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Progress

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Sequencing technologies to measure translation in single cells

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Abstract

Translation is one of the most energy-intensive processes in a cell and, accordingly, is tightly regulated. Genome-wide methods to measure translation and the translatome and to study the complex regulation of protein synthesis have enabled unprecedented characterization of this crucial step of gene expression. However, technological limitations have hampered our understanding of translation control in multicellular tissues, rare cell types and dynamic cellular processes. Recent optimizations, adaptations and new techniques have enabled these measurements to be made at single-cell resolution. In this Progress, we discuss single-cell sequencing technologies to measure translation, including ribosome profiling, ribosome affinity purification and spatial translatome methods.

Sections

Introduction

Ribosome profiling

Ribosome-associated transcripts

Spatially localized translation

Limitations and future improvements

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Introduction

Single-cell sequencing methods are revolutionizing our ability to characterize tissues across developmental stages, diseases and individuals, and following large-scale genetic perturbations[1,2]. Driven by an enormous community effort of developing technologies to measure mRNA, single-cell transcriptomes have quickly become the gold standard in delineating cell types. These technological breakthroughs, combined with parallel innovations in computational analysis techniques[3], are enabling efforts to collect and assemble single-cell transcriptome measurements into organism-scale atlases and to localize them into their spatial contexts.

However, gene expression does not begin or end with transcription. A major goal in cell biology is to understand how the myriads of gene regulation layers ultimately influence a cell type or state. Accordingly, technologies for single-cell analysis (reviewed in ref. 4) have expanded to include epigenetic modifications, chromatin accessibility, protein abundance and, more recently, translation and the processes that regulate it.

Translation is a dynamically regulated stage of gene expression. Many different molecular mechanisms have been described for modulating translation at its initiation[5], elongation[6], and termination and recycling[7] phases at the global and gene-specific levels. However, although specific instances have been identified where these mechanisms influence development[8,9], disease[10,11], cancer[12] and other cellular processes[13], limitations in existing technologies have ultimately restricted the systems in which these processes could be studied.

Recent technological advances have enabled measuring translation and studying its regulation genome-wide at the single-cell level. Although still in their infancy, these methods are beginning to reveal the broad extent to which translation dynamically regulates gene expression.

In this Progress, we discuss recent technologies that enable genome-wide measurements of translation in single cells. We first discuss methods centred around ribosome profiling that provide transcriptome-wide positioning of individual translating ribosomes. Next, we discuss methods that quantify transcripts bound by ribosomes. Finally, we discuss methods that spatially measure ribosome association, and future prospects. We also overview single-cell genomic technologies that measure translation regulators (Box 1).

Ribosome profiling

Ribosome profiling is a commonly used sequencing-based technique to measure in vivo translation genome-wide by mapping ribosome–mRNA interactions[14] (Fig. 1a). In appropriate conditions, ribosomes can be locked in place and remain stably associated with the mRNAs they are translating. By then using a nuclease to digest away any exposed RNA, and purifying and sequencing the remaining ribosome-protected fragments (RPFs; or 'footprints') of mRNA, both the precise locations and numbers of ribosomes on transcripts can be measured[15,16].

The position and quantitative information contained in the ribosome footprints can be used for various applications (reviewed elsewhere[15–17]). Ribosome profiling provides information on where and how ribosomes move along transcripts. These data identify translation-relevant features such as coding sequences, upstream open reading frames (uORFs), or translation pausing or frameshifting locations and can be used to measure properties of translation initiation, elongation and termination. When combined with parallel quantification of transcript abundance, ribosome profiling can be used to additionally measure ribosome density, which is often used as a proxy for protein production or translation efficiency. However, care should be taken when interpreting these proxy measurements for translational output as higher ribosome densities do not always result in higher protein production[18,19].

Several features of the standard, bulk ribosome profiling workflow[20] limit its sensitivity and scalability and make its application to single cells challenging (Fig. 1). First, the nuclease used for footprinting and the strength of the digestion are crucial parameters in all ribosome profiling methods[21]. For example, RNase I, the most used nuclease, has low sequence bias and, thus, produces precise mRNA cuts at the ribosome edges. Because each ribosome characteristically protects a ~30-nucleotide portion of the mRNA, a simple offset from the 5′ end of the footprint can be used to position the ribosome exit (E), peptidyl (P) and aminoacyl (A) sites within each read. The nuclease concentration, however, must be optimized for different sample types and abundances to balance the efficient creation of monosomes with over-digestion leading to monosome degradation. By contrast, micrococcal nuclease (MNase) maintains monosome integrity over a wider concentration range, is less sensitive to RNA secondary structures and is compatible with more organisms and cell types[21]; however, its strong sequence bias obscures the precise location of the ribosome active site.

Following footprinting (RNA digestion), monosomes are purified from the rest of the cell lysate, and the ribosome footprints are enriched from other RNA digestion products. Monosomes are typically purified based on their molecular weight by ultracentrifugation over a sucrose cushion or gradient. RNA is then extracted from the resulting monosome pellet or fraction, and RPFs are enriched based on their characteristic length (~30 nucleotides) using polyacrylamide gel electrophoresis. These laborious purification steps inherently limit the number of samples that can be processed in parallel. Additionally, when working with low-input samples, gel extractions typically have low recovery rates and high cross-contamination rates. The high losses and low throughput associated with these preprocessing steps are compounded by a scarcity of methods for efficient low-input small-RNA library construction. Adapters that are necessary for amplification and sequencing are commonly attached using single-stranded RNA ligations, which suffer from low efficiency, sequence and secondary structure biases, and high levels of contamination with adapter dimers in the resulting libraries[22,23].

Recent modifications to this general workflow by us and others have allowed measuring ribosome profiles in single cells. Although there are considerable differences between the specific techniques employed by each of these studies, in general, they all increase sensitivity by using different RPF enrichment strategies and efficient library preparation chemistries (Table 1 and Fig. 1b).

Single-cell ribosome sequencing (scRibo-seq)[24] integrates all processing steps from lysis to PCR amplification into a single-tube reaction. First, individual cells are sorted by fluorescence-activated cell sorting (FACS) into wells of a 384-well plate, lysed and digested with MNase. Digested lysates are dissociated, and individually barcoded libraries are then directly made on these unenriched and unpurified digestion products. Following RNA end repair, adapters are ligated to the RNA fragments using two successive single-stranded RNA ligations. These adapters are used as priming sites for cDNA synthesis and for indexing PCR, which adds cell and plate barcodes. Because the lengths of the adapters are known, following barcoding, amplification and pooling, RPFs can be enriched based on their characteristic length. Performing RPF enrichment at this stage avoids the high losses and cross-contamination issues that are endemic to low-input

Box 1 | Measuring regulators of translation in single cells

Global and gene-specific translation can be modulated throughout the mRNA lifecycle and at the different phases of translation by various molecular mechanisms. Genome-wide methods for assaying many of these mechanisms have been adapted to measuring single cells, including profiling transcript isoforms, RNA modifications, RNA secondary structures, RNA–protein interactions, and microRNAs (miRNAs) and their targets (see the figure). As translation ultimately revolves around the mRNA template, determining the quantity and state of these templates and how they interact with translation and other regulatory machinery is crucial to understanding translational regulation. Although the relevant methods, discussed below, have not yet been incorporated with concomitant measurements of translation in the same single cells, such multi-omics techniques would enable powerful functional correlations to be made between the mRNA state and translation.

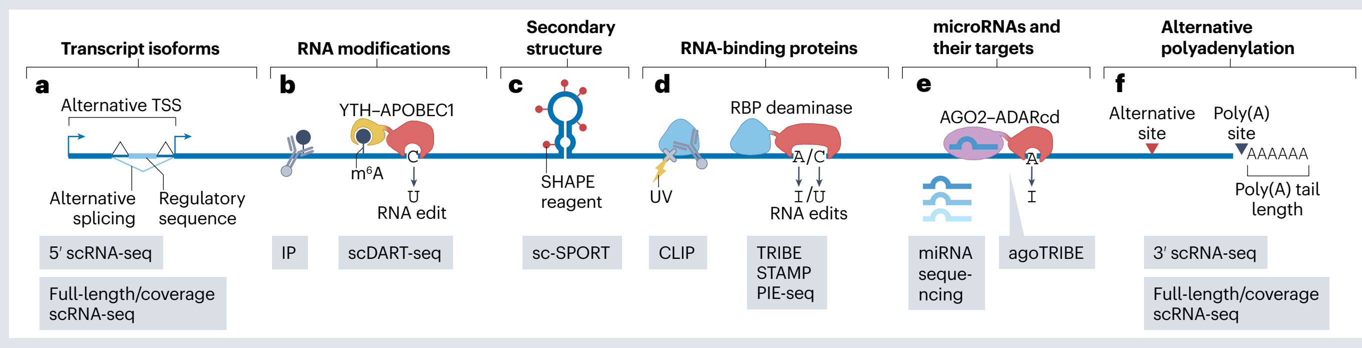
Production of transcript isoforms increases the complexity of the proteome, and can alter the stability of transcripts and the capacity to regulate them[55,56]. Fortunately, the large diversity of available methods for single-cell RNA sequencing (scRNA-seq) provides a selection of tools to quantify transcript isoforms[57]. The approaches used by these techniques range from providing coverage across the entire transcript[58,59] to specifically measuring transcription start sites (TSSs)[60,61] (see the figure, part **a**), sequencing full-length transcripts using long-read sequencing technologies[62,63], identifying alternative polyadenylation sites[64,65] and measuring poly(A) tail lengths[66] (see the figure, part **f**).

RNA molecules contain a diverse repertoire of chemical modifications that affect their stability, structure and interactions (reviewed elsewhere[67–69]). Genome-wide methods based on chemical reactivities, reverse transcriptase-induced misincorporations, immunoprecipitation (IP) or direct RNA sequencing (RNA-seq) allow these modifications to be mapped across the transcriptome in bulk[70]. Increases in sensitivity and reductions of non-specific background in RNA IP enabled measurement of N^6-methyladenosine (m^{6}A) in single cells[71,72]. Alternatively, in single-cell deamination adjacent to RNA modification target sequencing (scDART-seq), adaptations to the RNA-editing strategy originally developed for surveying targets by APOBEC-mediated profiling (STAMP)[33] (described below) measure m^{6}A-containing mRNAs in cell lines[73] and in vivo[74]. In scDART-seq, the YTH m^{6}A reader protein domain is fused to an APOBEC1 RNA-editing domain, so that cytosine-to-uracil (C-to-U) RNA edits are created in the vicinity of m^{6}A bases, which are read out using scRNA-seq[73] (see the figure, part **b**).

Secondary structures in mRNA can influence in *cis* its levels of translation, either by promoting direct interactions with the translation machinery or through secondary interactions with RNA-binding proteins (RBPs)[75]. Recent adaptations of bulk methods to probe RNA structures in vitro (reviewed in ref. 76) allowed RNA structure profiling in single cells using single-cell structure probing of RNA transcripts (sc-SPORT), which is based on selective 2′-hydroxyl acylation analysed by primer extension (SHAPE). In sc-SPORT, paired and unpaired RNA bases differentially react with chemical probes that cause mutations during cDNA synthesis; the mutation rates are thus a measure of each base’s reactivity that can be used to infer secondary structure[77] (see the figure, part **c**).

RBPs are a major class of proteins that interact with and orchestrate all aspects of RNA biogenesis, function and decay[78]. Because there are a wide variety of RNA–protein interaction mechanisms, RNA-centric and protein-centric techniques have been instrumental in describing RBPs and their binding partners[79]. In addition to adapting cross-linking immunoprecipitation (CLIP) sequencing (reviewed in ref. 80) to use in single oocytes[81], an alternative class of methods uses RNA editing to mark RNAs associated with specific RBPs. The basis of these methods is the use of transgenic gene fusions of an RBP of interest with an RNA-editing domain. Targets of RNA-binding proteins identified by editing (TRIBE)[82,83], STAMP[33] and PIE-seq[84] use the catalytic domain of the deaminases ADAR (ADARcd), APOBEC1 or both, respectively, to identify RNA–RBP interactions through the occurrence of the RNA edits adenosine-to-inosine (A-to-I), C-to-U or both, respectively (A/C-to-I/U; see the figure, part **d**). As these edits can be generated distant from the actual binding site, these techniques can only be used to identify binding partners and not binding sites.

A specific class of RNA–protein interactions is mediated by miRNAs, a class of small non-coding RNAs that post-transcriptionally regulate gene expression[85,86]. Mature miRNAs are incorporated into Argonaute proteins and direct mRNA destabilization and translation repression through the formation of miRNA–mRNA weak base-pair interactions. As these interactions are thought to be widespread[85], measuring miRNA expression levels and identifying the sets of genes they target are important for understanding these interactions. Despite the challenges associated with efficiently sequencing small RNAs, various efficient library preparation methods have been developed so that miRNAs can be sequenced in single cells[52,87–91]. Additionally, agoTRIBE builds on the RNA-editing approach used in TRIBE[82] to mark mRNAs that are interacting with any miRNA in complex with Argonaute 2 (AGO2), through the formation of A-to-I edits (see the figure, part **e**).



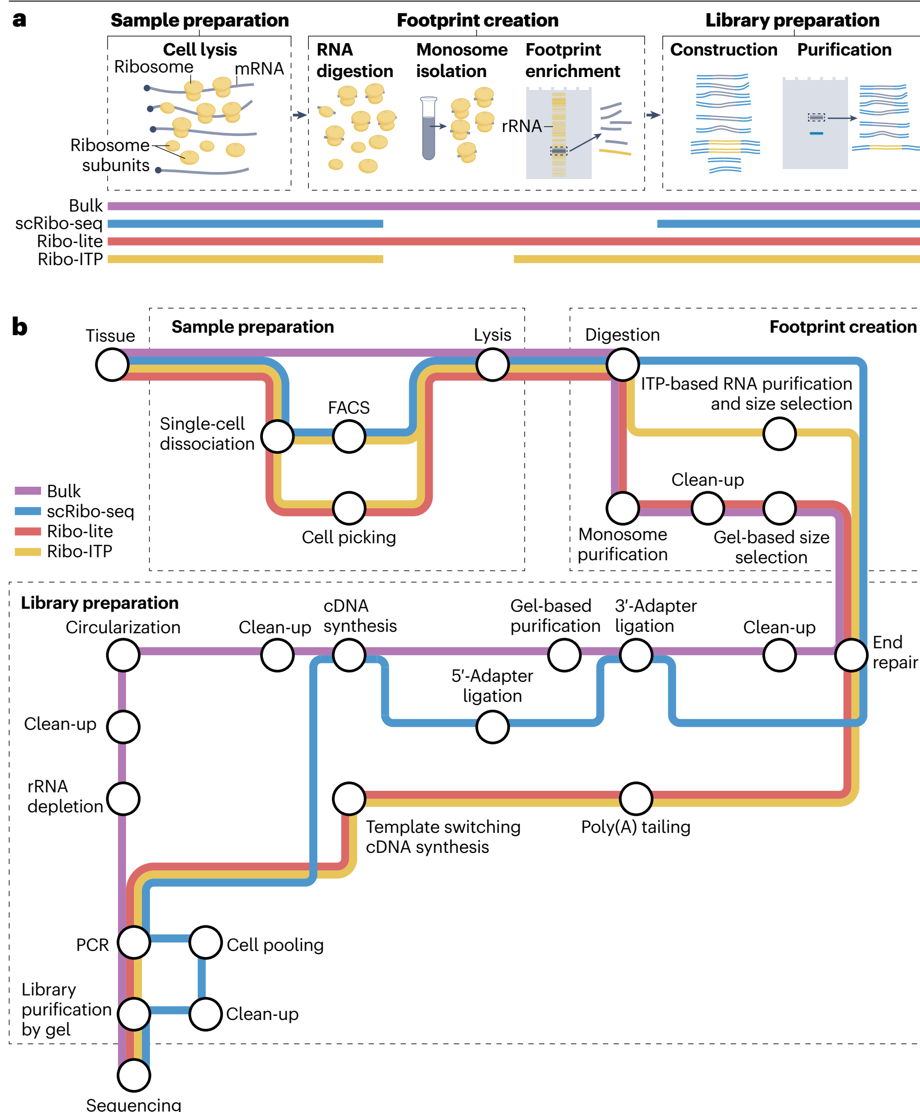


Fig. 1 | Comparison of bulk and single-cell ribosome profiling methods. a, The main steps of ribosome profiling. Cells are lysed in conditions that maintain the association between translating ribosomes and transcripts. A nuclease is then used to digest mRNA that is not protected by the ribosomes. The resulting monosomes are separated from other cell contents and individual ribosome subunits by ultracentrifugation over a sucrose gradient or cushion. The purified monosomes are denatured, and RNA corresponding to the characteristic ~30-nucleotide footprint size (grey) is purified from other, contaminating RNA (yellow). These ribosome 'footprints' are then made into sequencing libraries. Coloured bars indicate the general steps used by different methods. **b**, Workflow comparison of bulk ribosome profiling[20], single-cell ribosome sequencing (scRibo-seq)[24], ligation-free, ultralow-input and enhanced ribosome sequencing (Ribo-lite)[27] and Ribo-ITP[25]. All methods perform the general steps of sample preparation, footprint creation and library preparation, but different specific strategies are used to increase sample throughput and detection sensitivity. FACS, fluorescence-activated cell sorting; ITP, isotachophoresis; rRNA, ribosomal RNA.

gel purifications and allows efficient parallel purification of reaction products from many single cells.

Using scRibo-seq, we investigated translation elongation dynamics in single-codon resolution between single cells[24]. We used established tools for single-cell RNA sequencing (scRNA-seq) analysis to cluster cells and identify cell types based on the RPF counts per cell. As expected, the differential expression of marker genes commonly used to identify cell types based on scRNA-seq were also applicable to scRibo-seq data. Measuring elongation dynamics required positioning the location of the ribosome active site within each footprint. In order to correct the strong sequence bias introduced by MNase during footprint generation, we used a random forest classifier to predict the position of the aminoacyl site within each RPF. With these capabilities to measure each cell's identity and translation elongation dynamics, we measured the response to amino acid starvation and ribosome pausing along the unperturbed cell cycle and in rare primary mouse enteroendocrine cells. These observations were made at resolutions ranging from aggregating overall codons down to the specific codon level on individual transcripts[24].

Ribo-ITP[25] is a single-cell ribosome profiling method based on a high-yield microfluidics RNA purification and footprint enrichment system. Individual embryos, oocytes or cells are placed in wells of a microplate for lysis and MNase or RNase I digestion. The digestion products are then independently mixed with fluorescent markers and loaded into a custom microfluidics device that integrates isotachophoresis (ITP)-based RNA purification with polyacrylamide gel electrophoresis size selection. These integrated high-yield purification steps enrich RPFs based on their length from the nuclease-digested lysates. The resulting size-selected fragments are then dephosphorylated, and libraries are generated using an efficient one-tube strategy based on polyadenylation and template switching[26]. First, a poly(A) tail is added to the 3′ end of the RNA fragments, which is next used for poly(T)-primed template-switching cDNA synthesis, which adds a second adapter sequence. Following indexing PCR amplification, the final libraries are again size-selected before sequencing.

Ribo-ITP was applied to single oocytes and embryos from hybrid mice to characterize translation regulation during pre-implantation development[25]. In addition to ribosome profiling, RNA sequencing (RNA-seq) was performed on separate oocytes and embryos collected from the same developmental stages. Using RNA expressed from the paternal allele as a proxy for zygotic gene activation, the authors first measured how ribosomes engage with zygotic transcripts. In general, ribosome engagement with these paternal transcripts was concurrent with their expression level. Interestingly, a small set of genes was identified to have allele-specific changes in ribosome engagement and translation between different developmental stages. Next, transcript-specific changes in ribosome densities were compared between developmental stages, identifying sets of genes whose expression is translationally regulated. These changes in translation efficiency were used in subsequent analyses, which identified RNA-binding protein (RBP) motifs as putative translation regulators and were integrated with previously generated bulk measurements of poly(A) tail lengths in similar developmental stages, to show that poly(A) tail length and translation efficiency were coupled in the zygote, but not later stages[25].

Ligation-free, ultralow-input and enhanced ribosome sequencing (Ribo-lite) is a high-sensitivity version of conventional bulk ribosome profiling[27]. Although the focus of this study[27] was on analysing low-input bulk samples containing approximately 100 oocytes, single-oocyte sensitivity was also demonstrated. In Ribo-lite, single oocytes are isolated, lysed and digested with RNase I in microwells. The monosomes are separated from the lysate based on molecular weight by ultracentrifugation through a sucrose cushion. RPFs are then enriched from the monosomes based on length using a marker-free polyacrylamide gel. This upstream RPF enrichment allows the use of ligation-free methods of small-RNA library preparation that instead use polyadenylation and template switching to attach sequences used for amplification and sequencing[26]. Ribo-lite was applied in combination with parallel RNA-seq on 100-oocyte bulk samples to characterize global translation regulation during the mouse oocyte to embryo transition[27].

There are some important technical differences to consider between these single-cell ribosome profiling methods, which affect data quality and interpretation. First, as both scRibo-seq and Ribo-ITP do not purify monosomes and instead perform RPF enrichment only based on their characteristic length, lysis conditions should ideally destabilize unwanted RNA–protein, RNA–RNA or RNA–DNA interactions prior to footprinting. Maintaining these interactions may result in similarly sized RNA fragments that are not produced through ribosome

Table 1 | Summary of sequencing methods to profile translation in single cells

Method	Translation specificity: Footprinting nuclease	Translation specificity: RPF enrichment	Library construction	UMIs[a]	Cells per experiment	Specialized equipment and techniques	Measurement type	Demonstrated resolution	Demonstrated systems
Ribosome profiling									
scRibo-seq[24]	MNase	Fragment length	One-pot, integrated with footprinting	Yes	~1,000	FACS, nanolitre-scale liquid handling	RPFs	Codon and gene	Cell lines, primary cells
Ribo-ITP[25]	MNase and RNase I	Fragment length	Poly(A) tailing and template switching	Yes	~10	Custom microfluidic device	RPFs	Gene	Oocytes, cell lines
Ribo-lite[27]	RNase I	Monosome purification and fragment length	Poly(A) tailing and template switching	No	~10	Ultracentrifuge	RPFs	Codon and gene	Oocytes
Ribosome-associated transcripts									
T&T-seq[32]	Biotinylated puromycin analogue (3P)		Compatible with any scRNA-seq; uses SMART sequencing-based kit	No	~10	None	Transcript counts and ribosome-associated transcript counts	Gene	Oocytes
Ribo-STAMP[33]	*RPS2–APOBEC1*		Compatible with any scRNA-seq; uses 10×3′ v3	Yes	~10,000	*RPS2–APOBEC1* fusion transgene delivery and expression	Transcript counts and C-to-U edit frequencies	Gene	Cell lines
Spatial ribosome-associated transcripts									
RIBOmap[43,45]	Three probes to specifically amplify ribosome-bound transcripts		Ligation and RCA; SEDAL sequencing[44]	No	~10,000	Cryo-sectioning, spatial SEDAL sequencing	Spatially localized ribosome-associated transcript counts	Gene; restricted to selected probe sets	Cell lines, brain tissue sections

C-to-U, cytosine-to-uracil; FACS, fluorescence-activated cell sorting; ITP, isotachophoresis; MNase, micrococcal nuclease; RCA, rolling circle amplification; Ribo-lite, ligation-free, ultralow-input and enhanced ribosome sequencing; RIBOmap, ribosome-bound mRNA mapping; Ribo-STAMP, ribosome surveying targets by APOBEC-mediated profiling; RPF, ribosome-protected fragment; scRibo-seq, single-cell ribosome sequencing; scRNA-seq, single-cell RNA sequencing; SEDAL, sequencing with error-reduction by dynamic annealing and ligation; SMART, switching mechanism at the 5′ end of RNA template; T&T-seq, transcriptome and translatome sequencing. [a]A unique molecular identifier (UMI) is a unique sequence that is added to RNA or DNA fragments before amplification to identify that molecule. UMIs can be used to count original unique molecules and correct for amplification biases.

protection. Care should also be taken to ensure that the strength of nuclease digestion is high enough to completely remove unprotected mRNA, while still maintaining ribosome integrity[21]. Without explicit monosome purification, undesired RNA protection or inadequate digestion would result in contaminating the RPF fraction with fragments produced from different sources. Commonly used metrics such as coding-sequence enrichment, trinucleotide periodicity and frame bias in how the reads align, and fragment size distributions can be used to evaluate the contribution of these alternative fragment sources. Second, as the poly(A) tailing and template-switching reactions used by Ribo-ITP and Ribo-lite do not add defined sequences to the original fragments, simple length-based offsets to infer the decoding-site positions may not be sufficient[28]. The loss of this information does not limit the utility of these methods in applications that do not require codon-level resolution, such as measuring ribosome densities or identifying CDS regions.

Workflow differences also require considerations of experimental design. For example, despite both Ribo-lite and Ribo-ITP being attractive methods for studying very limited samples such as single embryos, the requirement to perform individual purifications for each cell ultimately limits the throughput to tens of cells per experiment. By contrast, because in scRibo-seq the reaction products from many cells can be pooled together prior to RPF enrichment, thousands of cells can be efficiently profiled in parallel.

Ribosome-associated transcripts

An alternative approach for measuring translation regulation is to quantify changes in the degree to which ribosomes are associated with transcripts. In bulk, these measurements are commonly done using either polysome profiling or affinity purification to purify ribosome-bound transcripts, which are then input to standard methods of mRNA quantification such as RNA-seq. These methods thus quantify the number of ribosome-bound transcripts, in comparison with ribosome profiling, which quantifies the numbers and positions of ribosomes on transcripts. As is the case with measurements of ribosome density made with ribosome profiling, the conflation of changes in ribosome-bound transcripts with translation efficiency can also be confounded by other mechanisms that increase density without increasing protein output, such as ribosome pausing.

In polysome profiling[29], ultracentrifugation over a sucrose gradient is used to fractionate ribosome-bound transcripts based on their ribosome density. The mRNA composition of each fraction can then be monitored using any standard quantification method, and, for example, a transcript transitioning from a low-ribosome-density fraction (that is, monosomes) to a high-density fraction (that is, polysomes) could represent an increase in ribosome density that is used to infer a change in translation efficiency.

In ribosome affinity purification, translated transcripts are enriched based on their association with ribosomes, such that mRNAs with higher ribosome densities are more strongly enriched in the pull-down over those with low densities. Thus, by comparing abundances between the pull-down and input fractions, changes in ribosome density can be quantified. Transgenic tagged ribosomal proteins[30] that are incorporated into functional ribosomes can be expressed in specific cell types and used to selectively profile their translation. Alternatively, in RiboLace, a biotin-functionalized puromycin analogue called 3P can be used as an affinity reagent[31] (Table 1). The puromycin moiety of 3P binds to the large subunit of actively translating ribosomes, which can then be pulled down with streptavidin beads using the biotin moiety.

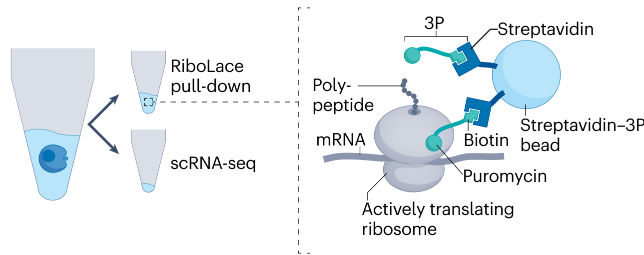
Similar to ribosome profiling, the issues of high-input requirements and scalability associated with these methods prohibits their direct application in measuring single cells. Single-cell techniques for measuring ribosome-associated transcripts thus use sensitive methods to pull down ribosomes or record ribosome–transcript interactions, and leverage the efficient library preparation workflows developed to measure single-cell transcriptomes.

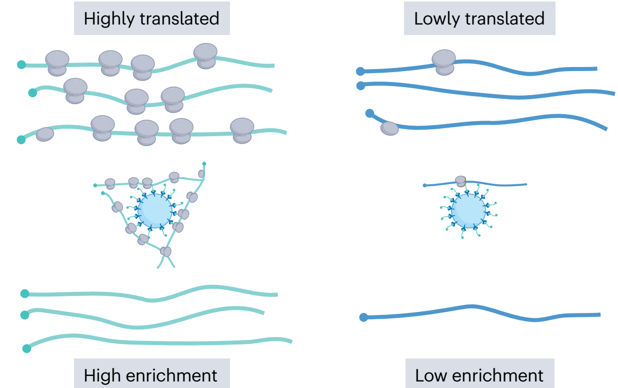
Transcriptome and translatome sequencing (T&T-seq) is a single-cell multi-omics method that measures both transcriptomes and ribosome-associated transcripts from the same single oocyte[32] (Fig. 2a). In T&T-seq, the lysate from each cell is split, with part being directly used for scRNA-seq and the remainder being used as input to a RiboLace ribosome pull-down[31] to enrich transcripts being translated. The result from this pull-down is then used without ribosome footprinting as input for scRNA-seq, which provides transcript abundances but not positional information. Comparing the mRNA abundances between the translated and transcriptome fractions provides a measure of ribosome association, which is used as a proxy to infer translation efficiency.

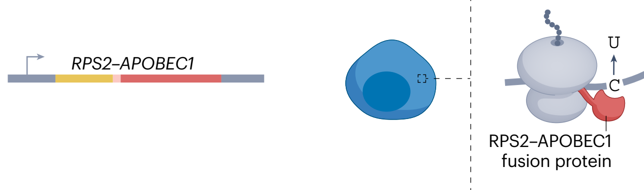
The ability of T&T-seq to profile paired transcriptomes and translatomes of very limited samples allowed the characterization of translation regulation during mouse and human oocyte maturation. Cross-species comparisons revealed shared and distinct translation regulation patterns and identified a factor that induces human oocyte maturation. This is currently the only described method of measuring both the transcriptome and the translatome of the same cell. However, the reduction in sensitivity that inherently accompanies sample splitting restricts the applicability of this method to single oocytes, which are considerably larger than other cell types. Furthermore, the necessity to individually split and pull down material from each cell ultimately limits the throughput to tens of cells per experiment.

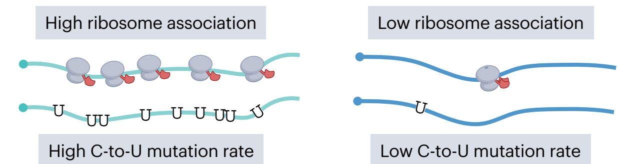
Surveying targets by APOBEC-mediated profiling (Ribo-STAMP) leverages RNA editing to measure ribosome association of transcripts in single cells[33] (Fig. 2b). In Ribo-STAMP, an inducible protein fusion of RPS2, a protein of the ribosomal small subunit, and APOBEC1, a cytosine deaminase, is transiently overexpressed, and ribosomes incorporate the tagged version of RPS2. Consequently, mRNAs being translated are brought near the deaminase and accumulate cytosine-to-uracil (C-to-U) edits. Following scRNA-seq, the number of C-to-U mutations is used as a measure of ribosome association; transcripts that are highly translated will have a higher mutation density than lowly translated ones. Single-cell Ribo-STAMP was validated in HEK293 cells, where it was observed that RPS2–APOBEC1 expression generated a higher proportion of CDS edits compared with fusions of APOBEC1 to other RBPs. Compared with single-cell ribosome profiling and T&T-seq, Ribo-STAMP is attractive because it can be directly used with existing scRNA-seq methods.

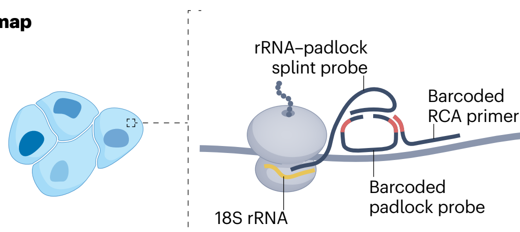
The wide variety of capabilities in throughput, coverage and sensitivity that single-cell RNA-seq methods offer (reviewed in ref. 2) allows researchers using Ribo-STAMP to select the sequencing strategy that is most suitable for answering their research question. However, as Ribo-STAMP requires the expression of an exogenous fusion protein, this method measures cumulative translation during the activity window rather than providing an instantaneous snapshot. Transgene induction and activity and the ribosome half-life all measure in days, and thus do not match the short timescales over which translation regulation can occur (minutes to hours); the effects of translation regulation mechanisms may thus be obscured when assayed in the highly dynamic biological systems that are ideally suited for single-cell analysis. Furthermore, use of the transgene prevents its direct application in vivo outside model systems. Finally, as previous ribosome tagging attempts











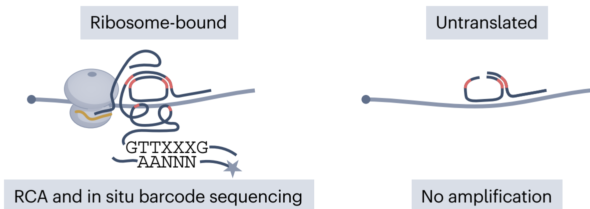


Fig. 2 | Single-cell technologies to measure the degree of ribosome association. a, Transcriptome and translatome sequencing (T&T-seq)[32] uses sample splitting to measure both mRNA abundance and mRNAs that are being translated. For the latter, T&T-seq uses the bifunctional biotinylated puromycin analogue (3P) developed in RiboLace[31] to pull down mRNA associated with actively translating ribosomes. Following pull-down, highly translated transcripts are enriched over lowly translated transcripts. **b**, Ribosome surveying targets by APOBEC-mediated profiling (Ribo-STAMP)[33] uses an inducible protein fusion between the ribosomal protein RPS2 and the deaminase APOBEC1 to mark translated transcripts. Translating ribosomes incorporating the RPS2–APOBEC1 fusion protein bring the deaminase into proximity of mRNAs, which then accumulate cytosine-to-uracil (C-to-U) mutations that can be quantified during sequencing. **c**, Ribosome-bound mRNA mapping (RIBOmap)[43] measures the spatial localization of mRNAs being translated in tissue slices. Three probes are used to ensure that rolling circle amplification (RCA) occurs only on ribosome-bound transcripts. The first probe provides specificity to transcripts associated with ribosomes by hybridizing to both 18S ribosomal RNA (rRNA) and a padlock probe. The padlock probe binds to specific mRNAs and is circularized by ligation when in close proximity to the rRNA probe. Circularized padlock probes can then be amplified by a barcoded RCA primer. scRNA-seq, single-cell RNA sequencing.

revealed that not all such protein fusions correctly incorporate into functional ribosomes[34], it is important to carefully characterize the functionality of these fusion proteins.

Spatially localized translation

Recent spatial transcriptomics technologies (reviewed elsewhere[35–37]) have provided genome-wide measurements of mRNA abundance across tissues, with some methods obtaining subcellular resolution. In addition to mRNA localization, the translation machinery and factors are also spatially distributed to help modulate gene expression patterns[38,39]. Localized translation is typically studied in single cells using microscopy, either by quantifying levels of global translation using metabolic labels of nascent protein synthesis[40] or by interrogating individual genes in live cells using single-molecule methodologies[41,42]. However, extending these spatially resolved measurements of translation to larger gene sets remains a challenge.

Ribosome-bound mRNA mapping (RIBOmap)[43] bridges the technological gap between measuring translation of individual genes with high spatial precision and genome-wide measurements carried out on dissociated or homogenized tissues to enable transcriptome-scale measurements of translation in tissue slices, with subcellular resolution. This technique builds on STARmap, the previously described method from the same research group for spatial transcriptome sequencing[44]. STARmap uses sets of gene-specific primer and padlock probes to amplify unique gene identifiers for sequencing. The primer probe acts both as a splint to circularize its corresponding

padlock probe during ligation and as a primer for rolling circle amplification (RCA) of a circularized padlock. Amplification thus occurs only when both probes are co-localized. In order to specifically measure ribosome-associated mRNAs with RIBOmap, this probe configuration was modified into a three-probe design (Fig. 2c). The first probe partially hybridizes to 18S ribosomal RNA (rRNA) and partially to a padlock probe, thus acting as a splint to circularize a padlock that is physically close to a ribosome. Next, the padlock probes target specific mRNAs and additionally contain a barcode for each gene that can be sequentially read out during imaging. Finally, a primer probe targets sequences adjacent to the padlock probes, and acts as a primer for RCA. Successful amplification of the gene barcodes can thus occur only when all three probes are in close proximity. Following amplification, the unique barcodes of each gene are sequenced in situ to identify where the different ribosome-associated transcripts are. STARmap and RIBOmap have both been expanded to enable profiling in thicker tissue slices[45].

Applying RIBOmap to cultured cells identified sets of genes whose subcellular translation location highly correlated with the functional location of the encoded proteins[43]. For example, mRNAs translated in the endoplasmic reticulum were enriched for membrane and secretion-pathway proteins. Extending these measurements to mouse brain sections revealed that non-neuronal cell types had a higher degree of translational regulation than neuronal cells, and revealed localized differences between neuronal cell bodies and peripheries.

Although RIBOmap is currently the only method capable of performing transcriptome-scale measurements of the spatially resolved translation of thousands of genes, it requires the a priori selection of probe sets. Additionally, as the three-probe assay quantifies transcripts that are associated with ribosomes, it may be difficult to distinguish transcripts with a high ribosome density from those with a low density. Finally, the highly specialized instrumentation and workflow of RIBOmap currently limits its adoption, but the development of commercial instruments with similar capabilities is likely to ameliorate these issues.

Glossary

Adapters
Sequences attached to a diverse set of RNA molecules, which are used to label, amplify and sequence those molecules.

Barcodes
Unique sequences that are added to all RNA fragments originating from the same source, thereby enabling pooling together of material from many samples for efficient sequencing, after which the barcodes are used to identify the original source of each read.

Isotachophoresis
(ITP). An electrophoretic technique for the selective separation and concentration of charged molecules.

Monosomes
Single ribosomes attached to an mRNA or mRNA fragment.

Polysome
Several ribosomes attached to an mRNA or mRNA fragment.

Random forest
A machine learning method that combines the output of multiple decision trees for classification and regression predictions.

Ribosome density
The number of ribosomes per mRNA.

Translation efficiency
The rate of polypeptide synthesis per mRNA per time.

Limitations and future improvements

Recently developed technologies for profiling translation in single cells are opening up previously inaccessible biological systems to genome-wide measurements of translation. Mirroring early discoveries in translation regulation[46], the majority of the first single-cell studies focused on early development. However, the increases in sensitivity provided by the new techniques have permitted the analysis of rare samples such as human oocytes and additionally provided genome-wide characterizations.

In addition to enabling analyses on limited samples, single-cell sequencing techniques are ideally suited to describing multicellular tissues and highly dynamic cell processes. Discrepancies between mRNA and protein abundances are the norm rather than the exception, and the observations of the variability of these correlations between different cell types[47] further suggests that modes of translation regulation are cell type and context dependent. There is now substantial evidence for the importance of translation regulation in systems varying from basic cell processes such as cell division or cell stress[13] to complex multicellular processes in development[8,9] and disease[10–12]. As many of these processes include short-lived intermediate cell states, the ability to capture and place these rare cell types along the developmental trajectories[48] will enable high-resolution characterizations. These detailed descriptions may be particularly revealing as the rapid timescales over which global and gene-specific translation can be modulated ideally position it as a first response to change.

However, these single-cell methods are still in their infancy and several gaps in their capabilities remain. Despite the impressive increases in sensitivity that single-cell ribosome profiling methods have achieved, they could benefit from increased cell throughput and reduced workflow complexity. Several sequential molecular steps are necessary to generate ribosome footprints before cell barcode sequences can be added and the labelled reaction products can be pooled for further processing. This requirement is not compatible with current commercial droplet-based systems for scRNA-seq analysis, which can only perform a single reaction. Wider availability of droplet systems that support multistep reactions may dramatically increase the throughput of single-cell ribosome profiling capabilities. Similarly, continued innovations in library preparation for sequencing small RNAs[28] will result in streamlined and less intensive workflows.

The specific methods used for ribosome profiling during the preparatory steps of lysis, footprint generation and library construction can introduce technical artefacts. A survey of sequence features that affect the density of local footprints in bulk ribosome profiling data revealed large differences between separate studies[49], which were later revealed to not be caused by cycloheximide pretreatment[50]. Compared with bulk-scale measurements, profiling single cells requires additional upstream sample preparation steps including dissociation and isolation, which can cause stress-induced artefacts in the transcriptome[51]. As rapid translation remodelling is part of the cellular response to stress[13], analysis into the potential contribution that these stress responses make to the resulting ribosome profiles will be important to consider as single-cell ribosome profiling is increasingly applied to primary tissues. Similarly, a benchmarking comparison of different single-cell small-RNA sequencing techniques revealed large differences in sensitivity, contamination levels and measured abundances[52]. Detailed reporting of sample preparation will help assess the influence of these potential biases and support comparative and integrative analyses.

With regard to quantifying translation levels at the gene level, the capabilities and limitations of methods to profile translation in single cells are similar to those of scRNA-seq or spatial transcriptomics. As the underlying data types, structures and dynamic ranges are very similar to those seen in single-cell transcriptomics, it is no surprise that analyses such as dimensionality reduction, data integration, clustering, pseudotime ordering, differential abundance analysis and visualization are all directly applicable to single-cell translatomics. Additionally, as transcript abundance is generally a major determinant of the translation level, common marker genes can be used to identify cell types. Another problem is sampling noise and technical variability between tubes, samples and experiments[53]. This technical noise, which is introduced during library preparation, and the necessity to correct for it during normalization or integration steps make it difficult to quantify global translation differences between single cells. Moreover, the sparsity in gene counts can make it difficult to quantify lowly expressed genes in rare cell types. The lower mappability of the short RPF reads and the high rRNA and tRNA contamination rates present in single-cell ribosome profiling libraries decrease the number of uniquely aligned protein-coding reads per cell compared with scRNA-seq. At similar sequencing depths, these smaller library sizes increase the sparsity in RPF count matrices[54], thereby limiting the ability to detect lowly expressed genes.

The positional information is a unique feature of ribosome profiling compared with transcriptomics. Features such as CDS enrichment and trinucleotide periodicity are characteristic of ribosome profiling. Accordingly, codon-specific ribosome distributions and elongation dynamics can be measured for individual transcripts at the single-cell level. Although data sparsity can restrict such analyses to highly expressed genes, aggregating similar cells or features (for example, RPF A-site codons) can expand the scope of these measurements.

A common goal of translation profiling is to quantify how much a gene is being translated. Determining ribosome densities and measuring translation regulation requires accounting for mRNA abundance to remove the effect of transcriptional changes. In bulk, these measurements are done by performing RNA-seq in parallel on a portion of the same input material. Without splitting the sample before ribosome footprinting for enrichment as done in T&T-seq[32], performing RNA-seq in parallel is fundamentally not possible at the single-cell level. To address this limitation, other cells of the same type may instead be used in specific situations such as early embryogenesis, where precise stages can be selected based on morphology, as was done in Ribo-ITP and Ribo-lite[25,27]. Further technological development of alternative methods to simultaneously measure the transcriptome and translatome within the same cell will enable measurement of ribosome density with single-cell resolution.

Numerous examples of specific molecular mechanisms that regulate translation have been identified. However, the sequence features that drive these mechanisms are still not well described. Separately measuring translational output, transcription or translation regulators (Box 1) can yield insight into how these processes vary across populations of cells. Yet the real power of such techniques becomes apparent when such measurements can be computationally integrated or simultaneously assayed in the same single cells. Inherent cell-to-cell variability combined with proper cell groupings can enable powerful correlation analyses. For example, measuring both chromatin accessibility and transcriptional output with single-cell resolution across tissues has deepened our understanding of enhancer-driven gene regulation. However, unlike chromatin accessibility and transcription, the lack of obvious shared features required for computational integration of, for example, RNA–protein interactions with translation activity, necessitates performing these measurements in the same single cell. New multi-omics methods to measure transcriptional and translational states in combination with translational regulators are thus needed to fulfil this need.

Genomics techniques to measure translation in single cells are in their infancy, but already they are making it possible to interrogate this understudied and highly dynamic layer of gene regulation in previously inaccessible biological systems.

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Author contributions

M.V. contributed to all aspects of the article. A.v.O contributed substantially to discussion of the content and reviewed and/or edited the manuscript before submission.

Competing interests

M.V. and A.v.O are inventors on a patent application related to measuring translation in single cells.

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