

Fluorescent Probes

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Fluorescence Visualization of Newly Synthesized Proteins in Mammalian Cells**

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Modern proteomic methods enable efficient identification of the hundreds or thousands of proteins present in whole cells or in isolated organelles.^[1] However, a thorough understanding of the proteome requires insight into protein localization

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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.^[2] Here we report that selective fluorescence labeling and imaging of newly synthesized proteins can be accomplished in a diverse set of mammalian cells.

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.^[3] However, a more global analysis of protein synthesis and transport requires a different approach: as 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 noncanonical amino acids provides a solution to this problem.^[4] Recently, bio-orthogonal noncanonical amino acid tagging (BONCAT) demonstrated the unbiased and selective enrichment and identification of newly synthesized proteins in a mammalian cell line.^[5] 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 noncanonical side chain enables facile labeling with fluorescent probes through selective transformations such as the Staudinger or azide-alkyne ligations.^[2,6-8] Azides and alkynes are essentially absent from mammalian cells, which makes the azide-alkyne ligation very selective, and the reaction rate can be enhanced by Cu^I catalysis or by ring strain.^[9]

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 ³⁵S-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.^[10] After incorporation, Hpg is susceptible to labeling with the membrane-permeant fluorogenic dye 3-azido-7-hydroxycoumarin for in situ imaging (Figure 1).^[11]

Initial experiments were performed with mouse embryonic fibroblasts that express a mitochondrially targeted GFP (MEF-mitoGFP).^[12] Cells were grown to confluence before passage into serum-free medium lacking Met (SFM). After incubation to deplete any residual Met, cultures were supplemented with 1 mM Met or Hpg for a 4 h pulse.

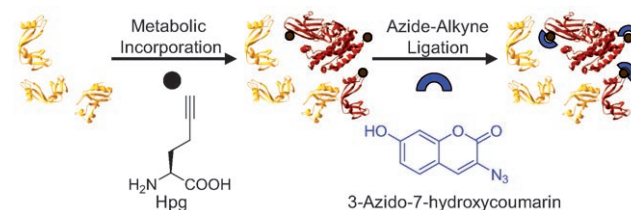


Figure 1. Bioorthogonal labeling of newly synthesized proteins for fluorescence visualization in mammalian cells.

Incorporation of Hpg into proteins did not appear to affect cell viability; staining with propidium iodide demonstrated that viability was similar when cells were pulse-labeled either with Met or with Hpg for 4 h (data not shown). During a 2 h chase, cells were incubated in SFM containing 1 mM Met. The 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.^[8] The 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. Microscopy observations using differential interference contrast (DIC) delineated the cells, and GFP fluorescence confirmed that the mitochondria had the proper morphology (Figure 2a). Images of coumarin fluorescence were acquired with identical acquisition settings for

cells labeled either with Hpg or with Met (Figure 2a, third column). Bright coumarin fluorescence was observed only for cells exposed to Hpg during the pulse. As a control, one of two inhibitors of protein synthesis, cycloheximide or anisomycin, was added to the medium 30 minutes 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 2a, bottom panels). The inhibitor control samples maintained low levels of fluorescence even when imaged by taking a sum of four coumarin scans (Figure 2a, inset). Flow cytometry was used to quantitate the fluorescence enhancement; cells treated with 1 mM Hpg were characterized by mean fluorescence that was 18-fold higher than that of cells pulse-labeled with Met (Figure 2b). 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.

Understanding the dependence of the observed fluorescence on Hpg concentration and on the Hpg/Met ratio should be useful for applications in which one wishes to control the extent of labeling. The optimal Hpg concentration was established by flow cytometry. The mean fluorescence increased twofold when the Hpg concentration was raised from 0.1 to 0.5 mM. There was no further enhancement in the range 0.5 to 2.0 mM Hpg, although there was some variability in the fluorescence levels at 0.5 mM. To ensure consistent fluorescence labeling, we used 1 mM Hpg for pulse-labeling. Reducing the Hpg/Met ratio in the medium from 1000:1 to 100:1 caused a decrease in the mean fluorescence (Figure 3a). Previous *in vitro* studies have shown that the specificity constant k_{cat}/K_m is reduced approximately 500-fold for Hpg relative to Met (for activation by the methionyl-tRNA synthetase derived from *E. coli*).^[10] 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.

To define the temporal resolution of the method, we examined pulse lengths ranging from 15 minutes to 6 hours. Flow cytometry showed that a 15-minute pulse with no chase yielded a fivefold enhancement in the mean fluorescence relative to Met controls. The mean fluorescence increased as the pulse length was extended to 4 hours but did not increase further at 6 hours.

The concentration of the CuSO_4 catalyst was varied from 50 to 200 μM (Figure 2b). The mean fluorescence at 50 μM CuSO_4 was comparable to that of cells treated only with Met. The mean fluorescence increased as the copper concentration was increased in this range. Copper concentrations of 250–500 μM resulted in only modest further enhancement of the fluorescence intensity.

The imaging strategy described here can be extended easily to a wide variety of cell types (Figure 4). Newly synthesized proteins in both transfected (MEF-mitoGFP, CHO- α_5 GFP) and nontransfected (MCF-10A, HUVEC) cells can be visualized when the cells are pulse-labeled with Hpg. The method works well on different cell types (fibroblasts,

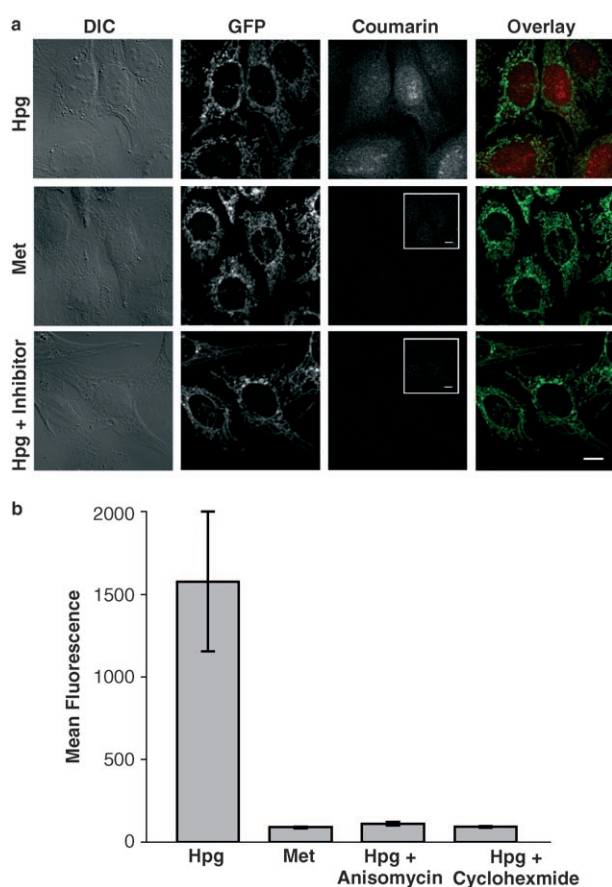


Figure 2. Fluorescence labeling of proteins in MEF-mitoGFP. a) Imaging of MEF-mitoGFP cells pulse-labeled in media containing 1 mM Hpg (top), 1 mM Met (middle), and 1 mM Hpg + 50 μM cycloheximide (bottom). The differential interference contrast (DIC) images are shown in the first column. Each set of images was obtained with identical conditions to capture either GFP (second column) or coumarin (third column) fluorescence. The insets for the coumarin fluorescence represent the sum of four scans. The final overlay contains the superposition of the GFP (green) and coumarin (red) images. Scale bars represent 10 μm . b) Mean fluorescence of cells obtained from flow cytometry. Each column represents three samples with 10000 events collected for each sample. Error bars represent one standard deviation.

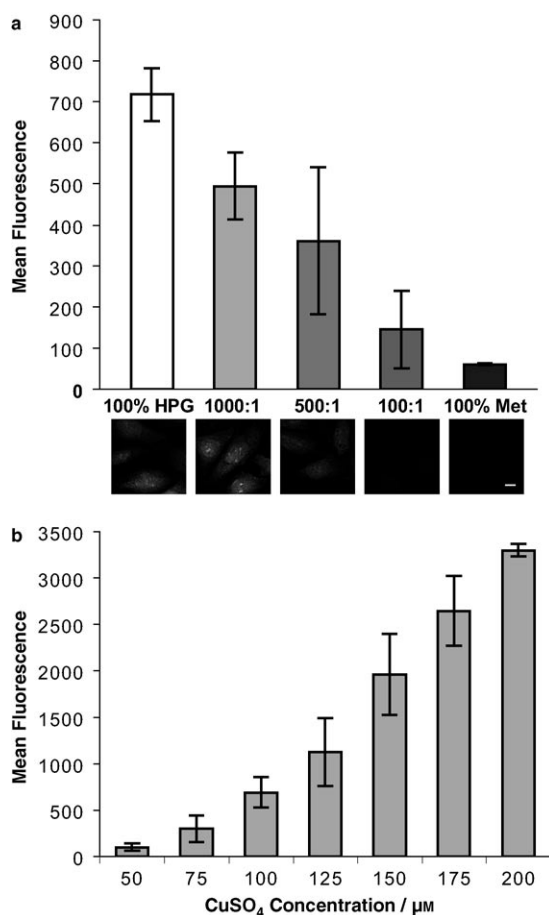


Figure 3. Flow cytometric analysis of pulse-labeling and dye-labeling conditions. a) Fluorescence of cells pulse-labeled with various Hpg/Met ratios. All Hpg-treated samples contained 1 mM Hpg, and the 100% Met sample contained 1 mM Met. Mean fluorescence was determined by flow cytometry. The same acquisition settings were used for all samples in the corresponding coumarin images. The scale bar represents 10 μm. b) Mean fluorescence of cells treated with various concentrations of CuSO₄. Each column represents three flow-cytometry samples with 10000 events collected for each sample. Error bars represent one standard deviation.

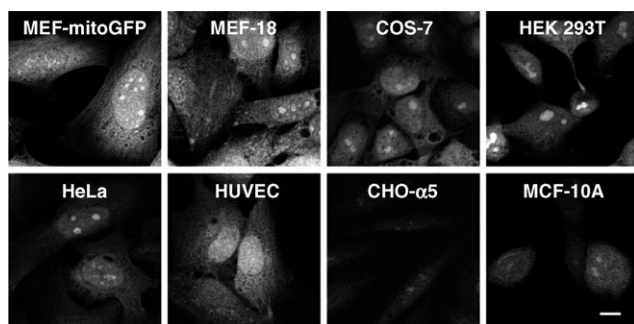


Figure 4. Coumarin labeling of newly synthesized proteins in a wide variety of cell types. The scale bar represents 10 μm.

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

In many of the cell types examined here, the most intense coumarin fluorescence appeared to be localized in nucleolar

structures (Figure 4). When HeLa and HEK293T cells were stained with an antinucleolar antibody, the areas of brightest coumarin fluorescence co-localized with the antibody (Figure 5). Nucleoli, sites of ribosomal biogenesis, are pro-

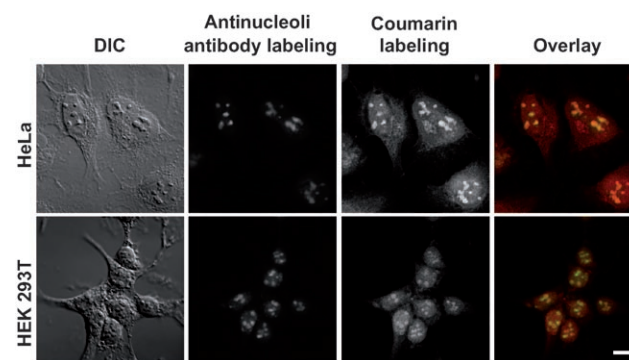


Figure 5. Fluorescence micrographs of nucleolar and coumarin labeling in HeLa and HEK293T cells. Nucleoli are clearly visible in the DIC image (first column) and were labeled with an antinucleolar antibody and detected with a Cy2-conjugated antibody (second column). Coumarin labeling shows intense fluorescence at the nucleoli (third column). The overlay combines the antibody (green) and coumarin (red) labeling. The scale bar represents 10 μm.

tein-rich (> 80% protein) and are the most dense part of the cell.^[13] They exchange proteins rapidly; dynamic analysis of HeLa nucleoli indicated that proteomic changes are observed in less than two hours.^[14] Furthermore, a recent proteomics study of HEK293 cells identified nucleolin and nucleophosmin (B23), major nucleolar proteins, as being synthesized during a two-hour pulse with azidohomoalanine.^[5,15] 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 four-hour window.^[16]

Noncanonical 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, for example, the hypothesis that synaptic plasticity is modulated by translation localized in dendrites.^[17] Moreover, other noncanonical amino acids can be metabolically incorporated and modified by the azide-alkyne or Staudinger ligations.^[2,7,18] Multicolor analysis should be possible by using multiple dyes to visualize subsets of the proteome expressed during sequential pulses.

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