Seminars in Cell and Developmental Biology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Imaging spatiotemporal translation regulation *in vivo*

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ARTICLE INFO

Keywords:
Single-molecule imaging
RNA
Translation regulation
Local translation
RNA granule

ABSTRACT

Translation is regulated spatiotemporally to direct protein synthesis when and where it is needed. RNA localization and local translation have been observed in various subcellular compartments, allowing cells to rapidly and finely adjust their proteome post-transcriptionally. Local translation on membrane-bound organelles is important to efficiently synthesize proteins targeted to the organelles. Protein-RNA phase condensates restrict RNA spatially in membraneless organelles and play essential roles in translation regulation and RNA metabolism. In addition, the temporal translation kinetics not only determine the amount of protein produced, but also serve as an important checkpoint for the quality of ribosomes, mRNAs, and nascent proteins. Translation imaging provides a unique capability to study these fundamental processes in the native environment. Recent breakthroughs in imaging enabled real-time visualization of translation of single mRNAs, making it possible to determine the spatial distribution and key biochemical parameters of *in vivo* translation dynamics. Here we reviewed the recent advances in translation imaging methods and their applications to study spatiotemporal translation regulation *in vivo*.

1. Introduction

Protein synthesis depends on an mRNA's abundance and translation efficiency, which is tightly regulated in both time and space. At the transcription level, genes are differentially expressed in different cell types. Additionally, mRNAs are post-transcriptionally regulated via translation, degradation, transportation to subcellular compartments, or sequestration in membraneless organelles. This allows cells to deploy specific proteins only where they are required, rapidly change their proteome in response to environmental cues, and shut down global translation at the time of stress. RNA localization and spatial translation regulation have been recognized since the 1980s [1]. They are required to establish subcellular structure and maintain local proteome homeostasis to sustain cell function. The leading edge of a fibroblast [2–4], the anterior and posterior pole of a developing embryo [5,6], and the neuronal dendrites or growth cones of axons [7–9] are examples of subcellular compartments that have distinct biochemical environments requiring translation of a set of localized mRNAs. Membraneless organelles, such as P granules [10], stress granules (SGs) [11,12] and processing-bodies (P-bodies) [13], were discovered in the early 2000s. It was proposed that they were phase condensates formed by RNAs and specific protein factors [14], and function in translation regulation, RNA transport, storage, and degradation [15–17]. Membrane-bound organelles are topologically distinct compartments from the cytosol that require trafficking of proteins through their membranes. The endoplasmic reticulum (ER) is the central hub for synthesis of membrane and secretory proteins. Mitochondria, the energy suppliers of the cell, rely on import of nuclear-encoded proteins. Local translation on these membrane-bound organelles has been studied extensively with biochemical approaches. However, the mRNA motility and translation dynamics on these organelles are not well understood. In comparison to spatial regulation, the temporal translation mechanism has been more thoroughly investigated through structural and functional studies. The ribosome structure has been solved at atomic resolution [18]; translation has been reconstituted *in vitro* [19], and studied at the single-molecule level [20]. However, inside cells, translation responds to diverse signaling pathways and adapts quickly to environmental cues. It becomes clear that the translation kinetic rate is coupled with quality control of ribosomes, mRNAs, and nascent peptides. Therefore, it is imperative to study spatial and temporal translation dynamics in the

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https://doi.org/10.1016/j.semcdb.2023.03.006
Received 23 March 2022; Received in revised form 8 March 2023; Accepted 15 March 2023

native environment.

Biochemical methods such as fractionation, polysome gradients, and ribosome profiling allow transcriptome-wide characterization of translation [21]. However, these methods require breakdown of tissue or cells and therefore do not retain precise spatial information. Fluorescence imaging, on the other hand, maintains the integrity of cellular structures and allows measurement *in situ*. In the past two decades, a plethora of innovative imaging methods have been developed to study translation globally as well as of specific transcripts. They range from metabolic labeling with direct fluorescent tagging [22–24], photoconversion of fluorescent proteins [25], amino acid or tRNA analogues [26–28], RNA biosensors [29], and nascent peptide labeling (NPL) [30–34]. These methods revealed rich posttranscriptional regulation mechanisms and are instrumental to understanding the complex life of mRNAs. Here, we review recent advances in translation imaging, and their applications in studying spatiotemporal translation regulation. For the spatial component, we focus on membrane-bound and membraneless organelles. We discuss the new discovery of translation factories and the innovative usage of artificial condensates for translational control. Regarding RNA localization and translation in neurons and embryos, we refer readers to excellent reviews in those topics [6–9]. As to temporal control, we discuss measurements of *in vivo* translation kinetic parameters and how they are coupled with quality control mechanisms.

2. Methods for *in vivo* translation imaging

We would like to make a distinction between translation imaging versus fluorescence reporters, commonly used to report overall gene expression including transcription, translation, mRNA decay, and protein degradation. Translation imaging focuses on the translation process itself, providing better spatiotemporal resolution. The translation process involves five different components: translation machinery (ribosomes and tRNA), the mRNA template, amino acids, the nascent peptides on ribosomes, and the newly synthesized proteins. Different technologies focus on each of these components to visualize various aspects of translation. Broadly speaking, translation imaging technologies can be divided into 4 categories: 1) Fluorescent tagging 2) Metabolic-labeling 3) RNA biosensors and 4) Nascent peptide labeling (NPL) (summarized in Fig. 1). Each method comes with its own advantages and caveats, which will be discussed here.

2.1. Fluorescent tagging

Pulse-chase is a powerful technique to selectively label newly synthesized proteins: once all existing proteins are saturated with one label, newly made proteins can be tagged with a different color. The pulse-chase approaches come in a variety of flavors (Fig. 1a). In the simplest form, a reporter fused directly with fluorescent protein (FP) is photobleached, and new protein is inferred from the emergence of fluorescent signals [35]. However, the bleaching is complicated by random switching of bright and dark states of fluorescent dyes, also known as blinking, or recovery from non-fluorescent states. Photoconvertible FPs ameliorate this problem. Wang and colleagues have applied Dendra2 to study local translation in an *Aplysia* neuron culture system [25]. FPs are generally less bright and stable than organic dyes. Dye-binding protein motifs can also be used in pulse-chase experiments. For example, the tetracysteine motif (CCXXCC) binds specifically to biarsenical dyes FLAsH/ReAsH to label translation sites [22]. The drawbacks of biarsenical dyes are that they are toxic, and the binding is reversible. Another class of tags includes HaloTag [23] and SNAP-tag/CLIP-tag [24], which covalently link to their respective fluorescent ligands. The bright and photostable Janelia Fluorophore comes in a wide variety of colors [36]. They have been successfully used in pulse-chase experiments to measure the distribution of newly synthesized protein in cultured neurons [37]. Unfortunately, the temporal resolution of the technique is poor due to the need for protein folding and accumulation. In addition, the labeling is irreversible and allows only a single chase step, precluding long-term tracking experiments.

2.2. Metabolic labeling

Non-canonical amino acid (NCAA) can be incorporated into proteins using native translation machinery (Fig. 1b). NCAAs themselves may contain detectable signals or can incorporate bioorthogonal chemistry to install fluorescent tags. FlUorescence Non-Canonical Amino acid Tagging (FUNCAT) uses an azide group containing AHA-NCAA (azidohomoalanine) [26]. A click-chemistry reaction installs alkyne-conjugated fluorophores to all newly synthesized proteins. To label a specific protein, an antibody is used in combination with NCAA-tagging. A proximity ligation reaction is then activated to generate fluorescence signals when both AHA and the antibody are present [38]. With FUNCAT, global translation and specific endogenous

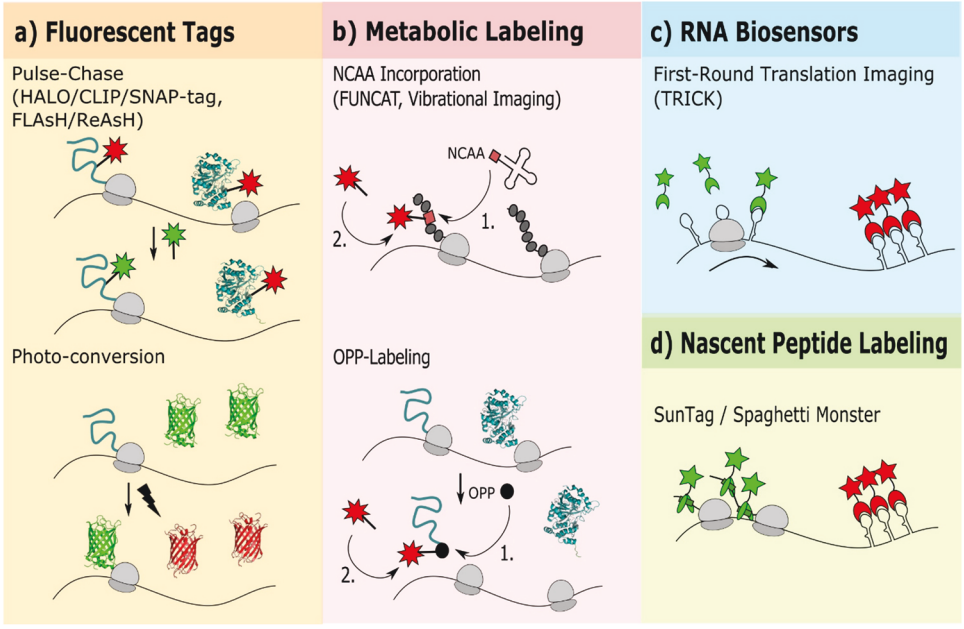

Fig. 1. Methods for translation imaging in vivo a) Directly conjugated fluorescent tags. Top: pulse-chase with two different fluorescent ligands allows differentiation between pre-existing and new proteins. Bottom: new photo-convertible fluorescent proteins (green) can track translation after the existing ones are converted (red). b) Metabolic labeling with non-canonical amino acid (NCAA, top) or O-propargyl puromycin (OPP, bottom). NCAA and OPP in new proteins can be labeled with fluorescent dyes by click chemistry or detected by immunofluorescence. c) RNA biosensors, such as the TRICK reporter, employ orthogonal stem loops placed in the ORF and 3' UTR which bind to different fluorescent coat proteins. Translating ribosomes will knock off the coat protein in the ORF, resulting in mRNAs labeled with a single color, indicating the first round of translation. d) Nascent Peptide Labeling uses multimerized epitopes expressed in the N-terminus of a protein which bind to fluorescent antibodies as soon as they emerge from the exit tunnel of the ribosome. RNA is labeled using stem loops and a colocalized RNA/nascent peptide signal indicates active translation.

protein can be labeled without genetic modification. However, this method is limited by the poor incorporation rate of AHA-NCAAs compared to native amino acids. Starvation of specific amino acids or high concentrations of AHA are required for efficient labeling. Additionally, cells need to be fixed to accommodate the click-chemistry catalysts and large size of the alkyne-conjugated fluorophores, so FUNCAT cannot be applied to live samples. Besides fluorescence, NCAAs can be detected by their distinct vibrational signatures through Stimulated Raman Scattering Spectroscopy [27].

O-propargyl puromycin (OPP) is another metabolic labeling method for nascent peptides. Puromycin, a t-RNA analogue, inhibits translation by conjugating nascent peptides and triggering their release from ribosomes. OPP is a derivative of puromycin with an alkyne group that can be conjugated to fluorescent azides, or directly detected by immunofluorescence (Fig. 1b) [28]. OPP-labeling has been used to identify subcellular localization of translation in several applications. Unexpectedly, one study suggested that translation may happen in nuclei [39]. Lately, the finding was challenged by two groups who warned that the "translation signal" may result from accumulated puromycylated peptides released from ribosomes, rather than the original translation sites [40,41]. Therefore, *in vivo* puromycylation assays should be carefully interpreted, especially for local translation.

2.3. RNA biosensors

A unique approach to measure the first round of translation, named Translating RNA imaging by Coat Protein Knockoff (TRICK), was developed by Halstead and colleagues [29]. The authors utilized two orthogonal RNA stem loop motifs in a biosensor: PP7 stem loops [42], placed in the coding region, and MS2 stem loops in the 3'UTR [43]. Under this design, untranslated mRNA would be labeled by two different colored coat proteins. During the first round of translation, ribosomes will remove the upstream PP7 coat proteins, rendering the translated mRNA to be a single color (Fig. 1c). The TRICK biosensor was generalized to study mRNA decay. An XRN1-resistant pseudoknot was placed between the two orthogonal stem loops such that the intact mRNA will contain two colors while the partially degraded mRNA will only harbor one color [44]. These RNA-based biosensors can assess translational and degradation status of mRNAs without visualization of nascent peptides.

2.4. Nascent peptide labeling

There have been tremendous efforts to visualize translation of single mRNAs in live cells. A breakthrough occurred in 2016 when five groups achieved this capability. The major challenge in visualizing translation sites is amplifying the weak single protein signal above background. The five groups used similar technologies to accomplish this. Four groups used the SunTag technology [45]: a tandem array of peptide epitopes is inserted at the N-terminus of the reporter, which are bound by co-expressed fluorescent antibodies to amplify the peptide signal [31–34]. Another group used the spaghetti monster [30]: a GFP backbone with multiple epitopes at the edge of the beta-barrel, which are labeled by fluorescent antibody fragments. To label mRNAs, these groups used different varieties of MS2 technologies [42,43,46]. When the multimerized epitopes emerge from the ribosome, the fluorescent antibodies bind to them immediately to illuminate the translation site (Fig. 1d). Because several ribosomes may translate on one mRNA, one expects a bright translation signal to colocalize with the mRNA. The major advantage of these NPL methods, compared with direct fluorescent tagging, is that the fluorescent antibody is already present and ready to bind, so there is no need to wait for protein folding or fluorophore maturation. Recently, NPL was generalized to multicolor imaging [47,48]. Orthogonal MoonTag and Frankenbody use a similar strategy as SunTag and Spaghetti Monster, respectively. With these orthogonal labeling techniques, one can measure translation of two different peptides in the same cell. More interestingly, these tags were combined in an alternating fashion to measure translation in two different frames on a single mRNA, resulting in the Moon And Sun Hybrid Tag (MASHTag) and Multi-Frame Tag (MF-tag). These hybrid tags have been used to reveal heterogeneous translation products due to frameshifting, alternative start site selection, or translation of upstream open reading frames (uORFs). While frameshifting and alternative start site selection were formerly deemed a consequence of ribosome infidelity, in some cases, this heterogeneity may be employed by cells or viruses as regulatory/proteome diversification tools. uORFs are found in about half of the human transcriptome and are known to repress downstream ORF protein yield by 30–80% [49]. uORF repression may result from several ribosome paths: translating the uORF then reinitiating on the downstream ORF, translating the uORF then failing to reinitiate, or skipping the uORF altogether. Boersma and colleagues explored these paths with multicolor translation imaging. It was found that the prevalence of each path was comparable, with skipping the uORF AUG being the least likely [47].

Since the inception of NPL techniques, they have been applied to study canonical and non-canonical translation events. For example, Wang and colleagues used SunTag to study the Repeat-Associated Non-AUG (RAN) translation of the GGGGCC repeats in the *C9ORF72* gene, which are the most common cause of the familial form of amyotrophic lateral sclerosis and frontotemporal dementia [50]. They identified that the substrate of RAN translation is the lariat form of an un-debranched intron. NPL has also been used to study RNA viruses, which is of particular significance now with the ongoing SARS-CoV-2 pandemic. Chen and colleagues applied SunTag to study translation in the HIV-1 RNA virus [51]. This study reported that individual viral RNA molecules serve as either a viral genome or translation template, but not both simultaneously. Likewise, Boersma and colleagues developed a single-molecule translation assay to monitor the early infection of the coxsackievirus B3 (CVB3) RNA virus [52]. They discovered that translation and replication of the viral genome are coordinated over several phases and uncovered extensive heterogeneity in cells' responses to viral infections. To this end, single-molecule translation assays provide important mechanistic information of RNA-related genetic diseases and pathogenic infections.

3. Spatial translation regulation

3.1. Translation on membrane-bound organelles

After an mRNA is processed and exported to the cytoplasm, it can diffuse in the cytosol (Fig. 2a) or anchor to specific regions of the cell to translate (Fig. 2b-d). Abundant evidence exists supporting local translation on membrane-bound organelles (Fig. 2b) [53,54]. Biochemical assays and genetic studies have uncovered the proteome targeted to membrane-bound organelles or secretory pathways. It was proposed that the Endoplasmic Reticulum (ER) not only plays an essential role to synthesize membrane and secretory proteins but is also the central hub for synthesizing most cytosolic ones [55,56]. The mitochondrion is a double-membrane-bound organelle with many of its proteins encoded in nuclear DNA. Unlike the ER-targeting proteins that are co-translationally targeted, it was proposed that mitochondrial proteins are imported post-translationally. Tracking mRNAs and their translation status in live cells can provide key information about transcript mobility and their relative distance to the organelles, which offers insight into the long-running dispute of local translation in the cytoplasm versus on membranes. In this section, we discuss the recent imaging studies of local translation on the ER and mitochondria.

Translation of proteins in secretory pathways on the ER surface is well established [57]. However, the dynamics of mRNA on the surface of the ER are poorly understood. Once mRNA is localized to the ER, how subsequent translation is initiated remains unclear. In addition, several studies have evidence that mRNAs coding for cytosolic proteins are also tethered to the ER [58,59]. This observation has contributed to a

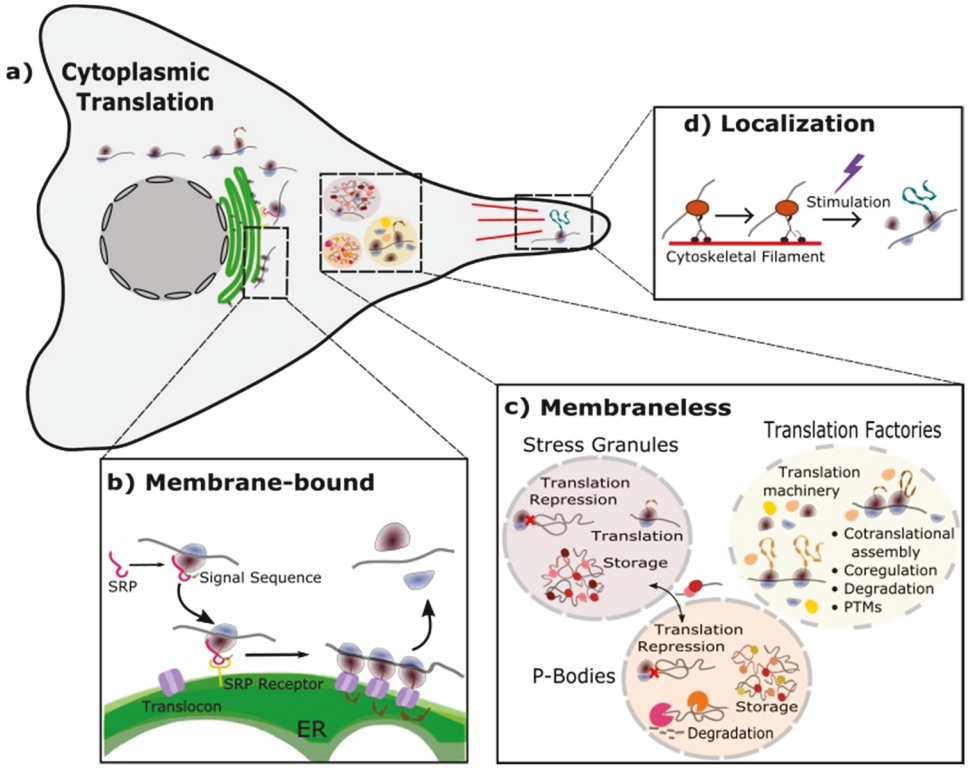

Fig. 2. Spatial regulation of translation occurs in various forms a) Cytoplasmic mRNAs may be transported, translated, degraded, or sequestered in membraneless organelles. b) Translation on membrane-bound organelles. Here, ER translation is depicted. Nascent peptides containing signal sequences are recognized by signal recognition particles (SRPs) to assist in tethering of mRNAs to the ER membrane. mRNAs undergo multiple rounds of translation on the surface of the ER. c) Membraneless organelles can regulate translation, mRNA storage, or decay. Translation factories are hot spots for abundant translation and Post-Translational Modifications (PTMs). d) Polarized cells, such as fibroblasts, neurons, intestinal epithelium, and embryos localize mRNAs to distal regions for local translation.

discussion about where the majority of transcripts are being translated. Through genome-scale ribosome footprinting studies of cytosolic and ER-associated ribosomes, Reid and colleagues observed mRNAs encoding cytosolic proteins holding similar ribosome densities on the ER compared to proteins with membrane-targeting signal sequences [55]. The authors concluded that most translation occurs in the ER through ER-bound ribosomes; mRNAs with bound ribosomes in the cytosol are involved in other processes such as storage or decay, but not primarily for translation. In contrast, Jan and colleagues used proximity ligation to pull down ribosomes on the surface of the ER [60]. Subsequent ribosome profiling identified transcriptome-wide local translation on the ER. They identified most proteins in the secretory pathway, with enrichment dependent on the position of the signal sequence in the open reading frame. Cytosolic mRNAs, on the other hand, are not enriched in the ER proximity pool, arguing against their translation on the ER surface. Recent imaging and single-molecule studies have shed light on this dispute. Wu and colleagues designed SunTag translation reporters targeted to the ER and the cytosol [31]. The ER-targeted mRNAs diffused 10 times slower when they were translating compared to non-translating. Puromycin treatment allowed the ER-targeted mRNAs to freely diffuse in 3D. This is consistent with the mechanism that nascent peptides tether the mRNA to the ER surface and slow down their diffusion. On the contrary, the diffusion constant of cytosolic mRNAs differed only slightly when they were translating or not (less than a 2-fold difference). These findings conclude that most cytoplasmic mRNAs are not translating on the ER surface. Another group reached a similar conclusion [61]. Furthermore, the translating ER-targeted mRNAs remained on the ER longer than the time required for translating a single peptide, signifying that translation can be re-initiated while the mRNA is tethered. Jan and Wu's studies support the co-translational translocation model: mRNAs begin to translate in the cytosol; once the signal sequence is synthesized and accessible outside of the ribosome, the nascent chain is then targeted to the ER membrane by a signal recognition particle (SRP). Once the complex is anchored to the ER, the mRNA can undergo multiple rounds of translation by polysomes (Fig. 2b).

Wu and colleagues consistently noticed a small subset of cytosolic mRNAs that displayed restricted mobility, which prompted more questions. Are they translating on the ER surface? Why and what causes their ER-localization remains unclear. Voigt and colleagues used smFISH to probe *Gapdh* mRNA, a well-known cytosolic mRNA, and found that up to 25% of *Gapdh* mRNAs localized to the ER in mammalian cells [62]. Application of puromycin revealed the ER-localization to be translation-dependent. To further investigate the localization mechanism, they performed a TRICK assay and showed that the ER-localization could happen before the first round of translation. In addition, they asserted that the ER-targeted transcripts can undergo multiple rounds of translation and then be released back to the cytosol. Fusing the SunTag to a cytosolic protein, they observed a higher occupancy of ribosomes on the ER-localized mRNAs compared to cytosolic ones. This agreed with Reid's earlier observation that cytosolic mRNAs acquire a higher ribosome density when anchored to the ER. Why might this be? One explanation is that the proximity of other ribosomes residing on the ER increases the translation initiation rate. Another explanation is that ribosomes have a slower elongation rate on ER-localized transcripts. Further single-molecule imaging assays to measure translation initiation and elongation speed are required to delineate these models.

Translation on the mitochondrial surface has been proposed as a mechanism for co-translational import of nuclear-encoded mitochondrial proteins [63]. It has been well established that mRNAs associate with mitochondria. However, only recently has evidence emerged of mRNAs undergoing translation on the mitochondrial surface [64]. Tsuboi and colleagues applied the MS2-MCP system to study transcripts with mitochondrial affinity in yeast [65]. They observed that mRNAs with low affinity for the mitochondria experienced an increase in localization when shifting from fermentative to respiratory conditions. The localization depends on both translation and the mitochondrial volume fraction. After slowing down elongation, they observed increased mRNA localization, agreeing with the co-translational translocation model [66]. Interestingly, the authors observed increased protein production when mRNAs were allowed to localize. The authors propose that the more significant role for localized translation on

mitochondria is to increase protein synthesis. Further evidence for the co-translational import model was seen in *Drosophila* ovaries. Zhang and colleagues used FUNCAT to visualize translation on mitochondria [64]. To investigate whether the nascent protein signal was coming from mitochondrial or cytoplasmic mRNAs, the authors treated the ovaries with chloramphenicol (inhibits mitochondrial ribosomes) and cycloheximide (inhibits cytoplasmic ribosomes). Treatment with cycloheximide eliminated FUNCAT labeling, while little effect was seen with chloramphenicol treatment, suggesting that cytosolic ribosomes can translate on the mitochondrial membrane. Like the ER membrane, mRNAs localized to the mitochondrial membrane showed an increase in ribosome density, implying that mitochondrial localization may improve translational efficiency. Further studies are needed to understand how local translation to the mitochondria is affected by the cellular state.

The rough ER has discrete areas with and without ribosomes, suggesting finer local translation control in sub-domains [67,68]. Mitochondrial ribosomes (or mitoribosomes) similarly exhibit localized translation in the inner membrane boundary and cristae membrane of the mitochondria to orchestrate the assembly of protein complexes [69,70]. Super-resolution imaging techniques are needed to study the translation on the sub-domains of these organelles.

3.2. Translation regulation by membraneless organelles or phase condensates

mRNAs often exist in single forms [71]. However, in certain developmental, physiological, and pathological conditions, RNAs may aggregate together to form protein-RNA granules, such as P-bodies, SGs, P granules (germline granules) [10], neuronal RNA granules [72], or repeat-induced RNA granules [73]. A common feature of these granules is that they are not enclosed in membranes. One proposed mechanism to form these granules is phase separation — a phenomena whereby specific proteins and RNAs enter a condensate phase with higher local concentrations [74]. These granules play important roles in mRNA metabolism, including translation activation, repression, RNA transport, storage, and degradation (Fig. 2c). The formation, transport, disassembly, and functional roles of these RNA granules have been extensively studied in the literature [12,13,16,17]. Here, we reviewed the recent progress in SGs, P-bodies, translation factories, and artificial condensates, particularly those studies involved in translation imaging.

3.2.1. Stress granules and P-bodies

Translation is the most resource-demanding process in a cell. During stress, cells shut down general translation of all non-critical processes. This results in sequestering mRNAs in SGs or P-bodies. By fluorescent labeling, Wilbertz and colleagues visualized the trafficking of mRNAs into these granules [61]. mRNAs were recruited to the granule at different speeds. For example, mRNAs with 5′ terminal oligo pyrimidine (5′ TOP) elements were recruited to granules faster than regular transcripts. Once incorporated into SGs, mRNAs rarely moved to P-bodies, in contrast to a previous model where SGs sort mRNAs into P-bodies for degradation [75]. While mRNAs in SGs are generally translationally repressed, a subset of mRNAs is upregulated [76]. Mateju and colleagues used the single-molecule NPL approach to visualize translation of ATF4, a transcription factor needed for stress response [77]. They found that the mRNAs shuttled between the cytoplasm and SGs. Contrary to previous understanding, the *Atf4* mRNAs kept translating even when localized to SGs. Surprisingly, mRNA containing the 5'TOP UTR also continued translation in SGs, albeit at the same repressed rate as in the cytoplasm. This has challenged prior assumptions that SG-localization is solely for repression. Additionally, mRNA's location within a SG substructure may play a role too. Moon and Mateju both demonstrated that SGs maintain different environments in their shell versus core [77,78] and *Atf4* mRNAs prefer translation near the SG boundary than the center.

P-bodies have multiple functions in mRNA metabolism. P-bodies are not the site of translation due to their lack of translation initiation factors and ribosomes [79], except for translation observed on the outer edge of P-bodies in *Drosophila* [80]. Instead, they may function in mRNA storage or decay (Fig. 2c) [81]. Aizer and colleagues identified RNAs accumulating in P-bodies during amino acid starvation [82]. Fluorescence recovery after photobleaching (FRAP) experiments reported rapid exchange of mRNAs between cytoplasm and P-bodies. After knocking down DCP2, a P-body-associated RNA decapping factor, the exchange rate decreased, and RNA decay was slowed. In another study, Horvathova and colleagues used the TREAT biosensor to observe the movement of mRNAs into P-bodies during stress, followed by stable association without degradation [44]. Likewise, artificially tethering AGO2, an RNAi factor, to an mRNA caused its translation to stall followed by localization to P-bodies, where it remained intact and untranslated for hours [83]. Altogether, these data suggest that P-bodies are sites for both mRNA storage and decay. Capturing the RNA decay using disappearance of a signal is challenging. It is worthwhile to develop single molecule methods to visualize RNA decay in P-bodies to elucidate this dual role.

What dictates the transcriptome in RNA granules is an open question. Firstly, it may depend on the type of stress applied. Wang and colleagues sequenced the RNA in P-bodies under different conditions. The P-body transcriptome under glucose starvation differs from the one in Na^{+} or Ca^{2+} stress; the RNA-binding protein PUF5P promoted storage for some transcripts, but decay for others [84], suggesting that both the localization and fate of an mRNA in P-bodies are context-dependent. In a multi-color tracking experiment, Moon and colleagues discovered that mRNAs could interact with granules stably or transiently, depending on their translational status, length, and the size of the granule [78]. Interestingly, factors involved in ribosome-associated quality control (RQC), normally called upon to clear collided ribosomes at stalling sites, are required to recruit mRNAs to SGs [85]. Moon and colleagues discovered a specific stress-activated RQC (saRQC) response [86]. The authors argue that ribosomes stall in the middle of an mRNA during stress-induced translation shutdown, requiring RQC factors to clear ribosomes before the mRNA can be recruited to SGs. In a meta-analysis of published P-body/SG transcriptome data, Courel and colleagues discovered that P-bodies are enriched in long AU-rich transcripts or mRNAs with low protein yield [87]. Matheny and colleagues concluded that the recruitment of mRNAs into SGs and P-bodies depends on their translation status [88]. Interestingly, mRNAs may change conformation before or after entering the granule. Adivarahan and colleagues used separate FISH probes to target the 5' and 3' ends of an mRNA and measure the distance between the two ends. Surprisingly, they found that mRNAs are in open conformations when they are actively translating, instead of a closed circular loop [89]. Furthermore, mRNAs assumed compacted conformations in the SGs. Overall, the makeup of an RNA granule is highly dynamic and context dependent. Future single-molecule imaging of RNAs targeted to membraneless organelles in various contexts will help to ascertain their multiple roles in further detail.

3.2.2. Translation factories: a hot-spot for translation activation

While the membraneless compartments discussed thus far are primarily involved in translational repression or mRNA decay, other compartments are found to promote translation. Translation factories, also known as translation 'hot-spots,' are locations where translation is highly abundant (Fig. 2c) [90]. Early smFISH experiments showed that mRNAs in neuronal dendrites are mostly singles [71], casting doubts on the translation factory hypothesis. It is unclear whether these translation factories are simply due to polysomes in close proximity or active phase separation playing regulatory roles. Methods capable of distinguishing between the two have been described [91]. One possibility is the requirement of co-translational assembly of protein complexes [92], as shown by ribosome profiling experiments [93,94]. Another possibility is

that the local biochemical environment promotes translation. Katz and colleagues found that *β-actin* mRNAs showed an increased translation rate when close to focal adhesions in mouse embryonic fibroblasts [95]. They used mRNA mobility as a proxy for polysome association, which is unfortunately unreliable as a readout of translation levels [31]. Recently, NPL methods have been used to tackle this problem [30,32, 96]. Pichon, Morisaki and colleagues found that mRNAs labeled with different colors can move together in a nascent peptide-dependent manner. Furthermore, Pichon and colleagues found that polysomes showed directed transport towards translation factories. In another study, Dufourt and colleagues investigated the spatial distribution of *Twist* mRNA translation in *Drosophila* embryos [96]. They found clusters of *Twist* reporters and endogenous *Twist* mRNAs colocalized with translation machinery- a perfect candidate for a translation factory. More excitingly, the clustering of mRNAs is inhomogeneous, preferentially concentrated at the basal perinuclear region. Previously, embryonic pattern development was mostly studied at the transcription level. This study demonstrated that spatial heterogeneous translation is also important.

The prevalence of translation factories is likely much higher than previously thought. Chouaib and colleagues screened more than 500 mRNAs [97]. Four were found to localize to potential translation factories. Chouaib and colleagues suggest that translation factories may have roles beyond co-translational assembly. For example, *β-catenin*, one mRNA in the factory, colocalized with protein degradation machinery. It is plausible that translation factories may prompt degradation, enrich chaperones for protein folding, or enhance post-translational modification activity. The exact role of translation factories and whether they are a mechanism for local translation in polarized cells (Fig. 2d) requires further investigation.

3.2.3. *Artificial phase separation as a tool for translation control*

Phase separation has been demonstrated to be a fundamental principle that cells use to organize biochemical processes [74]. Biotechnologists have taken advantage of this to create artificial condensates to control biological reactions. As an example, Reinkemeier and colleagues created an artificial membraneless organelle to improve the efficiency of orthogonal biosynthesis using genetic code expansion (GCE) technology [98]. This technology selectively incorporates modified amino acids capable of click chemistry into proteins of interest using amber stop codon (UAG) suppression [99]. However, the amber stop codon is found in 20% of mammalian transcripts, resulting in undesirable off-target incorporation into endogenous proteins. Reinkemeier and colleagues employed an intrinsically disordered "assembler" protein motif that readily forms phase condensates. They fused the assembler to tRNA synthetases for the amber stop codon, and to specific RNA-binding proteins, which formed specialized membraneless compartments. This artificial organelle brought together target mRNAs and specific translation machinery to form an orthogonal translating (OT) factory to efficiently synthesize specific modified proteins. In another study, Kim and colleagues created artificial condensates to repress translation of specific mRNAs [100]. They used the CRY2/CIB1 light-induced dimerizer [101] to tether an RNA-regulatory factor to target RNA. This optogenetic system reversibly sequestered mRNA into an RNP cluster and repressed its translation. Using this technique, the authors confirmed the need for local β-actin synthesis at the leading edge of fibroblasts for directed cell migration. Taken together, the artificial phase condensates may become invaluable tools for biotechnology and biomedical research.

4. Translation dynamics and temporal translation regulation

Translation is typically divided into three stages: initiation, elongation, and termination (Fig. 3a). Recent studies show that elongation and termination play an essential role in signaling and quality control. For example, the codon optimality of mRNA or the elongation speed is related to mRNA stability [102] and influences protein folding [103]. Global translation elongation inhibition leads to massive ribosome collisions and triggers general stress responses [104]. An emerging consensus is that the ribosome is not only a translation apparatus, but

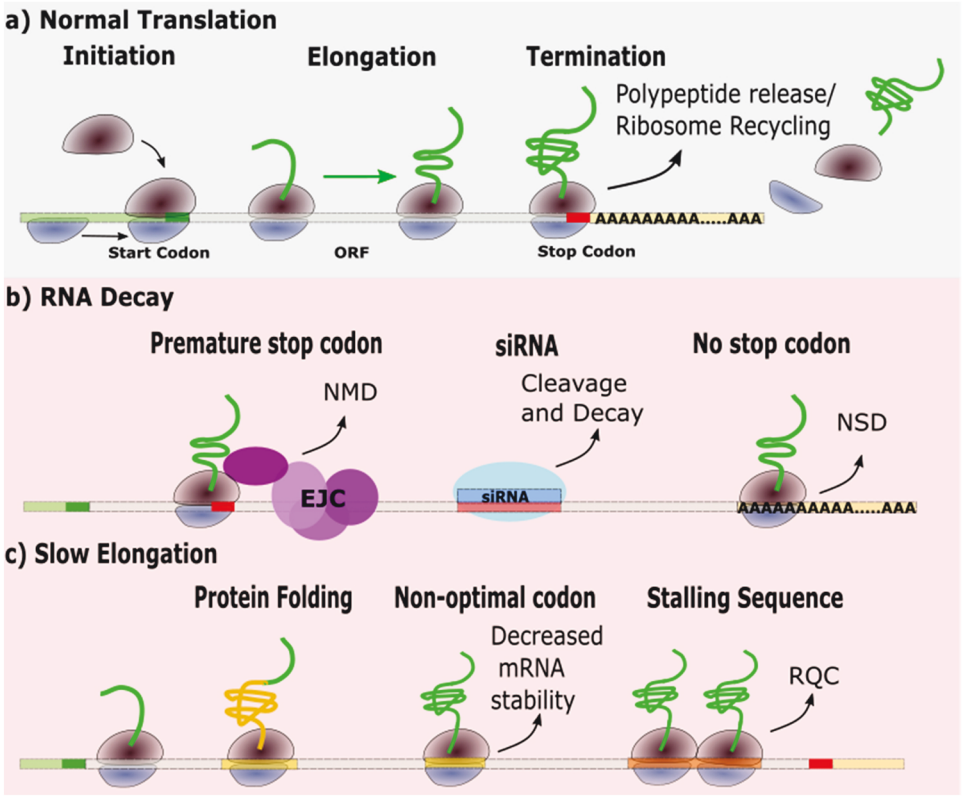

Fig. 3. Temporal control of translation couples with RNA metabolism
a) Normal translation is divided into initiation, elongation, and termination stages, followed by ribosome recycling. b) Ribosomes at the premature stop codon may interact with downstream factors (exon junction complex) to induce nonsense-mediated decay. Translation may open up the secondary structure of mRNA for siRNA to bind and degrade the RNA. Translation at the poly-A sequence leads to non-stop decay. c) Elongation is coupled with processing of nascent peptides and mRNAs. Programmed pausing is coupled with protein folding. Slow elongation due to non-optimal codons is related to decreased mRNA stability. Extended stalling leads to ribosome collisions and associated quality control.

also acts as a signaling hub. Quantitative measurements of these dynamic events are essential for a mechanistic understanding of how ribosomes perform these multifaceted functions. Single-molecule imaging in live cells revealed heterogeneous translation dynamics masked by ensemble average and elucidated how cells detect and resolve aberrant transcripts. In this section, we highlight recent single-molecule imaging studies on temporal translation regulations.

4.1. Translation dynamics

Through nascent peptide imaging, one can measure translation dynamics in live cells and even embryos [30–34,96]. Wu and colleagues showed that translating mRNAs diffuse slightly slower than non-translating ones. However, it is unreliable to differentiate the translation status of mRNAs solely based on its diffusion coefficient. On the other hand, mobility is an excellent indicator of translation for mRNAs targeted to membranes (see previous discussion). In these studies, translation elongation rates were extracted using different techniques and fell in the range of 3–10 amino acids/s, which coincide with ribosome profiling measurements of 5.6 amino acids/s [105]. Interestingly, these studies demonstrated that translation may not be a constitutive process, but rather exhibit bursty behavior with single mRNAs having multiple rounds of on-and-off translation [31,33]. What is the benefit and biological relevance of this bursty behavior and what controls it? There are still many questions to be answered.

4.2. Translation coupled with mRNA decay

Translation and decay of mRNA are closely coupled. Translation elongation depends on amino acid sequence, tRNA availability, and secondary structure of mRNA [106]. Cells have evolved strategies to handle heterogeneous elongation and termination kinetics, while also detecting anomalies and degrading defective mRNAs and proteins (Fig. 3b). Recent single-molecule studies have shed light on the importance of temporal dynamics of elongation and termination on mRNA decay and quality control pathways.

Nonsense-mediated decay (NMD) is triggered when a premature stop codon (PTC) is present upstream of a splicing-dependent exon junction complex (EJC) [107]. mRNAs subject to NMD are quickly targeted for decay [108]. It has been reported that the pioneering round of translation triggers NMD with nuclear cap-binding proteins CBP20/80 on the mRNA [109]. However, other studies show that NMD can occur during subsequent rounds of translation when cytoplasmic cap-binding protein eIF4E is on the mRNA [110,111]. Hoek and colleagues inserted a PTC after the SunTag to study the kinetics of translation and NMD [112]. They measured the timing between translation signal appearance and endonucleolytic cleavage of the mRNA. The resulting time distribution is inconsistent with the assumption that the first encounter of a ribosome with PTC leads to cleavage. Rather, it agrees with a model that each ribosome has ~10% probability of detecting the PTC and it takes ~8 trials to induce NMD. Is it possible that the pioneering round of translation marks the mRNA for degradation, but subsequent translation events are required for degradation to occur? By perturbing eIF4E binding, the authors conclude that NMD does occur on eIF4E-bound mRNAs, and there is no preference for CBC-bound mRNAs. In addition, there is no stalling of ribosomes at the PTC for endogenous NMD targets [113]. It is therefore unlikely that the pioneering round of translation causes the ribosome to stay at the PTC longer than a normal termination codon.

Small interfering RNA (siRNA) forms an RNA-induced silencing complex (RISC) and directs cleavage of its complementary target RNA. Target binding is critical for the function of the RISC complex. *In vivo*, the biochemical environment and target RNA structure renders the target search a non-trivial and complex process. Ruijtenberg and colleagues developed a single-molecule translation reporter with a siRNA target site incorporated [114]. By measuring the time it took for the translation signal to separate from mRNA, they inferred the cleavage time taken by RISC. The authors found that RNA secondary structure may mask the target site, inhibiting target search. They propose that translation stimulates siRNA cleavage by unfolding the RNA, promoting binding and cleavage by the RISC complex.

Slow elongation can signal defects within the mRNA or translation machinery. A stalling site in the open reading frame may induce ribosome collisions, triggering RQC (Fig. 3c) [115]. However, incidental collisions occur frequently, as exemplified by the widespread disomes distributed throughout the transcriptome [116]. In fact, programmed ribosome pausing is needed for protein folding in some cases [117]. It is unclear how cells differentiate these pausing events from prolonged stalling that invokes quality control. Goldman and colleagues designed a single-molecule RQC reporter by inserting a stretch of poly-A sequence between SunTag and the stop codon [118]. The protein level was reduced by ten-fold, but the mRNA changed only slightly, demonstrating that the expression level is mainly controlled by RQC, not by mRNA decay (no-go decay (NGD) or non-stop decay (NSD), Fig. 3b). By counting the number of ribosomes on the mRNA, the authors observed massive ribosome queues and collisions. When initiation is inhibited, it takes much longer to clear the existing ribosome queues than normal elongation and termination. When the key sensor for collided ribosomes, ZNF598, was knocked down, there was a dramatic increase in the time it took to clear the ribosome queue. This demonstrated that cells differentiate RQC substrates using kinetic information; prolonged stalling is subject to RQC, while transient pausing is ignored.

5. Conclusion and future directions

There has been significant progress in translation imaging *in vivo* over the last two decades. We now have a toolbox to visualize translation of endogenous proteins or reporters at the single mRNA level in live cells and developing embryos. These *in vivo* studies have revealed rich spatial and temporal regulation mechanisms that cells use to regulate gene expression post-transcriptionally, monitor mRNA quality, and couple translation with degradation of aberrant mRNAs and nascent peptides. Looking ahead, there are several technical areas that need to be further developed. *First*, improvements on mRNA tracking. Translation of a typical protein requires a few minutes. To understand the translation regulation at the single mRNA level, we need to track them for tens of minutes, or even hours. Bright and stable RNA labeling techniques are needed for this purpose. *Second*, enhancement of *in vivo* microscopy techniques. It is still challenging to visualize freely diffusing mRNAs in cells and tissues. Improving imaging technology such as light sheet microscopy or adaptive optics may allow single mRNA imaging in live animals. *Third*, development of optogenetic or small molecule perturbation techniques to control translation. By synchronous induction of translation or RNA degradation, one can observe the resulting phenotype with great spatial and temporal resolution. *Fourth*, establishing quantitative mathematical models that integrate single-molecule translation measurements with ensemble protein output. This will allow for better interpretation of reporter assays performed in general biology labs. *Finally*, investigation into how *cis* and *trans* regulatory elements influence translation dynamics. This requires us to label protein factors or mRNAs endogenously at the native expression level, made possible by the revolution in CRISPR/Cas gene editing tools. Taken together, translation imaging will be an indispensable tool to uncover the intricate regulation mechanisms cells use to deploy their proteome in space and time.

Author contributions

All authors conceptualized and reviewed the manuscript for publication.

Declaration of competing interest

The authors reported no competing interest.

Acknowledgements

This work was supported by the National Science Foundation (Grant# MCB1817447) and the National Institutes of Health (Grant# R01GM136897). L.A.B. and A.D. were supported by the National Institute of Health (Grant# T32 GM008403) and A.D. was supported by the National Science Foundation Graduate Research Fellowship (Grant# DGE-1746891).

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