Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Translation imaging of single mRNAs in established cell lines and primary cultured neurons

Malgorzata J. Latallo[a,b,1], Nathan M. Livingston[a,b,1], Bin Wu[a,b,c,*]

[a] Johns Hopkins School of Medicine, Department of Biophysics and Biophysical Chemistry, 855 N Wolfe Street Ste. 454, Baltimore, MD 21205, USA
[b] Johns Hopkins School of Medicine, Center for Cell Dynamics, Baltimore, USA
[c] Johns Hopkins School of Medicine, Solomon H. Snyder Department of Neuroscience, Baltimore, USA

ARTICLE INFO

Keywords:
Single-molecule
mRNA
Translation
Fluorescence microscopy

ABSTRACT

The central dogma of molecular biology reaches a crescendo at its final step: the translation of an mRNA into its corresponding protein product. This process is highly regulated both spatially and temporally, requiring techniques to interrogate the subcellular translational status of mRNAs in both living and fixed cells. Single-molecule imaging of nascent peptides (SINAPs) and related techniques allow us to study this fundamental process for single mRNAs in live cells. These techniques enable researchers to address previously intractable questions in the central dogma, such as the origin of stochastic translational control and the role of local translation in highly polarized cells. In this review, we present the methodology and the theoretical framework for conducting studies using SINAPs in both established cell lines and primary cultured neurons.

1. Introduction

The process of translation is highly regulated both spatially and temporally. Regulation at the translation level allows cells to rapidly respond to stimuli and tune protein production levels without *de novo* mRNA synthesis [1]. It is generally believed that translation initiation acts as the gatekeeper of overall protein output and translational control [2]. Determining when, where, and how translation takes place becomes even more important in highly polarized cellular systems, such as neurons, where many proteins are synthesized often at great distance from the cell body [3].

Despite the clear need to visualize translation in a native environment, much of what we know regarding translational control originates from ensemble measurements lacking precise spatial and temporal resolution. Genome-wide ensemble techniques such as ribosome profiling provide unprecedented codon resolution of ribosome occupancy but cannot identify *in vivo* translational kinetic parameters such as initiation, elongation, and termination rates with subcellular resolution [4]. Additionally, breakthroughs in *in vitro* single molecule techniques have provided critical insight about ribosome dynamics in both eukaryotic and prokaryotic systems, but these techniques are limited to short mRNAs and cannot recapitulate all behavior of native messenger ribonuclear particles (mRNPs) [5,6].[2]

To address the need for an *in vivo* single-molecule assay to study translation, our group and others have recently developed techniques to quantitatively image translation from single reporter mRNAs in their native cellular context [7–11]. Single molecule imaging of nascent peptides (SINAPs) relies on three pieces of core technology (Fig. 1a). First, we employ the MS2-tagging system to track single mRNAs [12,13]. We insert 24 MS2 (MS2v5) stem loops in the 3′ untranslated region of the reporter mRNAs and within the same cell co-express a MS2-coat protein (MCP) fused to a fluorescent moiety to label them. Second, we use the SunTag system to rapidly label the translating nascent peptide emerging at the ribosome exit channel [14]. The reporter mRNA sequence codes for 24 repeats of GCN4 epitope at the N-terminal end of the peptide, which are bound by a co-expressed single-chain variable fragment of an antibody fused to superfolder Green Fluorescent Protein (scFv-sfGFP) [14]. Because scFv-sfGFP is already

* Corresponding author at: Johns Hopkins School of Medicine, Department of Biophysics and Biophysical Chemistry, 855 N Wolfe Street Ste. 454, Baltimore, MD 21205, USA.
E-mail address: bwu20@jhmi.edu (B. Wu).

[1] Equal contribution.

[2] AID – auxin inducible degradation tag; IAA – indole-3-acetic acid; SINAPs – Single molecule imaging of nascent peptides; MCP – MS2-coat protein; FP – fluorescent protein; NLS- nuclear localization signal; osTIR1 – *Oryza sativa* F-box transport inhibitor response 1 protein; scFv-sfGFP – single chain variable fragment of antibody fused to superfolder Green Fluorescent Protein; smFISH – single-molecule fluorescence *in situ* hybridization; TLS – translation site; DIV – days *in vitro*; IRES: internal ribosome entry site.

https://doi.org/10.1016/j.ymeth.2019.03.021
Received 12 January 2019; Received in revised form 17 March 2019; Accepted 20 March 2019

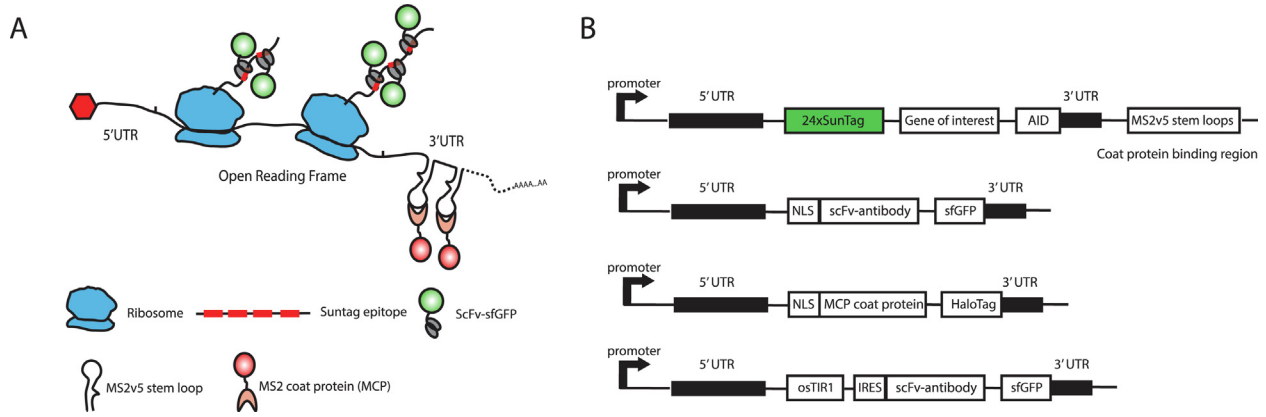

Fig. 1. Single molecule imaging of nascent peptides (SINAPs) enables quantitative imaging of translation dynamics in living and fixed cells. (A) SINAPs visualizes single mRNAs and their translation status simultaneously. The mRNA is labeled in the 3′UTR by a 24× array of MS2 (MS2v5) stem loops bound by a co-expressed MS2 coat protein (MCP). These binding sites can be substituted with the orthogonal PP7 stem-loop labeling system. The newly translated GCN4 "SunTag" epitopes are labeled by mature fluorescent scFv-sfGFP fusion proteins. (B) Schematic overview of plasmids used for SINAPs reporter imaging. AID: auxin induced degron; NLS: nuclear localization sequences; scFv: single chain variable fragment, osTIR: *Oryza sativa* F-box transport inhibitor response 1 protein; IRES: internal ribosome entry site).

mature and fluorescent, the fluorescence intensity at the translation site (TLS) is a real-time readout of ribosome occupancy along the transcript. This is advantageous compared with conventional fluorescent proteins because their slow maturation process fails to capture real-time translation dynamics. Third, in order to reduce the background from completed proteins in the cytoplasm and maintain scFv-sfGFP binding of nascent GCN4 peptides, we insert an auxin inducible degradation tag (AID) at the C-terminus, which, upon addition of indole-3-acetic acid (IAA), degrades completed proteins and frees individual scFv-sfGFP fusions to label other TLS [15,16]. In the following sections, we present general methods and considerations for conducting and interpreting experiments using SINAPs in both established cell lines and primary cultured neurons.

2. Single-molecule imaging of nascent peptides in live and fixed cells

2.1. Creating cell lines for SINAPs experiments

In order to visualize single translating mRNAs, three auxiliary components are required: an MCP-fluorescent protein fusion (MCP-FP), a scFv-sfGFP fusion protein, and the *Oryza sativa* F-box transport inhibitor response 1 protein (osTIR1), an E3 ubiquitin ligase required for the auxin-induced degradation system [16]. Stable lines expressing all three components are generated using lentiviral or retroviral transduction into U-2 OS human osteosarcoma cell lines as previously described in [9]. Techniques similar to SINAPs have also been performed in HeLa cells [10,11]. After establishing the necessary auxiliary components, these parent cell lines can then be used for stable expression or transient transfection of the SINAPs reporter. All publicly available variants on SINAPs reporters can be found in Table 1 (Fig. 1b).

2.1.1. RNA labeling considerations

MS2 stem-loop labeling has become the standard in live cell single-molecule tracking of mRNA and has been extensively reviewed elsewhere [12,13]. The PP7 system is orthogonal to MS2 and can be used for mRNA tracking and multi-color applications [7,10,17–19]. Both systems contain coat proteins that bind their respective target stem-loop as dimers [17,20,21]. Extensive engineering of both the target mRNA aptamer sequences and coat proteins have yielded optimized mRNA labeling by curtailing issues associated with repetitive sequences, non-uniform labeling, and interference in mRNA decay [19,22,23]. Table 2 outlines improvements to fluorescent proteins, dyes, and mRNA labeling strategies that assist in single mRNA visualization in live cells.

Despite improvements in stem-loop labeling technologies, mRNA tracking is often the limiting factor for translation imaging. Because translation takes place on polysomes, there are multiple peptide arrays each bound by up to 24 scFv-sfGFP. In contrast, there is only a single array of MS2 binding sites per TLS. Improvements in fluorescent proteins and organic dyes have significantly improved the ability to track these relatively dim species. MCP-HaloTag fusions offer the best option for robust mRNA labeling. HaloTag is a modified de-halogenase that can covalently conjugate cell-permeable, organic dyes [24]. Together with the recently developed Janelia Fluor® dyes, the HaloTag offers superior brightness and photostability when compared to conventional fluorescent proteins for single-molecule tracking [25,26]. To limit background fluorescence from unbound MCP in the cytoplasm, MCP fusion proteins possess a nuclear localization sequence (NLS). Using fluorescence correlation spectroscopy (FCS) to measure coat protein and mRNA concentration, tdMCP and tdPCP expression in the 100–400 nM range is adequate to confer uniform mRNA labeling [19] (Table 2). Because FCS is not widely available, the coat protein expression level is normally empirically determined by transfecting an MBS-containing reporter and the proper MCP expression level is isolated using clonal expansion or fluorescence activated cell sorting

Table 1
Publically available SINAPs components.

Construct	Description	Addgene Lookup	Citation
pUbC-OsTIR1-myc-IRES-scFv-sfGFP	Combined OsTIR1 and scFv-sfGFP	84563	Wu et al., 2016
pHR-scFv-GCN4-sfGFP-GB1-NLS-dWPRE	Independent scFv-sfGFP	60906	Tanenbaum et al., 2014
pBabe Puro osTIR1-9Myc	Independent OsTIR1	80074	Holland et al., 2012
pUbC-FLAG-24xSuntagV4-oxEBFP-AID-baUTR1-24xMS2V5-Wpre	Canonical SINAPs reporter with baUTR	84561	Wu et al., 2016
phage-ubc-nls-ha-tdMCP-gfp	Canonical MCP, exchangeable fluorophore	40649	Wu et al., 2012
pHR-PP7-2xmCherry-CAAX	PP7-coat protein fused to CAAX motif	74925	Yan et al., 2016

Table 2
Fluorescent protein and stem-loop mRNA labeling improvements.

Improvement	Description	Pros	Cons	Citation
HaloTag	33 kDa modified dehalogenase that can conjugate a wide range of fluorescent ligands	Can bind a range of spectrally unique, cell-permeable ligands	Requires additional dying and washing prior to imaging, slightly larger than conventional eGFP (27 kDa)	Los et al., 2008
Janelia Fluor (JF) 549 and 646	Improved Red and Far-Red dyes for single-molecule imaging in live cells. JF-549 and 646 possess quantum yields of 0.88 and 0.54 respectively. These dyes are ~3× as bright as previous generation TMRs and efficiently label HaloTag at low nanomolar concentration. JF-646 additionally demonstrate fluorogenic properties upon conjugation to the HaloTag that stabilize the fluorescently active state	Bright, photostable dyes enhance the signal-to noise ratio	Requires additional dying and washing prior to imaging	Grimm et al., 2015, 2016
Tandem Dimer MCP and PCP (tdMCP and tdPCP)	MCP and PCP molecules bind their respective stem-loops with a 2:1 stoichiometry. tdMCP and tdPCP express a tandem dimer and fluorescent protein on a single peptide.	Provides more uniform and complete mRNA labeling at lower concentrations of coat protein allowing for quantitative imaging of mRNA	Necessary to make synonymous modifications to faithfully subclone repetitive protein sequences	Wu et al., 2012, 2015
MS2v5 and MS2v6/v7	A non-repetitive versions of a 24× cassette of MS2 stem-loops that faithfully degrade in both yeast and mammalian cells	Resistant to truncations and deletions due to recombination during viral infection, bacterial proliferation, and homologous recombination after integration, do not affect mRNA degradation kinetics	Still requires large RNA aptamer insertion for single-molecule resolution	Wu et al., 2015; Tutucci et al. 2017

(FACS).

It takes minutes to translate a typical-sized protein. To measure translation dynamics, it is imperative to track single mRNAs for longer than the translation cycle. Because mRNAs diffuse rapidly in the cytoplasm, the molecules need to be continuously illuminated to track in three dimensions. To facilitate long-term tracking, the MCP or mRNA can be altered to anchor the mRNA to various cellular structures, such as the endoplasmic reticulum (ER) or the plasma membrane. A signal peptide can be added to the SINAPs reporter to target translating mRNA to the surface of the ER. Because the scFv is localized in the cytosol, the SunTag motif of the reporter must be exposed to the cytoplasmic side. For example, the cytochrome P450 signal peptide (cytERM) at the N-terminus is inserted into the membrane of the endoplasmic reticulum but leaves the rest of the protein exposed to the cytoplasm, which can be labeled by scFv-sfGFP [9,27]. The cytERM-SINAPs reporter mRNA exhibits restricted motility when actively being translated, but freely diffuses when not translating. This demonstrates that it is the translation activity and nascent peptides that localize the mRNA to the surface of the ER [9].

Alternatively, mRNA could be artificially tethered to the plasma membrane by coat proteins fused with a C-terminal CAAX motif [7]. The CAAX motif signals for the prenylation of the cysteine residue, which will insert into the inner leaflet of the plasma membrane and bind mRNAs containing complementary stem loops. mRNAs that are tethered to the membrane have very limited motility and require infrequent imaging for tracking. Although it has previously been shown membrane tethering has limited effect on translation, this modification prevents using SINAPs to study spatially regulated translational control. Methods and considerations for imaging membrane tethered mRNA will be discussed in detail in a later section and are comprehensively discussed in [28].

2.1.2. Expression of scFv-sfGFP

The SunTag system consists of two components, an array of linear yeast GCN4 epitopes attached to a protein of interest and a complementary scFv-sfGFP fusion protein [14]. To quantitatively interpret the fluorescence intensity of TLS between different cells, it is important to saturate the binding of scFv-sfGFP to the epitopes. The concentration of scFv to reach saturate binding will vary with the concentration of the expressed proteins, and the optimal scFv expression level must be isolated by FACS. Experimentally, one can measure the intensity of single mature proteins to determine whether binding is saturated. This can be accomplished using single-molecule fluorescence *in situ* hybridization (smFISH) coupled with immunofluorescence against the nascent peptide (described in detail in 2.2). In this assay, the intensity of single, released SunTag arrays can be determined, and this value should not vary from cell-to-cell when scFv binding is saturated.

One further consideration for the scFv component of SINAPs is the choice of fluorescent moiety. sfGFP was specifically chosen to assist proper protein folding and to resist aggregation [14]. Any substitution should be validated for faithful expression and labeling of SunTag epitopes.

2.1.3. Degradation of completed proteins

The mature SINAPs reporter proteins bind scFv-sfGFP and contribute to the diffusive background that decrease the signal/noise ratio of TLS. If the expression of the target protein is high enough, the available scFv-sfGFP will be depleted and the TLS will not be visible. We use the auxin-inducible degron (AID) to degrade the maturated protein in a drug dependent manner [9]. We reconstitute the plant SCF ubiquitin E3-ligase by stably expressing the F-box protein osTIR1 in the target cells. In the presence of indole-3-acetic acid (IAA), osTIR1 recognizes an AID containing protein, leading to its ubiquitination and degradation [16]. The C-terminal end of the SINAPs reporter peptide contains the AID sequence in order to degrade completed proteins and reduce cytoplasmic fluorescent background. This degradation

Table 3
smFISH-IF reagents.

Reagent	Supplier	Catalog number
10× Phosphate-Buffered Saline (PBS)	Corning	46-013-CM
20xSSC Buffer	Roche	11666681001
Bovine Serum Albumin	VWR	VWRV0332
Bovine Serum Albumin (Roche) 20 mg/mL	Sigma-Aldrich	10711454001
Calcium chloride dihydrate	Sigma-Aldrich	C5080-500G
Chicken anti-GFP (Green Fluorescent Protein) antibodies IgY	Aves Labs	GFP-1010
Dextran sulfate sodium salt (from Leuconostoc spp)	Sigma-Aldrich	D8906-100G
E. Coli tRNA	Sigma-Aldrich	10109541001
Formamide, 99.5%	Acros Organics	AC205821000
Glycine	Thermo Fisher Scientific	BP 381-1
Goat anti-Chicken IgY (H + L) Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A11055
Goat anti-Chicken IgY (H + L) Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific	A21449
Magnesium chloride hexahydrate	Sigma-Aldrich	M2670-500G
Molecular Biology Water	Quality Biological	351029131
MS2V5 custom probe	Biosearch	Sequence in Table 3
Nail polish	Electron Microscopy Sciences	50-949-071
Paraformaldehyde (20%)	Electron Microscopy Sciences	50-980-492
Sucrose	Sigma-Aldrich	S7903-1
Suntag Stv4 custom probe	Biosearch	Sequence in Table 4
TetraSpeck 100 nm Microspheres	Thermo Fisher Scientific	T7279
Triton X-100	Sigma-Aldrich	T8787

mechanism also acts to recycle unbound scFv-sfGFP for use in subsequent labeling of nascent peptides on translating ribosomes. Similar translation reporter systems have used destabilizing domains to degrade completed proteins [10].

2.1.4. Generating viral particles and infection of SINAPs auxiliary components

MCP variants are derived from the pHAGE backbone, a third-generation, replication deficient lentiviral expression vector [29]. The expression of the MCP is driven by a human ubiquitin C (UBC) promoter for ubiquitous expression in all cell types [19]. The original scFv-sfGFP is expressed from a third generation lentivirus backbone [14,30]. To reduce the number of lentiviral infections, we combined scFv-sfGFP and osTIR1 into a bi-cistronic viral construct using internal ribosome entry to drive the expression of scFv-sfGFP (Table 1, Fig. 1b). This is especially advantageous when working in cell populations that cannot be sorted by fluorescence or drug selection. Many of these MCP and scFv-sfGFP variants are publically available. To produce lentiviral particles and transduce target cells, standard protocols and safety procedures should be followed [30].

2.1.5. Generating reporter mRNA constructs in U-2 OS cells

The SINAPs mRNA reporter contains three elements: a 24xSunTag GCN4 epitope array at the N-terminus, an AID tag at the C-terminus of the protein of interest, and a non-synonymous 24xMS2 array in the 3′ untranslated region. The reporter is cloned into a lentiviral backbone, allowing for either stable integration or expression from transient transfection. Due to the length of the SINAPs reporter, lentiviral transduction is often inefficient and requires large amount of concentrated virus. Alternatively, one can transiently express the SINAPs reporter construct in a host cell line expressing all required auxiliary proteins. However, the interpretation of TLS intensity is complicated by heterogeneous scFv and reporter expression levels. The fluorescence fluctuation analysis is not sensitive to this cell-to-cell variability because the autocorrelation function is normalized by the mean TLS intensity [9,31].

Any protein of interest can be inserted in the coding sequence (CDS) between the SunTag array and AID tag. Previously, we have included blue fluorescent protein to determine the translation dynamics of the reporter [9]. The average TLS intensity scales linearly with the length of the CDS because longer CDS can pack more ribosomes. The 5′ and 3′ untranslated regions (UTRs) contain cis-regulatory elements that modulate the metabolism of the target mRNAs. One can incorporate these cis UTR elements into the SINAPs reporters to control mRNA localization and translation dynamics. For example, addition of the β-actin 3′UTR localizes the SINAPs reporter to the distal dendrites in primary cultured neurons and spatially regulates translatability [9].

2.2. Creating stable expression of SINAPs reporter in primary neuronal cells

Neurons are one of the most morphologically polarized cells displaying high degree of compartmentalization. RNA localization and localized translation plays important role in neuron development and function. It restricts protein production in specific compartments to establish the polarized morphology and allows neurons to quickly respond to stimulation and cues by synthesizing plasticity related proteins locally. Previous methods focus on RNA dynamics and distributions; the translation dynamics of single mRNA and its relationship with synaptic activity has not been studied. SINAPs, with its sensitivity to single messages, provides an ideal tool to address these issues. Here we will give detailed instructions of how to implement SINAPs in cultured primary neurons, with emphasis on issues specific to neurons.

As described in Section 2.1, to visualize mRNA and its translation signal, neurons must stably express all SINAPs accessory components: MCP-FP, scFv-sfGFP, osTIR1. These can be introduced to a neuron via lentiviral transduction. Unlike cell lines, neurons do not divide and cannot be sorted. To increase the likelihood of infecting a single neuron with all three components, we have constructed a bi-cistronic pHAGE plasmid expressing osTIR1 and scFv-sfGFP (Table 1, Fig. 1b), with the latter under the control of an IRES (internal ribosome entry site). In order to have adequate scFv-sfGFP concentration for translation site labeling at the distal dendrites, we have removed the NLS in the scFv-sfGFP. The SINAPs reporter is also packaged into lentivirus. However, the virus titer will be lower compared with other accessory virus due to its length [32]. So, the ratio between different virus should be tested and optimized in neuronal cultures. One should keep in mind that too high expression of either coat proteins or antibody will increase the diffusive background and decrease the signal/noise ratio.

2.3. Performing fixed cell SINAPs experiments

Although live cell SINAPs experiments provide a powerful tool for studying translation dynamics, the throughput is low and it is difficult to measure many cells. To compare with ensemble biochemical experiments, it is necessary to study many mRNAs in a large cell population. This can be accomplished in fixed cell experiments. smFISH is a

Table 4
smFISH probe sequences.

MS2v5 Probes		Stv4 Probes	
MBSV5_probe1	tgattgtgaagtgtcgggtg	SunTagV4_probe1	ccacttcgttctcaagatga
MBSV5_probe2	gatattcgggaggcgtgatc	SunTagV4_probe2	ccctttttcagtctagctac
MBSV5_probe3	acgcactgaattcgaaagcc	SunTagV4_probe3	aatttttgctcagcaactcc
MBSV5_probe4	attcgactctgattggctgc	SunTagV4_probe4	ttctttagtcgtgctacttc
MBSV5_probe5	ctcttcgcgaaagtcgactt	SunTagV4_probe5	tttcgagagtaactcctcac
MBSV5_probe6	taagaatggcgcgaaggctg	SunTagV4_probe6	ccacttcgttttcgagatga
MBSV5_probe7	gtaggggagagtgtggtttg	SunTagV4_probe7	acttccctttttaagcgtg
MBSV5_probe8	caggaacgctgatgctgttc	SunTagV4_probe8	tcttggatagtagctcttca
MBSV5_probe9	ttggggatgtattcttgggg	SunTagV4_probe9	acctcgttctcaagatgata
MBSV5_probe10	ttggtgctcggatgtgattt	SunTagV4_probe10	cggaacccttcttcaaacgc
MBSV5_probe11	aagaaacaacactccgagcc	SunTagV4_probe11	agttcttcgagagcagttcc
MBSV5_probe12	atggagggtttgtccagttg	SunTagV4_probe12	gatccctttttttaatcgagc
MBSV5_probe13	gtatgctcgagtgtttcgaa	SunTagV4_probe13	tgaaagtagttcctcaccac
MBSV5_probe14	gatcgtccacccaagaaata	SunTagV4_probe14	cttcgttttcgaggtggtaa
MBSV5_probe15	aattcgtgagagcatgggtg	SunTagV4_probe15	ccctgaacctttctttaatc
MBSV5_probe16	tcgtattggacgtggaacga	SunTagV4_probe16	tactcagtaattcttcaccc
MBSV5_probe17	tcgtgatcccgaaaggtaag	SunTagV4_probe17	tttcgatagcaactcttcgc
MBSV5_probe18	atcgtgcatgcttgaatgtc	SunTagV4_probe18	tttttgagcctagcaacttc
MBSV5_probe19	gttgagacttgtggagcatg	SunTagV4_probe19	ttttcgagagcaactcctcg
MBSV5_probe20	tgaacccatttggtagtttc	SunTagV4_probe20	acctcattttccaagtggta
MBSV5_probe21	tttggtatgttggaatgggc	SunTagV4_probe21	tttgctcaataactcctcgc
MBSV5_probe22	gatgctgtaccagtaattgt	SunTagV4_probe22	cgcgacttcgttctctaaat
MBSV5_probe23	tagtagtgagagatgtgggc	SunTagV4_probe23	ttcgataagagttcttcgcc
MBSV5_probe24	tgctgaacggtttggttttt	SunTagV4_probe24	ctcattttcgaggtggtagt
MBSV5_probe25	ttgatttttccgtgtgtacc	SunTagV4_probe25	agtggtagttcttgctcaag
MBSV5_probe26	gtctttcgtatttgtaaacc	SunTagV4_probe26	ttcaatctcgcgacctcatt
MBSV5_probe27	ttgcgctggacgaaagcgtg	SunTagV4_probe27	attcttgctgagcaattcct
MBSV5_probe28	ccgtcggatgtttttcgtaa	SunTagV4_probe28	cgacttcgttctccaaatga
MBSV5_probe29	ggttgtaagtttgtgggttg	SunTagV4_probe29	cgacttcattttccaagtgg
MBSV5_probe30	ctgaggtgtttgatgtacgg	SunTagV4_probe30	ttgctcaataactcttcgcc
MBSV5_probe31	tccacccttgtgtattgtac	SunTagV4_probe31	ttcgttctccaagtggtaat
MBSV5_probe32	tgtaatgtgtctggagggtg	SunTagV4_probe32	agttcttcgataagagctcc
MBSV5_probe33	gcttctgtttgattggattt	SunTagV4_probe33	gcgacttcattctctaagtg
MBSV5_probe34	gatggtgattccttgttgta	SunTagV4_probe34	ttcttgctcaagagctcttc
MBSV5_probe35	gtatattgcacagggaatcc	SunTagV4_probe35	cacctcattttccaagtggt
MBSV5_probe36	ttttcttgagttgggtactg	SunTagV4_probe36	ttagatagtaactcttcccc
MBSV5_probe37	tgatgctgcatggggacata	SunTagV4_probe37	cctcgttctcgagatgataa
MBSV5_probe38	tttgtcttgttggtgagagt	SunTagV4_probe38	gatagttcttcgacaggagt
MBSV5_probe39	ctgatgctgcttcgagaaga	SunTagV4_probe39	ccttttaagtcttgcaacc
MBSV5_probe40	tttgaggtaggagtgggttc	SunTagV4_probe40	ttactgagtagttcctcacc
MBSV5_probe41	ttgccagttttgtgggaaga	SunTagV4_probe41	ttcgttttccaggtggtaat
		SunTagV4_probe42	tcctgatcctttcttcaaac
		SunTagV4_probe43	cttttgagagcagttcttcg
		SunTagV4_probe44	gcaacctcattttccaaatg
		SunTagV4_probe45	tgccacttccctttttttaaa
		SunTagV4_probe46	tttcgacagaagttcctcac
		SunTagV4_probe47	gctacttcattctcgagatg
		SunTagV4_probe48	gagccagaaccctttttaag

standard method for visualizing single-mRNAs in fixed samples [13,33]. In smFISH, multiple fluorescently labeled, complementary DNA oligonucleotides allows detection of the target mRNA with single molecule resolution [34]. The translation sites can be detected with immunofluorescence using an antibody targeting GFP [9,35] (Fig. 2a). Targeting GFP is advantageous because it is already pre-bound to the nascent peptide. This mitigates the risk that the epitope array forms any sort of structure that may prevent binding of all GCN4 epitopes. Single scFv-sfGFP molecules are too dim to be detected using this method, but mature SINAPs protein with up to 24-bound scFv-sfGFP molecules is readily detected and can be used to calibrate the number of nascent peptides associated with the mRNA (Fig. 2b-c).

2.3.1. Sample preparation for U-2 OS cells

The smFISH-IF protocol is adapted from [35,9] and described as performed in U-2 OS cells.

- Prepare coverslips by base etching for 2 min in 3 M NaOH.
- Wash coverslips with DPBS for 5 min three times.
- Coat coverslips with 25 μg/mL collagen in 20 mM acetic acid for 30 min at 37 °C.
- Wash coverslips 2× with DPBS.
- Seed 3×10^4 cells per coverslip (18 mm circular).
- Incubate cells for overnight and up to 24 h.
- Add 500 μM IAA and incubate overnight or up to 24 h.

2.3.2. Sample preparation for primary neuronal cultures

Here we describe a procedure for conducting SINAPs experiment in mouse hippocampal primary cultured neurons (Fig. 3a). We used 12 mm circular cover slips for fixed samples and 35 mm imaging dishes with 14 mm circular coverglass bottom.

- Dissect hippocampi and dissociate neurons according to established protocols. Seed 50×10^4 cells of mouse hippocampal neurons per 12 mm coverslip or 75×10^4 cells per imaging dish.
- Cells are grown in Neurobasal A medium (supplemented with B-27, primocin and Glutamax) free of Phenol Red for live neuron imaging; or with Phenol Red for fixed neuron imaging.
- Infected neurons with viruses between DIV 5–10: Combine all viruses into a single tube and mixed with warm medium (100 μL per

Table 5
smFISH-IF protocol in U2-OS cells and primary neuronal cultures.

STEP (volume)	U-2 OS	Primary neuronal cultures
1. Wash before fixation (1 mL/well)	3× 5 min with warm PBSM (1× PBS with 5 mM MgCl2 warmed up to 37 °C)	Not recommended
2. Fixation (1 mL/well)	Immediately after wash, for 10 min in PBS + 4% paraformaldehyde + 5 mM MgCl2	On ice for 20 min with ice cold: PBS, 4% PFA, 4% sucrose w/v, 1 mM MgCl2 and 1 mM CaCl2
3. Wash (1 mL/well)	3× 5 min with PBSM	3× 5 min with ice cold PBSMC (1× PBS with 1 mM MgCl2 and 1 mM CaCl2) on ice
4. PFA Quenching (1 mL/well)	10 min wash with PBSM + 100 mM glycine	10 min with ice-cold PBSMC + 100 mM glycine on ice
5. Cell Permeabilization (0.5–1 mL/ well)	10 min incubation in PBSM supplemented with 0.1% Triton X-100, and 5 mg/mL BSA	10 min incubation on ice with ice cold PBSMC with 0.1% Triton X-100 and 5 mg/mL BSA
6. Wash (1 mL/well)	3× 5 min with PBSM	3× for 5 min with PBSMC
7. Change of buffer (1 mL/well)	10 min incubation with 2xSSC, 10% formamide, and 5 mg/mL BSA	30 min incubation with 2xSSC, 5 mg/mL BSA, 10% v/v deionized formamide
8. Hybridization (~50 µL/coverglass)	3 h incubation at 37 °C in: 2xSSC, 10% v/v formamide, 1 mg/mL tRNA, 0.2 mg/mL BSA, 10% w/v dextran sulfate, 50–100 nM smFISH probes targeting SINAPS reporter and an antibody targeting sfGFP (1:5,000 anti-GFP)	Same hybridization conditions as for U-2 OS cells, recommended addition of RVC in hybridization buffer
9. Fast Wash (1 mL/well)	3× with warm: 2xSSC + 10% formamide	Same as for U-2 OS cells
10. First incubation with secondary antibodies (1 mL/well)	20 min at 37 °C with the secondary antibody of choice (usually fluorescently labeled with Alexa 488 or Alexa 647) diluted 1:1000 in 2xSSC with 10% formamide	Same as for U-2 OS cells
11. Second incubation with secondary antibodies (1 mL/well)	Repeat step 10	Repeat step 10
12. Fast Wash (1 mL/well)	3×: 2xSSC	Same as for U-2 OS cells
13. Sample mounting	Mount sample with mounting media containing DAPI. Cure for 24 h prior to imaging Alternatively: Stain nucleus with DAPI (1 min, 0.5ug/mL in 2xSSC) followed up by 5 min wash with 2xSSC buffer, next mount with mounting media	Same as for U-2 OS cells

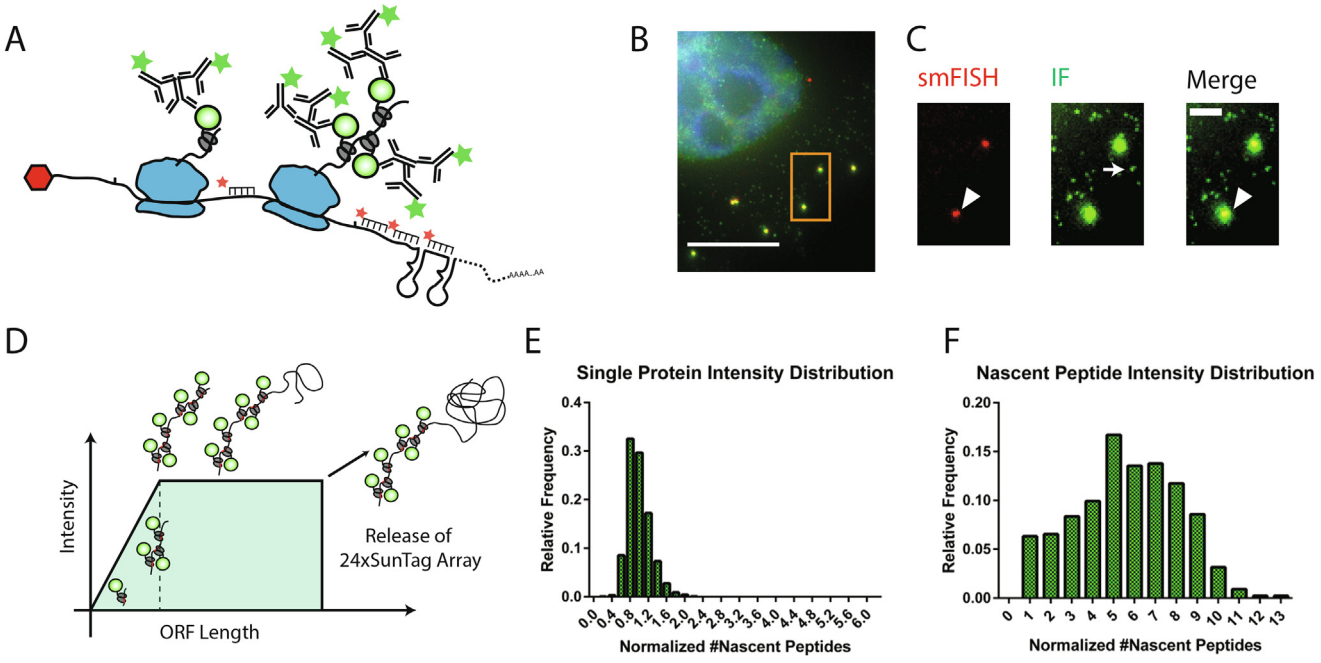

Fig. 2. smFISH-IF provides a quantitative translation readout for a population of single mRNAs. (A) smFISH-IF relies on the multivalent signal from fluorescently labeled DNA oligo probes targeting the mRNA and IF from an antibody against GFP (B) smFISH-IF performed in HeLa cells expressing a SINAPs reporter (Scale bar = 10 µm). (C) Enlarged image of box in (B) (Scale bar = 1 µm). Arrow: single protein. Arrow head: TLS (translation sites). (D–F) Analysis of translation site (TLS) intensity distribution. (D) Intensity profile of nascent peptide when the ribosome is at different positions along the mRNA. (E) Fluorescence intensity histogram of single mature proteins (IF spots not colocalized with mRNA). (F) Histogram of apparent number of ribosomes at TLS, determined by the TLS intensity normalized by the mean value of single mature proteins in Fig. 2E.

well or imaging dish). The virus mixture is added dropwise into the dish right on top of the coverslip. To evaluate the best conditions for integrating SINAPs reporter into neuronal culture, we recommend screening viral transduction conditions in U-2 OS cells prior to introducing them to neurons to check their efficiency. This can be done by infecting cells with a range of viral particles and assessing the expression level after transduction. Due to the nature of neuronal culture it is impossible to sort the cells post transduction. The viral transduction conditions have to be evaluated empirically.

- Culture neurons at least 5 days before live cell imaging or fixation. The most suitable days for performing an experiment on hippocampal neurons is between DIV 14–21.
- Prior to fixation or live cell imaging, treat neurons with 250 µM IAA from one hour to overnight to degrade the pre-existing proteins.

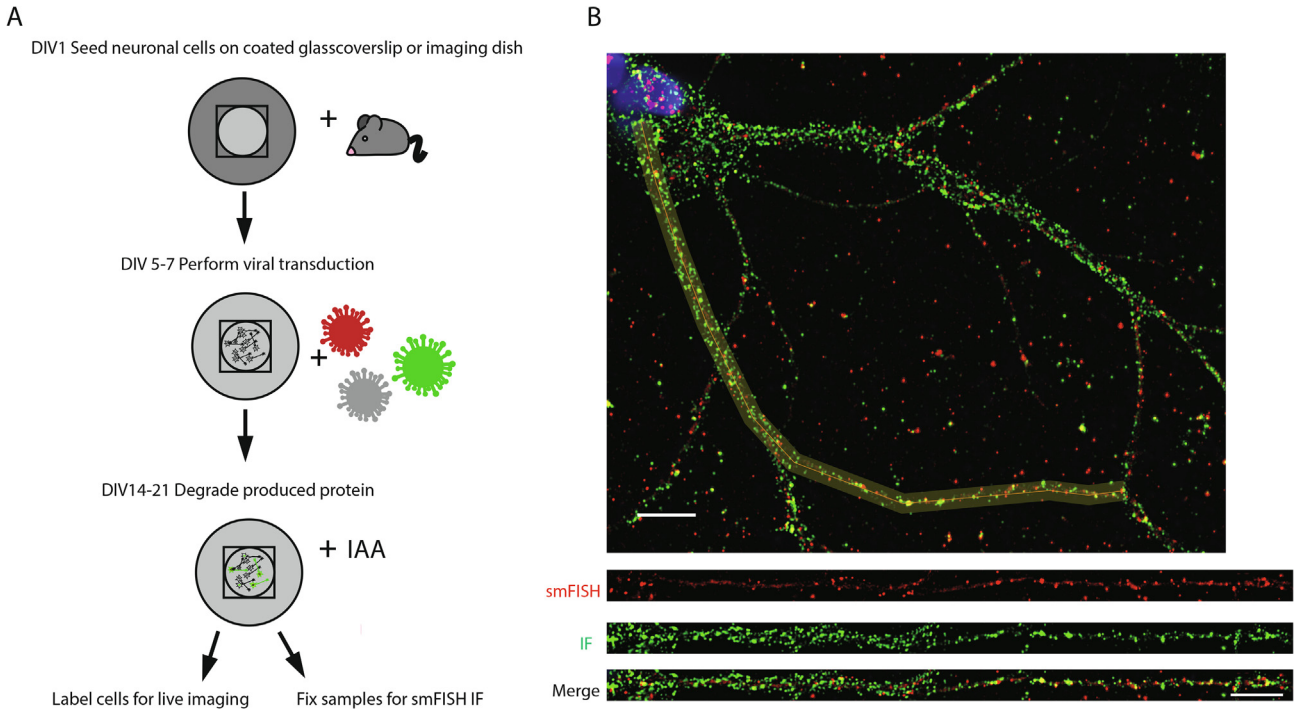

Fig. 3. SINAPs can be used in cultured primary neurons. (A) Work flow for live and fixed SINAPs experiments in neurons. (B) smFISH-IF image of infected neuron (protein channel), with selected dendrite for further analysis. Zoom in on selected dendrite which was straightened with imageJ. Green: protein (IF); red: mRNA (smFISH). Colocalization of both signals indicates a translation site (yellow, scale bar = 5 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3.3. smFISH IF protocol

U-2 OS and primary neuronal cultures require different sample preparations, however the two protocols for IF smFISH experiments are remarkably similar. Unlike U-2 OS cells, neurons should be fixed as quickly as possible after taking out from the incubator. After the quenching step samples can be carefully moved to another laboratory environment. We do not recommend moving them on ice in quenching buffer, as the samples are fragile and prone to rupture. All buffers should be well mixed prior to adding them to sample. We recommend to supplementing all buffers containing BSA with RNase inhibitor ribonucleoside vanadyl complex (RVC). Both smFISH-IF protocols are listed in Table 5.

2.4. Performing live cell SINAPs experiments

2.4.1. U-2 OS sample preparation

After establishing a host cell line expressing scFv-sfGFP, MCP-FP, and osTIR1, the workflow of live cell experiments is relatively straightforward. We use transient transfection in U-2 OS cells as an example. For stably integrated SINAPs reporter, the procedure is the same except the transient transfection step is omitted.

- On day 1, seed cells on a 35 mm, glass-bottomed imaging dish with 2 mL medium.
- On day 2, 24 h after seeding, transfect 1 μg of SINAPs mRNA reporter using X-tremeGENE 9 transfection reagent following the manufacturers protocol.
- 6 h following transfection, add IAA to a final concentration of 500 μM. IAA stock are prepared as 250 mM in ethanol.
- We label MCP-Halotag 24 h after transfection using a JaneliaFluor® dye. Remove 94 μL of medium from the imaging dish and add 2 μL 100 μM JF-646 (or 10 μM JF549) and 4 μL fresh 250 mM IAA. Add back the 100 μL solution and place the sample back in the incubator for 30 min. Wash the sample 3× with warm DMEM and allow the sample to incubate for at least one hour.
- When the incubation period is complete, change the medium to phenol red free Leibovitz-15 medium + 10% FBS (L-15) and add 4 μL of 250 mM IAA and bring the sample to the microscope. Other sodium bicarbonate buffered medium such as DMEM without phenol red can be substituted if the imaging conditions allow for CO_2 incubation.

2.4.2. Primary neuronal culture sample preparation

To ensure continued degradation of completed protein, medium in all the following steps should be supplemented with 250 μM IAA. IAA should be first diluted into 100 μL of original culture medium and added to culture dropwise (final concentration of 250 μM). During all washing steps, gently remove or apply solutions by placing the pipet tip on the wall of imaging dish without perturbing the neurons. The following protocol assumes that the sample is in a round 14 mm glass-bottom imaging dish with 2 mL culture medium.

- Prepare tube with 1 mL of fresh neurobasal phenol free medium, warm it up and re-suspend IAA and JF-Halo dye.
- Take out 1 mL of the original conditioned medium from the imaging dish, set aside. Add the 1 mL medium prepared above.
- Incubate cells in 37 °C for 30 min.
- Wash out the residual dye with fresh warm neurobasal medium 3 times.
- Incubate the neuron with 1 mL Neurobasal medium plus the 1 mL condition medium set aside previously in the incubator for at least one hour. The medium should not have phenol red, which increases the background noise [36].
- Image the neuron on microscope with incubator with temperature set at 37 °C and supplied with 5% CO2.

2.4.3. Microscopy setup

A microscope with single molecule sensitivity is required for SINAPs. A widefield microscope equipped with high-numerical aperture objective, sensitive EMCCD or sCMOS camera is recommended

[37]. To image freely diffusing mRNA and TLS, it is necessary to acquire both channels simultaneously. We use a dual-camera system for simultaneous exposure. A less expensive alternative is to use dual-view imaging system with a single camera. Depending on the choice of Halo-ligand, the mRNA can be imaged in either red or far red channels while the translation site is imaged in the green channel. To minimize chromatic aberration between colors, it is essential to use an apochromatically corrected objective lens. Because single mRNAs are diffraction limited spots, the pixel size needs to be at most half of the point spread function to satisfy the Nyquist sampling criterion. Additionally, it is important to maintain a humidified imaging environment at 37 °C.

When imaging plasma membrane tethered TLS, it is advantageous to use total internal reflection microscopy (TIRFM) [37,38]. Because the mRNAs are relatively stationary, they do not need to be imaged as frequently as freely diffusing molecules, thereby reducing photobleaching and phototoxicity. Additionally, restricted mRNA movement allows for the use of extended exposure times, reducing the required laser power and increasing the photon counts of *bona fide* localizations while decreasing the relative photon counts from rapidly diffusing, unbound particles. Typical illumination power ranges from 0.03 to 0.6 mW laser illumination, as measured at the objective lens, for both the mRNA and nascent peptide channels and exposure times can vary from 50 ms to 500 ms.

3. Data analysis

3.1. Interpretation of translation sites from fixed cell experiments

The mRNA and translation sites detected by smFISH-IF are diffraction limited spots. To detect the position and the fluorescence intensity, the fluorescence image is fitted to the point spread function (PSF), typically approximated as a Gaussian function (for details, see review [39]). We use two Matlab programs that are freely available online (Airlocalize and FISH-Quant [40,41]). The positions and intensity values of mRNA and TLS should be determined independently in each channel.

The positions of mRNA and TLS are used for colocalization analysis to match them with one another. We use the nearest neighbor approach to assign colocalized particles. This algorithm matches particles by minimizing the distance between colocalized particles in each channel. If there are many completed proteins in the cytoplasm, it is important to include an intensity threshold to eliminate false-positive translation sites. When colocalizing diffraction limited spots in different channels, chromatic aberration must be taken into account. Even with a Plan Apo corrected lens, there may still be a significant pixel shift between colors, especially at the edge of the field of view. To correct this, we perform calibration by imaging multicolor 100 nm TetraSpeck beads [35]. A transformation matrix is calculated assuming that the beads in each channel are perfectly colocalized [35]. Using this technique, the colocalization between the smFISH and IF signals can be determined with 30 nm precision [42,43]. This level of two color co-localization precision can be achieved on a conventional widefield microscopy setup.

Because most but not all of the completed SINAPs protein is degraded in the auxin degradation system, individually labeled scFv-sfGFP signals can be readily distinguished in the cytoplasm. Therefore, the IF detection channel will contain a bimodal intensity distribution consisting of the relatively dim single SINAPs proteins (Fig. 2e) and the generally more intense translation site signal (Fig. 2f). By fitting the intensity peak corresponding to the distribution of completed single proteins, we can calculate the number of nascent peptides present in the translation sites. However, because some of the ribosomes have not translated all the way through the SunTag array, we must correct the normalized translation site intensity to estimate the number of nascent peptides (Fig. 2d). If we assume that ribosomes are evenly distributed along the mRNA, we can estimate,

$$\#\ Ribosomes = \frac{N}{N - n/2}\frac{I_{TLS}}{I_{Single}}$$

where N is the total length of the protein, n is the SunTag length, I_{Single} is the intensity of a single protein and I_{TLS} is the intensity of the translation site. The assumption of even ribosome distribution may break down, for example when ribosomes are stalled, and only serves as a first order approximation of the number of nascent peptides associated with an mRNA [9].

3.2. Specific considerations for analysis of smFISH-IF in neurons

Neurons have an elaborate dendritic arbor. To analyze local translation of mRNA, it is important to measure the distance of translation sites to the soma. One can simply use the Straighten plug-in from ImageJ to straighten the dendrites starting from soma (Fig. 3b). The transformed images are analyzed by spot detection programs discussed previously. The drawback is that the point spread function might be altered during the straightening transformation. Alternatively, the distances of mRNA to soma can be calculated directly using morphological image analysis. To do that, the dendrite is outlined manually as the region of interest (ROI) starting from the soma. Morphological transformation of the dendrites will find the center of the dendrites as the "skeleton", from which the distance can be measured (Matlab function bwdistgeodesic).

3.3. Interpretation and data analysis for live cell experiments

We extract the position and intensity of both the mRNA and nascent peptide channels separately using single particle detection and tracking software (Airlocalize [41] for particle detection and u-track [44] for tracking) (Fig. 4a). This algorithm offers good performance when compared to multiple hypothesis tracking algorithms for heterogeneous diffusing particles and allows temporary track disappearance. After the tracks are defined, the mRNA and TLS are linked with custom MATLAB software (Fig. 4b).

Since the fluorescence intensity of TLS encodes the ribosome kinetics of the mRNA, SINAPs can be used to measure the translation elongation rate directly in live cells. The intensity of the translation site depends on the number of ribosomes and their locations on the mRNA. Each time a newly synthesized epitope leaves the ribosome exit tunnel, it is recognized by a scFv-sfGFP antibody and TLS intensity increases by one unit of GFP intensity until all epitopes are synthesized. This relationship can be used to measure the kinetics of a ribosome translating the SINAPs mRNA [9,45].

The TLS intensity of SINAPs is the convolution of nascent peptides originating from multiple ribosomes positioned along a given mRNA. One way to de-convolute these signals and extract kinetic parameters is to apply the principles of signal theory and autocorrelation (Fig. 4c). In simplistic terms, the autocorrelation function $G(\tau)$ is defined as the overlap between a signal with itself after an imposed time delay τ. This type of analysis has previously been established in the transcription field [31,46]. This analysis has also been generalized to fluorescence fluctuations originating of translations sites to yield initiation and elongation rates [9,31,45,46]. Autocorrelation analysis can account for cell to cell variability in scFv labeling because the fluctuation is normalized by the average intensity. The experimentally measured autocorrelation function is fit by a theoretical model describing the translation dynamics. The mathematical model and fitting is comprehensively covered in [9,31].

One approach to extract the kinetic parameter is fluorescence recovery after photobleaching (Fig. 4d). The TLS is bleached using focused laser light. As new ribosomes are loaded and the existing ones are translating further along the mRNA, new SunTag epitopes are produced, the TLS spot intensity recovers to its steady state intensity value. To track mRNA and TLS over the recovery time scale, the ER targeted or

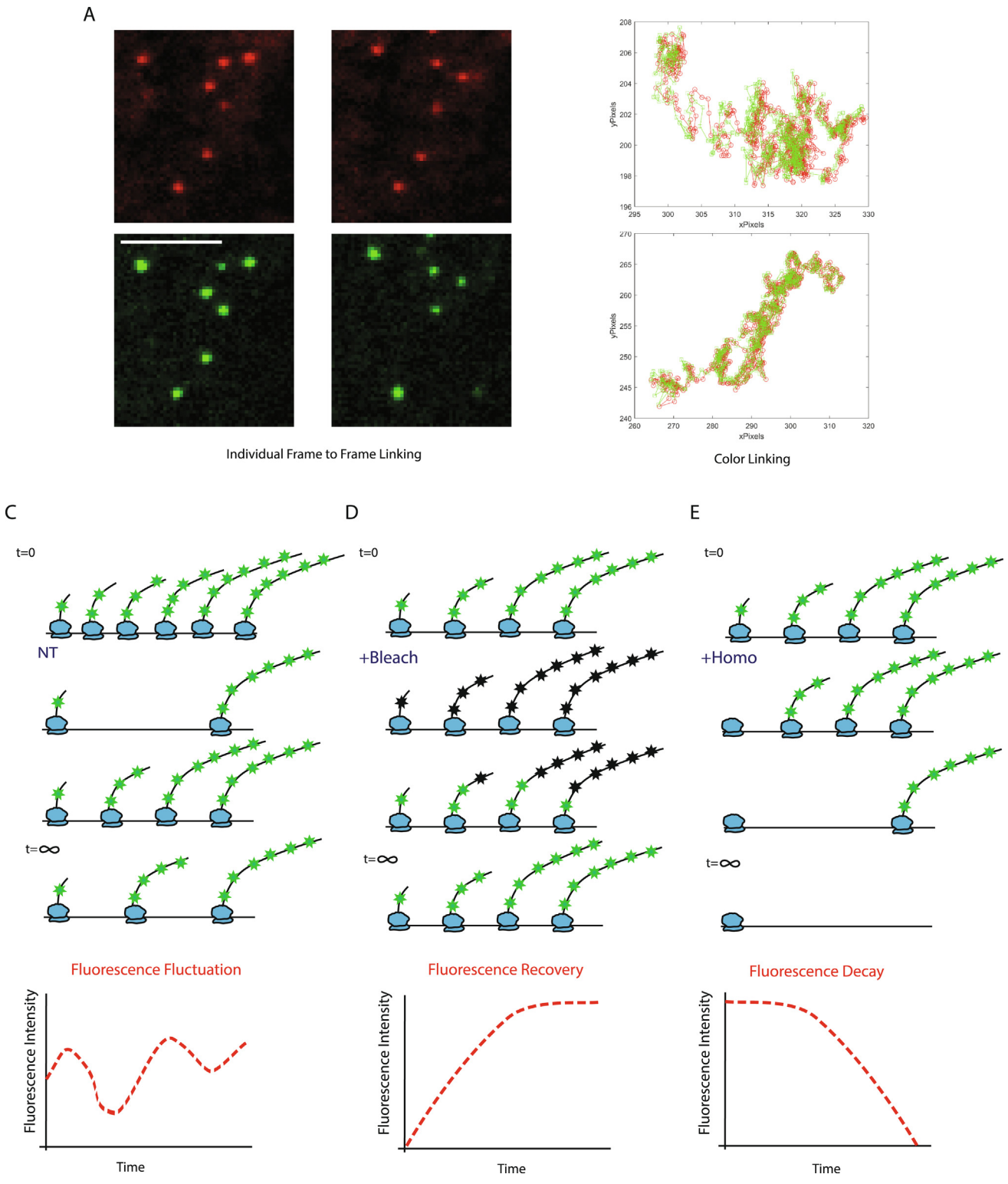

Fig. 4. Live cell SINAPs experiments to extract translation kinetic parameters. (A-B) mRNA (red) and TLS (green) are detected and tracked independently. The tracks in each channel are linked according to their spatial proximity (Scale bar = 5 μm). (C) SINAPs fluorescence intensity encodes translational kinetic parameters. As the mRNA is being translated, different number of ribosomes will be occupying it at each time point, causing a fluctuation of intensity. Autocorrelation analysis can extract kinetic parameters from the fluctuating TLS intensity. (D) Fluorescence recovery after photobleaching (FRAP). After illuminated with focused laser light, the GFP at the translation site are bleached. As existing ribosomes continue translating and new ribosomes initiate, new SunTag epitopes are synthesized, and the intensity of TLS recovers to the steady state level. (E) Runoff assay. Treatment with homoharringtonine stalls ribosomes at the first peptidyl transfer event but does not affect ribosomes already in the elongation stage. These remaining ribosomes will continue to translate and "run off" until termination. The elongation and termination rates can be measured with the run-off assay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

membrane tethered SINAPs reporters should be used. The experimental FRAP curve is fit to a theoretical equation to extract the elongation speed [9].

Another approach to measure elongation speed is to use ribosome runoff [7,10]. Cells are treated with the Homoharringtonine, which stalls ribosomes in the first round of peptide bond formation, effectively blocking initiation, but does not influence ribosomes in the elongation stage (Fig. 4e) [47]. As ribosomes finish translation and dissociate from mRNA, the TLS intensity gradually decreases. The fluorescence decay curve is fit to a kinetic model to extract translation elongation speed [7].

All of the above data analysis approaches must have theoretical modeling of translation dynamics. There are explicit and implicit assumptions built into these models and the limitations for interpreting the data must be carefully evaluated. For example, ribosomes are assumed to be statistically independent and one does not influence the other. This is valid only when the initiation rate is very low and the ribosome density along the mRNA is small. As more ribosomes pack on mRNA, they inevitably collide with one another and the assumption will break down. Another common assumption is that ribosomes initiate and translate with a constant rate. The assumption should be taken as a first order approximation, as many experiments have demonstrated the variability in initiation and elongation dynamics. For example, the observation of translational bursting indicates that the assumption of constant initiation rate indeed requires refinement [7,9]. Therefore, the measured speed should be interpreted as a coarse grained average value.

As the time to reliably track single mRNAs increases, more complicated translation phenomena such as bursting and non-equilibrium behavior will emerge, which calls for an updated theory to model ribosome dynamics in live cells. These models must take into account events such as ribosome interdependence and non-uniform ribosome distribution. Two current theoretical models with analytical solutions may provide the mathematical framework to describe these novel behaviors.

Translation involves three steps: initiation, elongation, and termination. Ribosomes move unidirectionally from 5′ to 3′ of mRNA, and can translocate only if there is no other ribosome present in the upcoming position. Mathematically, this is modeled as a totally asymmetric simple exclusion process (TASEP) [48]. TASEP analysis has been applied theoretically to study translation dynamics, even taking into account the ribosome size, pausing due to non-optimal codons, and mRNA looping [49–51]. An analytical solution to a TASEP exists for constant initiation, elongation and termination rates, which is valuable to validate simulation or approximation. Additional work must be done to account for an initiation rate that varies with time. This approach is computationally intensive and approximation is needed for effective data analysis. Ribosome flow model (RFM), incorporating aspects of TASEP, represents a deterministic and computationally tractable method to describe translational dynamics [52]. However, work is needed to use it to describe experimentally measurable quantities, such as the autocorrelation function or FRAP curve.

4. Concluding remarks

Translation has been mainly characterized by biochemical and structural assays, which have elucidated the roles of key players and molecular mechanisms. SINAPs provides a complimentary framework to address the spatiotemporal translation kinetics under physiological conditions. Significant questions remain unknown regarding the origins of stochastic translational control, such as translational bursting and its physiological role governing gene expression. Further theoretical work and assay refinement are needed to use SINAPs to answer these previously intractable questions in the central dogma.

5. Useful links

- U-track SPT program (https://github.com/DanuserLab/u-track)
- FISH-Quant (https://bitbucket.org/muellerflorian/fish_quant/src)
- Airlocalize (http://www.timotheelionnet.net/software/)

Acknowledgements

M.J.L, N.M.L and B.W. were supported by NSF Grant 129611. M.J.L. was supported by NIH Training Grant T32 GM 8403-28. N.M.L. was supported by NIH Training Grant T32 GM007445. B.W was supported by Pew Charitable Trust.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2019.03.021.

References

[1] B. Schwanhausser, D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen, M. Selbach, Global quantification of mammalian gene expression control, Nature 473 (7347) (2011) 337–342.
[2] N. Sonenberg, A.G. Hinnebusch, Regulation of translation initiation in eukaryotes: mechanisms and biological targets, Cell 136 (4) (2009) 731–745.
[3] C.E. Holt, E.M. Schuman, The central dogma decentralized: new perspectives on RNA function and local translation in neurons, Neuron 80 (3) (2013) 648–657.
[4] N.T. Ingolia, S. Ghaemmaghami, J.R.S. Newman, J.S. Weissman, Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling, Science 324 (5924) (2009) 218–223.
[5] R.A. Marshall, C.E. Aitken, M. Dorywalska, J.D. Puglisi, Translation at the single-molecule level, Annu. Rev. Biochem. 77 (2008) 177–203.
[6] J. Frank, R.L. Gonzalez, Structure and dynamics of a processive Brownian motor: the translating ribosome, Annu. Rev. Biochem. 79 (79) (2010) 381–412.
[7] X. Yan, T.A. Hoek, R.D. Vale, M.E. Tanenbaum, X. Yan, T.A. Hoek, R.D. Vale, M.E. Tanenbaum, Dynamics of translation of single mRNA molecules in vivo resource dynamics of translation of single mRNA molecules in vivo, Cell 165 (4) (2016) 976–989.
[8] T. Morisaki, K. Lyon, K.F. DeLuca, J.G. DeLuca, B.P. English, Z.J. Zhang, L.D. Lavis, J.B. Grimm, S. Viswanathan, L.L. Looger, T. Lionnet, T.J. Stasevich, Real-time quantification of single RNA translation dynamics in living cells, Science 352 (6292) (2016) 1425–1429.
[9] B. Wu, C. Eliscovich, Y.J. Yoon, R.H. Singer, Translation dynamics of single mRNAs in live cells and neurons, Science 352 (6292) (2016) 1430–1435.
[10] C. Wang, B. Han, R. Zhou, X. Zhuang, Real-time imaging of translation on single mRNA transcripts in live cells, Cell (2016).
[11] X. Pichon, A. Bastide, A. Safieddine, R. Chouaib, A. Samacoits, E. Basyuk, M. Peter, F. Mueller, E. Bertrand, Visualization of single endogenous polysomes reveals the dynamics of translation in live human cells, J. Cell Biol. 214 (6) (2016) 769–781.
[12] E. Bertrand, P. Chartrand, M. Schaefer, S.M. Shenoy, R.H. Singer, R.M. Long, Localization of ASH1 mRNA particles in living yeast, Mol. Cell 2 (4) (1998) 437–445.
[13] E. Tutucci, N.M. Livingston, R.H. Singer, B. Wu, Imaging mRNA in vivo, from birth to death, Annu. Rev. Biophys. (2018).
[14] M.E. Tanenbaum, L.A. Gilbert, L.S. Qi, J.S. Weissman, R.D. Vale, A protein-tagging system for signal amplification in gene expression and fluorescence imaging, Cell 159 (3) (2014) 635–646.
[15] A.J. Holland, D. Fachinetti, J.S. Han, D.W. Cleveland, Inducible, reversible system for the rapid and complete degradation of proteins in mammalian cells, Proc. Natl. Acad. Sci. USA 109 (49) (2012) E3350–E3357.
[16] K. Nishimura, T. Fukagawa, H. Takisawa, T. Kakimoto, M. Kanemaki, An auxin-based degron system for the rapid depletion of proteins in nonplant cells, Nat. Methods 6 (12) (2009) 917–U78.
[17] J.A. Chao, Y. Patskovsky, S.C. Almo, R.H. Singer, Structural basis for the coevolution of a viral RNA-protein complex, Nat. Struct. Mol. Biol. 15 (1) (2008) 103–105.
[18] S. Hocine, P. Raymond, D. Zenklusen, J.A. Chao, R.H. Singer, Single-molecule analysis of gene expression using two-color RNA labeling in live yeast, Nat. Methods 10 (2) (2013) 119–121.
[19] B. Wu, J.A. Chao, R.H. Singer, Fluorescence fluctuation spectroscopy enables quantitative imaging of single mRNAs in living cells, Biophys. J . 102 (12) (2012) 2936–2944.
[20] F. Lim, T.P. Downey, D.S. Peabody, Translational repression and specific RNA binding by the coat protein of the Pseudomonas phage PP7, J. Biol. Chem. 276 (25) (2001) 22507–22513.
[21] K. Valegard, J.B. Murray, P.G. Stockley, N.J. Stonehouse, L. Liljas, Crystal structure of an RNA bacteriophage coat protein-operator complex, Nature 371 (6498) (1994) 623–626.
[22] B. Wu, V. Miskolci, H. Sato, E. Tutucci, C.A. Kenworthy, S.K. Donnelly, Y.J. Yoon, D. Cox, R.H. Singer, L. Hodgson, Synonymous modification results in high-fidelity gene expression of repetitive protein and nucleotide sequences, Genes Dev. 29 (8)

(2015) 876–886.
[23] E. Tutucci, M. Vera, J. Biswas, J. Garcia, R. Parker, R.H. Singer, An improved MS2 system for accurate reporting of the mRNA life cycle, Nat. Methods 15 (1) (2018) 81–+.
[24] G.V. Los, L.P. Encell, M.G. McDougall, D.D. Hartzell, N. Karassina, C. Zimprich, M.G. Wood, R. Learish, R.F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D.H. Klaubert, R.F. Bulleit, K.V. Wood, HaloTag: a novel protein labeling technology for cell imaging and protein analysis, ACS Chem. Biol. 3 (6) (2008) 373–382.
[25] J.B. Grimm, B.P. English, H. Choi, A.K. Muthusamy, B.P. Mehl, P. Dong, T.A. Brown, J. Lippincott-Schwartz, Z. Liu, T. Lionnet, L.D. Lavis, Bright photoactivatable fluorophores for single-molecule imaging, Nat. Methods 13 (12) (2016) 985–988.
[26] J.B. Grimm, B.P. English, J. Chen, J.P. Slaughter, Z. Zhang, A. Revyakin, R. Patel, J.J. Macklin, D. Normanno, R.H. Singer, T. Lionnet, L.D. Lavis, A general method to improve fluorophores for live-cell and single-molecule microscopy, Nat. Methods 12 (3) (2015) 244–250 3 p following 250.
[27] L.M. Costantini, M. Fossati, M. Francolini, E.L. Snapp, Assessing the tendency of fluorescent proteins to oligomerize under physiologic conditions, Traffic 13 (5) (2012) 643–649.
[28] S. Ruijtenberg, T.A. Hoek, X. Yan, M.E. Tanenbaum, Imaging translation dynamics of single mRNA molecules in live cells, Methods Mol. Biol. 1649 (2018) 385–404.
[29] G. Mostoslavsky, A.J. Fabian, S. Rooney, F.W. Alt, R.C. Mulligan, Complete correction of murine Artemis immunodeficiency by lentiviral vector-mediated gene transfer, PNAS 103 (44) (2006) 16406–16411.
[30] H. Miyoshi, M. Takahashi, F.H. Gage, I.M. Verma, Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector, Proc. Natl. Acad. Sci. U.S.A. 94 (19) (1997) 10319–10323.
[31] A. Coulon, D.R. Larson, Fluctuation analysis: dissecting transcriptional kinetics with signal theory, Methods Enzymol. 572 (2016) 159–191.
[32] M. Kumar, B. Keller, N. Makalou, R.E. Sutton, Systematic determination of the packaging limit of lentiviral vectors, Hum. Gene Ther. 12 (15) (2001) 1893–1905.
[33] A.M. Femino, K. Fogarty, L.M. Lifshitz, W. Carrington, R.H. Singer, Visualization of single molecules of mRNA in situ, Method Enzymol 361 (2003) 245–304.
[34] A.M. Femino, F.S. Fay, K. Fogarty, R.H. Singer, Visualization of single RNA transcripts in situ, Science 280 (5363) (1998) 585–590.
[35] C. Eliscovich, S.M. Shenoy, R.H. Singer, Imaging mRNA and protein interactions within neurons, PNAS (2017) 201621440.
[36] M. Stadtfeld, F. Varas, T. Graf, Fluorescent protein-cell labeling and its application in time-lapse analysis of hematopoietic differentiation, Methods Mol. Med. 105 (2005) 395–412.
[37] Z. Liu, L.D. Lavis, E. Betzig, Imaging live-cell dynamics and structure at the single-molecule level, Mol. Cell 58 (4) (2015) 644–659.
[38] D. Axelrod, T.P. Burghardt, N.L. Thompson, Total internal reflection fluorescence, Annu. Rev. Biophys. Bioeng. 13 (1984) 247–268.
[39] B. Zhang, J. Zerubia, J.C. Olivo-Marin, Gaussian approximations of fluorescence microscope point-spread function models, Appl. Opt. 46 (10) (2007) 1819–1829.
[40] F. Mueller, A. Senecal, K. Tantale, H. Marie-Nelly, N. Ly, O. Collin, E. Basyuk, E. Bertrand, X. Darzacq, C. Zimmer, FISH-quant: automatic counting of transcripts in 3D FISH images, Nat. Methods 10 (4) (2013) 277–278.
[41] T. Lionnet, K. Czaplinski, X. Darzacq, Y. Shav-Tal, A.L. Wells, J.A. Chao, H.Y. Park, V. de Turris, M. Lopez-Jones, R.H. Singer, A transgenic mouse for in vivo detection of endogenous labeled mRNA, Nat. Methods 8 (2) (2011) 165–170.
[42] S. Adivarahan, N. Livingston, B. Nicholson, S. Rahman, B. Wu, O.S. Rissland, D. Zenklusen, Spatial organization of single mRNPs at different stages of the gene expression pathway, Mol. Cell 72 (4) (2018) 727–+.
[43] A. Khong, R. Parker, mRNP architecture in translating and stress conditions reveals an ordered pathway of mRNP compaction, J. Cell Biol. 217 (12) (2018) 4124–4140.
[44] K. Jaqaman, D. Loerke, M. Mettlen, H. Kuwata, S. Grinstein, S.L. Schmid, G. Danuser, Robust single-particle tracking in live-cell time-lapse sequences, Nat. Methods 5 (8) (2008) 695–702.
[45] D.R. Larson, D. Zenklusen, B. Wu, J.A. Chao, R.H. Singer, Real-time observation of transcription initiation and elongation on an endogenous yeast gene, Science 332 (6028) (2011) 475–478.
[46] D.R. Larson, C. Fritzsch, L. Sun, X.H. Meng, D.S. Lawrence, R.H. Singer, Direct observation of frequency modulated transcription in single cells using light activation, eLife 2 (2013).
[47] M. Fresno, A. Jimenez, D. Vazquez, Inhibition of translation in eukaryotic systems by Harringtonine, Eur. J. Biochem. 72 (2) (1977) 323–330.
[48] B. Derrida, An exactly soluble non-equilibrium system: the asymmetric simple exclusion process, Phys. Rep. 301 (1–3) (1998) 65–83.
[49] T. Chou, G. Lakatos, Clustered bottlenecks in mRNA translation and protein synthesis, Phys. Rev. Lett. 93 (19) (2004).
[50] T. Chou, Ribosome recycling, diffusion, and mRNA loop formation in translational regulation, Biophys. J. 85 (2) (2003) 755–773.
[51] A.R. Subramaniam, B.M. Zid, E.K. O'Shea, An integrated approach reveals regulatory controls on bacterial translation elongation, Cell 159 (5) (2014) 1200–1211.
[52] S. Reuveni, I. Meilijson, M. Kupiec, E. Ruppin, T. Tuller, Genome-scale analysis of translation elongation with a ribosome flow model, PLoS Comput. Biol. 7 (9) (2011) e1002127.