this system was a Leica DMi8, with confocal scanner unit Yokogawa CSU-W1, Andor Zyla 4.2 Plus sCMOS camera, 63x oil objective Leica 1.40 NA, and an ASI MS2000 stage. Each field of view was acquired with 1 µm z-steps for 14 or 15 z-slices, across 647 nm, 561 nm, 488 nm, and 405 nm fluorescent channels. Maximum intensity projections of the slices were used for example images in Figs. 5D and S11. For each brain, 1 z-slice with in-focus FISH signals (determined with mCerulean FISH signal to avoid bias), was then selected for downstream analysis.

Analysis of smFISH readout in D. melanogaster brain section
Fly cells were segmented manually. A custom Matlab program (available at (63)) was used to determine the barcode state, as with the mES cells. Clones which had at least one barcode inversion and at least 4 cells were chosen for downstream analysis.

**Gene expression analysis in brain section (Brain B1 as the example for the description below)**
The brain data set comprises gene expression, location, and intMEMOIR state for 5,332 individual cells. For each cell, we recorded the average pixel value as the expression level for the 8 endogenous genes. To investigate the structure of the gene expression space, we constructed a gene expression matrix $M_{m \times n}$, where $n = 8$ genes, and $m = 5,332$. Based on this matrix, the analysis pipeline we built to delineate gene expression clusters consists of several steps: 1. Scaling gene expression using a z-transform, such that all genes have mean=0 and standard deviation=1. 2. Denoising data by applying PCA (57) to the scaled data and retaining principal components accounting for 80% of the total variance (6 PCs). 3. The 6-dimensional data were then transformed into a UMAP embedding of 4 dimensions followed by the DBSCAN clustering algorithm (59) (sklearn DBSCAN with eps = 0.3) which resulted in a total of 20 distinct clusters. 4. For visualization, the 6-dimensional data set was transformed and projected into 2 dimensions using UMAP (58) (default parameters, Python UMAP v0.3.10). 5. Finally, we mapped the gene expression clusters (as color labels) into either the UMAP space (Fig. 5G) or physical space (Fig. 5I).

**Determining the relationship between clonality, physical distance, and gene expression distance**
The physical distance was calculated as the Euclidean distance between all pairs of barcoded cells chosen for analysis. This data set was then divided into two groups: pairs within the same clone or pairs from two different clones, and plotted as a cumulative histogram (Fig. 5F, ‘within clone’ and ‘between clone’, respectively). For gene expression space, the Euclidean distance was calculated between cell pairs using the UMAP coordinates (e.g. Fig. 5K and S16A). To disentangle the relative contribution of physical distance and lineage to gene expression, the aforementioned data was further binned by the physical Euclidean distance between cell pairs (Fig. 5M). Pearson correlation was also used as an alternative metric for gene expression distance (figs. S16B and S17).

**Lineage and statistical analysis**
Here we describe procedures for two types of lineage analysis (Fig. 1A). First, we discuss assignment of individual cells to clones, i.e. groups of cells that share a common ancestor at the time of editing (clonal classification). Second, we discuss the hierarchical assignment of cells or clones into multi-generational lineage trees (lineage tree reconstruction). In both cases, we describe an analytical framework and experimental validation using the data in Figure 3. These data were obtained from experiments in which Bxb1 was expressed for ~3 generations, followed by an additional ~1-2 generations of clonal expansion without Bxb1 induction (Fig. 2G).
Clonal classification

intMEMOIR can classify cells into clones based on shared array state inherited from a common ancestor that was uniquely labeled at a specific point in the past. Clonal analysis can be used both to address specific biological questions, and to provide the ‘leaves’ of more detailed lineage tree reconstruction (below).

Ideally, clonal classification should group cells in such a way that each cell is more closely related to other cells in its own group than to any cell in other groups. In general, a given lineage tree can generate multiple, distinct clonal classifications depending on which edits occurred at what point in the tree. For example, the tree in figure S6 could show multiple distinct sets of unique edit patterns (colors), all consistent with the true lineage.

An experimental clonal classification is made in a straightforward way by grouping cells with identical edit patterns into putative clones. To assess the accuracy of such a clonal classification, we must first determine if it is consistent with the ground truth lineage tree observed by direct time-lapse imaging (Fig. 3A), and, second, quantify the number and types of classification errors, if any (Fig. 3C).

The following algorithm assigns an accuracy score to a given putative clone, labeled R. To do so, it considers all subtrees (partitions) of the ground truth lineage tree and asks whether any subtree exactly matches the inferred clone. If such a subtree exists, then the clonal classification is considered accurate. If not, we identify the subtree that most closely matches the clone, which we label as S, and quantify its deviation from the putative clone. This deviation is computed by first classifying each cell in S as either a true positive (appears in both S and R), a false positive (appears in R but not S), or a false negative (appears in S, but not R). We then count the number of cells in each of these three categories and compute a clone score:

\[
\text{score} = \frac{TP}{TP + FP + FN}
\]

Here, \(TP\), \(FP\), and \(FN\) denote the number of cells that are true positive, false positive, or false negative, respectively. A higher score indicates a higher fraction of true positive cells (greater accuracy). Results from this analysis are plotted in Fig. 3D.

Lineage reconstruction

To reconstruct a multi-generation lineage tree from observed edit patterns, we first develop a relatedness metric for pairs of cells (or clones) based on their edit patterns. The metric is based on the likelihood of a sister relationship. We then use this metric to reconstruct lineage trees in such a way that cells that score higher on this sister likelihood metric are grouped more closely together on the reconstructed tree. Finally, we validate this procedure and quantify its accuracy.
To develop the metric, we start by modeling the molecular events that generate the final edit patterns. We assume each unedited memory element can be edited stochastically at a constant, empirically determined rate per cell generation per memory element, denoted $\mu_k$, where $k = 1, \ldots, 10$ indexes the memory element within the array (see Fig. 2I). We also incorporate the empirical transition probabilities for each of the two possible outcomes of each state (Fig. 2I). We then define the probability distribution $P_g$ for observing each of the 10-unit array states that occur in the colony of interest starting from an unedited array, after $g$ generations. $P_g$ is computed for each cell in the colony, and represents the probability of observing that cell’s specific array state, independent of the states of other cells in the colony.

Next, we define the pairwise distance metric for a single memory element. We denote the conditional probability of observing any two specific memory states in a pair of sister cells as $P^{sis}_{g}(i,j)$, where $i$ and $j$ index two specific, different cells ($i \neq j$). To convert this probability into a distance metric, we need to normalize it by comparing the likelihood of observing these two memory states in a pair of sister cells to the likelihood of them occurring independently in two unrelated cells. That is, we define the distance metric as

$$d_{i,j} \equiv \frac{P_g(i)P_g(j)}{P_g^{sis}(i,j)}.$$

Memory units edit independently (Fig. 2J). Therefore, it is possible to extend this distance metric for a single memory element to the level of a complete array in a straightforward manner, by replacing the single unit probabilities with products over all the units:

From this we obtain the $K = 10$ unit array distance metric:

$$d_{i,j}^{array} \equiv \frac{\prod_{k=1}^{10} P_g(i)_k P_g(j)_k}{\prod_{k=1}^{10} P_g^{sis}(i,j)_k}.$$

**Deriving probability distributions for the intMEMOIR system**

This distance metric is independent of many details of the recording system. To apply it to intMEMOIR data, we first need to derive expressions for the distributions $P_g$ and $P^{sis}_g$. The recording units have an initial state, denoted $1$, that can be edited irreversibly into either of two states, denoted $0$ and $2$. The probability that a given unit is edited during a cell division is $\mu_k$, and the probability that no edit happens during a cell division is $(1 - \mu_k)$. For simplicity, we first derive $P_g$ assuming only two possible states: unedited ($1$) and edited ($0$). For a given unit, the probability that no editing happens for $g$ generations (cell divisions) is then

$$P_g(1) = (1 - \mu)^g$$

The probability that an edit occurred at some point in the past is defined by the geometric distribution:
This expression considers all possible times at which the edit could have happened, e.g. in the first generation, the second generation, or even in the last generation. Once the edit occurs, the unit can no longer be edited. (Below, we will extend this analysis to the case of multiple edit outcomes.)

By applying the geometric series, we can show that $P_g$ is well defined as a probability distribution for all values of $g$ such that:

$$P_g(0) + P_g(1) = 1$$

(3)

We derive Eq. 3 by first expanding Eq. 2:

$$P_g(0) = \mu + (1 - \mu) \mu + (1 - \mu)^2 \mu + (1 - \mu)^3 \mu + \ldots + (1 - \mu)^{g-1} \mu$$

Combining eqs. 1 and 2 we obtain the total probability:

$$P_g(0) + P_g(1) = \mu [1 + (1 - \mu) + (1 - \mu)^2 + \ldots + (1 - \mu)^{g-1}] + (1 - \mu)^g$$

We then use the following identity for the geometric series:

$$\sum_{k=0}^{n-1} q w^k = q \frac{1 - w^n}{1 - w}$$

By setting $q = 1$, $w = 1 - \mu$ and $k = g$, we obtain:

$$P_g(0) + P_g(1) = \mu \frac{1 - (1 - \mu)^g}{\mu} + (1 - \mu)^g = 1$$

Which shows that $P_g$ is well-defined for all values of $g$.

**Three-state model**

We now extend the model by considering three possible edit outcomes: $\{1, 0, 2\}$. The probabilities of observing the recording unit in each of three possible states at generation $g$ become:

$$P_g(1) = (1 - \mu)^g$$

$$P_g(0) = \sum_{g=1}^{G} (1 - \mu)^g \mu \alpha$$

$$P_g(2) = \sum_{g=1}^{G} (1 - \mu)^g \mu (1 - \alpha)$$

(4)

Here, $\alpha$ denotes the probability of an edited unit going to state 0 and $1 - \alpha$ is the probability of it reaching state 2. Note that, in a similar way, this framework could also be generalized to larger
numbers of editing outcomes. The transition probability distribution \( P(z_{g-1} \rightarrow i) \) represents all the ways in which an individual unit can change state during a single cell division cycle:

\[
P(1 \rightarrow 1) = 1 - \mu \\
P(1 \rightarrow 0) = \mu \alpha \\
P(1 \rightarrow 2) = \mu (1 - \alpha) \\
P(0 \rightarrow 0) = 1 \\
P(2 \rightarrow 2) = 1
\]

(5)

The probabilities of all other transitions are zero, consistent with the irreversibility of intMEMOIR editing. Further, these transition rates are assumed to be time independent.

**Sister likelihood**

We now have all elements necessary to compute the sister likelihood scores that are the basis of our distance metric. We calculate the conditional probability that an unobserved parental state \( z \) in the previous generation transitioned into the observed states \( i, j \). For simplicity, first consider just a single cell state, \( i \):

\[
P_g(i|z_{g-1}) = P(z_{g-1} \rightarrow i|z_{g-1})P_{g-1}(z)
\]

(6)

Where \( P_{g-1}(z) \) is the probability of observing the parental state \( z \) in the previous generation and can be calculated using Eq. 4. Considering now two cells \( i, j \), this transition probability becomes the joint distribution:

\[
P_g(i, j|z_{g-1}) = P(z_{g-1} \rightarrow i|z_{g-1})P(z_{g-1} \rightarrow j|z_{g-1})P_{g-1}(z)
\]

(7)

Eq. 7 provides the probability that the observed states \( i, j \) came from the unobserved parental state \( z \). Since we don’t actually observe \( z \), we need to account for all possible parental states to obtain the total sister probability \( P_g^{sis} \):

\[
P_g^{sis}(i, j) = \sum_{z=0}^{2} P_g(i, j|z_{g-1})
\]

(8)

Finally, considering an array of \( k \) independent units we extend this and calculate the product:

\[
P_g^{sis}(C_i, C_j) = \prod_{k=1}^{10} P_g^{sis}(i, j)_k
\]

(9)

Where \( C_i, C_j \) represent a specific pair of array states, e.g. in two different cells or clones.

**Hierarchical lineage tree**
The calculations above enable us to compute the pairwise distance matrix \( d_{i,j}^{\text{array}} \), defined above, for any actual data set. As the final step in reconstruction, we built a dendrogram from \( d_{i,j}^{\text{array}} \) by applying divisive clustering, a top-down approach in which all observations start in one cluster, and two-way splits are performed recursively as one moves down the lineage tree, terminating at the leaves (individual cells or clones). Divisive clustering was implemented with the DIANA function from the R cluster package. Examples of the resulting trees are shown in Figures 3E to 3G. A complete list of reconstructed trees is provided in table S2 in the Newick tree format.

**Assessing tree accuracy**

To quantitatively assess reconstruction accuracy compared to ground truth (Fig. 3H), we used the Robinson-Foulds distance metric, as implemented in the R phytools package, to compute the difference between the reconstructed and ground truth trees. For this analysis, all cells sharing the same array state are collapsed into a single clonal tree “leaf.” Occasionally, analysis of ground truth trees revealed convergent edits producing identical array states in distantly related cells (false positive events; see discussion of clonal analysis, above). These events prevent one from unambiguously collapsing identical array states in the ground truth tree. In these cases, we randomly retain one of the array states.

**Calculation of entropy in colonies**

In applications where no ground truth is available, we would like to develop a predictive metric that could be used to enrich for colonies that are likely to reconstruct with greater accuracy. Multiple variables, including spatial arrangements of cells or morphological similarity could in principle be informative. However, we reasoned that the most useful and generalizable metric would be one based only on the observed edit patterns, since this information should be available in all applications independent of systems-specific biological features.

Shannon’s entropy provides an ideal metric to quantify information content in a discrete data set such as a list of edit states. To apply it to colonies, we pooled the edit states from all cells within each colony. We then constructed a \( 10 \times 3 \) matrix, \( \Gamma \) representing the frequency of observing each of the 10 recording units in each of the 3 edit states. We can then apply Shannon’s formula to each unit to obtain its individual entropy, e.g. \( H_k = - \sum_{i=0}^{2} \Gamma_{i,k} \log \Gamma_{i,k} \) is the entropy of the \( k^{\text{th}} \) unit. The total entropy of the colony is then obtained by adding the entropies of the individual sites: \( H_{\text{colony}} = \sum_{k=1}^{10} H_k \). Finally, we scaled the entropy by the fraction of edited sites in the colony, \( \pi \), to obtain an informative score of expected reconstruction quality. We confirmed that selecting colonies with higher normalized entropy enriched for better reconstruction (Figs. 3H and S8).
Supplementary Figures

(A) The central dinucleotide (red) of attP/B form base pairs during recombination, dictating specificity and orthogonality of the sites. In principle, out of the 16 possible dinucleotide combinations, 10 could confer orthogonality in an array: four are palindromic, and the two sets of six non-palindromic dinucleotides are reverse complements of one another, so only one set could be used. (B) Schematic of the 10-unit array as designed with annotated att sites and barcodes (BC).

Fig. S1. 10 central dinucleotide variants in the att site enable the arrangement of 10 independent memory units in one array.
**Fig. S2. Additional members of the serine integrase family function in mES cells.**

(A) To assess the activity of different serine integrases in mES cells (30), we integrated reporter constructs with an attP/B flanked unit followed by a barcode. Active integrase inverts the unit upon transfection, which can be detected via HCR-FISH. (B) Percentage of cells with inverted reporter after transfection. sPBc, phiC31, and wBeta are active in mES cells (n=91, 165, and 139 cells, respectively); R4 show weak activity (n=88 cells), and no activity was detected for phiBT1 and TP901 (n=172 and 171 cells, respectively). (C) Additional serine integrases can mediate inversion and deletion between att sites with palindromic dinucleotides. sPBc and wBeta reporter cell lines with a 4 unit array were transfected with their corresponding integrases, and their relative
edit frequencies analyzed via HCR-FISH (n=307 and 264 cells, respectively). (D and E) Fluorescent reporter assay demonstrates that other serine integrases operate orthogonally to Bxb1 att sites, opening the possibility for orthogonal recording in the same cell. This is true for phiC31 and R4, as shown in (D), and wBeta and sPBc, as shown in (E), with n=3 for each sample. (F) Schematic illustrating simultaneous lineage tracking and signal recording using orthogonal serine integrases.
Fig. S3. Two fluorescent in situ hybridization methods were used to read the intMEMOIR array.

(A) We used HCR v3.0 (42) to read the array state in the intMEM1 experiments (Figs. 2, G to J, 3, 4, S4, and S5) and to determine the effect of heat shock duration on *D. memoiphila* editing (Fig. 5B). 9 probe pairs were used for each unit, and the signals were subsequently amplified through HCR. The primary probe binding regions of each unit, the probe pairs’ HCR initiator ID, and the fluorophore used in the corresponding amplification hairpins are listed in table S3. We also used the HCR-FISH method, with different barcodes and corresponding probes, to test additional members of the serine integrase family (fig. S2, A to C) (B) We used automated smFISH (65) to read the array state and endogenous genes in *D. memoiphila* experiments (Fig. 5C onward, and S11 onward). 12-13 primary probes were used for each intMEMOIR unit, 15 probes were used for mCerulean, and 24 probes were used for each endogenous gene. The target-specific sequences, readout sequences and ID, and the fluorophore used with the corresponding readout probes are listed in table S4.
A Stain E-cadherin  

B Classify membrane in Ilastik  

C Watershed segmentation  

D Raw FISH Data  

<table>
<thead>
<tr>
<th>Barcode ID</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ab</td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>bc</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>abc</td>
</tr>
</tbody>
</table>

E Determine location of all 20 FISH probes  

F Sum all 20 FISH channels

G Locate areas with more than one signal

H Local maxima are array locations

I Count number of RNA arrays positive for each barcode

J Classify barcodes as unedited, inverted, or deleted

K Barcode state determination

<table>
<thead>
<tr>
<th>Barcode ID</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unedited</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
</tr>
<tr>
<td>Inverted</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
</tr>
</tbody>
</table>

Legend:  
- Unedited  
- Deletion  
- Inversion
Fig. S4. Cell segmentation and barcode determination.

Here we show the workflow for classifying array states in individual cells. (A) In order to segment individual cells, we acquired image stacks of cells stained with an E-cadherin antibody that localizes to cell membranes. (B) We trained the image analysis program Ilastik to classify pixels in these images as membrane or non-membrane. (C) A 3-dimensional watershed algorithm (Matlab) was used to segment cells from the pixel classification stack, using the final annotated cell positions from the movie as watershed seeds. Obvious segmentation errors were fixed by adding additional seeds and cutting joined cells. After the watershed, stacks were converted to 2-dimensional images by maximum projection. Here, cells ‘a’, ‘b’ and ‘c’ were shaded and labeled for subsequent panels. (D) Maximum intensity projections of the images for the 20 array channels. (E) RNA molecule locations were detected for each HCR-FISH probe set by finding the local maximum after applying a Laplacian filter. The points were dilated to account for small errors in localization. (F) Dilated points from all 20 channels were summed to identify locations with two or more barcodes as barcode arrays. Blue locations contain only a single detected dot and were discarded. (G) Locations with multiple detected unit reads are shown in magenta and white, and considered validated array signals. (H) Each local maximum was designated as an array location. For visualization, green boxes were drawn around the array locations. (I) The presence or absence of HCR-FISH signal (magenta dots) was determined for each barcode array location (green boxes) and tallied for each cell. Cells with greater than 50 validated unit reads were retained for downstream analysis. (J) Cells were considered positive for a given unit when a signal was present in at least 25% of the array locations in the cell. (K) From these results, the final array states were determined for each cell.
Fig. S5. The four HCR-FISH fluorescent channels show minimal crosstalk. Each row shows a portion of an intMEM1 cell positive for a single channel. Only the true positive channel shows significant fluorescent signal above background. Fluorescent crosstalk would have appeared as signal in the negative channel, at the same location as the true positive channel. Each column is shown with the same exposure, brightness, and contrast.
Fig. S6. A given lineage tree can generate multiple, distinct clonal classifications. Seven possible subtree assignments can accurately describe this simple five-cell tree, where all cells within a clone are more closely related to each other than to any cell outside of the clone. In one extreme, each individual cell can be classified as its own clone. At the other extreme, in the absence of editing, all cells are grouped into a single clone.
Fig. S7. Lineage simulation and reconstruction based on maximum likelihood of sister relationships.

(A) Schematic of the lineage simulation method. We define a two-parameter stochastic model where \( \mu \) equals the edit rate in units of edits per site per generation, and \( \alpha \) denotes the probability that the edit goes to state 2. The model assumes that cells divide synchronously and at a constant rate such that at generation G the lineage comprises \( 2^G \) cells. (B) For a constant \( \mu \), the number of new edits appearing in each generation decays exponentially as dictated by the equation

\[
E[\mu, t] = (1 - \mu)^g \mu
\]

where \( g \) is the number of generations (cell divisions) and \( E[\mu, t] \) is the expected fraction of edited sites. For intMEMOIR, the experimental value of \( \mu \) is \( 0.1 - 0.3 \) (C) Schematic of the reconstruction approach. We first compute the probability that a trit is in either of the three possible states at generation G, combining the transition probabilities shown in A and the equation from B and called this distribution (blue cell). For sister likelihood, we compute the probability that two cells share a parent cell in the previous generation. (D) Equations for reconstructing lineages based on sister likelihood.

1. Probability of two states i, j sharing an parent z in G-1

\[
P^{2^G}_{ij}(i,z) = \sum_{i=0}^{2^G} P^(i \rightarrow i) \times P(z \rightarrow j) P^{i-1}_{G-1}(z)
\]

2. Likelihood ratio as distance for an array of 10 units

\[
d_{ij} = \prod_{k=1}^{10} P^{2^G}_{ij}(i_k,z_k)
\]

3. Schematic of the reconstruction approach. We first compute the probability that a trit is in either of the three possible states at generation G, combining the transition probabilities shown in A and the equation from B and called this distribution \( P_G \) (blue cell). For sister likelihood, we compute the probability that two cells \((i, j)\) share a parent \(z\) in the previous generation. (D) Equations for reconstructing lineages based on sister likelihood. 1. The probability that a recording unit is in either of the three possible states at generation G, independently of the other cells. 2. The probability that a parent cell \(z\) at \( G - 1 \) transitions into the states i, j. This equation assumes that the daughter cells \((i, j)\) inherit the state of \(z\) and then edit with probability \( \mu \). Since the recording is irreversible, the only valid transitions are \( 1 \rightarrow 0 \) and \( 1 \rightarrow 2 \); once a cell reaches either state 2 or 0, all its daughters will inherit that state with \( \text{Pr} = 1 \). We finally sum over all possible states of the
3. We can then calculate the joint probability for the 10 units as the product of the probabilities of each unit. And compare this number to the probabilities of observing the states \((i, j)\) assuming no sister relationship, which are just the product of their \(P_G\) probabilities in the numerator. This ratio quantifies the likelihood of observing a given pair of array states for two sister cells compared to two unrelated cells. 4. This likelihood provides a pairwise distance metric that we then use to reconstruct the lineage tree. (E) Once we computed the likelihood ratio for all pairs of cells, we can cluster the matrix using divisive hierarchical clustering, which starts by partitioning the data set into the most distinct groups, then it proceeds to partition each subgroup into two groups iteratively until each group contains only one cell. Ideally, each partition of the algorithm would correspond to a cell division event.
Fig. S8. Barcode entropy enriches for colonies that reconstruct with greater accuracy.

We computed the entropy for a lineage as the sum of the individual entropies for each trit, using Shannon’s formula. The normalized entropy is then computed as the lineage’s entropy times the fraction of edited sites for that lineage, scaled by the maximum such that the metric has a range from [0,1]. This simulated dataset comprises 3000 lineages. (A) The fraction of lineages with normalized entropy larger than the threshold. (B) The fraction of perfectly reconstructed lineages for increasing thresholds of normalized entropy. For a given threshold value, we split the dataset and calculated the fraction of perfect trees in the high-entropy set. Note that the number of lineages analyzed decreases with increasing entropy thresholds, as shown in (A). (C) As an example, using a threshold of 0.6, we obtain a high-accuracy set of colonies that exhibit a fraction of perfect trees > 0.4 (compare high entropy colonies, ‘H MEMOIR’, with low entropy colonies, ‘L MEMOIR’). Note that the threshold is arbitrary and can be tuned to maximize the numbers of colonies and minimize the false discovery rate.
Fig. S9. Additional intMEMOIR arrays increase reconstruction accuracy and depth.

(A) Accuracy in the reconstruction of simulated lineages using increasing numbers of recording units. Parameters were estimated from experimental data. The structure of the lineage trees used in the simulation are those observed experimentally. Using 40 units arranged as 4 intMEMOIR arrays, more than 50% of lineages can be reconstructed perfectly. (B) For a given accuracy (90%), the number of recording units necessary for reconstruction scales with the depth of the lineage. The calculation assumes binary lineages with no cell death.
Fig. S10. Example of stitched microscope images.
(A) Data shown in Figure 4 were derived from four overlapping microscope positions (white squares) that were digitally combined. (B) Microscope images shown in Figure 5 and S11 were similarly derived from five positions (white squares).
Fig. S11. Probing the expression of 8 endogenous genes in an adult *Drosophila* brain section with smFISH.

In addition to the intMEMOIR array, we probed for the expression of 8 endogenous genes in the same brain section: tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), fruitless (Fru), short neuropeptide F precursor (sNPF), glutamic acid decarboxylase (Gad1), vesicular glutamate transporter (VGlut), serotonin transporter (SerT), and tyramine β-hydroxylase (Tbh). Endogenous genes and DAPI signals are shown in magenta and gray, respectively (scale bar, 30 µm).
Fig. S12. intMEMOIR recovers clone sizes *in vivo*.
Cells in the adult *Drosophila* brain section were segmented, their array states determined (see Materials and Methods), and clones with ≥four cells and at least one unit inverted were chosen for downstream analysis. Clones 1, 2, and 3, as shown in Fig. 5E, are highlighted in their corresponding colors.
Fig. S13. Brain B2, a section of D. melanogaster antenna lobe. 

(A) A section of brain B2 stained with DAPI. (B and C) Cells in the same clone were closer in physical space than cells in different clones, as seen on the spatial map (B) and in the cumulative distributions (C). In (B), segmented cells are colored by the analyzed clones. Grey cells were excluded from analysis (see Materials and Methods) (scale bar, 30 µm). (D and E) Cells within a clone were more similar in gene expression space than cells in different clones, as seen on the UMAP (D) and in the cumulative distributions (E). (F) Within a clone, but not between different clones, cell pairs exhibited spatially graded cell type similarity (cf. Figure 5).
**Fig. S14. Brain B3, a section of a D. melanogaster brain.**

(A) A section of brain B3 stained with DAPI. Downstream analyses were done on stitched images from two microscope positions (white squares). (B and C) Cells in the same clone were closer in physical space than cells in different clones, as seen on the spatial map (B) and in the cumulative distributions (C). In (B), segmented cells are colored by the analyzed clones. Grey cells were excluded from analysis (see Materials and Methods) (scale bar, 30 µm). (D and E) Cells within a clone were more similar in gene expression space than cells in different clones, as seen on the UMAP (D) and in the cumulative distributions (E). (F) This brain does not show a spatially graded dependence on cell fate similarity within clones, likely due to its reduced diversity of captured cell types (fig. S17). Cf. Figure 5.
Fig. S15. Brain B4, a section of a D. melanogaster brain.

(A) A section of brain B4 stained with DAPI. Downstream analyses were done on a single microscope position capturing the central brain region (white square). (B and C) Cells in the same clone were closer in physical space than cells in different clones, as seen on the spatial map (B) and in the cumulative distributions (C). In (B), segmented cells are colored by the analyzed clones. Grey cells were excluded from analysis (see Materials and Methods) (scale bar, 30 µm). (D and E) Cells within a clone were more similar in gene expression space than cells in different clones, as seen on the UMAP (D) and in the cumulative distributions (E). (F) Within a clone, but not between different clones, cell pairs exhibited spatially graded cell type similarity (cf. Figure 5).
Fig. S16. Intra-lineage spatial distribution of cells predicts fate similarity in subsamples or with alternative distance metric.

(A) To determine if the relationship observed in Fig. 5M was skewed by the large Kenyon cell clone, we repeated the analysis omitting those cells. The results still showed a strong role for lineage in cell fate determination at close distances. (B) Clonal dependence of cell type similarity at short distances is observed when we use Pearson correlation to measure gene expression similarity, demonstrating that it is robust to the choice of distance metric.
Fig. S17. Brain B3 displays the least diversity within clones.

The Pearson correlation was used to determine the amount of diversity within and between clones in each brain and is shown as a cumulative histogram. Here, the correlation distance is defined as 1 - correlation coefficient. For Brain B3, 95% of cell pairs within the captured clones have a correlation distance of less than 0.06, showing very little diversity within clones. 95% of cell pairs within clones in Brains B1, B2, and B4 have correlation distances of less than 0.84, 0.81, and 0.77, respectively.
Table S1. List of constructs used in the manuscript.

Table S2. Ground truth and reconstructed lineage trees.

Table S3. HCR probe binding regions and information.

Table S4. Automation smFISH probe sequences and information.

Movies S1 to S3. Time-lapse imaging and tracking of intMEM1 cells used for lineage reconstruction in Figure 3, E, F, and G. Cells were imaged as shown in Fig. 3A. Ground truth lineage trees were constructed by manually tracking the cells in the time-lapse images using a modified version of the EasyTrack software developed by Yaron Antebi (freely available at (63) and https://github.com/AntebiLab/EasyTrack/tree/Memoir). Tracked cells and division events are indicated with open and filled squares, respectively. Cells were primarily tracked by their CFP fluorescence. Arbitrary cell numbers in the final frame were assigned for subsequent analysis.
References and Notes


13. H. Zong, J. S. Espinosa, H. H. Su, M. D. Muzumdar, L. Luo, Mosaic analysis with double...


27. J. Bonnet, P. Subsoontorn, D. Endy, Rewritable digital data storage in live cells via


40. M. Iwamoto, T. Björklund, C. Lundberg, D. Kirik, T. J. Wandless, A general chemical

41. Detailed materials and methods are available as supplementary materials.


