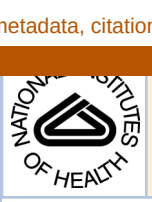

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A Bodipy-Cyclooctyne for Protein Imaging in Live Cells

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Proteomic studies are designed to elucidate the nature, causes and consequences of spatial and temporal variation in the protein contents of cells. Chemical biologists have made important contributions to proteomic research through the creation of new reactive metabolites and selective bioconjugation reactions, both of which have enhanced our ability to characterize subsets of the proteome.[1] An expanding set of reactive analogues of cellular metabolites (e.g., amino acids,[2] glycans,[3] and lipids[4]) has been described; appropriately designed analogues can be inserted into biomolecules in either wild-type or genetically-altered cells. After incorporation, analogues are ligated to affinity tags or biophysical probes through bioorthogonal reactions. In the last few years, the preeminent reactions for tagging biomolecules have been the copper-catalyzed[5] or strain-promoted[6] azide-alkyne ligations. For studies of live cells, the strain-promoted ligation is often preferred because of concerns about the toxicity of copper. Early work on the strain-promoted ligation introduced a set of reactive cyclooctyne probes for labeling of cell-surface glycans.[6] But many proteomic changes occur within the cell, and study of such processes requires probes that can label intracellular targets. In this communication, we describe a membrane-permeant bodipy-cyclooctyne for imaging azide-tagged proteins in live cells.

Metabolic labeling of proteins is readily accomplished by treatment of cells with the reactive methionine (Met) analogue azidohomoalanine (Aha).[7] During a defined exposure, or “pulse,” addition of Aha to Met-depleted medium allows insertion of Aha into cellular proteins in response to Met codons. Recently we reported a set of coumarin-cyclooctyne dyes for labeling of Aha-tagged proteins in live cells.[8] Good selectivity for newly synthesized proteins was observed; however, the 800 nm (two-photon) excitation source used for imaging of coumarin-labeled proteins is inaccessible to some researchers, and many imaging systems are insensitive to coumarin fluorescence. Coumarins can be imaged after excitation with ultraviolet light, but ultraviolet light has poor tissue penetration and prolonged exposure to ultraviolet radiation can damage live cells.[9] The limitations of the coumarin fluorophores prompted us to search for alternative probes for intracellular labeling of proteins. We identified the small, bright fluorophore Bodipy[10], which can be imaged on most standard fluorescence microscopes owing to its similarity in excitation and emission to the widely used green fluorescent protein.[11] Here we report the use of bodipy-cyclooctyne (BDPY) to capture images of Aha-tagged proteins in live mammalian cells.

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

Fluorescence imaging of Rat-1 fibroblasts by confocal microscopy provided an initial assessment of the specificity of labeling by BDPY (Figure 1). Cells were pulse-labeled with Aha for 4 h before 10 min of dye-labeling at 37 °C with 10 μM BDPY and counterstaining with a nuclear dye, Hoechst, and MitoTracker Red. MitoTracker Red localizes to functional mitochondria and serves as a viability indicator. Fluorescence micrographs of live cells stained with BDPY showed rapid and selective labeling of newly synthesized proteins. Minimal fluorescence was observed in BDPY-treated control cultures incubated with Met or with Aha plus the protein synthesis inhibitor anisomycin (Aha+aniso).

BDPY labeling of azide-tagged proteins was examined further by in-gel fluorescence imaging. After a 4 h Aha pulse, cells were labeled with 10 μM BDPY for 30 min. Labeled cells were fractionated to separate proteins into four fractions. Proteins localized in the cytosol (C) were separated from those derived from the plasma membrane and membrane-bound organelles (M; e.g., mitochondria and endoplasmic reticulum), the nuclear membrane and nucleus (N), and a final fraction that contained primarily cytoskeletal and insoluble (I) proteins. Equal amounts of each protein fraction were separated by SDS polyacrylamide gel electrophoresis, and protein bands were detected in-gel by fluorescence imaging of BDPY (Figure 2). Distinct fluorescent bands could be detected in all four fractions isolated from cells exposed to Aha. Although the most intense fluorescence was observed in the membrane fraction, proteins isolated from the cytosol, nucleus, and cytoskeleton also showed clear evidence of BDPY labeling. There was little detectable fluorescence for protein fractions isolated from cells labeled with Met or with Aha plus anisomycin (Supporting Information, Figure S2).

Live-cell labeling with BDPY was characterized quantitatively by flow cytometry. After a 4 h Aha pulse, cells were incubated for 30 min with BDPY at concentrations ranging from 0.5 to 50 μM (Figure 3). The mean fluorescence enhancement (the ratio of mean fluorescence observed for cells labeled with Aha to that of cells treated only with Met) increased from 4 to 26 with increasing concentrations of BPDY.

To determine the effect of the Aha pulse length on the intensity of labeling with BDPY, we treated cells with 1 mM Aha or Met for intervals ranging from 30 min to 6 h (Figure 4). Following the pulse, cells were labeled with 5 μM BDPY for 30 min at 37 °C. After 30 min of exposure to Aha, the mean fluorescence enhancement was 3.8; increasing the pulse length raised the mean fluorescence enhancement to 21 at 6 h.

Variations in the conditions under which cells were dye-labeled were also examined. Cells were labeled with Aha for 4 h before incubation for 10 min or 30 min with three different concentrations of BDPY (0.5, 5, and 50 μM). As shown in Supporting Information (Figure S4), 10 min treatment with BDPY enabled selective dye-labeling under each set of conditions.

The methods described here do not appear to compromise cell viability. MitoTracker Red was used to ensure that mitochondrial morphology remained normal during imaging, and counterstaining with propidium iodide confirmed that the cellular membrane remained intact after labeling with BDPY (Supporting Information, Figure S5). Cells remained well spread after fluorescence imaging.

BDPY is a reactive, membrane-permeant fluorophore that can be imaged and evaluated on standard fluorescence equipment. Live-cell imaging demonstrates that BDPY permits rapid and selective visualization of Aha-tagged proteins inside living cells. Protein fractionation and in-gel imaging confirm that cytosolic and nuclear proteins are labeled by BDPY. In combination with Aha and other azide-tagged metabolites, BDPY should find many applications in cellular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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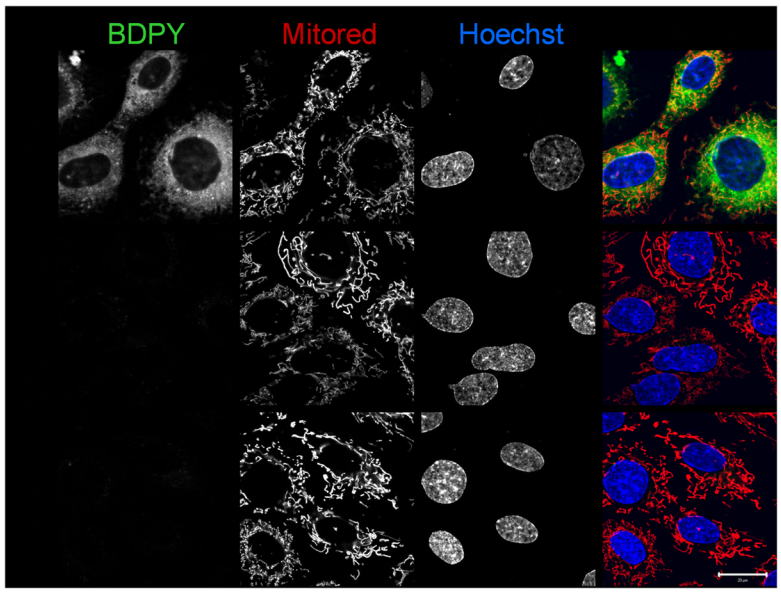

Figure 1.
Fluorescence labeling of proteins with BDPY in Rat-1 fibroblasts. Cells were cultured in media containing 1 mM Aha (top row), 1 mM Aha+anisomycin (middle row), or 1 mM Met (bottom row) before dye-labeling with 10 μM BDPY. Cells were counterstained with MitoTracker Red (Mitored) and Hoechst before imaging. The overlay (last column) contains superimposed images of the BDPY (green), MitoRed (red), and Hoechst (blue) fluorescence. Scale bar represents 20 μm.

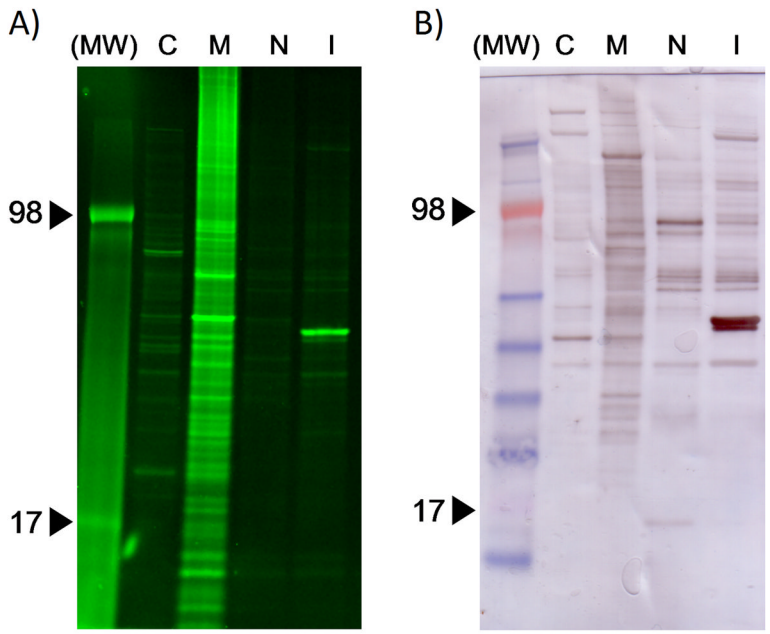

Figure 2.

SDS polyacrylamide gel electrophoresis (SDS PAGE) of fractionated proteins from cells labeled with BDPY. Cells were pulse-labeled 4 h with Aha before 30 min dye-labeling. Cells were fractionated to isolate cytosolic proteins (C), membrane proteins (M), nuclear proteins (N), and cytoskeletal and insoluble proteins (I). Equal amounts of protein were loaded in each lane. A) Protein fractions were imaged in-gel by collecting BDPY fluorescence (excitation at 488 nm). B) The protein gel was transferred to a nitrocellulose membrane and protein bands were revealed by staining with India Ink. The first lane in each image contains a molecular weight (MW) ladder, with bands at 98 kDa and 17 kDa indicated. Cells pulse-labeled with Aha+aniso or with Met were also fractionated and imaged (Supporting Information, Figure S2).

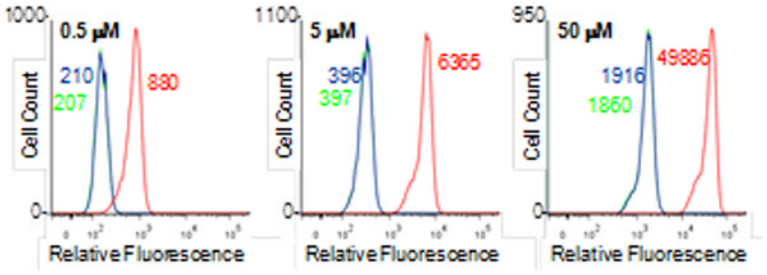

Figure 3.

Histograms of BDPY fluorescence as a function of BDPY concentration for live cells. The mean fluorescence for cells pulse-labeled 4 h with 1 mM Aha (red), [1 mM Aha+aniso] (green), or 1 mM Met (blue) is indicated. Cells were dye-labeled with 0.5, 5, or 50 μM BDPY (30 min at 37 °C) before analysis by flow cytometry. For each sample, 20,000 events were collected.

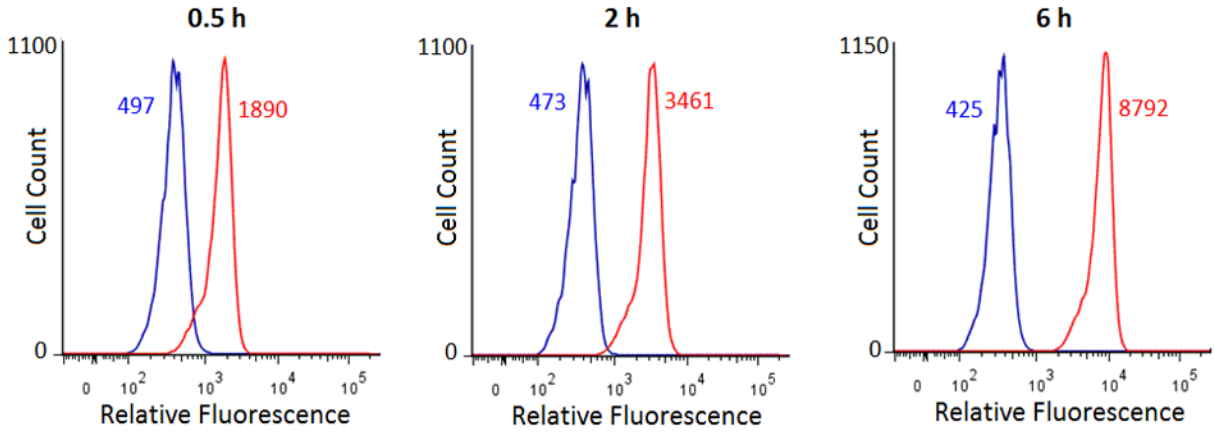

Figure 4.

Histograms of BDPY fluorescence as a function of Aha pulse length. The mean fluorescence for cells pulse-labeled from 30 min to 6 h with 1 mM Aha (red) or 1 mM Met (blue) is indicated. Cells were dye-labeled with 5 μM BDPY (30 min at 37 °C) before analysis by flow cytometry. For each sample, 20,000 events were collected. Histograms corresponding to Aha pulses of 1 and 4 h are shown in the Supporting information, Figure S3.