A beacon in the cytoplasm: Tracking translation of single mRNAs

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Translation is carefully regulated to control protein levels and allow quick responses to changes in the environment. Certain questions about translation in vivo have been unattainable until now. In this issue, Pichon et al. (2016. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201605024) describe a new technique to allow real-time monitoring of translation on single mRNAs.

Cellular protein levels are largely regulated by translation (Schwanhäusser et al., 2011). Translation regulation is fast and reversible, compared with transcription and mRNA processing, allowing cells to quickly adapt to environmental changes. The trans factors bound to an mRNA, to form a messenger RNP (mRNP) complex, will affect whether or not an mRNA is translated. For example, during efficient translation, polysomes, which are mRNAs bound by two or more translating ribosomes, form. Alternatively, during many stresses, translation is strongly repressed and mRNAs bind translation repression factors. Understanding how translation is regulated in the complexity of the cell by trans factors, mRNA localization, and cell signaling would be enhanced by the ability to track translation in real time on single mRNAs.

Translation efficiency can be either positively or negatively affected by the localization of mRNA. For example, in budding yeast, *ASH1* mRNA localizes to the bud tip during late anaphase. During transport to the bud, *ASH1* mRNA is bound by proteins that inhibit translation. Once delivered to the bud tip, the *ASH1* mRNP associates with membrane-bound kinases that trigger translation repressors to exit the mRNP, allowing *ASH1* mRNA to be translated (Martin and Ephrussi, 2009). Several other mRNAs are localized to subcellular domains where they are translated locally, including actin mRNA at the leading edge of a cell, mRNAs in developing embryos, or mRNAs at the synapse of a neuron (Martin and Ephrussi, 2009). Localized translation is an efficient way to spatially organize proteins. However, spatial regulation of mRNA localization and translation is often assumed to be relevant only in either specialized cases or unique, highly polarized cell types. The ability to monitor the correlation between localization and translation of single, endogenous RNAs will test this assumption.

Other patterns of localization correlate with translation repression. For example, mRNAs that accumulate in stress granules and processing bodies (P-bodies) are repressed for translation. Under stresses such as oxidative stress and glucose starvation, translation is strongly repressed and mRNAs accumulate in P-bodies and stress granules within minutes. Upon relief of stress, at least some mRNAs can return to translation. The timing of translation repression and the direction of mRNA flow between translation, P-bodies, and stress granules is still debated (Decker and Parker, 2012). The ability to track both single mRNAs and their translation would clarify how mRNAs move between active translation and storage in these cytoplasmic foci.

Investigating translation regulation has made great leaps in the past several years as a result of the development of important new techniques. For example, ribosome profiling measures in vivo translation of the entire transcriptome, allowing calculation of mean ribosome occupancy and elongation rates (Ingolia et al., 2009). However, because this technique requires lysis of the cells, it provides a single snapshot of translation and averages the translation of the mRNAs from many cells. Fluorescent-based assays to monitor translation in vivo require the folding and maturation of the newly translated fluorescent proteins (e.g., see Wang et al., 2009), which means that newly translated protein is detectable well after translation. Fortuitously, new advances in microscopy that allow the visualization of individual RNAs (Buxbaum et al., 2015) and individual proteins (Tanenbaum et al., 2014) have provided the tools needed to visualize translation in real time.

In this issue, Pichon et al. present a new technique to visualize real-time translation of single mRNAs in live cells. At the N terminus of an ORF, they insert several repeats of an epitope that recruit multiple copies of a GFP fusion protein, amplifying the fluorescence to allow detection of a single nascent peptide (Fig. 1). Specifically, they modify the SunTag system (Tanenbaum et al., 2014) by inserting 32–56 repeats encoding a Gcn4 peptide epitope into the N terminus of the ORF of interest. These epitopes are recognized by a high-affinity, genetically encoded antibody fused to GFP (scFv-sfGFP). The scFv-sfGFP antibody binds an epitope that does not naturally occur in the model system, allowing highly specific detection of nascent SunTag-containing polypeptides. As the scFv-sfGFP protein is constitutively expressed, a matured population of the fluorescent protein is available to bind the SunTag as it emerges from the ribosome, allowing detection of nascent peptides as they are translated. Furthermore, this system allows detection of polypeptides that are translated at low levels, as the individual polypeptide fluorescent signal is detectable because of the multivalent nature of the reporter. Pichon et al. (2016) can track

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The Rockefeller University Press $30.00
J. Cell Biol. Vol. 214 No. 6 649–652
www.jcb.org/cgi/doi/10.1083/jcb.201608075

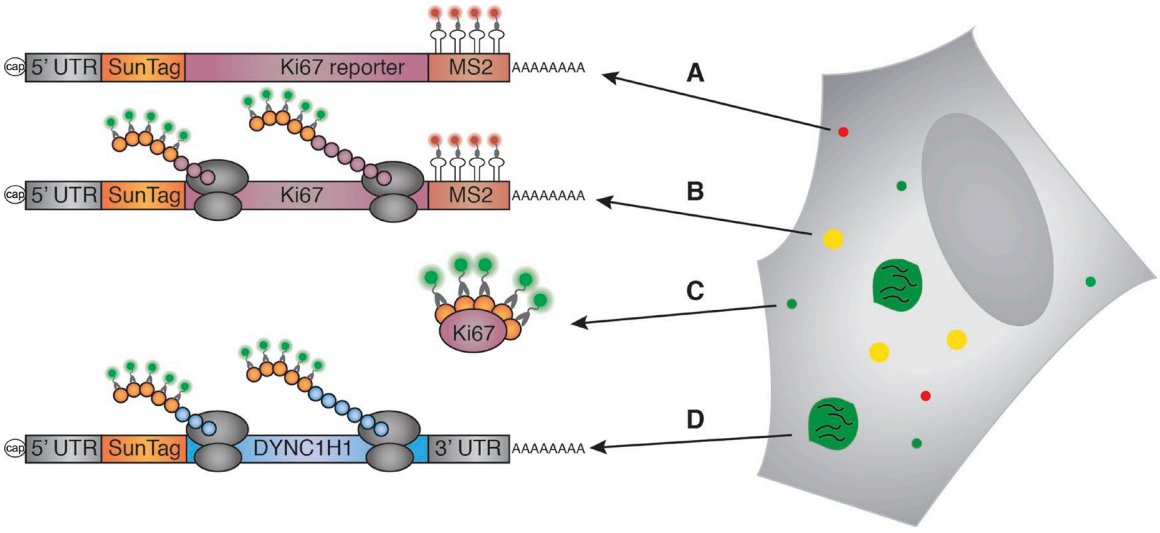

Figure 1. **Real-time detection of translation on single mRNAs.** Pichon et al. (2016) developed the Ki67 reporter (A), which contains 56 SunTag repeats at the N terminus of the ORF and several MS2 motifs in the 3′ UTR. MCP-RFP fusion protein (red) will bind the 3′ UTR, allowing visualization of the mRNA (red foci). If the mRNA is translating (B), scFv-sfGFP fusion (green) will bind the SunTag epitopes as they emerge from the ribosome. These yellow foci mark translating polysomes. Free Ki67 protein (C, small green foci) still bound to scFv-sfGFP is fainter than the translation sites, as they represent only one peptide and do not colocalize with the mRNA signal. In separate experiments, incorporating the SunTag into the endogenous DYNC1H1 transcript (D) revealed the presence of single polysomes (not depicted) and blobs containing three to seven DYNC1H1 polysomes. Pichon et al. (2016) show proof of concept that translating and nontranslating mRNAs can be tracked simultaneously in vivo. They also show that the SunTag can be used to track endogenous mRNAs, such as DYNC1H1.

SunTag translation in live cells over time. Alternatively, they can fix cells to analyze colocalization of the scFv-sfGFP translation sites and SunTag mRNA, visualized by single molecule hybridization to the SunTag coding sequence (Femino et al., 1998).

Pichon et al. (2016) validated their method using a SunTag reporter construct encoding a nuclear protein, Ki67 (SunTagx56-Ki67). In HeLa cells expressing this SunTag reporter and scFv-sfGFP, they observed both faint and bright GFP foci. The bright foci colocalized with individual reporter mRNAs and only the bright foci disappeared upon treatment with a translation inhibitor. These results suggested that the faint foci were single Ki67 proteins bound by scFv-sfGFP proteins, whereas the bright foci were sites of active translation (Fig. 1). Using the mean intensity of the faint foci as the signal for a single Ki67 peptide, they were able to determine the number of nascent peptides, and therefore ribosomes, on each mRNA. By examining live cells, Pichon et al. (2016) could track individual polysomes over time and throughout the cell. In a few cases, translation sites would appear or disappear, whereas the other mRNAs translated consistently. By monitoring fluorescent recovery after photobleaching, Pichon et al. (2016) could calculate the in vivo elongation rate on this reporter.

An advantage of this technique is the ability to monitor single translation events within a population of mRNAs, rather than averaging them together. Pichon et al. (2016) found a wide range of both ribosome densities on reporter mRNAs (8–27 nascent proteins) and percentage of translating mRNAs within single cells (from 0–100% with a mean of 47%), suggesting wide variability of translation both among mRNAs from the same gene and between cells. This wide distribution is a novel finding from this technique. Endogenous mRNA from the same gene can exhibit alternative splicing or polyadenylation (de Klerk and 't Hoen, 2015), differences in mRNP composition (Mitchell and Parker, 2014), or varied localization (Jung et al., 2014), any of which could influence translation. Future studies using the SunTag approach will illuminate the range of variation on endogenous mRNAs.

Other groups have simultaneously developed similar techniques based on the SunTag system to visualize single translating mRNAs (Wang et al., 2016; Wu et al., 2016; Yan et al., 2016). Each group created their own variation of a reporter transcript with interesting 5′ or 3′ UTRs and elements to decrease background fluorescence, such as nuclear localization signals or degrons. A caveat with all of the SunTag approaches is the insertion of a 2–4.4-kb tag to the ORF. The insertion of such a large tag may disrupt the regulation of some mRNAs or the localization and function of the encoded protein. Another group uses a smaller tag to recruit fluorophores; this technique requires transfection of antibodies conjugated to fluorophores, rather than genetic expression of the fluorescent marker (Morisaki et al., 2016). The method from Pichon et al. (2016) is unique in two critical ways. First, they used CRISPR to add the SunTag to endogenous RNAs, showing proof-of-principle that this technique can be used to track endogenous transcripts. Second, they can visualize translation on these endogenous RNAs without adding degrons or nuclear localization signals to the endogenous transcript. By keeping the endogenous RNAs unaltered, except for the insertion of the SunTag, their data are more likely to reflect the behavior of the endogenous mRNAs.

Pichon et al. (2016) introduced the SunTag to two different endogenous mRNAs: mRNAs coding for the large subunit of RNA polymerase II (POLR2A) and dynein heavy chain (DYNC1H1). As one may expect for a housekeeping gene, nearly all of the POLR2A mRNA is translated and there is less variation among cells, compared with the Ki67 reporter. POLR2A mRNA has a density of 1.3 ribosome/kb, whereas DYNC1H1 mRNA has about twice that density. Although ribosome density can be measured by ribosomal profiling, the SunTag translation system uniquely allows one to determine the

percentage of mRNAs that are actively translating and whether translation correlates with localization.

This technique revealed interesting movement patterns of DYNC1H1 polysomes. A portion of the DYNC1H1 polysomes showed rectilinear movement dependent on microtubules, suggesting that these translating mRNPs can be moved by motors along microtubules. Many localized mRNAs, including neuronal mRNAs and *ASH1*, are predicted to be translationally repressed during transport based on the presence of translation repression proteins (Martin and Ephrussi, 2009). Pichon et al. (2016) demonstrate that some types of transported mRNPs are translated, consistent with observations of other SunTag translation reporters (Wang et al., 2016).

Interestingly, Pichon et al. (2016) also find that DYNC1H1 mRNAs accumulate in cytoplasmic "blobs" containing three to seven DYNC1H1 mRNAs. These blobs do not colocalize with P-bodies or stress granules, which contain non-translating mRNAs. In fact, DYNC1H1 mRNAs in blobs translate with slightly more frequency than individual DYNC1H1 polysomes. Translation is required for the accumulation of the mRNA blobs, as treatment with a translation inhibitor reduces blobs. These results suggest that DYNC1H1 mRNA, unlike POLR2A, can localize in aggregates that allow translation. Pichon et al. (2016) suggest that these blobs might represent "translation factories," which would be an exciting and novel connection between translation regulation and mRNA localization. The SunTag system provides a method to explore the function of these DYNC1H1 blobs and to search for other mRNAs that form these structures.

By allowing the real-time monitoring of spatial and temporal dynamics of endogenous translation of single mRNAs, this modified SunTag system has the potential to generate novel data that can radically broaden our understanding of translation regulation. First, one can examine whether individual mRNAs expressed from the same gene show variable translation characteristics. Second, differences in translation rate between individual cells are now tractable, allowing investigation of variability between similar cells or between distinct cell types. Third, by exploring the translation of mRNA from unique genes, we can learn how in vivo translation frequency, initiation rate, and elongation rate varies among mRNAs with different sequences. Characterizing the translation dynamics of more mRNAs will likely reveal interesting new phenomena, like the translation-dependent blobs of DYNC1H1 mRNAs. The authors' initial investigation suggests there is wide variability within and between mRNA populations. Whether this variability is stochastic or represents differential regulation among the individual mRNAs remains to be seen. However, these new techniques provide the means for such mechanistic studies.

Combining the SunTag technique with long term tracking of polysomes addresses previously unattainable questions. For example, how often does a polysome stop or restart translation? Do many polysomes move through the cells? If so, where and by what means? Upon induction of an miRNA or a stress that reduces translation, what are the kinetics of that translation repression? Upon relief of repression, how quickly does the population recover translation? Do changes in translation correlate with localization of the mRNA? Pichon et al. (2016) make great progress in this area, tracking some mRNPs for tens of minutes, but the number and movement of mRNPs made consistent tracking difficult. Combining the SunTag approach with advances in lattice light-sheet microscopy (Chen et al., 2014), for example, may allow more consistent tracking of mRNPs over time, at least for mRNAs at low copy number. As tracking of single molecules by fluorescence microscopy gets more attainable, three-color studies tracking a transcript, its translation, and trans factors will lead to great insight into the function and timing of trans factors in regulating translation.

To understand the connection between RNA localization and translation, in vivo imaging of both the mRNA and the nascent peptide would be valuable. Pichon et al. (2016) show that this colocalization in live cells is technically difficult when using MS2-RFP fusions to track mRNA because of bleaching and weak signal of red fluorescence protein. However, the development of other fluorescent tagging systems that allow bright, stable labeling of mRNA in vivo (Wu et al., 2016) might make in vivo colocalization more feasible. This combined approach allows one to address whether localization of a particular mRNA is correlated with a change in translation.

Certain methods open new doors in a field and allow previously elusive phenomena to become tractable. Pichon et al. (2016) have contributed an important new tool to fill a long standing need and have shown the utility of this method on endogenous mRNAs. As shown by their proof of concept, the ability to measure in vivo translation in real time on individual mRNAs will create an influx of new data that was previously unattainable in the study of translation.

Acknowledgments

We thank Phil Hilliker for assistance with Fig. 1.

A.K. Hilliker is funded by the Thomas F. and Kate Miller Jeffress Trust and the University of Richmond. H.V. Pingali is funded by the University of Richmond.

The authors declare no competing financial interest.

Submitted: 22 August 2016
Accepted: 22 August 2016

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