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Two-color labeling of temporally defined protein populations in mammalian cells

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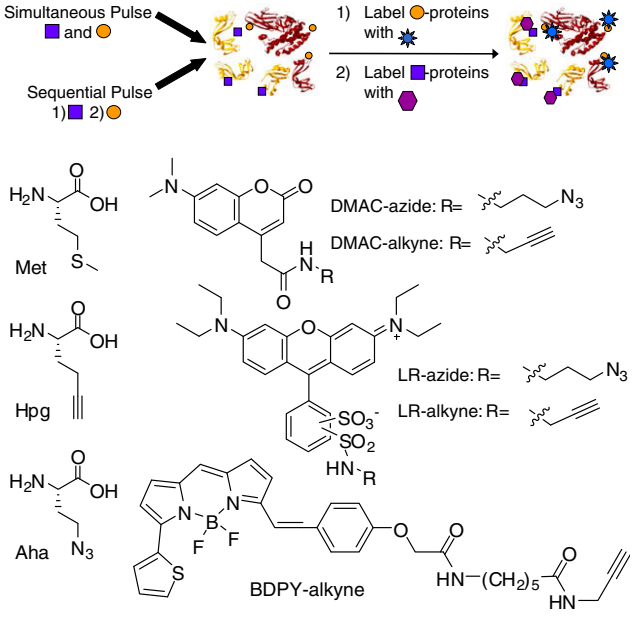
ABSTRACT

The proteome undergoes complex changes in response to disease, drug treatment, and normal cellular signaling processes. Characterization of such changes requires methods for time-resolved protein identification and imaging. Here, we describe the application of two reactive methionine (Met) analogues, azidohomoalanine (Aha) and homopropargylglycine (Hpg), to label two protein populations in fixed cells. Reactive lissamine rhodamine (LR), 7-dimethylaminocoumarin (DMAC), and bodipy-630 (BDPY) dyes were prepared and examined for use in selective dye-labeling of newly synthesized proteins in Rat-1 fibroblasts. The LR and DMAC, but not BDPY, fluorophores were found to enable selective, efficient labeling of subsets of the proteome; cells labeled with Aha and Hpg exhibited fluorescence emission three- to sevenfold more intense than that of control cells treated with Met. We also examined simultaneous and sequential pulse-labeling of cells with Aha and Hpg. After pulse-labeling, cells were treated with reactive LR and DMAC dyes, and labeled cells were imaged by fluorescence microscopy and analyzed by flow cytometry. The results of these studies demonstrate that amino acid labeling can be used to achieve selective two-color imaging of temporally defined protein populations in mammalian cells.

Metabolic labeling provides a powerful approach to the characterization of changes in the cellular proteome. Proteins in complex biological mixtures can be labeled with ketones,[1–4] azides,[5–8] or alkynes[9–11] and subsequently affinity-tagged through a variety of bioorthogonal transformations.[1,2,6,12–16] Metabolic labeling strategies have enabled the identification of proteins containing many different post-translational modifications, including glycosylation,[17–19] phosphorylation,[20,21] farnesylation,[22] and fatty acylation.[23–25]

Similarly, reactive amino acid analogues can be used to track spatial and temporal changes in protein synthesis.[26–31] In previous work, the Met analogues Aha and Hpg were used both to identify[32] and to visualize[33] temporally defined subsets of the proteome (Scheme 1). The amino acid tagging method is reminiscent of conventional pulse-labeling with radioactive amino acids; the endogenous cellular machinery places a reactive Met analogue at sites normally occupied by Met within polypeptide chains. The newly synthesized proteins, which contain either Aha or Hpg, are then labeled with a fluorophore or affinity purification tag by selective copper-catalyzed azide-alkyne ligation.[14,15]

Fluorescent proteins or tetracysteine tags can be genetically fused to proteins to enable microscopic analysis of one or more pre-selected proteins inside cells.[34–36] Genetic fusions have been



Scheme 1. Two-dye labeling strategy and structures of Met, Hpg, Aha, and the reactive fluorophores.

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used to image many cellular processes, including organelle dynamics[37,38] and biogenesis,[39] protein phosphorylation,[40] and protein trafficking.[36,41] Recently, Tsien and coworkers described a new method, 'TimeSTAMP', which can define the age of genetically modified proteins by clever use of drug-controlled, protease cleavage of epitope tags.[42]

Two-color labeling of protein populations in cells can provide new insight into global processes that rely on spatial and temporal control of protein synthesis,[31] such as bacterial infection,[43] cancer,[44] or secretion.[45,46] The method described here relies on the simultaneous or sequential addition of two distinct reactive metabolites to enable the fluorescent tagging of two protein populations within cells (Scheme 1). The first demonstration of two-dye labeling of metabolically tagged cells was described in 2007 by Chang and coworkers,[47] who used flow cytometry to show that cells treated with two reactive sugars could be labeled with distinct fluorophores.

Here, we report selective fluorophore-labeling of two temporally defined sets of proteins in mammalian cells. We prepared three types of reactive, spectrally distinct fluorophores based on the rhodamine, coumarin, and bodipy dye scaffolds (Scheme 1). The reactive fluorophores were prepared by coupling 3-azidopropylamine[48] or propargylamine to commercially available amine-reactive dyes. The combined use of two reactive fluorophores (e.g., LR and DMAC) to dye-label proteins displaying Aha or Hpg enables two-color fluorescence imaging of cells. We used fluorescence microscopy to evaluate each fluorophore for selective dye-labeling of a single population of newly synthesized proteins in Rat-1 fibroblasts. Cells were pulse-labeled for 3 h in media supplemented with 1 mM amino acid (Aha, Hpg, or Met). Cells were also pre-treated with the protein synthesis inhibitor anisomycin (aniso) prior to pulse-labeling to determine the contribution to labeling from free amino acid. After pulse-labeling, cells were washed, fixed, and blocked before dye-labeling. Cells were dye-labeled in PBS (pH 7.5) containing 1 mM $CuSO_4$, 1 mM triscarboxyethylphosphine (TCEP), 100 μM tris((1-benzyl-1H-1,2,3-triazol-4-yl)-methyl)amine (TBTA),[8,49] and an optimized concentration of each reactive dye: 10 μM DMAC-alkyne; 50 μM LR-alkyne; 10 μM BDPY-alkyne; 50 μM DMAC-azide; 50 μM LR-azide. Cells were washed before imaging on a confocal fluorescence microscope.

The reactive DMAC and LR fluorophores each provided bright, selective labeling of newly synthesized proteins inside cells (Fig. 1). The dyes had access to both the nucleus and cytoplasm, and both types of fluorophore appeared to brightly stain the protein-rich nucleoli.[50] We did not observe dye-specific accumulation in any particular organelles (e.g., mitochondria or lysosomes). Both of the DMAC dyes and the LR-azide dye enabled consistent staining of cells, while the LR-alkyne gave more varied levels of labeling among different cells in a population. We recommend use of LR-azide, rather than LR-alkyne, for this reason.

Flow cytometry was used to determine the extent of fluorescence enhancement. Cells treated with Aha or Hpg for 5 h were characterized by a mean fluorescence three- to sevenfold higher than that of cells pulse-labeled with Met (see Supporting Information, Fig. S2). Addition of anisomycin to cells prior to addition of the reactive analogue reduced the mean fluorescence intensity to the level observed for the Met controls.

Flow cytometry suggested that BDPY-alkyne also labels newly synthesized proteins with good selectivity; Aha-treated cells exhibited a 6.5-fold enhancement in mean fluorescence compared to Met-treated cells (Fig. S2). In contrast, imaging experiments revealed two problems. First, we observed large differences in the level of staining from cell to cell. Second, and more problematic, is the fact that the dye stained the same cytoplasmic structures in control cells treated with Met or with [Aha+aniso] as in cells treated with Aha. BDPY-alkyne might be useful for other applications, such as labeling of purified azide-tagged proteins, but we cannot recommend it for labeling azide-treated cells. We are unsure why BDPY-alkyne appears to be selective by flow cytometry but not by microscopy.

As described above, our preliminary experiments showed that the LR and DMAC fluorophores enable selective labeling of newly synthesized proteins in cells. Simultaneous pulse-labeling with two reactive amino acids provides the simplest means of introducing two distinct tags into cells.[47] Rat-1 fibroblasts were pulse-labeled for 5 h with 1 mM Met and several ratios of Aha to Hpg. Different ratios of Aha to Hpg were evaluated because the kinetics of aminoacylation probably differ for the two amino acids. We found previously, using purified *Escherichia coli* methionyl-tRNA synthetase, that k_{cat}/K_M for Aha is approximately 25% larger than that for Hpg.[51] After the pulse, cells were labeled for 1 h with 50 μM LR-azide, washed, and labeled for 1 h with 10 μM DMAC-alkyne before imaging. Examination of individual cells by fluorescence microscopy revealed that it is possible to selectively dye-label both Aha and Hpg residues in newly synthesized proteins (Fig. 2). Because the analogues were added simultaneously, the

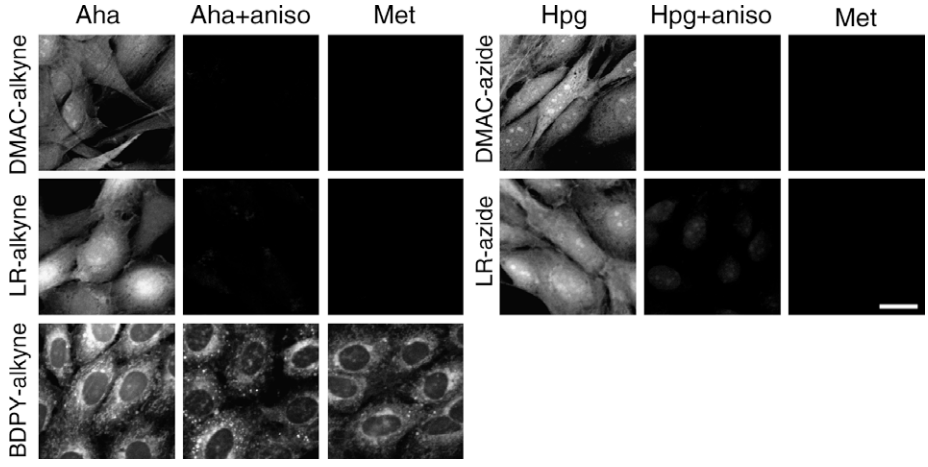

Figure 1. Selective dye-labeling of newly synthesized proteins using azide or alkyne fluorophores. Confocal fluorescence imaging of Rat-1 fibroblasts grown for 3 h in media containing 1 mM Met, 1 mM Aha, or 1 mM Hpg. Control cells were pre-treated with the protein synthesis inhibitor anisomycin (aniso) prior to pulse-labeling. Cells were dye-labeled with 10 μM DMAC-alkyne, 50 μM LR-alkyne, 10 μM BDPY-alkyne, 50 μM DMAC-azide, or 50 μM LR-azide. Scale bar represents 20 μm. Corresponding DIC images can be found in the Supporting Information (Fig. S1).

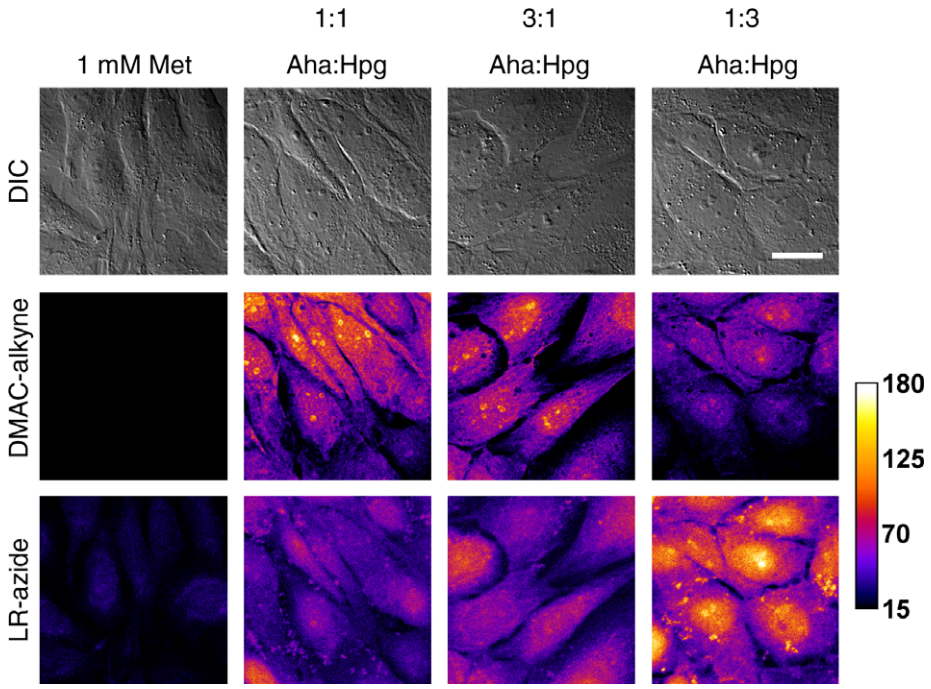

Figure 2. Fluorescent images of Rat-1 fibroblasts simultaneously pulse-labeled with two reactive amino acids. Cells were pulse-labeled for 5 h with 1 mM Met, 1 mM Aha, and 1 mM Hpg (1:1), 3 mM Aha and 1 mM Hpg (3:1), or 1 mM Aha and 3 mM Hpg (1:3). Cells were fixed and blocked before dye-labeling with LR-azide and DMAC-alkyne. Images were false-colored in ImageJ. Scale bar represents 20 μm.

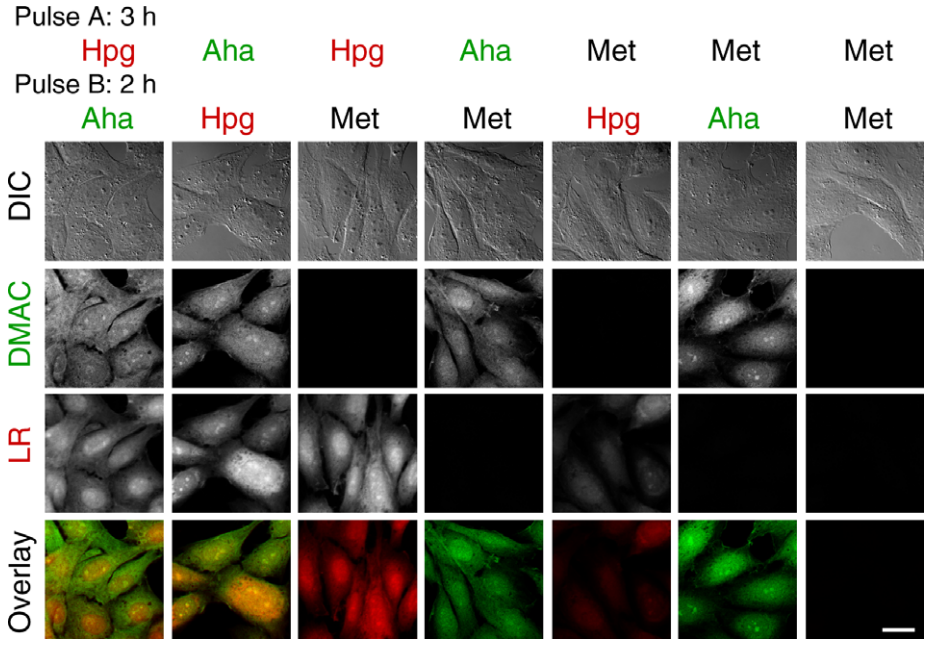

Figure 3. Fluorophore labeling of two distinct populations of proteins in Rat-1 fibroblasts. Cells were pulse-labeled for 3 h with an amino acid (Pulse A), washed, and then pulse-labeled for 2 h with a second amino acid (Pulse B). Cells were fixed and blocked before dye-labeling for 1 h with 50 μM LR-azide and 1 h with 10 μM DMAC-alkyne. The overlay shows both the DMAC (green) and LR (red) fluorescences. Scale bar represents 20 μm.

distribution of labeling should be similar for the two fluorophores. The false-colored micrographs indicate that both dyes label the entire interior of the cell, although LR-azide appears to stain the nucleus more brightly than DMAC-alkyne. Background labeling by LR-azide is also slightly higher than that observed for DMAC-alkyne. While ideally the dyes would behave identically, we are satisfied that we have identified two reactive fluorophores that stain similarly.

We sought to label two temporally defined populations of proteins by sequential addition of two amino acid analogues. Rat-1 fibroblasts were pulse-labeled for 3 h in media supplemented with either Aha, Hpg, or Met. At the end of the first pulse, cells were washed and incubated 15 min to deplete the first amino acid. The medium was replaced, and the fresh medium was supplemented with one of the three amino acids for the second pulse (2 h). As described above, fixed cells were dye-labeled for 1 h with

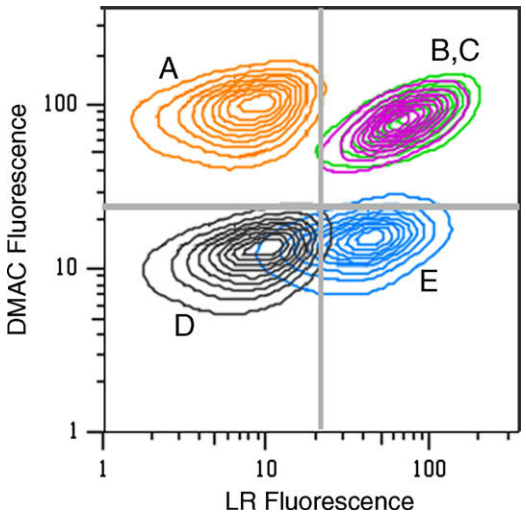

Figure 4. Flow cytometry contour plot of two-dye labeled fibroblasts. Cells were pulse-labeled for 3 h with 1 mM amino acid (Pulse I), washed, and then pulse-labeled for 2 h with 1 mM of a second amino acid (Pulse II). Cells were fixed and blocked before dye-labeling with LR-azide and DMAC-alkyne. A: Cells were pulse-labeled with 1 mM Aha, followed by 1 mM Met [Orange: Aha→Met]. B: [Magenta: Aha→Hpg]. C: [Green: Hpg→Aha]. D: [Gray: Met→Met]. E: [Blue: Hpg→Met]. For each sample, 50,000 total events were collected. Dead cells and debris were excluded from analysis using forward scatter and side scatter.

LR-azide followed by 1 h with DMAC-alkyne. Individual cells were examined for two-color labeling using confocal fluorescence microscopy (Fig. 3). For cells treated with both reactive analogues, bright LR and DMAC fluorescences were observed. As expected, for cells treated with Met and Hpg or with Met and Aha, only LR fluorescence or DMAC fluorescence, respectively, was detected. For cells pulse-labeled twice with Met, only background fluorescence was observed. The imaging results were validated by flow cytometry of cells sequentially pulsed with two analogues (Fig. 4). Compared to cells pulse-labeled with Met, cells pulse-labeled with both Aha and Hpg showed substantial LR and DMAC labelings. Cells treated with a single reactive amino acid were labeled by a single dye.

We obtained similar results upon reversing the order of addition of the dyes (i.e., DMAC-alkyne for 1 h, then LR-azide for 1 h). We also tested two-dye labeling with DMAC-azide and LR-alkyne, and observed efficient labeling by fluorescence microscopy and flow cytometry (Figs. S3 and S4). As mentioned above, LR-azide is a more reliable reactive fluorophore than LR-alkyne for one-dye labeling, and this holds true for two-dye labeling. Finally, it should be noted that two-dye labeling of proteins worked in every cell type examined. Notably, the rat exocrine cell line AR42J enabled sequential pulse-labeling with shorter (1 h) pulse lengths (Fig. S5).

The work described here introduces reactive LR and DMAC dyes that enable two-color labeling of temporally defined protein populations in mammalian cells. Labeling is easily observed by flow cytometry and fluorescence microscopy. The dyes and methods developed in this work should find many uses in studies of the temporal and spatial character of protein translation.

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Supplementary data

Experimental protocols and additional data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.046.

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