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One Message, Many Translations: Heterogeneity Revealed with Multicolor Imaging

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mRNA translation and its regulation shape the human proteome. Lyon et al. (2019) and Boersma et al. (2019) introduce new orthogonal peptide-tagging systems to study translation in multiple reading frames. Both groups discovered diverse translation behavior of single mRNAs derived from the same genes.

The human genome only codes for roughly 20,000 protein-coding genes, yet the proteome consists of millions of distinct proteoforms (Ponomarenko et al., 2016). Many processes throughout the central dogma account for this difference: alternative transcription start sites, splicing, and polyadenylation produce distinct mRNA isoforms and greatly enhance the genomic coding capacity. The potential for generating diverse peptides from single mRNA species, however, has not been well appreciated. Recent genome-wide approaches, such as ribosome profiling and mass spectrometry, have identified widespread translation events beyond annotated open reading frames (Ingolia et al., 2019). Noncanonical translational events, such as the translation of upstream open reading frames (uORF), alternative start-codon selection, frameshifting, stop codon readthrough, and internal ribosome entry site initiation, contribute to diverse proteoforms from the same mRNAs (Kwan and Thompson, 2019). Although ensemble measurements are superb in establishing averages of translation across many mRNAs, mechanistically, it is still unclear how ribosomes produce several unique peptides from a single mRNA. Now, two new studies (Boersma et al., 2019; Lyon et al., 2019) report methods to visualize translation of multiple reading frames of the same mRNAs in live cells. Both groups have identified surprisingly heterogeneous dynamics during these unconventional translation events (Figure 1).

The two groups built upon recently developed technologies of single-molecule translation imaging (Morisaki et al., 2016; Yan et al., 2016). In short, multivalent peptide epitopes are placed upstream of the gene of interest and are rapidly detected by fluorescent antibodies as soon as they emerge from the ribosome exit channel. The 3′ untranslated regions of mRNAs contain bacteriophage-derived stem loops bound by fluorescent coat proteins to visualize mRNA and translation simultaneously. To expand the palette of nascent peptide imaging, both groups combined different antibody-epitope pairs. Boersma et al. identified a genetically encoded nanobody that folds efficiently in mammalian cytoplasm and binds robustly to its epitope array. Importantly, the new antibody-epitope pair, named MoonTag, is orthogonal to the original SunTag system. With MoonTag encoding an open reading frame (ORF) and SunTag placed after the stop codon, the authors identified mRNAs expressing both SunTag and MoonTag signals, indicating stop codon readthrough events. To measure translation originating from different reading frames, the authors combined the two epitopes in an alternating fashion, which they named Moon and Sun Hybrid Tag (MashTag). Similarly, Lyon et al. combined repetitive FLAG and SunTag epitopes in two reading frames to form the Multi-Frame (MF) tag.

The MashTag and MF tag allow the authors to study noncanonical, out-of-frame (OOF) translation and frameshifting at the single-molecule level. While these events are usually considered consequences of ribosome infidelity, the groups' findings suggest that these processes could be highly regulated and hold a greater role in gene expression than previously thought. Boersma et al. used MashTag as a reporter for OOF translation, identifying alternative start site selection as its main mechanism, rather than frameshifting. They also used MashTag as a real-time sensor for uORF translation, whose short peptide product has been difficult to capture (Starck et al., 2016). The authors were able to find detailed transition probabilities of various translation pathways, such as uORF translation only, re-initiation, and leaky scanning. Lyon et al. applied the MF tag to investigate the frameshifting behavior of an HIV frameshifting sequence (FSS). They found that some frameshifting mRNAs formed multimers and that FSSs caused ribosome pauses leading to traffic jams. Remarkably, they discovered that frameshifting exhibits bursting behavior. A common feature between these studies is heterogeneity; although the mRNAs were derived from the same sequence, they demonstrated inhomogeneous behavior. Further biochemical and genetic studies are required to find the origin of this heterogeneity.

There are numerous avenues to pursue following the advent of these single-molecule tools. By placing tags at different positions on the same mRNA, one can now directly measure elongation speed, differences in cap-dependent and cap-independent translation, kinetics of self-cleaving peptides, and nascent peptide translocation into the endoplasmic reticulum. Furthermore, it is unclear whether noncanonical translation events are controlled spatiotemporally. Single-molecule techniques are promising to address these previously inaccessible problems *in*

situ. When the tags are incorporated into separate mRNAs, one can observe their regulation spatially. For example, certain subunits of supramolecular complexes are co-translationally assembled (Shiber et al., 2018), and the coupling between translation and the assembly process can now be tracked in live cells.

There are still challenges to effectively use these tools to study translation regulation. First, a typical protein translates in minutes; therefore, it is necessary to track translation of single mRNAs for hours, or even throughout the entire cell cycle. The limiting factor is RNA tracking. There are exciting opportunities to develop novel labeling tools for long-term RNA imaging. Second, it is still challenging to visualize translation of endogenous mRNAs *in vivo*. The revolution in gene editing using CRISPR technology will allow knockin of these fluorescent tags at endogenous loci. Further development of microscopy techniques and fluorescent probes will make it possible to directly observe single mRNA translation in live animals. Third, as these studies demonstrate, translation dynamics are not homogeneous. The initiation, elongation, and termination rates may be subject to complex regulation. Theoretical modeling and rigorous data analysis are required to fully uncover the hidden information in single-molecule translation measurements. These challenges will surely inspire further technological developments to better understand translation in physiological and pathological contexts.

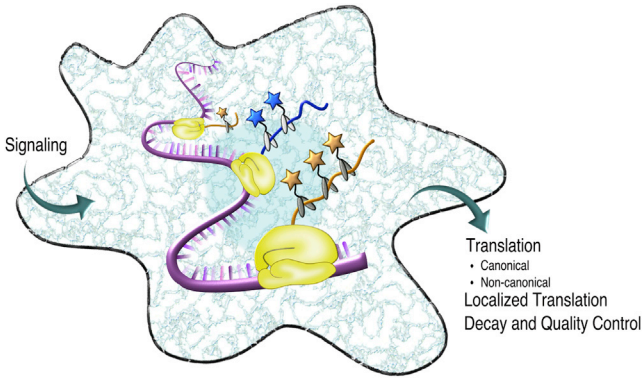

Figure 1. Multicolor Single-Molecule Imaging Can Be Used to Study Many Aspects of Translation in Live Cells

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