Chapter 4

Measuring Bulk Translation Activity in Single Mammalian Cells During the Integrated Stress Response

Alyssa M. English and Stephanie L. Moon

Abstract

The attenuation of global translation is a critical outcome of the integrated stress response (ISR). Consequently, it is important to effectively detect and measure protein synthesis in studies seeking to evaluate the ISR. This chapter details two methods, surface sensing of translation (SUnSET) and fluorescent noncanonical amino acid tagging (FUNCAT), to measure global translation activity in individual cells using fluorescence microscopy as a read-out. Detecting bulk translation activity in single cells is advantageous for the concurrent observation of newly synthesized proteins and other cellular structures and to identify differences in translation activity among individuals within a population of cells.

Key words Translation, Puromycin, Puromycylation, Surface sensing of translation, SUnSET, Fluorescent noncanonical amino acid tagging, FUNCAT, Immunofluorescence, Click chemistry, Integrated stress response

1 Introduction

The suppression of bulk translation activity is one of the first events to occur during the integrated stress response (ISR). Stress-sensing kinases phosphorylate the major translation initiation factor eukaryotic initiation factor 2α (eIF2α), which reduces ternary complex formation and suppresses translation activity [1, 2]. Thus, protein synthesis is a critical process to monitor as a central readout of ISR activity. Several approaches are available to measure bulk translation activity at the single-cell and population levels. This chapter focuses on two separate methods, surface sensing of translation (SUnSET) [3] and fluorescent noncanonical amino acid tagging (FUNCAT) [4, 5], to quantify the translation activity of single cells by fluorescence microscopy.

Classically, protein synthesis is measured by the incorporation of radioactive amino acids, typically methionine and cysteine, into newly synthesized proteins during translation elongation. This

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powerful technique is typically used to quantify the translation activity of a population of cells, as nascent proteins from cell lysates are commonly visualized following their separation on SDS-PAGE gels [6–8] . More recently, additional methods to evaluate protein synthesis have been developed including SUnSET [3] and FUNCAT [4, 5] that permit facile detection of bulk translation activity in single cells. SUnSET and FUNCAT identify newly synthesized proteins by fluorescence-based techniques and do not require the use of radioactive materials. A primary advantage of these methods is that they detect the translation activity of single cells. This not only allows for the assessment of the subcellular location of newly synthesized proteins but also permits the simultaneous observation of newly synthesized proteins and other fluorescently labeled biomolecules or subcellular structures such as organelles. Additionally, it enables visualization of the range of translation activity within a population of cells. Thus, SUnSET and FUNCAT are advantageous methods that are highly suitable for investigating pathways that impact translation, including the ISR, in individual cells, and we detail these methods below.

SUnSET is an easily accessible, rapid, and sensitive method to measure bulk translation activity in situ [3, 7]. SUnSET utilizes puromycin, a protein synthesis inhibitor that resembles the 3′ end of an aminoacylated tyrosyl-tRNA (Fig. 1a) [9, 10]. In a process known as puromycylation, puromycin binds to the ribosomal A-site and the nascent polypeptide chain is transferred to it (Fig. 1a). This results in translation termination and release of the polypeptide chain from the ribosome (Fig. 1a) [9–12]. Puromycylated proteins can be detected by anti-puromycin antibodies and secondary antibodies labeled with fluorophores using immunofluorescence microscopy (Fig. 1a). Alternatively, newly synthesized proteins can be labeled with O-propargyl puromycin (OPP) and detected by click chemistry instead of immunofluorescence [13]; however, this approach will not be discussed in this article. The benefits of using SUnSET are that it is easy, sensitive, and fast—nascent polypeptide labeling occurs within 5 min [3]. Further, SUnSET can be combined with other methods such as immunofluorescence microscopy or fluorescent fusion protein detection to concurrently visualize bulk translation activity and subcellular structures [7, 14] or markers of specific cell or tissue types within a population [15]. However, three limitations are associated with the use of this assay to quantify protein synthesis activity. Namely, (1) puromycin disrupts translation machinery by reacting with nascent polypeptide chains, causing them to be ejected from ribosomes along with mRNA [16–18], (2) two steps are needed to detect puromycylated polypeptides, and (3) puromycylation generates truncated proteins [9, 12] that may activate protein quality control pathways or otherwise perturb cellular protein metabolism. Thus,

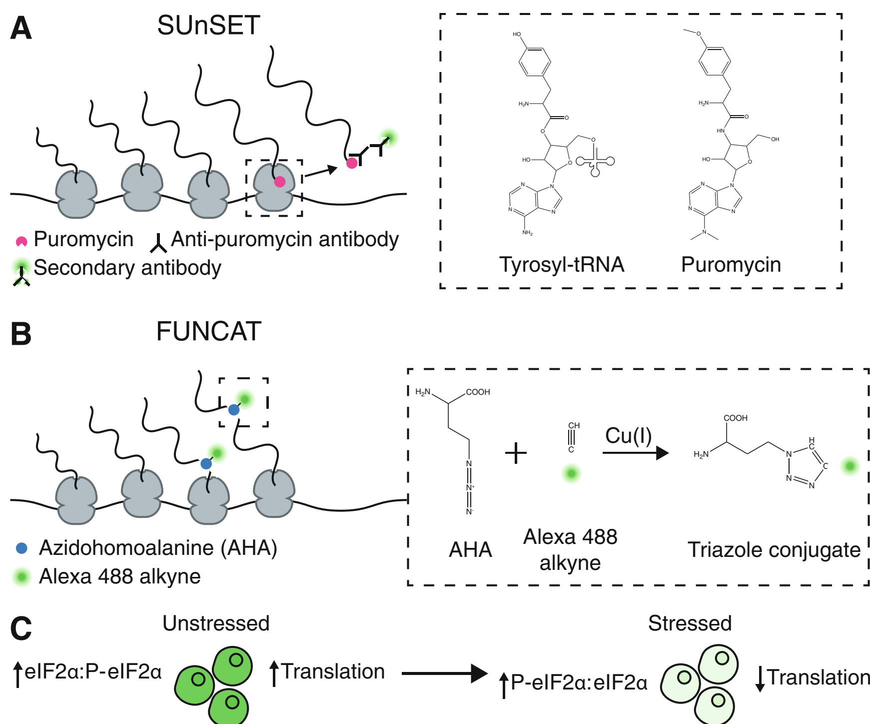

Fig. 1 Surface sensing of translation (SUnSET) and fluorescent noncanonical amino acid tagging (FUNCAT) enable visualization of nascent protein translation in situ in mammalian cells. (**a**) Puromycin is incorporated into nascent polypeptide chains and causes them to be ejected from the ribosome (left). At right, the structure of puromycin resembles the structure of the tyrosyl-tRNA, (adapted from [9, 16]). (**b**) The bioorthogonal methionine analog azidohomoalanine is incorporated into nascent polypeptide chains (left). At right, the click reaction between azidohomoalanine and Alexa 488 alkyne results in a stable triazole conjugate. (**c**) Both assays will result in a read-out of global translation activity in individual cells (green), which is reduced during the integrated stress response when the ratio of P-eIF2α:eIF2α is high

care should be taken when designing and interpreting experiments in order to appropriately use SUnSET.

FUNCAT is a second useful method to detect bulk translation activity via metabolic labeling of growing nascent polypeptide chains [4, 5]. This approach relies on the incorporation of bioorthogonal alkyne or azide-containing amino acids into newly synthesized proteins (Fig. 1b) [19]. Upon addition of an alkyne- or azide-containing fluorophore, the fluorophore covalently attaches to its complementary alkyne- or azide-containing amino acid ortholog by click chemistry (Fig. 1b). The click reaction is a simple, high-yield linking reaction that occurs rapidly at room temperature in conditions amenable to labeling and purifying biomolecules [20–22]. Nascent proteins labeled via FUNCAT are detected by fluorescence microscopy, and this method can be combined with immunofluorescence microscopy or the detection of fluorescent fusion proteins to simultaneously visualize other cellular structures

[5, 23], specific cell types [5, 24], and cells expressing certain proteins [4]. In contrast to SUnSET, FUNCAT does not interfere with the translation machinery and, as a result, does not create truncated, aberrant proteins. Three disadvantages of the FUNCAT method are: (1) it is costlier than SUnSET, (2) it requires two steps to detect newly synthesized proteins through a click reaction, and (3) similar to radiolabeling nascent proteins, FUNCAT is typically performed following a short-term amino acid deprivation period, which can increase the sensitivity of the assay at the expense of potentially altering cell metabolism. Therefore, it is important to consider the limitations of FUNCAT when designing and interpreting experiments.

Together, the SUnSET and FUNCAT fluorescence-based approaches permit a wide range of scientific questions regarding translation activity in single cells and cell populations to be explored. These methods are extensively used to investigate protein synthesis in a variety of cell types [4, 7, 13, 24–26], tissues [13, 26–29], and whole organisms [5, 16, 30]. Specifically, SUnSET and FUNCAT can be used to explore translation activity during the ISR (Fig. 1c) [7, 23]. Additionally, because puromycylation produces truncated proteins, SUnSET can be exploited to study the outcome of defective ribosomal products (DRiPs) [31, 32]. It is important to note that, although these techniques identify nascent proteins, they do not necessarily label sites of active translation [16]. This chapter will focus on the use of SUnSET and FUNCAT with a fluorescence microscopy read-out in mammalian cell culture systems to study translation activity regulation during the ISR in the context of arsenite stress.

2 Materials

2.1 SUrface SEnsing of Translation (SUnSET) Assay

1. Adherent tissue culture cells, such as U-2 OS cells.
2. Humidified cell culture incubator with 5% CO_2.
3. Biosafety cabinet.
4. Hemocytometer.
5. Light microscope with 10× objective for cell counting.
6. Trypsin–EDTA solution (0.25%).
7. Phosphate-buffered saline (PBS).
8. Cell culture maintenance medium: Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% streptomycin/penicillin, 2 mM glutamine (glutaMAX).
9. 12-well tissue culture plate(s).
10. Glass coverslips (circular #1, 18 mm diameter).
11. Sodium arsenite solution (100×): 50 mM in sterile water.

12. Puromycin (1000×): 10 μg/μL in sterile water.
13. Paraformaldehyde: 4% in PBS.
14. Triton X-100: 0.5% in PBS.
15. Bovine serum albumin (BSA): 3% in PBS.
16. Bovine serum albumin (BSA): 0.3% in PBS.
17. 12-well uncoated plate(s) (sterile).
18. Primary mouse anti-puromycin antibody (Millipore Sigma, MABE343) (1:1000).
19. Secondary anti-mouse antibody (e.g., goat anti-mouse IgG FITC, Abcam ab97022, 1:1000).
20. Glass slides.
21. VECTASHIELD® Antifade Mounting Medium with DAPI (Vector laboratories).
22. Wide-field fluorescence microscope with deconvolution software.

2.2 Fluorescent Noncanonical Amino Acid Tagging (FUNCAT) Assay

1. Adherent tissue culture cells, such as U-2 OS cells.
2. Humidified cell culture incubator with 5% CO_2.
3. Biosafety cabinet.
4. Hemocytometer.
5. Light microscope with 10× objective for cell counting.
6. Trypsin–EDTA solution (0.25%).
7. Phosphate-buffered saline (PBS).
8. Cell culture maintenance medium: Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% streptomycin/penicillin, 2 mM glutamine (glutaMAX).
9. Labeling medium: Dulbecco's Modified Eagle Medium lacking methionine, 10% dialyzed fetal bovine serum, 1% streptomycin/penicillin, 2 mM glutamine (glutaMAX).
10. 12-well tissue culture plate(s).
11. Glass coverslips (circular #1, 18 mm diameter).
12. Sodium arsenite solution (100×): 50 mM in sterile water.
13. Methionine (1000×): 50 mM in dimethyl sulfoxide.
14. Azidohomoalanine (Click-iT AHA C10102) (1000×): 50 mM in dimethyl sulfoxide.
15. Deionized sterile water.
16. Paraformaldehyde: 4% in PBS.
17. Triton X-100: 0.5% in PBS.
18. Bovine serum albumin (BSA): 3% in PBS.
19. Click-iT™ Cell Reaction Buffer Kit (Invitrogen, cat. C10269).

20. Alexa fluor 488 alkyne (1000×): 2 mM in dimethyl formamide.
21. Glass slides.
22. VECTASHIELD® Antifade Mounting Medium with DAPI (Vector laboratories).
23. Wide-field fluorescence microscope with deconvolution software.

3 Methods

All reagents and solutions should be sterile and protease-free when possible.

3.1 SUnSET Assay

1. In a biosafety cabinet, place one glass coverslip per condition (for unstressed and stressed conditions, plate two coverslips) into each well of a 12-well plate. Wash twice with 70% ethanol and once with cell culture maintenance medium.
2. Split and count cells using a hemocytometer. Resuspend cells in cell culture maintenance medium to plate them in a 1-mL volume on the glass coverslips to achieve ~50% confluency the next day. Remove any trapped air beneath the cover slip by pressing them down gently with a sterile pipette tip.
3. The next day, remove the medium and replace it with 1 mL of warm (37 °C) fresh medium without or with the stressor (e.g., 0.5 mM sodium arsenite). Incubate in the humidified cell culture incubator (5% CO_2, 37 °C) for at least 20 min to evaluate translation at 30 min post-stress, or 50 min to evaluate translation for 60 min post-stress.
4. Ten minutes prior to the desired post-stress time-point, add enough puromycin to reach 10 μg/mL (1 uL) to each well. Incubate for 10 min in the cell culture incubator (*see* **Note 1**).
5. In a fume hood, rinse the cells twice with 1 mL PBS to remove free puromycin. Add 250 μL of 4% paraformaldehyde (*see* **Note 1**) to each well and incubate for 10 min (*see* **Note 2**).
6. Rinse the cells with 1 mL PBS and permeabilize the cells with 0.5% Triton X-100 for 5 min.
7. Wash the cells with 1 mL PBS for 5 min. Incubate the cells in 3% BSA for 1 h or at 4 °C overnight (*see* **Note 3**).
8. Wash cells thrice with 1 mL PBS for 10 min each.
9. Incubate with primary antibody against puromycin (1:1000) in 0.3% BSA for 1 h at room temperature by placing coverslips upside down onto a ~100 μL droplet of antibody solution in a petri dish humidified chamber (*see* **Note 4**).

10. Place coverslips into the wells of an uncoated 12-well dish, cell-side up. Wash cells thrice with 1 mL PBS for 10 min each.
11. Incubate with secondary anti-mouse antibody (e.g., goat anti-mouse IgG FITC) at 1:1000 in 0.3% BSA for 1 h at room temperature (*see* **Note 5**).
12. Place coverslips into new wells of an uncoated 12-well dish, cell-side up. Wash cells thrice with 1 mL PBS for 10 min each.
13. Remove coverslips from the 12-well plate, and briefly dry by turning them upside down onto a fresh kimwipe. Place the dry coverslip on top of ~15 μL mounting medium (e.g., VECTA SHIELD®) containing DAPI nuclear stain on a glass slide, cell side down.
14. Allow to cure overnight in the dark.
15. Proceed to imaging and image analysis of cells, Subheading 3.3.

3.2 FUNCAT Assay

1. In a biosafety cabinet, place one glass coverslip per condition (for one unstressed and one stressed condition, plate three coverslips to include a methionine labeling unstressed control, **Note 6**) into each well of a 12-well plate. Wash coverslips twice with 70% ethanol and once with maintenance medium.
2. Split and count cells using a hemocytometer. Resuspend cells in cell culture maintenance medium to plate them in a 1 mL volume on the glass coverslips to achieve ~50% confluency the next day. Remove any trapped air beneath the cover slip by pressing them down gently with a sterile pipette tip.
3. Prepare and store Click-iT™ Cell Reaction Buffer and Click-iT™ Cell Buffer Additive according to the Click-iT™ Cell Reaction Buffer Kit manufacturer's instructions.
4. The next day, replace the cell culture maintenance medium with 1 mL pre-warmed (37 °C) labeling medium. Incubate for 30 min in the cell culture incubator.
5. Replace labeling medium with at least 0.5 mL fresh pre-warmed (37 °C) labeling medium containing either azido-homoalanine (0.5 μL of 1000× stock per dish) or methionine (0.5 μL of 1000× stock per dish) in the presence or absence of stressor (e.g., 0.5 mM sodium arsenite) (*see* **Note 1**). Incubate in the cell culture incubator for 30 min to 1 h (*see* **Note 7**).
6. In a fume hood, remove the medium and replace with ~250 μL paraformaldehyde (*see* **Note 1**). Incubate for 10 min.
7. Wash cells once with PBS.
8. Permeabilize cells with Triton X-100 (0.5% in PBS) for 5 min.
9. Incubate cells with 3% BSA in PBS for 10 min.

10. While cells are incubating (**step 9**), prepare a 1.5 mL master mix of Click-iT™ cell reaction cocktail by combining 1.32 mL 1× Click-iT™ reaction buffer, 30 μL copper sulfate, 150 μL cell buffer additive, and 1.5 μL of Alexa Fluor 488 alkyne.
11. Incubate the cells under 0.5 mL each of Click-iT™ reaction cocktail for 30 min protected from light with rocking (*see* **Note 8**).
12. Incubate cells for 10 min with 3% BSA in PBS (*see* **Note 9**).
13. Mount coverslips by first drying by gently placing upside down on a kimwipe, and placing onto a droplet (~15 μL) of mounting medium (e.g., VECTASHIELD® plus DAPI nuclear stain).
14. Allow samples to cure overnight in the dark.
15. Proceed to imaging and image analysis, *see* Subheading 3.3.

3.3 Imaging and Image Analysis

Samples can be visualized using widefield fluorescence microscopy such as with a DeltaVision Elite microscope equipped with a 100× objective and a PCO Edge sCMOS camera. Choose a system with an appropriate wavelength of light for excitation and mirrors/filters to detect emission of selected fluors, e.g., in the FITC channel.

1. Determine imaging parameters (i.e., exposure time and intensity) empirically to obtain fluorescence read-outs of all samples within the dynamic range, and avoid over-saturated pixels in micrographs of cells with high FUNCAT or SUnSET labeling.
2. Determine the number of Z sections and step sizes empirically. For U2-OS cells, 15 Z sections at 0.2 μm intervals are adequate to span the entire cell volume.
3. Acquire photomicrographs using the same imaging parameters for each sample.
4. Deconvolve image data using a non-destructive method, such as the softWoRx DeltaVision software.
5. The open-source software Fiji/ImageJ [33, 34] can be used to create maximum intensity projections. Use the Fiji/ImageJ Analyze and Set Measurements tools to obtain pixel intensities in cells demarcated as regions of interest (ROI) to obtain a read-out of relative translation activity in individual cells. Useful resources for Fiji/ImageJ analysis are the online forum https://forum.image.sc/ and the website https://imagej.net/ImageJ.

4 Notes

1. Puromycin-containing medium should be inactivated by autoclaving or properly disposed of as a toxic chemical product

[35]. Arsenite and paraformaldehyde solutions must be properly disposed of as hazardous chemical waste.

2. Incubations are performed at room temperature unless otherwise noted.
3. Incubations can be either at 1 h room temperature or overnight at 4 °C. Overnight incubations should be done by sealing the coverslips in a humidified chamber to reduce evaporation. Samples stored in 12-well plates can be humidified by wrapping them in wet paper towels and covering in plastic wrap. Alternatively, a petri dish incubator can be assembled to reduce the amount of antibody solution needed (*see* **Note 4**).
4. Assemble a petri dish humidifier by placing a layer of 3–5 paper towels cut to fit inside the bottom dish. Wet the paper towels with sterile water, and place a piece of parafilm cut such that it is slightly smaller than the paper towel on top. Mark the parafilm at the edges with a marker to identify samples. Gently place the coverslip onto a droplet of antibody solution (100 μL). Close the petri dish and seal with parafilm if incubating overnight.
5. It is not advised to incubate samples in secondary antibody overnight.
6. A methionine control is highly recommended. This control condition is fed methionine instead of azidohomoalanine and should be used to determine the non-specific fluorescence contribution to observed fluorescence intensities in FUNCAT conditions.
7. For brevity, we describe FUNCAT using azidohomoalanine and the fluor-labeled alkyne Alexa Fluor 488 alkyne. However, other bioorthogonal amino acids such as L-homopropargylglycine, and other fluor-labeled alkynes such as Alexa Fluor 594 alkyne, are available.
8. Copper sulfate must be properly disposed of as a toxic chemical product.
9. To perform FUNCAT labeling and immunofluorescence microscopy, proceed after Subheading 3.2.11 to the primary antibody incubation step using a standard immunofluorescence labeling protocol.

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