

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated
- Clearly defined error bars
State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on [statistics for biologists](#) may be useful.

Software and code

Policy information about [availability of computer code](#)

Data collection

Nikon Ti Elements software was used to capture the microscope images and image data converted in TIFF format.

Data analysis

Computer code is freely downloadable from GitHub, including image processing algorithms and Monte Carlo simulations, at <https://github.com/marcottelab/FluorosequencingImageAnalysis>.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All single molecule imaging datasets and an image processing tutorial are available via Zenodo (10.5281/zenodo.782860).

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences Behavioural & social sciences Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/authors/policies/ReportingSummary-flat.pdf](https://www.nature.com/authors/policies/ReportingSummary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Not Applicable. However for most data presented, the number of image fields were between 50 - 100, as described in each figure text.
Data exclusions	p. 15, lines 1-6; p. 15, lines 27-30
Replication	Yes, all data was replicated multiple times independently. p 13, lines 40-42, All peptide fluorosequencing results were independently confirmed on two separate microscopes by two independent investigators. Replicates are listed in Supp. File 1.
Randomization	Not applicable. No experimental trials requiring randomization were performed
Blinding	Not applicable. Due to the nature of the experiments performed, blinding studies are not applicable, and results were computed algorithmically.

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	<input type="text"/>
Research sample	<input type="text"/>
Sampling strategy	<input type="text"/>
Data collection	<input type="text"/>
Timing	<input type="text"/>
Data exclusions	<input type="text"/>
Non-participation	<input type="text"/>
Randomization	<input type="text"/>

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	<input type="text"/>
Research sample	<input type="text"/>
Sampling strategy	<input type="text"/>
Data collection	<input type="text"/>
Timing and spatial scale	<input type="text"/>
Data exclusions	<input type="text"/>
Reproducibility	<input type="text"/>
Randomization	<input type="text"/>

Blinding

Did the study involve field work? Yes No

Field work, collection and transport

Field conditions

Location

Access and import/export

Disturbance

Reporting for specific materials, systems and methods

Materials & experimental systems

- | | | |
|-------------------------------------|--------------------------|-----------------------------|
| n/a | <input type="checkbox"/> | Included in the study |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Unique biological materials |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Antibodies |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Eukaryotic cell lines |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Palaeontology |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Animals and other organisms |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Human research participants |

Methods

- | | | |
|-------------------------------------|--------------------------|------------------------|
| n/a | <input type="checkbox"/> | Included in the study |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | ChIP-seq |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | Flow cytometry |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> | MRI-based neuroimaging |

Unique biological materials

Policy information about [availability of materials](#)

Obtaining unique materials

Antibodies

Antibodies used

Validation

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

Authentication

Mycoplasma contamination

Commonly misidentified lines
(See [ICLAC](#) register)

Palaeontology

Specimen provenance

Specimen deposition

Dating methods

 Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

Wild animals

Field-collected samples

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics

Recruitment

ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

Files in database submission

Genome browser session

(e.g. [UCSC](#))

Methodology

Replicates

Sequencing depth

Antibodies

Peak calling parameters

Data quality

Software

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Instrument

Software

Cell population abundance

Gating strategy

 Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Design specifications

Behavioral performance measures

Acquisition

Imaging type(s)

Field strength

Sequence & imaging parameters

Area of acquisition

Diffusion MRI

 Used Not used

Preprocessing

Preprocessing software

Normalization

Normalization template

Noise and artifact removal

Volume censoring

Statistical modeling & inference

Model type and settings

Effect(s) tested

Specify type of analysis:

 Whole brain ROI-based BothStatistic type for inference
(See [Eklund et al. 2016](#))

Correction

Models & analysis

n/a | Involved in the study

 Functional and/or effective connectivity Graph analysis Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Graph analysis

Multivariate modeling and predictive analysis