Translatome profiling: methods for genome-scale analysis of mRNA translation

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Abstract

During the last decade, there has been a rapidly increased appreciation of the role of translation as a key regulatory node in gene expression. Thereby, the development of methods to infer the translatome, which refers to the entirety of mRNAs associated with ribosomes for protein synthesis, has facilitated the discovery of new principles and mechanisms of translation and expanded our view of the underlying logic of protein synthesis. Here, we review the three main methodologies for translatome analysis, and we highlight some of the recent discoveries made using each technique. We first discuss polysomal profiling, a classical technique that involves the separation of mRNAs depending on the number of bound ribosomes using a sucrose gradient; and which has been combined with global analysis tools such as DNA microarrays or high-throughput RNA sequencing to identify the RNAs in polysomal fractions. We then introduce ribosomal profiling, a recently established technique that enables the mapping of ribosomes along mRNAs at near nucleotide resolution on a global-scale. We finally refer to ribosome affinity purification techniques that are based on the cell-type specific expression of tagged ribosomal proteins, allowing the capture of translatomes from specialised cells in organisms. We discuss the advantages and disadvantages of these three main techniques in the pursuit of defining the translatome, and we speculate about future developments.

Introduction

The development of sophisticated ’omics’ technologies paved the way for a systems-level understanding of gene expression. Among the modern ‘omics’ approaches, the measurement of the level of the entire set of RNAs (the transcriptome) became very popular because simple and reliable tools are available for RNA extraction and global quantification with DNA microarrays or high-throughput RNA sequencing (RNA-seq). Although transcriptome analysis gives a good indication about changes in gene expression instigated by drugs, hormones or in disease, the significance is limited by the fact that mRNA levels do not necessarily correlate with the levels of proteins they encode [1-3]. Besides protein degradation, the observed variability is likely attributed to the control of protein synthesis. Thus, the analysis of the translatome, which refers to all mRNAs recruited to ribosomes for protein synthesis [4], can reveal important regulatory cues and discover relevant pathways linked to disease [5-7].

Protein synthesis is mediated by the ribosome, which is a large ribonucleoprotein complex. The eukaryotic ribosome is comprised of four ribosomal RNAs (rRNAs) and 79 ribosomal proteins (RPs), which are shared by two subunits referred to as the large (60S) and small (40S) ribosomal subunit [8, 9]. The process of translation can be divided into three steps: initiation, elongation and termination [10]. During initiation, which is thought to be the primary target for translational control, translation initiation factors (eIFs) recruit the mRNA to the small ribosomal subunit (40S subunit). The so-formed initiation complex then scans the mRNA from 5’ to 3’ until the initiation codon is reached. At this point, the 60S subunit joins the complex leading to the formation of a fully assembled 80S ribosome. Of note, ribosomes can also be recruited cap-independently to some viral and cellular mRNAs by direct binding of the small ribosomal subunit to internal RNA structures, termed IRES [11].

During the elongation phase, the ribosome and another set of accessory proteins termed elongation factors (eEFs), move along the coding region of the mRNA and synthesise the encoded polypeptide, with multiple ribosomes covering the mRNA to form polysomes. Finally, at the termination codon, peptide chain-release factors (eRFs) assist the release of the nascent polypeptide and dissociate ribosomes from the mRNA [10].

Translation can be controlled on a global level or on specific mRNAs (reviewed in [10, 12]). Global control can be achieved by phosphorylation of eIFs, such as of eIF2α that reduces the amount of active initiation complexes and leads to a rapid reduction of the translation of most mRNAs. Global control is also achieved by controlling the bioavailability of eIFs through interacting proteins, for example eIF4E is controlled by 4E-binding proteins (4E-BPs) that compete for binding of eIF4E with eIF4G, and thus inhibit association of the small ribosomal subunit with the mRNA [12]. Conversely, the translation of specific mRNAs can be regulated by *trans*-acting RNA-binding proteins (RBPs), which often bind to sequence or structural elements in untranslated regions (UTRs) of the mRNA and, for instance, repress or activate translation via interactions with eIFs [13]. Likewise, certain classes of non-coding RNAs (ncRNAs), such as microRNAs (miRNAs; small RNAs of 22 nucleotides in length) can repress translation via base pairing to sequences located in 3'-UTR of target mRNAs [14]. Finally, translation is also controlled by other types of *cis*-acting elements residing in the mRNA that more directly interact with ribosomes such as IRESs (see above [11]), upstream ORFs (uORFs) [15] or sites of programmed frame-shifting within coding sequences [16].

The increased recognition of the richness and impact of translation regulation for cell function and disease has come along with the development of sophisticated tools that allow the investigation of translation and the underlying regulatory events on a global-scale.

Herein, we provide a brief overview of the main methodologies currently used to profile the translatome; starting with polysomal profiling developed in the 1960s and proceeding to the more recently evolved ribosomal profiling and affinity-capture techniques.

Polysomal profiling – the traditional "gold standard"

A reliable measure for translation of a cellular mRNA is the degree of its association with ribosomes. Since the rate of initiation usually limits translation, most translational responses will alter the ribosome density on a given mRNA. During polysomal profiling, actively translated mRNAs bound by several ribosomes (polysomes) are separated from the "free" RNA, the small (40S) and the large (60S) ribosomal subunits and the 80S monosomes by sucrose gradient centrifugation (Figure 1; for a recent polysomal profiling protocol see [17]). After isolation of RNA from fractions of the gradient, the distribution of specific mRNAs in the gradient can be monitored by Northern blot or reverse-transcription quantitative PCR (RT-qPCR), or on a global level using cDNA microarrays, or more recently with RNA-seq (for RNA-seq examples see [18, 19]). Of note, polysomal profiling likewise enables monitoring of proteins associated with initiation complexes and/or ribosomes by immunoblot-blot analysis and/or proteomics [20].

The global profiling of mRNAs in each fraction of the polysomal gradient allowed the drafting of the first high-resolution maps of ribosome occupancy and densities on all individual mRNAs expressed in healthy "normal" cells [21-23]. Nevertheless, most genome-wide studies to date have used polysomal profiling to compare the translational status of mRNAs in different cell-types (e.g. healthy *vs.* cancer cells) or of cells subjected to intrinsically or extrinsically induced conditions in order to reveal translationally regulated messages. The analysis is thereby based on the well-established assumption that a shift of a mRNA in the polysomal gradient is indicative of an altered translational status of the

respective mRNA. To simplify the analysis, mostly “low-resolution profile” analysis has been performed, such that mRNA contents of high sucrose gradient fractions (polysomes) were compared with fractions from low sucrose gradient fractions (the pool of non-translated mRNAs) (Fig. 1, left). In parallel, changes in the levels of total RNA are often measured to study the relationship between transcription/decay and translation under different conditions. Early examples refer to investigations of global changes of translatome under different physiological conditions in yeast [24], and the approach was also adapted to mammalian cells to investigate, for instance, the response to diverse stressors/stress-situations such as apoptosis [25], endoplasmic reticulum (ER)-stress [26], hypoxia [27], and viral infection [28]. More recent examples refer to the translational response of cells exposed to ultra-violet (UV) irradiation which induces the DNA-damage response, and cells appear to cope by the selective recruitment of mRNA encoding for DNA-repair enzymes to the polysomal fractions [29, 30]. Translationally deregulated mRNAs were also identified during the inflammatory response in several studies [29, 31]: this includes the contribution of translational regulation to the early phase of the macrophage response from mouse macrophages stimulated with lipopolysaccharide [29]; or the co-culturing of breast tumour cells with conditioned medium of activated monocyte-derived macrophages which revealed novel cellular functional IRES located in the 5’UTR of mRNAs [31].

Besides the application of diverse stress conditions, likewise analysis has been carried out to study drug action: for example, a recent global study on the translational targets of canonical mTOR inhibitors (mTOR is major pathway controlling signals of nutrient availability) revealed that the antidiabetic drug metformin preferentially controls selective translational suppression of mRNAs coding for cell-cycle regulators and thus, could possibly inhibit mRNAs coding for proteins that promote neoplastic transformation [32]. As such, the study could facilitate investigation for the use of this drug in cancer prevention

and treatment, and highlights the advantages of translational profiling for studies on drug action [32]. Numerous studies have further applied polysomal profiling to test the effect of mutants or overexpression of global or specific translational regulators in yeast [33], mammalian cells [34]; or to investigate the impact of ncRNAs, such as miRNAs in translation [35, 36]. Finally, the comparative analysis of cell lines such as cancer cells (e.g. leukaemia [37]); or translational reprogramming during cell-differentiation, as for instance during adipogenic differentiation [18]; suggested new potential targets that could open the door for future drug development and medical intervention. Noteworthy, although most studies were conducted in yeast and mammalian cells, global polysomal profiling has also been amended to other cells or organisms, such as the study of translational regulation events upon stress in bacteria [38] and plants [39], or of developmental programs in the fruitfly *Drosophila melanogaster* [40].

Although sucrose density fractionation has for a long time been recognised as the ‘‘gold standard” to monitor active translation, there are some drawbacks that may have hindered its integration by the wider research community (Table 1). It requires specialised and expensive equipment (e.g. ultracentrifuge, gradient fractionation system) that may not be available in every laboratory and the procedure is labour intensive and does not allow handling of many samples in parallel. Furthermore, the samples are usually diluted in sucrose solution containing heparin (a potent RNase inhibitor) making more elaborate precipitation steps necessary to isolate RNA of sufficient quality for microarray/ RNA-seq analysis. Additionally, polysomal fractions may be contaminated with other high molecular weight complexes that are not an integral part of ribosomes, such as lipid rafts, processing body components or pseudo-polysomes [41]. Finally, we wish to note that polysomal as well as ribosome profiling (see below) requires a relatively large sample size, in the range of ten million cells, to obtain sufficient RNA for microarray/ RNA-seq analysis. Thus, the

methods are less amenable to samples or tissues of low abundance, such as cancer patient tissue biopsies.

Ribosomal profiling – determining the position of ribosomes at codon resolution

The advances in sequencing technology inspired the development of the ribosomal profiling technique, which provides a global measure of translation at near nucleotide resolution [42]. The technique is based on deep sequencing of ribosome protected RNA fragments (RPFs) that persist after treatment of a cell lysate with RNaseI, and thus enables the exact measurement of ribosome positions and densities along all RNA molecules present in a cell (Figure 1; for a detailed protocol [43]). In analogy to polysomal profiling, the analysis is based on the approximation that reading of the average ribosome density per mRNA correlates to the synthesis level of the corresponding protein, assuming that elongation rates are constant. In addition, a triplet periodicity should be seen in the sequencing data with peaks at the first nucleotide position, as the ribosome moves along the mRNA in a step-wise fashion one codon at a time beginning at the start codon [43-45]. In order to define the translatome, total mRNA extraction and sequencing is performed in parallel, to normalise RPFs to mRNA abundance.

The ribosomal profiling technique was originally developed in the Weissman lab in the yeast *Saccharomyces cerevisiae,* where ribosomal profiles of cells grown under rich and amino-acid starvation conditions were compared [42]. It was found that one third of genes underwent a change in translational efficiency between the two conditions; thereby mRNA encoding proteins involved in ribosome biogenesis were prevalent in the translationally repressed fraction during starvation conditions [42]. Besides the systematic identification of translationally regulated messages - which has been previously achieved with “traditional” polysomal profiling (see above) - the true strength of the technique is the global acquisition

of positional information in regard to the location of where the ribosomes are positioned along the mRNA. In this respect, the study by Ingolia *et al.* disclosed previously undiscovered uORFs engaged with ribosomes, and numerous potentially novel non-AUG start codons [42].

Since then, the technique became very rapidly adapted to different cellular systems and organisms to investigate translational control during diverse stress conditions, drug action or fundamental cellular processes (for a detailed review [44, 45]). Examples include the translational response of yeast to oxidative stress [46]; the response of mammalian cells to heat shock [47] and proteotoxic stress [48]; the (re-)investigation of drug action, namely inhibitors of the mTOR pathway in mouse embryonic fibroblasts or prostate cancer cells [49, 50]; and deciphering translational control during yeast meiosis [51] or cell-cycle progression in human cultured cells [52]. Ribosomal profiling has also been adapted to study the impact of specific *trans*-acting factors on translation such as miRNAs and RBPs [53-55]. Besides further modification of the biochemical procedure, the establishment of rigorous bioinformatic analysis of the ribosome footprint sequencing data was key to deciphering novel and surprising insights into translational control. This includes the discovery of features related to translation initiation such as uORFs [42, 56], non-AUG initiation codons [56, 57], novel coding transcripts [51], or novel protein isoforms arising from N-terminal extensions/truncations [57, 58]. It also enabled the accurate measurements of elongation rates or decoding speeds of individual codons [59, 60], mapping of translational pause sites [61], and the study of co-translational folding [62].

To date, two major variants of the technique can be distinguished which relate to the preferred mapping of either elongating and/or initiating ribosomes. To map the position of all ribosomes on mRNAs, cells are commonly treated with cycloheximide (CHX), which is

an antibiotic that rapidly diffuses in to cells and stalls both initiating and elongating ribosomes. However, recently it was claimed that treatment of cells with CHX can introduce prominent artefacts in ribosome profiling data [63]. In particular, there is evidence that treatment of stressed cells with the most commonly used concentration of CHX (100 µg/ml) can cause the accumulation of RPFs shortly downstream of the start codon, an effect not seen using either no CHX or high concentrations [63]. This finding challenges conclusions drawn from ribosomal profiling studies applying CHX which suggest that accumulation of ribosomes around the start site of coding sequences (the so-called ribosome “ramp”) is due to either slow elongation [64] or to the change in recruitment of ribosome-associated chaperones [48].

To accurately map initiating ribosomes, antibiotics other than CHX are preferentially used that specifically stall ribosomes at translation initiation sites (TIS) [65]. In a first application, mouse embryonic stem cells were treated with harringtonine, a compound that binds to free 60S subunits and is thought to effectively inhibit translation initiation [61]. Indeed, harringtonine caused a profound accumulation of RPFs at the beginning of CDS, and its application uncovered an unexpected number of alternative translational initiation codons and uORFs with regulatory potential, whose ribosome coverage and translation changed after differentiation [61]. Because the full extent to which harringtonine blocks translation initiation is not well resolved, Lee and colleagues used a combination of CHX and lactimidomycin (LTM) to differentiate between elongating ribosomes and initiating ribosomes, respectively [56] . Like CHX, LTM binds in the E-site of the ribosome, but due to its size, LTM is only recruited to ribosomes lacking tRNA thereby specifically inhibiting translation initiation. As with the previous study, they also identified an unexpectedly high number of novel initiation sites and uORFs in HEK293 and a mouse embryonic fibroblast cell line. In fact, only 51% of initiation events occurred at AUG codons in HEK293 cells, with

the remainder occurring at other triplets including CUG [56]. Fritsch and colleagues performed likewise analysis to obtain a transcriptome-wide map of TISs in a human monocytic cell line. Thereby, cells were initially treated with the drug puromycin, which releases elongating ribosomes from the mRNA, and then with CHX to stall the remaining initiating ribosomes [57]. Once more, it was found that only 47% of initiation events occurred at annotated AUG codons in ORFs, and suggested the existence of numerous novel uORFs and alternative TISs leading to potential N-terminally extended proteins. Importantly, a consequence of alternative TIS within a mRNA has recently been revealed for a protein involved in interferon signalling, *MAVS*, leading to the production of either a full length or a shorter isoform [58]. Thus, the short isoform of the protein interferes with interferon production induced by full-length MAVS, whereas both proteins positively regulate cell death [58]. Overall, these studies are intriguing as they suggest that translation start sites are much less-well defined than previously anticipated, generally strengthening the theory of the importance of translational control at initiation for cellular physiology.

Whilst many ribosomal profiling studies were conducted in yeast or cultured mammalian cells, the technique also became adapted to other species including viruses [66, 67], bacteria [68], worms [69], and zebrafish [70]. For instance, in a recent study, ribosomal profiling was carried out to accurately quantify absolute protein synthesis rates in the bacteria *Escherichia coli*, owing to the fact that the average half-life of a protein in *E. coli* is longer than the doubling time [68]. Ribosomal profiling allowed the discovery of key principles for protein synthesis in bacteria, such as proportional synthesis, whereby translation of proteins in a multi-protein complex encoded on the same polycistronic mRNA are precisely controlled at the level of translation to reflect the stoichiometry of the complex [68].

Although ribosome profiling has proven to be powerful in defining ribosome positions on the entire transcriptome, nevertheless the technique also has some challenges: one major issue is that ribosomal profiling, like polysomal profiling, relies on fractionation of cell extracts through a sucrose gradient/ cushion; and it requires a large amount of starting material (10 million cells) to get a reasonable amount of RNA for downstream analysis. Moreover, the method is labour intensive (~7 days) and thus not suitable for high-throughput applications. There are also issues that could lead to artefacts and misinterpretation of data. For example, pseudoRPFs could arise from structured double stranded regions of RNA, as RNaseI only degrades single stranded RNA, thus double stranded RNA is artificially protected as an RPF. The shortness of RPF sequences also obscures the analysis of distinct mRNAs subpopulations (e.g. splice forms, alternate 5' and 3' UTRs) that may be translated at different levels and could be occupied by different numbers of ribosomes (e.g. splice isoforms, alternate 5' and 3' UTRs).

Ribosome affinity purification – capturing the translatome of specialised cells

Gene expression studies from particular cells types are limited by difficulties of isolation without substantial contamination from other surrounding cells or tissues. Ribosome affinity purification (RAP) or translating RAP (TRAP) has thus become increasingly popular as a new tool to monitor gene expression in specific cell-types such as neurons and stem cells [71]. Therefore, genetically modified cells/organisms are constructed which express an affinity-tagged ribosomal protein of the large (60S) ribosomal subunit *in vivo*. Importantly, the expression of the tagged RPs can be controlled by a tissue-specific promotor, such as the Gal4-UAS system in *D. melanogaster* or the Cre-lox system in mice. The tissue is then collected and tagged ribosomes are recovered by affinity selection, capturing only those ribosomes that are expressed in the cells of interest (Figure 1). Finally, the RNA is isolated

from the captured ribosomes and quantitatively measured with microarrays/ RNA-seq. Of note, since entire ribosomes are captured, which includes monosomes and polysomes, RAP/TRAP does not provide a high-resolution map of the translational status of a mRNA as polysomal profiling does. Nevertheless, because translation is primarily regulated at the initiation step, before the 80S ribosomes are formed on the mRNA, RAP and translatome analysis gives a very good approximation of the translation status of mRNAs; and if combined with transcriptome analysis, it can unravel translational regulation [4, 72].

In a first application, Inada and colleagues used FLAG-$(His)_6$-tagged ribosomal protein L25 (Rpl25p) to capture monosomes and polyribosomes (polysomes) from yeast *Saccharomyces cerevisiae* extracts with an anti-FLAG agarose affinity resin [73]. Although ribosomal proteins, ribosomal RNAs (rRNAs) and mRNAs were successfully co-purified with the Rpl25p bait, large polysomes (> 5 ribosomes) were underrepresented and hence, it was concluded that the strategy may not adequately reflect the translational status of mRNAs. However, a selection screen for tagged RPs that fully integrate into translating polysomes, as well as biochemical improvements (use of microbeads instead of agarose/sepharose for affinity isolation), enabled the efficient capture of ribosome associated RNA for translatome analysis in yeast [74]. Thereby, affinity-tagged Rpl16a was used to profile alterations of the translatome upon different stress conditions, and comparison of translatome changes with that of the transcriptome revealed that RAP is suitable to detect translational regulation events with high sensitivity [4]. In particular, it was found that severe stress to cells imposed highly co-ordinate programs between the transcriptome and the translatome, whereas mild stress lead to a non-correlated response preferentially changing the translatome, providing further evidence that translational regulation events are key for rapid adaptation to changing environmental conditions [74].

Whereas the study of the translatome in unicellular organisms like yeast provides a valuable alternative to classical polysomal profiling with diverse applications, e.g. [75], RAP has now become of great interest to profile gene expression of specific cell-types in both plants and animals. In plants, RAP was initially established in *Arabidopsis thaliana* by the Bailey-Serres laboratory [76]. They engineered plants that allowed for cell-specific expression of FLAG-tagged-Rpl18 via developmentally regulated promotors, and quantified differentially expressed mRNAs of 21 different cell-types under normal and hypoxic conditions with DNA microarrays [77]. The study provided a first atlas of translated mRNAs within a cell population of seedlings and explored cell-specific adjustments in response to hypoxia. RAP was also implemented to profile gene expression in multiple zones of the *Arabidopsis* floral meristem and developing flowers [72]. The latter study also compared the translatome with the transcriptome using a deep sequencing approach, which revealed widespread post-transcriptional regulation at both the intron-splicing and translational stages; and they identified a new class of non-coding RNAs associated with polysomes [72].

In animals, RAP was first established in mice [78, 79]. The Heintz lab used bacTRAP transgenic mice to drive expression of enhanced green fluorescent protein (eGFP)–tagged-*Rpl10a* in different neuronal cell types, followed by the affinity purification of eGFP-labelled ribosomes with anti-GFP antibodies and subsequent microarray analysis of associated mRNAs [78]. To further validate their approach, which they referred to as TRAP, they profiled the translatomes of twenty-four CNS cell populations, identifying thousands of cell-specific mRNAs that could not be detected in whole-tissue microarray studies, and which demonstrated the benefits of TRAP for comparative analysis of gene expression across different cell-types [80]. While these initial studies were based on the tissue specific expression of GFP-L10a using BAC transgenes such that each lineage of interest required

construction of a new transgenic mouse line, several TRAP mouse lines have now been constructed in which lineage-selective Cre activates expression of tagged *Rpl10a*. Given the large number of available Cre driver lines, such conditional TRAP mouse models now greatly broadens the applicability of the technology; for instance to profile for markers and pathways in mouse disease models [81-83]. For example, EGFP-L10a was targeted into the ubiquitously expressed *Rosa26* locus in murine embryonic stem cells (ES) and TRAP was conducted to identify genes that are differentially expressed in a cardiomyocyte disease model [81]. Likewise, the distinct cellular responses in an ischemia reperfusion injury mouse model of acute kidney injury (AKI) were investigated [82]; and translatome profiling in myofibroblasts during kidney fibrosis revealed new potential biomarkers [84]. In order to define changes in the translatome versus changes in the transcriptome, a commonly used method is to compare the translatome from the tagged-ribosome cell population, with total RNA isolated from the entire tissue [78, 80, 83]. An alternative approach, explored by Hupe and colleagues, involved comparing TRAP-mRNAs from the tagged cell of interest with TRAP-mRNAs from the whole tissue containing the tagged cell of interest, but this requires the generation of two different Cre driver lines, and was found to be important only when studying abundant cell populations [83].

Recently, TRAP has also been implemented for cancer studies, in particular to study the radiation response of glioma cells in a mouse model of proneuronal glioblastoma [85]. A recent innovative study modified TRAP by tagging *Rpl10a* with a camelid nanobody raised against GFP [86]. The nanobody fused to ribosomes stably binds to intracellular GFP, which then allows for ribosome capture from cell-lysates with anti-GFP antibodies that recognise a different epitope than the nanobody. The approach was used to capture translating mRNAs from neurons injected with a retrogradely transported tracing GFP-tagged virus (canine adenovirus type 2), allowing the identification of markers which

delineate between cell-types projecting into the nucleus *accumbens* [86]. Finally, TRAP in non-transgenic animal has recently been demonstrated through viral infection of Purkinje cells in mice [87]. Combining micro-dissection with TRAP on cytoplasmic and rough endoplasmic reticulum (ER) provided an advanced map of the expression landscape of Purkinje cells and its dendrites [87].

Besides yeast, plants and mice, RAP/TRAP became has also been adapted to other model organisms: in the fruitfly *D. melanogaster*, the use of the GAL4/UAS system allows for profiling of almost any tissue/cell-type. For example, TRAP was employed to quantify neuronal expression in heads and to capture the translated RNAs from small populations of neuro-secretory cells in adult brains [88]. In zebrafish *(Danio rerio)*, TRAP was applied to profile translating RNAs in cardiomyocytes during heart regeneration [89], and to measure the impact of a short heat-shock on the expression of selected mRNAs in neutrophils, macrophages, and epithelial cells [90]. Finally, TRAP was used in frogs (*Xenopus laevis*) [91] to isolate mRNAs from retinal ganglion cell axons and rod photoreceptors [92].

Conclusions and perspectives

Polysomal profiling was the first method which hinted at the scope of translational control; it allowed us to recognise for the first time that just because a mRNA is transcribed, it doesn't mean that it is "automatically" translated, and that a mRNA may be translated under one particular condition only. However, it wasn't until the advent of ribosomal profiling that the understanding of the complexity of translational control really began to gather pace. The power of the positional information conferred by ribosomal profiling is revealing not only dynamic changes to the translatome during different conditions, but also the *cis*- and *trans*-acting control mechanisms by which these changes can be conferred. Furthermore, parallel development of affinity-tagged approaches allowed for cell/tissue-type specific profiling of

ribosomes from complex heterogeneous samples. This category of translatome analysis method also tends to be reliable, quick and efficient, as it relies on a robust immunoprecipitation against a well-defined tag.

Nonetheless, refinement and further development of the current methods for translational profiling are in need as all three methods are not without fault (Table 1). Whereas polysomal and ribosomal profiling require relatively large sample size, and involve laborious biochemical purification on a sucrose density gradient prone to contamination; the affinity-purification techniques often require the expression of exogenously tagged RPs and thus, capture only a fraction of all ribosomes in cells. The combination and further iteration of the three main methodological-themes bears the potential to cope with the issues related to each individual approach. Indeed, two recent papers reported the combination of either affinity purification or polysomal fractionation, with ribosomal profiling: Juntawong and colleagues combined ribosome affinity purification and ribosomal profiling to map the translatome of *Arabidopsis* during normoxic and hypoxic conditions [93]; and Aspden and colleagues profiled translation in Drosophila S2 cells using “poly-ribo-seq”, whereby polysomal fractions obtained from sucrose density gradients are used for subsequent ribosomal profiling [94].

An additional consideration when measuring the translatome to infer the proteome, is the post-translational regulation of produced proteins; if a protein is produced then rapidly degraded, the translational status of that protein may not correlate with the protein level. For example, the hypoxia inducible factor 1 alpha (HIF1α) is translated and rapidly degraded during normoxic conditions [95]. In such instances, it may be useful to measure the proteome in parallel with the translatome, and indeed, recent work using a combination of LC-MS/MS and ribosomal profiling was able to demonstrate the presence of several N-

terminally extended proteins that either method alone was not able to reliably identify [96, 97].

Looking forward, further advancement of translatome analysis is likely to require the development of entirely new biochemical methods that enable easy, rapid, and reliable isolation of ribosomes from small numbers of cells or even a single cell. Likewise, improved and simplified bioinformatics will be key for streamlined analysis of the fast data generated from RNA-seq and to generate models (e.g. [98]). If this could be accomplished, translatomics may become as widely used as transcriptomics and become applicable for integration in high-throughput functional genomics screens.

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Key Points

- Translational control is known to play a pivotal role in the gene expression programme, and as such, analysis of the translatome (the set of mRNA undergoing translation) is critical for the understanding of gene expression.
- Three main approaches for translatome analysis are currently used; polysome profiling, ribosome profiling and ribosome-affinity techniques.
- Polysome profiling separates mRNA based on the number of associated ribosomes on a sucrose gradient, but suffers from the need for specialised equipment, large sample size requirements and has potential for contamination with other macromolecular complexes.
- Ribosomal profiling involves the sequencing of ribosome-protected mRNA fragments (RPFs) and generates highly informative ribosome-positional information, but requires a large sample size and has potential for pseudo-RPF contamination.
- Ribosome affinity purification involves the isolation of affinity-tagged ribosomes and allows for cell-type specific translatome analysis, but suffers from the requirement for genetic manipulation.

Figure legend

Figure 1: Experimental approaches to study the translatome

Cell-extracts are usually prepared in the presence of cycloheximide (CHX), which is a potent inhibitor of translational elongation. (Left) Polysomal profiling; extracts are separated by ultracentrifugation through a linear 10–50% sucrose density gradient. The gradient is then fractionated while continuously monitoring the absorbance at 254 nm (A_{254}) allowing the separation of ‘‘free” RNA, the small (40S) and large (60S) ribosomal subunits, monosomes (80S), and polysomes. RNA is isolated from individual gradient fractions and pooled for subsequent microarray or RNA-seq analysis. The relative position of a message in this profile is an indicator of its translational activity. (Middle) Ribosomal profiling; extract is treated with RNaseI to digest unprotected and non-ribosome bound regions in the mRNAs. The ribosomes are further enriched through a sucrose cushion, and ribosome protected fragments (RPFs) of RNA are size-fractionated by gel electrophoresis. RPFs of approximately 30 nucleotides are recovered and ligated to sequencing adaptors for reverse transcription, amplification and high-throughput RNA-seq. Additionally, cDNAs generated from rRNA are usually depleted before amplification. (Right) RAP procedure; affinity-tagged (e.g. GFP, protein A) ribosomes are captured from extracts with specific antibodies or ligands coupled to a matrix. After several stringent washes, the ribosomes and associated RNAs are released from the matrix and captured RNAs are analysed with DNA microarrays or by RNA-seq. It is possible to combine either polysomal profiling (left), or affinity purification (right), with ribosomal profiling (centre), using either of the aforementioned methods as a vehicle for enriching the sample with ribosomes before isolating ribosome protected fragments.

Table 1

Method	Advantages	Disadvantages	Optimal applications
Polysomal profiling (Figure 1, left)	• Ribosome density measurement • Fractionation by ribosome occupancy on mRNA	• Specialised equipment • Large number of cells (~10^7) • Labour intensive • Pseudopolysome contamination • Lack of cell-type specificity	• Cultured cells/ tissues • Initial screen for translational changes • To track the translational status of a given mRNA / particular set of mRNAs whose identity is known
Ribosomal profiling (Figure 1, centre)	• Ribosome density measurement • Ribosome positional information • Discovery of; new uORFs, alternative start codons, alternative protein isoforms, decoding speeds, translational pause sites, translational dynamics	• Specialised equipment • Large number of cells (~10^7) • Labour intensive • PseudoRPF contamination • Expensive (deeper sequencing due to fragment size) • Extensive bioinformatics – annotation of small sequencing fragments • Lack of cell-type specificity	• Cultured cells/ tissues • Dissection of initiation and elongation events. • Determination of molecular mechanisms of translational control
Ribosome affinity purification (Figure 1, right)	• Smaller number of cells (<10^6) • Cell/tissue-type specific • Simple, cheap, robust	• Genetic modification e.g. transgenes to express tagged RPs • Lower resolution	• Samples with genetically tagged ribosomes