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Minireview

Eyes on Translation

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Translation is a fundamental biological process by which ribosomes decode genetic information into proteins. The regulation of this process plays a key role in tuning protein levels, allowing cells to respond rapidly to changes in the environment and to synthesize proteins with precise timing and at specific subcellular locations. Despite detailed biochemical and structural insight into the mechanism of protein synthesis, translational dynamics and localization in a cellular context are less well understood. Here, we summarize recent efforts to quantify and visualize translation, focusing on four publications (Morisaki et al., 2016; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016) describing novel approaches to imaging in real time the synthesis of nascent peptides from individual mRNAs in living cells.

Introduction

The expression of genes can be considered a two-stage process, beginning with transcription and the biogenesis of an mRNA, and followed by translation of that mRNA into protein by ribosomes. In the past decades, genetics, reconstituted expression systems, and structural biology provided molecular insights into the fundamental reactions of protein synthesis. Moreover, single-molecule experiments were used to study the kinetics of ribosome movement in vitro by following single ribosomes translating individual tethered mRNAs (Wen et al., 2008). Yet studies of single-molecule mRNA translation in living cells, which could provide a quantitative view of the kinetics and localization of protein synthesis in vivo, were lacking.

In recent years, a variety of techniques have been developed to measure translational efficiency and visualize sites of protein synthesis in a cellular context. Table 1 provides an overview of these approaches grouped based on the experimental readout method. Approaches relying on RNA sequencing (RNA-seq) and mass spectrometry (MS) are powerful and yield transcriptome-wide quantitative results. However, these methods offer little or no spatial information on localized mRNA translation and cannot detect translation heterogeneity of single mRNA molecules within a cell population. In contrast, imaging-based techniques give insight into subcellular location of protein synthesis and allow monitoring of translation of individual mRNA molecules.

Two sequencing-based approaches, TRAP/Ribo-tag (Heiman et al., 2008; Sanz et al., 2009) and ribosome profiling (Ingolia et al., 2009), measure the steady-state and aggregate association of ribosomes with mRNAs, using RNA-seq of ribosome-bound mRNAs or mRNA fragments protected by translating ribosomes in a cell population (Figure 1). To some extent, these methods give clues to where translation takes place, if combined with a spatially restricted biotin ligase to label ribosomes at specific cellular locations (proximity-specific ribosome profiling; Jan et al., 2014; Williams et al., 2014) or using tissue-specific promotors for expression of epitope-tagged ribosomes (Heiman et al., 2008; Sanz et al., 2009).

Pulsed SILAC (stable isotope labeling by amino acids in cell culture) (Schwanhäusser et al., 2009), PUNch-P (puromycin-associated nascent chain proteomics) (Aviner et al., 2013), and BONCAT/QuaNCAT (bio-orthogonal/quantitative non-canonical amino acid tagging) (Dieterich et al., 2006; Eichelbaum et al., 2012; Howden et al., 2013) are based on labeling of newly synthesized proteins with stable amino acid isotopes, reactive amino acids, or aminoacyl-tRNA analogs, respectively, which are enriched and subsequently quantified by MS. Similarly, FUNCAT (fluorescent non-canonical amino acid tagging) (Dieterich et al., 2010) and SUnSET (surface sensing of translation)/ribopuromycylation apply labeling of nascent peptides; however, the newly synthesized proteins are detected by imaging in fixed cells (David et al., 2012; Schmidt et al., 2009). Both methods reveal the subcellular location of mRNA translation but are not transcript specific. In contrast, FUNCAT-PLA (proximity ligation assay) and Puro-PLA (tom Dieck et al., 2015) combine noncanonical amino acid tagging and puromycylation, respectively, with the PLA to directly visualize specific newly synthesized proteins and to monitor their origin in fixed cells.

TRICK (translating RNA imaging by coat protein knock-off) is a tool for monitoring the kinetics of the first round of translation in living cells (Halstead et al., 2015). In TRICK, an mRNA is labeled in the coding region as well as the 3′ UTR using site-specific RNA-binding proteins fused to fluorescent proteins. However, after the fluorescent protein is displaced by ribosomes during the first round of protein synthesis, the translation dynamics of the reporter mRNA cannot be monitored anymore.

Notwithstanding the success of these approaches, the different methods only look into certain aspects of translation. Monitoring translation dynamics of single mRNAs for over longer periods of time had not been achieved. After all, the challenge was to simultaneously label fluorescently the nascent peptide and its cognate mRNA template. mRNA reporters encoding fluorescent proteins could not serve this purpose, since the fluorescent signal would only be detectable after the complete protein is synthesized and folded properly, i.e., when translation is long over. Recently, the laboratories of Robert Singer, Tim Stasevich, Marvin Tanenbaum, and Xiaowei Zhuang have independently overcome these problems by generating mRNA reporters encoding proteins with arrays of epitopes for recognition by fluorescently labeled antibody fragments (Figure 2D; Morisaki

Table 1. Overview of Methods Used to Quantify and Visualize Translation

Method	Spatial Resolution	Detection Method	Scale	Short Description	References
RNA-Seq- or MS-Based Methods to Quantify Translation					
Proximity-specific ribosome profiling	allows one to obtain ribosome profiles for specific organelles (ER, mitochondria)	RNA-seq	genome-wide and gene specific	Method enables global analysis of translation in defined subcellular locations by combining several approaches. (1) Tagging ribosomes with biotin acceptor peptide (AviTag). (2) Expressing biotin ligase (BirA) fused to a localization element that targets it to a specific subcellular location, for example, ER or mitochondria. BirA recognizes and biotinylates Avi-tagged ribosomes at this location. (3) Isolation of biotinylated ribosomes followed by conventional ribosome profiling.	Williams et al., 2014; Jan et al., 2014
Ribosome profiling	no			RNA-seq of ribosome-protected fragments that allows genome-wide quantification of translation efficiencies and identification of ribosome stalling sites and uORFs.	Ingolia et al., 2009
TRAP (translating ribosome affinity purification technique) and Ribo-tag	tissue specific			Methods rely on stably expressing tagged ribosomal proteins (eGFP-L10 or HA-L22) in a tissue-specific manner, followed by pull-down of tagged ribosomes and microarray analysis (or RNA-seq) of ribosome-associated mRNAs.	Heiman et al., 2008; Sanz et al., 2009
BONCAT and QuaNCAT (bio-orthogonal/quantitative non-canonical amino acid tagging)	no	MS		Methods rely on incorporation of a modified methionine analog, azidohomoalanine (AHA), in newly synthesized proteins. Biotin is covalently attached to AHA-labeled proteins using "click chemistry," and then biotinylated proteins are purified and analyzed by MS using label-free quantification (BONCAT) or SILAC (QuaNCAT).	Dieterich et al., 2006; Howden et al., 2013; Eichelbaum et al., 2012
PUNch-P (puromycin-associated nascent chain proteomics)	no			Method is based on incorporation of biotin-puromycin (a mimetic of the 3′ end of aminoacylated tRNA) into the nascent polypeptide chains, followed by isolation of newly synthesized tagged proteins with streptavidin beads and MS analysis.	Aviner et al., 2013
pSILAC (pulsed stable isotope labeling by amino acids in cell culture)	no			MS-based approach that detects differences in protein abundance using stable isotope-labeled amino acids. pSILAC is a variation of the technique that involves short incubation (several hours) of cells with labeled amino acids and thus allows the monitoring of differences in de novo protein synthesis.	Schwanhäusser et al., 2009

(*Continued on next page*)

Table 1. ***Continued***

Method	Spatial Resolution	Detection Method	Scale	Short Description	References
Imaging-Based Methods to Visualize Translation					
SINAPS (single-molecule imaging of nascent peptides), NCT (nascent chain tracking), and others	yes	imaging live cells	single molecule	Methods are based on the technology for fluorescent signal amplification (Suntag or 10xFLAG co-expressed with fluorescently labeled anti-FLAG antibody) that allows the labeling of the nascent peptide and visualization of translation dynamics in live cells.	Morisaki et al., 2016; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016
TRICK (translating RNA imaging by coat protein knock-off)	yes			Method is based on the engineered transcript that bears phage PP7 and MS2 stem loops upstream and downstream of the stop codon, and thus allows the labeling of coding sequence and 3′ UTR with two spectrally distinct fluorescent proteins: (1) GFP fused with PP7 coat protein that binds within coding sequence and (2) RFP fused with MS2 coat protein that binds to the 3′ UTR. Using this approach, untranslated mRNAs (labeled with both GFP and RFP) can be distinguished from mRNA that have undergone the first round of translation (labeled with RFP only, as GFP is displaced by translating ribosomes).	Halstead et al., 2015
FUNCAT-PLA and Puro-PLA	yes	imaging fixed cells		Method allows visualization of newly synthesized proteins in situ (similarly to FUNCAT and RPM/SUnSET), but in a protein-specific manner. It is based on a PLA to detect coincidence of two antibodies: (1) antibody to specific protein of interest and (2) antibody that detects newly synthesized proteins tagged with FUNCAT or ribopuromycinylation/SUnSET.	tom Dieck et al., 2015
FUNCAT (fluorescent non-canonical amino acid tagging)	yes		bulk translation	Direct in situ visualization of newly synthesized proteins based on incorporation of a modified methionine analog AHA, followed by fluorescence tagging of AHA-labeled proteins using “click chemistry.” An “imaging version” of BONCAT.	Dieterich et al., 2010
SUnSET (surface sensing of translation) and RPM (ribopuromycylation)	yes			Method is based on incorporation of puromycin, a mimetic of the 3′ end of aminoacylated tRNA, into the nascent polypeptide chains, followed by immunostaining with anti-puromycin antibodies.	Schmidt et al., 2009; David et al., 2012

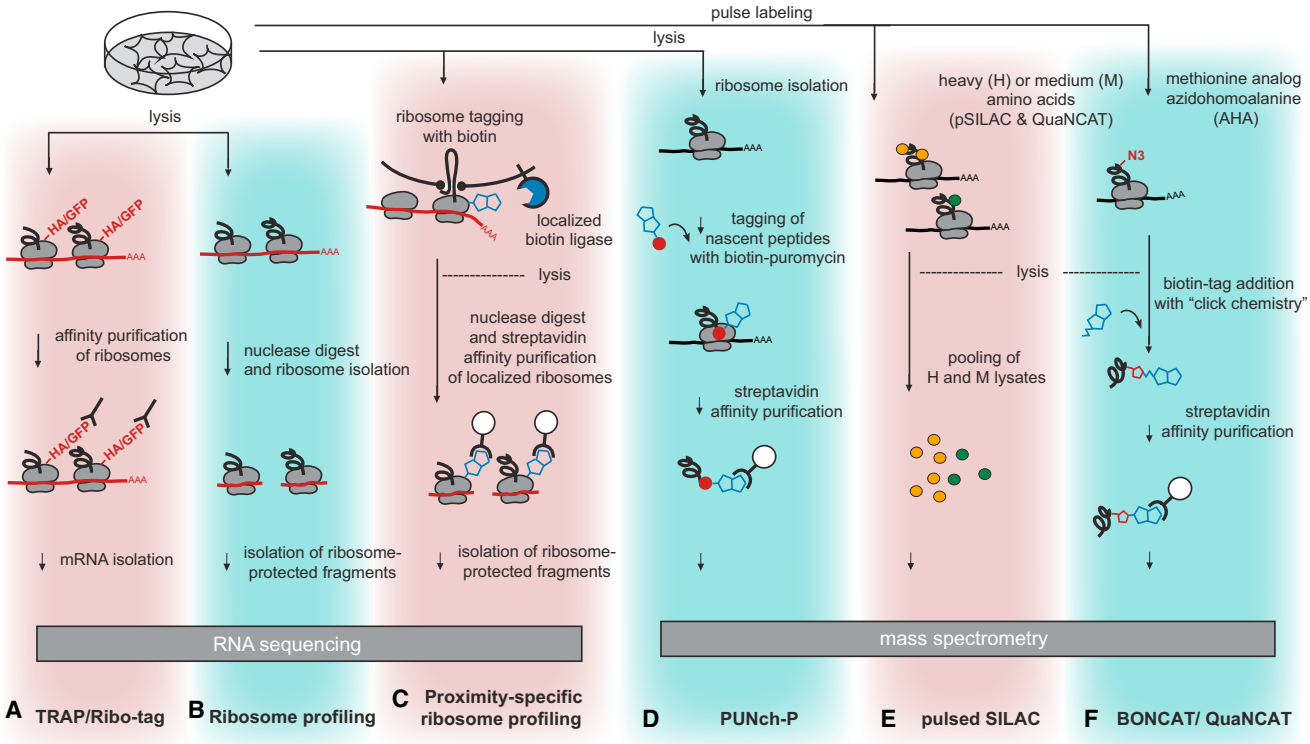

Figure 1. Overview of RNA-Seq- and MS-Based Methods Used to Quantify Translation

(A) TRAP/Ribo-tag relies on tissue-specific expression of epitope-tagged ribosomes, followed by affinity purification and sequencing of associated ribosome-bound mRNAs.

(B) Ribosome profiling involves nuclease treatment to fragment mRNAs. Ribosome-protected mRNA fragments are isolated and sequenced.

(C) Proximity-specific ribosome profiling uses biotinylation of ribosomes at a specific subcellular location with a localized biotin ligase. Biotinylated ribosomes are isolated on streptavidin beads and analyzed as in (B).

(D) PUNch-P relies on tagging of growing peptide chains with biotin-puromycin, followed by streptavidin affinity purification and MS.

(E) Pulsed SILAC (pSILAC) involves labeling of newly synthesized proteins with stable isotopes and quantification by MS.

(F) In BONCAT, newly synthesized proteins are labeled with methionine analog azidohomoalanine (AHA), followed by biotin tagging, isolation on streptavidin beads, and detection by MS. QuanCAT combines BONCAT with pulsed SILAC, enabling more accurate quantification of newly synthesized proteins. Affinity purification steps employed in (D) and (F) allow the reduction of the background of pre-synthesized proteins.

See Table 1 for more details and references.

et al., 2016; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016). When these multimerized epitopes are synthesized, several copies of the cognate fluorescent antibody bind to the nascent peptide, leading to amplification of fluorescent signal and rendering the translation sites visible above the background. Simultaneously, the authors follow the reporter mRNAs with fluorescent RNA-binding proteins, allowing for real-time imaging of nascent peptide synthesis from single mRNAs in living cells. Wu et al. (2016) coined their approach SINAPS, for single-molecule imaging of nascent peptides, whereas Morisaki et al. (2016) named it NCT, for nascent chain tracking.

The two core detection elements of the novel reporter systems, employed in all four studies, are peptide- and mRNA-labeling tags. To mark the nascent peptide, two types of epitope-tags were used: SunTag (Wang et al., 2016; Wu et al., 2016; Yan et al., 2016) and spaghetti monster (SM; Morisaki et al., 2016). SunTag peptides provide a high-affinity binding site for the single-chain variable fragment (scFV) of the anti-GCN4 antibody (Tanenbaum et al., 2014). By expressing scFV fused to GFP, the nascent polypeptide could be visualized. Alternatively, the SM tag consists of tandem 10xFLAG epitope array, enabling the interaction with fluorescently labeled anti-FLAG antibody fragments (Fab-Cy3). In addition to the peptide-labeling tag at the N terminus, phage MS2 or PP7 coat proteins (MCP or PCP) RNA binding sites were introduced in the 3′ UTR to label mRNA transcripts with MCP or PCP fused with fluorescent proteins, allowing the simultaneous detection of mRNA molecules and their corresponding nascent peptides.

What Can Be Measured by Nascent Peptide Imaging?

The newly developed approach of nascent peptide imaging and co-tracking of mRNAs provided a direct quantitative readout of initiation, elongation, and location of translation (summarized in Table 2).

To measure translation elongation rate, ribosome run-off (Wang et al., 2016; Yan et al., 2016) and fluorescence recovery after photobleaching (FRAP; Morisaki et al., 2016; Wu et al., 2016) were used. For these run-off experiments, cells were

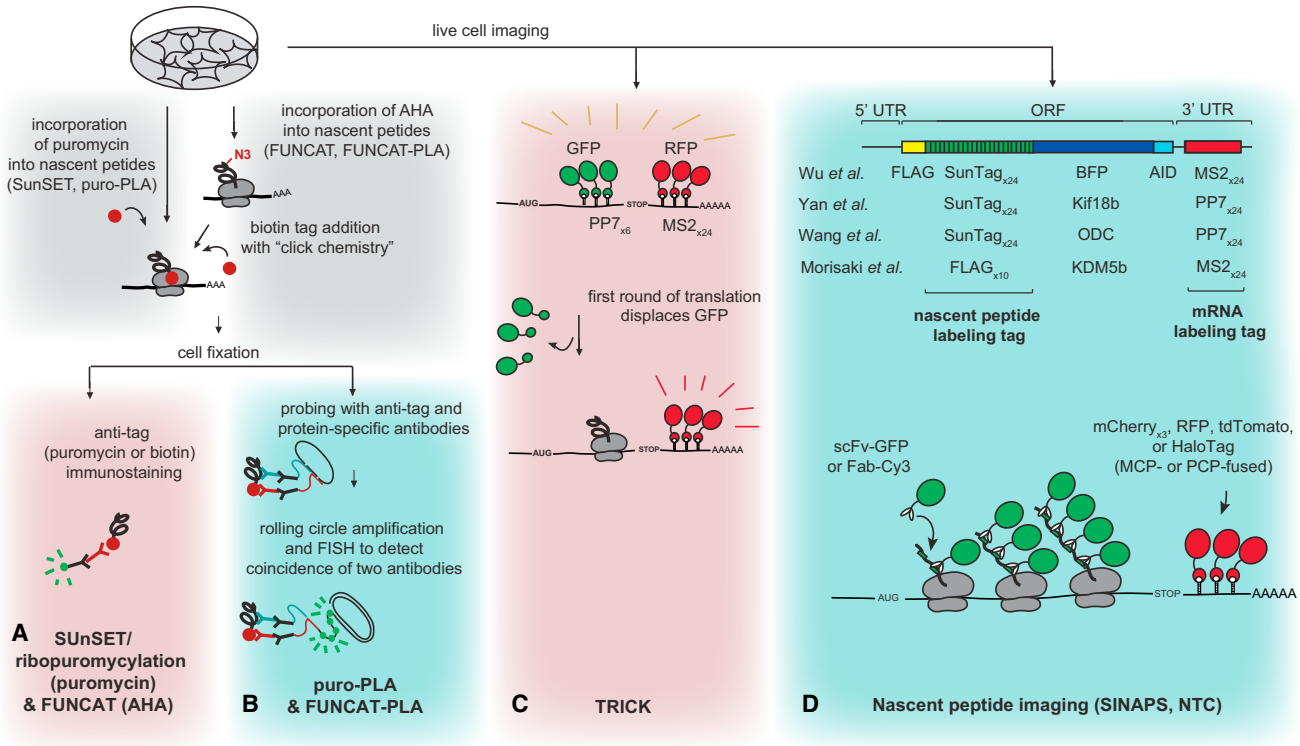

Figure 2. Overview of Imaging-Based Methods Used to Quantify and Visualize Translation

(A) Overall translation can be monitored by the labeling of newly synthesized proteins with puromycin (SUnSET/ribopuromycylation) or the methionine homolog AHA (FUNCAT), followed by biotin tagging and immunostaining with antibodies directed against the tag.

(B) To visualize specific newly synthesized proteins, a proximity ligation assay (PLA) is used to detect the spatial coincidence of a protein-specific antibody and an antibody against the tag introduced by SunSET/ribopuromycylation (puro-PLA) or FUNCAT (FUNCAT-PLA).

(C) TRICK distinguishes between mRNAs that have undergone the first round of translation and mRNAs that have never been translated by tagging both ORF and 3′ UTR with phage-derived RNA hairpins that bind distinct fluorescent proteins.

(D) Nascent peptide imaging (SINAPS, NTC, and others) visualizes and quantifies translation in real time. Each construct contains a tag for labeling the nascent peptide (SunTagx24 or FLAGx10 in the N-terminal part of the coding sequence) and a tag for labeling the mRNA (MS2 or PP7 stem loops in the 3′ UTR). Co-expressed scFv-GFP or bead-loaded Fab-Cy3 (interacting with SunTag and FLAG peptide, respectively) serves to visualize the nascent peptide. Co-expressed mCherry, td-Tomato, or RFP, fused with the MS2 or PP7 coat proteins (MCP or PCP), binds to the 3′ UTR and enables mRNA tracking. Alternately, bead-loaded MCP-HaloTag (JF646 fluorophore) was used. AID, auxin-induced degron, was used in one study to reduce background fluorescence (Holland et al., 2012).

See Table 1 for more details and references.

treated with harringtonine. Harringtonine inhibits the initial cycles of elongation but does not interact with polysomes, thereby allowing translating ribosomes to complete translation and leading to ribosome run-off (Fresno et al., 1977). The decay kinetics of the GFP signal coming from nascent peptides allowed the authors to measure the transit time (i.e., time that the ribosome needs to translate the entire mRNA) and estimate average elongation rate. Similarly, in FRAP experiments pre-existing nascent peptides were photobleached and time required for fluorescence recovery was assessed. Remarkably, the four laboratories came up with similar measurements of elongation rates between 3 and 10 aa/s, which is comparable to ~5–6 aa/s measured previously using metabolic labeling in Hep G2 cells (Boström et al., 1986) and ribosome profiling in mouse embryonic stem cells (Ingolia et al., 2011). Moderate variability of measurements between different studies likely resulted from differences in construct design, reflected in different codon usage and mRNA folding. Indeed, Yan et al. (2016) observed somewhat faster elongation for a codon-optimized reporter. Based on the GFP signal intensity, the amount of nascent peptides (= translating ribosomes) on each mRNA was estimated and found to be one ribosome per 200–900 nt (Morisaki et al., 2016) or 200–400 nt (Yan et al., 2016). Taking the elongation rate and ribosome frequency into consideration, the different labs were able to derive similar initiation rates of 1.4–3.6 min^{-1} (Yan et al., 2016), 1.3–2.1 min^{-1} (Wu et al., 2016), and ~0.5 min^{-1} (Morisaki et al., 2016), depending on the reporter mRNA and cell type used.

By simultaneously tracking mRNA and nascent peptides, Morisaki et al. (2016) observed different translation efficiencies for distinct mRNAs. In fact, the percentage of translated mRNAs encoded by different genes varied quite significantly—between 4% and 86%—depending on the specific transcript studied. Moreover, Morisaki et al. (2016) and Wu et al. (2016) examined if translated mRNAs can be distinguished from untranslated based on their diffusion rates. Interestingly, the association of mRNA transcripts with the translation machinery only slightly affected their mobility, suggesting that diffusion rate is not an exact predictor of the mRNA translation status. Surprisingly, tracking

Table 2. Measurements Possible with the Nascent Peptide Imaging

• Simultaneously track location/movement of mRNA and the nascent peptides produced from this mRNA, and measure ratio and diffusion rate of translated and untranslated mRNAs
• Measure initiation and elongation rates
• Estimate number of ribosomes/nascent peptides per mRNA
• Evaluate polysome shape
• Detect heterogeneity in translation efficiencies of single mRNA molecules
• Detect ribosome stalling
• Track translation of multiple mRNAs with multi-color imaging

of translation in real time also revealed polysome movement in dendrites (Wang et al., 2016; Wu et al., 2016), in contrast to the generally accepted view that translation of localized mRNAs is repressed during transport (reviewed by Besse and Ephrussi, 2008). These findings suggest that mRNA transport and association with the translation machinery are not mutually exclusive and highlight the complexity of translational regulation in neurons. As expected, polysomes interacting with cellular structures, such as the endoplasmic reticulum, showed lower mobility than free cytoplasmic polysomes (Wang et al., 2016; Wu et al., 2016). Furthermore, Morisaki et al. (2016) concluded that polysomes are globular in shape rather than elongated, based on the observation that distance between protein- and mRNA-labeling fluorophores was largely unaffected by the length of the coding sequence.

By monitoring single transcripts over several hours, Wu et al. (2016) observed fluctuations between translating and non-translating states of mRNAs in primary neurons. In proximal dendrites, mRNAs were translated, but repressed in distal dendrites. Interestingly, these mRNAs displayed "bursting" translation behavior: protein synthesis was interspersed with long periods of no translation.

Several studies previously reported that translation kinetics can be influenced by RNA sequences with regulatory functions (Yanagitani et al., 2011), strong secondary structure (Wen et al., 2008), or chemical modifications of nucleotides (Simms et al., 2014). Yan et al. (2016) showed that real-time translation imaging could recapitulate stalling of the translation machinery on the ribosome pausing sequence of the stress-related transcription factor Xbp1 mRNA. Interestingly, most ribosomes were only briefly delayed at the Xbp1 pause site, but a small subset of ribosomes remained stalled for an extended period of time. Furthermore, Yan et al. (2016) demonstrated that chemical damage to mRNA stalls ribosome elongation. The authors also studied the transcript-specific translational regulation of Emi1, a key cell-cycle regulatory protein. Emi1 possesses at least two splicing isoforms that differ in their 5′ UTR sequences. While mRNAs with a short 5′ UTR are translated, the majority of Emi1 reporter transcripts with the long 5′ UTR showed no detectable translation. Interestingly, only a tiny fraction of these molecules (~2%) underwent robust translation, indicating substantial heterogeneity in translational efficiency among different mRNAs in the same cell.

Translational activity is also influenced by changes in the immediate cellular environment (Sonenberg and Hinnebusch, 2009). To illustrate the utility of the translation imaging approach, Wang et al. (2016) monitored the dynamics of translational regulation in response to stress. Although translation is typically repressed under such conditions, certain genes are upregulated, like ATF4, which is controlled by two upstream open reading frames (uORFs). Notably, the authors observed that different stress conditions resulted only in transiently increased translation of ARF4 reporter mRNAs, suggesting that uORFs can exert a temporal regulation of their downstream genes in response to certain environmental signals.

Lastly, Morisaki et al. (2016) went one step further and employed multi-color imaging to track translation of two mRNAs simultaneously. To this end, they generated an additional peptide-labeling tag consisting of 10xHA at the N terminus of an mRNA reporter. The co-transfection of 10xHA and 10xFLAG reporters and anti-HA and anti-FLAG antibody fragments labeled with different fluorophores allowed them to monitor translation of two reporters at the same time. Using this approach, Morisaki et al. (2016) found that only ~5% of polysomes formed complexes, suggesting that most polysomes function independently of each other rather than as parts of higher-order "translation factories" containing multiple mRNA species.

Future Perspectives

Undoubtedly, the imaging of nascent peptide synthesis opens new perspectives in understanding both molecular mechanisms of translation and translational regulation in a variety of physiological conditions. For mechanistic studies, it would be particularly exciting to assess the translation status of a single mRNA along with measurements of regulatory protein-RNA interactions using fluorescence fluctuation spectroscopy (FFS; Wu et al., 2015). FFS allows the quantification of binding and stoichiometry of interactions. Wu et al. (2015) applied this approach to measure the association between fluorescently labeled ribosomes and β-actin mRNAs labeled using MS2 system. It should be feasible to use FFS with labeled translation factors and regulatory *trans*-acting proteins to track their association with translated and non-translated mRNAs and determine effects on initiation and elongation. Since internal ribosome entry sites (IRESs) differ in their requirement for translation factors and accessory proteins, it would be interesting to visualize engagement of *trans*-acting factors with IRESs and measure the resulting initiation rates. Another exciting application of this technique would be to measure how interaction of specific RNA-binding proteins with their *cis*-regulatory elements in untranslated mRNA regions affects protein synthesis. This approach could also be used to understand the mechanism behind the bursting translation pattern observed by Wu et al. (2016) and Wang et al. (2016),

when translation of individual mRNAs was periodically "on" and "off." By labeling the ribosomal subunits and translation initiation factors with spectrally different fluorophores, it should be possible to unravel which steps of translation are rate limiting and cause translation to cease during "off" periods.

Nascent peptide imaging will be relevant to study translational regulation in various biological contexts, such as metabolic changes and different stages of development and differentiation, in disease and in response to drug treatment. To this end, it will be important to tag endogenous loci using genome-editing tools to measure translation in a physiological context (Sternberg and Doudna, 2015). Moreover, multi-color imaging (Morisaki et al., 2016) will enable tagging and monitoring translation of multiple mRNA species in the same cell. This approach will be particularly advantageous to studying compartmentalized translation in highly polarized cells, such as neurons and oocytes. These cells are subjected to tight spatial regulation, with many mRNAs localized and translated at specific sites of the cell (reviewed by Besse and Ephrussi, 2008). On the other hand, multi-color nascent peptide imaging should reveal whether different mRNAs are co-assembled in larger mRNA granules to be transported together and translated in a localization-dependent manner. Likewise, effects of various signals, such as synaptic stimulation and growth factors, on translation of localized mRNAs could be determined.

Defects in translational regulation underlay a number of human pathologies, including neurological diseases and cancer. For example, the eukaryotic translation initiation factor 4E (eIF4E), which mediates the recruitment of the small ribosomal subunit to mRNA, is frequently targeted in cancers (reviewed by Siddiqui and Sonenberg, 2015). Overexpression or increased activity of eIF4E stimulates translation of mRNAs involved in cellular transformation and metastasis. Nascent peptide imaging would be an invaluable tool for monitoring the effects of eIF4E overexpression on translation and testing the efficiency of therapies in reversing this effect.

In summary, multiple methods have been developed to quantify translation efficiencies and visualize translation in situ (Table 1; Figures 1 and 2). The choice of the method is defined by the desired outcome—transcriptome-wide or single-molecule, bulk or localization-specific readout of translation. The recent advances of real-time imaging of nascent peptide synthesis undoubtedly offer many exciting possibilities in studying the regulatory mechanisms of translation and will lead to a more complete understanding of this paramount cellular process.

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