

Reporting Summary

Nature Portfolio 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 Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on 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 <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ 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 statistical parameters 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. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ 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 <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

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

Data collection	The NovaScope pipeline (https://github.com/seqscope/NovaScope ; https://doi.org/10.5281/zenodo.18292675 ; v1.1.0)
Data analysis	The NEDA package (https://github.com/seqscope/NovaScope-exemplary-downstream-analysis ; https://doi.org/10.5281/zenodo.18291721 ; v1.0.0) and Spatula match-tag (https://github.com/seqscope/spatula ; v1.1.0)

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 and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [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 description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The Seq-Scope-X datasets generated from this study were deposited to GEO under accession code GSE316811 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE316811>] and to Deep Blue Data repository [<https://doi.org/10.7302/kj26-7j44>] as raw sequences and spatially annotated digital gene expression (sDGE) matrix. The datasets used for benchmarking and distortion analyses are provided as Supplementary Data 1. Publicly available datasets used in this study include GEO accession codes GSE230402 (Bulk) [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE230402>], GSE165141 (ST) [<https://www.ncbi.nlm.nih.gov/geo/query/>

acc.cgi?acc=GSE165141], GSE192741 (Visium) [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192741], GSE169706 (Seq-ScopeMISEQ) [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169706], and GSE207843 (xDBiT) [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE207843], as well as data available at Deep Blue Data (Seq-ScopeNOVASEQ) [https://doi.org/10.7302/tw62-4f97], LISTA database (Stere-Seq) [https://db.cngb.org/stomics/lista/download/], Broad Single Cell Portal (Slide-Seq) [https://singlecell.broadinstitute.org/single_cell/study/SCP354/slide-seq-study], and MERFISH Mouse Liver Map [REF31: https://info.vizgen.com/mouse-liver-data]. The source image files corresponding to all microscopy figures are available in Figshare [https://doi.org/10.6084/m9.figshare.31093639].

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Human tonsil tissue was obtained from the UCLA Pathology Biobank as a discarded surgical specimen from an adult patient diagnosed with chronic tonsillitis. The sample was fully de-identified prior to acquisition and is classified as IRB-exempt under institutional and federal guidelines. No identifiable patient information, including sex and gender was accessible, and no additional ethical review or consent was required.

Reporting on race, ethnicity, or other socially relevant groupings

Race, ethnicity, and other social groupings were not recorded for this fully de-identified surgical specimen and did not play any role in the study design or analysis.

Population characteristics

One specimen from an adult patient with chronic tonsillitis was analyzed; no additional demographic information was available.

Recruitment

No participants were recruited. Tissue was obtained as a fully de-identified discarded surgical specimen.

Ethics oversight

Use of the de-identified tonsil tissue was classified as IRB-exempt under institutional guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is 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/documents/nr-reporting-summary-flat.pdf

Life sciences study design

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

Sample size

No statistical method was used to predetermine sample size. Sample sizes were chosen based on practical considerations and prior experience with the experimental system.

Data exclusions

No data were excluded from the analyses except through standard quality control procedures for single-cell and spatial data.

Replication

Unless otherwise indicated, experiments were performed once for each application to demonstrate technical feasibility of the method; where indicated, experiments were repeated independently with similar results and representative data are shown.

Randomization

The experiments were not randomized.

Blinding

The investigators were not blinded to allocation during experiments or outcome assessment.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	TotalSeq™-A Human Universal Cocktail (Company: BioLegend, Cat#: 399907, Dilution: 1:3), TotalSeq™-A Mouse Universal Cocktail (Company: BioLegend, Cat#: 199901, Dilution: 1:3), anti-COL1A1 (Company: Cell Signaling, Cat#: 72026, Dilution: 1:300), anti-ACTA1 (Company: Proteintech, Cat#: 17521-1-AP, Dilution: 1:200), anti-p-4E-BP1 (Company: Cell Signaling, Cat#: 2855, Dilution: 1:200), biotinylated anti-rabbit secondary antibody (Company: Vector laboratories, Cat#: BA-1000, Dilution: 1:200), Streptavidin-Horseradish Peroxidase (Company: BD Biosciences, Cat#: 554066, Dilution: 1:300), FITC-labeled CD21-CD35 (Company: BioLegend, Cat#: 123407, Dilution: 1:100), Alexa488-labeled CD4 (Company: BioLegend, Cat#: 100532, Dilution: 1:100), Alexa594-labeled CD8b (Company: BioLegend, Cat#: 126635, Dilution: 1:33).
Validation	All commercially sourced antibodies used in this study were validated by the manufacturers according to their standard quality control procedures, as described on the Cell Signaling Technology and BioLegend websites. In addition, key immune markers, including CD4, CD8b, and CD21-CD35, were independently validated in this study by comparing Seq-Scope-X proteomic signals with corresponding immunofluorescence or immunohistochemical staining on adjacent tissue sections, demonstrating concordant spatial expression patterns (Supplementary Fig. 9d, 9e).

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Mouse tissues, including liver, colon, and spleen, were collected from 8-week-old male <i>Mus musculus</i> C57BL/6J wild-type mice (The Jackson Laboratory), and brain tissue was obtained from a 6-month-old male C57BL/6J wild-type mouse. Liver from 8-week-old Alb-Cre/Tsc1F/F/Depdc5F/F female mice and gastrocnemius (GTN) skeletal muscle from 10-week-old CKM-Cre/Tsc1F/F/Depdc5F/F male mice, both in the C57BL/6J background, were also used. Mice were housed under specific pathogen-free conditions with a 12 h light and 12 h dark cycle, ambient temperature maintained at 22 ± 2 °C, and relative humidity maintained at 40–60%, with food and water provided ad libitum.
Wild animals	N/A
Reporting on sex	Both male and female mice were used, and the sex of each animal is specified.
Field-collected samples	N/A
Ethics oversight	All mouse experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	N/A
Novel plant genotypes	N/A
Authentication	N/A