Long-term imaging of individual mRNA molecules in living cells

Graphical abstract

Highlights

- SunRISER is an approach for imaging single cytoplasmic mRNAs in living cells
- SunRISER-labeled mRNAs show high signal intensity and are resistant to photobleaching
- SunRISER is amenable to short stem-loop arrays without compromising mRNA detection
- Mitotic mRNA partitioning during stress increases diversity between sister cells

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In brief
In this issue, Guo et al. use computational and experimental approaches to optimize an mRNA-labeling system that enables long-term imaging of single mRNA molecules in living cells. By tracking mRNA inheritance in cells undergoing mitosis, they show that environmental stresses promote diversity between mother-daughter and sister-cell pairs.
Long-term imaging of individual mRNA molecules in living cells

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SUMMARY

Single-cell imaging of individual mRNAs has revealed core mechanisms of the central dogma. However, most approaches require cell fixation or have limited sensitivity for live-cell applications. Here, we describe SunRISER (SunTag-based reporter for imaging signal-enriched mRNA), a computationally and experimentally optimized approach for unambiguous detection of single mRNA molecules in living cells. When viewed by epifluorescence microscopy, SunRISER-labeled mRNAs show strong signal to background and resistance to photobleaching, which together enable long-term mRNA imaging studies. SunRISER variants, using 8- and 10-stem-loop arrays, demonstrate effective mRNA detection while significantly reducing alterations to target mRNA sequences. We characterize SunRISER to observe mRNA inheritance during mitosis and find that stressors enhance diversity among post-mitotic sister cells. Taken together, SunRISER enables a glimpse into living cells to observe aspects of the central dogma and the role of mRNAs in rare and dynamical trafficking events.

INTRODUCTION

Messenger RNA (mRNA) molecules interact with RNA-binding proteins throughout their lifespan to carry genetic information and provide precise spatiotemporal regulation within cells. Live-cell single-molecule imaging techniques have enabled in-depth characterization of dynamics for mRNA-processing steps, including transcription, translation, splicing, export, degradation, and interactions with ribonucleoprotein (RNP) granules (Bertrand et al., 1998; Buxbaum et al., 2014; Czapinski, 2017; Horvathova et al., 2017; Katz et al., 2016; Khuperkar et al., 2020; Larson et al., 2019; Mo et al., 2010; Sato et al., 2020; Tu et al., 2018; Vargas et al., 2011; Vitor et al., 2019; Wan et al., 2021; Wang et al., 2016; Wilbertz et al., 2019; Wu et al., 2016; Yan et al., 2016). However, continuous imaging of single mRNAs has numerous challenges coupled to low imaging sensitivity that is exacerbated by rapid photobleaching (Pichon et al., 2018; Tutucci et al., 2018a). Typically, live-cell detection of single mRNAs requires sophisticated imaging approaches and trade-offs that restrict spatial and temporal aspects of imaging experiments.

Bacteriophage-derived MS2 and PP7 stem loops are extensively used for labeling mRNA molecules. In many applications, the reporter mRNA is tagged with 24-stem-loop copies in the 3′ UTR, and the corresponding coat protein (MCP or PCP) is fused with a fluorescent protein (FP). When co-expressed in the same cell, dimers of FP-fused coat proteins bind to each stem loop, enabling visualization of mRNAs and active transcription sites by fluorescence microscopy (Braselmann et al., 2020; Fusco et al., 2003; Rath and Rentmeister, 2015; Sato et al., 2020; Tutucci et al., 2018a; Vera et al., 2016). For most applications, coat proteins are also fused to a nuclear localization signal (NLS) to deplete unbound FP-MCP and -PCP from the cytoplasm, thereby increasing image contrast for mRNAs labeled in the cytoplasm (Ben-Ari et al., 2010; Ferguson and Larson, 2013; Lenstra and Larson, 2016; Spille and Kubitscheck, 2015;...
Tutucci et al., 2018c; Wu et al., 2012). Since the NLS on unbound FP-M/PCP will favor a nuclear localization, the reduced availability of free coat proteins will limit cycling of FPs on cytoplasmic mRNAs. Consequently, depletion of fluorescence signals on cytoplasmic mRNA molecules resulting from dissociated and photobleached FP-M/PCP molecules is difficult to recover, which can constrain cytoplasmic mRNA detection over longer timescales. Although variants with increased stem-loop numbers enhance imaging sensitivity (up to 128 copies of extended MS2 repeats [Forero-Quintero et al., 2021; Golding and Cox, 2004; Tantale et al., 2016]), the bulky mRNA extension has the potential to perturb mRNA dynamics, and the reporter still suffers from photobleaching and limited resolution for single mRNAs.

Here, we develop and characterize SunRISER (SunTag-based reporter for imaging signal-enriched mRNA), an approach for long-term imaging of mRNA in living cells. SunRISER implements SunTag (Tanenbaum et al., 2014) as a scaffold to achieve fluorescence signal amplification of coat proteins and enhance contrast of mRNAs. Although the naive design is impractical, with inconsistent fluorescent properties that complicate mRNA detection, we optimize the approach using computational and synthetic biology to achieve robust and unambiguous detection of individual mRNAs. We show that SunRISER-labeled mRNAs are resistant to photobleaching over at least 7,000 exposures, and it is generalizable for robust whole-cell mRNA imaging experiments using PP7, MS2 (Chao et al., 2008), MS2V6, and MoonTag (Boersma et al., 2019; Tutucci et al., 2018b) as orthogonal components. We demonstrate that SunRISER variants using shorter 8× and 10× stem-loop arrays (SunRISER SRv.1.1 and SRv.1.2, respectively) result in consistent mRNA labeling and detection while reducing the size of alterations to target mRNA sequences. As an application of SunRISER, we characterize mitotic inheritance of mRNA molecules during a variety of stresses. When observed over the period of cell doubling time, we observe that mitotic mRNA inheritance is equally partitioned in standard growth conditions and that inflammatory stress or nutrient limitation can enhance diversity among postmitotic sister cells. Our results demonstrate that SunRISER is an effective single-cell approach to investigate dynamics of mRNA molecules over long periods of time.

**RESULTS**

**Design and model-based optimization of SunRISER**

To facilitate long-term imaging of mRNAs in live cells, we set out to develop a signal-enrichment approach that circumvents several limitations associated with contemporary mRNA imaging techniques. To amplify fluorescence intensity of labeled coat proteins, our design employs SunTag, an array of GCN4 peptide epitopes that recruit multiple antibody molecules (Tanenbaum et al., 2014). The GCN4 antibody-peptide pair bind rapidly and serve as a robust scaffold for protein recruitment with a complex half-life in the order of minutes (Morfill et al., 2007; Weber-Bornhauser et al., 1998). Specifically, SunRISER comprises two stages of signal amplification: (1) 24× stem-loop copies are inserted in the 3′ UTR of mRNA and (2) the corresponding coat protein is fused with up to 24× SunTag peptides. With co-expression of an FP-fused single-chain antibody (scFv-GFP or codon-optimized scAB-GFP [Voigt et al., 2017]) that bind GCN4 epitopes, each SunTag coat protein can be labeled with up to 24 scFv-FPs, resulting in a theoretical upper bound of over 1,000 FP molecules per mRNA (24× stem loops, 2× coat proteins per stem loop, 24× SunTag arrays, 24 × 24 × 2 = 1,152 [Figure 1A]). In contrast with previous approaches, we reasoned that a two-stage approach where SunTag-fused coat proteins and scAB-GFP are both expressed throughout the cell would bolster active cycling of nascent coat proteins and antibody-FPs, providing resistance to photobleaching.

Our initial design for live-cell imaging of mRNAs was first aimed at circumventing problems associated with signal detection and cytoplasmic depletion of FP-fused coat proteins. We expressed components of the naïve design in HeLa cells using 24 copies for both PP7 stem loops and SunTag arrays under control of the cytomegalovirus (cmv) promoter and imaged cells by 3D epifluorescence microscopy. As a generic transcript for mRNA detection, detection plasmids expressed a cyan FP (CFP) open reading frame, followed by 24×PP7 inserted after the stop codon in the 3′ UTR. Detection plasmids were transfected together with two additional plasmids expressing 24×SunTag-PCP and scFv-GFP.

Although diffraction-limited spots were visible, we observed spot-to-spot variability in size and intensity (Figures 1B and S1A). It was anticipated that two-stage signal amplification has non-linear systems properties, e.g., limiting scFv-GFP or excessive SunTag-PCP concentrations will both influence signal intensity dependent on mRNA concentration. We therefore developed a mathematical model (STAR Methods; Table S2) to estimate the number of FP molecules bound to mRNA for different expression levels of SunRISER components, scAB-GFP, SunTag-PCP, and PP7-tagged mRNA. Using protein-binding kinetics for SunRISER components and sampling across parameter combinations, we found broad variability in the expected intensity and signal to background (Figures 1C and S1), in some cases leading to a quantized distribution of single mRNA intensities (e.g., parameter combination 10). Variability between spots will complicate accurate identification and measurement of single mRNA molecules. By inspection, we found that a 5:1 ratio between scAB-GFP and SunTag-PCP with high-abundance expression yields uniform signal-intensity distributions in cells expressing up to 1,000s of mRNAs (Figure 1C). We also simulated 5×, 10×, and 24× SunTag-PCP variants. Although longer variants produce more intense signals, all had comparable signal-to-background ratios (Figure S1).

**Refinement of SunRISER for whole-cell mRNA imaging**

Guided by simulations, we designed SunRISER variants and assayed quantitative properties of mRNA spots. To establish an approximately 5:1 ratio of abundance for SunRISER protein components, we compared different constitutive promoters in HeLa cells (Figure S2A) predicted to have different strengths (Qin et al., 2010). Comparing cmv and ubc, promoters for strong and weak (respectively) mammalian gene expression, we found that the abundance of GFP expressed from the cmv promoter is approximately 5 times that of ubc (Figure S2A). To reduce the size of labeled mRNA complexes without significantly
compromising signal to background (Figure S1D), we validated model predictions using 10xSunTag-PCP. HeLa cells were co-transfected with SunRISER components, approximating parameter combinations 1, 4, 6, and 9 (Figures 1 and S1). Consistent with simulations, an approximately 5:1 ratio achieved by cmv-scAB-GFP and ubc-SunTag-PCP (Figure S2C) enhanced signal intensity and signal-to-background values compared to sub-optimal ratios (Figures S2B, S2D, and S2E) that fail to reliably and unambiguously label single transcripts driven by the same cmv promoter. Using single-molecule fluorescence in situ hybridization (smFISH) against PP7 stem-loop sequences (Heinrich et al., 2017) in SunRISER-expressing cells, we confirmed co-localization of cytoplasmic mRNAs between SunRISER and smFISH. However, smFISH revealed nuclear mRNAs that were not detected by SunRISER (Figure S3A). Further live-cell and fixed-cell assays demonstrated that GFP-SunTag-PCP is excluded from the nuclear compartment (Figures S3B and S3C), suggesting that SunTag-PCP required further optimization for whole-cell mRNA detection.

To alleviate nuclear-export effects from repeats of the GCN4 epitope and cytoplasmic sequestration of anti-GCN4 (Wörn et al., 2000), we continued with the smaller 5xSunTag variant. Next, we focused on modifications to 5xSunTag-PCP for homogeneous expression throughout the cell and for detection of nuclear and cytoplasmic mRNAs. We also considered that the ornithine decarboxylase (ODC) tag (Kahan et al., 2005) fused to SunTag-PCP while under the control of the cmv promoter as an alternative approach to establish a 5:1 deficit of SunTag-PCP that may also alter its subcellular distribution. ODC is one of the most short-lived proteins, which can facilitate fast degradation of tagged proteins. Although ODC tagging is not as precise as controlling transcript abundance using promoters, ODC
fusion of SunTag-PCP is a viable approach to limit its expression relative to scAB-GFP. Although several variants enable whole-cell mRNA detection, an optimized design was eventually achieved by switching the fusion order of PCP and 5’ SunTag, in addition to inserting a 5’ NLS (Figures S3D and S3E; Table S1). We note that the addition of a 5’ NLS without switching the fusion order to PCP-SunTag still fails to detect nuclear mRNAs when expressed with other SunRISER components.

The optimized SunRISER version 1 (SRv.1) design consists of three plasmids (cmv-GFP-GB1-scAB, phage-nls-PCP-5’xSunTag and cmv-stGFP-GB1-scAB) and a detection plasmid expressing mRNA tagged with 24XPP7) and has an overall molecular weight comparable with MS2x128 (Table S3). SunRISER faithfully labels single mRNAs in the nucleus and cytoplasm with uniform fluorescence intensity and high signal to background, allowing for long-term imaging (Figure 2; Video S1). We also compared fluorescence distributions of single cytoplasmic mRNAs detected by smFISH in cells expressing either the detection plasmid only or the complete SunRISER system. We found that expression of SunRISER components does not significantly alter the fluorescence intensity and signal to background of mRNA spots (Figure 2D), suggesting that each SunRISER spot represents a single mRNA molecule. Similarly, detection plasmids using weak and strong promoters to express low and high mRNA numbers result in expected mRNA abundances, consistent fluorescence intensity, and signal to background, in line with expectations from the model (Figures S4A–S4C). For some cell lines, calibration of promoters for protein components may be necessary to ensure optimal SunRISER labeling. Nevertheless, even though promoter activity will vary by cell type, expression of SRv.1 components in A549 cells produced qualitatively similar results even though A549 cells are phenotypically distinct from HeLa by many criteria (Figure S4D). Furthermore, the same optimization can be applied to orthogonal stem loops and antibody-epitope pairs, such as MS2, MS2V6 (Tutucci et al., 2018b), and MoonTag (Boersma et al., 2019) (Figures 3 and S5).

Characterization of optimal SunRISER system
To compare SunRISER directly with widely used approaches, we imaged cells expressing the 24xpp7 detection plasmid with
PCP-GFP. Signal-to-background for standard PP7-labeling was near the detection limit throughout, and most cytoplasmic mRNAs fell below detectable signal levels within 10 min of imaging (Figure S6). By contrast, signal intensity and signal-to-background for SunRISER remained strong and consistent between single cells throughout the 10-min experiments (Figures S6C and S6D). To characterize photobleaching properties of SunRISER in more challenging imaging conditions, cells were exposed to a rapid time-lapse experiment where 7200 consecutive epifluorescence images were collected within 1 h (Figure 4). Although signal intensity values decreased to approximately 40% by the end of the experiment, the signal-to-background was reduced only modestly, and mRNAs were robustly detected in single cells throughout (Figures 4B and 4C). Taken together, we conclude that SunRISER is resistant to photobleaching and provides greater robustness for detection of mRNAs when compared with the well-established PP7-PCP systems.

Next, we compared functional properties of SunRISER-labeled mRNA with expectations from unlabeled mRNA, as well as previous reports from the literature. Comparing mCherry fluorescence expressed from a detection plasmid in single cells from a transcript with 243PP7 stem loops, SunRISER labeling did not have significant effects on protein production (Figure S7A). SunRISER-labeled mRNA maintained a mean ratio of 5 between nuclear and cytoplasmic compartments (Figure S7B), which is consistent with mean value expectations of 3.8–6.5 based on RNAseq of subcellular fractions (Bahar Halpern et al., 2015), suggesting the subcellular distribution of SunRISER-labeled mRNA is unaltered. Remarkably, SunRISER-labeled mRNA numbers in response to transcription

Figure 3. Design principles of SunRISER are generalizable to orthogonal stem loops and protein-tagging systems
(A) HeLa cells transfected with detection plasmids phage-cmv-CFP-24×MS2 (top) and phage-cmv-CFP-24×MS2V6 (bottom) stem-loop variants of SunRISER with ubc-nls-MCP-5×SunTag show similar characteristics and intensity distributions, quantified in histograms (right, top: n = 30 for cell numbers and n = 8,227 for spots numbers; bottom: n = 21 for cell numbers and n = 4,627 for spots numbers).
(B) HeLa cells transfected with detection plasmid phage-cmv-cfp-24×pp7 with cmv-sfGFP-GB1-Nb-gp41 and ubc-nls-PCP-12×MoonTag, quantified in histograms (right, n = 19 for cell numbers and n = 4,534 for spots numbers). We note that the MoonRISER example can be further optimized as it uses a longer 12×MoonTag and a nanobody that has different binding properties. Cells were imaged with 60× wide-field microscope 24 h after transfection and quantified with dNEMO (Kowalczyk et al., 2021) Scale bar: 10 μm.
See also Figure S5.
inhibition revealed mRNA half-lives with significant cell-to-cell variability (Figure S7C). Single cell mRNA half-lives were consistent with previous results, spanning the divide that separates median mRNA half-lives expected of stable and unstable mRNA molecules (Schwanhäusser et al., 2011; Tani et al., 2012). Although this may represent a source of cellular heterogeneity, cells with particularly long mRNA half-lives can also indicate partial escape from transcriptional inhibition through enhanced chemical efflux or other mechanisms that also vary between cells. Finally, extended extensions of widely used RNA tags have the potential to alter diffusion rates, so we also measured diffusive properties of SunTag-labeled mRNA. Since the SunRISER-labeled mRNA complex is larger than 24×PP7-PCP (Table S3), it is expected to result in lower single-mRNA diffusion rates within the cell. When measured from high-frequency time-lapse images, diffusive motion of SunRISER-labeled mRNA molecules was 0.19 μm²/s (Figures S7D–S7F), which is lower yet within the expected range of values (0.15–0.72 μm²/s) measured from 24×PP7-PCP-labeled endogenous mRNAs (Wu et al., 2012). Taken together, although the SunRISER mRNA-protein complex is bulkier, it does not significantly alter mRNA function beyond expectations from previous analysis of conventional fluorescent mRNA reporters (Braselmann et al., 2020).

SunRISER variants with small stem-loop arrays and reduced plasmid requirements

Most mRNA-tagging applications using bacteriophage-derived stem loops use a 24× copy array or larger for signal amplification. Shorter stem-loop arrays exist but are often used in CRISPR-based genome imaging, where multiple copies of target sites are present to detect bright foci, or are engineered to reduce impact on general mRNA metabolism (Katz et al., 2021; Ma et al., 2018; Saroufim et al., 2015). We therefore asked whether SunRISER can be used to label single mRNAs using shorter stem-loop arrays.

To establish versatility of our approach, we developed SunRISER configurations using shorter PP7 stem-loop arrays (8×, 10×, 12×) and varying lengths of PCP-SunTag arrays (5×, 10×, 12×, and 24×). For each array combination, we measured median and variance of signal to background for distributions of single mRNA molecules (Figure 5 and S8A). We found that although longer SunTag arrays tend to correspond with greater signal to background, they also result in greater inter-spot variance, which reduces mRNA-detection efficiency (Figures 5A and 5B). Of all combinations tested, we found two SunRISER variants capable of labeling mRNAs with low variance, comparable signal to background, and comparable mRNA detection numbers to SunRISER (Figures 5A, 5B, S8B, and S8C). We refer to these as SRv.1.1 and SRv.1.2 respectively for 8×PP7 with 10×SunTag and 10×PP7 with 12×SunTag. Although mRNA labeled with SRv.1.1 and SRv.1.2 have comparable molecular weights to MS2x128 (Table S3), these alternative designs provide flexibility in SunRISER applications to label shorter mRNAs without compromises to mRNA detection.

Finally, to further enhance versatility of the SunRISER approach, we considered whether the protein components of SunRISER can be expressed from a single plasmid with multiple promoters. We generated a plasmid with two components to independently regulate expression of GFP-scAB and PCP-5×ST, thereby in combination with the detection plasmid reducing the system to two plasmids (referred to as SRv.1-2P; Figure 5C). Single mRNAs detected using the SunRISER variant
SRv.1-2P again showed comparable imaging and detection properties to SRv.1 (Figures 5D, 5E, and S8D). As expected, SRv.1.1-2P and SRv.1.2-2P also produce results that are indistinguishable from their 3-plasmid counterparts.

Variability of mitotic mRNA inheritance between sister cells

Random partitioning of biomolecules between daughter cells during cell division is a contributing source to non-genetic heterogeneity (Huh and Paulsson, 2011a, 2011b). The symmetry of mRNA inheritance during mitosis is tightly regulated during embryonic development and tissue homeostasis as the distribution of specific mRNAs controls cell-signaling pathways and subsequent cell-fate decisions (Shlyakhtina et al., 2019; Skamagki et al., 2013; Stahl et al., 2019; Sunchu and Cabernard, 2020; Varela et al., 2016).

However previous studies on mRNA division have been performed mostly across populations and in fixed samples, therefore lacking the resolution to examine mRNA partitioning in single mother and daughter cells in real time. Since SunRISER is theoretically capable of imaging mRNAs over timescales of cell division, we set out to quantify mRNA partitioning between single mother-daughter- and sister-cell pairs in various growth conditions. 

Enabled by the long-term mRNA imaging capability of SunRISER, we imaged HeLa cells transfected with cmv-promoter-driven mRNAs labeled with SunRISER for 24 h and quantified mRNA abundance during cell division with and without cellular stress (Figures 6A and 6B; Video S2). SRv.1-labeled mRNAs that encode CFP were used to examine the mitotic partitioning mechanisms for a generic mRNA species that is not subject to particular mitotic regulation mechanisms. The relative difference in mRNA levels between sister cells was used as a metric for symmetry of mRNA partitioning. We observed significant variability between pairs of sister cells in the same culture condition (Figures 6C and S9). To classify differences between sister-cell pairs, we used information criteria and k-means clustering (Figures S9A and S9B) and found 4 clusters that we refer to as “symmetric,” “weak asymmetry,” “asymmetric,” and “strong asymmetry.” Cells cultured in the presence of tumor necrosis factor (TNF; 15 ng/mL) or low-serum conditions (5% FBS) showed a significant shifts favoring asymmetric with the emergence of rare sister-cell pairs with strong asymmetry (Figures 6C and S9B). We further compared mRNA ratios between the sum of daughter cells and their corresponding mother (Figure 6D) and observed evidence of significant mitotic transcription (defined as mRNA ratio significantly greater than 1 for the sum of daughter cells divided by the mother cell) induced by cells exposed to lithium chloride (LiCl) as well as low-serum conditions (Figures 6D, S9C, and S9D). Notably, although LiCl promoted escape from silencing of mitotic transcription, it only had modest effects on
asymmetric mRNA inheritance between post-mitotic sister cells (Figures 6 and S9D). For all environmental stresses, distributions of mRNAs allocated to daughter cells shifted significantly away from the binomial distribution observed for cells dividing in the growth medium condition (Figure S10A). Although R² values suggest that partitioning of mRNA between daughter cells is partly explained by distribution of total cellular mass (Figure S10B), our data suggest that other factors also contribute to the observed diversification between sister cells.

DISCUSSION

The lifespan of mRNA molecules in mammalian cells occurs over timescales of hours to days, during which mRNAs participate in highly dynamic processes that are tightly regulated in time and space. Bacteriophage-derived stem loops and FP-tagged coat proteins are current state-of-the-art approaches to detect single mRNA molecules in live cells; however, there are still significant limitations of these seminal reporter systems. Furthermore, to take full advantage of typical stem-loop- and coat-protein-labeling systems requires highly customized microscopy equipment. For example, two-photon fluorescence fluctuation spectroscopy can provide accurate measurement of single mRNAs up to several minutes, but these measurements cannot be sustained over longer durations (Tutucci et al., 2018a). By contrast, SunRISER enables long-term investigation of dynamical mechanisms of mRNAs over timescales of at least 24 h using standard epifluorescence microscopy.

To optimize SunRISER, simulations were used to explore non-linear interrelations between the components of the two-stage reporter system. As a static model, we assume that all binding events in the system have reached equilibrium. The steady-state assumption allows conversion of binding affinity to binding probabilities, which reduces the computational load while preserving key features of the system. Selected binding constants for our simulations were chosen specific to stem-loop, coat-protein, and antibody-epitope pairs used in this study (summarized in Table S2). With appropriate modifications to the kinetic parameters, the mathematical model (https://github.com/recleelab/SunRISER_SupplementalModel) is generalizable to any two-stage molecular amplification reporter and is extensible to higher-order systems.
Direct comparison between SunRISER and PP7-PCP systems showed significant improvements to signal intensity, stability, and signal-to-background ratios. Furthermore, SunRISER minimally perturbs normal mRNA function and is highly resistant to photobleaching. Although our stress-test and long-term experiments concluded after 7,200 consecutive images and 24 h, respectively, we expect that these represent lower limits and that mRNA signals will remain detectable over longer repeat-exposure conditions. We also note that several “sub-optimal variants” of the PCP-SunTag component of SunRISER that were tested in our synthetic biology approach showed an exclusively cytoplasmic localization (Table S1). Although these sub-optimal variants of the reporter do not identify nuclear mRNAs, they may still have value in certain experimental settings where selective labeling of only cytoplasmic mRNAs is preferred. Finally, SunRISER variants SRv.1, SRv.1.1, and SRv.1.2 offer flexibility to label shorter mRNAs by balancing the reporter’s mRNA:protein composition, reducing sequence perturbations on mRNAs without compromising signal to background and detection.

Asymmetric mitotic mRNA inheritance is an essential mechanism to control the maintenance and emergence of specialized cellular phenotypes during development (Skamagki et al., 2013; Varela et al., 2016). Previous studies on mRNA partitioning during mitosis typically required cell fixation and chemical synchronization, with mRNA levels assessed via bulk cell measurements that are incapable of providing dynamical single-cell information. Here, we used SunRISER to visualize the mRNA partitioning during mitosis in single cells without chemical perturbations associated with synchronization. We observed significant heterogeneity between sister cells in terms of mRNA partitioning during both TNF stimulation and in low-serum conditions. Remarkably, upon serum starvation, a distinct partitioning during both TNF stimulation and in low-serum conditions concluded after 7,200 consecutive images and 24 h, respectively, we expect that these represent lower limits and that mRNA signals will remain detectable over longer repeat-exposure conditions. We also note that several “sub-optimal variants” of the PCP-SunTag component of SunRISER that were tested in our synthetic biology approach showed an exclusively cytoplasmic localization (Table S1). Although these sub-optimal variants of the reporter do not identify nuclear mRNAs, they may still have value in certain experimental settings where selective labeling of only cytoplasmic mRNAs is preferred. Finally, SunRISER variants SRv.1, SRv.1.1, and SRv.1.2 offer flexibility to label shorter mRNAs by balancing the reporter’s mRNA:protein composition, reducing sequence perturbations on mRNAs without compromising signal to background and detection.

In summary, SunRISER enables unambiguous detection of mRNA molecules in living cells. By using an optimized two-phase design, our reporter system is robust to photobleaching over long-term experiments, and the approach is generalizable to other stem-loop and peptide arrays using the accompanying computational tool. We anticipate that this approach will facilitate studies of dynamical properties for single mRNAs and biological variability between single cells, cell types, and eventually in tissues, with applications across biological disciplines.

LIMITATIONS OF THE STUDY

While SunRISER presents a solution for robust labeling of single mRNAs in mammalian cells, there are limitations to be considered. Optimal mRNA labeling with SunRISER requires an expression ratio of protein components using promoters that may be diversely regulated in different cell types and may also vary in different environmental conditions. When applying SunRISER to a new cell line, a stable cell line, or to certain environmental conditions that impact mRNA detection, it may be necessary to test different promoters to ensure the optimal expression ratio is achieved. In our application of SunRISER to study mRNA partitioning during mitosis, a generic transcript was used to examine passive mechanisms of mitotic inheritance of mRNAs. Expectations for mRNA distributions between daughter cells may change significantly for mRNAs associated with specific functions such as mitosis, development, and cell-fate specification. Furthermore, SunRISER-labeled transcripts comprise a large complex that does not significantly alter normal function of mRNAs assessed here but may still affect other dynamical properties of mRNAs, for example, structural complexes or particular biological processes. When mRNA translation and stability are important aspects of a study, careful controls should be performed to verify there are no specific effects from SunRISER labeling on the particular mRNA species. Therefore, other mRNA-labeling methods such as 24 x PP7/PCP-GFP may be more appropriate for applications that do not require imaging of cytoplasmic mRNAs, long-term processes, or rapid assemblies such as transcriptional start sites. When SunRISER is used to study mRNA-mRNA interaction or stoichiometry relations, verification via orthogonal approaches will be necessary. Finally, we note that simulations predict SunRISER will perform poorly at extremely high mRNA-expression levels (Figure 1C). Caution on interpretation should be taken when mRNA numbers exceed 1,000s per cell or for applications where large numbers of mRNAs are bundled in close proximity.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2022.100226.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.G. and R.E.C.L.; methodology, Y.G. and R.E.C.L.; investigation, Y.G.; software, Y.G.; formal analysis, Y.G.; writing – original Draft, Y.G. and R.E.C.L.; writing – review & editing, Y.G. and R.E.C.L.; visualization, Y.G. and R.E.C.L.; funding acquisition, R.E.C.L.; supervision, R.E.C.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| monoclonal antibody to GCN4 | Absolute Antibody | C11L34 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Recombinant human TNF-α | Peprotech | Cat#: 300-01A |
| Lithium chloride solution | Sigma-Aldrich | Cat#: L-7026 |
| Fugene HD | Promega | Cat#: E2311 |
| **Critical commercial assays** | | |
| Stellaris® Design Ready probes mCherry with Quasar® 670 Dye | Biosearch™ technologies | VSMF-1031-5 |
| **Experimental models: Cell lines** | | |
| Human: HeLa cell | ATCC | RRID: CVCL_0030 |
| Human: A549 lung carcinoma cells | ATCC | RRID: CVCL_0023 |
| **Recombinant DNA** | | |
| cmv-sfgfp-gb1-scAB | This paper | Addgene #185794 |
| cmv-mCherry-8×PP7 | This paper | Addgene #185795 |
| cmv-mCherry-10×PP7 | This paper | Addgene #185796 |
| ubc-nls-pcp-5×SunTag (SRv.1) | This paper | Addgene #185797 |
| ubc-nls-pcp-10×SunTag (SRv.1.1) | This paper | Addgene #185798 |
| ubc-nls-pcp-12×SunTag (SRv.1.2) | This paper | Addgene #185799 |
| cmv-sfgfp-gb1-scAB-ubc-nls-pcp-5×SunTag (SRv.1-2P) | This paper | Addgene #185800 |
| cmv-sfgfp-gb1-scAB-ubc-nls-pcp-10×SunTag (SRv.1.1-2P) | This paper | Addgene #185801 |
| cmv-sfgfp-gb1-scAB-ubc-nls-pcp-12×SunTag (SRv.1.2-2P) | This paper | Addgene #185802 |
| phage-cmv-cfp-24×ms2 | (Wu et al., 2012) | Addgene #40651 |
| phage-cmv-cfp-24×pp7 | (Wu et al., 2012) | Addgene #40652 |
| UbC-NLS-HA-MCP-YFP | (Grunwald and Singer, 2010) | Addgene #31230 |
| phage-ubc-nls-ha-pcp-gfp | (Halstead et al., 2015) | Addgene #64539 |
| phage UbiC scAB-GFP | (Voigt et al., 2017) | Addgene #104998 |
| Nb-gp41-Halo | (Boersma et al., 2019) | Addgene #128603 |
| 12×MoonTag-12×SunTag-kif18b-24×PP7 | (Boersma et al., 2019) | Addgene #128606 |
| pcDNA4TO-5×GCN4_v4-kif18b-24×PP7 | (Yan et al., 2016) | Addgene #74927 |
| pcDNA4TO-mito-mCherry-10×GCN4_v4 | (Tanenbaum et al., 2014) | Addgene #60914 |
| pET259-pUC57-24×MS2V6 | (Tutucci et al., 2018a, 2018b, 2018c) | Addgene #104391 |
| pH-sgRNA-Sirius-8×PP7 | (Ma et al., 2018) | Addgene #121940 |
| CMV-10×PP7 oligo-library based | (Katz et al., 2021) | Addgene #158199 |
| pDZ645 pKAN 1x-mCherry-12xPP7 V4 | (Saroufim et al., 2015) | Addgene #73173 |
| **Software and algorithms** | | |
| ImageJ | (Schneider et al., 2012) | https://imagej.nih.gov/ij/ |
| dNEMO | (Kowalczyk et al., 2021) | https://github.com/recleelab |
| Python | Python Software Foundation | https://www.python.org |
| SunRISER modeling scripts | This paper | https://doi.org/10.5281/zenodo.6512459 |
HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% streptomycin/penicillin and 1% L-glutamine at 37°C with 5% humidified CO₂.

**METHOD DETAILS**

**Plasmid construction**

The 24×SunTag-PCP plasmids were constructed with 24×GCN4 repeats flanked by HindIII and BamHI sites and coat protein flanked by BambHI and EcoRI sites in a pcDNA3 vector. Ubc promoter was PCR amplified from plasmid ubc-nls-ha-pp-gfp (Addgene #64539; Halstead et al., 2015) and inserted between MluI and HindIII sites to make ubc-n by BamHI and EcoRI sites in a pcDNA3 vector. Ubc promoter was PCR amplified from phage-ubc-nls-ha-pcp-gfp (Addgene #60914; Tanenbaum et al., 2014) and inserted between EcoRI and XbaI for various modifications. The ubc-nls-PCP-5×SunTag plasmids were created by replacing gfp sequence in phage-ubc-nls-ha-pp-gfp with 5′-GCACAGCAG-3′ and inserting ubc-nls-PCP fragment between SpeI and HindIII sites in front of 12×GCN4 (40×GCN4_v4; Tutucci et al., 2018b) and inserting ubc-nls-MCP-5×SunTag were obtained by insertion of 5′-GCN4 to UbC-NLS-HA-MCP-YFP (Addgene #31230; Grünwald and Singer, 2010) after digestion of XbaI and BsrGI restriction enzymes.

**Live cell imaging and quantification of single mRNA spots with dNEMO**

HeLa cells were plated on 96-well glass bottom plates (Matriplate) at the density of 8×10³ per well. Transient transfection was performed with Fugene HD (Promega) 24 hrs later according to manufacturer’s protocol. A mixture of plasmids comprising SunTag system with equal amount (50 ng) for each was first made in Opti-MEM then Fugene HD was added and incubated for 15 mins at room temperature. The amount of DNA and Fugene HD can be optimized accordingly. 24 hrs after transfection cells were washed with Opti-MEM and dNEMO was added to the plates. After 15 mins, the plates were imaged with a Nikon TiE microscope equipped with a spinning disk confocal head and a Photometrics ERS camera and AxioVision software. Images were acquired using a 20× objective and a 320×320 pixel binning. The images were analyzed using Cellomics software to quantify the number of mRNA spots.
were imaged using a DeltaVision Elite microscope with a 60× objective (1.42 NA; Olympus) and temperature-matched oil in an environmentally controlled chamber (37°C, 5% CO₂). Z-stacks of 5 images with 1 μm interval were acquired for quantification with dNEMO. Cell segmentation was manually performed in dNEMO and the spot detection parameters are set as default. In comparison of SunRISER and MS2-MCP, stacks of 4 planes with a z-spacing of 0.5 μm were obtained for high frame rate (one 3D stack per 2s). For comparison of promoters, mean fluorescence intensity was measured using ImageJ for a fixed region in cytoplasmic area.

**Stress treatments**

Cells were plated on 96-well glass bottom plates (Matriplate) for fixed-cell and live cell imaging experiments. For perturbation of mitosis, cells were cultured in DMEM with 15 ng/mL TNF, 10 mM LiCl or 5% FBS.

**smFISH probes and image acquisition**

Five 3’ Cy5 fluorescently labeled DNA oligos (Heinrich et al., 2017) (ggcaattagggtccttagg, catatgcttctctcttcc, gagtcgacctgccaggag, atatcatcgtctggttcc, atatgctctgctgcctttc) as smFISH probes against PP7 stem-loops were synthesized by Genewiz. Stellaris® Design Ready probes mCherry with Quasar® 670 Dye (VSMF-1031-5) against mCherry CDS were synthesized by Biosearch™ technologies. HeLa cells were plated on 96-well glass bottom plates and transfected with SunRISE components for 24 hrs. Cells were then fixed with 3.7% formaldehyde, washed three times in 1xPBS for 5 mins each and permeabilized in 70% (v/v) EtOH overnight at 4°C. The hybridization was then performed overnight at 37°C with 100 nM probes in 2XSSC with 10% formamide and 10% dextran sulfate. Nuclei were labeled in the wash step after the hybridization. Cells were finally imaged in Glox buffer (Raj and Tyagi, 2010) using a 60× objective on a DeltaVision microscope. Z-stack images of both FITC channel (SunRISER) and Cy5 channel (smFISH) were collected.

**Fixed-cell immunofluorescence**

HeLa cells were plated on 96-well glass bottom plates and transfected with 10×SunTag-PCP or the combination of scAB-GFP and 10×SunTag-PCP for 24hrs. Cells were then fixed with 3.7% formaldehyde for 10 minutes, rinsed three times in 1×PBS for 5 mins each and incubated in 100% methanol for 10 min. Cells were washed three times in PBST (1XPBS 0.1% Tween 20) for 5 mins each and then a primary antibody α-GCN4 (Absolute Antibody C11L34) diluted 1:100 in 3% BSA PBST was applied and incubated overnight at 4°C. After several washes, cells were incubated with secondary antibody (3% BSA PBST with 1:1000 Alexa594-conjugated anti-mouse IgG antibody) for one hour at room temperature. Nuclei were stained in the wash step after secondary antibody. Cells were finally imaged using a 60× objective on a DeltaVision microscope.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis**

Levene test was performed to calculate p values using raw data from Figure 6B, demonstrating significant changes in the variance of the indicated distributions. Binomial test was used to determine if partitioning of mRNA into daughter cells follows binomial distribution. Two-tailed t tests were performed for calculation of p values for other figures. All analysis is performed with Scipy stats packages in Python.

In Figures 5A, 5B and S8, the same data set is analyzed with the following cell numbers for each condition: 8×PP7-5×ST: n = 8; 8×PP7-10×ST: n = 12; 8×PP7-12×ST: n = 14; 8×PP7-24×ST: n = 9; 10×PP7-5×ST: n = 11; 10×PP7-10×ST: n = 11; 10×PP7-12×ST: n = 10; 10×PP7-24×ST: n = 11; 12×PP7-5×ST: n = 11; 12×PP7-10×ST: n = 14; 12×PP7-12×ST: n = 15; 12×PP7-24×ST: n = 17. In Figures 6C, 6D, S9 and S10, the same dataset is analyzed with the following numbers for mother-daughter pairs: growth medium, LiCl, TNF, low serum are 31, 30, 40 and 32, respectively.

Other number of samples are all listed in figure legends or marked in figures.

**Half-life and diffusion constant measurement**

HeLa cells transfected with SunRISER for 24hrs was treated with 50μM DRB (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside, Sigma-Aldrich) and imaged for 10hrs with frame rate of 10 mins. The time course of mRNA counts was fitted to exponential function to extract mRNA half-life $t_{1/2} = \ln 2 \times r$. For mRNA tracking, fast movie (frame rate of 1.1s) of HeLa cells transfected with SunRISER was taken 24 hrs after transfection and mRNAs were identified with dNEMO and tracked with u-track (Jaqaman et al., 2008). The diffusion constant is calculated as:

$$D = \frac{\sigma^{2/3}}{6}$$

Where $\gamma$ and $\sigma$ are intercept and slope from linear fit to log-log representation of mean square displacement provided by u-track.
mRNA inheritance during mitosis analysis

HeLa cells were transfected with SunRISER for 24 hrs and then cultured in DMEM with TNF (15 ng/mL; Peprotech), LiCl (10 mM; Sigma-Aldrich) or 5% FBS and imaged for 24 hrs after media replacement with a frame rate of 10 mins. mRNA counts are extracted by dNEMO and mother-daughter pairs are manually assigned. The last frame where mother cell remains as a single cell with a semi-detached circular appearance was set as frame 0. The frames −2, −1, 0, of mother cell and 1, 2, 3, of daughter cells are discarded for mRNA quantification because mRNA counts are not accurate during this period due to morphological changes and temporary detachment of the dividing cell. mRNA counts from frames −5, −4, −3, of mother cell and 4, 5, 6 of daughter cells were averaged to represent mRNA abundance before and after division. mRNA relative difference between sister cells is defined as

\[
\text{mRNA relative differences} = \frac{\text{daughter}1 - \text{daughter}2}{\text{daughter}1 + \text{daughter}2}
\]

It is noted that daughter1 and daughter2 are randomly assigned so we use absolute value of the difference. mRNA ratio between the sum of post-mitotic sister cells and mother cell is defined as daughter1 + daughter2.

Molecular weight calculations

RNA molecular weight was calculated with Quest Calculate™ RNA Molecular Weight Calculator (AAT Bioquest Inc., 2022) and protein molecular weight was calculated with The Sequence Manipulation Suite (Stothard, 2000).

Model-based calculation of signal intensity and signal-to-background values

In the calculation, we assume that all dynamic processes, e.g. the expression of proteins, the transcription of mRNAs and the binding/unbinding of proteins, have reached equilibrium. The dissociation constant of scFv and GCN4, can be written as

\[
k_{D1} = \frac{[\text{scFv}] \cdot [\text{GCN4}]}{[\text{scFv} \cdot \text{GCN4}]} = \frac{(c1 - p1N1c2)(1 - p1)N1c2}{p1N1c2}
\]

where \(c1\) is the molar concentration scFv, \(c2\) is the molar concentration of PCP-SunTag, \(N1\) is the number of GCN4 peptides on the PCP-SunTag, \(p1\) is the probability of a GCN4 binding site occupied by the scFv. Thus, we obtain

\[
p1 = \frac{1}{2N1c2} \left( k_{D1} + N1c2 + c1 - \sqrt{(k_{D1} + N1c2 + c1)^2 - 4c1N1c2} \right)
\]

Similarly, the probability of a PP7 binding site occupied by a PCP is

\[
p2 = \frac{1}{2N2c2} \left( k_{D2} + N2c3 + c2 - \sqrt{(k_{D2} + N2c3 + c2)^2 - 4c2N2c3} \right)
\]

Where \(c3\) is the molar concentration of mRNA, \(N2\) is the number of PP7 stem-loops on the mRNA, \(k_{D2}\) is the dissociation constant between PCP and PP7.

The number of scFv binding to a single PCP-SunTag satisfies a binomial distribution

\[
B1(n) = \binom{N1}{n} p1^n (1 - p1)^{N1 - n}
\]

Similarly, the number of PCP-SunTag binding to a single mRNA molecule satisfies

\[
B2(m) = \binom{N2}{m} p2^m (1 - p2)^{N2 - m}
\]

The average number of scFv-GFP on a single mRNA molecule is thus

\[
\bar{n} = \sum_{m=0}^{N2} \left( m \cdot B2(m) \cdot \sum_{n=0}^{N1} n \cdot B1(n) \right) = N1p1N2p2
\]

To calculate the distribution of number of scFv-GFP binding to a single mRNA molecule, we first calculate the probability of \(n\) scFv-GFP binding to \(m\) PCP-SunTag, which is

\[
B1^*(n) = \binom{mN1}{n} p1^n (1 - p2)^{mN1 - n}
\]
Then the probability of \( n \) scFv-GFP on a single mRNA molecule is the sum of all possible \( m \), \( B_m^m(n) \) which is

\[
P(n) = \sum_{m=0}^{N_2} B_2^m(m) B_m^m(n) = \sum_{m=0}^{N_2} \binom{N_2}{m} \left( \frac{p_2}{1 - p_2} \right)^{m_2 - m} \left( \frac{m N_1}{n} \right) p_1^m (1 - p_1)^{m_1 - n} = (1 - p_2)^{N_2} \left( \frac{p_1}{1 - p_1} \right) \sum_{m=0}^{N_2} \binom{N_2}{m} \left( \frac{m N_1}{n} \right) \]

\[
\times \left( \frac{p_2}{1 - p_2} \right)^m (1 - p_1)^{m_1}
\]

Although dissociation constants for variant scF fragments vary significantly, a value of 0.38 nM (Wörn et al., 2000) was selected for scFV-GCN4 in simulations. We note that modeling results showed similar patterns with optimization at a 5:1 ratio of system components across orders of magnitude in parameter sweeps of the dissociation constant for scFV-GCN4. The dissociation constant for PCP/PP7 is 1 nM (Lim and Peabody, 2002). The dissociation constants for Nb-gp41/MoonTag and MCP/MS2V6 are 30 nM (Lutje Hulsik et al., 2013) and 2.4 nM (Tutucci et al., 2018b).

Considering that the signal from one mRNA molecule will spread to an ellipsoidal area, of which the size is determined by the Rayleigh radius, so we calculate the fluorescence intensity of this area in the presence and without the presence of a single mRNA molecule to calculate signal to noise ratio (SNR). Here, the volume we chose for calculation is \( V = 200 \times 200 \times 500 \) nm. If there is no mRNA in the area, the intensity is defined as the background or noise intensity, \( I_B \). Assuming that the intensity of single GFP-scFv is 1, the background intensity is the sum of free scFv-GFP molecules and the GFP-SunTag-PCP complex

\[
I_B = N_A V (c_1 - N_1 p_1 c_2) + N_A V (c_2 - N_2 p_2 c_3) N_1 p_1
\]

where \( N_A \) is Avogadro constant.

With the intensity of an mRNA molecule as \( I_{RNA} = N_1 p_1 N_2 p_2 \) the SNR is

\[
SNR = \frac{I_{RNA}}{I_B} = \frac{N_1 p_1 N_2 p_2}{N_A V (c_1 - N_1 p_1 c_2) + N_A V (c_2 - N_2 p_2 c_3) N_1 p_1}
\]
Supplemental information

Long-term imaging of individual mRNA molecules in living cells

Yue Guo and Robin E.C. Lee
Figure S1

A

B

PCP-5xSunTag:scFv-sfGFP
- 5:1
- 1:1
- 1:5

PCP-10xSunTag:scFv-sfGFP
- 5:1
- 1:1
- 1:5

C

D

Signal intensity

Signal-to-background ratio
Figure S1. Quantification of naive design with 24xSunTag-PCP and simulations to identify expression ratios of SunRISER components for consistent and high signal intensity, related to Figure 1.

(A) Histograms of signal intensity and signal-to-background ratio of individual foci in cells expressing phage-cmv-cfp-24xPP7, cmv-24xSunTag-PCP and cmv-sfGFP-GB1-scAB quantified with dNEMO (Kowalczyk et al., 2021). Signal intensity is defined as the average of background-corrected pixel values within the area of each detected spot. Size is measured by number of pixels comprising the spot. Signal-to-background ratio is calculated as the ratio of average pixel intensity within an mRNA spot divided by the average intensity of background pixels in an annular ring surrounding the spot. Inset numbers indicate the coefficient of variation (CV) for each distribution (n=30 for cell numbers and n=5179 for spots numbers). (B) and (C) Parameter sweeps in the space spanned by number of molecules for scFv-GFP and ‘n’xSunTag-PCP (left) using a computational model to calculate the number of GFP molecules per mRNA. Parameter combinations 1-10 were selected to represent different ratios between scFv-GFP and ‘n’xSunTag-PCP (solid line 1:1, dotted line 5:1 and dash-dotted line 1:5) in plots of frequency versus intensity of GFP labeling (right). Different concentrations of mRNAs per cell are distinguished by different colors. (A) 5xSunTag; (B) 10xSunTag; See also, Figure 1 for 24xSunTag). (D) Heatmaps of mean number of GFP molecules per mRNA (left) and signal-to-background ratio (right) in the parameter space spanned by the number of molecules for scFv-GFP and ‘n’xSunTag-PCP (from left to right, 5x, 10x and 24x). Signal-to-background ratio is calculated as ratio of scFv-sfGFP molecules bound to an mRNA divided by the average intensity of unbound scFv-sfGFP in the background. Heatmaps were calculated assuming 100 mRNA molecules per cell.
Figure S2. Varying promoters for scAB-GFP and 10xSunTag-PCP corroborates model predictions, related to Figure 1. 
(A) HeLa cells transfected with cmv-sfGFP-GB1-scAB, ubc-sfGFP-GB1-scAB, or EF1A-sfGFP-GB1-scAB were imaged 24 hours after transfection and mean GFP fluorescence intensity was measured in single cells. Boxplots (median and interquartile ranges) show expression variability for indicated promoters. The expression of GFP driven by the cmv promoter is approximately 5-times and 7-times that by ubc and EF1A promoters respectively (number for cells in each condition: cmv: n=57; ubc: n=60; EF1A: n=67). (B)-(E) HeLa cells transfected with phage-cmv-cfp-24xpp7 and indicated constructs were imaged by 60x wide-field microscopy 24 hours after transfection and imaged with identical settings, representative maximum intensity projections are shown. Images were analyzed with dNEMO (Kowalczyk et al., 2020) to quantify mRNA spots. Signal intensity is defined as the average of background-corrected pixel values within the area of each detected spot. Signal-to-background ratio is calculated as ratio of signal intensity divided by the average intensity of background pixels in an annular ring surrounding the spot. Note that images in (D) and (E) were contrast-enhanced for visualization. The combination of cmv-sfGFP-GB1-scAB and ubc-10xSunTag-PCP enabled robust detection of diffraction-limited mRNA spots and shows the highest signal intensity and signal-to-background ratio. (B) n=16 for cell numbers and n=502 for spots numbers; (C) n=5 for cell numbers and n=504 for spots numbers; (D) n=3 for cell numbers and n=25 for spots numbers; (E) n=4 for cell numbers and n=45 for spots numbers. Scale bar: 10 μm.
Figure S3. The preliminary version of SunRISER labels only cytoplasmic mRNA because SunTag-PCP is excluded from nuclei and modifications to SunTag-PCP alter sub-cellular distribution of SunRISER components, related to Figure 2.

(A) Maximum intensity projection images of smFISH performed with probes against the pp7 stem-loops on HeLa cells transfected with indicated constructs are shown. The combination of cmv-sfGFP-GB1-scAB and ubc-10xSunTag-PCP allows visualization of cytoplasmic mRNA, but not mRNAs in nucleus. From left to right, FITC CY5, and merged channels are shown.

(B) HeLa cells were transfected with cmv-sfGFP-GB1-scAB only (top) or co-transfected with a combination of cmv-sfGFP-GB1-scAB and ubc-10xSunTag-PCP (bottom) and imaged after 24 hours. Co-expression of scAB-GFP and SunTag-PCP depletes scAB-GFP from the nucleus through interaction with SunTag-PCP.

(C) HeLa cells transfected with cmv-sfGFP-GB1-scAB and ubc-10xSunTag-PCP (left) and ubc-10xSunTag-PCP only (right) were stained for SunTag using a α-GCN4 antibody. Since sfGFP-scAB competes with the α-GCN4 antibody for the GCN4 epitope, co-expressing cells are lower intensity in the A594 channel (top left). In both conditions, 10xSunTag-PCP is predominantly cytoplasmic regardless of whether sfGFP-scAB is expressed in the same cell.

(D)-(E) HeLa cells transfected with cmv-sfGFP-GB1-scAB, phage-cmv-cfp-24xpp7 and indicated constructs were imaged and quantified 24 hours after transfection. Signal intensity and Signal-to-background ratio is calculated as described in Figure 2. n>18 cells and n>4500 spots for each condition; Coefficient of variation (CV) is the ratio of the standard deviation to the mean; ODC ornithine decarboxylase; nls, nuclear localization signal; nes, nuclear export signal. Scale bar: 10 μm. See also Table S1.
Figure S4. SunRISER-labeled mRNAs comparing weak and strong promoters in the detection plasmid, and expression in A549 cells, related to Figure 2.

(A-C) Comparison of signal intensity (A), signal-to-background ratio (B), and mRNA numbers (C), between mRNAs expression driven by cmv and ubc promoters in the detection plasmid. Blue bars are cmv-driven (n=26 for cell numbers and n=8713 for spots numbers) and orange bars are ubc-driven (n=28 for cell numbers and n=2744 for spots numbers) SRv1-labeled mRNA. (D) Maximum intensity projection images of A549 cells transfected with SunRISER SRv1 phage-cmv-cfp-24xpp7, ubc-nls-PCP-5xSunTag and cmv-sfGFP-GB1-scAB are shown. Note that A549 cells were imaged with longer exposure times to HeLa for similar overall image intensity, possibly related to the large size of A549 cells. Scale bar: 10 µm.
Figure S5. Model calibrated to orthogonal RNA hairpin and coat protein systems are comparable with previous observations for PP7, related to Figure 3.

(A) Parameter sweeps in the space spanned by the number of molecules for scFV-GFP and 5xSunTag-MCP (left) using a computational model to calculate number of GFP molecules per mRNA. Parameter combinations 1-10 were selected to represent different ratios between scFV-GFP and 5xSunTag-MCP (solid line 1:1, dotted line 5:1 and dash-dotted line 1:5) and different expression levels and frequency plots are shown (right). Different concentrations of mRNAs per cell are distinguished by different colors. (B) Parameter sweeps in the space spanned by the number of molecules for Nb-gp41-GFP and 12xMoonTag-PCP (left) using a computational model to calculate number of GFP molecules per mRNA. Parameter combinations 1-10 were selected to represent different ratios between Nb-gp41-GFP and 12xMoonTag-PCP (solid line 1:1, dotted line 5:1 and dash-dotted line 1:5) in plots of frequency versus intensity of GFP labeling (right). Different concentrations of mRNAs per cell are distinguished by different colors.
Figure S6. Comparison between mRNAs labeled with SunRISER SRv1 and PP7-PCP-GFP, related to Figure 4.
(A) Representative maximum intensity projection images of HeLa cells transfected with phage-cmv-cfp-24xPP7 and phage-ubc-nls-ha-pcp-gfp. Cytoplasmic region (yellow box) was zoomed in and shown in time series. (B) Time course of signal-to-background values for SunRISER SRv1 (green; n=12 for cell numbers) and PCP-GFP (grey; n=12 for cell numbers) labeled mRNAs. Imaging conditions were selected to minimally facilitate detection of single mRNA spots while at the same time minimizing phototoxicity for the two reporter systems. (C) Time course of detected mRNA numbers with SunRISER SRv1 (green) and PCP-GFP (grey). (D) Boxplot for ratio of mRNAs detected with SunRISER SRv1 (left) and PCP-GFP (right) between t=10min and t=0. The mRNA numbers were calculated as average of 50 frames at specified time points. P-value was calculated using a 2-tailed t-test.
Figure S7. Characterization of translation, half-life, cytoplasm-to-nucleus ratio, and diffusion for SunRISER-labeled mRNA, related to Figure 4.

(A) Boxplots of mCherry fluorescence intensity in Hela cells transfected with SunRISER labeled mCherry-24xPP7 (left) and mCherry-24xPP7 only (right) quantified 24 hours after transfection. The p-value (0.17) of a 2-tailed t-test suggests that SunRISER does not significantly alter protein expression from labeled mRNAs. (B) Histogram of cytoplasm:nucleus mRNA ratios for SunRISER labeled mRNAs in Hela cells transfected with SunRISER labeled CFP-24xPP7 (n=56 for cell numbers). (C) Boxplot of mRNA half-lives for SunRISER labeled CFP-24xPP7 mRNAs measured in single cells 24 hours post-transfection. Transcription was inhibited via treatment with 50μM DRB and cells were imaged with 10-minute intervals over a period of 10 hours. To calculate half-lives, single-cell mRNA time courses were fit to exponential functions. Shaded area approximates the half-lives measured for global mRNAs (Schwanhäusser et al., 2011; Tani et al., 2012). (D) Maximum intensity projection images from a representative high frame rate movie of SunRISER labeled mRNAs. Spots were detected by dNEMO (Kowalczyk et al., 2021) and tracked by u-track (Jaqaman et al., 2008) to establish single-mRNA time course trajectories. Individual trajectories are distinguished by different colors. Scale bar: 10 μm. (E) Representative histogram of diffusion constants calculated from mRNA trajectories within a single cell. Mean and standard deviation are extracted from Gaussian fit (n=495 for spots numbers). (F) Boxplot of mean diffusion constants from 16 cells.
Figure S8. Quantification and representative images of different configurations for SunRISER, related to Figure 5. (A) Distribution of signal-to-background ratio for different stem-loops paired with different SunTag array lengths. Different color groups indicated different stem-loops and darker shades represent larger SunTag array. Vertical lines mark median values for different combinations. (B) Representative maximum intensity projection images of HeLa cells transfected with cmv-mCherry-8xpp7, phage-nls-pcp-10xSunTag, cmv-sfGFP-GB1-scAB (SR v1.1). (C) Representative maximum intensity projection images of HeLa cells transfected with cmv-mCherry-10xpp7, phage-nls-pcp-12xSunTag, cmv-sfGFP-GB1-scAB (SR v1.2). (D) Representative maximum intensity projection images of HeLa cells transfected with phage-cmv-cfp-24xpp7 and single construct encoding phage-nls-pcp-5xSunTag and cmv-sfGFP-GB1-scAB (SR v1-2P). Scale bar: 10 μm.
Figure S9. Symmetry and asymmetry of mitotic mRNA partitioning identified by k-means clustering and active transcription during mitosis, related to Figure 6.

(A) AIC/BIC calculation for determining the number of clusters (k) used for subsequent k-means clustering on mRNA symmetry between post-mitotic sister cells. Vertical line indicates the optimal cluster number as 4 for both analyses. (B) Box plots for relative difference of mRNA between post-mitotic sister cells when cultured in media without or with indicated stress. Black dots represent individual sister pairs. Red lines mark the boundaries of k-means clustering. (C) Box plots for mRNA ratio between the sum of daughters and mother in different symmetry groups identified by k-means clustering on mRNA relative difference. (D) P-value table for t-tests between mRNA ratio between the sum of daughters and mother for each cluster and growth media group shown in panel (C). P-values indicate significant evidence of mitotic transcription in LiCl and low serum conditions.
Figure S10. Mitotic mRNA partitioning between daughter cells during stress, related to Figure 6. (A) Histograms for ratios of mRNAs allocated to daughter cells when cultured in media without or with indicated stress. Binomial distributions are overlayed with grey lines. P-values for binomial tests are marked, showing that partitioning deviates significantly from a binomial distribution in all stress conditions. (B) Scatter plots for the ratios of mRNA numbers and the ratios of areas between daughter cells when cultured in media without or with indicated stress. R² values are marked. Coefficients of determination suggest that asymmetry of mRNA inheritance is partially, but not completely, explained by variance in cellular mass distribution between daughter cells.
Table S1. Summary of SunTag/PCP plasmid variants, related to Figure 2

SunTag-PCP sequence modifications include varying promoters (ubc and cmv), nuclear localization sequences (nls), nuclear export sequences (nes) and ornithine decarboxylase (ODC). Note that “Limited” indicates that significant cell-to-cell variability or lower number than expected was observed for the associated property.

| PCP/SunTag variants                      | Nuclear GFP | Nuclear spots | Cytoplasmic spots |
|------------------------------------------|-------------|---------------|-------------------|
| ubc-10xSunTag-PCP-nls                    | No          | Limited       | Yes               |
| ubc-10xSunTag-PCP-nls-nes                | No          | Limited       | Yes               |
| cmv-10xSunTag-PCP-ODC                    | Yes         | No            | Yes               |
| cmv-10xSunTag-PCP-nls-ODC                | Yes         | Limited       | Yes               |
| cmv-10xSunTag-PCP-nls-nes-ODC            | Yes         | Limited       | Yes               |
| ubc-10xSunTag-PCP-ODC                    | Limited     | No            | Limited           |
| ubc-10xSunTag-PCP-nls-ODC                | Yes         | Limited       | Limited           |
| ubc-10xSunTag-PCP-nls-nes-ODC            | Limited     | No            | Limited           |
| cmv-5xSunTag-PCP-ODC                     | Limited     | No            | Yes               |
| cmv-5xSunTag-PCP-nls-ODC                 | Yes         | No            | Yes               |
| cmv-5xSunTag-PCP-nes-ODC                 | No          | No            | Yes               |
| ubc-5xSunTag-PCP-nls                     | No          | Limited       | Yes               |
| ubc-nls-5xSunTag-PCP                     | Limited     | Limited       | Yes               |
| ubc-nls-5xSunTag-PCP-nls                 | No          | Limited       | Yes               |
| ubc-nls-5xSunTag-PCP-2xnls               | Yes         | No            | Limited           |
| ubc-2xnls-5xSunTag-PCP-2xnls             | Yes         | No            | No                |
| ubc-PCP-5xSunTag                         | Limited     | Limited       | Yes               |
| ubc-nls-PCP-5xSunTag                     | Yes         | Yes           | Yes               |
| ubc-nls-PCP-5xSunTag-nes                 | Limited     | Limited       | Yes               |
Table S2. Summary of parameters used in simulation, related to Figures 1 and 3.

List of tunable parameters used in models simulating different variants of SunRISER with orthogonal stem-loops and antibody-epitope pairs. The volume of HeLa cell is from Bionumbers database (ID 103725), which can be adjusted for different cell types or modified for use with other measurements of cell volume. The volume of Rayleigh criterion is calculated as 200nmx200nmx500nm (lateral resolution for light microscope is 200nm and axial resolution is 500nm). Note that Kd for anti-GCN4 scFv can range from $10^{-6}$ to $10^{-11}$ M.

|                        | Kd of antibody-peptide pair (mol/L) | Kd of coat protein and stem-loop pair (mol/L) | # of epitopes on coat protein |
|------------------------|--------------------------------------|-----------------------------------------------|-------------------------------|
| PCP-5xSunTag +mRNA-24xPP7 | 0.38e-9                              | 1e-9                                          | 5                             |
| PCP-10xSunTag +mRNA-24xPP7 | 0.38e-9                              | 1e-9                                          | 10                            |
| PCP-24xSunTag +mRNA-24xPP7 | 0.38e-9                              | 1e-9                                          | 24                            |
| MCP-5xSunTag +mRNA-24xMS2 | 0.38e-9                              | 1e-9                                          | 5                             |
| MCP-5xSunTag +mRNA-24xMS2V6 | 0.38e-9                              | 2.4e-9                                        | 5                             |
| PCP-12xMoonTag +mRNA-24xPP7 | 30e-9                                | 1e-9                                          | 12                            |
| Common constants       | Volume of whole cell = 1745 μm³     | Volume of Rayleigh criterion = 0.02 μm³     | # of stem-loops = 24           |
Table S3. Molecular weight for SunRISER variants and other mRNA labeling techniques for labeling single mRNAs, related to Figures 2 and 5.
Molecular weight calculations for fully assembled mRNA labeled complexes, including associated coat proteins fused to GFP. Calculated weight for mRNA includes only the stem loops and does not consider an associated ORF. RNA molecular weight is calculated with Quest Calculate™ RNA Molecular Weight Calculator (AAT Bioquest, 2022) and protein molecular weight is calculated with The Sequence Manipulation Suite (Stothard, 2000).

| Variants                  | Molecular weight of mRNA stem-loops | Molecular weight of protein cargos | Total molecular weight |
|---------------------------|--------------------------------------|-----------------------------------|------------------------|
| 24xPP7                    | 426.34kDa                            | 2100.48kDa                        | 2526.82kDa             |
| MS2x128                   | 2233.6kDa                            | 10931.2kDa                        | 13164.8kDa             |
| SunRISER-V1 (24xPP7-5xST) | 426.34kDa                            | 15528.48kDa                       | 15954.82kDa            |
| SunRISER-V1.1 (8xPP7-10xST)| 134.86kDa                            | 10085.76kDa                       | 10220.62kDa            |
| SunRISER-V1.2 (10xPP7-12xST)| 154.17kDa                           | 15062kDa                          | 15216.17kDa            |