nature research

Corresponding author(s): Hao Wu

Last updated by author(s): Jun 27, 2020

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.

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 (*n*) 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 *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 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. *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

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	No software was used for data collection.
Data analysis	STAR (version 2.5.2a), sam2tsv (version ec2c2364), Drop-Seq analysis pipeline (version 1.1.2). Seurat (version 2.3.4, 3.0.0 and 3.1.4), dropEst (version 0.8.5), SCENIC (version 1.1.2.2), dynamo (https://github.com/aristoteleo/dynamo-release, commit:9871d78), R (version 3.5.1), R packages: ggplot2 (version 3.3.0), cowplot (version 1.0.0), dplyr (version 0.8.5), tidyr (version 1.0.2), reshape2 (version 1.4.3), pheatmap (version 1.0.12), RColorBrewer (version 1.1.2), MASS (version 7.3.51.5), viridis (version 0.5.1). All scripts for figure generation are available at https://github.com/wulabupenn/scNT-seq.

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. 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

Raw and processed data have been deposited in NCBI Gene Expression Omnibus (GEO) database under accession number GSE141851.

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 procedures were used to select a sample size, but rather, cell numbers typically used in the field were used based on previous studies (Macosko et al, 2015, Cell; Hu et al, 2017, Mol Cell). More than 400 cells were used for two-species mixing experiment. More than 2,000 cells were profiled at each time-point for cortical neuronal culture and mESC pulse-chase experiments. 4,633 WT cells and 2,319 Tet-TKO cells were profiled after 4 hour labeling (from two biological replicates). More than 400 cells were profiled at each of 6 experiments for benchmarking the library complexity of second strand synthesis scNT-Seq protocol.
Data exclusions	Data were not excluded.
Replication	Two biologically independent replicates were included for mESC scNT-Seq experiments (wild-type and Tet-TKO). For mESC pulse-chase experiments, two biologically independent replicates were performed. For other large-scale, high-throughput sequencing datasets, no replication was performed for reasons of cost. Reproducibility of scNT-Seq is accessed in Fig. 5e, Extended Data Fig. 1c and 6b.
Randomization	For estimation the fraction of new transcripts (θ) in each experiment, we randomly sampled 10,000 UMIs to estimate global substitution probabilities p and q based on the binomial mixture model. For PCA analysis of new RNAs, old RNAs, total RNAs and new-to-total RNA ratios, 200 excitatory neurons or non-neuronal cells were randomly sampled from each of two time points. No other randomization strategies were applied.
Blinding	Not applied. Blinding was not relevant since sample identities were encoded into experiment design, and then subjected to scNT-Seq analysis.

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
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

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

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	K562 (ATCC, CCL-243) and wild-type mESC line (J1, ATCC, SCRC-1010) were originally purchased from ATCC. Tet-TKO mESC (J1) line was derived in house by CRISPR-Cas9 genome edited using previously validated sgRNAs (Wang et al, Cell, 2013). The Tet-TKO mESC line was verified as noted below.
Authentication	Genotypes of Tet-TKO mESCs (J1) were verified by both Sanger sequencing and single-cell RNA-seq at Tet1/2/3 loci. The lack of 5-hydroxymethylcytosine (5hmC) was confirmed by mass spectrometry as previously described (Schutsky et al, Nat Biotech, 2018). K562 (ATCC, CCL-243) and wild-type mESC (J1 line, ATCC, SCRC-1010) were originally obtained from ATCC and no additional authentication was performed.
Mycoplasma contamination	Not tested.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Embryonic day 16 (E16) C57BL/6 embryos of mixed sex (Charles River).
Wild animals	This study did not involve wild animals.
Field-collected samples	This study did not involve field-collected samples.
Ethics oversight	Experiments were conducted in accordance with the ethical guidelines of the National Institutes of Health and with the approval of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Animals were solely used for collection of material, and no animal experiments were performed.

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