Chapter 7

FLUORESCENCE VISUALIZATION OF NEWLY SYNTHESIZED PROTEINS IN MAMMALIAN CELLS

7.1 Abstract

Modern proteomic methods enable efficient identification of the hundreds or thousands of proteins present in whole cells or in isolated organelles.[1,2] But a thorough understanding of the proteome requires insight into protein localization as well as protein identity. Recently, visualization of newly synthesized proteins in bacterial cells was demonstrated through co-translational introduction of an alkynyl amino acid followed by selective Cu(I)-catalyzed ligation of the alkynyl side chain to the fluorogenic dye 3-azido-7-hydroxycoumarin.[3] Here we report that selective fluorescence labeling and imaging of newly synthesized proteins can be accomplished in a diverse set of mammalian cells.

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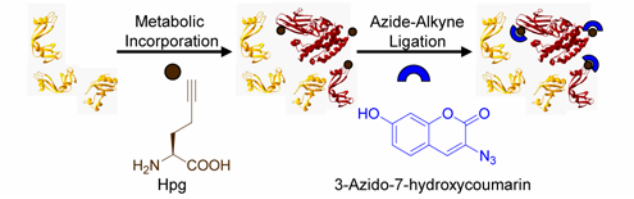
[†]These authors contributed equally to this work.

7.2 Introduction

Fluorescence microscopy provides the most convenient means of visualizing cellular proteins. Protein tagging with green fluorescent protein (GFP) or with tetracysteine motifs has provided powerful tools for tracking individual proteins in intact cells.[4,5] But a more global analysis of protein synthesis and transport requires a different approach; because the identities of the proteins of interest may not be known *a priori*, a labeling strategy without genetic manipulation is needed.

Co-translational incorporation of non-canonical amino acids provides a solution to this problem.[6] Susceptibility to amino acid tagging is determined not by the identity of the protein, but rather by the spatial and temporal character of its synthesis, and proper design of the non-canonical side chain enables facile labeling with fluorescent probes via selective transformations such as the Staudinger or azide–alkyne ligations.[3,7-14] Azides and alkynes are essentially absent from mammalian cells, making the azide–alkyne ligation very selective, and the reaction rate can be enhanced by Cu(I) catalysis or by ring strain.[15-17]

Here we describe the use of homopropargylglycine (Hpg) for tagging and fluorescence visualization of newly synthesized mammalian proteins. Protein tagging with Hpg is operationally similar to conventional pulse-labeling with ^{35}S-methionine; the absence of Met synthesis in mammalian cells and the promiscuity of the methionyl-tRNA synthetase make it straightforward to incorporate Hpg into mammalian proteins in competition with Met.[18,19] After incorporation, Hpg is susceptible to labeling with the membrane permeant fluorogenic dye 3-azido-7-hydroxycoumarin for *in situ* imaging (Scheme 7.1).[20]



Scheme 7.1 Bio-orthogonal labeling of newly synthesized proteins for fluorescence visualization in mammalian cells.

7.3 Materials and Methods

7.3.1 Cell Culture

Untransfected mouse embryonic fibroblasts (MEF18) and MEF transfected with Su9-GFP (MEF-mitoGFP), a mitochondrially-localized enhanced GFP, were a gift from D.C. Chan (California Institute of Technology, Pasadena, CA).[21] Human mammary epithelial cells (MCF-10A), human embryonic kidney cells (HEK 293T), and Chinese hamster ovary cells transfected with the human α_5 integrin subunit fused to GFP (CHO-α_5)[22] were provided by A.R. Asthagiri (Caltech). HeLa cells and African green monkey kidney cells (COS-7) were gifts from C.D. Smolke (Caltech) and E.M. Schuman (Caltech), respectively. Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex BioSciences (Walkersville, MD). MEF18, MEF-mitoGFP, HEK 293T, HeLa, and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 50 U/mL penicillin, and 50 μg/mL streptomycin. CHO-α_5 cells were

maintained in a similar medium supplemented with 1% (v/v) non-essential amino acid solution (Sigma-Aldrich, St. Louis, MO) and 1 mM sodium pyruvate. Near-confluent cells were passaged with 0.05% trypsin in 0.52 mM EDTA. HUVECs were grown in Endothelial Growth Medium-2 (EGM-2, 2% serum, Cambrex BioSciences) and passaged nonenzymatically by treatment with 0.61 mM EDTA.

7.3.2 Preparation of Cells for Fluorescence Microscopy

Near-confluent cells in 100 mm Petri dishes were rinsed twice with 5 mL warm phosphate-buffered saline (PBS). Cells were detached with either EDTA (HUVECs) or trypsin in EDTA and treated with 2 mL of SFM [DMEM, with 1 mg/mL bovine serum albumin (BSA, fraction V, Sigma-Aldrich), without Met] containing 2.3 mg/mL soybean trypsin inhibitor (Sigma-Aldrich). The cells were pelleted via centrifugation (200*g*, 3 min), washed with 3 mL of SFM, and counted. Cells were added at a density of $0.25 \times 10^6 – 1 \times 10^6$ cells per well to prepared slides ($0.36 \times 10^6 – 1.43 \times 10^6$ cells/cm^2).

Lab-Tek II Chamber Slides (8-well, Nalge Nunc International, Rochester, NY) were prepared by treatment with fibronectin solution (10 μg/mL) at 4 °C overnight. The wells were rinsed three times with PBS, blocked with a 2 mg/mL solution of heat-inactivated BSA for 30 min at room temperature, and rinsed with PBS.

All cells were incubated for 30 min in SFM to deplete intracellular Met stores. Cycloheximide (50 μM) or anisomycin (40 μM) was added to control cells during this time to inhibit protein synthesis. After incubation, either 1 mM Met or 1 mM Hpg was added to the medium. After 4 h, wells were rinsed once with PBS and the medium was replaced with SFM containing 1 mM Met for the chase.

After a 2 h chase, cells were rinsed three times with warm PBS, fixed with 3.7% paraformaldehyde solution for 10 min, and rinsed twice with PBS. Cells that were stained with anti-nucleolar antibodies were permeabilized with 0.1% Triton X100 for 3 min. The cells were treated with a blocking solution [10% (v/v) fetal calf serum (Cambrex BioSciences), 50 mg/mL sucrose, 20 mg/mL BSA] for at least 30 min at room temperature and rinsed twice with PBS. For nucleolar staining, cells were incubated with antibody clone 125-10 (Chemicon, Temecula, CA) at a dilution of 1:40 for 1 h at room temperature. After rinsing three times with PBS, cells were incubated with a secondary antibody solution containing 3% BSA and 12.5 μg/mL Cy2-conjugated affinity-purified goat anti-mouse secondary antibody (Chemicon) for 1 h at room temperature. Cells were then rinsed three times with PBS.

Cells were dye-labeled as previously described.[3] Chamber wells were filled to the top with >1.2 mL of PBS (pH 7.5) containing 200 μM $CuSO_4$, 400 μM TCEP, 200 μM triazole ligand, and 25 μM 3-azido-7-hydroxycoumarin.[11] The wells were sealed with polyolefin tape (Nalge Nunc), wrapped in foil, and inverted. Inversion was necessary to prevent debris from forming on the slide surface. Slides were allowed to react on a waver at room temperature overnight.

After reaction, cells were washed four times with PBS (1% Tween 20, 0.5 mM EDTA) and once with water. Slides were agitated for 1 min between washes. Chamber walls were removed from the slide. Mounting medium was added, and a cover slip was attached before visualization.

7.3.3 Preparation of Cells for Flow Cytometry

As described above, pulse-labeling was performed directly in the 35- or 60-mm tissue culture polystyrene dishes in which cells were grown. After the chase, cells were washed twice with PBS and detached using 0.05% trypsin in EDTA. Cells were centrifuged (200*g*, 3 min), lightly fixed in a 1% paraformaldehyde solution for 10 min at room temperature, washed with PBS, and treated with a blocking solution for at least 30 min at room temperature. Cells were incubated at 4 °C overnight in PBS supplemented with 200 μM $CuSO_4$, 400 μM TCEP, 200 μM triazole ligand, and 25 μM 3-azido-7-hydroxycoumarin. For optimization of $CuSO_4$ concentration, cells from 60-mm plates were split and supplemented with 50–500 μM $CuSO_4$.

7.3.4 Fluorescence Microscopy

Fixed cells were imaged on a confocal microscope (Zeiss LSM 510 Meta NLO, Thornwood, NY) at Caltech's Biological Imaging Center. Each set of images was obtained with identical conditions to capture either GFP or coumarin fluorescence. To visualize GFP fluorescence or Cy2-labeled nucleoli, cells were excited at 488 nm (Argon laser) and emission was passed through a bandpass filter (500–550 nm) before imaging. Coumarin fluorescence was obtained by two-photon excitation at 800 nm (Ti:sapphire laser) with emission collected between 376–494 nm. Coumarin fluorescence varied among the cell lines and the settings were optimized for each cell type through comparison to labeling from Met or Hpg–cycloheximide control cells. The final coumarin image represents the average of two scans. For insets (Figure 7.1a), the sum of four scans was taken. The GFP and coumarin images were false-colored and

superimposed on a differential interference contrast (DIC) image. All images were acquired with a Plan-Apochromat 63×/1.4 oil objective (Zeiss) and analyzed with Zeiss LSM software.

7.3.5 Flow Cytometry

After overnight treatment with the coumarin dye, cells were washed once with PBS (1% Tween 20, 0.5 mM EDTA), resuspended in a total volume of 500 μL of PBS, and filtered through a 50 μm Nytex nylon mesh screen (Sefar, Depew, NY). Cells were analyzed on a BD Bioscience FACSAria flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) at Caltech's Flow Cytometry Facility. Coumarin fluorescence was excited by a 407 nm violet laser and detected after passage through a 450/40 bandpass filter. GFP fluorescence was excited by a 488 nm blue laser and detected after passage through a 530/30 bandpass filter. Unlabeled MEF18, coumarin-labeled MEF18, and MEF-mitoGFP without coumarin were analyzed to ensure minimal cross-over fluorescence in each channel. Three samples were prepared for each experiment, and 10,000 events were collected for each sample. Forward- and side-scatter properties were used to exclude doublets, dead cells, and debris from analysis. FlowJo 5.7.1 software (Tree Star, Inc., Ashland, OR) was used for data analysis. The mean fluorescence of each population was averaged to give the reported mean fluorescence. The error bars represent one standard deviation.

7.4 Results and Discussion

Initial experiments were performed with mouse embryonic fibroblasts that express a mitochondrially-targeted GFP (MEF-mitoGFP).[21] Cells were grown to confluence before passaging into serum-free medium lacking Met (SFM). After incubation to deplete residual Met, cultures were supplemented with 1 mM Met or Hpg for a 4 h pulse. Incorporation of Hpg into proteins did not appear to affect cell viability; propidium iodide staining demonstrated that viability was similar when cells were pulse-labeled either with Met or with Hpg for 4 h. During a 2 h chase, cells were incubated in SFM containing 1 mM Met. Cells were washed, fixed, and blocked before reaction with 200 μM $CuSO_4$, 400 μM triscarboxyethylphosphine (TCEP), 200 μM tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine (triazole ligand), and 25 μM 3-azido-7-hydroxycoumarin.[11] Cells were treated overnight at room temperature in the dark and then washed before visualization.

Individual cells were examined by confocal microscopy and flow cytometry. Microscopic observations of GFP fluorescence delineated the cells and confirmed proper mitochondrial morphology (Figure 7.1a, bottom panels). Images of coumarin fluorescence were acquired with identical acquisition settings for cells labeled either with Hpg or with Met (Figure 7.1a, top panels). Bright coumarin fluorescence was observed only for cells exposed to Hpg during the pulse. As a control, one of two protein synthesis inhibitors, cycloheximide or anisomycin, was added to the medium 30 min prior to pulse-labeling with 1 mM Hpg. Cells labeled in this medium exhibited levels of coumarin fluorescence comparable to the background levels observed for Met-labeled cells (Figure 7.1a, right panels). The inhibitor controls maintained background levels of fluorescence

even when imaged by taking a sum of four coumarin scans (Figure 7.1a, inset). Flow cytometry was used to quantitate the fluorescence enhancement; cells treated with 1 mM Hpg were characterized by mean fluorescence 18-fold higher than that of cells pulse-labeled with Met (Figure 7.1b). Addition of cycloheximide or anisomycin to cells prior to addition of 1 mM Hpg reduced the mean fluorescence to the level observed for the Met control. Both microscopy and flow cytometry indicate that fluorescence labeling is highly selective for newly synthesized proteins that contain Hpg.

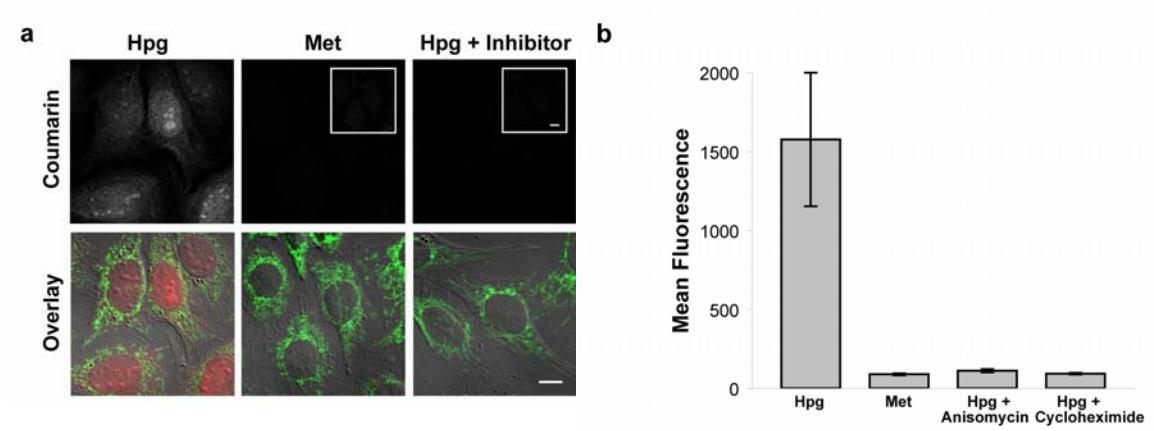


Figure 7.1 Fluorescence labeling of proteins in MEF-mitoGFP. (a) Imaging of MEF-mitoGFP cells pulse-labeled in media containing 1 mM Hpg (left), 1 mM Met (middle), and 1 mM Hpg + 50 μM cycloheximide (right). The coumarin images were acquired under identical conditions. The insets for the coumarin fluorescence represent the sum of four scans. The final overlay contains the superposition of the GFP (green), coumarin (red), and differential interference contrast (DIC) images. Scale bars represent 10 μm. (b) Mean fluorescence of cells obtained from flow cytometry. Each bar represents three samples with 10,000 events collected for each sample. Error bars represent one standard deviation.

Understanding the dependence of the observed fluorescence on Hpg concentration and on the ratio of Hpg to Met should be useful for applications in which one wishes to manipulate the extent of labeling. The optimal Hpg concentration was established by flow cytometry. Mean fluorescence increased 2-fold when the Hpg concentration was raised from 0.1 to 0.5 mM. There was no further enhancement in the range of 0.5 to 2.0 mM Hpg, although there was some variability in fluorescence levels at 0.5 mM. To ensure consistent fluorescence labeling, we used 1 mM Hpg for pulse-labeling. Reducing the ratio of Hpg to Met in the medium from 1000:1 to 100:1 caused a decrease in mean fluorescence (Figure 7.2a**)**. Previous *in vitro* studies have shown that the specificity constant k_{cat}/K_m is reduced ca. 500-fold for Hpg as compared to Met (for activation by the MetRS derived from *E. coli*).[18,19] In accord with those studies, we find that coumarin fluorescence could be discerned by confocal microscopy when the Hpg:Met ratio was 500:1, but not at a ratio of 100:1.

In order to define the temporal resolution of the method, we examined pulse lengths ranging from 15 min to 6 h. Flow cytometry showed that a 15 min pulse with no chase yielded a 5-fold enhancement in mean fluorescence as compared to Met controls. The mean fluorescence increased as the pulse length was extended to 4 h but did not increase further at 6 h.

The concentration of the $CuSO_4$ catalyst was varied from 50 to 200 μM (Figure 7.2b). At 50 μM $CuSO_4$, the mean fluorescence was comparable to that of cells treated only with Met. As the copper concentration was increased in this range, the mean fluorescence increased. Copper concentrations of 250–500 μM resulted in only modest further enhancement of the fluorescence intensity.

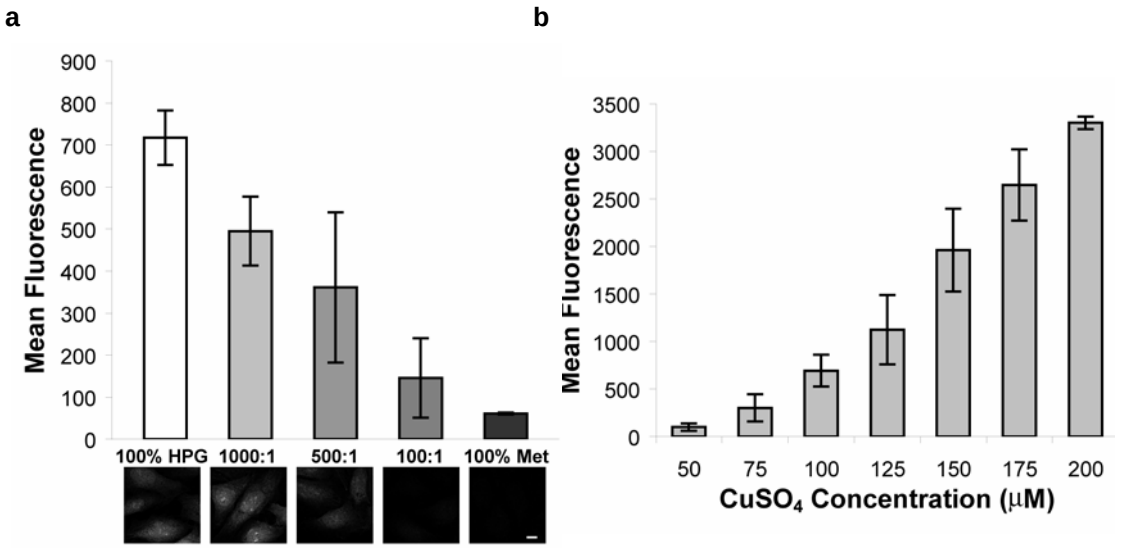


Figure 7.2 Flow cytometric analysis of pulse-labeling and dye-labeling conditions. (a) Fluorescence of cells pulse-labeled with various ratios of Hpg to Met. Mean fluorescence was determined via flow cytometry. In the corresponding coumarin images, the same acquisition settings were used for all experiments. The scale bar represents 10 μm. (b) Mean fluorescence of cells treated with various concentrations of $CuSO_4$. Each bar represents three flow cytometry samples with 10,000 events collected for each sample. Error bars represent one standard deviation.

The imaging strategy described here can be extended easily to a wide variety of cell types (Figure 7.3). Newly synthesized proteins in both transfected (MEF-mitoGFP, CHO-α_5GFP) and non-transfected (MCF-10A, HUVEC) cells can be visualized when the cells are pulse-labeled with Hpg. The method works well on different cell types

(fibroblasts, endothelial and epithelial cells) and on cells derived from numerous species (human, mouse, monkey, hamster).

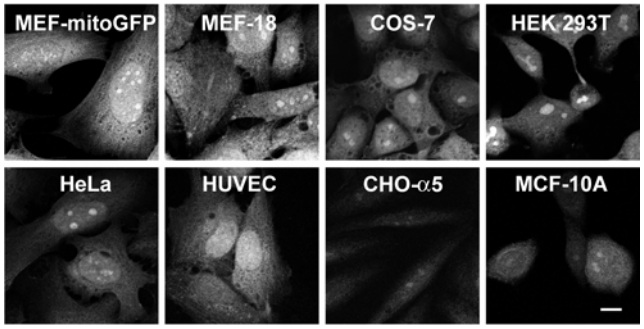


Figure 7.3 Coumarin labeling of newly synthesized proteins in a wide variety of cell types. The scale bar represents 10 μm.

In many of the cell types examined here, the most intense coumarin fluorescence appeared to be localized to nucleolar structures (Figure 7.3). When HeLa and HEK 293T cells were stained with an anti-nucleolar antibody, the areas of brightest coumarin fluorescence co-localized with the antibody (Figure 7.4). Nucleoli, sites of ribosomal biogenesis, are protein-rich (>80% protein) and are the most dense part of the cell.[23,24] They exchange proteins rapidly; dynamic analysis of HeLa nucleoli indicated that proteomic changes are observed in less than 2 h.[25,26] Evidence that there is rapid nucleolar assembly and protein turnover is consistent with our observation that a subset of newly synthesized proteins localize in nucleoli during a 4 h window.[27,28]

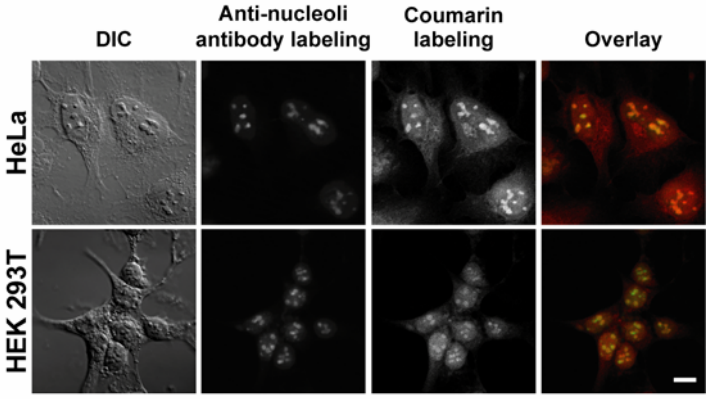


Figure 7.4 Fluorescence micrographs of nucleolar and coumarin labeling in HeLa and HEK 293T cells. Nucleoli are clearly visible in the DIC image (first column) and were labeled with an anti-nucleolar antibody and detected with a Cy2-conjugated antibody (second column). Coumarin labeling shows intense fluorescence at the nucleoli (third column). The overlay combines the antibody (green) and coumarin (red) labeling. The scale bar represents 10 μm.

7.5 Conclusion

Non-canonical amino acid tagging offers a facile means of labeling newly synthesized proteins in mammalian cells. Since labeling is determined solely by the timing (and in principle by the location) of the Hpg pulse, it is possible to visualize proteins of unknown sequence, structure, or function. We suggest that this method will be useful for elucidating complex processes involving spatially localized protein

translation, e.g., the hypothesis that synaptic plasticity is modulated by translation localized in dendrites.[29] Moreover, other non-canonical amino acids can be metabolically incorporated and modified by the azide–alkyne or Staudinger ligations.[3,9,10,14,30] Multi-color analysis should be possible by using multiple dyes to visualize subsets of the proteome expressed during sequential pulses.

7.6 Acknowledgments

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