nature protocols

https://doi.org/10.1038/s41596-025-01248-3

Protocol

Check for updates

Spatially resolved in situ profiling of mRNA life cycle at transcriptome scale in intact cells and tissues using STARmap PLUS, RIBOmap and TEMPOmap

Jingyi Ren[1,2,3], Hu Zeng[1,2,3], Jiahao Huang[1,2,3], Jiakun Tian[1,2], Morgan Wu[2], Hailing Shi[1,2], Xin Sui[1,2], Connie Kangni Wang[1,2], Haowen Zhou[1,2], Zefang Tang[1,2], Shuchen Luo[1,2] & Xiao Wang[1,2]

Abstract

Controlled gene expression programs have a crucial role in shaping cellular functions and activities. At the core of this process lies the RNA life cycle, ensuring protein products are synthesized in the right place at the right time. Here we detail an integrated protocol for imaging-based highly multiplexed in situ profiling of spatial transcriptome using antibody-based protein comapping (STARmap PLUS), spatial translatome mapping (RIBOmap) and spatiotemporal transcriptome mapping (TEMPOmap). These methods selectively convert targeted RNAs, ribosome-bound mRNAs or metabolically labeled RNAs to DNA amplicons with gene-unique barcodes, which are read out through in situ sequencing under a confocal microscope. Compared with other methods, they provide the analytical capacity to track the spatial and temporal dynamics of thousands of RNA species in intact cells and tissues. Our protocol can be readily performed in laboratories experienced in working with RNA and equipped with confocal microscopy instruments. The wet lab experiments in preparing the amplicon library take 2–3 d, followed by variable sequencing times depending on the sample size and target gene number. The spatially resolved single-cell profiles enable downstream analysis, including cell type classification, cell cycle identification and determination of RNA life cycle kinetic parameters through computational analysis guided by the established tutorials. This spatial omics toolkit will help users to better understand spatial and temporal RNA dynamics in heterogeneous cells and tissues.

Key points

- This protocol for highly multiplexed in situ profiling of spatial transcriptome uses STARmap PLUS, RIBOmap or TEMPOmap to selectively convert targeted RNAs, ribosome-bound mRNAs or metabolically labeled RNAs to DNA amplicons with gene-unique barcodes, which are read out through imaging-based in situ sequencing.
- These new methodologies provide the analytical capacity to track the spatial and temporal dynamics of thousands of RNA species and their translational status in intact cells and tissues.

Key references

Zeng, H. et al. *Nat. Neurosci.* **26**, 430–446 (2023): https://doi.org/10.1038/s41593-022-01251-x

Zeng, H. et al. *Science* **380**, eadd3067 (2023): https://doi.org/10.1126/science.add3067

Ren, J. et al. *Nat. Methods* **20**, 695–705 (2023): https://doi.org/10.1038/s41592-023-01829-8

Shi, H. et al. *Nature* **622**, 552–561 (2023): https://doi.org/10.1038/s41586-023-06569-5

Chen, H. et al. *Nat. Biotechnol.* **43**, 194–203 (2025): https://doi.org/10.1038/s41587-024-02174-7

[1]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, USA. [2]Broad Institute of MIT and Harvard, Cambridge, MA, USA. [3]These authors contributed equally: Jingyi Ren, Hu Zeng, Jiahao Huang. e-mail: xwangx@mit.edu

Introduction

Background

Cell state and function are shaped by the spatiotemporal regulation of gene expression. This intricate pattern of expression is, in part, attained through the precise regulation of RNA transcription, transport and translation within individual cells across spatial and temporal dimensions, ensuring protein products are synthesized in the right place at the right time. In the era of single-cell biology, the scientific community has charted single-cell and spatial atlases that unraveled the transcriptome landscape of cells across a variety of tissues and longitudinal time scales, identifying rare cell populations and cell state diversity with high spatial resolution[1,2]. Currently, most spatial RNA and cell profiling methods capture static snapshots of the transcriptome. Yet, RNA transcription is a dynamic process, which may impact how we define and study cell types and states. Therefore, it is critical to methodically study the spatially resolved RNA dynamics at the single-cell and single-molecule levels in cells and intact tissues. Moreover, our comprehension of RNA translational regulation in diverse cellular types and states will be greatly enriched by the examination of the spatially resolved RNA translatome. In addition, a deeper understanding of how intracellular RNA dynamics influences cell–cell interactions and tissue microenvironments opens a wide range of opportunities in investigating disease progression and can inspire new therapeutic modalities.

To fully understand spatiotemporal RNA dynamics and protein synthesis at the genomic scale, new spatial sequencing technologies are required. Several breakthrough spatial transcriptomics technologies[3–10] have achieved imaging-based highly multiplexed in situ RNA detection in single cells and intact tissues, enabling high-throughput molecular profiling of cell types and states with subcellular resolution. However, these techniques solely detect overall RNA expression and copy numbers, falling short of directly capturing RNA dynamics and their controlled translation within subcellular domains. More importantly, despite the current consensus of using mRNA quantification to infer protein expression, previous works have shown only modest correlations of transcriptome and proteome in single cells[11], indicating complex interplay during post-transcriptional and translational controls. On the contrary, the recent single-cell RNA sequencing-based approaches quantitatively track RNA dynamics through integrating with metabolic labeling or measure RNA translation efficiency by sequencing ribosome footprints at the whole transcriptome level[12–14]. However, these methods necessitate tissue lysis and cell dissociation, consequently missing the spatial dimension needed to establish connections in subcellular RNA regulation, cell–cell interaction and tissue organization at large. Thus, there exists a pressing need for a comprehensive tracking of the whole RNA life cycle, including key stages such as synthesis, transport, translation and degradation at the transcriptome level for a holistic understanding of RNA-regulated cellular function and tissue physiology. Here, we describe STARmap (spatially resolved transcript amplicon readout mapping) with protein localization and unlimited sequencing (STARmap PLUS)[15], ribosome-bound mRNA mapping (RIBOmap)[16] and temporally resolved in situ sequencing and mapping (TEMPOmap)[17], providing detailed experimental procedures and computational pipelines, starting with highly-multiplexed gene-targeting probe design, DNA library preparation and in situ sequencing and imaging, followed by image preprocessing and downstream data analysis (Fig. 1).

Overview of the protocol

Here, we introduce three image-based in situ sequencing methodologies that spatially resolve: (1) metabolically labeled nascent RNA dynamics (TEMPOmap), (2) ribosome-bound mRNA (RIBOmap) and (3) steady-state RNA with protein detection (STARmap PLUS), respectively. Utilizing different, technique-specific proximity-based oligonucleotide probe designs (Fig. 2 and 'Splint probe design' section), the three methods enable selective amplification of the RNAs with properties of interest for optical detection, as detailed below.

STARmap PLUS is a method that integrates a previously developed targeted in situ RNA sequencing approach called STARmap[10] with antibody-based protein detection and improved

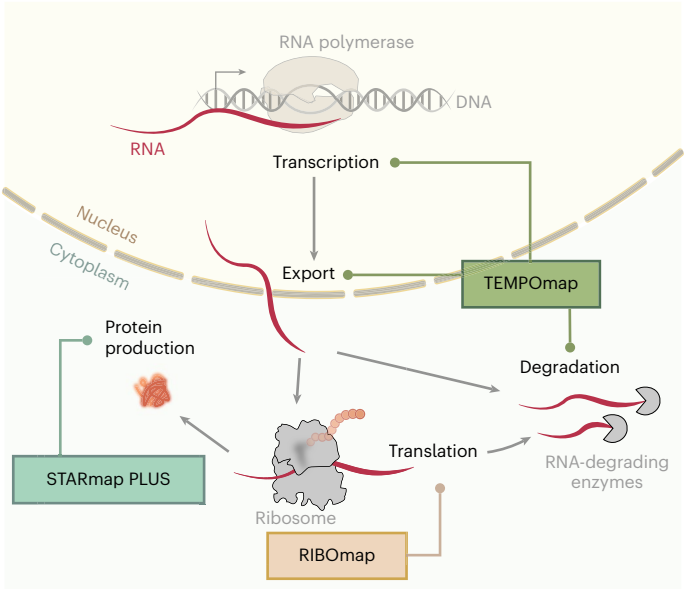


Fig. 1 | Overview of spatial omics technologies that track the RNA life cycle. The key events during the RNA life cycle include RNA transcription, nuclear export, translation and degradation. These events in their native spatial context in single cells could be individually tracked and quantified by TEMPOmap and RIBOmap with highly multiplexed, near-transcriptome scale. In addition, the protein localization and the transcriptome snapshots can be mapped by STARmap PLUS.

probe design, achieving simultaneous detection of the transcriptome and a limited number of proteins in the same tissue slice, with the number of detectable proteins constrained by the number of spectrally distinct imaging channels available in the microscopy setup. STARmap PLUS utilizes a di-probe strategy to detect and amplify mRNAs of interest (Fig. 2a): (1) a padlock probe targets specific mRNA species of interest and encodes a gene-specific barcode and (2) a primer probe targets the adjacent site to the one targeted by the padlock probe. The primer serves as the template for the ligation to circularize the padlock probe as well as the primer for rolling circle amplification (RCA) to generate a DNA nanoball (amplicon) that contains multiple copies of the cDNA complementary to padlock sequences.

RIBOmap and TEMPOmap are built upon a tri-probe design strategy to selectively detect and amplify ribosome-bound mRNAs or metabolically labeled RNAs, respectively (Fig. 2b): (1) a splint DNA probe hybridizes to ribosomal RNAs (rRNAs) in RIBOmap or is covalently attached with the 5-ethynyl uridine-labeled RNAs in TEMPOmap and serves as a template for proximity ligation to circularize the adjacent padlock probe; (2) a padlock probe targets mRNA molecules of interest and encodes a gene-specific barcode; and (3) a primer probe targets the adjacent site on the same RNA to serve as a primer for RCA to generate an amplicon.

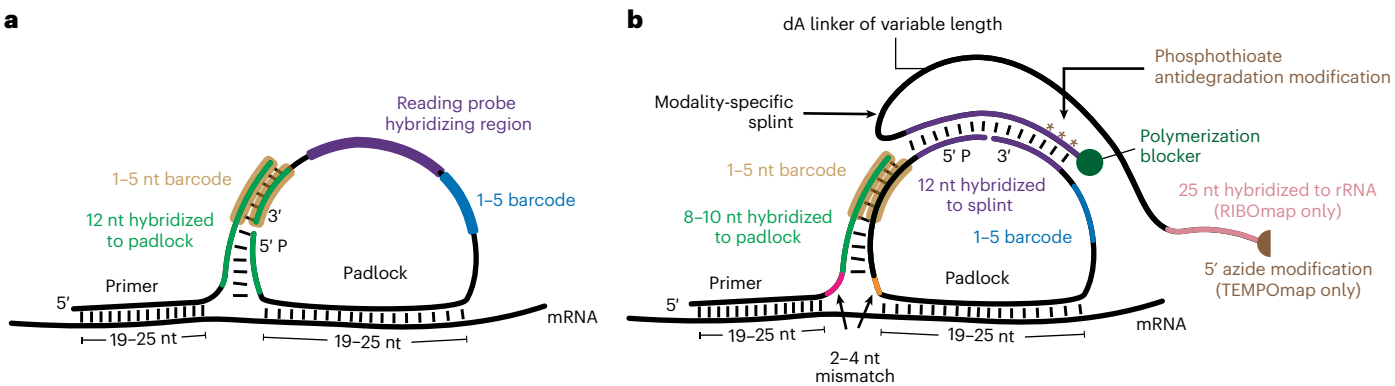


Fig. 2 | Probe design. a,**b**, Illustrations of probe design for the di-probe strategy for STARmap and STARmap PLUS (**a**) and the tri-probe strategy for RIBOmap and TEMPOmap (**b**). 5′ P, phosphate modification at the 5′ end of the probe.

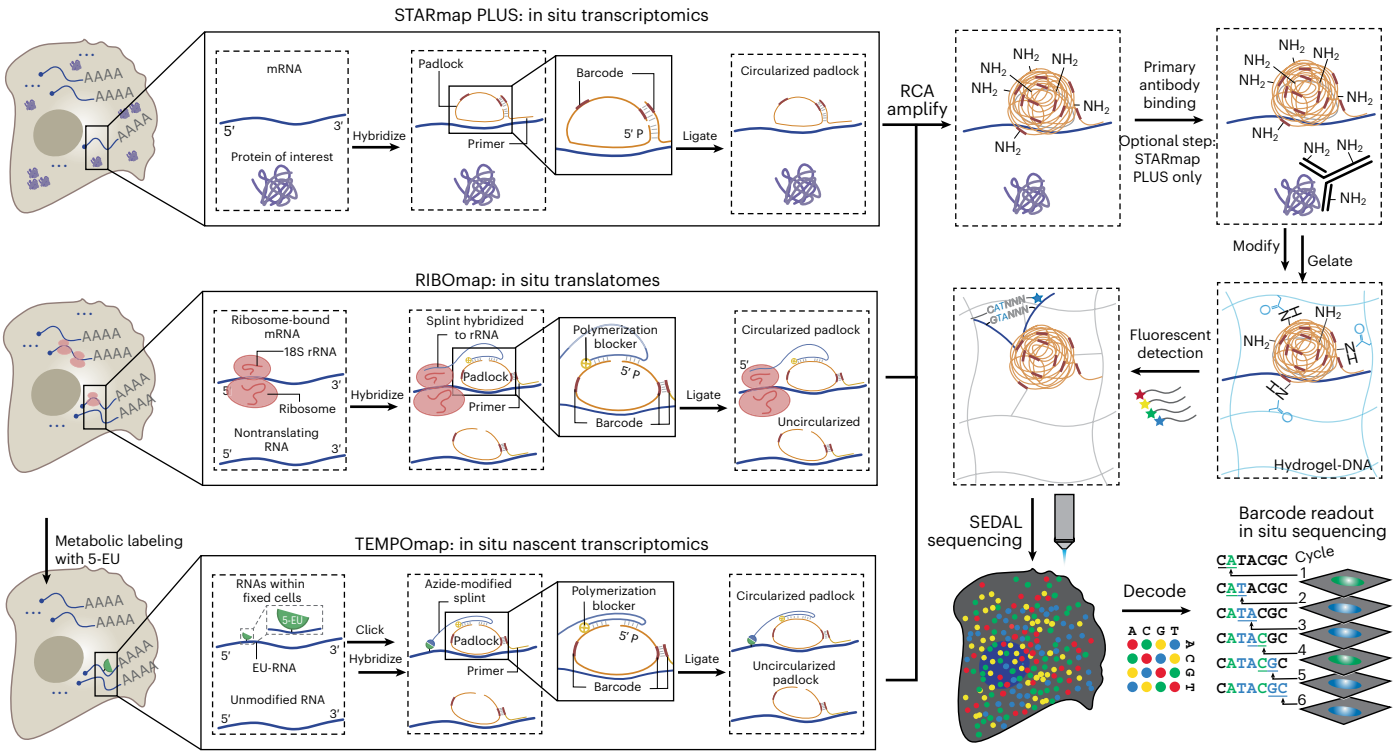


Fig. 3 | Schematic diagrams of STARmap PLUS, RIBOmap and TEMPOmap. After the cells or tissues are fixed, a paired barcoded padlock and primer probe hybridize to a targeted intracellular RNA. Meanwhile, the splint probe is hybridized to the rRNA of ribosomes (in the case of RIBOmap) or conjugated to metabolically labeled nascent RNA through click chemistry (in the case of TEMPOmap). The splint probe is used as a template for the proximity ligation to circularize the padlock probe. The circularized padlock probe is amplified to generate amine-modified DNA amplicons in situ, leaving the uncircularized probe behind. Next, these DNA amplicons are crosslinked into hydrogel for fluorescent detection and (or) in situ SEDAL sequencing. The gene-unique barcode sequence (red) in the cDNA amplicons (yellow) is read out by cyclic in situ sequencing (detailed in Fig. 4). Note that RCA amplification, gelation, fluorescent detection and SEDAL sequencing apply to all three methods. The primary antibody binding only applies to STARmap PLUS (therefore skipped for RIBOmap and TEMPOmap). 5′ P, phosphate modification at the 5′ end of the probe. The figure was adapted from ref. 17, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

Despite serving different purposes, the three methods share some common technical procedures to provide single-molecule and single-cell resolved readout. The experimental procedures include RNA targeting by technique-specific oligo probe sets (Steps 15–20), target-specific amplification of padlock probes to generate a DNA library (Steps 21–27), followed by in situ sequencing (Steps 50–61) (see Fig. 3 and 'Experimental design' section for details). A key step in DNA library preparation is hydrogel-amplicon crosslinking. The DNA amplicons are covalently embedded into a tissue hydrogel for sample mechanical stability and optical transparency (Steps 31–41) when combined with tissue clearing (Steps 42–43). This is achieved by incorporating primary amine groups into DNA amplicons via an aminoallyl-dUTP spike-in in the RCA reaction. The DNA amplicons are then modified by methacrylic acid *N*-hydroxysuccinimide esters (MA-NHS) and copolymerized with acrylamide and bis-acrylamide monomers to form a hydrogel-DNA amplicon network. Finally, for protein comapping in STARmap PLUS, primary antibody incubation can be performed after the RCA step and before the MA-NHS reaction, resulting in the primary antibodies being also chemically modified and copolymerized in the hydrogel matrix (Steps 28–30).

For gene-specific identification, a unique barcode sequence is designed on each padlock probe (hereby referred to as a 'barcode'). This results in a 'barcode-gene' codebook, a list of barcode-to-gene correspondences, which can be used for later decoding (converting the imaged barcodes back to gene identity for gene expression analysis). After RCA, hydrogel embedding and dephosphorylation, the barcode region in the DNA amplicon is read out

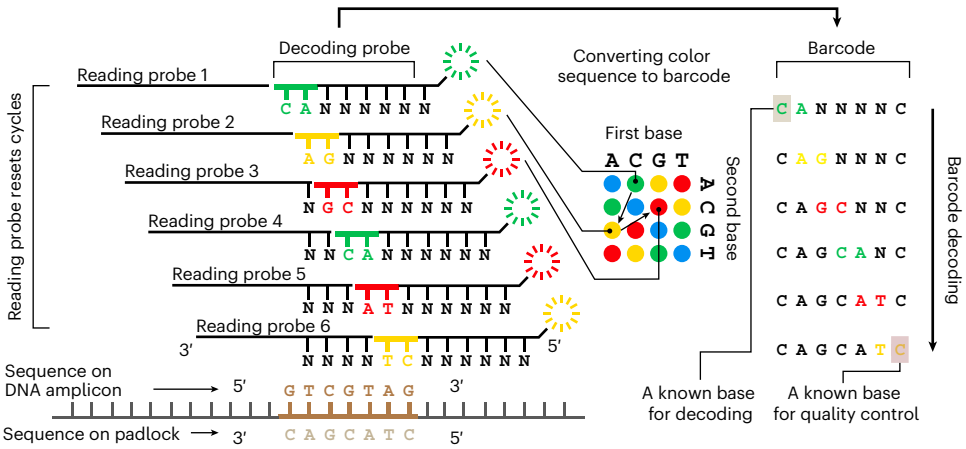


Fig. 4 | Schematic overview of the SEDAL color-coding and decoding principle. The number of sequencing cycles depends on the total number of genes that are targeted. For example, six rounds of sequencing can decode a five-base barcode (hereby profiling up to 1,024 genes (4^5)). As the sequencing cycle progresses, the reading probe contains an increasing length of degenerate bases (*N* representing an equal molar mixture of A, T, C and G), with phosphate at the 5′ end to set the starting position. A mixture of 16 fluorophore-conjugated decoding probes color-codes all 16 combinations of dinucleotide at the 3′ end. A successful ligation of reading probe and decoding probe happens only when both probes are perfectly complementary to the DNA amplicon sequences, enabling each amplicon to be detected by distinct fluorescence with confocal microscopy. After each imaging cycle, the ligated probes are stripped away to reset for sequencing with a new reading probe. The barcode of each amplicon can then be decoded from sequential rounds of colors. As a quality control for decoding barcodes, a known base should be used upstream and downstream of the varying region of the barcode, respectively.

through in situ sequencing with error reduction by dynamic annealing and ligation (SEDAL)[10]. In each cycle of SEDAL (Step 50), T4 DNA ligase enzymatically conjugates two short, degenerate probes using the DNA amplicon as the template (Fig. 4): a reading probe (11-nt long) that is hybridized to the common sequence in all amplicons and sets the base positions to be sequenced and a mix of 16 fluorescent decoding probes (8-nt long) corresponding to all possibilities of dinucleotide base combinations to decode the two bases downstream on the amplicon binding site (Fig. 4). This room-temperature enzymatic step and the following washing steps can be directly performed on the sample plate on the microscope stage without taking it off to minimize the movement of the imaged plate. The details of imaging setup are covered in Steps 54–59. After imaging, the ligated fluorescent probes (19-nt long) are stripped from the amplicons using a formamide-based stripping buffer (see 'Reagent setup' section). The SEDAL procedure is then repeated by adding a new reading probe binding one-base downstream on the amplicon, therefore sequencing the two bases one-base downstream in the barcode region. For a 1,000-gene experiment, this SEDAL cycle is repeated up to five times as needed to cover every sequential dinucleotide in the barcode region, followed by one last round of sequencing for quality control purposes. For STARmap PLUS, fluorescent secondary antibodies or small-molecule dyes are applied to read out the protein signal in the same imaging regions after all rounds of in situ sequencing are completed (Steps 62–63).

The raw images are first processed with image deconvolution to reduce the background noise (Steps 64–66). Image clarity is then enhanced by intensity normalization and histogram equalization across the channels and imaging rounds based on the assigned reference channel and round (by default, the first sequencing round). To adjust for any shifts and distortions that occurred during imaging sessions and accurately and robustly identify the barcode of each cDNA amplicon, we perform a global registration and a nonrigid registration to align the positions of the same amplicon across each round of sequencing using first-round images as reference (Step 74–75). After registration, individual amplicons are identified in the images of the first round of sequencing, and the colors of each amplicon across all registered rounds are determined. The color sequence is then converted to the gene-unique barcode following

a two-base decoding scheme (Fig. 4) and then to gene identity according to the barcode-gene codebook. Both the physical locations and gene identities of the amplicons are utilized for downstream analyses, including cell segmentation, cell clustering and spatial gene expression analysis.

Comparison with other methods

Spatial transcriptomic methods can be broadly divided into imaging-based and sequencing-based methods, with a detailed comparison to be found elsewhere[18]. In brief, imaging-based methods such as STARmap PLUS, RIBOmap and TEMPOmap enable highly multiplexed profiling of RNA copy numbers, translation status and transcriptional timestamp at subcellular resolution.

STARmap PLUS has differences as well as advantages compared with other methods that spatially profile transcriptome-scale RNA expression with low-plex protein detection[19–21]. Three of these methods are sequencing-based, and they provide spatial transcriptome sequencing in an untargeted manner and enable the detection of multiple proteins through sequencing oligo-barcoded antibodies[19,20] or a combination with direct immunofluorescence[21]. Yet, the spatial resolution of sequencing-based approaches remains inherently limited (near to single-cell resolution). On the other hand, PLAYR[22] enables imaging-based comapping of RNA and proteins, though it is limited in gene multiplexity (detecting ~10–20 RNA species). STARmap PLUS offers robust single-cell and subcellular RNA profiling on a near-transcriptome scale, with the added capacity and potential for multiplexed protein detection through in situ sequencing of oligo-indexed proteins.

Methods with purposes similar to RIBOmap can be categorized into single-cell sequencing-based ribosome profiling or imaging-based in situ detection of translation events. Single-cell sequencing-based methods such as scRIBO-seq[13] and Ribo-ITP[14] can unbiasedly sequence the whole transcriptome in a nontargeted manner and resolve subcodon information in ribosome and polysome footprints. However, they need to isolate intact single cells for subsequent sequencing, and thus, the spatial resolution is lost. Furthermore, it remains technically challenging to isolate intact single cells without altering cellular translation state or losing cellular fragments for different tissues, such as the brain with interwoven synaptic networks. On the contrary, RIBOmap intrinsically provides single-cell detection of highly multiplexed ribosome-bound RNA species together with the dimension of spatial resolution, yet it lacks subcodon information of ribosome footprints. Therefore, RIBOmap is a promising tool that provides the spatial information complementary to the current single-cell sequencing methods. Other previously published imaging-based detection methods, including FLARIM[23] and FUNCAT[24], are limited in multiplexing capabilities.

Methods with purposes similar to TEMPOmap are mainly single-cell sequencing-based methods integrated with various RNA metabolic labels, including scSLAM-seq[25], scEU-seq[12], sci-fate[26], NASC-seq[27] and scNT-seq[28]. The major advantage of metabolic labeling and sequencing is that these methods are useful for identifying newly transcribed RNAs on a transcriptome scale in an unbiased manner. By contrast, TEMPOmap requires a curated panel of genes for profiling but in addition provides the first spatial and temporal RNA profiling methodology, tracking the dynamics of RNA at the subcellular resolution. Thus, these different techniques answer different questions and are all important in combination. However, two major limitations of all the methods involving metabolic labeling, including TEMPOmap, are that these experiments are generally performed within in vitro cell culture models and may be biased toward RNA enriched with metabolically labeled bases.

Scope of method applications

STARmap PLUS and RIBOmap can be readily applied to human cell lines and a variety of fresh-frozen mammalian tissues (such as tissues from mouse, marmoset and human). The two methods have been previously demonstrated in brain[15,29,30], liver[31], lymph nodes[32] and placenta tissues[33], as well as in vitro models such as organoids[34]. In addition, we expect the methods will be broadened to detecting transcriptomes in thin slices (~20 µm) from other tissue types, including human samples. Future development of these methods may also create opportunities in processing thicker tissues[35] and formalin-fixed paraffin-embedded (FFPE) samples.

Given the necessity of the nucleotide-analog labeling step, TEMPOmap can be readily applied to various cell lines, including primary cells. Although challenges remain to implement this method in vivo for detecting nascent transcripts in intact tissues, we expect that TEMPOmap has great potential to profile RNA dynamics in metabolically active tissue types (such as in liver) given the existing success of in vivo metabolic RNA labeling[36].

To ensure that the experiments work in the cell or tissue type of interest, we strongly suggest an initial test detecting highly abundant and/or subcellularly localized RNAs as positive controls. In the 'Reagent setup' section, we include the primer and padlock probe sequences for di-probe and tri-probe strategies to simultaneously detect RNAs of four genes (*ACTB*, *MALAT1*, *vtRNA1-1* and *GAPDH*).

Limitations

Since all three spatial RNA mapping techniques are targeted approaches, they require a predefined list of gene targets. Such targeted profiling may be subject to biases due to various choices in gene selection. On the flip side, the targeted approach eliminates the concerns from overabundance of housekeeping RNA species and can be a major advantage for profiling transcriptomes of a focused gene panel, especially when informed by gene expression data from other single-cell RNA sequencing studies.

The second shared limitation is that, since the gene readout solely relies on the identification of the corresponding barcode, the data output does not provide single-nucleotide resolution. Thus, unlike next-generation RNA sequencing, these approaches do not offer robust identification of single-nucleotide polymorphisms and sequence variations necessary for evolutionary and lineage tracing studies. Nevertheless, depending on the purpose of the experiment (for example, profiling RNA splicing variants), in theory, the primer and padlock probes can be designed to specifically target RNA loci of interest, and variations in one loci can be differentiated using distinct barcodes[37]. Similarly, one intrinsic limitation of RIBOmap is that the gene readout does not provide the sequences of the ribosome footprints with subcodon resolution.

The third limitation is that different methods need to be performed on adjacent tissue slices (rather than the same slice) when utilizing those approaches for multimodality studies. Yet, single-cell correspondence across tissues is challenging, which complicates the multiomic analyses. For example, when estimating translational efficiency of different cell types on brain slices, it remains challenging to compare RIBOmap and STARmap data at the single-cell resolution across two tissue slices. Nevertheless, this issue will potentially be addressed with the development of new computational methods on the horizon to robustly align single cells across batches and data modalities[38].

STARmap PLUS in its current form is limited in the multiplexity of protein detection, as the workflow relies on usage of primary antibodies and fluorescent secondary antibodies. This limitation can be improved in the future by integrating STARmap with highly multiplexed protein detection methodologies (for example, CODEX[39]). It is also to be noted that the detection efficiency of TEMPOmap may have sequence biases for U-rich sequences since it requires the labeling of U analogs with click-chemistry moiety to generate amplicons from nascent RNAs.

At a practical level, all three methods require a confocal microscope for high-resolution, five-color imaging (four colors for sequencing and one color for nuclei), potentially incurring high expenses of initial setup (also detailed in the following paragraph). To ensure uninterrupted sequencing and accurate registration across sequencing cycles, the majority of users may find it necessary to book the microscope for a continuous block. For example, using a typical point-scanning confocal microscope, acquisition of one round of imaging for profiling a 10-µm mouse brain hemisphere coronal tissue section takes ~15 min per square millimeter per cycle with 140 nm × 140 nm × 350 nm voxel resolution in two sequential scans (see 'Imaging and SEDAL sequencing' section for details). The imaging speed can be substantially improved by using faster optics, such as spinning-disk confocal microscopes (around tenfold faster) and light-sheet microscopy.

Lastly, the cost of setting up these experiments can vary depending on the size of gene libraries and the volume of samples used. Besides the cost of imaging and computation, a major expense is associated with the primer and padlock probes required for highly multiplexed gene detection. When ordered as pooled probes, these probes are produced and sold in batches and can be used for 15–30 experiments in a 12-well-plate setup (that is, an imaging area of 12–18 mm in diameter). We typically request quotes from Integrated DNA Technology (IDT) oPools service, but the cost of probes can vary depending on the synthesis scale and manufacturer. For accurate pricing, please consult the specific manufacturers for pooled oligo quotes.

Experimental design

Gene panel selection

Owing to the nature of targeted profiling, gene panel selection is one of the most important steps that determines the success of an imaging-based spatial profiling experiment. Here, we provide some general criteria for selecting genes for a high-plex profiling. Please note that, depending on the tissue sample and the purpose of the experiment, the criteria can be fine-tuned.

(1) We recommend starting with the genes tailored to specific biological questions or tissue types. A general guidance is to exclude housekeeping genes with above 2,000 transcripts per million from bulk RNA sequencing (RNA-seq) data to avoid abundant genes that may compromise the detection efficiency of other genes expressed at lower levels. Nevertheless, abundant genes that are biologically relevant can be included.

(2) In parallel to point 1, existing scRNA-seq, scNuc-seq and bulk-RNA-seq data as reference datasets can be informative for gene selection, particularly in the case of complex cellular types and heterogeneity. Here are some criteria to consider:
   (a) Cell-type-specific expression: choose genes that are specifically expressed in cell types of interest within the tissue (for example, top-ranked most variable genes). scRNA-seq can identify marker genes that are characteristic of particular cell populations, cell states or regulatory processes, which are essential for cell-type-specific identification and analyses in spatial transcriptomics.
   (b) Differential expression (expression consistency and variability): focus on genes that show differential expression across conditions or time points in scRNA-seq datasets. These genes can provide insights into dynamic biological processes or distinguishable cell type markers.
   (c) Biological relevance and functional annotations: consider selecting genes based on previous gene ontology analyses.
   (d) For cell cultures, we recommend to select transcripts that have diverse subcellular locations[40] and (or) can be cell-cycle markers[41].

(3) Besides traditional gene selection and manual curation, advanced gene-panel selection methods (data-driven, unsupervised) are being actively developed[42] and can be explored.

Padlock and primer probe design

The schematics for padlock and primer probe design are shown in Fig. 2. Please note that the primer design is applicable to all technologies, whereas the padlock design varies by method. In short, the primer probe sequence is composed of (1) a 19–25-nt gene-targeting region (more details described below) and (2) a 12-nt sequence (containing a 1–5-nt gene-specific barcode) that hybridizes to its paired padlock probe. For STARmap and STARmap PLUS, a padlock probe sequence is composed of (from 5′ to 3′, Fig. 2a) (1) a 5′ phosphate; (2) 6 nt at the 5′ end and 6 nt at the 3′ end, complementary to its paired primer (containing 1–5-nt gene-specific barcode if needed); (3) a 19–25-nt gene-targeting region; (4) a 1–5-nt gene-specific barcode; (5) a 12-nt reading probe-hybridizing region (see the 'Reagent setup' section for the reading probe sequence). For RIBOmap and TEMPOmap, padlock probe sequence is composed of (from 5′ to 3′) (Fig. 2b) (1) a 5′ phosphate; (2) 8–10 nt complementary to its matched primer (containing 1–5-nt gene-specific barcode if needed) with a 2–4 nt mismatch; (3) a 19–25-nt gene-targeting region; (4) a 1–5-nt barcode; (5) 12 nt for both reading probe-hybridizing and splint-hybridizing region (see 'Reagent setup' section for splint probe sequence and see below for information on design).

The padlock and primer probes target genes of interest and their specificity is essential for the reliability of STARmap PLUS, RIBOmap and TEMPOmap (Fig. 2a,b). The design of the hybridization regions of the padlock and primer probes for these three targeted spatial technologies is similar, which contains the following steps: (1) extracting the isoforms of each gene from the entire transcriptome and consensus coding sequence databases of the target species; (2) designing 40–46-nt-long hybridization sequences for each padlock-primer pair with specific constraints (GC%, melting temperature (T_m) ranges and so on) using Picky 2.2 software[43]. Here, we recommended the range of GC% to be between 30% and 70% and the T_m between 56 °C and 70 °C at oligo concentration of 5 nM and at salt concentration of 300 mM. Moreover, the preselected primer and padlock hybridization regions for human and mouse transcriptomes are included in Supplementary Tables 1 and 2, respectively. (3) Dividing the resulting hybridization sequences into two segments, each ranging from 19–25 nt in length, with an interspace of 1–2 nt, while ensuring the optimal matching of T_m between these segments and (4) eliminating sequences with overlaps and containments using the Dedupe tool from the BBmap aligner[44], ultimately generating the definitive sequence pool for the target species.

We design gene-specific barcodes using a combinatorial approach based on the four nucleotides (A, T, C and G). For example, using a sequence of 4 nt in length can provide 4^4 (=256) unique barcode sequences meaning we can profile 256 genes, while a 5-nt barcode enable us to profile 1,024 genes (4^5). When the barcodes exceed 5 nt (to enable more than 1,024 genes to be profiled), we recommend designing the sequence so that it can be read in two parts, with each being sequenced separately by orthogonal reading probes (SeqA and SeqB) (see 'Reagent setup' section for the reading probes). The barcode length solely depends on the number of genes each researcher wants to profile for their project. It is worth pointing out that barcode sequences that produce the same fluorescent color during each imaging round (Fig. 4) or contain dinucleotide repeats should be avoided when possible (for instance, C<u>CCCCC</u>C, C<u>ACACA</u>C, C<u>GCGCG</u>C, C<u>TCTCT</u>C, barcode sequence underlined).

After generating the full probe sequence library, we recommend filtering the probes that form strong secondary structures or interactions with other probes. Strong secondary structures as well as probe heterodimers may decrease the efficiency of the probes or generate spurious signals. We use NUPACK[45] to predict secondary structures and filter out probes with strong secondary structures. We perform BLAST[46] on each probe against the minus strand of the whole probe library to search for and filter out probe heterodimers. Typically, 10–20% of probes are filtered out by this pipeline. Therefore, we recommend designing more probes per gene (ideally more than six probe pairs) to ensure that each gene has enough probes after probe filtration. Typically three to six pairs of primer and padlock probes are suggested for one RNA transcript to balance budgetary concerns, yet there is no limit of the maximal number of probe pairs, and the detection sensitivity increases as the number of probes per gene increases.

Splint probe design

The RIBOmap splint probe comprises three distinct segments (Fig. 2b): (1) a 25-nt sequence at the 5′ terminus, which hybridizes with 18S ribosomal RNA (rRNA), (2) a central 50- or 10-nt section composed of deoxyadenosine nucleotides (dA) and (3) a 12-nucleotide splint-padlock annealing sequence at the 3′ end. We found that, in some cases, the splint probe with a 10-nt dA linker offers better specificity than the one with a 50-nt dA linker in some cases due to its shorter proximity-ligating radius (unpublished data, J.T.). We chose areas within the 18S rRNA that exhibit relatively weak secondary structures and high accessibility as the splint probe hybridization region[23]. However, if the users intend to exclude the reads from 43S translation initiation complexes, we also included the splint probes targeting 28S rRNA, which may better represent translating ribosomes. The 3′ end of the splint probe is modified with three consecutive phosphorothioates for enhanced stability and a 3′ terminal inverted dT (invdT) to obstruct it from serving as a primer in the downstream RCA.

The TEMPOmap splint probe contains a 5′ azide modification followed by two regions: a linker containing 50-nt dA connected to a 12-nt splint-padlock annealing sequence. Similar to the RIBOmap splint, the TEMPOmap splint contains phosphorothioate modifications on the last three nucleotides at the 3′-end of the oligo and a 3′ terminal invdT.

Preparation of cell and tissue samples

We have applied STARmap PLUS and RIBOmap to both cell line and tissue samples. For TEMPOmap, we have applied it to a wide variety of cell line samples (in this protocol, we use HeLa cells as an example), while application of this method to tissue samples may be limited by the low efficiency and defined temporal resolution of in vivo metabolic labeling. For high-resolution imaging, we use poly-D-lysine-coated glass-bottom plates (#1.5 cover glass). The fixation step is similar for cell line and tissue sample except that we use a higher paraformaldehyde (PFA) concentration for tissue sample. After fixation, the sample is permeabilized with cold methanol at −20 °C or −80 °C or Triton in 1× phosphate-buffered saline (PBS) when preservation of fluorescence protein signal is desired.

In situ amplification

We use a pair of padlock probe and primer probe to target mRNAs of interest, and the padlock probe contains a gene specific barcode. After circularization of the padlock probe via ligation, the primer uses the circularized padlock probe as template for RCA to convert mRNA into DNA amplicon with gene-unique barcodes. Figure 3 provides the schematic diagram of the experimental workflows for preparing DNA library and the subsequent in situ sequencing for all three methodologies.

Dephosphorylation

We use a sequencing-by-ligation method for in situ sequencing. To minimize background fluorescence caused by nonspecific ligation of fluorescent decoding probes, we perform dephosphorylation before the sequencing steps to remove endogenous phosphate groups in the sample, especially in fragmented genomic DNA.

SEDAL sequencing

We generally use Leica TCS SP8 or Stellaris 8 (with minimal in-house engineering) point-scanning confocal microscopes for image acquisition in the lab. However, other confocal microscopes can potentially be used for the same purposes, such as spinning-disk confocal microscopes (see 'Equipment' section for details). Notably, we have observed a ~10× enhancement in imaging speed when utilizing spinning-disk confocal microscopy compared with its laser-scanning counterpart while still maintaining voxel resolution, expediting data acquisition when a large imaging area is required.

In our experimental setup, we recommend the plate is taped onto the microscope stage. The implementation of an automated stage with adaptive focus control functionality of the Leica microscope is critical, ensuring a consistent focal plane throughout the imaging process and permitting precise z-stack acquisition across multiple x–y tiles and across imaging cycles. Such consistent z-stack acquisition is important for image registration ('Image processing' section) and thus gene identity decoding. Without adaptive focus control, deviations in the z-plane between rounds would lead to insufficient number of overlapping z-steps among sequencing rounds, rendering fewer decodable amplicons and the comparisons and analysis of acquired data inaccurate and challenging.

Image deconvolution

We use three-dimensional (3D) deconvolution to further minimize out-of-focus background and enhance the accuracy of read calling in the imaging data of in situ sequencing. Since high-quality 3D deconvolution demands additional computational iterations, a long duration ranging from few hours to days depending on the number of sequencing rounds and fields of view (FOVs) (that is, ~8 h for a dataset with six rounds of sequencing and 100 FOVs) of the deconvolution process should be expected. The microscopic parameters (that is, aperture and channel emission wavelength) during image acquisition should be noted. Follow the guidance provided by your chosen deconvolution software for optimizing image quality. Specifically, if using Huygens deconvolution software, we suggest optimizing the 'signal-to-noise ratio' value with a subset of images. Moreover, using a measured point spread

function specific to the microscopy system, rather than a computed theoretical point spread function, provides better results in the deconvolution process.

Image registration, reads calling and cell segmentation

Upon desired deconvolution outcome, we suggest arranging the image files by sequencing round label and FOV ID, as detailed in the 'Procedure' section. We developed a software package, Starfinder (https://github.com/wanglab-broad/starfinder), to correct chromatic aberrations, enhance signals, register images and extract reads (that is, amplicons). The output files contain detailed single-molecule information, including spatial coordinates (x, y and z, in the unit of pixel), nucleotide barcodes (or 'gene-unique identifier') and gene identities. Cell segmentation can be conducted using various techniques or software, including Distance Transformed Watershed, StarDist[47], CellProfiler[48] and ClusterMap[33]. It's crucial to fine-tune segmentation processes based on visual inspection and preliminary cell typing for different tissue types and cell populations, ensuring the generation of high-quality expression profiles. To aid in troubleshooting, we provide examples of typical data outputs and screen logs related to our bioinformatics software ('Data availability' section).

Data analysis

Our software produces comma-delimited text files with single-cell level reads quantification. For effective preprocessing, dimensionality reduction, cell clustering and differential gene expression analysis, we recommend using Seurat[49], Scanpy[50] or Squidpy[51] with the latest version. We provide a sample Jupyter notebook as an introduction to the downstream analysis (https://github.com/wanglab-broad/starfinder/tree/main/example/downstream).

A complete overview of experimental and computational procedures can also be found in Fig. 5.

Materials

Biological materials

- HeLa cells (ATCC Cat. no. CCL-2, RRID:CVCL_0030) or any other adhesive cells of interest, growing in culture
  ▲ **CAUTION** The cell lines used in your research should be regularly tested for mycoplasma contamination and authenticity.
- Mice (The Jackson Laboratory, cat. no. C57BL/6) or any other animals of interest
  ▲ **CAUTION** Any experiments involving live mice must conform to relevant Institutional and National regulations. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Broad Institute of MIT and Harvard. From our experience, IACUC procedure approval takes ~4 months.

Reagents

- IDTE buffer pH 7.5 (IDT, cat. no. 11-01-02-02)
- Tissue-Tek OCT compound (SAKURA, cat. no. 4583)
- 3-(Trimethoxysilyl) propyl methacrylate (Bind-Silane) (Sigma, cat. no. M6514)
  ▲ **CAUTION** Bind-Silane is an irritant to eyes, respiratory systems and skin. Use appropriate personal protective equipment (PPE) and adequate ventilation when handling the chemical. Store in a cool, dry place.
- Acetic acid (Millipore Sigma, cat. no. 1000631011)
- Poly-D-lysine (Sigma-Aldrich, cat. no. A-003-M)
- 16% PFA (Electron Microscope Sciences, cat. no. 15710-S)
  ▲ **CAUTION** PFA is an irritant to eyes, respiratory systems and skin. It is also toxic when ingested and can be harmful if inhaled. Use appropriate PPE and adequate ventilation. Store in a cool, dry place or according to the manufacturer's instructions.
- Methanol (Sigma-Aldrich, cat. no. 34860-1L-R)

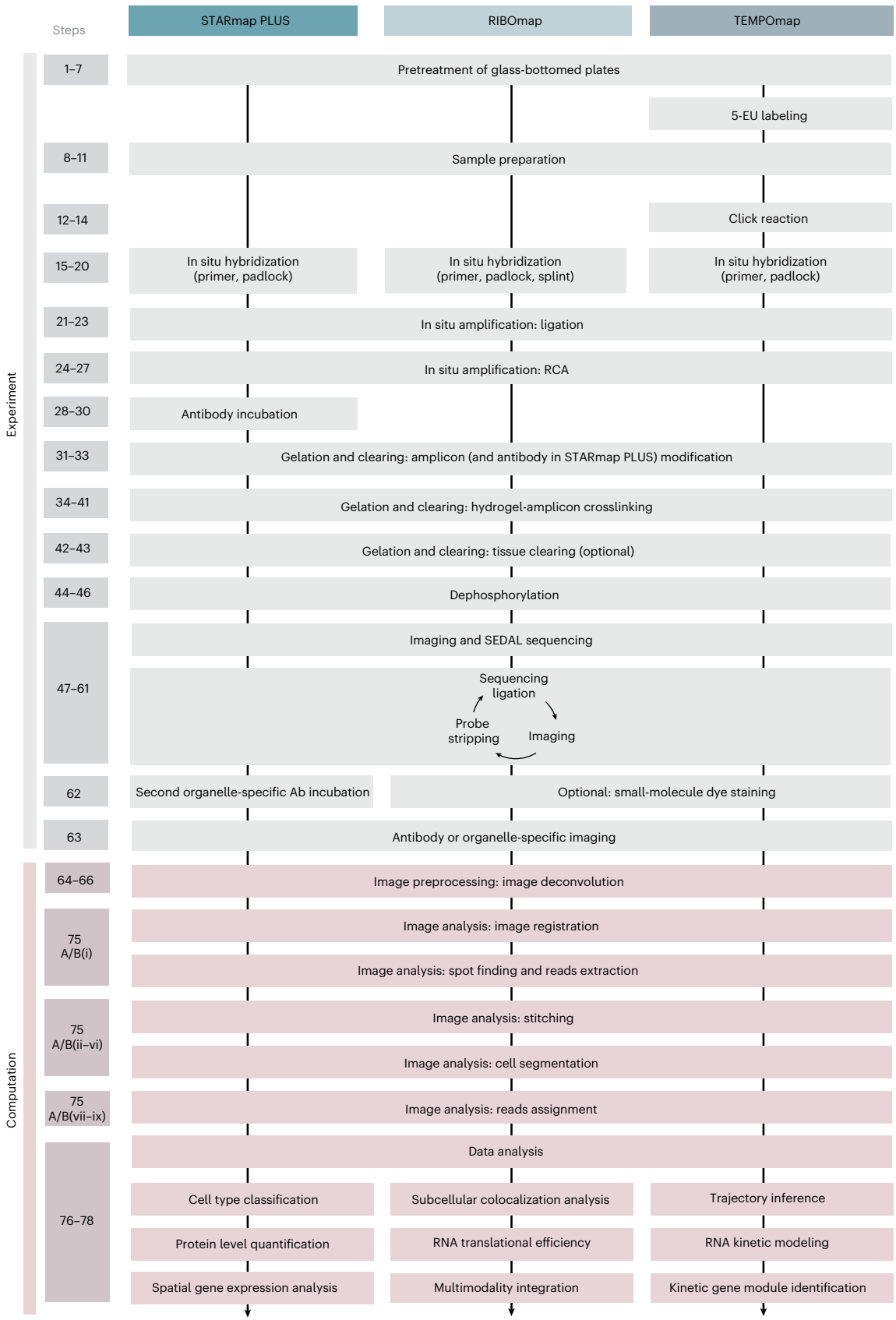

Steps
STARmap PLUS
RIBOmap
TEMPOmap
Experiment
1–7
Pretreatment of glass-bottomed plates
5-EU labeling
8–11
Sample preparation
12–14
Click reaction
15–20
In situ hybridization (primer, padlock)
In situ hybridization (primer, padlock, splint)
In situ hybridization (primer, padlock)
21–23
In situ amplification: ligation
24–27
In situ amplification: RCA
28–30
Antibody incubation
31–33
Gelation and clearing: amplicon (and antibody in STARmap PLUS) modification
34–41
Gelation and clearing: hydrogel-amplicon crosslinking
42–43
Gelation and clearing: tissue clearing (optional)
44–46
Dephosphorylation
47–61
Imaging and SEDAL sequencing
Sequencing ligation
Probe stripping
Imaging
62
Second organelle-specific Ab incubation
Optional: small-molecule dye staining
63
Antibody or organelle-specific imaging
Computation
64–66
Image preprocessing: image deconvolution
75 A/B(i)
Image analysis: image registration
Image analysis: spot finding and reads extraction
75 A/B(ii–vi)
Image analysis: stitching
Image analysis: cell segmentation
75 A/B(vii–ix)
Image analysis: reads assignment
Data analysis
76–78
Cell type classification
Subcellular colocalization analysis
Trajectory inference
Protein level quantification
RNA translational efficiency
RNA kinetic modeling
Spatial gene expression analysis
Multimodality integration
Kinetic gene module identification

Fig. 5 | Complete overview of experimental and computational procedures of STARmap PLUS, RIBOmap and TEMPOmap. The procedure consists of pretreatment of plates, sample preparation, DNA library preparation, imaging acquisition, imaging processing and downstream data analysis.

- PBS (Gibco, cat. no. 10010-023)
- UltraPure DNase/RNase-Free Distilled Water (ThermoFisher Scientific, cat. no. 10977015)
- Tween-20, 10% solution (Teknova, cat. no. T0710)
- Yeast tRNA (ThermoFisher Scientific, cat. no. AM7119)
- Glycine (Sigma aldrich, cat. no. 50046-250G)
- SUPERase In RNase Inhibitor (ThermoFisher Scientific, cat. no. AM2696)
- 20× saline sodium citrate (SSC) (Sigma-Aldrich, cat. no. S6639)
- Formamide (Calbiochem, cat. no. 655206)
  ▲ **CAUTION** Formamide is a known reproductive toxin and can cause irritation to the eyes, skin and respiratory system. It is also combustible and can produce irritating or toxic fumes in a fire. Always use PPE in adequate ventilation when handling the chemical. Store in a cool, dry place, separated from incompatible substances such as oxidizing agents, acids and bases.
- Ribonucleoside vanadyl complex (RVC; New England Biolabs, cat. no. S1402S)
- T4 DNA ligase, 5 Weiss U/µl (ThermoFisher Scientific, cat. no. EL0012)
- Phi29 DNA polymerase (ThermoFisher Scientific, cat. no. EP0094)
- 10 mM dNTP mix (ThermoFisher Scientific, cat. no. 18427089)
- UltraPure BSA (ThermoFisher Scientific, cat. no. AM2618)
- Recombinant albumin, molecular biology grade (NEB, cat. no. B9200S)
- 5-(3-aminoallyl)-dUTP (ThermoFisher Scientific, cat. no. AM8439)
- Methacrylic acid NHS ester, 98% (Sigma-Aldrich, cat. no. 730300)
- DMSO, anhydrous (Molecular Probes, cat. no. D12345)
- Sodium bicarbonate (Sigma aldrich, cat. no. S5761-500G)
- Acrylamide solution, 40% (Bio-Rad, cat. no. 161-0140)
  ▲ **CAUTION** Acrylamide is toxic if swallowed, inhaled or absorbed through skin. It also potentially causes damage to the nervous system and organs through prolonged exposure. It is classified as a probable human carcinogen and can cause skin and eye irritation. Always use PPE in adequate ventilation when handling the chemical. Store the acrylamide solutions in a cool, dry place. Avoid creating aerosols.
- Bis solution, 2% (Bio-Rad, cat. no. 161-0142)
- Ammonium persulfate (Sigma-Aldrich, cat. no. A3678)
- *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Sigma-Aldrich, cat. no. T9281)
- OminiPur SDS, 20% (Calbiochem, cat. no. 7991)
- Proteinase K Solution, RNA grade (ThermoFisher Scientific, cat. no. 25530049)
- Antarctic Phosphatase (New England Biolabs, cat. no. M0289L)
- 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, cat. no. D1306)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. 93443)
- Concanavalin A, Alexa Fluor 594 Conjugate (ThermoFisher Scientific, cat. no. C11253)
- Flamingo Fluorescent Protein Gel Stain (Bio-Rad, cat. no. 1610490)
- Azidobutyric acid NHS ester (Lumiprobe Corporation, cat. no. 63720)
- 1.5× Click buffer (Lumiprobe, cat. no. 61150)
- L-ascorbic acid (Sigma-Aldrich, cat. no. A5960)
- 5-Ethynyl uridine (5-EU) (Invitrogen, cat. no. E10345)
- Uridine (Millipore Sigma, cat. no. U3750)
- DAPI (Invitrogen, cat. no. D1306)
- Concanavalin A conjugates (Invitrogen, cat. no. C11253)
- Flamingo Fluorescent Protein Gel Stain (Bio-rad, cat. no. 1610490)
- Gel Slick Solution (Lonza, cat. no. 50640)

Probes

- TEMPOmap splint probe: `/5AzideN/AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA TATCTTTAGT*G*T*/3InvdT/` (IDT, '/5AzideN/' denotes azide modification

Table 1 | RIBOmap splint probes

Splint probe set 1	18S 50A splint probe 1	ACAAAATAGAACCGCGGTCCTATTCAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	18S 50A splint probe 2	CATCGTTTATGGTCGGAACTACGACAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	18S 50A splint probe 3	AGGTTTCCCGTGTTGAGTCAAATTAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	18S 50A splint probe 4	TGTTATTGCTCAATCTCGGGTGGCTAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	18S 50A splint probe 5	AGATAGTCAAGTTCGACCGTCTTCTAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
Splint probe set 2	18S 10A splint probe 1	ACAAAATAGAACCGCGGTCCTATTCAA AAA AAA AA TAT CTT TAG T*G*T* /3InvdT/
	18S 10A splint probe 2	CATCGTTTATGGTCGGAACTACGACAA AAA AAA AA TAT CTT TAG T*G*T* /3InvdT/
	18S 10A splint probe 3	AGGTTTCCCGTGTTGAGTCAAATTAAA AAA AAA AA TAT CTT TAG T*G*T* /3InvdT/
	18S 10A splint probe 4	TGTTATTGCTCAATCTCGGGTGGCTAA AAA AAA AA TAT CTT TAG T*G*T* /3InvdT/
	18S 10A splint probe 5	AGATAGTCAAGTTCGACCGTCTTCTAA AAA AAA AA TAT CTT TAG T*G*T* /3InvdT/
Splint probe set 3	28S 50A splint probe 1	CCGTTCCCTTGGCTGTGGTTTCGCTAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	28S 50A splint probe 2	AAGCGACGTCGCTATGAACGCTTGGAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	28S 50A splint probe 3	TCCCTTACGGTACTTGTTGACTATCAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	28S 50A splint probe 4	ATTCCGACTTCCATGGCCACCGTCCAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
	28S 50A splint probe 5	CTCCAGCGCCATCCATTTTCAGGGCAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAT CTT TAG T*G*T* /3InvdT/
Splint probe set 4	28S 10A splint probe 1	CCGTTCCCTTGGCTGTGGTTTCGCTAAAAAAAAAA TAT CTT TAG T*G*T* /3InvdT/
	28S 10A splint probe 2	AAGCGACGTCGCTATGAACGCTTGGAAAAAAAAAA TAT CTT TAG T*G*T* /3InvdT/
	28S 10A splint probe 3	TCCCTTACGGTACTTGTTGACTATCAAAAAAAAAA TAT CTT TAG T*G*T* /3InvdT/
	28S 10A splint probe 4	ATTCCGACTTCCATGGCCACCGTCCAAAAAAAAAA TAT CTT TAG T*G*T* /3InvdT/
	28S 10A splint probe 5	CTCCAGCGCCATCCATTTTCAGGGCAAAAAAAAAA TAT CTT TAG T*G*T* /3InvdT/

Four sets of 18S and 28S rRNA splint probe sequences are included. The probes are applicable to all experimental conditions. All the probes contain phosphorothioate bonds (code '*' in IDT's catalog) and 3′ invdT modification (code '/3InvdT/' in IDT's catalog).

at the 5′ end of the probe and the probe contains phosphorothioate bonds (code '*' in IDT's catalog) and 3′ invdT modification (code '/3InvdT/' in IDT's catalog). Researchers can order the exact sequence as provided with standard purification

- Fluorescent detection probe: `/5Alex647N/CATACACTAAAGATAACAT` (IDT, '/5Alex647N/' denotes Alexa Fluor 647 modification at the 5′ end of the probe). Researchers can order the exact sequence as provided
- For RIBOmap splint probes, IDT, see Table 1. Researchers can order the exact sequence as provided with standard purification
- For STARmap primers and padlock probes, IDT, see Table 2. If additional genes are to be targeted, researchers must design new probe sequences (see Fig. 2 for probe design guidelines)
- For RIBOmap and TEMPOmap primers and padlock probes, IDT, see Table 3. If additional genes are to be targeted, researchers must design new probe sequences (see Fig. 2 for probe design guidelines)
- For SEDAL sequencing reading probes, IDT, see Table 4. Researchers can order the exact sequence as provided
- For SEDAL fluorescent decoding probes, IDT, see Table 5. Researchers can order the exact sequence as provided

Equipment

- Glass bottom 24-well plates (Cellvis, cat. no. P24-1.5H-N)
- Glass bottom 12-well and 24-well plates (MatTek, cat. nos. P12G-1.5-14-F and P24G-1.5-13-F)

Table 2 | Example STARmap primers and padlock probes targeting human *ACTB* mRNA, *MALAT1*, *vtRNA1-1* and *GAPDH* mRNAs

STARmap_ACTB_padlock 1	/5Phos/ACATTAGGGATAGCACAGCCTGGATAGCAAAATTATTACTGAA**A**CATACACTAAAGATA
STARmap_ACTB_padlock 2	/5Phos/ACATTAAGCGCGGCGATATCATCATCAATTATTACTGAA**A**CATACACTAAAGATA
STARmap_ACTB_padlock 3	/5Phos/ACATTAACCATCACGCCCTGGTGCCAATTATTACTGAA**A**CATACACTAAAGATA
STARmap_ACTB_primer 1	TGGTACGGCCAGAGGCGTATAATGTTATCTT
STARmap_ACTB_primer 2	CGGAGCCGTTGTCGACGACTAATGTTATCTT
STARmap_ACTB_primer 3	TAGGAATCCTTCTGACCCATGCTAATGTTATCTT
STARmap_MALAT1_padlock 1	/5Phos/ACATTACTTCAGACCTTCTGAACCGGAATTATTACTGAA**T**CATACACTAAAGATA
STARmap_MALAT1_padlock 2	/5Phos/ACATTACGATTATGGATCATGCCCAAATTATTACTGAA**T**CATACACTAAAGATA
STARmap_MALAT1_padlock 3	/5Phos/ACATTAAATCGGCCTACGTCCCCATAATTATTACTGAA**T**CATACACTAAAGATA
STARmap_MALAT1_primer 1	CTGTGTTATGCCTGGTTAGGTATGTAATGTTATCTT
STARmap_MALAT1_primer 2	ACACCATCGTTACCTTGAAATAATGTTATCTT
STARmap_MALAT1_primer 3	AGAGAAACCTACAACACCCGGTAATGTTATCTT
STARmap_vtRNA1-1_padlock	/5Phos/ACATTAAACTGTCGAAGTAACCGCTAATTATTACTGAA**C**CATACACTAAAGATA
STARmap_vtRNA1-1_primer	AGACAGGTTGCTTGTTTCAATTAATAATGTTATCTT
STARmap_GAPDH_padlock 1	/5Phos/ATCCACGAGCTTGACAAAGTGGTCGTATTACCTCG**G**CATACACTAAAGATAAACTACCC
STARmap_GAPDH_padlock 2	/5Phos/ATCCACGTTCAGCTCAGGGATGACCTATTACCTCG**G**CATACACTAAAGATAAACTACCC
STARmap_GAPDH_padlock 3	/5Phos/ATCCACCCACGACGTACTCAGCGCCAGCATTACCTCG**G**CATACACTAAAGATAAACTACCC
STARmap_GAPDH_primer 1	CCAAATTCGTTGTCATACCAGGAAGTGGATGGGTAG
STARmap_GAPDH_primer 2	GCCATGCCAGTGAGCTTCCGTGGATGGGTAG
STARmap_GAPDH_primer 3	TGGTGGTGAAGACGCCAGTGGAGTGGATGGGTAG

In the sequence, '/5Phos/' denotes phosphorylation at the 5′ end of the padlock probe. The specific annotations for primer and padlock probe design are shown in Fig. 2a. If all four genes are simultaneously targeted using the probes listed in this table and sequenced using SeqA1 reading probe (Table 4) and 16-mix fluorescent decoding probes (Table 5), we expect *ACTB* signals to be Alexa 546, *MALAT1* Alexa 594, *vtRNA1-1* Alexa 488 and *GAPDH* Alexa 647 (gene-specific barcode is in bold in the probe sequence) (see Fig. 4 for color-decoding scheme).

- 4 °C, −20 °C and −80 °C storage units (VWR, cat. nos. 10819-650 and 10819-894 and Thermo Scientific, cat. no. TDE60086FD)
- Micro coverglass, 12 mm diameter (Electron Microscope Sciences, cat. no. 72226-01)
- Cryostat (Leica, cat. no. CM1950)
- Plasma systems (Diener electronic, cat. no. ATTO B)
- VACUSIP Aspiration System (INTEGRA Biosciences, cat. no. 159000)
- Analog Rocker (VWR, cat. no. 10127-876)
- Analog Vortex Mixer (VWR, cat. no. 10153-838)
- Desiccator cabinet, Dry-Keeper, with gas port (SP Bel-Art, cat. no. H42053-0002)
- Dry block heaters (VWR, cat. no. 75838-270)
- Rocking Hybridization Oven/Incubator (Boekel Scientific, cat. no. 136400)
- Cell incubator at 37 °C, with regulated humidity and CO_2 (Thermo Scientific, cat. no. 51030284)
- Point-scanning confocal (for example, Leica, SP8/Stellaris; Nikon; Zeiss Airyscan) or spinning-disk confocal microscope (for example, Nikon; Andor, Dragonfly)

Software

- Access to a high-performance computing cluster (remote host)
- Fiji/ImageJ (http://fiji.sc/Fiji)
- MATLAB 2023b or newer (http://www.mathworks.com) on the remote host
- Python 3.9 (https://www.python.org/) on the local or remote host
- Conda package manager (https://docs.conda.io/en/latest/) on the remote host
- Starfinder (https://github.com/wanglab-broad/starfinder)
- ClusterMap (https://github.com/wanglab-broad/ClusterMap)
- Windows PC or Mac with 16 GB RAM minimum
- Optional: SVI Huygens 3D deconvolution software (commercial)

Table 3 | Example RIBOmap and TEMPOmap primers and padlock probes targeting human *ACTB* mRNA, *MALAT1*, *vtRNA1-1* and *GAPDH* mRNAs

Tri-probe_ACTB_padlock 1	/5Phos/AAGATAAACATCGTAGACTAGCAAAGGCGAGGCTCTGTGCTCAGG**A**CATACACTA
Tri-probe_ACTB_padlock 2	/5Phos/AAGATAAACATCGTAGACTACACCATCACGCCCTGGTGCCTCAGG**A**CATACACTA
Tri-probe_ACTB_padlock 3	/5Phos/AAGATAAACATCGTAGACTAGGGATAGCACAGCCTGGATAGCAATCAGG**A**CATACACTA
Tri-probe_ACTB_primer 1	GGTGTGGACGGGCGGCGGATGTGTCTACGATG
Tri-probe_ACTB_primer 2	TAGGAATCCTTCTGACCCATGCGTGTCTACGATG
Tri-probe_ACTB_primer 3	TGGTACGGCCAGAGGCGTAGTGTCTACGATG
Tri-probe_MALAT1_padlock 1	/5Phos/AAGATAAACATCGTAGACTACTTCAGACCTTCTGAACCGGTCAGG**T**CATACACTA
Tri-probe_MALAT1_padlock 2	/5Phos/AAGATAAACATCGTAGACTACGATTATGGATCATGCCCATCAGG**T**CATACACTA
Tri-probe_MALAT1_padlock 3	/5Phos/AAGATAAACATCGTAGACTAAATCGGCCTACGTCCCCATTCAGG**T**CATACACTA
Tri-probe_MALAT1_primer 1	CTGTGTTATGCCTGGTTAGGTATGGTGTCTACGATG
Tri-probe_MALAT1_primer 2	ACACCATCGTTACCTTGAAAGTGTCTACGATG
Tri-probe_MALAT1_primer 3	AGAGAAACCTACAACACCCGGGTGTCTACGATG
Tri-probe_vtRNA1-1_padlock	/5Phos/AAGATAAACATCGTAGACTAAACTGTCGAAGTAACCGCTTCAGG**C**CATACACTA
Tri-probe_vtRNA1-1_primer	AGACAGGTTGCTTGTTTCAATTAAGTTGCTACGATG
Tri-probe_GAPDH_padlock 1	/5Phos/AAGATAAACTACCCATCCTAGAGCTTGACAAAGTGGTCGTATTACCTCG**G**CATACACTA
Tri-probe_GAPDH_padlock 2	/5Phos/AAGATAAACTACCCATCCTAGTTCAGCTCAGGGATGACCTATTACCTCG**G**CATACACTA
Tri-probe_GAPDH_padlock 3	/5Phos/AAGATAAACTACCCATCCTACCACGACGTACTCAGCGCCAGCATTACCTCG**G**CATACACTA
Tri-probe_GAPDH_primer 1	CCAAATTCGTTGTCATACCAGGAAGTGGATGGGTAG
Tri-probe_GAPDH_primer 2	GCCATGCCAGTGAGCTTCCGTGGATGGGTAG
Tri-probe_GAPDH_primer 3	TGGTGGTGAAGACGCCAGTGGAGTGGATGGGTAG

In the sequence, '/5Phos/' denotes phosphorylation at the 5′ end of the padlock probe. The specific annotations for primer and padlock tri-probe design are shown in Fig. 2b. Note that in the RIBOmap experiment, *ACTB* and *GAPDH* mRNA serve as positive controls, whereas noncoding RNAs *MALAT1* and *vtRNA1-1* as negative controls. If all four genes are simultaneously targeted using the probes listed in this table and sequenced using SeqA1 reading probe (Table 4) and 16-mix fluorescent decoding probes (Table 5), we expect *ACTB* signals to be Alexa 546 and *GAPDH* Alexa 647. Minimal RIBOmap signals should be seen in *MALAT1* Alexa 594 or *vtRNA1-1* Alexa 488 (gene-specific barcode is in bold in the probe sequence) (see Fig. 4 for color-decoding scheme).

Table 4 | SEDAL sequencing reading probes

SeqA1	/5Phos/ATACACTAAAG/3InvdT/
SeqA2	/5Phos/CATACACTAAA/3InvdT/
SeqA3	/5Phos/(N:25252525)CATACACTAA/3InvdT/
SeqA4	/5Phos/(N:25252525)(N)CATACACTA/3InvdT/
SeqA5	/5Phos/(N:25252525)(N)(N)CATACACT/3InvdT/
SeqA6	/5Phos/(N:25252525)(N)(N)(N)CATACAC/3InvdT/
SeqB1	/5Phos/CGTAGACTANN/3InvdT/
SeqB2	/5Phos/TCGTAGACTAN/3InvdT/
SeqB3	/5Phos/(N:25252525)TCGTAGACTA/3InvdT/
SeqB4	/5Phos/(N:25252525)(N)TCGTAGACT/3InvdT/
SeqB5	/5Phos/(N:25252525)(N)(N)TCGTAGACT/3InvdT/
SeqB6	/5Phos/(N:25252525)(N)(N)(N)TCGTAGACT/3InvdT/

'/5Phos/' denotes phosphorylation at the 5′ end of the padlock probe and '/3InvdT/' denotes 3′ invdT modification. The reading probe sequences contain random ribonucleotides (denoted as code (N:25252525) or (N) in IDT's catalog), which should be ordered as standard mixed bases with an equal ratio of A/T/C/G.

Reagent setup

▲ **CRITICAL** Unless indicated, all reagents can be stored at 23 °C (room temperature) for up to 3 months.

Bind-Silane coating solution

Prepare 50 ml of Bind-Silane coating solution by adding 5 ml acetic acid and 0.5 ml of Bind-Silane into 44.5 ml of 95% ethanol. Make fresh before use.

Table 5 | SEDAL fluorescent decoding probes

AA-488	/5Alex488N/(N:25252525)(N)(N)(N)(N)(N)AA
CC-488	/5Alex488N/(N:25252525)(N)(N)(N)(N)(N)CC
GG-488	/5Alex488N/(N:25252525)(N)(N)(N)(N)(N)GG
TT-488	/5Alex488N/(N:25252525)(N)(N)(N)(N)(N)TT
AC-546	/5Alex546N/(N:25252525)(N)(N)(N)(N)(N)AC
CA-546	/5Alex546N/(N:25252525)(N)(N)(N)(N)(N)CA
TG-546	/5Alex546N/(N:25252525)(N)(N)(N)(N)(N)TG
GT-546	/5Alex546N/(N:25252525)(N)(N)(N)(N)(N)GT
AG-594	/5Alex594N/(N:25252525)(N)(N)(N)(N)(N)AG
GA-594	/5Alex594N/(N:25252525)(N)(N)(N)(N)(N)GA
CT-594	/5Alex594N/(N:25252525)(N)(N)(N)(N)(N)CT
TC-594	/5Alex594N/(N:25252525)(N)(N)(N)(N)(N)TC
AT-647	/5Alex647N/(N:25252525)(N)(N)(N)(N)(N)AT
TA-647	/5Alex647N/(N:25252525)(N)(N)(N)(N)(N)TA
GC-647	/5Alex647N/(N:25252525)(N)(N)(N)(N)(N)GC
CG-647	/5Alex647N/(N:25252525)(N)(N)(N)(N)(N)CG

The 16 decoding probe sequences contain random ribonucleotides (denoted as code (N:25252525) or (N) in IDT's catalog), which should be ordered as standard mixed bases with an equal ratio of A/T/C/G. '/5Alex488N/', '/5Alex546N/', '/5Alex594N/' and '/5Alex647N/' denote 5′ modification with Alexa Fluor 488, 546, 594 and 647, respectively. The probes should be ordered with high-performance liquid chromatography (HPLC) purification.

PBST
Prepare 50 ml of PBS-Tween (PBST) by adding 500 µl of 10% Tween-20 solution to 49.5 ml of 1× PBS. Can be stored at room temperature for 3 months.

PBSTR
Prepare 10 ml of PBS-Tween-RNaseIn (PBSTR) by adding 50 µl of SUPERase·In RNase Inhibitor to ~10 ml of PBST. Make fresh before use.

PBS-Triton
Prepare 50 ml of PBS-Triton by adding 500 µl of 10% Triton X-100 to 49.5 ml of 1× PBS. Can be stored at room temperature for 3 months.

30% sucrose–PBS
Prepare 50 ml of 30% sucrose–PBS solution by dissolving 15 g of sucrose in 35 ml of 1× PBS. Fill up the solution to a final volume of 50 ml with additional 1× PBS. Filter the solution through a 0.22-µm filter unit. Make fresh before use.

Quenching buffer A
Prepare 10 ml of quenching buffer by dissolving 75 mg of glycine in PBSTR, adding 100 µl of Yeast tRNA, 500 µl of ribonucleoside vanadyl complex (RVC) and adjusting the volume to 10 ml. Make fresh before use.

Quenching buffer B
Prepare 10 ml of permeabilization and quenching buffer by dissolving 75 mg of glycine in PBSTR, adding 1 ml of 10% Triton-X-100, 100 µl of yeast tRNA, 500 µl of RVC and adjusting the volume to 10 ml. Make fresh before use.

High salt buffer
Prepare 10 ml of high salt buffer by adding 2 ml of 20× SSC to 8 ml of PBSTR. Make fresh before use.

Stripping buffer
Prepare 10 ml of stripping buffer by adding 6 ml of formamide and 100 µl of 10% Triton X-100 to 3.9 ml of Ultrapure DNase/RNase-Free distilled water. Make fresh before use.

Washing and imaging buffer
Prepare 10 ml of washing and imaging buffer by adding 1 ml of 20× SSC solution and 1 ml of Formamide to 8 ml of Ultrapure DNase/RNase-Free distilled water. Make fresh before use.

DAPI working solution
Prepare 5 mg/ml DAPI in H_2O as stock (long-term storage at −20 °C). Prepare 100 ng/ml as working concentration by dissolving the stock in PBS or washing and imaging buffer. Can be stored at room temperature for 3 months. Protect from light.

RIBOmap and TEMPOmap splint probe stock preparation
Dissolve each RIBOmap splint probe (Table 1) or TEMPOmap splint probe (Reagents) in Ultrapure DNase/RNase-Free distilled water or IDTE buffer to produce a 100 µM stock solution. Prepare mixtures of each of the four sets of RIBOmap splint probes by combining the stock solutions of each of the five probes of the same set in equal volumes. The dissolved probe stock can be stored at −80 °C for 1 year.
▲ **CRITICAL** We recommend testing each of the four sets of RIBOmap splint probes and then using only one set going forwards.

STARmap primer and padlock probe stock preparation
For single-gene probe stocks, dissolve each probe (Table 2 and 'Reagents' section) in Ultrapure DNase/RNase-Free distilled water or IDTE buffer to produce a 100 µM stock solution. Prepare probe mixture for each gene by combining the stock solutions of each of the six probes of the same gene in equal volumes.

For large-scale probe pools (probes targeting multiple genes), dissolve the pool in IDTE buffer to a stock concentration of 50–100 nM per probe. The dissolved probe stock can be stored at −80 °C for 1 year. Aliquot the dissolved probes to avoid freeze–thaw cycles if necessary.

RIBOmap/TEMPOmap primer and padlock probe stock preparation
Dissolve each probe (Table 3 and 'Reagents' section) in Ultrapure DNase/RNase-Free distilled water or IDTE buffer to produce a 100 µM stock solution. Prepare probe mixture for each gene by combining the stock solutions of each of the six probes of the same gene in equal volumes.

For large-scale probe pools (probes targeting multiple genes), dissolve the pool in IDTE buffer to a stock concentration of 50–100 nM per probe. The dissolved probe stock can be stored at −80 °C for 1 year. Aliquot the dissolved probes to avoid freeze–thaw cycles if necessary.

SEDAL reading probe and fluorescent decoding probe stock preparation
Dissolve each probe (Tables 4 and 5) in pH 7.5 IDTE buffer to produce a 100 µM stock solution. Prepare the fluorescent decoding probe mixture by combining the stock solutions of the 16 probes in equal volumes. The dissolved probe stock can be stored at −80 °C for 1 year. Aliquot the dissolved probes to avoid freeze–thaw cycles if necessary.

Procedure

Pretreatment of glass-bottomed plates

● **TIMING** 1 d

1. Expose the glass-bottom plates to 100 W, 2% O_2 plasma for 45 s to 5 min. Immediately after plasma activation, treat the plates with Bind-Silane (3-(trimethoxysilyl) propyl methacrylate) coating solution. Use a total of 300 µl per well for 24-well plates and 600 µl per well for 12-well plates.

▲ **CRITICAL STEP** Bind-Silane is prone to hydrolysis. Make the Bind-Silane working solution immediately before use.

2. Keep the plate in the chemical hood at room temperature for 2 h.
3. Aspirate and wash the plate 3 times with 1 ml 95% ethanol per well.
4. Air-dry the plate at room temperature overnight.
5. Coat the plate with 600 µl 50 µg/ml poly-D-lysine in water per well at room temperature for at least 1 h.
6. Aspirate and wash the plate three times with 1 ml water per well.
7. Air-dry the plate at room temperature for at least 2 h. Store the plate at 4 °C for later usage.
   ■ **PAUSE POINT** The coated plate can be stored at 4 °C for several months, if needed.

Sample preparation

● **TIMING 1–2 d**

8. (TEMPOmap only) Seed HeLa cells to ~60% confluence on a pretreated glass-bottom plate. Allow the cells to grow at 37 °C for 12–18 h to 70–80% confluence. Replace with media containing 200 µM to 1 mM 5-EU for 30 min to 2 h, followed by media washes supplemented with 400 µM uridine for 1 to 6 h, depending on the experimental design for pulse-chase analysis. Skip this step and directly proceed to Step 9 if TEMPOmap is not performed.
   ▲ **CRITICAL STEP** Depending on the transcriptional activities of cells, the necessary 5-EU concentration in the media differs for different cell types and may require optimization for each sample. We recommend starting with 200 µM 5-EU as the minimal concentration and 1 mM 5-EU as the maximal concentration. Please note that minimal concentration of 5-EU is recommended (200–400 µM). A short labeling duration (within 2 h) is advised. Since DMSO is used to dissolve 5-EU, note that the total volume of DMSO in cell media should not exceed 1:1,000 dilution.
9. For sample preparation, follow option A for cultured adherent cells or follow option B for tissue sections.
   (A) **Cultured adherent cells on a 24-well glass-bottom plate**
      (i) Fix the cells by 400 µl 1.6% PFA in 1× PBS buffer per well at room temperature for 15 min.
   (B) **Tissue sections on a 12-well glass-bottom plate**
      (i) Perfuse mouse with PBS transcardially under anesthesia before collecting organs from wild-type mice to preserve cell morphology. Perfuse mouse with 20 ml of PBS followed by 20 ml of 4% PFA at a constant speed of ~1 ml per 5 s. Collect the tissues from the perfused animal.
      (ii) Post-fix the collected tissues in 4% PFA for 3–4 h at 4 °C and incubate in 30% sucrose–PBS cryoprotectant overnight at 4 °C until tissues drop to bottom of the vial.
      (iii) Immerse a metal block (from heating blocks) in liquid nitrogen until temperature equilibrium. Remove excess liquid nitrogen to expose the upper surface of the metal blocks. This process can take ~0.5 h.
      (iv) Embed freshly collected tissue samples immediately in OCT and flash-freeze on top of the metal blocks. Leave the tissue block on the metal block for at least five minutes after the surface OCT turns white.
         ■ **PAUSE POINT** Tissue blocks in O.C.T. can be kept at −80 °C for up to 2 years.
      (v) Transfer the tissue block to cryostat and cut 20-µm slices. Mount the slices in the pretreated glass-bottom 12-well plate, one slice per well.
      (vi) Fix the tissue slices with 600 µl 4% PFA in 1× PBS buffer per well at room temperature for 15 min.
         ◆ **TROUBLESHOOTING**
10. For sample permeabilization and quenching, follow option A for regular sample processing or option B for sample processing to preserve endogenous fluorescence
   (A) **Regular sample processing**
      (i) Aspirate fixative and add 600 µl prechilled −20 °C methanol per well and keep the plate at −20 °C for 1 h or at −80 °C for 1 h.
         ■ **PAUSE POINT** The sample can be stable at −80 °C in methanol for up to 1 week.

(ii) Bring the sample in methanol from −20 °C or −80 °C fridge to room temperature for 5 min.
(iii) Aspirate the methanol completely.
(iv) Quench the samples with 600 µl quenching buffer A at room temperature for 5 min.
(B) **Permeabilizing and quenching samples while preserving endogenous fluorescence.**
(i) Aspirate fixative and add 600 µl quenching buffer B at room temperature for 10 min.

11. Briefly rinse the samples with PBSTR. Proceed directly to Step 15 if TEMPOmap is not being performed.

▲ **CAUTION** Minimize handling time between PFA fixation and hybridization (Step 15).

Click reaction (for TEMPOmap only)

● **TIMING 2 h**

12. Prepare a click reaction mixture as follows:

Component	Amount (µl)	Final
40 µM TEMPOmap splint probe (Reagents)	50	5 µM
1.5× Lumiprobe click buffer	266.6	1×
10 mM dNTP	20	500 µM
50 mM ascorbic Acid	6.4	800 µM
20 U/µl SUPERase·In	8	0.4 U/µl
1× PBS	49	
Total	400	

▲ **CRITICAL STEP** The reaction should be initiated by adding ascorbic acid last.

13. Incubate samples with the click reaction mixture at 37 °C for 1 h with gentle shaking.
14. Wash the samples twice with PBSTR at 37 °C for 10 min each.

In situ hybridization

● **TIMING 1–2 d**

15. Heat the primer and padlock probe mixtures ('Reagent setup' section) at 90 °C for 5 min and immediately store on ice.
16. To prepare a hybridization mixture for STARmap, RIBOmap or TEMPOmap probes, preheat and dissolve RVC stock solution at 55 °C, mix the rest of the reagents before adding RVC to create the hybridization buffer (see table below). Then add the heated probe mixtures (from Step 15) to a final concentration of 100 nM in hybridization buffer unless specified.

Component	Amount (µl)	Final concentration
20× SSC buffer	40	2×
Formamide	40	10% (vol/vol)
RVC	40	20 mM
10% Tween-20	40	1% (vol/vol)
10 mg/ml tRNA	4	0.1 mg/ml
20 U/µl SUPERase·In RNase Inhibitor	4	0.2 U/µl
20 µM RIBOmap splint probe mixture (5-probe mix)	2	100 nM per probe
100 µM Primer probe	0.4 per probe	100 nM per probe
100 µM Padlock probe	0.4 per probe	100 nM per probe
H_2O	Adjusted according to the probe number	
Total	400	

▲ **CRITICAL STEP** For detection of more than 100 genes, dilute the heated stock padlock and primer probes to a final concentration of 1–5 nM per probe in hybridization buffer. Optimal final concentration varies by tissue type and requires optimization for each project. For tissue slices, Tween-20 in the hybridization mixture can be replaced by the same concentration of Triton X-100 depending on tissue type. Please note that RIBOmap splint probe mixture is only required for RIBOmap.

17. Remove PBSTR and add 400 µl hybridization mixture per well to the samples.
18. Incubate the samples in the hybridization mixture in a 40 °C oven with shaking and parafilm wrapping for 12–36 h.
    ▲ **CRITICAL STEP** Seal the plate with parafilm carefully to avoid buffer evaporation and sample dry-out. Add a water reservoir in the oven for prolonged hybridization.
19. Aspirate and wash the samples with 600 µl PBSTR twice and 600 µl high salt buffer once at 37 °C for 20 min each wash.
20. Rinse the samples briefly with 600 µl PBSTR at room temperature once.

In situ amplification

● **TIMING 5 h**

21. Prepare a ligation mixture as follows:

Component	Amount (µl)	Final
10× T4 DNA ligase buffer	40	1×
50 mg/ml UltraPure BSA	4	0.5 mg/ml
20 U/µl SUPERase·In RNase Inhibitor	4	0.2 U/µl
5 U/µl T4 DNA ligase	8	0.1 U/µl
H_2O	344	
Total	400	

    ▲ **CRITICAL STEP** For detection of more than 100 genes, use a 2.5-fold higher T4 DNA ligase concentration.
    ▲ **CRITICAL STEP** T4 DNA ligase buffer should be thawed at room temperature, instead of at 37 °C to prevent breakdown of ATP or at 4 °C to avoid precipitation of DTT.
22. Incubate the samples with 400 µl ligation mixture per well at room temperature for 2 h.
    ▲ **CRITICAL STEP** Seal the plate with parafilm to protect the ligation reaction from oxygen in the open air.
23. Aspirate and wash the samples with 600 µl PBSTR twice for 5 min each.
24. Prepare an RCA mixture as follows:

Component	Amount (µl)	Final
10× Phi29 DNA polymerase buffer	40	1×
10 mM dNTP mixture	10	250 µM
4 mM 5-(3-aminoallyl)-dUTP	2	20 µM
50 mg/ml BSA	4	0.5 mg/ml
20 U/µl SUPERase·In RNase Inhibitor	4	0.2 U/µl
10 U/µl Phi29 DNA polymerase	8	0.2 U/µl
H_2O	332	
Total	400	

    ▲ **CRITICAL STEP** For detection of more than 100 genes, use a 2.5-fold higher Phi29 DNA polymerase concentration.
25. Add this mixture to the samples (400 µl per well) and preincubate at 4 °C for 30 min.
26. Incubate the reaction in a 30 °C humidified oven with shaking and parafilm wrapping for 2 h.
27. Aspirate and wash the samples with 600 µl PBST twice for 5 min each. Proceed directly to Step 31 if STARmap PLUS is not being performed.

Primary antibody binding (for STARmap PLUS only)

● **TIMING 2 h**

28. Block the sample with 400 µl 5 mg/mL UltraPure BSA in PBS-Triton (BSA blocking buffer) at room temperature for 30 min.
29. Incubate with a primary antibody in the BSA blocking buffer.
    ▲ **CRITICAL STEP** The incubation temperature and time are antibody dependent.
30. Wash with 600 µl PBS-Triton 3 times for 5 min each.

Gelation and tissue clearing

● **TIMING 4–5 h**

31. Prepare a modification mixture as follows:

Component	Amount (µl)	Final
250 mM MA-NHS in DMSO	40	25 mM
1 M sodium bicarbonate (pH of ~8.4)	40	100 mM
H_2O	320	
Total	400	

▲ **CRITICAL STEP** The half-life of NHS esters in sodium bicarbonate buffer is on the order of minutes. Prepare the solution right before the reaction. MA-NHS should be stored in a desiccated environment at 4 °C and need to be equilibrated to room temperature before use.

32. Incubate the samples with 400 µl modification mixture at room temperature for 1 h.
33. Aspirate and briefly rinse the samples with 600 µl PBST once.
34. Prepare a monomer buffer as follows:

Component	Amount (µl)	Final
40% Acrylamide solution	100	4% (vol/vol)
2% Bis solution	100	0.2% (vol/vol)
20× SSC	100	2×
10% TEMED in water	20	0.2% (vol/vol)
H_2O	680	
Total	1,000	

35. Incubate the samples with 400 µl monomer buffer at room temperature for 25 min.
36. Coat coverslips in Gel Slick coating solution.
37. Prepare a polymerization mixture as follows:

Component	Amount (µl)	Final
Monomer buffer	100	
10% Ammonium persulfate	2	~0.2% (vol/vol)
Total	102	

38. Aspirate the monomer buffer from the samples in Step 35 and add 35 µl polymerization mixture from Step 37 per well for a 12-well plate (14 mm glass diameter, see 'Equipment' section for product specifics) or 25 µl per well for 24-well plate (13 mm glass diameter) to the center of the samples.
39. Cover the samples with Gel Slick-coated 12-mm coverslips from Step 36 immediately.
40. Incubate the samples in a desiccator cabinet with purged nitrogen at room temperature for 1 h.
    ◆ **TROUBLESHOOTING**
    ▲ **CRITICAL STEP** Ensure that the gelation reaction is performed in an oxygen-free environment.
41. Remove the coverslips and wash the samples with 600 µl PBST twice for 5 min each.
    ◆ **TROUBLESHOOTING**
    ■ **PAUSE POINT** The samples can be kept in PBST at 4 °C for up to 3 months after this step.
42. Prepare a proteinase K mixture as follows and digest the tissue–gel hybrid samples with 400 µl reaction mixture at 37 °C for 1 h.

Component	Amount (µl)	Final
20× SSC	40	2×
20% SDS	20	1% (vol/vol)
20 mg/ml proteinase K	4	0.2 mg/ml
H_2O	336	
Total	400	

43. Aspirate and wash the sample with 600 µl PBST three times for 5 min each.
▲ **CRITICAL STEP** Skip Steps 42–43 for STARmap PLUS or other experiments if protein signals need to be obtained. By omitting proteinase K treatment, DNA amplicon imaging may require a higher laser intensity than proteinase K-treated samples. No substantial impact was observed on the quantification of RNA counts between proteinase K-treated and untreated samples.

Dephosphorylation

● **TIMING 1.5 h**

44. Prepare a dephosphorylation mixture as follows:

Component	Amount (µl)	Final
10× Antarctic phosphatase buffer	40	1×
50 mg/ml UltraPure BSA	4	0.5 mg/ml
5 U/µl Antarctic phosphatase	20	0.25 U/µl
H_2O	336	
Total	400	

45. Incubate the samples with 400 µl dephosphorylation mixture at 37 °C for 1 h (optional: wash the samples with 1× Antarctic phosphatase buffer before adding this. This is because phosphate ion is an inhibitor for phosphatase).
46. Aspirate and wash the samples with 600 µl PBST three times for 5 min each.
47. Wash the samples with the stripping buffer twice at room temperature for 10 min each.
48. Aspirate and wash the samples with 600 µl PBST three times for 5 min each.

Imaging and SEDAL sequencing

● **TIMING 1–20 d**

49. Detection by fluorescent oligos (for a single gene test, perform this step and then proceed directly to Step 54; alternatively, for a multiplexed gene test, skip this step and proceed directly to Step 50). To detect single genes or perform quality control prior to sequencing, dilute a 19-nt fluorescent oligonucleotide ('fluorescent detection probe', 'Reagents' section) complementary to the DNA amplicon to a concentration of 100 nM in washing and imaging buffer. Incubate the sample with the detection probe solution at room temperature for 1 h. Following incubation, aspirate the sample and wash it three times with 600 µl of washing and imaging buffer for 5 min each time before proceeding to imaging.
◆ **TROUBLESHOOTING**
50. For a multiplexed gene test, prepare the sequencing mixture as follows and incubate the samples with 400 µl of this mixture at room temperature for a minimum of 3 h.

Component	Amount (µl)	Final
10× T4 DNA ligase buffer	40	1×
50 mg/ml UltraPure BSA	4	0.5 mg/ml
100 µM reading probe (Table 4)	40	10 µM
100 µM fluorescent decoding probe mixture (Table 5 and 'Reagent setup' section)	20	5 µM
5 U/µl T4 DNA ligase	15	0.1875 U/µl
H_2O	281	
Total	400	

51. Wash the samples with 600 µl washing and imaging buffer three times at room temperature for 10 min each.
52. Prepare 100 ng/ml of 400 µl DAPI from 5 mg/ml stock in the washing and imaging buffer and stain the samples for 3 h.
▲ **CRITICAL STEP** The DAPI signal is critical for cell segmentation and incubation duration varies depending on tissue type. Overnight staining might be needed to achieve a better signal-to-noise ratio for some organs.

53. Wash the samples with washing and imaging buffer two times at room temperature for 5 min each. Then, immerse the samples in the washing and imaging buffer for imaging.
▲ **CRITICAL STEP** Larger volumes of washing and imaging buffer are required for long sequencing sessions (>4 h). Add water to neighboring unused wells to keep the sequencing wells moist.
54. Affix the sample securely to the microscope stage to mitigate any potential sources of movement, ensuring stability throughout the imaging process.
55. Configure a four-channel microscope with the appropriate excitation light sources and emission filters. For imaging on the Leica TCS SP8 confocal microscope with a 405nm laser, a white light laser and four HyD detectors, use the bidirectional sequential scan (optional) mode between lines. In sequential scan 1, use a 495 nm laser line and set the HyD 1 detector to the 508–555 nm range to image Alexa Fluor 488; use a 590 nm laser line and set the HyD 3 detector to 605–655 nm to image Alexa Fluor 594. In sequential scan 2, use a 551 nm laser line and set the HyD 2 detector to the 560–600 nm range to image Alexa Fluor 546; use a 653 nm laser line and set the HyD 4 detector to the 665–745 nm range to image Alexa Fluor 647. Finally, in sequential scan 3, use a 405 nm laser line and HyD 1 detector to image DAPI.
▲ **CRITICAL STEP** It is expected that individual amplicons appear in a single color. Prior to finalizing imaging settings, make sure to preclude channel crosstalk, a phenomenon where a fluorescence channel's signal erroneously appears in another. To evaluate, deactivate the laser for a specific dye, and then, monitor whether the corresponding detector captures signals from other dyes before the start of each imaging session. Addressing channel crosstalk is crucial as it can compromise data integrity, leading to potentially false or misleading results.
56. Utilize a lower magnification objective (for example, 10× or 20×) to scan the entire sample, and then, switch to higher magnification objective (for example, 40× or 63×) to assess the density and distribution of the amplicons. The amplicons should appear as round-shaped entities under 40× and 63×, each with a diameter on the order of hundreds of nanometers. Avoid the use of too strong ultraviolet light to prevent DAPI photoconversion.
57. For data collection, we typically use a 40× NA 1.3 oil-immersion objective to examine tissue sections and cultured cell monolayers. However, when imaging at high amplicon densities, such as when visualizing over 1,000 genes simultaneously, we use a 63× NA 1.4 oil-immersion objective for high-resolution imaging. It is recommended to ensure that the x–y pixel size remains below 0.15 µm, and the z-step size is less than 0.35 µm to maintain optimal imaging conditions.
58. Adjust the laser intensity meticulously to ascertain that the fluorescence signal is within the dynamic range of the detector, thereby preventing image overexposure and ensure accurate spot finding and quantitative analysis.
▲ **CRITICAL STEP** Overexposure of the image can result in the merging of intensity profiles of proximate amplicons. This confluence impedes the ability to distinguish between closely situated amplicons.
59. Acquire a 10-µm-thick (or any thickness within the working distance of the objective) volumetric image of the targeted region. Acquiring data in 3D can help to mitigate the spatial crowding of amplicons and increase transcript detection efficiency and accuracy, especially at the scale of thousands of genes.
▲ **CRITICAL STEP** Perform imaging within a darkroom setting to eliminate ambient light interference, ensuring the acquisition of high-fidelity fluorescence signals.
60. Upon validating the imaging quality of the finished acquisition, carefully aspirate the wash and imaging buffer to minimize disruption to the gel matrix and uphold the stability of the plate. Subsequently, gently perform a thorough wash using the stripping buffer twice for 10 min each. Then wash with PBST three times for 10 min each.
▲ **CRITICAL STEP** Avoid removing the plate from the microscope when possible.
61. Repeat Steps 50–51 and 58–60 using different reading probes (SeqA1-6, SeqB1-4).
62. After all sequencing rounds, incubate the sample with the appropriate secondary antibody with an Alexa fluorophore (combination of antibodies can be used with spectrally distinct fluorophores) at room temperature for at least 12 h. We recommend a high concentration of secondary antibodies (such as 1:100 dilution from the antibody stock). For RIBOmap

and TEMPOmap, optional small-molecule staining (for example, DAPI, concanavalin A, Flamingo[16,17]) can be used for cell segmentation and organelle detection purposes.

▲ **CRITICAL STEP** Steps 62–63 are for STARmap PLUS specifically. Antibody concentration and the incubation time are dependent on the specific antibodies.

63. Wash the sample three times with PBST for 5 min each. Proceed to imaging following Steps 58–59.

Image processing

● **TIMING 1–10 d**

64. Extract microscopic imaging parameters from a small, cropped test image of the experiment by importing it to Huygens Essential software. Users can save the parameters as a template for future reference.
65. Determine the optimal deconvolution parameters with the same cropped test image. In Huygens Essential, we typically use the CMLE mode, ten iterations and a signal-to-noise ratio from 5 to 10. See the images of cells processed by RIBOmap before and after deconvolution (Fig. 6a).
66. Deconvolve all images and save images as single 3D stacks in the uint8 format in a folder named 'sample-dataset'. The output format is specified in Huygens Essential with the option 'TIFF, 8-bit, single file, scaled'.
67. Put the gene-to-barcode conversion table as a 'genes.csv' file in the sample-dataset folder.
68. Organize images according to the sequencing round label and FOV ID as shown in the file tree below:

```
# Folder Name (related annotation)
sample-dataset (Dataset ID):
--round1 (sequencing round number)
       --FOV1 (FOV ID)
            --ch01.tif (3D image stack for individual channel)
            --ch02.tif
            --ch03.tif
            --ch04.tif
            --...
       --FOV2
       --...
--round2
--...
--genes.csv
```

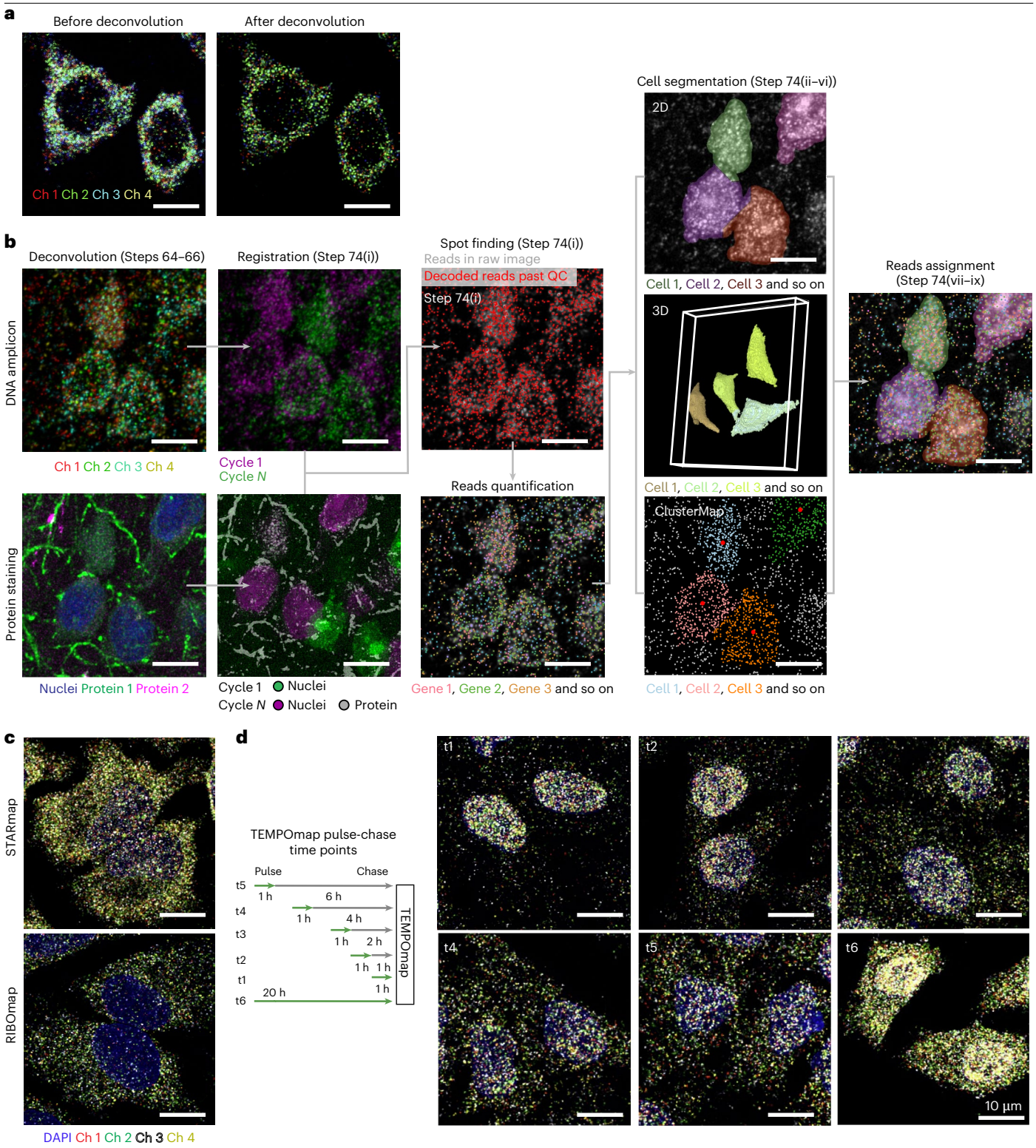
▲ **CRITICAL STEP** Users who have little or no background in bioinformatics can benefit from the introduction of common computational environments and tools. It is recommended to seek additional assistance from experienced users, network administrators and other online resources.

▲ **CRITICAL STEP** The example workflow here is for analyzing each FOV sequentially and independently using a sample dataset we provide. Users can run the workflow on either a remote host or a local workstation as long as they meet the minimum system memory requirements (Step 71). It is possible to parallelize and accelerate the computation by creating array jobs if supported by the high-performance computing cluster. Contact your network administrator for assistance if needed.

69. Download Starfinder package (https://github.com/wanglab-broad/starfinder) and copy it to a remote host using a command-line terminal:

```
local:~$ scp starfinder.zip <user@remote_host_name:~/>
```

▲ **CRITICAL STEP** Ask the network administrator at your institution if one needs an account to a designated remote host.



70. Download and unzip sample-dataset.zip (https://doi.org/10.5281/zenodo.11176779). Copy the sample-dataset folder to the remote host:

```
local:~$ scp -r ~/sample-dataset/ <user@remote_host_name:~/>
```

Fig. 6 | Data processing pipeline and representative images showing the anticipated results of STARmap PLUS, RIBOmap and TEMPOmap. **a**, An example of RIBOmap fluorescent image of two cells before and after deconvolution. **b**, A diagram showing data processing pipeline of STARmap PLUS to extract the gene expression from the decoded amplicons reads in the raw images of 64 genes in mouse brain tissue (see the 'Procedure' steps indicated)[15]. DNA amplicons should appear as punctas with a relatively high intensity profile compared with the background. After quantifying the reads, we recommend visualizing the spatial pattern of representative gene markers across all FOVs to confirm accurate signal detection. Note that the cells shown in the 3D segmentation panel do not correspond to the cells in other steps of the workflow. QC, quality control. **c**, Mapping of ribosome-bound RNAs (981 genes) using RIBOmap and the same group of RNAs using STARmap[16]; for RIBOmap data, DNA amplicon is not expected to be enriched in the nucleus compared with STARmap. **d**, Pulse-chase experiment design (left) on HeLa cells and the images of in-process TEMPOmap at each pulse-chase time point (right)[17]. All the cell images show amplicons in one cycle of sequencing. In the TEMPOmap data, the amplicon density in different cellular compartments varies based on the pulse-chase experiment duration. As shown previously[17], a longer chase experiment results in decreased amplicon density in the nucleus. t1–6, pulse-chase time series 1–6.

▲ **CRITICAL STEP** Analyzing large volumes of image stacks requires additional disk space. Contact your network administrator for assistance if needed.

71. Connect to the remote host and initiate an interactive session with high memory by submitting a job request, as follows:

```
local:~$ ssh -r <user@remote_host_name>
remote:~$ qrsh -q interactive -now n -l h_vmem=70G
```

▲ **CRITICAL STEP** It is recommended to allocate a minimum of 70 GB of memory

72. Unzip starfinder.zip and change the working directory to starfinder:

```
remote:~$ unzip starfinder.zip
remote:~$ cd starfinder
```

73. Create a starfinder python conda environment and install related software packages with conda:

```
remote:~/starfinder$ conda env create --file config/environment.yaml
```

74. Start MATLAB and add a search path:

```
remote:~/starfinder$ matlab
>>addpath('./code-base/src', './example/sequential_workflow')
```

75. To perform the complete preprocessing procedures, use option A for a tissue section example or option B for a cell culture example with different cell segmentation strategies.

(A) **Preprocessing: tissue section example**

(i) Run the image registration and reads extraction script as follows. The script also creates two-dimensional (2D) projections of protein staining images and sequencing composite images for stitching.

```
>>rsf_workflow_example('~/sample-dataset/tissue-2D/dataset-info.
json')
```

▲ **CRITICAL STEP** As an example workflow, it only processes one FOV with the default setting. To complete process the whole dataset, please modify the 'number_of_fovs' parameter in dataset-info.json file accordingly.

(ii) Use Fiji/ImageJ Stitching plugin (Plugins > Stitching > Grid/Collection stitching) to merge projections located at 'images/protein' and 'images/ref_merged' folders from workflow output. Dataset specific grid configuration can be found in the corresponding dataset-info.json file. Once the fusion process is finished, save fused images to folder sample-dataset/tissue-2D/images/fused.

▲ **CRITICAL STEP** The grid configuration is determined during image acquisition. Additional image rotation to every FOV may be necessary to correctly align the input images with the stitching order. We recommend users to first stitch the images of the nucleus staining channel to test whether the stitching order and image orientation are correct.

(iii) Use the TileConfiguration.registered.txt file generated from the previous step to merge detected amplicon signals of each FOV:

```
>>reads_stitching('~/sample-dataset/tissue-2D/dataset-info.json')
>>quit()
```

(iv) Use Fiji/ImageJ StarDist plugin (Plugins > StarDist 2D) to segment fused DAPI images from Step 75A(ii). We typically use the 'Versatile (fluorescent nuclei)' model with a probability threshold of 0.4–0.6 and an overlap threshold of 0.3–0.6, depending on sample histology.

▲ **CRITICAL STEP** Processing large, fused images requires additional memory space. Scale down the image or increase the 'Number of Tiles' parameter to improve the performance.

▲ **CRITICAL STEP** StarDist segmentation performance might vary depending on cell morphology and image resolution. We suggest training a customized model to achieve the best performance. For tutorial of advanced StarDist segmentation or model training, please refer to GitHub at https://github.com/stardist/stardist.

(v) Open the fused DAPI staining and sequencing image in Fiji/ImageJ (Step 75A(ii)) and create a composite image (Image > Color > Merge Channels) with these two, then convert it into 8-bit format (Image > Type > RGB color > 8-bit).

▲ **CRITICAL STEP** Users can adjust the intensity profile of each channel (Image > Adjust > Color Balance) in the composite image from 'Merge Channels' option so that it can better describe the cell boundaries.

(vi) Enhance the contrast of the composite image with Fiji/ImageJ (Process > Enhance Contrast, Saturated Pixels: 1%, Normalize) and save it as overlay.tif in the sample-dataset/tissue-2D/images/fused folder. This composite image will later be used as input for cell boundary segmentation.

(vii) Activate starfinder conda environment and run reads assignment in python:

```
remote:~/starfinder$ conda activate starfinder
remote:(starfinder)~/starfinder$ python example/sequential_
workflow/reads_assignment.py ~/sample-dataset/tissue-2D/dataset-
info.json
```

(viii) Alternatively, for reads assignment with ClusterMap (https://github.com/wanglab-broad/ClusterMap), the user can skip Step 75A(v–vi), activate starfinder conda environment, and then run the following script in python:

```
remote:~/starfinder$ conda activate starfinder
remote:(starfinder)~/starfinder$ python example/sequential_
workflow/run_clustermap.py ~/sample-dataset/tissue-2D/dataset-
info.json
```

▲ **CRITICAL STEP** User needs to install ClusterMap by following the provided tutorial via GitHub (https://github.com/wanglab-broad/ClusterMap).

(ix) Stitch ClusterMap reads assignment results:

```
remote:(starfinder)~/starfinder$ python example/sequential_
workflow/stitch_clustermap.py ~/sample-dataset/tissue-2D/
dataset-info.json
```

(B) **Preprocessing: cell culture example**

(i) Run image registration and reads extraction script:

```
>>rsf_workflow_example('~/sample-dataset/cell-culture-3D/
dataset-info.json')
```

▲ **CRITICAL STEP** System memory requirements vary depending on the dataset, and users should adjust accordingly.

▲ **CRITICAL STEP** A minimum of 40 GB system memory is required.

(ii) Use Fiji/ImageJ Stitching plugin (Plugins > Stitching > Grid/Collection stitching) to stitch 3D images of the DAPI staining and Flamingo staining and save fused images to folder sample-dataset/cell-culture-3D/images/fused. Grid configuration should follow the previous image acquisition setting (see Step 75A(ii)).

(iii) For each fused image, create a downsampled copy with a downsampling factor of 0.5 for the following 3D cell segmentation steps. Then create an additional maximum projection image for each scaled-down image for the following CellProfiler segmentation workflow.

▲ **CRITICAL STEP** Users can refer to the examples in the expected outcomes in Fig. 6.

(iv) Use the TileConfiguration.registered.txt file to stitch detected amplicon signals of each FOV:

```
>>reads_stitching('~/sample-dataset/cell-culture-3D/dataset-
info.json')
```

(v) Create reference segmentation masks with the provided CellProfiler workflow starfinder/example/sequential_workflow/create_reference_segmentation.cpproj.

(vi) Run 3D segmentation script to generate segmentation masks for each cellular compartment:

```
>>create_3d_segmentation('~/sample-dataset/cell-culture-3D/
dataset-info.json')
>>quit()
```

(vii) Activate starfinder conda environment and run reads assignment in python:

```
remote:~/starfinder$ conda activate starfinder
remote:(starfinder)~/starfinder$ python example/sequential_
workflow/reads_assignment_cell_culture.py ~/sample-dataset/
cell-culture-3D/dataset-info.json
```

Data analysis

● **TIMING 1–3 d**

▲ **CRITICAL** Downstream analysis can be done with any single-cell transcriptomics software package, but Scanpy in Python is recommended and integrated into the workflow. A Jupyter lab/notebook may be more intuitive for novice users compared to the command-line interface. We offer a couple of sample notebooks and expected outcomes from the workflow for an easier start (https://github.com/wanglab-broad/starfinder/tree/main/example/downstream).

```
remote:(starfinder)~/starfinder$ cd example/downstream && jupyter-lab
```

76. We recommend consulting published datasets for protocol-specific analysis, especially for STARmap PLUS (https://github.com/wanglab-broad/mAD-analysis, https://github.com/wanglab-broad/mCNS-atlas), RIBOmap (https://github.com/wanglab-broad/RIBOmap-analysis) and TEMPOmap (https://github.com/wanglab-broad/TEMPOmap). Example

scripts are available (Step 75 and GitHub sites) for various analyses within each protocol: cell type classification, protein level quantification, and spatial gene expression analysis in STARmap PLUS; covariational gene module detection, colocalization analysis and multi-modal dataset integration in RIBOmap; trajectory inference and RNA kinetic modeling in TEMPOmap. Moreover, we recommend that users refer to several state-of-the-art tutorials implemented in both python (https://www.sc-best-practices.org/preamble.html) and R (https://bioconductor.org/books/release/OSCA/).

77. To start cell type classification in tissue samples, users should exclude low-quality cells using metrics such as total reads per gene, number of genes per cell and cell segmentation volumes. Next, apply preprocessing steps such as cell-level counts normalization, logarithmic transformation, gene-level standardization and batch effect removal to the filtered gene expression profiles. Then utilize specific gene sets (for example, cell type markers) and conduct dimensionality reduction with techniques such as principal component analysis to extract eminent gene expression features from noise. Clustering algorithms, such as the Leiden community detection algorithm, could be applied for cell classification. The principal component analysis matrix can be used to create a *k*-nearest neighbor graph connecting cells based on the similarity in the high-dimensional latent space. The Leiden community detection algorithm was applied over the *k*-nearest neighbor graph to detect cell clusters. Finally, cell clusters can be annotated based on canonical markers.
78. For other downstream analysis such as cell cycle phase identification, users can subset the expression profiles of cells restricted to cycle marker genes and calculate cell cycle scores as illustrated in these tutorials (for python, https://nbviewer.org/github/theislab/scanpy_usage/blob/master/180209_cell_cycle/cell_cycle.ipynb; for R, https://satijalab.org/seurat/articles/cell_cycle_vignette).

Troubleshooting

Troubleshooting advice can be found in Table 6.

Table 6 | Troubleshooting

Step	Problem	Possible reason	Solution
9B	Tissues detached from the plate	Poly-D-lysine coating is not optimal; small contact area between sample and glass or not enough adherence Water condenses and turns into ice on the coverslip in the Crystat	Store the plate with mounted tissues at room temperature for 3–4 min to allow OCT melting and tissue adherence; make sure the plate is completely dried. Prewarm the coverslip with thumbs to thaw and evaporate ice on the coverslip before attaching tissue slices
40–41	Hydrogel detached	Plate is not properly coated with Bind-Silane	Prolong Bind-Silane treatment
	Hydrogel not formed	Hydrogel reagents have deteriorated	Check the reagent stocks and make sure all reagents are within the expiration date. We recommended dissolving ammonium persulfate fresh each time before the reaction; Degas monomer buffer in nitrogen-purged box
49	Amplicons limited to the edges of tissue	Poor cell permeabilization or limited enzyme penetration	Change the permeabilization step to 0.5–1% Triton in PBS supplemented with SUPERase Inhibitor
	Amplicons too large	RCA duration too long	Reduce the reaction time of RCA
	High background in tissues	Nonspecific ligation by endogenous phosphorylated DNA fragments; amplicons are dim; amplicons are not well-anchored in the hydrogel; autofluorescence in the tissue	Extend the dephosphorylation reaction at 37 °C to overnight; extend RCA step; check the reagent quality for hydrogel gelation; remove autofluorescence by proteinase K and/or photobleaching
	Amplicons outside tissue	Excessive permeabilizations or RNA degradation; the poly-D-lysine coating did not get quenched after PFA fixation and thus crosslinked with the probes	Reduce the time of the permeabilization step and (or) decrease the concentration of detergent in hybridization step; check if there is RNase contamination in reagents; use RNase free reagents

Timing

Steps 1–48, DNA amplicon library construction: 2–3 d
Steps 49–63, sequencing and imaging: 1–20 d
Steps 64–75, image processing: 1–10 d
Steps 76–78, data analysis: 1–3 d
The total timeline required for sequencing and image acquisition varies depending on a range of factors. Some critical factors include (1) the microscopy parameters for image acquisition, such as the magnification objective and imaging resolution (here, we recommend keeping *xy* pixel size below 0.15 µm, and *z* pixel size ~350 nm); (2) the number of targeted genes (directly related to the number of sequencing rounds); (3) the size of the tissue slices to be imaged (*x*–*y* dimensions) and the thickness of tissue slices (the number of *z*-steps to be acquired); (4) other variations, including the type of confocal microscope (point scanning versus spinning disk), number of detectors or cameras, time duration of ligation reaction between each imaging cycle. Therefore, we strongly recommend that researchers estimate the total time needed before planning data collection. A specific example of acquisition time for acquiring a 10-µm mouse brain hemisphere coronal tissue can be found in the 'Limitations' section.

Anticipated results

The raw and deconvolved images of DNA amplicon libraries of cell cultures are shown (Fig. 6a). Meanwhile, we have also included raw images in both intact cells and brain tissues (Fig. 6b–d). It is likely that the capacity for detecting protein signals is varied depending on the proteins or the nature of the imaged samples in the workflow of STARmap PLUS. Therefore, we strongly recommend testing the performance of antibodies against the proteins of interest with and without the experimental procedure of STARmap PLUS. In addition, we anticipate subcellular patterns for RIBOmap and TEMPOmap signals. RIBOmap amplicons are mostly devoid of cell nuclei (Fig. 6c). TEMPOmap amplicons, when pulsed and chased in a properly timed manner, should yield a general cell nucleus-to-cytoplasm localization trend across chase times (Fig. 6d).

Regardless of the methodology, the size of each amplicon is ~200 nm at 40× NA 1.3 after image deconvolution. We anticipate that the typical amplicons are 20–50 times brighter than single-molecule FISH[52] and usually require less than 5% laser intensity to image each color channel using Leica TCS SP8 and Stellaris 8 systems with no crosstalk. A good amplicon library of >64 genes should comprise RNAs with a wide dynamic range of gene expression and RNAs that represent well-validated cell-type or subcellular marker genes. With >64 genes mapped in a library, one should see bright amplicons with a high signal-to-noise ratio that can roughly outline cell boundaries and are distinct from background fluorescence outside the cells or tissues. The fluorescence intensity in four color channels in each round of imaging should be evenly balanced from visual inspection and may require further adjustment of laser intensities across different imaging cycles. The amplicon density may vary depending on the abundance of targeted genes. We expect that the fluorescence of amplicons is completely removed when the sequencing-by-ligation products are stripped (Step 47). We also recommend leaving the ligation reaction longer (3 h to overnight) for later sequencing cycles because the effective concentration of reading probes drops as the sequencing cycle progresses and the number of degenerative bases in the reading probe increases.

The size of an image stack with multiple optical planes can vary from hundreds of megabytes to several gigabytes, depending on the microscope objective, spatial resolution and image bit depth. Upon analysis and processing by our software, the system produces a comma-separated file detailing each signal's spatial location and gene ID. The corresponding log files, inclusive of statistical metrics such as the percentage of reads correctly matching the codebook, can assist in quality evaluation. We also advise a preliminary data review by visualizing a gene that demonstrates a typical spatial pattern and comparing it with existing ISH data. The optimal

cell segmentation result is a 16-bit label image, with unique scalar values differentiating cellular regions. After the reads assignment, the data—comprising a gene expression matrix, cell annotation table and gene annotation table—are preserved in three comma-separated files. These files are compatible for subsequent analysis with tools such as Scanpy[50] in Python or Seurat[53] in R.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The datasets mentioned and discussed in this protocol are available in the supporting primary research articles[15–17]. All of the processed sequencing data are available in the Single Cell Portal (STARmap PLUS: https://singlecell.broadinstitute.org/single_cell/study/SCP1375, https://singlecell.broadinstitute.org/single_cell/study/SCP1830; RIBOmap: https://singlecell.broadinstitute.org/single_cell/study/SCP1835; TEMPOmap: https://singlecell.broadinstitute.org/single_cell/study/SCP1792) and Zenodo (STARmap PLUS: https://doi.org/10.5281/zenodo.7332091, https://doi.org/10.5281/zenodo.8327576; RIBOmap: https://doi.org/10.5281/zenodo.8041114; TEMPOmap: https://doi.org/10.5281/zenodo.7803716). The demo dataset for tutorial purposes is available via Single Cell Portal at https://singlecell.broadinstitute.org/single_cell/study/SCP2637 and via Zenodo at https://doi.org/10.5281/zenodo.11176779 (ref. 54). Additional information is available at the Wang Lab website (https://www.wangxiaolab.org). Additional raw images or data are available for research purposes upon request from the corresponding author.

Code availability

All codes and analyses are available via GitHub (STARmap PLUS: https://github.com/wanglab-broad/mAD-analysis, https://github.com/wanglab-broad/mCNS-atlas; RIBOmap: https://github.com/wanglab-broad/RIBOmap-analysis; TEMPOmap: https://github.com/wanglab-broad/TEMPOmap) and Zenodo (STARmap PLUS: https://doi.org/10.5281/zenodo.7332091; RIBOmap: https://doi.org/10.5281/zenodo.8041114; TEMPOmap: https://doi.org/10.5281/zenodo.7803716). Probe design is available via GitHub at https://github.com/wanglab-broad/probe-design. Starfinder analysis tool will be maintained and updated at https://github.com/wanglab-broad/starfinder. ClusterMap segmentation method is available at https://github.com/wanglab-broad/ClusterMap. Additional requests can be made by contacting the corresponding author.

Received: 21 May 2024; Accepted: 25 June 2025;
Published online: 30 September 2025

References

1. Tabula Sapiens Consortium*. et al. The Tabula Sapiens: a multiple-organ, single-cell transcriptomic atlas of humans. *Science* **376**, eabl4896 (2022).
2. Elmentaite, R., Domínguez Conde, C., Yang, L. & Teichmann, S. A. Single-cell atlases: shared and tissue-specific cell types across human organs. *Nat. Rev. Genet.* **23**, 395–410 (2022).
3. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* **348**, aaa6090–aaa6090 (2015).
4. Eng, C.-H. L. et al. Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH. *Nature* **568**, 235–239 (2019).
5. Borm, L. E. et al. Scalable in situ single-cell profiling by electrophoretic capture of mRNA using EEL FISH. *Nat. Biotechnol.* **41**, 222–231 (2023).
6. Gyllborg, D. et al. Hybridization-based in situ sequencing (HybISS) for spatially resolved transcriptomics in human and mouse brain tissue. *Nucleic Acids Res.* **48**, e112 (2020).
7. Sun, Y.-C. et al. Integrating barcoded neuroanatomy with spatial transcriptional profiling enables identification of gene correlates of projections. *Nat. Neurosci.* **24**, 873–885 (2021).
8. Lee, J. H. et al. Highly multiplexed subcellular RNA sequencing in situ. *Science* **343**, 1360–1363 (2014).
9. Alon, S. et al. Expansion sequencing: spatially precise in situ transcriptomics in intact biological systems. *Science* **371**, eaax2656 (2021).
10. Wang, X. et al. Three-dimensional intact-tissue sequencing of single-cell transcriptional states. *Science* **361**, eaat5691 (2018).
11. Schwanhüusser, B. et al. Global quantification of mammalian gene expression control. *Nature* **473**, 337–342 (2011).
12. Battich, N. et al. Sequencing metabolically labeled transcripts in single cells reveals mRNA turnover strategies. *Science* **367**, 1151 (2020).
13. VanInsberghe, M., van den Berg, J., Andersson-Rolf, A., Clevers, H. & van Oudenaarden, A. Single-cell Ribo-seq reveals cell cycle-dependent translational pausing. *Nature* **597**, 561–565 (2021).
14. Ozadam, H. et al. Single-cell quantification of ribosome occupancy in early mouse development. *Nature* **618**, 1057–1064 (2023).

15. Zeng, H. et al. Integrative in situ mapping of single-cell transcriptional states and tissue histopathology in a mouse model of Alzheimer's disease. *Nat. Neurosci.* **26**, 430–446 (2023).
16. Zeng, H. et al. Spatially resolved single-cell translatomics at molecular resolution. *Science* **380**, eadd3067 (2023).
17. Ren, J. et al. Spatiotemporally resolved transcriptomics reveals the subcellular RNA kinetic landscape. *Nat. Methods* **20**, 695–705 (2023).
18. Ren, J., Luo, S., Shi, H. & Wang, X. Spatial omics advances for in situ RNA biology. *Mol. Cell* **84**, 3737–3757 (2024).
19. Liu, Y. et al. High-plex protein and whole transcriptome co-mapping at cellular resolution with spatial CITE-seq. *Nat. Biotechnol.* **41**, 1405–1409 (2023).
20. Ben-Chetrit, N. et al. Integration of whole transcriptome spatial profiling with protein markers. *Nat. Biotechnol.* **41**, 788–793 (2023).
21. Vickovic, S. et al. SM-Omics is an automated platform for high-throughput spatial multi-omics. *Nat. Commun* **13**, 795 (2022).
22. Frei, A. P. et al. Highly multiplexed simultaneous detection of RNAs and proteins in single cells. *Nat. Methods* **13**, 269–275 (2016).
23. Burke, K. S., Antilla, K. A. & Tirrell, D. A. A fluorescence in situ hybridization method to quantify mRNA translation by visualizing ribosome–mRNA interactions in single cells. *ACS Cent. Sci.* **3**, 425–433 (2017).
24. tom Dieck, S. et al. Direct visualization of newly synthesized target proteins in situ. *Nat. Methods* **12**, 411–414 (2015).
25. Erhard, F. et al. scSLAM-seq reveals core features of transcription dynamics in single cells. *Nature* **571**, 419–423 (2019).
26. Cao, J., Zhou, W., Steemers, F., Trapnell, C. & Shendure, J. Sci-fate characterizes the dynamics of gene expression in single cells. *Nat. Biotechnol.* **38**, 980–988 (2020).
27. Hendriks, G.-J. et al. NASC-seq monitors RNA synthesis in single cells. *Nat. Commun.* **10**, 3138 (2019).
28. Qiu, Q. et al. Massively parallel and time-resolved RNA sequencing in single cells with scNT-seq. *Nat. Methods* **17**, 991–1001 (2020).
29. Shi, H. et al. Spatial atlas of the mouse central nervous system at molecular resolution. *Nature* **625**, 552–561 (2023).
30. Maher, K. et al. Mitigating autocorrelation during spatially resolved transcriptomics data analysis. Preprint at *bioRxiv* https://doi.org/10.1101/2023.06.30.547258 (2023).
31. Chen, H. et al. Branched chemically modified poly(A) tails enhance the translation capacity of mRNA. *Nat. Biotechnol* **43**, 194–203 (2025).
32. Chen, H. et al. Chemical and topological design of multicapped mRNA and capped circular RNA to augment translation. *Nat. Biotechnol* **43**, 1128–1143 (2025).
33. He, Y. et al. ClusterMap for multi-scale clustering analysis of spatial gene expression. *Nat. Commun.* **12**, 5909 (2021).
34. Li, Q. et al. Multimodal charting of molecular and functional cell states via in situ electro-sequencing. *Cell* **186**, 2002–2017.e21 (2023).
35. Sui, X. et al. Scalable spatial single-cell transcriptomics and translatomics in 3D thick tissue blocks. Preprint at *bioRxiv* https://doi.org/10.1101/2024.08.05.606553 (2024).
36. Jao, C. Y. & Salic, A. Exploring RNA transcription and turnover in vivo by using click chemistry. *Proc. Natl Acad. Sci. USA* **105**, 15779–15784 (2008).
37. Rothschild, D. et al. Diversity of ribosomes at the level of rRNA variation associated with human health and disease. *Cell Genom* **4**, 100629 (2024).
38. Tang, Z. et al. Search and match across spatial omics samples at single-cell resolution. *Nat. Methods* **21**, 1818–1829 (2024).
39. Goltsev, Y. & Nolan, G. CODEX multiplexed tissue imaging. *Nat. Rev. Immunol.* **23**, 613 (2023).
40. Fazal, F. M. et al. Atlas of subcellular RNA localization revealed by APEX-seq. *Cell* **178**, 473–490.e26 (2019).
41. Tirosh, I. et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* **352**, 189–196 (2016).
42. Zhang, Y. et al. Gene panel selection for targeted spatial transcriptomics. *Genome Biol.* **25**, 35 (2024).
43. Chou, H.-H. Shared probe design and existing microarray reanalysis using PICKY. *BMC Bioinformatics* **11**, 196 (2010).
44. Bushnell, B. BBMap: a fast, accurate, splice-aware aligner. *US Department of Energy Office of Scientific and Technical Information* https://www.osti.gov/biblio/1241166 (2014).
45. Zadeh, J. N. et al. NUPACK: analysis and design of nucleic acid systems. *J. Comput. Chem.* **32**, 170–173 (2011).
46. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402 (1997).
47. Weigert, M. et al. Star-convex polyhedra for 3D object detection and segmentation in microscopy. in *Proc. 2020 IEEE Winter Conference on Applications of Computer Vision* 3655–3662 (IEEE, 2020).
48. Stirling, D. R. et al. CellProfiler 4: improvements in speed, utility and usability. *BMC Bioinformatics* **22**, 433 (2021).
49. Hao, Y. et al. Dictionary learning for integrative, multimodal and scalable single-cell analysis. *Nat. Biotechnol.* **42**, 293–304 (2024).
50. Wolf, F. A., Angerer, P. & Theis, F. J. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol.* **19**, 1–5 (2018).
51. Palla, G. et al. Squidpy: a scalable framework for spatial omics analysis. *Nat. Methods* **19**, 171–178 (2022).
52. Lee, J. H. et al. Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. *Nat. Protoc.* **10**, 442–458 (2015).
53. Stuart, T. et al. Comprehensive integration of single-cell data. *Cell* **177**, 1888–1902.e21 (2019).
54. Ren, J. et al. Example dataset and expected outcomes of Spatially resolved in situ profiling of mRNA life cycle at transcriptome scale in intact cells and tissues. *Zenodo* https://doi.org/10.5281/zenodo.11176779 (2024).

Acknowledgements

H.S. is supported by Helen Hay Whitney Foundation postdoctoral fellowship. X.W. gratefully acknowledges support from the Searle Scholars Program, Thomas D. and Virginia W. Cabot Professorship, Edward Scolnick Professorship, Ono Pharma Breakthrough Science Initiative Award, Packard Fellowship for Science and Engineering, Merkin Institute Fellowship, NIH DP2 New Innovator Award, and Stanley Center gift from the Broad Institute. We thank Y. Zhou, W. X. Wang, M. Wu, Q. Zhang and Y. He for their helpful suggestions to the manuscript.

Author contributions

J.R. and H. Zeng. designed the protocols and performed the experiments. J.H., X.S., C.K.W., H. Zhou, M.W., Z.T. and S.L. performed the computational analysis. J.H. and M.W. cleaned up the starfinder package. J.R., H. Zeng and J.H. wrote the manuscript. H.S., J.T. and X.S. provided critical comments for the manuscript. X.W. supervised the study. All authors critically reviewed and revised the manuscript.

Competing interests

X.W. is a scientific cofounder of Stellaromics. X.W., J.R. and H. Zeng are inventors on patent applications (International Application No. PCT/US2022/031275, No. PCT/US2022/035271 and No. PCT/US2022/028012) related to STARmap PLUS, RIBOmap and TEMPOmap. The other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-025-01248-3.

Correspondence and requests for materials should be addressed to Xiao Wang.

Peer review information *Nature Protocols* thanks Junyue Cao and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

nature portfolio

Corresponding author(s): Xiao Wang

Last updated by author(s): Jun 30, 2025

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 (*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	Leica LAS-X microscope imaging software 3.5.5.19976 was used during data acquisition. Huygens 20.10 was used for image deconvolution.
Data analysis	All codes and analyses are available on Github (STARmap PLUS: https://github.com/wanglab-broad/mAD-analysis, https://github.com/wanglab-broad/mCNS-atlas; RIBOmap: https://github.com/wanglab-broad/RIBOmap-analysis; TEMPOmap: https://github.com/wanglab-broad/TEMPOmap) and Zenodo (STARmap PLUS: https://doi.org/10.5281/zenodo.7332091; RIBOmap: https://doi.org/10.5281/zenodo.8041114; TEMPOmap: https://doi.org/10.5281/zenodo.7803716). Probe design is available at https://github.com/wanglab-broad/probe-design. Starfinder analysis tool will be maintained and updated at https://github.com/wanglab-broad/starfinder. ClusterMap segmentation method is available at https://github.com/wanglab-broad/ClusterMap.

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 datasets mentioned and discussed in this protocol are available in the supporting primary research articles (cited in the article). All of the processed sequencing data are available in the Single Cell Portal (STARmap PLUS: https://singlecell.broadinstitute.org/single_cell/study/SCP1375, https://singlecell.broadinstitute.org/single_cell/study/SCP1830; RIBOmap: https://singlecell.broadinstitute.org/single_cell/study/SCP1835; TEMPOmap: https://singlecell.broadinstitute.org/single_cell/study/SCP1792) and Zenodo (STARmap PLUS: https://doi.org/10.5281/zenodo.7332091, https://doi.org/10.5281/zenodo.8327576; RIBOmap: https://doi.org/10.5281/zenodo.8041114; TEMPOmap: https://doi.org/10.5281/zenodo.7803716). The demo dataset for tutorial purposes is available at Single Cell Portal (https://singlecell.broadinstitute.org/single_cell/study/SCP2637) and Zenodo (https://doi.org/10.5281/zenodo.11176779). Additional information is available at the Wang Lab website (https://www.wangxiaolab.org). Additional raw images or data are available for research purposes upon request from the corresponding author.

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	Sex and gender are not applicable for this protocol manuscript.
Reporting on race, ethnicity, or other socially relevant groupings	Race, ethnicity, or other socially relevant groupings are not applicable for this protocol manuscript.
Population characteristics	Population characteristics are not applicable for this protocol manuscript.
Recruitment	Recruitment is not applicable for this protocol manuscript.
Ethics oversight	Ethics oversight is not applicable for this protocol manuscript.

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	Not applicable because this is a protocol manuscript with no particular study design.
Data exclusions	Not applicable because this is a protocol manuscript with no particular study design.
Replication	Not applicable because this is a protocol manuscript with no particular study design.
Randomization	Not applicable because this is a protocol manuscript with no particular study design.
Blinding	Not applicable because this is a protocol manuscript with no particular study design.

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

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	Human HeLa cell line: purchased from ATCC (CCL-2, RRID:CVCL_0030, https://scicrunch.org/resolver/CVCL_0030)
Authentication	The cell line has been authenticated by the STR method.
Mycoplasma contamination	The cell lines were confirmed as mycoplasma negative by DAPI (4",6-diamidino-2-phenylindole) or Hoechst DNA staining and microscope imaging.
Commonly misidentified lines (See ICLAC register)	None.

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	The Jackson Laboratory, C57BL/6
Wild animals	None.
Reporting on sex	Not applicable.
Field-collected samples	Not applicable.
Ethics oversight	All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Broad Institute of MIT and Harvard.

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

Plants

Seed stocks	Not applicable.
Novel plant genotypes	Not applicable.
Authentication	Not applicable.