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Critical Reviews and Perspectives

Next generation sequencing technologies to address aberrant mRNA translation in cancer

Ángel-Carlos Román*, Dixan A. Benítez, Alba Díaz-Pizarro, Nuria Del Valle-Del Pino, Marcos Olivera-Gómez, Guadalupe Cumplido-Laso, Jose M. Carvajal-González and Sonia Mulero-Navarro*

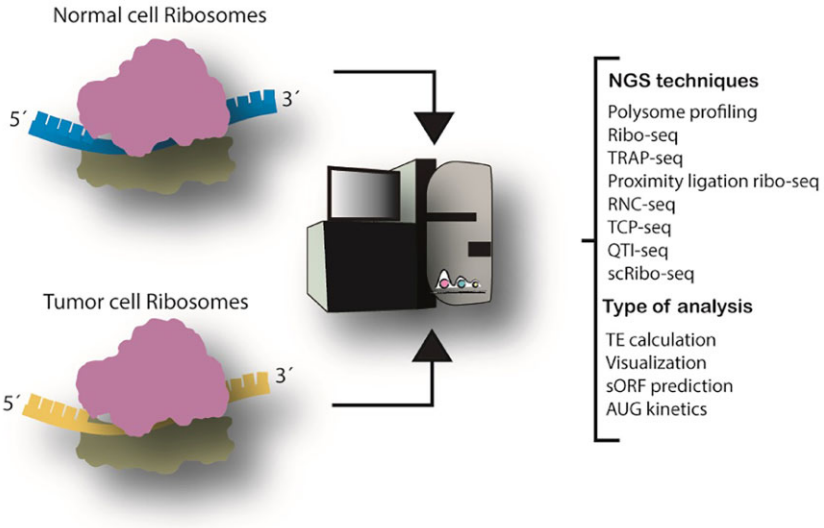
Departamento de Bioquímica y Biología Molecular y Genética, Universidad de Extremadura. Avda. de Elvas s/n, 06071 Badajoz, Spain

*To whom correspondence should be addressed. Tel: +34 924 289 653; Email: acroman@unex.es
Correspondence may also be addressed to Sonia Mulero-Navarro. Email: smmulero@unex.es

Abstract

In this review, we explore the transformative impact of next generation sequencing technologies in the realm of translatomics (the study of how translational machinery acts on a genome-wide scale). Despite the expectation of a direct correlation between mRNA and protein content, the complex regulatory mechanisms that affect this relationship remark the limitations of standard RNA-seq approaches. Then, the review characterizes crucial techniques such as polysome profiling, ribo-seq, trap-seq, proximity-specific ribosome profiling, rnc-seq, tcp-seq, qti-seq and scRibo-seq. All these methods are summarized within the context of cancer research, shedding light on their applications in deciphering aberrant translation in cancer cells. In addition, we encompass databases and bioinformatic tools essential for researchers that want to address translatome analysis in the context of cancer biology.

Graphical abstract



Introduction

Proteins play a myriad of essential roles in cellular functions, ranging from structural components to enzymes. The central dogma of molecular biology elucidates the flow of information from DNA to RNA through transcription, and from RNA to protein through translation. Over the past few years, next generation sequencing (NGS) technologies have revolutionized transcriptomic analysis, providing a rapid and cost-effective means to explore large-scale data (1–3). NGS has become an indispensable tool in biomedicine and cancer research, facilitating a comprehensive understanding of gene expression responses to various cellular states (4,5). The high-throughput nature of NGS allows simultaneous analysis of millions of RNA sequences, yielding extensive information previously unattainable with traditional sequencing or PCR-based methods.

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Despite the central dogma's expectation of a strong correlation between mRNA and protein content, additional regulatory mechanisms, such as mRNA stability, quality assessment, ribosome heterogeneity and translation elongation rate, impact mRNA and protein abundance (6–8). Consequently, the direct use of RNA-seq is not an appropriate tool for measuring protein abundance. This is even exacerbated in the case of cancer studies, due to the existence of multiple DNA copy number variations affecting gene expression that are then compensated at the protein level, reducing the correlation between mRNAs and proteins (reviewed in 9).

This review explores the adaptation of RNA-seq and other NGS methods in the study of protein synthesis, encompassing the emerging field known as 'translatomics' (10). Emphasis will be placed on studies utilizing these methods to investigate aberrant translation in cancer. Finally, we will provide a summary of essential resources and databases for cancer researchers delving into translatomics. It's important to note that, due to space constraints, we will exclude epitranscriptomic methods such as m6A-seq or MeRIP-seq. These methods allow the identification and quantification of modified RNA molecules, affecting indirectly protein translation through their impact on mRNA stability and interactions with the ribosomal machinery.

Polysome profiling

Protein synthesis relies on the ribosome's ability to translate mRNA into peptides. During the elongation phase, multiple ribosomes associate with different coding regions of the same mRNA molecule, forming a structure known as a polyribosome or polysome. Analyzing mRNAs recruited to these polysomes serves as a valuable proxy for protein abundance. A traditional technique for isolating actively translated mRNAs bound by polysomes involves sucrose gradient centrifugation (11). The mRNAs bound to a different number of ribosomes can be separated in this gradient. Subsequently, these mRNAs can be analyzed using various techniques such as RT-qPCR, microarrays or RNA-seq, collectively referred to as polysome profiling. In order to stabilize ribosomes before the experiment, a cycloheximide treatment (that arrest ribosomal elongation) is generally used in multiple translatomic methods. Several reports have showed that this cycloheximide treatment can produce artifacts or impact in gene expression (12,13). While other options as flash freezing the samples are available, cycloheximide treatments remain as the most standardized method to stabilize ribosomes.

Polysome profiling analyses typically compare mRNA levels obtained in polysomal fractions to those in non-polysomal fractions (i.e. non-translated mRNAs). This translational efficiency (TE) becomes the rate of mRNA translation into protein. When it is compared across different conditions identifies mRNAs that undergo changes in translation status. This is particularly relevant in the case of oncogene-driven cancers are they are characterized by elevated ribosome biogenesis. Thus, this analytical approach has revealed translatome signatures in conditions such as glioblastoma or leukemia patients (14,15). Additionally, it has been employed to study the impact of ionizing radiation on the translatome of glioblastoma cell lines (16) and assess the molecular effects of novel cancer drugs (17). In this case, the combined mechanistic effect of rRNA synthesis inhibitors with other drugs was analyzed, leading to the relation between metabolic activity and pro-survival signaling in cancer.

Despite certain limitations associated with polysome profiling (see Table 1), modified versions, such as high-resolution polysome profiling followed by sequencing of the 5′ ends of mRNAs (HP5) (18), have been proposed. HP5, with its calculation of the mean ribosome load using spike-in RNA standards, allows the distinction of mRNA isoforms based on their transcriptional start site (TSS) usage.

Ribosome profiling (Ribo-seq)

While polysome profiling assesses mRNAs actively undergoing the elongation phase of protein synthesis, it lacks the single-nucleotide resolution needed for certain aspects, such as determining untranslated regions (UTRs) or identifying pauses and variations in translational elongation rates. Ribosome profiling, or ribo-seq, capitalizes on the ribosome-protected mRNA footprint (∼30 nt) resulting from nuclease digestion (19). Specifically, cell samples treated with cycloheximide undergo RNAse I treatment, followed by ribosome retrieval through density in sucrose gradient or sucrose cushion. The mRNA fragments are isolated using a small RNA purification kit and separated by electrophoresis in order to extract 26–34nt footprints. Then, the mRNA footprints are dephosphorylated, subjected to linker ligation and reverse transcribed. Finally, the cDNA products are circularized, depleted from rRNA and amplified by PCR with a barcode addition, prior to their analysis by NGS (20).

Analyzing ribo-seq data follows a similar approach to polysome profiling, involving the ratio of translated to total mRNA for each mRNA, with subsequent comparison between different conditions. This technique has been applied across various tumor types, including liver cancer, to identify differentially translated genes alongside differentially expressed genes from transcriptomics (21,22). Beyond this, ribosome profiling serves a critical role in globally identifying novel open reading frames (ORFs) and upstream ORFs (23,24), particularly evident in cancer samples such as lymphomas (25).

Adapting ribo-seq analysis for TE calculation involves generalized linear or logistic regression models between individual ribo-seq and bulk RNA-seq reads (26,27). This method allows the computation of fold changes in TE and corrected P-values in the transcriptome, proving useful in detecting TE alterations between poorly and highly metastatic breast cancer cells (27). Furthermore, ribo-seq data offers single-base resolution for identifying the reading frame which was currently translated at the time of cycloheximide treatment (28). In the context of cancer, these data can serve to multiple uses. For example, neoantigens (tumor-specific mutation-derived antigens) can be developed from ribo-seq data (29). In addition, codon analyses showed how kidney cancer cells depend on the amount of proline and the tumor progression was linked to PYCR1 expression (30). Ribo-seq data also clarified the mechanism by which FKBP10 enhances lung cancer progression. This protein altered the translation elongation rates at the beginning of ORFs; specifically, reading frame occupancy significantly increased in the four proline codons compared to others after FKBP10 knockdown. This result suggests that FKBP10 favors the elongation of proline-coding codons (31). Similar analyses even allow the definition of specific stages within the elongation phase due to distinct populations of ri-

Table 1. Summary of the NGS methods that have been used for translatomic analyses described in the review

Name	Relative input material	Relative technical expertise	Relative cost	Main advantage	Main limitation	Used in cancer studies
Polysome profiling	Mid	Low	Low	Standardized, ability to discriminate between monosomes and different polysomes	Low nucleotide resolution without modified procedures	Yes
Ribo-seq	Low	Mid	Low	Main standard in literature, multiple modifications of the technique	General approach that does not discriminate between different ribosomes	Yes
TRAP-seq	Low	High	High	Ideal for *in vivo* (mouse) cancer models	Mostly limited to model organisms	Yes
Proximity-specific ribo-seq	High	High	High	Adequate to study translation associated to cancer metabolism or ER stress	Limited to transgenic cancer cell lines	No
RNC-seq	Mid	Mid	Low	Ideal for study circRNA translation in cancer	Not adequate for TE ratios	Yes
TCP-seq	High	Mid	Low	Ideal for sORF and AUG kinetics prediction in cancer	Not much literature, not applied to cancer yet	No
QTI-seq	High	Mid	Low	Ideal for pulse-and-chase studies in cancer cell lines	Not much literature, not as standard as ribo-seq	Yes
scRibo-seq	Very low	High	Very High	Interesting for human cancer samples	Expensive, not much literature	No

bosomal footprints (32) and the determination of translation elongation speed (33).

Translating ribosome affinity purification (TRAP-seq)

TRAP-seq distinguishes itself through its ribosome purification method, achieved by expressing a tagged ribosomal protein in the cellular sample. Subsequently, the tagged ribosomes are immunopurified for subsequent analysis. A notable advantage of this technique lies in the utilization of specific promoters that control the expression of the tagged gene, enabling a cell type-specific measure of translation in tissues or complex co-cultures (34–36). This innovation has led to the creation of mouse lines, such as the RiboTag mouse, with labeled ribosomes in different cell types using Cre recombinase-expressing lines (37). Furthermore, a modified TRAP method employing the enhancer-trap technique allows the generation of zebrafish lines suitable for tissue-specific translation studies (38). RiboTag mice have been instrumental in determining the translational landscape of brain tumors; it was used not only to identify specific genes related to cell adhesion and extracellular matrix that maintain higher translational rates in brain tumors compared to normal brains, but also to find that the translation efficiency is reduced in gliomas (39).

However, it's important to note that transgene expression in samples is a requisite for TRAP-seq, potentially making it more challenging or time-consuming compared to other techniques that utilize endogenous or chemically-treated samples, and limiting its use to model organisms. The analysis of TRAP ribosomes typically involves ribo-seq, following RNAse I treatment for ribosomal footprint detection. While TRAP methods traditionally rely on transgene expression, new adapted methods employ endogenous ribosomal epitopes for purification, exemplified by riboPLATE-seq (40).

Proximity-specific ribosome profiling

While Ribo-seq and other techniques traditionally purify the entire cellular content of ribosomes, recent years have shed light on the significant heterogeneity of ribosomes. This diversity depends on factors such as rRNA, protein stoichiometry, and epitranscriptomic and post-translational modifications (41). Such heterogeneity can give rise to specialized ribosomes, ultimately influencing the translation of specific mRNAs. Although TRAP-seq can detect different ribosomal subtypes based on expressed protein variants (42), its application is time-consuming and necessitates prior knowledge of the variants.

In contrast, proximity-specific ribosome profiling offers an intriguing method for assessing heterogeneous ribosomal footprints and understanding translation at defined subcellular locations (43). This technique utilizes a spatially-restricted biotin ligase to label ribosomes with a biotin acceptor peptide in live cells. For example, an exogenous biotin ligase can be designed to localize in the endoplasmic reticulum (ER) using a construct with Sec1 protein. After a biotin pulse, only the ribosomes that are associated to this organelle will be modified and then isolated during streptavidin pulldown. Afterwards, the procedure aligns with a standard ribo-seq experiment (43). The non-biotinylated fraction of footprints can then be compared with the subcellularly-enriched fraction.

Other methods rely upon other techniques to isolate specific subcellular ribosomes. In the case of mitochondrial ribosomes, MitoRiboSeq, explores subcellular translation (specifically, mitochondrial translation) through ultracentrifugation (44); in addition, a FLAG-tagged mitochondrial protein can

lead to mitochondrial ribosomes purification after immunopurification (45). The advantage of MitoRiboSeq resides in the use of non-genetically engineered cell samples, as it is also in the case of proximity-specific ribosome profiling.

These subcellular-restricted translatomic techniques have successfully characterized translation associated with endoplasmic reticulum stress and the unfolded protein response in yeast, pointing to the role of ER membrane complex as the regulator of the multipass membrane protein synthesis (46). Proximity-labeling of mitochondrial ribosomes has been also done in human cells (47). Anticipated in the coming years is the exploration of how proximity labeling can unravel translational mechanisms linked to cancer progression, using cancer cell lines.

Ribosome-nascent chain complex (RNC-seq)

The ribosome-nascent chain complex (RNC) encompasses molecules attached to a polypeptide during synthesis, including mRNA and ribosomal components. RNC-seq involves isolating full length RNCs for subsequent purification of mRNAs subjected to deep sequencing. Cell lysates, cleared of debris, are transferred to a high sucrose cushion (not gradient), and pellets obtained after ultracentrifugation yield purified RNCs (48). In contrast with polysome profiling, that uses sucrose gradient, RNC-seq does not discriminate between different mRNAs bound by a different number of ribosomes. Since whole (poly-A) mRNAs are present in the RNCs, poly-dT selection can be employed for mRNA purification. A limitation derived from this method is that initiating ribosomes (ribosomes at 5′ position of the coding sequence) are not detected by RNC-seq. Unlike ribo-seq, which requires specific exclusion of rRNA, RNC-seq calculates the translation ratio for each transcript by comparing RNC-fraction mRNA to total mRNA. A key analytical distinction from ribo-seq is that in RNC-seq, full-length translating mRNAs are sequenced, improving the efficiency of alternative spliced transcript detection. Interestingly, this feature of RNC-seq becomes useful for the analysis of protein-coding circRNAs due to backsplicing identification.

Using human cell lines, RNC-seq demonstrated approximately two times higher estimation of protein isoforms compared to ribo-seq for the same number of valid reads (49). Sequencing technologies enabling longer reads enhance the detection of known and novel isoforms (49). RNC-seq holds promise for studying circular RNAs (circRNAs) and their role during translation (50), with applications in detecting circRNAs bound to ribosomes in glioblastoma. Specifically, a novel peptide translated from LINC-PINT circRNA interacts with the RNA polymerase II machinery to inhibit transcriptional elongation of several oncogenes, while circMET (a circular transcript from MET oncogene) self activates MET signaling (51,52).

Translation complex profile (TCP-seq)

TCP-seq could be considered an intermediate method between ribo-seq and RNC-seq. The main distinction lies in the formaldehyde crosslinking step preceding ribosomal sedimentation (53,54). Subsequently, RNAse I treatment generates additionally protected mRNA footprints. During centrifugation, polysomal, ribosomal, and SSU (small subunit of the ribosome) fractions can be separated before deep sequencing (53,54). SSU footprints are notably enriched in 5′UTRs and start codons, enhancing information about translation initiation. Extensions of this method allow the analysis of yeast and human 40S and 80S fractions through immunoprecipitation (55,56). This resulted in the determination of differences in eukaryotic AUG recognition kinetics (56). To our knowledge, TCP-seq has not yet been employed for determining translational mechanistic features associated with UTRs or start codons in the context of cancer models, though it is especially interesting for sORF and uncanonical 5′ UTR identification.

Quantitative translation initiation (QTI-seq)

Ribo-seq technology excels at estimating translation efficiency, but initiation rates can be influenced by elongation speed, which, in turn, impacts ribosome density on mRNAs. Moreover, alternative translation events within the same mRNA are often obscured in the data. To address these challenges, treatments with initiation-specific translation inhibitors like harringtonine or lactimidomycin have been employed instead of cycloheximide. However, these treatments can also introduce artifacts due to the incubation period (20,57). As mentioned earlier, TCP-seq also contributes to our understanding of translation initiation (53).

QTI-seq is another method designed to detect start codon selection and quantify translation initiation sites and rates (58). This technique aims to preserve initiating ribosomes with minimal perturbation, achieved through sequential treatments with lactimidomycin or harringtonine and puromycin. Lactimidomycin (or harringtonine) stabilizes initiating ribosomes, while puromycin triggers the dissociation of elongation ribosomes. Consequently, QTI-seq selectively retrieves mRNA footprints from initiating ribosomes, specifically focusing on detecting translation initiation sites (58). By combining RNA-seq, ribo-seq and QTI-seq, a pausing index representing the dwell time of initiation ribosomes can be calculated (59). This analytical technique, akin to the one designed for promoter-bound RNA Pol II pausing, revealed alterations in pausing during RASG12V oncogenic transformation (59). This pausing index has been correlated with m6A modification of 5′ UTRs (59), though a recent report questions the role of this modification on translation initiation (60). Nevertheless, METTL3 knockdown also promoted tumorigenesis in RASG12V cancer samples (59).

Single-cell ribosome profiling (scRibo-seq)

In recent years, NGS technologies have undergone advancements to facilitate the assessment of transcriptomic or epitranscriptomic content at the single-cell level (61–64). This approach proves invaluable for deciphering cellular heterogeneity, as well as identifying and characterizing cell types or subtypes within complex tissues (65). In the realm of translatome studies, single-cell technology has been adapted for ribo-seq (66).

Specifically, single, live cycloheximide-treated cells are sorted and lysed. Subsequently, either exposed or free RNA is digested with micrococcal nuclease, and the protein content of each cell is degraded using a guanidium thiocyanate-containing proteinase K solution. Ribosome protected fragments of RNA from each cell are processed to generate indexed libraries, and purified pools are subjected to deep sequencing (66). To mitigate micrococcal nuclease preferences

Table 2. Summary of bioinformatic tools and databases that can be used for translatome analysis

Name	Usage	Technical expertise	Reference
TranslatomeDB	Database; Retrieval and simple comparison of NGS (translatomic) experiments	Low (webpage)	(68)
MetamORF	Database of short ORFs	Low (webpage)	(69)
sORFs.org	Database of short ORFs	Low (webpage, with the possibility of advanced queries)	(70,71)
XPRESSyourself	Complete analysis and visualization of translatome data	Mid (Github code)	(73)
RIVET	Visualization and differential analysis	Low (R shiny)	(74)
Riborex	Differential analysis	Mid (R package)	(75)
RiboDiff	Differential analysis	Low (code and galaxy server)	(76)
RiboDoc	Differential analysis	High (Docker image)	(77)
RiboToolkit	From data cleaning to multiple analyses	Low (webserver)	(78)
RiboMiner	From QC to differential analysis	High (Python code)	(79)
RiboStreamR	Data preprocessing, analysis and visualization	Low (R Shiny)	(80)
Rp-Bp	Prediction of translated ORFs	High (Python code)	(81)
Ribo-TISH	Prediction of translated ORFs	High (Python code)	(82)
RiboTaper	Prediction of translated ORFs	High (R and Linux code)	(83)
RiboGalaxy	Integrated suite of tools	Low (Galaxy server)	(84)

for cutting positions, a random forest classifier algorithm has been developed for single-codon resolution of single-cell footprints (66). A similar single-cell technique has been employed to characterize ribosomal occupancy in oocytes (67). In the near future, we anticipate studies utilizing scRibo-seq for the analysis of translation in the context of cancer heterogeneity. Nevertheless, this technique is strongly limited due to the amount of mRNA fingerprints that can be analyzed (only the most expressed/translated genes) and the heterogeneity arisen from the ribo-seq method.

Databases and resources for translatome analysis

As demonstrated in the preceding paragraphs, translatomic methods are continuously evolving to enhance accuracy and detect specific molecular features of translation (Table 2). However, the escalating volume of generated data presents an opportunity for future re-analysis or meta-analysis. The TranslatomeDB is a notable database that integrates both published and user-generated translatome sequencing data (68). Other databases, such as MetamORF (69) and sORFs.org (70,71), focus specifically on characterizing novel ORFs. Though the existence of these databases is positive, it is important to remark that those are laboratory-centric initiatives, and the participation of international consortia is required in order to develop curated, stable databases for translatome studies.

As of our knowledge, there are no additional translatomic standard repositories consolidating data from various sources specifically in the context of cancer, despite the National Cancer Institutes emphasizing the importance of uniformly processed NGS and clinical data (72). It is also imperative to include translatomic data alongside other data types, including genomic, epigenomic or transcriptomic, to ensure cancer databases contain accurate information on protein translation. Standardizing analytical NGS toolkits for translation, as demonstrated by the recent XPRESSyourself pipeline (73), could facilitate the sharing and integration of this information. In addition, several other software tools have been developed for different laboratories to analyze (determine changes in TE efficiency per transcript) and visualize (represent data in IGV or similar genomic browsers) translatomic data (74–80). These tools use distinct programming languages and methods of accession, and in Table 2 we present not only their usage but also their simplicity for non-computational biology users. A specific field that is specially interest in the case of cancer is the prediction of short translated ORFs from translatomic data. In this case, there are specific tools that are available to perform this analysis (81–83, see Table 2). Finally, we consider that RiboGalaxy (84) can become a very useful resource for biologists and oncologists that perform sporadically ribo-seq or other translatomic experiments, because it maintains a suite of different tools (as the previously mentioned) in a cloud-based environment that reduce the difficulty of installation and use of lab-made software.

Data availability

No new data were generated or analysed.

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Conflict of interest statement

None declared.

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