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Assessment of de novo Protein Synthesis Rates in *Caenorhabditis elegans*

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Abstract

Maintaining a healthy proteome is essential for cell and organismal homeostasis. Perturbation of the balance between protein translational control and degradation instigates a multitude of age-related diseases. Decline of proteostasis quality control mechanisms is a hallmark of ageing. Biochemical methods to detect de novo protein synthesis are still limited, have several disadvantages and cannot be performed in live cells or animals. *Caenorhabditis elegans,* being transparent and easily genetically modified, is an excellent model to monitor protein synthesis rates by using imaging techniques. Here, we introduce and describe a method to measure de novo protein synthesis in vivo utilizing fluorescence recovery after photobleaching (FRAP). Transgenic animals expressing fluorescent proteins in specific cells or tissues are irradiated by a powerful light source resulting in fluorescence photobleaching. In turn, assessment of fluorescence recovery signifies new protein synthesis in cells and/or tissues of interest. Hence, the combination of transgenic nematodes, genetic and/or pharmacological interventions together with live imaging of protein synthesis rates can shed light on mechanisms mediating age-dependent proteostasis collapse.

Introduction

Protein synthesis and degradation is essential for organismal homeostasis. A multitude of age-related diseases are instigated by defective protein production[1,2]. In order to measure global protein translation rates, there are biochemical techniques such as ribosomal profiling, which involves deep sequencing of ribosome-protected mRNA fragments to monitor expression as well as novel protein synthesis[3]. This method, besides being an indirect readout of translational rates, as increased RNA association to ribosomes does not necessarily mean increased translation, has technical disadvantages, such as high cost and a requirement of a large amount of starting material. On the other hand, proteomics-based methods allow for direct protein quantification by pulse metabolic profiling followed by mass spectrometry analysis[4,5]. However, this is a semi-quantitative approach with limited temporal resolution that

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cannot be easily used in vivo. Moreover, labelling of the protein can be unequally distributed in the animal/tissue of interest. Importantly, both these methods can conceal tissue-specific or cell-specific variations in protein translation rates in whole animals or tissues, respectively.

Caenorhabditis elegans is an easy-to-use model organism that can be grown in large numbers[6]. Additionally, its genetic amenability and transparency allow for live imaging in vivo. This protocol describes the methodology to detect protein synthesis rates using fluorescence recovery after photobleaching (FRAP). We take advantage of the transgenic expression of fluorescent proteins, either in the whole organism or in specific tissues/cells. Transgenic animals may either express GFP using the promoter of a gene, which is widely/globally expressed or a tissue-specific promoter to target specific cell types. This technique can be extended to a specific promoter to examine protein synthesis rates of a specific protein.

Protocol

NOTE: Both transcriptional and translational fusions to fluorophores can be used to evaluate de novo protein translation in several tissues. Protein synthesis rates can be assessed for multiple protein families that localized in different cellular compartments, including cytoplasm, mitochondria and nucleus[7]. The following strains are used to monitor global and neuronal protein synthesis rates, N2;*Ex*[p$_{ife-2}$GFP, pRF4], *ife-2(ok306)*;*Ex*[p$_{ife-2}$GFP, pRF4], N2;*Ex*[p$_{unc-119}$GFP, pRF4], *edc-3(ok1427)*;*Ex*[p$_{unc-119}$GFP, pRF4], N2;*Ex*[p$_{sod-3}$GFP; pRF4]

1. Maintenance, synchronization and preparation of transgenic nematodes for monitoring de novo protein synthesis

1. Use a dissecting stereomicroscope to assess the developmental stages and growth of wild type (wt) and mutant transgenic nematodes.
2. Day 1: Use a worm pick to select and transfer 10 L4 larvae of wt and mutant transgenic animals carrying the desired fluorescent reporter onto Nematode Growth Media (NGM) plates seeded with *Escherichia* coli (OP50) (**Table 1**).
    1. Make a worm pick by attaching a platinum wire into the tapered end of a glass Pasteur pipette by melting the glass at the contact site on a Bunsen burner. Then, flatten the end of the platinum wire into a spatula shape by using any tool (e.g., pincer or light hammer).
    2. Inoculate a single colony of *E. coli* (OP50) bacteria into a flask containing 50 mL of Luria-Bertani (LB) liquid medium and grow for 10 hours at 37 °C in a shaking incubator (200 rpm; **Table 1**). Then, seed NGM plates with 200 µL of bacterial culture. Incubate the seeded plates at room temperature overnight to allow the growth of the bacterial lawn.
3. Incubate and grow the nematodes at the standard temperature of 20 °C.
4. Day 5: The plates contain a mixed population of transgenic worms. Select and transfer 15 L4 larvae of each strain on freshly seeded OP50-NGM plates.
5. Day 6: Perform FRAP assay and monitor protein synthesis rate on day 1 of adulthood.
6. Prepare and use cycloheximide containing NGM plates as a positive control:

NOTE: Prepare a stock solution of cycloheximide by diluting in water to a concentration of 10 mg/mL. Keep stock solution at 4 °C.

1. Kill bacteria by exposing the seeded NGM plates for 15 minutes with UV light (222 μW/cm^2 intensity).
2. Add cycloheximide on top of bacterial-seeded plates to 500 μg/mL final concentration in the agar volume.
3. Allow plates to dry at room temperature for 30 minutes.
4. Transfer transgenic nematodes expressing p*sod-3GFP* on vehicle and cycloheximide-containing plates.
5. Incubate animals for 2 h at the standard temperature of 20 °C.

2. FRAP assay using transgenic animals expressing somatic tissue reporters p*ife-2*GFP and p*sod-3*GFP

NOTE: Monitor global protein synthesis rate in the whole animal body. These transcriptional reporters present different expression levels and are ubiquitously expressed in multiple somatic tissues, including intestine, pharynx and body wall muscles.

1. Pick and transfer 1-day-adult transgenic animals to individual NGM plates seeded with a 20 μL OP50 drop in the center.
2. Remove the lid and place each plate under a 20x objective lens of an epifluorescence microscope.
3. Focus and capture a reference image before photobleaching (pre-bleach).
4. Photobleach each sample for 10 minutes.

NOTE: Stop the photobleaching when the fluorescent signal is quenched to 30 – 50% intensity relative to pre-bleaching image. The light intensity and the duration of the bleaching period should be adjusted accordingly for the specific fluorophore, animal stage and cell or tissue under examination. The appropriate duration of irradiation required to reach an adequate extent of photobleaching, for different specimens should be experimentally determined.

5. Capture an image after photobleaching (bleach).
6. Keep animals in individual NGM plates and let them to recover.
7. Image and record the recovery of each fluorescent reporter every 1 hour for at least 6 hours at a fluorescence stereomicroscope.
8. Alternatively, perform fluorescent recovery assessment by using an epifluorescence microscope (e.g., AxioImager Z2).
9. Carefully evaluate the animal healthspan per animal by monitoring their locomotion, touch sensitivity, reproductive capacity and survival over the next 3 days. Nematodes showing signs of damage after photobleaching were excluded from further analysis.

3. Mounting the samples and perform FRAP assay using transgenic nematodes expressing pan-neuronally cytoplasmic GFP, p*unc-119*GFP

NOTE: Monitor pan-neuronal protein synthesis by targeted photobleaching at the head region, where nerve ring is located.

1. Prepare 2% agarose pads.
2. Add a 10 μL drop of M9 buffer to the center of the agarose pad.

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3. Transfer 5 transgenic nematodes expressing pan-neuronally cytoplasmic GFP into a drop of M9 buffer.
4. Use an eyelash to spread the liquid.
   NOTE: Animals display reduced movements within 2 minutes because of M9 absorbance into the agar.
5. Change nematode position to avoid overlapping by using an “eyelash” pick.
6. Place the sample under a 40x objective lens of an epifluorescence microscope.
   NOTE: Do not use a coverslip.
7. Focus and capture a reference image (pre-bleach).
8. Photobleach the targeted area of interest for 90 seconds.
   NOTE: Stop the photobleaching when the fluorescent signal is quenched to 30 – 50% intensity relative to pre-bleaching image. The light intensity and the duration of the bleaching period should be adjusted accordingly for the specific fluorophore, animal stage and cell or tissue under examination. The appropriate duration of irradiation required to reach an adequate extent of photobleaching, for different specimens should be experimentally determined.
9. Capture an image after photobleaching (bleach).
10. Add a 10 µL drop of M9 buffer on photobleached nematodes.
11. Let the animals recover for 5 minutes.
12. Use the “eyelash” pick or a pipette to transfer the nematodes to individual NGM plates seeded with 20 µL OP50 drop in the center.
13. Capture an image of each sample every 1 hour under an epifluorescence stereomicroscope.
    NOTE: Count t=0 for the recovery time after photobleaching.

4. Data analysis of images capture during the FRAP assay

1. Analyze the acquired images using software (e.g., Fiji).
2. Open images with the software.
3. Select the **Split Channels** command via the **Image** and **Color** drop-down menu.
   NOTE: Keep the green channel image.
4. Use the **Freehand Selection** tool to manually set the fluorescent region of interest (e.g., whole body, head, intestine).
5. Select the **Measurement** command via the **Analyze** drop-down menu to measure mean pixel intensity of the area of interest for each time point.
   NOTE: The percentage of fluorescence recovery is calculated by using the reference images captured after photobleaching.

5. Report statistical analysis

1. Use at least 20 – 25 nematodes of each strain and/or condition.
   NOTE: Three biological replicates should be performed.
2. Perform student *t*-test (comparison between two groups) or ANOVA (comparison among multiple groups) for statistical analysis with $P < 0.05$ as significant using statistical analysis software.

Representative Results

Using the procedure presented here, wild type and mutant transgenic nematodes expressing the following somatic reporters, $p_{ife\text{-}2}$GFP, $p_{sod\text{-}3}$*GFP* and $p_{unc\text{-}119}$GFP, were used to assess protein synthesis rates according to the respective protocols. Particularly, wild type and *ife-2* mutant

worms expressing cytoplasmic GFP throughout their somatic tissues by using the *ife-2* promoter were compared before, immediately after photobleaching and 5 hours post-recovery. Representative images and quantification show that wild type animals fully recover while *ife-2* mutants have diminished recovery capacity (**Figure 1A**). Thus, wild type worms initiate de novo protein biosynthesis after photobleaching while worms lacking mRNA translation initiation factor IFE-2 are unable to do so, indicating that the rate of fluorescence recovery illustrates the rate of protein synthesis in vivo[7]. Moreover, cycloheximide, a specific inhibitor of mRNA translation, can be used as a positive control for protein translation inhibition. Indeed, cycloheximide-treated transgenic animals expressing cytoplasmic GFP under the *sod-3* promoter, do not recover their fluorescence upon photobleaching (**Figure 1B**).

We also applied this method to detect how mRNA processing bodies (P bodies) affect the rate of protein translation[8]. Wild type and *edc-3(ok1427)* mutant nematodes expressing GFP pan-neuronally were examined for their recovery capacity upon targeted photobleaching at the head region. Fluorescence recovery is much slower in EDC-3 deficient animals compared to wild type (**Figure 1C**). This indicates that EDC-3-mediated mRNA turnover hinders novel protein synthesis and ultimately suppresses protein translation initiation[9].

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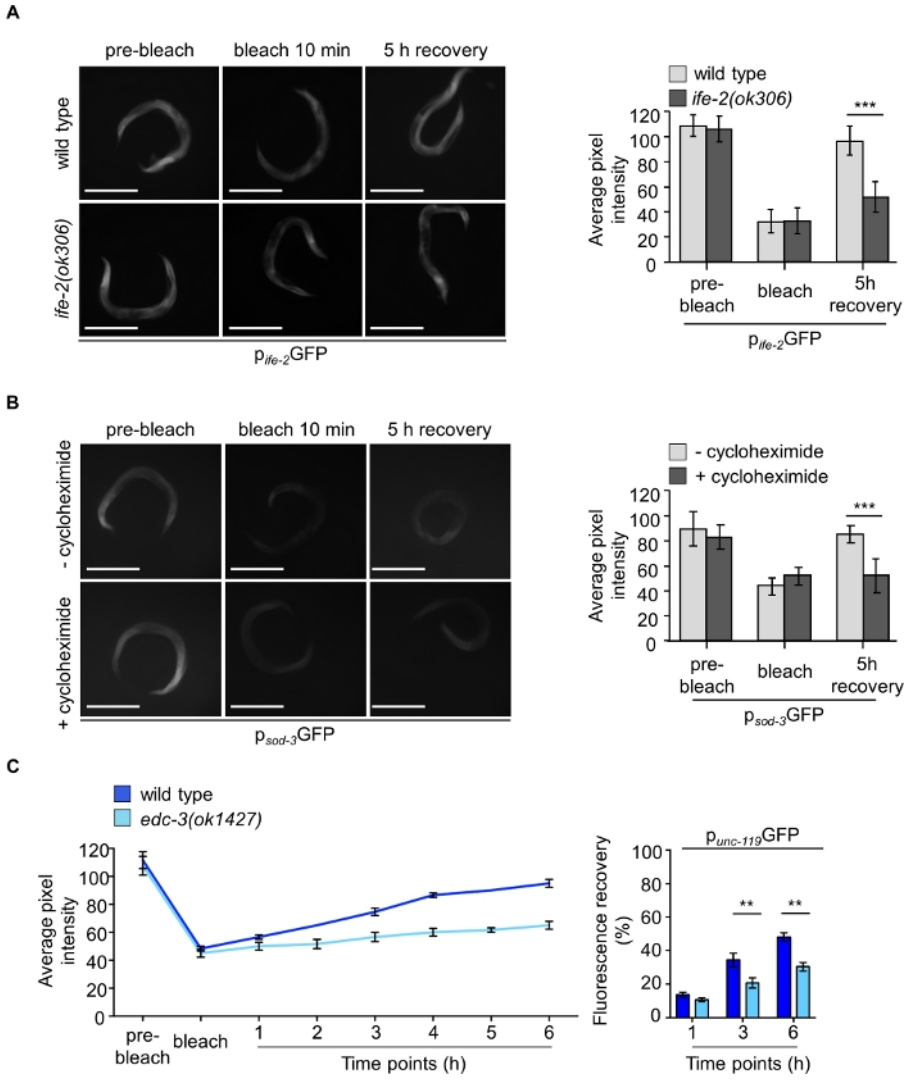

Figure 1: In vivo assessment of de novo protein synthesis in *C. elegans*. (**A**) FRAP analysis in both wild type and IFE-2 deficient nematodes expressing cytoplasmic GFP under the promoter of *ife-2*. Transgenic animals are subjected to whole-animal photobleaching. The GFP signal is reduced to 30-50% of initial intensity. Fluorescence is recorded before photobleaching (pre-bleach), following the end of photobleaching period (10 min; bleach) and 5 hours post recovery. (**B**) Cycloheximide treatment inhibits fluorescence recovery of transgenic animals expressing cytoplasmic GFP under the promoter of *sod-3*. Transgenic animals are subjected to whole animal photobleaching. The GFP signal is reduced to 30-50% of initial intensity. Fluorescence is recorded before photobleaching (pre-bleach), following the end of photobleaching period (10 min; bleach) and 5 hours post recovery (A, B: AxioImager Z2 microscope; objective lens: 20x, numerical aperture 0.8; high-power light source, HBO 100; 100 Watt mercury arc lamp; excitation/ emission filter sets, Zeiss Set 38 Endow GFP shift free). Scale bars, 500µm. (**C**) EDC-3 deficient nematodes display decreased de novo protein synthesis rates in neuronal cells. Fluorescence signal of both wild type and *edc-3(ok1427)* animals expressing pan-neuronally cytoplasmic GFP is

measured before photobleaching (pre-bleach) as well as following the photobleaching period (90sec; bleach). Transgenic animals are subjected to photobleaching at head region (nerve ring). The GFP signal is reduced to 30-50% of initial intensity (AxioImager Z2; objective lens: 40x, numerical aperture 0.75; 30% luminescent power of the fluorescent illumination source (FI illumination System X-Cite 120 XL FL PC, 120W metal halogenide lamp) and fully opened iris). Average pixel intensity of fluorescence recovery is measured at one hour time intervals. 20 animals were quantified per strain in each of three independent experiments. Data represent mean S.E.M., ***P < 0.05, unpaired *t*-test. Please click here to view a larger version of this figure.

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Reagent	Recipe
2% Agarose pads	1. Weigh 0.5 g o fagarose in a cylindrical glass beaker
	2. Add 25 mL of M9 buffer
	3. Heat in a microwave until close to boiling. Take out, stir with a pipette tip and boil again. Repeat until the agarose is dissolved.
	4. Place an empty microscope slide on the bench.
	5. Put a drop (~ 50 µL) of fresh 2% agarose solution in the middle of the slide.
	6. Take a second microscope slide and place it on top of the agarose drop. Gently press down to flatten the drop.
	7. Let the agarose harden for 30 seconds and remove gently the top microscope slide.
	8. Immediately proceed with the sample preparation, since the agarose pads will start drying within approximately 5 minutes.
	Tip: Leave the top microscope slide as a cover to preserve the humidity longer (~ 1 hour). Thus, several agarose pads can be prepared and used swiftly during the experiments.
LB liquid medium	1. Dissolve 10 g of Bactotryptone, 5 g of Bacto Yeast Extract, 5 g of NaCl in 1 L distilled water and autoclave.
M9 buffer	1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g NaCl in 1 L distilled water and autoclave.
	2. Let cool and add 1 mL of 1 M $MgSO_4$ (sterile).
	3. Store M9 buffer at 4 °C.
Nematode growth medium (NGM) agar plates	1. Mix 3 g of NaCl, 2.5 g of bactopeptone, 0.2 g of streptomycin, 17 g of agar and add 900 mL of distilled water. Autoclave.
	2. Let cool to 55-60 °C.
	3. Add 1 mL of cholesterol stock solution, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of nystatin stock solution, 25 mL of sterile 1 M phosphate buffer, pH 6.0, and distilled sterile water up to 1 L.

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	4.	Pipette 10 mL of medium per Petri dish and leave to solidify.
	5.	Store the plates at 4 °C until used.

Table 1: Recommended recipes for reagents used. All the reagents recipes used in the presented protocol are outlined here.

Discussion

Protein synthesis modulation is essential for organismal homeostasis. During ageing, global as well as specific protein synthesis is perturbed. Recent studies reveal the fact that protein translation balance directly controls senescence and aging is not merely a byproduct of the aging process. In particular, core components of the translation machinery such as eukaryotic initiation factor 4E (eIF4E), which facilitates mRNA capping during translation initiation, and thus the rate of cap-dependent protein translation, induces oxidative stress and accelerates the aging process[1] . Moreover, neuron-specific EDC-3, which interacts with mRNA processing bodies, P bodies and increases the rate of mRNA decapping, also accelerates the aging process[8] . Specifically, EDC-3 suppression allows for IFE-2 sequestration into P bodies, ultimately reducing protein translation levels, delaying neuronal aging[9] .

FRAP is a technique initially developed to investigate protein dynamics, localization, interactions and activity with non-invasive fluorescent tagging using live imaging in vivo, making it a powerful tool to study protein synthesis rates in real time[8 , 10] . However, there are certain critical steps that require attention and troubleshooting. We provide alternative solutions to these potential obstacles or limitations encountered during the experimental procedure.

One issue concerns worm movement as the worm can escape the light source during photobleaching. This issue can be partially overcome by using transgenic animal expressing the *rol-6(su1006)* allele, which causes animals to move in a sinusoidal manner. This way, animal photobleaching can be performed continuously. Moreover, the experimenter can follow the movement of the worm that moves outside the plane of view much slower. Nevertheless, agar pads can also be used as an alternative to fully immobilize animals.

The optimal duration of photobleaching is an additional critical factor that should be determined and kept stable throughout the experiments. Insufficient photobleaching due to untargeted photobleaching or short duration can be encountered. This can be surpassed by increasing (a) the magnification objective and numerical aperture, (b) the light intensity and/or (c) photobleaching duration.

If no significant fluorescence recovery is detected, there are potential parameters that can be altered. If photobleaching is excessive, reduce photobleaching duration as specimen damage has occurred. Damage to the worm can be assessed by observing life traits such as touch sensitivity, locomotion, egg laying, pharyngeal pumping and reproductive capacity. If kinetics of protein translation recovery are slow, allow longer recovery period. Protein synthesis recovery varies between different protein families and depends on the reporter fusions length[7] .

Promoter activity could influence kinetics of protein synthesis. If the promoter is inactive during recovery, change the promoter used. Promoter activity might be altered upon

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stress conditions, such as photodamage. Use stable activity promoters to monitor either global (e.g. *let-858, ife-2*, *sod-3*) or tissue-specific (e.g. body wall muscles; *myo-3, unc-54*; pharynx: *myo-2*; pan-neuronal: *unc-119, rab-3*; mechanosensory neurons: *mec-4*; intestine: *vha-6, ges-1*) protein translation rates.

If inadequate protein translation inhibition by cycloheximide occurs, increasing pre-incubation time of the nematodes would overcome this problem. A caveat that should be taken into consideration is the use of transgenic worms overexpressing proteins. Using CRISPR/Cas9-generated transgenic nematodes expressing the fluorescent tagged proteins of interest at physiological levels will provide a more accurate picture of protein kinetics and turnover.

Total protein levels detected are the result of their protein degradation and synthesis rates. Protein degradation invariably occurs at basal levels in all eukaryotic cells. Thus, protein fluorescence recovery rates, which are measured in this protocol, are not absolute but relative to the conditions being compared. In essence, the fluorescence recovery in the presence of protein degradation is measured. Therefore, it is assumed that all conditions have equal protein degradation rates after photobleaching. In order to measure absolute protein translation rates, protein degradation-deficient mutant worm strains should be used. Specifically, mutations in genes such as *rpn-10* of the proteasome or *lgg-2* for autophagy can be used as a control[11]. Alternatively, *RNAi* knockdown of proteasomal and autophagic proteins can be performed. Additionally, autophagic substrates such as SQST-1::GFP can be used to detect the contribution of autophagic degradation under the experimental conditions selected[12].

In both experimental settings, this FRAP technique is non-radioactive, non-invasive, which detects de novo protein synthesis rates in vivo in different tissues in *C. elegans.* It allows for live imaging before and during photobleaching in an in vivo setting and subsequent monitoring of the animals for their whole lifetime. Perturbation of translation initiation decelerates aging, hence measuring global protein synthesis rates by FRAP could be used as a direct read-out of the aging process. This protocol can be optimized and used on a larger scale to screen genes and/or chemicals related to the aging process or age-related disease. Alternatively, total or specific protein degradation rates can be measured by photobleaching under different genetic backgrounds using the same protocol in combination with cycloheximide. As protein translation is inhibited by cycloheximide, the rate of protein degradation will be detected. This protocol will aid in better understanding protein degradation pathways in genetic models of protein aggregation diseases[13].

Disclosures

The authors declare no competing interests.

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