


# Quantification of mRNA translation in live cells using single-molecule imaging

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mRNA translation is a key step in gene expression. Proper regulation of translation efficiency ensures correct protein expression levels in the cell, which is essential to cell function. Different methods used to study translational control in the cell rely on population-based assays that do not provide information about translational heterogeneity between cells or between mRNAs of the same gene within a cell, and generally provide only a snapshot of translation. To study translational heterogeneity and measure translation dynamics, we have developed microscopy-based methods that enable visualization of translation of single mRNAs in live cells. These methods consist of a set of genetic tools, an imaging-based approach and sophisticated computational tools. Using the translation imaging method, one can investigate many new aspects of translation in single living cells, such as translation start-site selection, 3'-UTR (untranslated region) translation and translation-coupled mRNA decay. Here, we describe in detail how to perform such experiments, including reporter design, cell line generation, image acquisition and analysis. This protocol also provides a detailed description of the image analysis pipeline and computational modeling that will enable non-experts to correctly interpret fluorescence measurements. The protocol takes 2–4 d to complete (after cell lines expressing all required transgenes have been generated).

## Introduction

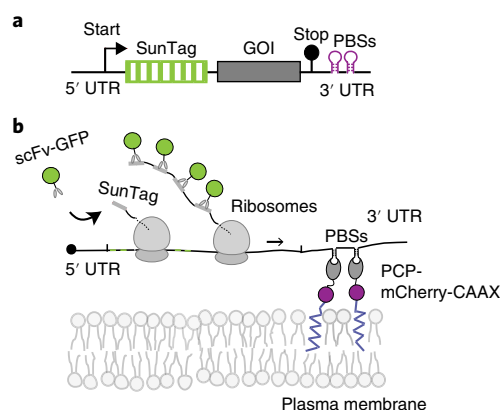
Post-transcriptional regulation of gene expression tunes protein expression levels and as such is critical to many biological processes. There are several different types of post-transcriptional gene regulation, including regulation of mRNA translation and decay. Control of gene expression through translational regulation and mRNA decay enables rapid changes in gene expression, and these types of regulation are often used in cases in which fast temporal changes in protein synthesis are required<sup>1,2</sup>. Furthermore, post-transcriptional gene regulation enables spatial control of protein synthesis, for example, during early development<sup>3,4</sup> and in highly polarized cells, such as neurons<sup>5</sup>. Finally, post-transcriptional gene regulation enables an additional level of RNA quality control that ensures that only functional, full-length protein products are synthesized<sup>6</sup>.

### mRNA translation and decay

Eukaryotic mRNA translation by the ribosome occurs through a number of well-orchestrated steps. First, the ribosome small subunit is recruited to the 5' end of the mRNA by translation initiation factors. Next, the small subunit of the ribosome scans along the 5' UTR in search of a translation initiation site, usually an AUG sequence. Once identification of the translation initiation site has occurred, the large subunit of the ribosome is recruited and translation can be initiated. The ribosome then translates the entire mRNA sequence in 3-nt steps (each step representing a codon) until a termination codon is encountered. During translation termination at the stop codon, the nascent polypeptide is released and the small and large ribosomal subunits split apart and detach from the mRNA. Because each codon consists of 3 nt, each mRNA sequence contains three 'reading frames', each of which encodes a distinct amino acid sequence. To ensure that the correct amino acid sequence is synthesized, the ribosome must initiate translation in the correct reading frame and maintain the reading frame throughout the translation elongation phase.

The majority of mRNAs have a half-life of several hours, much shorter than the length of a cell cycle and/or lifetime of a typical cell, so mRNAs are constantly degraded in cells<sup>7</sup>. There are three

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**Fig. 1 | An assay for visualization of translation using the SunTag system.** **a**, Schematic of a standard translation-imaging reporter with the SunTag upstream of a GOI. **b**, Schematic of the translation imaging method using the SunTag system. mRNAs are fluorescently labeled and at the same time tethered to the plasma membrane using the PP7 system through insertion of PBSs into the 3' UTR of the reporter mRNA. For simplicity, only 2 of 24 PBSs are shown. Nascent polypeptides are labeled through insertion of the SunTag sequence into the coding sequence of the reporter mRNA. As soon as the SunTag peptides emerge from the ribosome exit tunnel, they are bound by the SunTag antibody fused to GFP.

different pathways of mRNA decay: (i) 5'-to-3' exonucleolytic decay by the exonuclease XRN1 (XRN1-dependent mRNA decay is usually preceded by mRNA decapping, because the 5' mRNA cap protects mRNAs from XRN1-dependent decay); (ii) 3'-to-5' exonucleolytic decay by the exosome complex, and (iii) endonucleolytic cleavage of the mRNA (followed by exonucleolytic decay of the two cleavage fragments).

Interestingly, mRNA degradation is frequently coupled to translation; in many cases, mRNA decay is initiated on mRNAs that are still actively translated<sup>8,9</sup>. Furthermore, several quality control pathways that assess the quality of an mRNA molecule and degrade erroneous mRNAs occur co-translationally. For example, mRNAs with a premature termination codon (PTC) in their coding sequence are recognized during translation and are degraded through a process called nonsense-mediated mRNA decay (NMD)<sup>10</sup>. Other defects in the mRNAs, such as the lack of a stop codon, can also lead to mRNA degradation during translation<sup>6</sup>.

### The SunTag translation imaging system

We have recently established a method to visualize mRNA translation of single mRNA molecules in real time in living cells (Fig. 1)<sup>11,12</sup>. Similar methods have been developed in parallel by several other groups<sup>13–16</sup>. Our method makes use of a fluorescence-tagging system that we developed, called the SunTag<sup>17</sup>, which consists of an array of short linear peptides that are recognized by a single-chain variable fragment (scFv) antibody fused to GFP (referred to as the scFv-GFP). To enable visualization of translation, the SunTag system is used to fluorescently label nascent polypeptides. The sequence encoding the SunTag peptide epitopes is inserted upstream of a gene of interest (GOI), and the mRNA encoding the SunTag–GOI complex is expressed in a cell. The synthesis of the SunTag peptide array by ribosomes leads to recognition and binding of the co-expressed SunTag scFv-GFP to the nascent SunTag peptides, resulting in strong GFP accumulation at the site of a translating mRNA, which can be visualized as a bright green spot using fluorescence microscopy. In addition to nascent peptide labeling, individual mRNA molecules are also labeled using the PP7 system<sup>18</sup>. The PP7 system consists of the PP7 coat protein (PCP), a viral RNA-binding protein that binds to a short RNA hairpin sequence (referred to as the PP7 binding site (PBS)) and is fused to a fluorescent protein (e.g., mCherry). Insertion of multiple copies (usually 24×) of the PBS into the mRNA of interest enables bright fluorescent labeling of the mRNA by co-expressed PCP-mCherry. To track mRNA molecules for extended periods of time, which is essential for understanding many aspects of post-transcriptional gene expression control, we have devised a system to tether mRNAs to the plasma membrane through attachment of the PCP to the inner leaflet of the plasma membrane<sup>11</sup>. Using this mRNA and translation imaging method, quantitative measurements of translation initiation and elongation can be obtained for single mRNA molecules with high spatial and temporal resolution.

## Further development of the translation imaging system

### Assays and genetic tools

The original SunTag translation imaging approach provided only a single readout of mRNA translation (i.e., translation of the main open reading frame (ORF)). However, translation can be very complex; each mRNA can, in principle, be translated in three different reading frames, and translation initiation can occur at different start codons, resulting in N-terminally extended or truncated proteins or generating short peptides from upstream ORFs (uORFs)<sup>19,20</sup>. To visualize such translational complexities, we recently developed a second, orthogonal nascent chain-labeling system, called the MoonTag system<sup>21</sup>. In this system, translation is visualized using a MoonTag-nanobody that is fused to a fluorescent protein and binds to nascent MoonTag peptides. Combining the SunTag and MoonTag systems in a multicolor single-molecule imaging approach enables two simultaneous readouts of translation, as well as visualization of many complex aspects of mRNA translation. For example, the MashTag (MoonTag and SunTag hybrid tag) contains SunTag and MoonTag peptides positioned in different reading frames, which enables monitoring of translation from two different reading frames for a single mRNA. A similar system for visualizing complex translation dynamics was developed in parallel by Lyon and colleagues<sup>22</sup>. Furthermore, we have recently established a new assay to visualize translation-coupled mRNA decay; we have used this assay to study mRNA quality control pathway NMD<sup>23</sup>.

### Computational tools

Sophisticated image analysis is key to using the translation imaging systems. Information on the number of translating ribosomes on an mRNA, the position of individual ribosomes along the mRNA, the speed at which ribosomes are moving and the rate at which ribosomes are initiating on an mRNA molecule are difficult to obtain without advanced image analysis and computational approaches. We have developed a user-friendly graphical user interface (GUI)-based image-analysis software package called TransTrack (written in MATLAB and freely available through GitHub: <https://github.com/TanenbaumLab/TransTrack>). In addition, we have developed a computational algorithm called RiboFitter that can use fluorescence intensity-time traces of single translating mRNAs to calculate the number of ribosomes on a single mRNA molecule and to precisely determine the moment of translation initiation of each translating ribosome over time. Calibration experiments, which will be covered in this protocol, are required to determine the parameters used for running RiboFitter.

In this protocol, we will briefly summarize the general SunTag translation imaging approach, after which we will focus on three technological developments of this assay: (i) use of multi-color translation imaging to study translational heterogeneity; (ii) visualization of translation-coupled mRNA decay and (iii) quantitative image analysis using custom-developed software for mRNA tracking and quantitative analysis of translation signals.

## Applications of the method and comparisons with other methods

Various methods that have been used to study mRNA decay and translational control, such as qPCR, northern blot, luciferase assays and RNA-seq/ribosome profiling are ensemble assays in which thousands of mRNAs are pooled together for analysis<sup>24–26</sup>, so these methods do not have single-cell or single-mRNA resolution. To overcome these limitations, additional methods have been developed previously for visualizing protein synthesis in single cells; these include methods for (i) measuring genome-wide protein synthesis rates in single cells<sup>27–31</sup>, (ii) providing a snapshot of translation of single mRNAs by staining nascent polypeptides with small-molecule dyes<sup>32</sup>, (iii) visualizing the first round of translation<sup>33</sup> and (iv) localizing subcellular sites of translation by co-localizing mRNAs and ribosomes<sup>34,35</sup>. However, these previous methods do not enable real-time measurements of translation initiation and elongation dynamics of single mRNAs, and thus, provide limited insight into translation heterogeneity among different mRNA molecules. Heterogeneity among mRNA molecules can arise from differences in mRNA sequence, for example, caused by alternative splicing or alternative transcription start-site usage, or by differential post-transcriptional modifications. In addition, heterogeneity among mRNA molecules can be due to distinct phases of the life cycle that different mRNAs are in. For example, all mRNA molecules are degraded at some point in their lifetime, but only a very small fraction of mRNAs are undergoing degradation at any given time. The translation imaging system is uniquely suited to gain insights into translation dynamics, heterogeneity and

regulation at the single-transcript level and is complimentary to other ensemble methods for studying post-transcriptional gene regulation.

The SunTag translation imaging systems can be applied to many biological questions. In recent work, we have used this system to study heterogeneity in mRNA decoding, as well as to study translation-coupled mRNA decay due to PTCs<sup>21,23</sup>. Both types of applications will be outlined in this protocol.

### Limitations

The translation imaging method has a number of limitations. First, the method is low throughput, limiting the number of mRNAs that can be investigated. Second, the method requires insertion of both the SunTag and PBS sequences into the mRNA of interest. These relatively large sequences could potentially interfere with endogenous regulation of the mRNA (although we have not observed any effects of these sequences in the assays we have performed so far). Therefore, it is important to test whether native regulation is still observed on a reporter mRNA after inserting the SunTag and PBS sequences. Third, we have used only exogenously expressed reporter mRNAs for translation imaging so far. It is challenging to study endogenous genes using the translation imaging method, because this requires targeted integration of relatively large DNA sequences (e.g., SunTag, PBS) into the native gene locus. Moreover, two rounds of targeting may be required, because the SunTag is usually inserted at the 5' end of the coding sequence, whereas the PBSs are inserted into the 3' UTR. In addition, constitutive expression of SunTag proteins (as would occur upon endogenous gene tagging) may result in high expression levels of 'mature' SunTag protein (i.e., proteins encoded by the reporter mRNA containing the SunTag for which synthesis is completed and which have been released from the ribosome). Because mature SunTag proteins remain bound to the SunTag antibodies, antibodies from the cytoplasm will be sequestered by the mature proteins, limiting the amount of SunTag antibody available for nascent chain labeling (referred to as 'cytoplasmic antibody depletion' in this protocol). Nonetheless, endogenous tagging has already been achieved for at least one gene<sup>16</sup>, providing hope that other genes can be tagged at the native locus as well.

### Experimental design

To apply the translation imaging method described in this protocol, the following steps are required: (i) design of mRNA reporters (Step 1 of the Procedure); (ii) generation of cell lines and expression of reporter mRNAs (Steps 2–14); (iii) image acquisition (Steps 15–24); (iv) image analysis (Steps 25–33) and (v) data quantification (Steps 34–46) (Fig. 2). In this protocol, we will describe five different applications of the translation imaging system (see below). Although many steps are similar for all applications, we will also highlight key differences.

The protocol will cover the following applications of the translation imaging system:

- 1 Translation imaging of a single GOI using the SunTag;
- 2 Translation imaging of two different mRNAs in the same cell;
- 3 Analysis of 3'-UTR translation;
- 4 Analysis of translation start-site selection; and
- 5 Quantification of NMD.

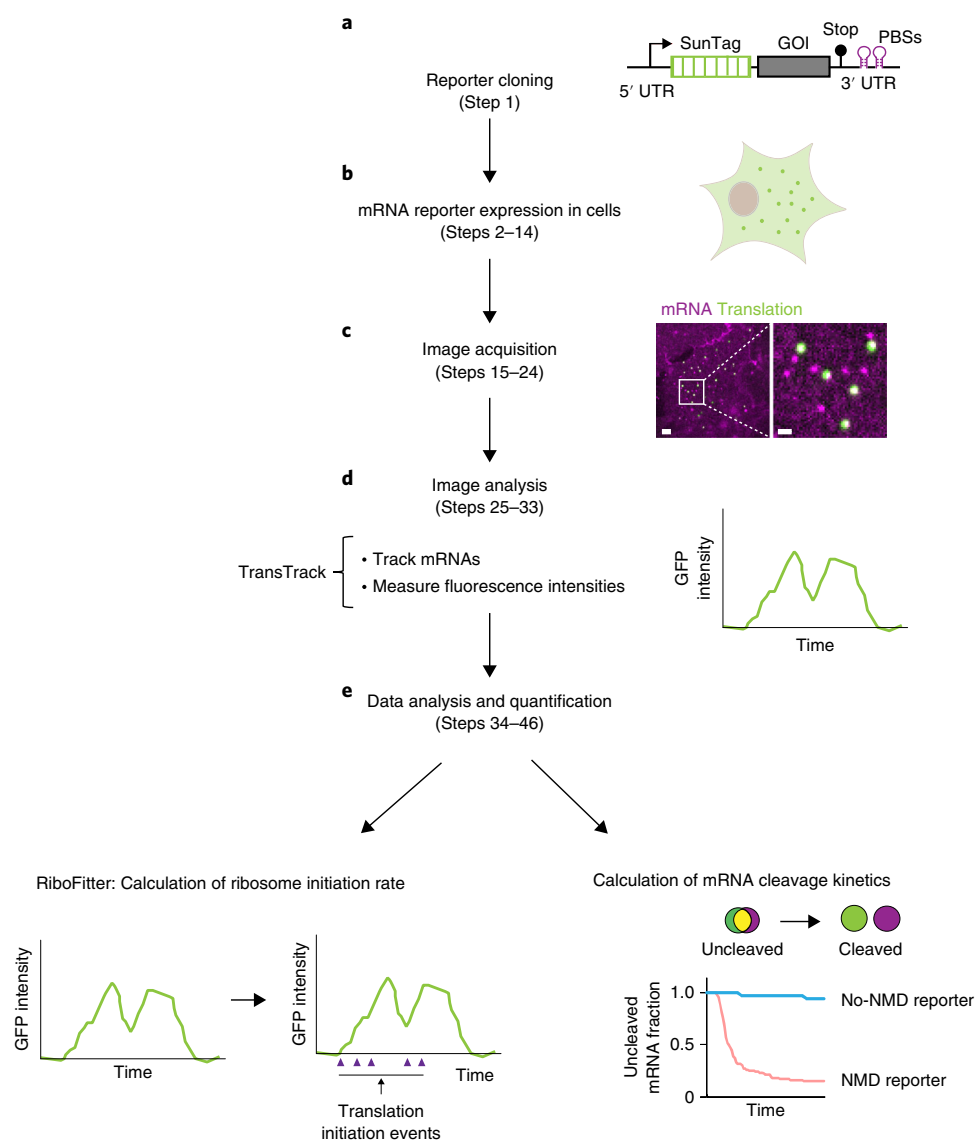
### Design of mRNA reporters (Step 1)

In general, two key components are required for translation imaging of reporter mRNAs (Fig. 3):

- 1 An array of peptide epitopes (SunTag, MoonTag, or MashTag) to visualize translation, generally located at the 5' end of the coding sequence; and
- 2 PBSs in the 3' UTR (generally 24 copies) for mRNA labeling and, where applicable, tethering to the plasma membrane.

In addition, a GOI is often inserted downstream of the peptide array. In some cases, a specific 5' or 3' UTR is also inserted into the reporter. In cases in which the 5' and 3' UTRs are not relevant to the research question, it is possible to use the default UTRs that are present in the expression vector.

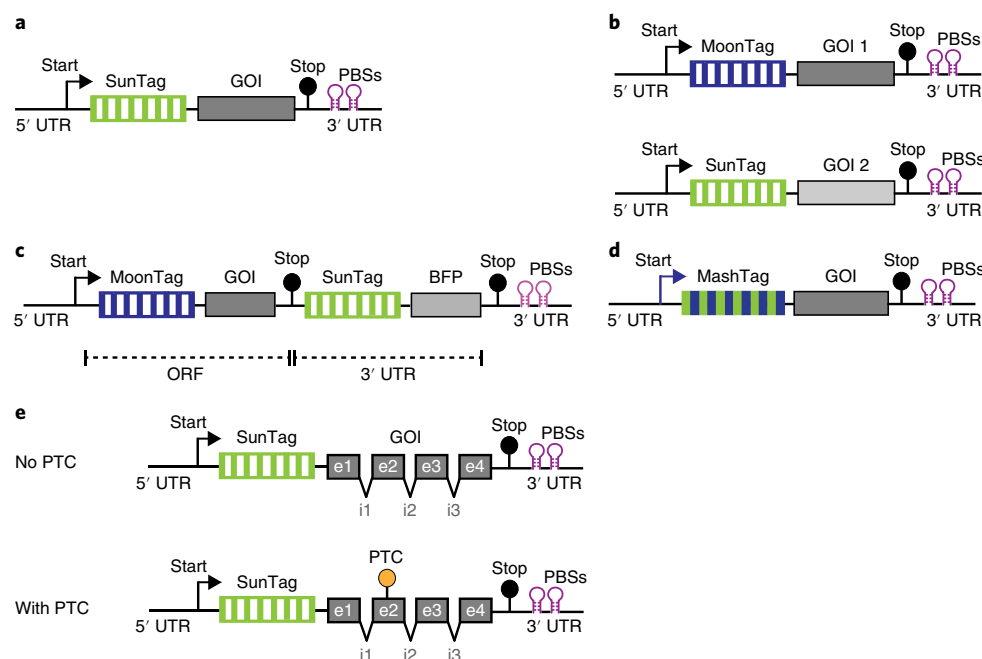
We usually use a tetracycline-inducible promoter to drive the expression of the reporter mRNA. Reporter mRNA expression is generally induced 15–30 min before the start of imaging. Inducible expression of the reporter mRNA is important, because constitutive expression results in a large accumulation of mature protein and cytoplasmic antibody depletion. To overcome the problems associated with an excess of mature SunTag protein, other groups have fused degrons to the protein encoded by the reporter mRNA<sup>14,15</sup>. In our experience, the mature 24× SunTag peptide array bound



**Fig. 2 | Overview of the experimental flow.** Schematic of the workflow of the protocol. **a**, mRNA reporters are designed and cloned. **b**, mRNA reporters are expressed in mammalian cells. **c**, Cells are imaged. Scale bars, 3  $\mu\text{m}$  (left); 1  $\mu\text{m}$  (right). **d**, Image analysis is performed using TransTrack; individual translating mRNAs are tracked and fluorescence intensities of translation signal are measured over time to generate a fluorescence intensity–time trace for each translating mRNA molecule. **e**, Data analysis on TransTrack-generated intensity–time traces is performed using a custom-built computational algorithm, RiboFitter, which determines the number of translating ribosomes and the moment of translation initiation of each ribosome for each mRNA molecule. The data are then further analyzed in different ways, depending on the goal of the experiment. For example, the translation initiation rate can be calculated for each reporter mRNA (left). For experiments addressing NMD, the kinetics of NMD are determined by assessing the frequency and timing of endonucleolytic cleavage, which is observed as a spatial separation of mRNA (mCherry) and translation (GFP) foci (right).

by scFvs is highly resistant to degradation by conventional degrons, so we have chosen instead to work with an inducible promoter.

To generate cell lines stably expressing the reporter mRNA, we have introduced an antibiotic resistance gene, driven by a separate promoter, into the plasmid encoding the reporter mRNA. To generate cell lines stably expressing the reporter, transient transfection of the reporter plasmid, followed by antibiotic selection, is preferable to introduction of the reporter by lentivirus, because the large size and partially repetitive nature of the SunTag/MoonTag/MashTag and PBS arrays make them challenging to package into lentiviral vectors. Moreover, use of lentiviral vectors limits the choice of the 3'-UTR/polyadenylation sequence. Although the basic design of the reporters (as outlined above) is the



**Fig. 3 | Reporter design.** **a–e**, Schematics of different reporters used for translation imaging experiments. For simplicity, only 2 of 24 PBSs are shown. All SunTag and MoonTag arrays contain 24 copies of the peptide array, whereas, for the MashTag, 36 copies each of the SunTag and MoonTag peptides are used to increase the fluorescence signal. For simplicity, not all 24 copies of SunTag or MoonTag are shown. Translation start and stop codons are indicated. **a**, Schematic of a standard translation imaging reporter containing the SunTag sequence upstream of a GOI. **b**, Translation of two different GOIs can be visualized simultaneously in a single cell: one GOI is fused to the MoonTag and the other is fused to the SunTag. Both contain PBS arrays for mRNA labeling. **c**, Schematic of a 3' UTR translation reporter containing the MoonTag upstream of a GOI in the coding region, followed by a stop codon and SunTag sequence. BFP is added downstream of the SunTag sequence as a filler sequence to lengthen the coding sequence downstream of the SunTag, which increases the duration required for translation of the SunTag ORF and facilitates detection of single ribosomes translating the 3' UTR. **d**, Schematic of a MashTag reporter in which the MoonTag sequence is in-frame with the AUG start codon and the SunTag sequence is positioned in the –1 frame relative to the MoonTag. Stop codons are present in both reading frames. **(e)** Schematic of NMD reporters that can be used to visualize endonucleolytic cleavage kinetics triggered by NMD. Two matched reporters are used: one contains a PTC within the coding region, which triggers NMD, whereas the other does not. e, exon; i, intron.

same for all the different applications of the translation imaging system, below we summarize a number of modifications to the reporter that are required for specific applications.

### Translation imaging of a single GOI

For imaging translation of a GOI, one can place the GOI sequence downstream of the SunTag or MoonTag peptide array, followed by PBSs in the 3' UTR, for mRNA labeling and tracking (Fig. 3a).

### Translation imaging of two different mRNAs in the same cell

For simultaneous analysis of two different mRNAs, one can use the SunTag system for one reporter mRNA and the MoonTag system for the other reporter mRNA (Fig. 3b; ref. <sup>21</sup>). We have used the PP7 system to label the mRNA of both reporters, and we distinguish the two genes solely on the basis of the translation signal. A downside of this approach is that mRNAs that do not undergo translation throughout the entire experiment cannot be assigned to either gene. To overcome this issue, one could also combine the SunTag and MoonTag systems with the PP7 system, for labeling of one of the two mRNAs, and the MS2 labeling system, to label the other mRNA<sup>15,36</sup>. Combining the MS2 and PP7 systems does, however, require fluorescence labeling and imaging in four different channels and thus requires multiple dyes and/or fluorescent proteins that are suitable for imaging of weak fluorescence signals.

### 3'-UTR translation

Translation of the 3' UTR of mRNAs can be caused by stop codon read-through, which is regulated during development and can cause formation of C-terminally extended proteins<sup>37</sup>. In addition,



translation of the 3' UTR can also be caused by reinitiation of translation after termination, which is observed, for example, when ribosome recycling is inhibited<sup>38</sup>. For imaging translation of the 3' UTR, we use a translation reporter that contains the MoonTag sequence in the ORF, followed by a stop codon and the SunTag sequences in the 3' UTR (Fig. 3c). We found that the main ORF is generally translated by multiple ribosomes simultaneously, whereas the 3' UTR is often translated by only a single ribosome at a time, thus resulting in a dimmer fluorescence signal. Therefore, the SunTag system is used for visualizing the 3'-UTR translation, because the SunTag system results in brighter fluorescence labeling than the MoonTag, owing to the higher binding affinity of the SunTag scFv to the SunTag peptides<sup>21</sup>. The SunTag sequence can be placed either in-frame with the stop codon (to visualize stop codon read-through and translation reinitiation) or out of frame with the stop codon to visualize translation reinitiation only.

#### Translation start-site selection

For studying translation start-site selection on an mRNA of interest, we developed a reading frame sensor called the MashTag<sup>21</sup>. The MashTag consists of SunTag and MoonTag peptides that are fused in an alternating fashion, such that all the sequences encoding the MoonTag peptide epitope are positioned in the same reading frame, whereas all the sequences encoding the SunTag peptide are encoded in the  $-1$  frame relative to the MoonTag peptides (Fig. 3d). The MashTag is designed such that (i) no AUG start codons or stop codons (TGA, TAA, or TAG) are present in any reading frame within the MashTag sequence and (ii) the nucleotide sequences of individual repeats of the SunTag and MoonTag peptides are minimally repetitive (different codons were used for same amino acids in different repeats of the peptide). To study translation start-site selection for a 5'-UTR sequence of interest using the MashTag, one can insert the 5'-UTR sequence upstream of the MashTag in a translation reporter and assess the frequency of translation at start sites present in the two reading frames.

#### Analysis of NMD

For visualizing NMD, one needs to introduce mRNA sequences into the reporter that stimulate NMD. Such sequences generally consist of a PTC and, optionally, one or more introns, at least one of which is positioned  $>55$  nt downstream of the stop codon. As a control, an mRNA reporter can be designed with an identical mRNA sequence but lacking a PTC or introns (Fig. 3e).

#### Generation of cell lines and expression of reporter mRNAs (Steps 2–14)

We have performed the majority of our experiments in U2OS cells (human osteosarcoma cells), but similar SunTag translation imaging experiments have been successfully performed in other cell types, such as HeLa cells<sup>14,16</sup> and neurons<sup>14,15</sup>. The main criteria to consider when choosing a suitable cell line are (i) whether it is possible to introduce the required components (e.g., SunTag antibody, reporter mRNAs) into the cells and (ii) whether the cell type can be imaged using high-resolution microscopy. Flat, adherent cells are generally more suitable for imaging than thick cells (e.g., oocytes) or cells that grow in suspension. However, use of thick cells may be possible when applying an appropriate microscopy method (e.g., light-sheet microscopy).

Preferably, cell lines are generated that stably express the SunTag and/or MoonTag antibodies, PCP-mCherry-CAAX (or PCP-mCherry, if the mRNAs should not be tethered to the plasma membrane) and the TetR transcription repressor for inducible expression<sup>39</sup> (plasmids encoding these proteins can be obtained from Addgene (Materials; see also Table 1). Cell lines stably expressing these proteins can be generated through lentiviral transduction (Steps 2–13). After lentiviral transduction, cells with appropriate expression levels of fluorescently labeled proteins are selected by flow cytometry. We generally generate monoclonal cell lines with a range of expression levels of the fluorescently labeled SunTag/MoonTag antibodies and PCP-mCherry (-CAAX) and functionally test the cell lines by introducing a reporter mRNA and performing a translation imaging experiment. The clone with the best signal-to-noise ratio for mRNAs and translation site fluorescence is selected for further experiments. Because optimal signal-to-noise ratio during imaging is very sensitive to the expression levels of both the antibodies and PCP-mCherry protein, many clones should be screened. For the SunTag antibodies, optimal expression levels are as high as possible while still enabling detection of translating mRNAs and mature SunTag proteins over the background when imaging using mature protein settings (Step 22B). For PCP-mCherry, we find that very low expression often results in the best signal-to-noise ratio. Because PCP-mCherry can accumulate in lysosomes during

**Table 1 | Addgene plasmids**

Plasmid name	Addgene no.	Application
<b>Plasmids used for cell line generation</b>		
SunTag-scFv	60907	For nascent chain labeling using SunTag
PCP-mCherry-CAAX	74925	mRNA labeling and membrane tethering
PCP-mCherry	74926	mRNA labeling for untethered mRNAs
MoonTag-Nb-HaloTag	128603	For nascent chain labeling using MoonTag-nanobody
MoonTag-Nb-GFP	128602	For nascent chain labeling using MoonTag-nanobody
SunTag-CAAX	134833	For measuring the fluorescence intensity of single mature proteins
<b>Reporter plasmids</b>		
Standard SunTag translation imaging reporter	74928	General SunTag translation assay
MoonTag translation reporter	128604	General MoonTag translation assay
3'-UTR translation reporter	128605	3'-UTR translation assay
MashTag translation reporter	128607	Translation start-site selection assay
NMD reporter (TPI-PTC160)	130698	mRNA cleavage assay
NMD control reporter (TPI-WT)	130697	mRNA cleavage assay

generation of stable cell lines, a clone should be selected that has minimal mCherry signal in lysosomes.

Translation reporters can be either transiently transfected or stably integrated into the genome of the cells. Stable integration can be performed, either by transient transfection and antibiotic selection, which results in random integration into the genome, or by targeting a specific locus in the genome (e.g., the AAVS1 locus)<sup>21</sup>. Transient transfection is faster, but generation of stable cell lines is easier if the reporter is imaged many times, especially in combination with siRNAs, because we have found that transfection of cells with both plasmids and siRNAs is sometimes less efficient. In our experience, mRNAs expressed from transiently transfected plasmids and stably integrated plasmids show similar translation kinetics. Furthermore, generating monoclonal cell lines after stable integration of the reporter results in more homogeneous expression of the reporter mRNA among cells, which can be useful if the cell line will be used very frequently or when applying other treatments that target only a subset of cells (e.g., expression of a transgene).

### Imaging acquisition (Steps 15–24)

We perform imaging experiments on a temperature-controlled spinning-disk confocal microscope with a 100×, numerical aperture (NA) 1.49, oil-immersion objective and an electron-multiplying charge-coupled device (EMCCD) or back-illuminated scientific complementary metal-oxide-semiconductor (sCMOS) camera to obtain high-resolution images with very sensitive detection. We prefer spinning-disk confocal imaging over total internal reflection fluorescence (TIRF), highly inclined and laminated optical sheet (HILO) microscopy or wide-field illumination because of the homogeneous illumination of spinning-disk confocal imaging over the entire field of view. Homogeneous illumination results in homogeneous fluorescence intensity values throughout the entire image, thus facilitating image quantification. Spinning-disk confocal imaging is preferred over point-scanning confocal microscopy because of its higher speed and improved detection sensitivity when combined with an EMCCD or back-illuminated sCMOS camera. For light-sensitive applications, such as imaging of single ribosomes or imaging at high time resolution in 3D, a microscope optimized for detection sensitivity is essential. However, for imaging a highly translated mRNA labeled with the SunTag system, an epifluorescence wide-field microscope or point-scanning confocal may also be sufficient, especially when the mRNAs are tethered to the plasma membrane.

### Image analysis (Steps 25–33)

After image acquisition, careful image analysis is required to correctly interpret the fluorescence images. For most experiments, analysis consists of tracking individual mRNA molecules and measuring fluorescence intensities over time. In this protocol, we will describe use of a user-friendly software package, TransTrack, designed for mRNA tracking and intensity measurements.



**Data analysis and quantification (Steps 34–46)**

After measuring fluorescence intensities over time for each mRNA molecule, additional analyses are usually required to determine translation initiation and elongation rates, as well as to perform more sophisticated analyses, including determining the initiation rates at multiple translation start sites or calculating the fraction of ribosomes translating the 3' UTR of an mRNA. Interpretation of fluorescence intensities is facilitated by a computational algorithm called RiboFitter, which uses fluorescence intensities of translation signals to determine the number of ribosomes translating an mRNA, as well as the moment at which each ribosome initiated translation. The first time that RiboFitter is used, several control experiments are required to determine the precise fluorescence intensity produced by a single ribosome translating the reporter mRNA, because the total translation signal associated with an mRNA translated by multiple ribosomes is a compound of multiple such single ribosome translation profiles. These control experiments will be described in Steps 41 and 42. After running RiboFitter, additional downstream analyses are often required as well; these are also described. For mRNA endonucleolytic cleavage through NMD, analysis consists mainly of scoring the moment of mRNA cleavage for each mRNA molecule, although additional analyses can be performed.

**Materials****Biological materials**

**! CAUTION** The cell lines used should be checked for authenticity and regularly tested for mycoplasma contamination.

- Cell line used for imaging: human U2OS cells (ATCC, cat. no. HTB-96, RRID: CVCL\_0042)
- Cell line used for lentivirus generation: HEK293T cells (ATCC, cat. no. CRL-3216, RRID: CVCL\_0063)

**Reagents**

- Lentiviral-production plasmids (Table 1) **! CAUTION** Lentiviruses can be a biological hazard, so all lentiviral work should be performed in biosafety level 2 labs with appropriate precautions and measures taken for usage and disposal of lentiviral samples.
- mRNA reporter plasmids (Table 1)
- Lentiviral packaging vectors psPAX2 (Addgene, cat. no. 12260) and pMD2.G (Addgene, cat. no. 12259)
- DMEM (high glucose with GlutaMAX supplement; Thermo Fischer Scientific, cat. no. 31966021)
- Leibovitz's L15 medium (no phenol red; Thermo Fischer Scientific, cat. no. 21083-027)
- Opti-MEM (reduced-serum medium; Thermo Fisher Scientific, cat. no. 11058021)
- Fetal bovine serum (FBS; Thermo Fisher Scientific, cat. no. F7524)
- FuGENE 6 transfection reagent (Promega, cat. no. E231A)
- DMSO (dimethyl sulfoxide; Sigma-Aldrich, cat. no. D8418-1L)
- PEI (polyethylenimine for plasmid transfection; Polysciences, cat. no. 23966)
- Penicillin-streptomycin (10,000 U/ml; Thermo Fischer Scientific, cat. no. 15140-122)
- Polybrene (Santa Cruz Biotechnology, cat. no. sc-134220) **! CAUTION** Polybrene is harmful. Wear gloves and avoid contact with eyes and skin.
- Doxycycline (Sigma-Aldrich, cat. no. D9891-1G)
- Puromycin (Thermo Fisher Scientific, cat. no. 12122530) **! CAUTION** Puromycin is harmful. Wear gloves and avoid contact with eyes and skin.
- Cycloheximide (Sigma-Aldrich, cat. no. C4859) **! CAUTION** Cycloheximide is harmful. Wear gloves and avoid contact with eyes and skin.
- Harringtonine (Cayman Chemical, cat. no. 15361)
- Janelia Fluor 646 HaloTag dye (Promega, cat. no. GA1120)
- Zeocin (Invitrogen, cat. no. R25001) **! CAUTION** Zeocin is harmful. Wear gloves and avoid contact with eyes and skin.
- HEPES (Sigma Aldrich, cat. no. H3375-500G)
- (Optional) LysoTracker (Invitrogen, cat. no. L12492)

**Equipment****Cell culture**

- Cell culture hood (Telstar BioVanguard, Green line)

- Nitrile gloves (Kimtech)
- Cell culture incubator (5% CO<sub>2</sub>, humidified, 37 °C; Panasonic, model no. MCO-170AICUVH-PE)
- Serological pipettes: 5 ml, 10 ml and 25 ml (Fisher Scientific, cat nos. GPN5E1, GPN10E1 and GPN25E1)
- Tissue culture plate (CellStar, 6 well; Greiner Bio-One, cat. no. 657160)
- Tissue culture plate (CellStar, 100 mm; Greiner Bio-One, cat. no. 664160)
- Falcon conical centrifuge tubes (CellStar, 15-ml; Greiner Bio-One, cat. no. 188271)

### Imaging

- Glass-bottom imaging plates (Matriplates, 96 well; Brooks Life Science Systems, cat. no. MGB096-1-2-LG-L)
- Confocal spinning-disk microscope: inverted microscope (Nikon, TI model with Perfect Focus System) equipped with a spinning disk (Yokagawa, model no. CSU-X1), a PlanApo oil immersion objective (100×, 1.49 NA) and an EMCCD camera (Andor, model no. iXon Ultra 897) or Prime95B camera (Photometrics). The microscope should be equipped with an incubation chamber that ensures control of temperature and humidity

### Flow cytometry

- Flow cytometry system (BD, model no. FACS Aria II)

### Software

- Micromanager for microscope control (Micro-Manager v.1.4.22: <https://micro-manager.org>)<sup>40</sup>
- ImageJ (<https://imagej.nih.gov/ij/>)
- NIS-Elements Imaging Software (Nikon HC 5.11.01)
- GraphPad Prism v.7 (GraphPad Software, <https://www.graphpad.com/scientific-software/prism>)
- MATLAB v.R2012b (MathWorks: <https://nl.mathworks.com/products/matlab.html>)
- R v.3.5.1 (R Project for Statistical Computing: <http://www.r-project.org>)
- TransTrack (written in MATLAB; GitHub: <https://github.com/TanenbaumLab>)
- RiboFitter (<https://github.com/TanenbaumLab>)

### Reagent setup

#### Complete DMEM

Complete DMEM is made by adding 5% FBS and 1% penicillin–streptomycin to DMEM. The complete medium should be stored at 4 °C and can be used for ~3–4 months.

## Procedure

### Design of reporter mRNA(s) ● Timing 2–5 d

- 1 Generate a reporter according to the instructions provided in the ‘Experimental design’ section. For standard translation imaging experiments (i.e., experiments in which only translation of the main ORF is visualized), several plasmids are available from the Tanenbaum lab (Addgene, plasmid nos. 74928, 128604, 128605 and 128607; Table 1). Alternatively, use molecular cloning to generate appropriate reporters suitable for your experiments. For example, for translation imaging of a GOI using the SunTag and PP7 systems, subclone the GOI into the standard SunTag translation reporter available on Addgene (plasmid no. 74928) using the AgeI and EcoRV sites, which removes the GOI (*KIF18B*) from this plasmid.

### Generation of cells lines suitable for translation imaging ● Timing 10–25 d

▲ **CRITICAL** To create a cell line that can be used for translation imaging, you need to deliver the plasmids encoding fluorescently labeled proteins (e.g., SunTag-scFv; see ‘Experimental design’ section) to the cells by lentiviral transduction. See the ‘Generation of cell lines and expression of reporter mRNAs’ section for a discussion of considerations for selecting a suitable cell type.

- 2 *Production of lentiviruses (Steps 2–8).* Seed HEK293T cells in a 6-well plate at 20–25% confluency (~3 × 10<sup>5</sup> cells per well).
- 3 Transfect the cells 16–24 h after plating with the lentiviral plasmid along with lentiviral packaging vectors psPAX2 and pMD2.G using PEI. First, mix 10 µl of PEI and 50 µl of Opti-MEM per reaction.

- 4 Then mix 1  $\mu\text{g}$  of lentiviral plasmid (e.g., SunTag-scFv) with 0.4  $\mu\text{g}$  of pMD2.G and 0.6  $\mu\text{g}$  of psPAX2 in an Eppendorf tube. Add 60  $\mu\text{l}$  of PEI + Opti-MEM mix to each tube with DNA, mix by pipetting and incubate for 5–15 min at room temperature (20 °C). Then add the transfection mix to the cells and gently swirl the plate to mix.
- 5 Twenty-four hours after transfection, replace the medium with fresh medium. Check the cells, using a fluorescence microscope to determine transfection efficiency. 25–75% of cells should be fluorescent.  
**▲ CRITICAL STEP** Be gentle while changing the medium, pipette into the corner of the well, not directly onto the cells, because HEK293T cells easily detach from the plate. 20 mM HEPES can be added to the medium when the cells are very dense to prevent changes in the pH of the medium, which can affect virus titers.
- 6 Harvest the virus-containing medium 3 d after transfection. Check the cells again, using a fluorescence microscope to determine virus production efficiency. All cells should be fluorescent because the newly produced virus will have infected all non-transfected cells as well. Collect the medium into 1.5- or 2 ml tubes.
- 7 Spin down tubes at 3,500g at 25 °C for 3 min. Gently remove ~1.5 ml of viral supernatant. Leave at least 100–200  $\mu\text{l}$  in the tube so as not to take along any cells or cell debris. The viral supernatant can also be filtered to ensure complete removal of the cell debris.
- 8 Use the virus-containing medium immediately for infection; store at 4 °C for up to ~1 week or store the virus in aliquots at –80 °C for up to several months.  
**▲ CRITICAL STEP** Making aliquots of the virus is important, because each freeze–thaw cycle can reduce the titer of the virus batch.
- 9 *Lentiviral transduction in U2OS cells (Steps 9–13).* Seed U2OS cells in a 6-well plate at 20–25% confluency ( $\sim 2 \times 10^5$  cells per well).
- 10 Sixteen to 24 h after plating, add 100–500  $\mu\text{l}$  of virus-containing medium to the cells (the amount of virus required for efficient transduction may vary for different cell types). Make sure that the volume of cell culture medium does not exceed 1 ml. Add Polybrene to each well (10  $\mu\text{g}/\text{ml}$ ).
- 11 Spin the 6-well plate containing the U2OS cells and virus for 90–120 min at 800g at 25 °C for increased transduction efficiency (spin infection).
- 12 After spin infection, replace the cell culture medium with fresh medium and grow the cells for at least 2 d before any further analysis or processing to obtain steady-state expression levels of the virally transduced proteins.
- 13 FACS-sort the cells on the basis of the expression of fluorescent proteins. Compare infected cells with uninfected control cells (that do not express any fluorescent proteins) and set gates that exclude all control cells. We recommend sorting single cells to make monoclonal cell lines, although polyclonal cell lines can also be used (see ‘Generation of cell lines and expression of reporter mRNAs’ in the ‘Experimental design’ section).  
**▲ CRITICAL STEP** Ensure that expression of the fluorescently labeled proteins is homogeneous among cells. If expression levels are variable, a correction may need to be applied during analysis of fluorescence images to correct for differences in the degree of antibody binding to the peptide epitopes.

### Expression of the reporter mRNA

- 14 For expression of reporter mRNAs using transient transfection, follow option A. For stable integration into the host cell genome, follow option B. See ‘Generation of cell lines and expression of reporter mRNAs’ in the ‘Experimental design’ section for a discussion of the advantages and disadvantages of these approaches.
  - (A) **Transient transfection** ● **Timing 2 d**
    - (i) Seed cells in a 96-well glass-bottom dish at 20–25% confluency ( $\sim 2 \times 10^4$  cells per well).
    - (ii) Sixteen to 24 h after plating, transfect the cells using an appropriate transfection reagent according to manufacturer’s guidelines. We transfect U2OS cells using FuGENE 6 according to the manufacturer’s guidelines.
    - (iii) Image the cells 24–48 h after transfection.
  - (B) **Stable integration** ● **Timing 12–20 d**
    - (i) Seed U2OS cells in 6-well plate at 20–25% confluency ( $\sim 2 \times 10^5$  cells per well).
    - (ii) Sixteen to 24 h after plating, transfect cells using an appropriate transfection reagent according to the manufacturer’s guidelines. We transfect U2OS cells using FuGENE 6 according to the manufacturer’s guidelines.

**Table 2 | Drugs used in the protocol**

Drug	Final concentration	Mode of action	Use	Stock solution made in
Doxycycline	1 µg/ml	Activates Tet-on system for gene expression	To induce transcription of reporters	Water
Puromycin	100 µg/ml	Translation inhibitor; dissociates nascent chains and ribosomal subunits from mRNA	To verify that GFP spots are translation sites	DMSO
Harringtonine	3 µg/ml	Translation inhibitor; blocks translocation of ribosome at initiation codon, without affecting downstream ribosomes	To estimate ribosome elongation rates on mRNA	DMSO

All dilutions from stock solutions are made in Leibovitz's L15 medium.

- (iii) Twenty-four hours after transfection, replace the medium with fresh medium and grow the cells in medium containing antibiotics (for plasmids with the pcDNA4/TO vector backbone, which have Zeocin as an antibiotic selection marker, we use 0.4 mg/ml Zeocin) for 10–15 d for selection.
- (iv) After integration of the reporter into the genome of the cells, prepare for imaging by seeding the cells at 40–50% confluency ( $\sim 4 \times 10^4$  cells per well) 16–24 h before the start of imaging.

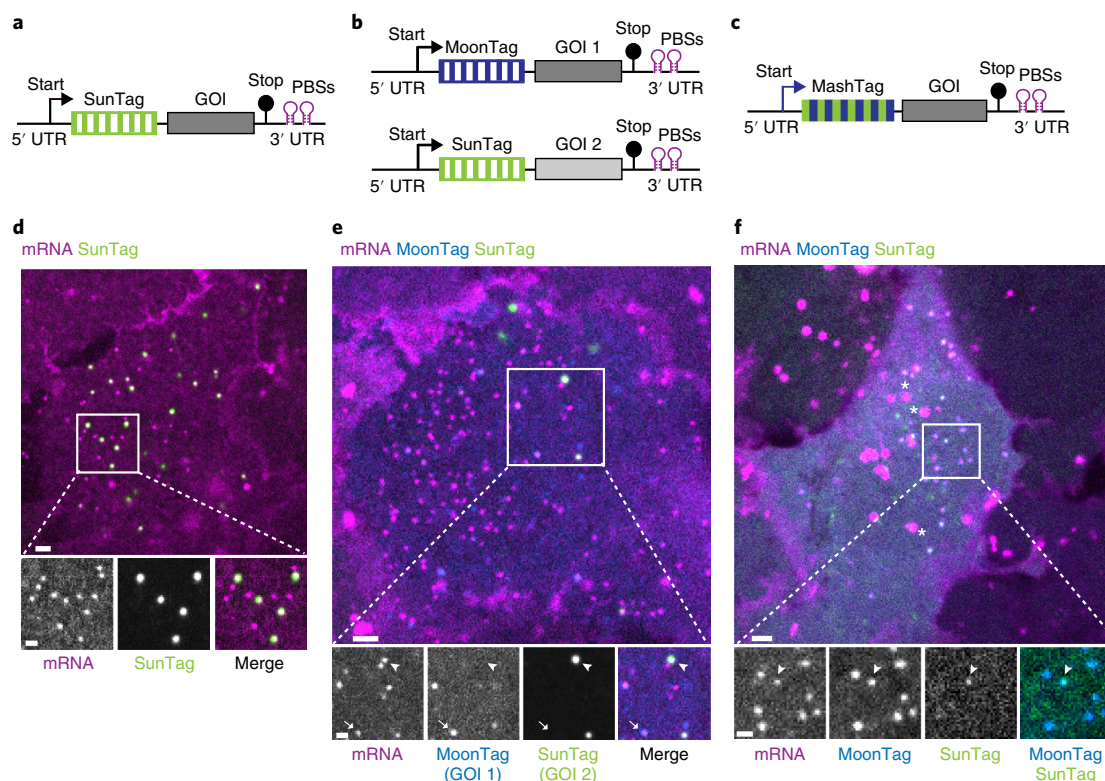
### Sample preparation for imaging ● Timing 1–2 d

- 15 Prepare the cells as described in Step 14.
- 16 Set the microscope temperature to 37 °C and wait until the correct temperature is reached.  
▲ **CRITICAL STEP** Changes in temperature can influence translation dynamics. Therefore, it is very important to ensure that temperature control is accurate during imaging.
- 17 Replace the cell culture medium with pre-warmed (37 °C) Leibovitz's L15 medium.  
▲ **CRITICAL STEP** A CO<sub>2</sub> perfusion chamber is not required when using Leibovitz's L15 medium during imaging but should be used when imaging in other media (e.g., DMEM). Use cell culture medium lacking phenol red, because phenol red in the medium can reduce image quality.  
▲ **CRITICAL STEP** For imaging experiments in which one of the antibodies is fused to a HaloTag, label the HaloTag by incubation with 50 nM Janelia Fluor 646 HaloTag<sup>41</sup> in pre-warmed Leibovitz's L15 medium (37 °C) for 1 h. After Janelia Fluor 646 HaloTag ligand incubation, wash the cells three times with Leibovitz's L15 medium.
- 18 Add doxycycline (final concentration 1 µg/ml of Leibovitz's L15 medium) to the cells to induce transcription of the reporters (See Table 2).
- 19 Start imaging 15–30 min after doxycycline addition.  
▲ **CRITICAL STEP** We recommend keeping a lid on the imaging plate when imaging for a long duration (> 1 h) to prevent evaporation of the medium.

### Image acquisition ● Timing 1–2 h

- ▲ **CRITICAL** Image acquisition settings will vary depending on the requirements of the experiment. Here, we provide settings that we use frequently for our experiments.
- 20 Select the *x*, *y* positions to be imaged. Representative images illustrating selection of appropriate *x*, *y* positions are shown in Fig. 4.  
▲ **CRITICAL STEP** Selection of the best *x*, *y* positions is critical, and important considerations to take into account for selection of positions are explained in Box 1.
  - 21 Find the correct focal plane using the mCherry channel.  
▲ **CRITICAL STEP** Because the PCP-mCherry is tethered directly to the plasma membrane, whereas the SunTag-scFv is tethered indirectly to the membrane (via the nascent chain, mRNA, and PCP-mCherry), the GFP signal is often located slightly above the mCherry fluorescence. Therefore, it is helpful to focus the objective slightly above the optimal *z*-position used to observe the PCP-mCherry signal such that both the mCherry and GFP signals are observed as crisp foci. Because the fluorescence intensity of GFP foci depends strongly on the precise focal plane, we recommend using an autofocus system (e.g., Nikon Perfect Focus System) to prevent fluctuations in the focal plane during time-lapse experiments or between different experiments. In the case of untethered reporter mRNAs, it is useful to focus on a region of the cytoplasm just below the nucleus, because mRNA mobility is often slightly lower in this region, facilitating long-term tracking of individual mRNAs.





**Fig. 4 | Representative imaging data for SunTag, MoonTag and MashTag reporters. a–c,** Schematics of indicated translation imaging reporters. **d–f,** Representative images of U2OS cells expressing SunTag-scFv (green), PCP-mCherry-CAAX (mRNA; magenta), MoonTag-nanobody-HaloTag labeled with Janelia Fluor 646 HaloTag (blue; **e,f**), and indicated reporters are shown. Top images show the entire cell; bottom images show a zoomed-in view of the boxed areas of the cells. In **e**, the arrowheads and arrows indicate SunTag and MoonTag translation, respectively. In **f**, the arrowheads indicate mRNA with out-of-frame (SunTag frame) translation. Large, bright fluorescence foci labeled with asterisks in **f** indicate fluorescent lysosomes. Scale bars, 2  $\mu$ m (top images); 1  $\mu$ m (bottom images).

### Box 1 | Considerations for selecting *x, y* positions during image acquisition

We generally image multiple *x, y* positions simultaneously to acquire data from a larger number of cells in each experiment. The number of *x, y* positions that can be imaged simultaneously depends on a number of variables, including the time interval of imaging, the speed of the microscope and the number of channels that are imaged. For imaging SunTag reporters, we generally select 10–15 *x, y* positions to image simultaneously. We select positions on the basis of the following considerations:

- Mature SunTag proteins.** Presence of mature SunTag proteins diffusing in the cytoplasm is the first criterion for selection of cells for imaging, because the presence of mature SunTag proteins indicates that the cell is expressing the reporter plasmid. Alternatively, in the case of transient transfection, co-transfect a plasmid encoding any fluorescent marker (e.g., BFP) as a method to identify transfected cells (see Troubleshooting).
- Translation sites.** When mRNAs that are present from the start of the movie will be analyzed, select cells for imaging that contain ~10–40 translation sites. However, when the experiment requires visualization of the first round of translation, only mRNAs that initiate translation during the movie can be included in the analysis. In this case, start imaging earlier (~15 min after doxycycline addition) and select cells with fewer translation sites (3–10). In all cases, exclude cells with >40 translation sites, because at high density, tracking of mRNAs is challenging and the translation signal will decrease because of cytoplasmic antibody depletion (see Troubleshooting).
- Fluorescent lysosomes.** PCP-mCherry can accumulate in lysosomes when stably expressed in cells (see Fig. 4f). Select cells with few fluorescent lysosomes because they interfere with mRNA tracking. In our experience, mCherry-positive lysosomes are visible in most cells at all expression levels of PCP-mCherry. Lysosomes can easily be distinguished from mRNAs on the basis of fluorescence intensity and movement; lysosomes are generally much bigger, show very high fluorescence and move faster (and in a directed fashion) than tethered mRNAs (see Troubleshooting).
- Aggregation of mature SunTag proteins.** Avoid cells that show aggregation of mature SunTag proteins. We found that cells containing SunTag aggregates can be easily identified on the basis of the presence of bright, largely immobile GFP-positive aggregates. Aggregation is not observed when using reporters encoding the SunTag only, but does occur when MashTag reporters are expressed at high levels. Aggregation probably occurs because of the different linker that is used in between peptide epitopes in the MashTag as compared with the SunTag. While selecting *x, y* positions, always use the lowest possible laser power and minimize the amount of time that cells are exposed to laser light to limit phototoxicity and photobleaching.

- 22 Set appropriate laser power and exposure time for imaging. Follow option A for standard translation imaging and option B for imaging of single mature SunTag proteins (see Step 41B).  
**▲ CRITICAL STEP** The precise laser power and exposure time will need to be optimized for each microscope and experiment.  
(A) **Standard translation imaging**  
(i) For translation imaging experiments, use a 500-ms exposure time for GFP, mCherry, and Janelia Fluor 646 HaloTag.  
**▲ CRITICAL STEP** Using a long exposure time results in motion blurring of the fast-moving mature SunTag and/or MoonTag proteins, resulting in a more homogeneous background fluorescence, which facilitates detection of weak translation signals. Aim for a laser power that results in ~10–50% photobleaching over the duration of the entire experiment (~60–120 time points). Images for different fluorescence channels can be obtained sequentially with a single camera. Good co-localization between GFP and mCherry foci is observed when images are acquired within ~1 s of each other. When available, a two-camera setup can also be used to reduce the time between images.  
**? TROUBLESHOOTING**  
(B) **Mature protein imaging**  
(i) For measuring intensities of mature SunTag array proteins bound to SunTag-scFvs, use an exposure time of 20 ms, because this prevents motion blurring of fast-moving mature proteins. Use a laser power that is as high as possible without saturating pixel intensities on the camera.  
**? TROUBLESHOOTING**
- 23 Set a time interval for the imaging experiment  
**▲ CRITICAL STEP** In our experience, a 30-s time interval enables good tracking of tethered mRNAs and captures most translation dynamics. However, for some other applications, such as tracking untethered mRNAs or visualizing rapid translation dynamics, other time intervals are required.
- 24 Start image acquisition  
**▲ CRITICAL STEP** When required, harringtonine (or other drugs) can be added during image acquisition. Prepare a solution containing 24 µg/ml harringtonine in pre-warmed Leibovitz's L15 medium. Add 50 µl of drug solution to the 350 µl of medium and pipette up and down a few times (final concentration, 3 µg/ml). Table 2 describes multiple drugs that can be used to assess translation dynamics, as well as their mechanisms of action and optimal concentrations for human cells.  
**▲ CRITICAL STEP** Be careful not to touch the microscope or the edges of the well when adding drugs, because this might cause a shift in the focal plane. Also, try to minimize the time during which the microscope chamber is open to limit changes in temperature.  
**? TROUBLESHOOTING**

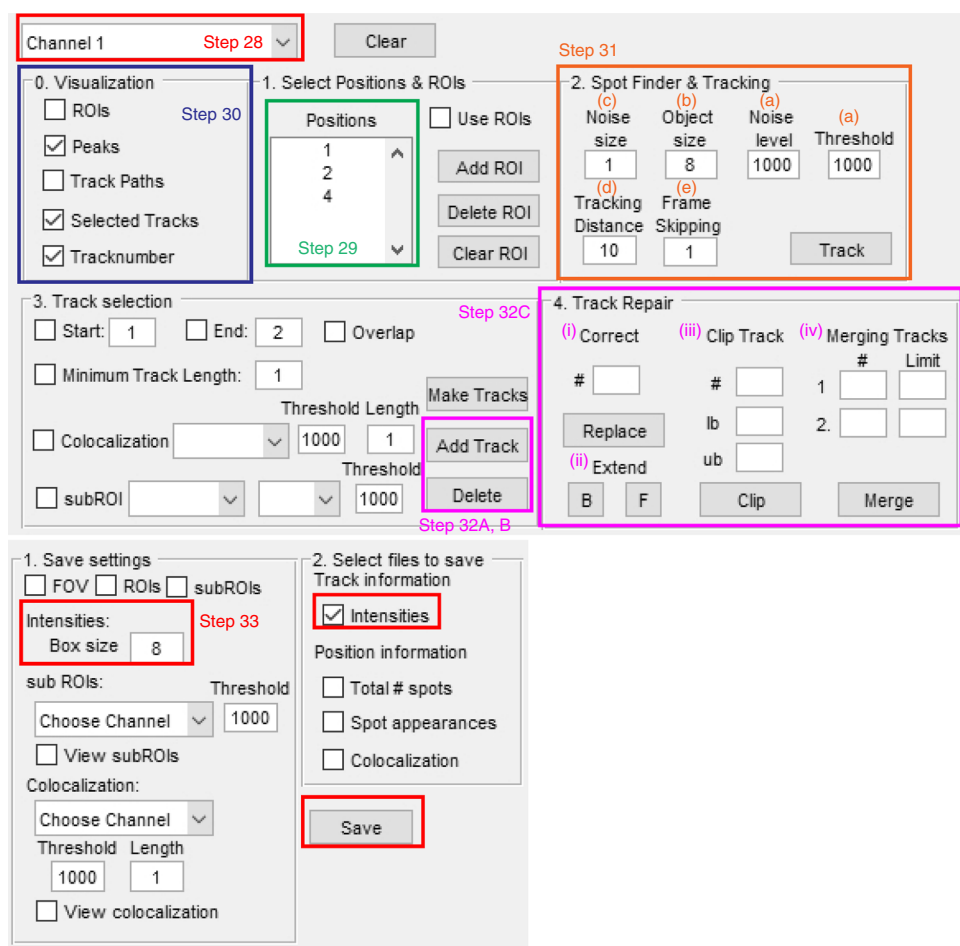
### Image analysis: tracking single mRNAs and measuring fluorescence intensities of translating mRNAs over time with TransTrack ● **Timing** 1–6 h

**▲ CRITICAL** We developed TransTrack, a MATLAB-based software package, to track single translating mRNAs and measure the intensity of the translation signal over time. TransTrack was developed to track membrane-tethered mRNAs but may also work for non-tethered mRNAs. Tracking of translating mRNAs is performed in a semi-automated manner, in which tracks are generated automatically, but the user can manually select, reject or adjust individual tracks. A tutorial, an example dataset and expected outcomes of TransTrack analysis are included in Supplementary Data 1.

- 25 Load a time-lapse movie of a single z-plane or maximum-intensity z-projection of one or multiple x, y positions into TransTrack. Go to 'File' → 'Open'. Indicate how many fluorescence channels are present in the data and select the folder in which the files are saved. All image files in the folder will now be loaded into TransTrack (Fig. 5).

**▲ CRITICAL STEP** TransTrack recognizes only .tif file extensions. If microscopy images are acquired in another format (e.g., .nd2, as is the case for Nikon Elements software), files must first be converted to .tif format. This conversion can generally be performed using the image acquisition software or ImageJ. While saving or exporting files to the .tif format, take the following considerations into account: (i) each .tif file should contain a single x, y position; (ii) the .tif file should contain at most six channels and (iii) the image can consist of only a single z-plane (in the case of a 3D stack of images, it is necessary to make 2D maximum-intensity projections first).





**Fig. 5 | Screenshot of TransTrack software.** Screenshot of the GUI of TransTrack. Options in the software are numbered in accordance with the corresponding steps in the Procedure. Different colors of boxes indicate different steps in the procedure.

- 26 Perform a correction for photobleaching. Select 'Analysis' → 'Photobleach correction' → 'Correction'.  
**▲ CRITICAL STEP** The mean intensity of the entire image is determined for each channel at each time point, and an exponential function is fit to determine the rate of bleaching. Next, a correction is applied to the fluorescence intensity values of all channels in which fluorescence intensities will be measured. The bleach correction will not work optimally when a large part of the image contains no signal. In this case, a manual bleach correction can be performed after obtaining intensity values from TransTrack.
- 27 Open the 'single particle localization and tracking' menu by selecting 'Analysis' → 'Single Channel Tracking'.
- 28 Select a channel for single-particle localization and tracking (Fig. 5). Tracking of translating mRNAs can be performed on the basis of the mRNA signal (mCherry channel) or the translation signal (GFP or Janelia Fluor 646 HaloTag channel). Spot intensities can also be determined when the data consist of a single time point, in which case tracks will consist of only a single value.  
**▲ CRITICAL STEP** In our experience, single-particle tracking is more accurate when using translation signal for tracking, because the signal-to-noise ratio in the translation channel is usually superior compared to that of the mRNA channel. However, the translation signal cannot be used for tracking when mRNAs are in an untranslated state part of the time. In this case, use the mRNA signal for single-particle tracking.
- 29 When multiple  $x, y$  positions are loaded into TransTrack, indicate the number(s) of the  $x, y$  position(s) that will be analyzed.
- 30 Visualize tracks that are detected by the algorithm and tracks that are manually selected in the Visualization menu by checking the boxes 'Peaks', 'Selected Tracks' and 'Track number'. Note that

## Box 2 | Parameters to be optimized during single-particle tracking when using TransTrack

'Noise level' and 'Threshold'. These parameters set the brightness threshold of particles that are detected. If some mRNAs are not detected when clicking 'Track', lower the value for these parameters. If too many spots are detected (including spots that do not represent translating mRNAs), increase the value of these parameters.

'Object size'. This is the size of detected particles in pixels. The default size of 8 pixels is based on a pixel size of 135 nm. Adjust this value according to the pixel size of your microscope so that the object size is  $\sim 1 \mu\text{m}$ .

'Noise size'. This can generally be left at 1, but the user can experiment with different noise size values to see if a better distinction between translating mRNAs and background fluorescence foci is achieved.

'Tracking distance'. The maximal distance (in pixels) that a particle can move between two consecutive frames while still being linked into a single track. The value for the tracking distance depends on the pixel size, the diffusion kinetics of an mRNA molecule and the time interval between frames. If translation sites diffuse rapidly (e.g., when they are not tethered) or when using a long time interval between images, a higher value is required here.

'Frame skipping'. A track will be ended when the particle is not detected for a selected number of time points. When set to 1, particles are connected only when they are detected in all consecutive frames. We generally use a frame skipping value of 1, because faulty tracks can be generated when using frame skipping values  $>1$ . The frame skipping value that can be used depends mainly on the particle density and brightness. At lower particle density and brightness, frame skipping values  $>1$  may result in improved tracking.

visualization of all tracks detected by the algorithm ('Peaks' box) can require a lot of computational power and may make the software performance slower. If this happens, uncheck the 'Peaks' box.

- 31 Localize and track single particles. Track particles by clicking the 'Track' button. This function detects particles at each time point, and links them together in tracks. During single-particle tracking, some parameters must be optimized; this is explained in Box 2.

**▲ CRITICAL STEP** More information about the algorithms used in TransTrack can be found on GitHub (<https://github.com/TanenbaumLab/TransTrack>).

- 32 After automated tracking has generated all the tracks, the tracks can be manually curated in a number of ways; follow the instructions in option A for adding tracks, option B for deleting tracks and option C for correcting tracks.

### (A) Adding tracks

- (i) Select tracks by clicking on the 'Add Track' button, clicking on the mRNAs that should be included, and pressing 'enter'.

### (B) Deleting tracks

- (i) If incorrect tracks have been manually selected, remove them by clicking on 'Delete' and then clicking on the track that is to be removed.

### (C) Correcting tracks

**▲ CRITICAL** Verify that each selected track contains the correct mRNA at every time point. If a mistake is observed, the following corrective actions can be taken:

- (i) *Replacing an incorrectly tracked frame.* Go to the frame in which the mRNA is incorrectly tracked, enter the track number at '#' and click 'Replace'. Now click in the image on the correct location of the mRNA that is being tracked.
  - (ii) *Extending the track.* Enter the track number at '#' and click 'B' to extend backward or 'F' to extend forward. The image will now go to the first frame in which the particle was not tracked anymore. Clicking on the particle will add it to the track and move the image to the next frame. When finished extending, press 'enter'.
  - (iii) *Clipping the track.* This option should be used if the track includes frames at either the start or end that should be excluded. Indicate the track number at '#' (below 'Clip Track') and enter the lower bound ('lb') and upper bound ('ub') of the frames that should be included in the track.
  - (iv) *Merging tracks.* Sometimes, a single mRNA is split into two tracks, for example, when the particle moves more than the maximally allowed tracking distance. In this case, two tracks can be merged. Indicate the numbers of the tracks at 'Merging Tracks - #' and indicate the last frame of the first track and the first frame of the second track in 'Limit'. If there is a gap between the merged tracks, the missing frames need to be added manually.
- 33 Save the tracks from TransTrack by clicking on 'File'  $\rightarrow$  'Save'. In the 'Save' menu, two settings should be selected: (i) the 'Box size', which is the pixel size of the region of interest (ROI) that is used for measuring fluorescence intensities. This ROI is centered around the location of the tracked particle. We typically set a box size of  $\sim 1 \mu\text{m}$ . Because images on our microscope have a

pixel size of 135 nm, we used a Box size of 8 pixels. Ensure that all the fluorescence signal that needs to be measured is within the selected box size. (ii) In the 'Save' menu, select the 'Intensities' checkbox. Tracks are saved and intensities are quantified and saved by TransTrack when the 'Save' button is clicked.

**▲ CRITICAL STEP** The results will be stored in an Excel file that contains all track information. For each fluorescence channel, a separate sheet is generated in Excel that contains the intensities of all tracks. These intensities are background-corrected and can be used for downstream analyses.

### Data analysis: measuring translation elongation rates ● Timing 2 h

**▲ CRITICAL** To measure translation elongation rates, a small molecule inhibitor of translation, harringtonine, can be used. Harringtonine stalls ribosomes on the start codon but does not affect translating ribosomes downstream of the start codon. Thus, addition of harringtonine to cells results in a progressive loss of fluorescence signal associated with translating mRNAs, as ribosomes on the mRNA complete translation one by one (referred to as ribosome run-off). The speed at which the fluorescence translation signal is lost reports on the translation elongation speed. Below, we will describe how to perform ribosome run-off experiments and the analysis steps required to calculate the translation elongation speed from these experiments.

34 *Image acquisition (Steps 34 and 35).* Prepare the cells and acquire images as described in Steps 15–24. During cell selection (Steps 20 and 21 and Box 1), select cells that have 10–40 translation sites. Use imaging conditions as described in Step 22A.

35 Five minutes after the start of imaging, add harringtonine (3 µg/ml final concentration) as described in Step 24 and Table 2.

36 *Image analysis.* Use TransTrack to measure the GFP intensity of nascent SunTag peptides associated with translating mRNAs over time. Track translating mRNAs on the basis of the GFP channel. Make sure that the tracks continue until the GFP signal has entirely disappeared; if the track stops before the GFP signal has disappeared completely, add additional time points (Step 32A(i)).

**▲ CRITICAL STEP** Select mRNAs that were already being translated at the start of the image acquisition. mRNAs that initiated translation shortly (1–2 min) before harringtonine addition have ribosomes only on the 5' part of the mRNA, which increases the complexity of the analysis as the translation signal first increases when ribosomes translate the SunTag sequence.

37 *Data analysis (Steps 37–40).* Normalize the intensity of each trace to the intensity of that trace in the frame before harringtonine addition.

38 For each translating mRNA, the total translation time is given by the time from harringtonine addition until complete disappearance of the GFP signal.

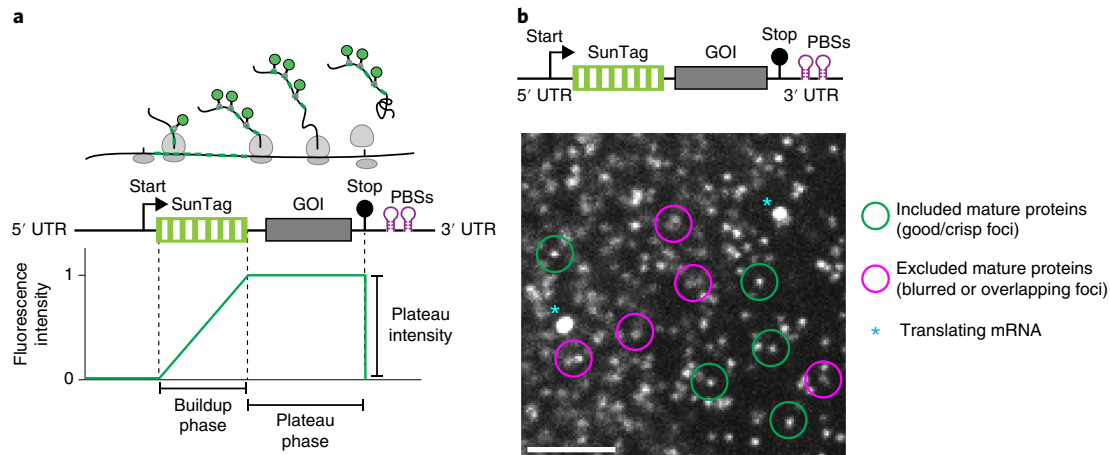
39 Determine the elongation rate by dividing the reporter length by the total translation time.

40 Calculate the average elongation rate of all mRNAs.

**▲ CRITICAL STEP** The translation elongation value gives the average translation elongation rate over the entire transcript. It is possible that certain sections of the mRNA will be translated faster than others.

### Data analysis: quantifying translation initiation kinetics using RiboFitter

**▲ CRITICAL** Generally, multiple ribosomes contribute to the total fluorescence intensity of a translating mRNA for our reporter mRNAs, and changes in fluorescence intensities are caused by a complex combination of translocating ribosomes in different positions along the mRNA. RiboFitter is a computational algorithm that deconstructs fluorescence intensity traces to determine the number of ribosomes translating an mRNA, as well as the moment at which each ribosome initiated translation. To run RiboFitter, one needs to know the intensity profile that is associated with a single ribosome that translates the mRNA. Because the peptide array is located at the 5' end of the coding sequence, the single-ribosome intensity profile consists of two phases: (i) a phase in which the peptide epitopes are translated ('buildup phase'; during this buildup phase, the fluorescence intensity increases over time as the ribosome translates the SunTag peptides one by one) and (ii) a phase in which the ribosome translates sequences downstream of the peptide array, during which the fluorescence intensity remains constant ('plateau phase') (Fig. 6a). Dedicated experiments are required to determine the intensity profile of a single ribosome before applying RiboFitter. Below, we discuss how to perform these dedicated experiments and how to run RiboFitter software.



**Fig. 6 | Fluorescence intensity profile of a single ribosome translating the SunTag reporter.** **a**, The intensity profile of a single ribosome translating a SunTag reporter consists of three phases: (i) a phase in which the ribosome is not associated with fluorescence. During this first phase, the sequence upstream of the peptide array and the first peptide epitope is translated. (ii) A buildup phase, in which the fluorescence intensity increases linearly over time. This phase starts and ends when the first and last peptide epitope have emerged from the ribosome exit tunnel, respectively. (iii) A plateau phase, in which all peptide epitopes have been synthesized and labeled with the SunTag-scFv. The plateau intensity is equal to the intensity of a mature SunTag protein labeled with SunTag-scFv. After completion of the third phase, the ribosome terminates translation and releases the nascent polypeptide, resulting in a rapid loss of fluorescence associated with the mRNA. **b**, A U2OS cell expressing SunTag-scFv, PCP-mCherry-CAAX and indicated translation reporter was imaged with mature protein imaging settings (Step 22B(i)). Green circles show examples of mature proteins that could be included in the analysis; these foci are crisp (not motion blurred) and not overlapping with other foci. Magenta circles show examples of mature proteins that should be excluded from analysis, as they are motion blurred or overlap with other foci. Blue asterisks indicate translating mRNAs. Scale bar, 1  $\mu\text{m}$ .

**▲ CRITICAL** To use RiboFitter, the following input is required: (i) bleach-corrected fluorescence intensity–time traces of translating mRNAs (which can be generated with TransTrack, Steps 25–33), (ii) the image acquisition time interval (in seconds) and (iii) the intensity profile of a single ribosome (Fig. 6a). The intensity profile of a single ribosome is calculated on the basis of the buildup time (in seconds), the plateau time (in seconds) and the plateau intensity (this is the intensity associated with a single translating ribosome that has completed synthesis of the entire peptide epitope array and is equal to the intensity of a single mature SunTag protein).

41 To calculate buildup and plateau times, follow option A. To determine the plateau intensity of a SunTag and MoonTag peptide array, follow options B and C, respectively. For running RiboFitter, follow option D.

**(A) Calculation of the buildup and plateau times ● Timing 10 min**

- (i) Determine the translation elongation rate in a set of dedicated experiments (Steps 34–40).
- (ii) Determine the number of nucleotides of the peptide array and the number of nucleotides downstream of the peptide array.

**▲ CRITICAL STEP** When analyzing stop codon read-through-based 3′-UTR translation, the length of the plateau phase depends on whether a ribosome translates the 3′ UTR or not. In this case, calculate the plateau length based on a situation without 3′-UTR translation and correct for 3′-UTR translation after running RiboFitter (Step 42B).

**▲ CRITICAL STEP** Because the plateau phase starts when the last peptide has fully exited the ribosome exit tunnel, the number of nucleotides in the plateau phase equals the number of nucleotides after the peptide array minus the length of the ribosomal exit tunnel (~30 amino acids = 90 nt).

- (iii) Calculate the buildup time (in seconds) by dividing the number of nucleotides of the peptide array by the elongation rate (in nucleotides per second).
- (iv) Calculate the plateau time (in seconds) by dividing the number nucleotides in the plateau phase by the elongation rate (in nucleotides per second).

**(B) Calculation of the plateau intensity of a SunTag peptide array ● Timing 30 min**

**▲ CRITICAL** The plateau intensity of a ribosome translating the SunTag peptide array is equal to the intensity of a single mature SunTag protein. The intensity of single mature proteins can be measured with distinct image acquisition settings (Step 22B) in cells expressing the translation reporter. The intensity of single mature proteins will be compared to the intensity

**Box 3 | An independent method to measure the fluorescence intensity of single mature proteins**

We recommend using an independent approach to validate the brightness of mature proteins when mature proteins are measured for the first time. This is done by expressing a plasmid encoding SunTag-CAAX instead of a translation imaging reporter. Mature SunTag-CAAX proteins made from this plasmid should have brightness equal to that of mature proteins expressed from a translation reporter, because they contain the same 24× SunTag peptide array. However, the mature proteins are tethered to the plasma membrane through prenylation of the CAAX domain and therefore diffuse more slowly, facilitating accurate measurements of their brightness. Measure the intensity of mature SunTag-CAAX proteins by performing Step 41B(i–vii). The mean intensity calculated in Step 41B(vii) should be comparable to the mean intensity calculated for mature proteins expressed from the translation reporter.

of translation sites. However, the two intensities are determined using different acquisition parameters, so a calibration step is required to convert the fluorescence intensities between the two image acquisition methods, which is described below:

- (i) *Image acquisition (Step 41B(i,ii))*. Prepare the cells and acquire images as described above (Steps 15–24). Select 10–25  $x, y$  positions that have 3–15 translation sites and show crisp mature protein foci (see Fig. 6b). Acquire images using mature protein imaging settings (Step 22B). Imaging a single time point suffices for this analysis.  
**▲ CRITICAL STEP** Perform imaging before, or shortly after, doxycycline addition (<15 min), because fewer mature proteins will have been produced at this time, which facilitates detection of individual mature proteins.  
**▲ CRITICAL STEP** To validate the fluorescence intensity of mature proteins, we recommend performing a parallel experiment in which mature proteins are tethered to the plasma membrane, which facilitates intensity measurements because of reduced diffusion of mature proteins (see Box 3).
- (ii) Acquire a second image at the same  $x, y$  positions using standard translation imaging settings (Step 22A). Acquire this second image as quickly as possible after acquisition of the previous image to ensure that the translation signal associated with the individual mRNAs does not substantially change between the images acquired in Step 41B(i) and Step 41B(ii).
- (iii) *Image analysis (Step 41B(iii–v))*. Use TransTrack to measure the intensity of mature proteins (Step 25–33). Because the data contain only a single time point, TransTrack will make ‘tracks’ of a single time point. Manually select 10–20 mature protein spots in each cell. Note the ‘Box size’ used when saving the data.  
**▲ CRITICAL STEP** Select foci that appear as crisp spots, because these are not motion blurred and are in focus. Mature proteins that are slightly out of focus appear blurred. We found that single mature proteins can most easily be identified in regions with few SunTag proteins present (e.g., the region of the cell under the nucleus or in the periphery of the cell).
- (iv) Use TransTrack to measure the intensity of the translation signal of translating mRNAs for images acquired using mature protein imaging settings (Steps 25–33). When saving the data (Step 33), use the same ‘Box size’ that was used in Step 41B(iii).
- (v) Use TransTrack to measure the intensity of the translation signal of translating mRNAs for images acquired using standard translation imaging settings (Steps 25–33). When saving the data (Step 33), use the same ‘Box size’ that was used in Step 41B(iii).
- (vi) *Data analysis (Step 41B(vi–viii))*. Determine the relative intensity of translation signals with the two imaging conditions by dividing the mean intensity of translation sites measured with standard imaging conditions (Step 41B(v)) by the mean intensity of translation sites measured with mature protein imaging conditions (Step 41B(iv)).
- (vii) Calculate the mean intensity of all mature proteins.
- (viii) Multiply the mean intensity of a single mature protein by the calculated relative intensity of translation signals (Step 41B(vi)). This value is the intensity of a single mature SunTag protein when using standard imaging conditions, which represents the plateau intensity of a single ribosome translation profile (Fig. 6a).  
**▲ CRITICAL STEP** Although we recommend RiboFitter for analysis of the number of ribosomes per mRNA, the number of ribosomes that translate an mRNA at any given time point can also be estimated using the fluorescence intensity of a translating mRNA (Step 41B(iv)) and the mean intensity of a mature protein (Step 41B(vii)). We discuss this alternative method in detail in Box 4.



**Box 4 | An alternative method to calculate the number of ribosomes translating an mRNA at any given time point without using RiboFitter**

In this method, the number of ribosomes that translate an mRNA at any given time point is estimated using the fluorescence intensity of a translating mRNA (Step 41B(iv)) and the mean intensity of a mature protein (Step 41B(vii)). This method is somewhat faster than running RiboFitter, but it is less accurate and does not provide temporal information about translation initiation events. An assumption is made that the mRNA is homogeneously covered by ribosomes. To calculate the number of ribosomes translating the mRNA, a correction is applied to account for ribosomes that have not completed translation of the entire SunTag peptide array and thus are associated with a lower level of fluorescence:

- 1 Calculate the correction factor (CF) for the intensity profile of a translating ribosome using the following equation:

$$CF = \frac{(0.5 \times L(\text{buildup})) + L(\text{plateau})}{L(\text{total})}$$

In this equation, CF is the correction factor,  $L(\text{buildup})$  is the nucleotide length of the SunTag array (Step 41A(ii)),  $L(\text{plateau})$  is the number of nucleotides translated during the plateau phase (Step 41A(iii)), and  $L(\text{total})$  is the nucleotide length of the complete coding sequence.

- 2 Divide the fluorescence intensity of a translating mRNA (Step 41B(iv)) by the mean intensity of a mature protein (Step 41B(vii)).
- 3 Divide the value obtained in step 2 by the correction factor (CF) calculated in step 1 to determine the number of ribosomes translating the mRNA at a given time point.

**(C) Calculation of the plateau intensity of a MoonTag peptide array** ● **Timing 30 min**

▲ **CRITICAL** Single mature MoonTag proteins are not bright enough for reliable detection using spinning-disk confocal microscopy when imaging MoonTag or MashTag reporters. Therefore, the brightness of a single mature MoonTag protein needs to be determined indirectly. This is done by calculating the number of ribosomes on a SunTag translation reporter and comparing the SunTag reporter with a MoonTag translation reporter with an identical promoter, 5' UTR, and translation start site. An assumption is made that these matched SunTag and MoonTag reporters have similar translation initiation rates and ribosome densities. Therefore, one can deduce the average number of ribosomes on the MoonTag translation reporters from the ribosome density on the SunTag reporters. The ribosome density on the MoonTag reporters, combined with the fluorescence intensity of translating MoonTag mRNAs, can be used to determine the intensity of single mature MoonTag proteins. Below, we provide a detailed protocol for these analyses. The analysis is described for MoonTag reporters but is identical when using MashTag reporters.

- (i) Acquire images and calculate the average number of ribosomes on SunTag-containing reporter mRNAs (e.g., the standard SunTag translation imaging reporter; see Fig. 3a) as described in Step 41B (perform Step 41B(i–vii) and see Box 4).
- (ii) Calculate the average number of ribosomes on MoonTag-containing reporter mRNAs. To do this, first divide the length of the MoonTag reporter coding sequence by the length of the SunTag reporter coding sequence.
- (iii) Then multiply the value obtained in Step 41C(ii) by the average number of ribosomes on a SunTag-containing reporter mRNA (Step 41C(i)). This value is the average number of ribosomes on a MoonTag-containing reporter mRNA.
- (iv) *Image acquisition.* In a parallel experiment, prepare cells expressing a MoonTag- or MashTag-containing reporter (see Fig. 3b,d and Table 1) and acquire images as described in Steps 15–24. Select 10–25 cells that have 3–15 translating mRNAs and acquire images using standard imaging settings for MoonTag (Step 22A).
- (v) *Image analysis.* Use TransTrack to measure the intensity of the translation signal of the translating mRNA (Steps 25–33).
- (vi) *Data analysis (Step 41C(vi–ix)).* Calculate the mean fluorescence intensity of translating mRNAs measured in Step 41C(v).
- (vii) Calculate the correction factor for the MoonTag reporter (see Box 4).
- (viii) Divide the mean intensity of translating MoonTag mRNAs (Step 41C(vi)) by the correction factor (Step 41C(vii)).
- (ix) Divide the corrected mean intensity (Step 41C(viii)) by the average number of ribosomes on the reporter mRNA (Step 41C(iii)). This value is the intensity of a single mature MoonTag protein, which represents the plateau intensity of a single ribosome translation profile.



**(D) Running RiboFitter ● Timing 1–180 min**

▲ **CRITICAL** Once the buildup time, plateau time and plateau intensity of a single translating ribosome are known, RiboFitter can be applied to fluorescence intensity–time traces of single translating mRNAs. Two example traces, as well as the parameters required for running RiboFitter and the expected outcome are included in Supplementary Data 2.

- (i) Create a comma-delimited (.csv) file containing the bleach-corrected intensity traces. This can be done in Excel by selecting ‘Save as’, and then selecting ‘CSV (comma delimited)’ in the ‘Save as type’ menu. Each column should contain one intensity trace. The first column is used to indicate the time (in frames).

▲ **CRITICAL STEP** When using intensity traces of both SunTag and MoonTag, make two separate files; the first file will contain SunTag intensities and the second file will contain MoonTag intensities. Ensure that, for each mRNA molecule, the SunTag and MoonTag data are in the same column in their respective files.

- (ii) Open the RiboFitter script in R or RStudio. Set the correct buildup time (Step 41A(iii)), plateau time (Step 41A(iv)), plateau intensity (Step 41B or 41C), and acquisition interval for the SunTag in lines 5–9 of the script.

▲ **CRITICAL STEP** When analyzing dual-color translation imaging data, set the parameters for the MoonTag in lines 15–17 of the RiboFitter script.

- (iii) Select the entire script and press the ‘Ctrl + R’ buttons in the R interface. A dialog box will appear.
- (iv) Select the file containing the intensity traces of the SunTag signal. A pop-up window will appear with the question of whether a fit for a second channel should be included. When selecting ‘No’, RiboFitter ignores the second channel and starts fitting translation initiation events for the first channel only. If ‘Yes’ is ticked, a new dialog will appear and the file containing the intensity traces of the second channel can be selected.
- (v) RiboFitter will now initiate the iterative process of fitting intensity traces to identify the most probable number of ribosomes that have translated the mRNA, as well as the timing of each translation initiation event. If many highly translated mRNAs are analyzed, running the script can take several hours. Once the script has finished the calculations, the following message will appear in the console window of the R interface:

```
[1] "Finished"
```

- (vi) A dialog box will appear. Select a folder in which to save the output files.

▲ **CRITICAL STEP** As output files, RiboFitter provides graphs of intensity traces, along with their optimal fits, as well as Excel files with intensity values. See Box 5 for more details about these output files.

**Data analysis: interpretation of RiboFitter data ● Timing 10 min**

▲ **CRITICAL** To quantitatively compare the translation rates of (i) two different mRNA reporters (one containing the SunTag and the other containing the MoonTag), (ii) a single reporter containing both the SunTag and MoonTag (e.g., the 3′-UTR translation reporter) or (iii) a reporter containing the MashTag, we recommend using RiboFitter to determine the exact number of translation initiation events in each channel (i.e., SunTag or MoonTag) and performing the downstream analysis described below.

- 42 For analyzing experiments containing two different reporters, follow option A. For determining the fraction of ribosomes undergoing 3′-UTR translation, follow option B and for calculating the fraction of ribosomes translating in different frames on MashTag reporters, follow option C. An example RiboFitter output is shown in Fig. 7.

**(A) Analysis of experiments containing two different reporters**

- (i) When SunTag and MoonTag are expressed simultaneously in a cell, calculate the translation initiation rate of each reporter independently by dividing the total number of ribosomes that translated the mRNA (column 2 or 5 of RiboFitter output; see Box 5) by the duration of the mRNA trace. The rate of translation initiation of the two reporters can be compared directly.

**(B) Calculation of the fraction of ribosomes translating the 3′ UTR of a reporter**

- (i) Determine the number of ribosomes that translated the MoonTag sequence and the SunTag sequence for each translating mRNA (columns 2 and 5 of RiboFitter output, see Box 5).

# Box 5 | Output files generated by RiboFitter

RiboFitter generates two output files:

## Plots.pdf

Each page of this .pdf file contains a plot of an experimentally determined intensity trace and its optimal fit as determined by RiboFitter (Fig. 7). In the box below the intensity traces, the moment of each translation initiation event on the mRNA is indicated by colored triangles. The total number of translating ribosomes, as well as the goodness of fit (measured by the root-mean-square error (RMSE)), is displayed on the right-hand side of the box. The vertical dotted line in the graph with intensity traces indicates the start of image acquisition. Translation initiation events can also be positioned to the left of this line by RiboFitter if a translation signal was already present at the start of image acquisition. We recommend visually inspecting whether the fits match the data well.

## All\_results.csv

All\_results.csv is a text file containing the information listed below for the best fit obtained for the iterative fitting process. This file can be opened in Excel. Data for each translating mRNA are given in a single row and contain the following information:

Column 1: mRNA ID

Column 2: The total number of translating ribosomes in the entire time trace for the first fluorescence channel (SunTag)

Column 3: The time of initiation (seconds) for each ribosome in the first fluorescence channel (SunTag)

Column 4: The RMSE of the fit to the data for the first fluorescence channel (SunTag)

Column 5: The total number of translating ribosomes in the entire time trace for the second fluorescence channel (MoonTag)

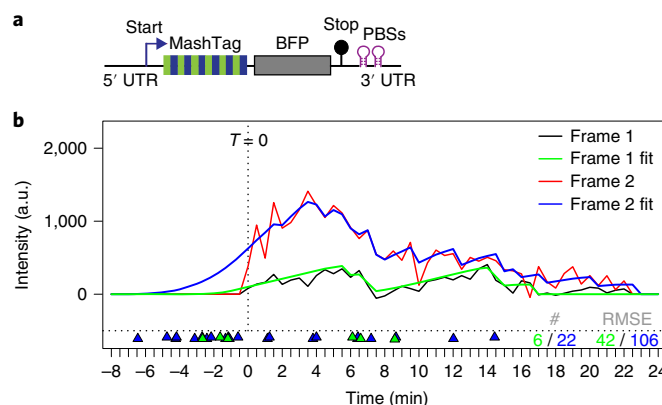
Column 6: The time of initiation (seconds) for each ribosome in the second fluorescence channel (MoonTag)

Column 7: The RMSE of the fit to the data for the second fluorescence channel (MoonTag)

Column 8: The time (in seconds) since the start of the fluorescence intensity-time trace

Column 9: The number of ribosomes translating the mRNA in the first translation frame at each time point

Column 10: The number of ribosomes translating the mRNA in the second translation frame at each time point



**Fig. 7 | Example output of RiboFitter.** **a**, Schematic of the reporter used to generate the fluorescence intensity-time traces shown in **b**. The reporter contains a single AUG translation initiation sequence, which is positioned in the MoonTag reading frame. **b**, Analysis by RiboFitter of fluorescence intensity-time traces of an example mRNA of the reporter indicated in **a**. Fluorescence intensity-time traces are shown for SunTag (black line) and MoonTag (red line) signals. Best fits obtained using RiboFitter are shown (green and blue lines for SunTag and MoonTag, respectively). Colored triangles below the traces indicate translation initiation events. Green and blue numbers and triangles represent SunTag and MoonTag, respectively. The total number of translation initiation events (#) and the root mean squared error (RMSE) (which indicates the goodness-of-fit) are indicated on the right.  $T = 0$ , time at start of the trace.

**▲ CRITICAL STEP** When the SunTag is placed in-frame with the stop codon and the predominant SunTag translation signal is caused by stop codon read-through, a correction is required for the number of MoonTag ribosomes; when a ribosome reads through the stop codon, the duration of the plateau phase of ribosomes that translated the MoonTag is increased as a longer nucleotide sequence downstream of the MoonTag array is translated. Correct for this using the following steps.

- (ii) Determine the number of ribosomes that translated the 3' UTR. This is equal to the number of ribosomes that translated the SunTag peptide array (Step 42B(i)).
- (iii) Calculate the plateau time of a ribosome that translates the 3' UTR (Step 41A).

- (iv) Calculate the integrated fluorescence intensity (FI) produced for a ribosome that translates only the annotated coding sequence, as well as the integrated fluorescence intensity produced for a ribosome that translates both the annotated coding and 3'-UTR sequences, using the following equation:

$$FI = (0.5 \times \text{buildup time}) + \text{plateau time}$$

Note that the buildup and plateau times (without 3'-UTR translation) have been calculated previously in order to run RiboFitter (Step 41A). The plateau time (when 3'-UTR translation occurs) should be calculated as described in Step 41A.

- (v) Calculate the additional integrated fluorescence intensity produced by each ribosome that translated the 3' UTR. This is given by the following equation:

$$FI(\text{additional}) = \frac{(FI(3' \text{ UTR}) - FI(\text{No } 3' \text{ UTR}))}{FI(\text{No } 3' \text{ UTR})}$$

In this equation, 'FI(additional)' is the extra fluorescence produced when the 3' UTR is translated, normalized to the number of ribosomes, and FI(3' UTR) and FI(No 3' UTR) are the integrated fluorescence intensities calculated in Step 42B(iv).

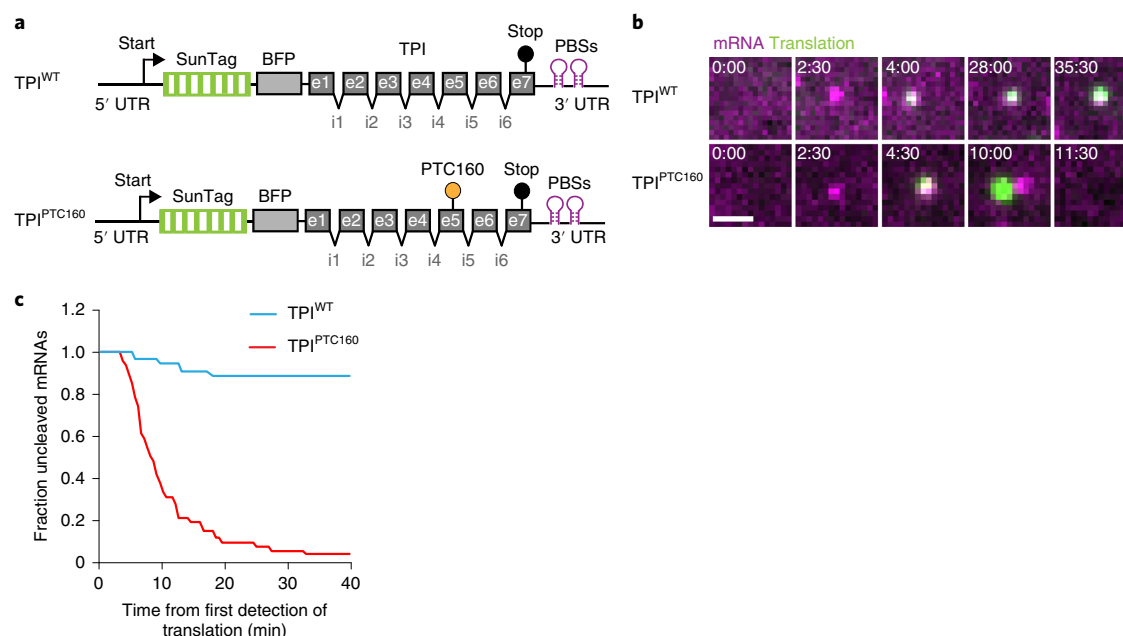
- (vi) Multiply FI(additional) by the number of ribosomes that translated the 3' UTR (Step 42B(ii)).
- (vii) Subtract the value determined in Step 42B(vi) from the total number of ribosomes determined by RiboFitter to obtain the corrected number of MoonTag ribosomes.
- (viii) Divide the number of SunTag ribosomes by the corrected number of MoonTag ribosomes to obtain the fraction of ribosomes that have translated the 3' UTR.
- (C) **Calculation of the fraction of ribosomes translating either the MoonTag or SunTag reading frame on MashTag mRNAs**
- (i) Determine the number of ribosomes that translated either the MoonTag sequence or the SunTag sequence for each translating mRNA (columns 2 and 5 of RiboFitter output, see Box 5).
- (ii) Divide the number of ribosomes that translated the SunTag sequence by the total number of ribosomes (SunTag + MoonTag) to obtain the fraction of ribosomes that translated the SunTag reading frame.

**▲ CRITICAL STEP** In the steps above, the fraction of ribosomes translating the SunTag and MoonTag frame is calculated. In these calculations, the ribosomes translating the third frame (referred to as the 'blank' frame), which are not visible, are not taken into account.

### Data analysis: analysis of translation-coupled mRNA degradation (NMD) ● Timing 1–3 h

**▲ CRITICAL** We recently developed an assay to visualize endonucleolytic cleavage of single mRNA molecules using translation imaging<sup>23</sup>. Because NMD frequently results in endonucleolytic cleavage, we can observe the moment of NMD induction using this assay. Below, we provide a detailed protocol for this NMD imaging assay.

- 43 **Image acquisition.** Select cells with 3–10 translation sites (Steps 15–24) and acquire images with standard imaging settings (Step 22A).
- 44 **Image analysis (Steps 44 and 45).** For each mRNA that appears in the field of view during the movie, collect the following information: (i) the moment that translation signal can first be observed on the mRNA and (ii) either the moment of mRNA cleavage (Fig. 8b) or, in the case that the mRNA molecule does not undergo cleavage during the movie, the last time point at which the mRNA could be detected/tracked.
- ▲ CRITICAL STEP** There are multiple possible reasons why cleavage may not be observed for a particular mRNA molecule. For example, an mRNA can move out of the field of view, an mRNA can no longer be reliably tracked because it crosses paths with another mRNA or the end of the movie is reached. In addition, because NMD is coupled to mRNA translation, it is critical that only mRNA molecules be included in the analysis for which all translating ribosomes are observed. Therefore, only mRNAs for which the first round of translation could be observed are analyzed. To ensure that the first round of translation is observed, include only newly transcribed mRNAs that first appear in the field of view in the absence of associated translation signal<sup>23</sup>.
- 45 Prevent bias in selection of the mRNAs that will be analyzed. If a subset of mRNAs in the cell are 'randomly' selected for analysis, the selection of mRNAs can inadvertently introduce a bias, for example, because mRNAs associated with a bright GFP signal are more easily detected than mRNAs associated with a dim GFP signal. To prevent an analysis bias, either analyze all mRNAs in



**Fig. 8 | Representative imaging data and analyses for experiments on NMD.** **a**, Schematic of indicated NMD reporter constructs containing the triosephosphate isomerase (*TPI*) gene. PTC160 indicates a PTC at amino acid 160. **b**, Representative images of U2OS cells expressing SunTag-scFv (green), PCP-mCherry-CAAX (magenta) and the NMD reporters indicated in **a** are shown. Scale bar, 1  $\mu$ m. Time is shown as minutes:seconds. **c**, The time from first detection of translation until separation of GFP and mCherry foci (i.e., mRNA endonucleolytic cleavage) was quantified. Note that little cleavage is observed during the first 3 min from the start of translation, which represents the time it takes for the first ribosome to reach the stop codon. Note that a fraction of mRNA molecules (~10%) appear to be insensitive to NMD-dependent mRNA cleavage. **d**, WT, wild type. **e**, exon:intron:WT, wild type.

a cell that match the criteria listed above, or, if the number of mRNAs in a cell is too large, create an ROI within a cell and analyze all mRNAs in the ROI.

**▲ CRITICAL STEP** In addition to mRNA cleavage, other parameters can be scored as well, including the relative intensity of the translation signal, the fraction of mRNAs that disappear during the movie (which could indicate exonucleolytic cleavage), and the time from mRNA cleavage to the disappearance of the 3' cleavage fragments (the mCherry foci after cleavage), which represents exonucleolytic decay of the 3' cleavage fragment by XRN1<sup>23</sup>.

46 **Data analysis.** For each mRNA molecule, calculate the time from first detection of GFP until cleavage or until the last time point that the mRNA could be detected/tracked (if the mRNA is not cleaved during the experiment).

**▲ CRITICAL STEP** We typically represent the results as a Kaplan–Meier plot. This plot type takes into account the track length of both cleaved and uncleaved mRNAs and shows the fraction of uncleaved mRNAs over time (relative to the start of translation). We have included a template for making a Kaplan–Meier plot in Excel in Supplementary Data 3.

## Troubleshooting

Troubleshooting advice can be found in Table 3.

### Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
22A(i)	No mRNA foci are detected	Expression level of PCP-mCherry(-CAAX) is too high	Sort cells with lower PCP-mCherry expression levels
22B(i), Box 1a	No mature protein detected	Cells do not express the reporter	Ensure transfection of the reporter, for example, through co-transfection with a plasmid expressing any fluorescent marker (such as BFP)
	Mature proteins have low signal-to-noise ratio	Expression level of SunTag-scFv is too high; mature protein not visible above background	Sort cells with lower SunTag-scFv expression levels

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
24	Mature proteins blurred	Expression level of SunTag-scFv is too low; there are not enough antibodies to bind to mature protein	Sort cells with higher SunTag-scFv expression levels
		Too much mature protein present in the cell	Induce expression of reporter for shorter time
		Signal below detection limit	Use higher laser power or more sensitive camera, if available
		Exposure time is too long	Reduce exposure time or image in region underneath the nucleus, where diffusion is sometimes slower
Box 1b	Translation signal disappears during image acquisition	If background signal also disappears, it is a result of a high amount of photobleaching	Reduce laser power
		If background signal remains constant, it is a result of cytoplasmic antibody depletion	Select cells with fewer translating mRNAs or sort cells with higher antibody expression
		Cells do not express the reporter	Ensure transfection of the reporter, for example, through co-transfection with a plasmid expressing any fluorescent marker (such as BFP)
		Induction of transcription of the reporter is slow	Image cells later, after doxycycline addition
Box 1c	No translating mRNAs detected	Expression of SunTag-scFv is too high	Sort cells with lower SunTag-scFv expression levels
		Expression of mature protein is too high as a result of cytoplasmic antibody depletion	Induce transcription for a shorter time
		Protein aggregates can sometimes also appear as fluorescence foci	Add puromycin (see Table 2); the translation signal should disappear within 1 min
		It is unclear whether foci in translation channel represent translating mRNAs	Determine whether the translation signal co-localizes with an mRNA
Box 1c	It is unclear whether mCherry foci are mRNAs or lysosomes	Lysosomes can also appear as foci in the mRNA channel	Use LysoTracker dye to ensure that foci do not co-localize with LysoTracker

## Timing

Step 1, design of reporter mRNA: 2–5 d  
 Steps 2–13, generation of cells lines suitable for translation imaging: ~10–25 d  
 Step 14A, transient transfection for expression of reporter mRNA: 2 d  
 Step 14B, stable integration of reporter plasmid: ~12–20 d  
 Steps 15–19, sample preparation for imaging: 1–2 d  
 Steps 20–24, image acquisition: 1–2 h  
 Steps 25–33, image analysis: 1–6 h  
 Steps 34–40, data analysis—measuring translation elongation rates: 2 h  
 Step 41A, calculation of the buildup and plateau times: 10 min  
 Step 41B, calculation of the plateau intensity of a SunTag peptide array: 30 min  
 Step 41C, calculation of the plateau intensity of a MoonTag peptide array: 30 min  
 Step 41D, running RiboFitter: 1–180 min  
 Step 42, interpretation of RiboFitter data: 10 min  
 Steps 43–46, analysis of translation-coupled mRNA degradation (NMD): 1–3 h

## Anticipated results

### Fluorescence images

In this protocol, we describe several applications of the SunTag- and MoonTag-based translation imaging systems. Using this protocol, translation rates of different mRNA reporters, or of different regions of a single mRNA, can be quantitatively compared. In Fig. 4, typical images are shown of cells expressing reporters containing the SunTag and/or MoonTag peptide array. Note that some mRNA molecules are not associated with translation signal. Although a subset of these mRNAs may undergo

translation at a later time point, we usually find a subset of mRNAs that are never associated with translation signal. The reason for this is unclear, but it is possible that differences in transcription start site selection or cryptic splicing events have altered the coding sequence of these mRNAs such that the peptide epitope array is no longer present or cannot be translated. These mRNAs are usually excluded from analysis. Signal-to-noise ratios for mRNA and translation foci are often reduced if cells have incorrect expression levels of the SunTag/MoonTag antibody or PCP-mCherry(-CAAX), or if reporter mRNAs are expressed at very high levels, which results in cytoplasmic antibody depletion or aggregation of mature proteins (see Troubleshooting).

### Data analysis

As discussed in this protocol, image analysis and data quantification are usually required for interpretation of fluorescence images. In many cases, image analysis consists of measurements of fluorescence intensities of translating mRNAs using TransTrack (Fig. 5), followed by analysis of ribosome number and translation initiation timing using RiboFitter (Fig. 7). We have provided a tutorial, a dataset for image analysis using TransTrack, and the expected outcome in Supplementary Data 1. To facilitate the use of RiboFitter, two example traces, fitting parameters, and the expected RiboFitter output are included in Supplementary Data 2. Typical analyses include comparison of the translation initiation rate of two reporters or of one reporter under two different conditions, the frequency of 3'-UTR translation compared to main ORF translation of the same mRNA, and the frequency of translation initiation in different translation reading frames. When assessing NMD, comparisons are typically made between the rates of NMD and the fractions of mRNA molecules sensitive to NMD for two different reporters (Fig. 8). More in-depth analyses can also be performed and have been described previously<sup>21,23</sup>.

Because all the analyses described in this protocol quantify parameters of single mRNA molecules, one can also assess the heterogeneity between cells and between mRNAs within a cell. For example, we found that the frequency of out-of-frame translation can vary among different mRNA molecules derived from the same (reporter) gene<sup>21</sup>.

### Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability

Example datasets for analysis are provided in Supplementary Data 1 and 2.

### Code availability

The analysis software (RiboFitter and TransTrack) is included in Supplementary Data 1 and 2, and is also freely available through GitHub (<http://github.com/TanenbaumLab/RiboFitter> and <http://github.com/TanenbaumLab/TransTrack>, respectively). The code in this manuscript has been peer-reviewed.

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## Author contributions

D.K., T.A.H. and S.S. made the figures in the paper. D.K., T.A.H. and M.E.T. wrote the manuscript with input from S.S., B.M.P.V. and S.B.

## Competing interests

The authors declare no competing interests.

## Additional information

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