Chapter 6

AN EXPANDED SET OF FLUOROPHORES FOR LABELING NEWLY SYNTHESIZED PROTEINS IN LIVE CELLS

6.1 Abstract

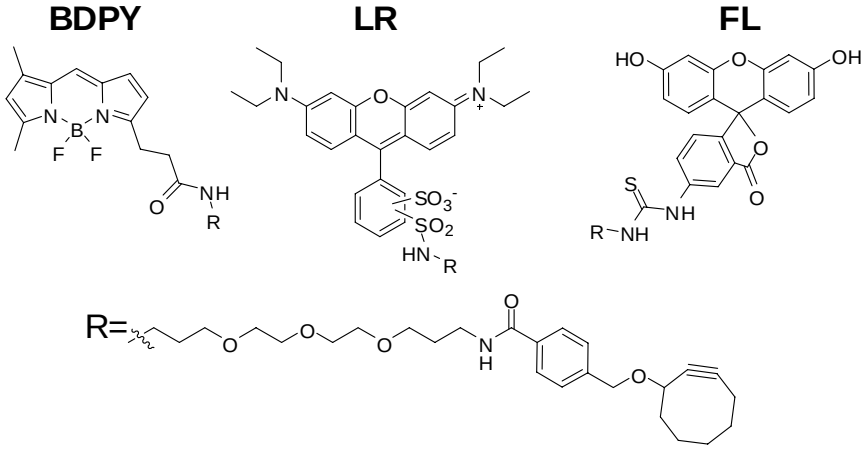
In Chapter 5, we described the use of a strain-promoted ligation between azidohomoalanine (Aha) and a set of coumarin-cyclooctynes for labeling newly synthesized proteins in living cells. Now we have synthesized and characterized bodipy (BDPY), fluorescein (FL), and lissamine rhodamine (LR) conjugated cyclooctynes to provide access to a broader range of the visible spectrum. Each dye was evaluated for its ability to selectively dye-label tagged proteins in fixed cells. Fluorescence micrographs indicated that Aha-treated cells had enhanced labeling compared to methionine-treated cells with both BDPY and LR, but not with FL. Furthermore, live cell imaging demonstrated that BDPY permits selective dye-labeling of Aha-tagged proteins inside living cells. Flow cytometric analysis of living cells was used to further characterize dye-labeling with BDPY. Fluorescence micrographs of labeling with LR and FL indicated that both dyes are excluded from the interior of live cells, preventing these dyes from selectively labeling intracellular proteins. BDPY, LR, and FL are being evaluated for labeling other azide-containing metabolites (e.g., azido-glycans). **

This project is an ongoing collaboration between Baskin JM, Beatty KE, Bertozzi CR, Fisk JD, Hangauer MJ, and Tirrell DA. ******The synthesis of the dyes (BDPY, LR, and FL) and the tagging of other reactive metabolites will be described elsewhere.

6.2 Introduction

The azide-alkyne ligation has emerged as a selective and sensitive method for attaching probes to proteins. Proteins are first tagged by the incorporation of small metabolite analogues. Reactive amino acids[1-8], glycans[9-16], and lipids[17-21] have all been used to tag proteins with an azide or alkyne using the endogenous cellular machinery. Next, the tagged protein is ligated to a probe using either a copper-catalyzed[22-24] or strain-promoted[25-27] azide-alkyne ligation. The probe can be an affinity purification tag or a small fluorescent dye. For fluorophore labeling in live cells, the strain-promoted ligation is preferable because it does not require a toxic copper catalyst. In Chapter 5, we described a new set of coumarin-cyclooctyne fluorophores for the selective dye-labeling of newly synthesized proteins inside mammalian cells. Briefly, proteins synthesized during a defined exposure, or "pulse," with azidohomoalanine (Aha) were tagged by this reactive methionine (Met) analogue[4, 28, 29]. The simple addition of this analogue to Met-free media is the only requirement for endogenous cellular enzymes to efficiently substitute Aha for Met within proteins[30]. After tagging, proteins were labeled using a strain-promoted ligation between Aha and a coumarin-cyclooctyne. Labeling was confirmed by fluorescence microscopy and flow cytometric analysis. Despite the selectivity of labeling with the coumarin-cyclooctynes, the 800 nm (two-photon) light used to excite these dyes makes imaging these fluorophores inaccessible to some researchers. The coumarin can be imaged after excitation with ultraviolet light; however, low wavelength light has poor tissue penetration and prolonged exposure to such high energy light can damage live cells[31].

Because of the imaging limitations for coumarin fluorophores, we are now evaluating labeling of azide-containing metabolites in live cells using new bodipy (BDPY), fluorescein (FL), and lissamine rhodamine (LR) conjugated cyclooctynes (**Scheme 6.1**). BDPY and FL are similar to green fluorescent protein in excitation (488 nm) and emission (~500-550 nm), enabling their visualization on most standard fluorescence microscopes[32]. LR is excited at a longer wavelength (~570 nm), which can improve signal-to-noise because there is less cellular autofluorescence from excitation at wavelengths above 500 nm[31, 33]. Each fluorophore-cyclooctyne will be examined for tagging several distinct azide-displaying metabolites with known cellular localization patterns because an ideal fluorophore will enable the selective and efficient labeling of any reactive metabolite, regardless of the metabolite's cellular localization. The evaluation of these fluorophores for labeling reactive metabolites other than Aha will be described elsewhere.



Scheme 6.1. Structures of the reactive fluorophore-cyclooctynes: BDPY, LR, and FL.

6.3 Results and Discussion

For imaging the proteome, an ideal fluorophore should have access to any protein tagged with Aha, whether it is on the surface of the cell, in the cytoplasm, or in the nucleus. This requires that fluorophores be membrane permeant, although dyes that cannot cross intact membranes would allow very selective labeling of metabolites found predominantly on the surface of cells. In the current work, we have characterized BDPY, LR, and FL for dye-labeling reactive metabolic analogues. Each of these dyes has been evaluated for selective labeling of proteins displaying the amino acid analogue Aha.

Although the final objective is to expand the set of available reactive fluorophore-cyclooctynes for live-cell labeling, fluorescence imaging of fixed cells by confocal microscopy provided an initial assessment of the specificity of the dyes (**Figure 6.1**). The mammalian Rat-1 fibroblast cell line was pulse-labeled for 4 h with either 1 mM Aha, Met, or Aha pre-treated with anisomycin [Aha+aniso], a protein synthesis inhibitor. The [Aha+aniso] control was included to assess the contribution of free Aha to the overall fluorescence in cells. After the pulse, cells were fixed and blocked before dye-labeling with 50 μM BDPY, LR, or FL for 30 min at 37 °C. Individual cells were then imaged. Cells that were pulse-labeled with Aha and dye-labeled with BDPY or LR exhibited brighter fluorescence labeling than control cells (Met or Aha+aniso). Dye-labeling with FL was non-specific, and fluorescence labeling was observed at nearly the same level in control cells as for cells treated with Aha.

Dye-labeling of fixed cells was also examined by flow cytometry using two concentrations of reactive-cyclooctyne, 50 or 10 μM (**Figure 6.2**). All cells treated with

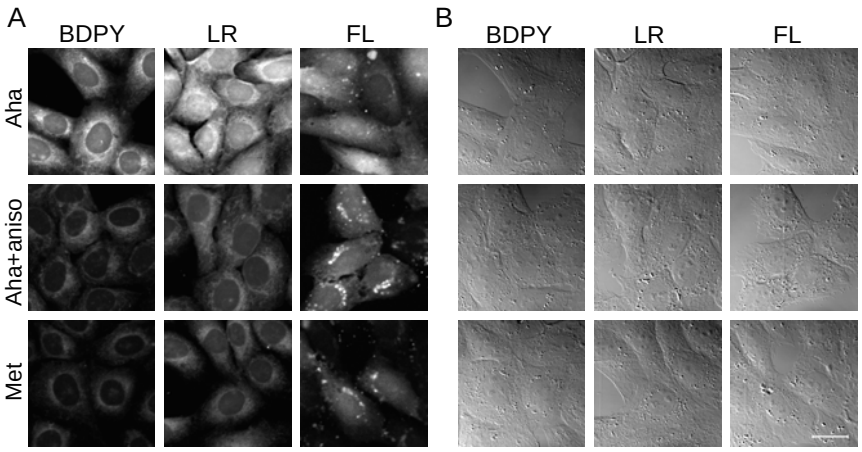


Figure 6.1. Fluorescence micrographs of fixed Rat-1 fibroblasts dye-labeled with BDPY, LR, or FL. Fibroblasts were grown 4 h in media containing 1 mM Aha (top row), 1 mM Aha pre-treated with the protein synthesis inhibitor anisomycin (Aha+aniso; middle row), or 1 mM Met (bottom row). After the pulse, cells were fixed, blocked, and dye-labeled (30 min at 37 °C) with 50 μM BDPY (first column), LR (second column), or FL (third column). **A.** Fluorescence images. The set of three images for each dye were acquired with identical conditions. **B.** Corresponding differential interference contrast (DIC) images. Scale bar represents 20 μm.

Aha had enhanced labeling compared to Met-treated cells, although only BDPY showed a substantial azide-dependent labeling enhancement. Compared to Met-treated cells, Aha-treated cells were characterized by an 8.3- or 6.4-fold enhancement in mean fluorescence after dye-labeling with 50 or 10 μM BDPY, respectively. LR and FL showed only slight labeling enhancements; Aha-treated cells were characterized by mean fluorescences only 1.3- to 1.9-fold higher than those of cells pulse-labeled with Met. The low fluorescence enhancement for LR might be attributed to the sub-optimal wavelength (514 nm) used to excite the dye during analysis. The low labeling enhancement with FL is consistent with the observations from fluorescence microscopy. Overall, fixed cell

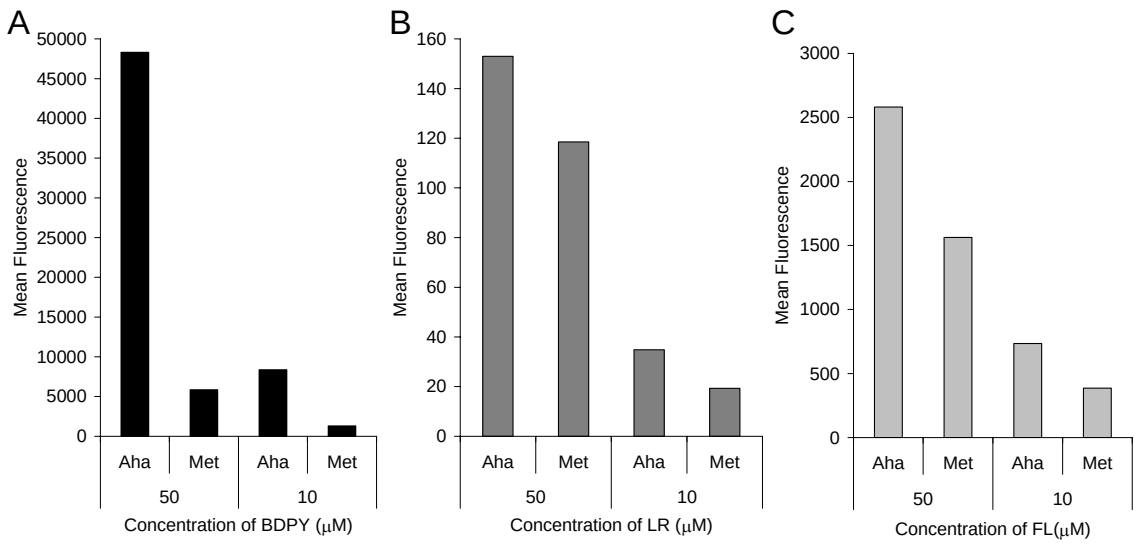


Figure 6.2. Flow cytometric analysis of fixed Rat-1 fibroblasts dye-labeled with BDPY, LR, or FL. Cells were pulse-labeled 4 h with 1 mM Aha or Met, fixed, and blocked. Cells were then dye-labeled 30 min at 37 °C with 50 or 10 μM of reactive fluorophore-cyclooctyne. **A.** BDPY. **B.** LR. **C.** FL. Each bar represents more than 10,000 events.

imaging of Aha-tagged proteins in cells indicated that the enhancements in labeling for each dye were not large, but that BDPY and LR are candidates for live-cell labeling of reactive metabolites.

Confocal fluorescence microscope images were used to evaluate dye-labeling with LR and FL in living cells (**Figure 6.3**). Cells were pulse-labeled for 4 h before 30 min of dye-labeling at 37 °C with 50 μM FL or LR. Before imaging, cells were counterstained with the nuclear stain Hoechst. Both FL and LR appear to be membrane impermeant, with the fluorescence staining pattern of cells treated with Aha being remarkably similar to cells treated with Met. In all images, labeling was limited to bright, punctate structures in the interior of cells. Further investigations could elucidate whether

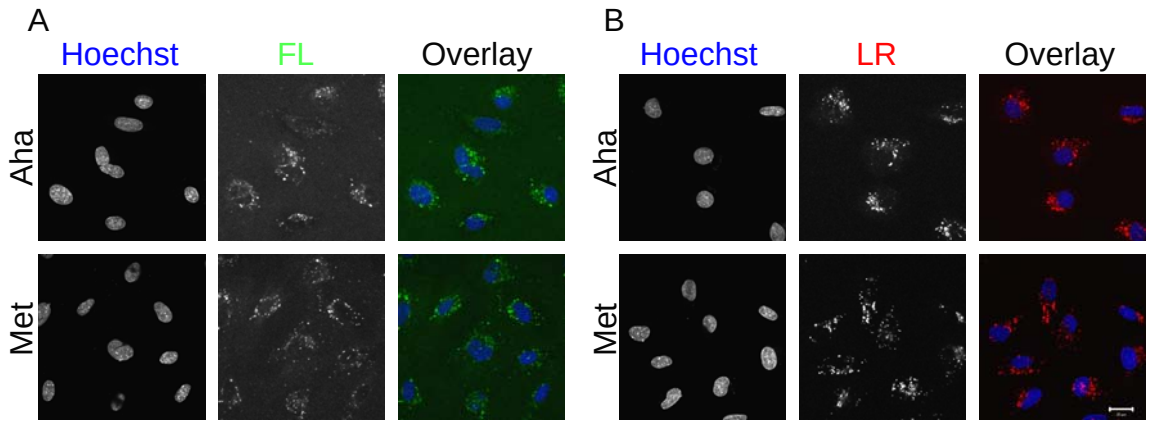


Figure 6.3. Fluorescence labeling of proteins with FL and LR in Rat-1 fibroblasts. Confocal fluorescence imaging of fibroblasts grown 4 h in media containing 1 mM Aha (top row) or 1 mM Met (bottom row). After the pulse, cells were dye-labeled for 30 min with 50 μM FL (**A**) or LR (**B**). Cells were counterstained with Hoechst before imaging. The overlay (last column) includes the FL (green) or the LR (red) fluorescence with the Hoechst (blue) fluorescence. Scale bar represents 20 μm.

these dyes are non-specifically endocytosed. Flow cytometric analysis of cells labeled with FL confirmed that this dye did not allow selective labeling of Aha-treated cells (data not shown).

Fluorescence microscopy was used to image cells labeled with BDPY (**Figure 6.4**). Cells were pulse-labeled 4 h before 10 min of dye-labeling at 37 °C with 10 μM BDPY and counterstaining with Hoechst and MitoTracker Red. MitoTracker Red, which localizes to functional mitochondria, enables the visualization of mitochondria as an indicator of cell viability. Fluorescence micrographs of live cells stained with BDPY indicated that this dye yielded rapid and selective labeling of newly synthesized proteins. Substantial fluorescence was observed in the cytoplasm of Aha-treated cells, while the nuclear region remained dim. A similar staining pattern was observed for the non-

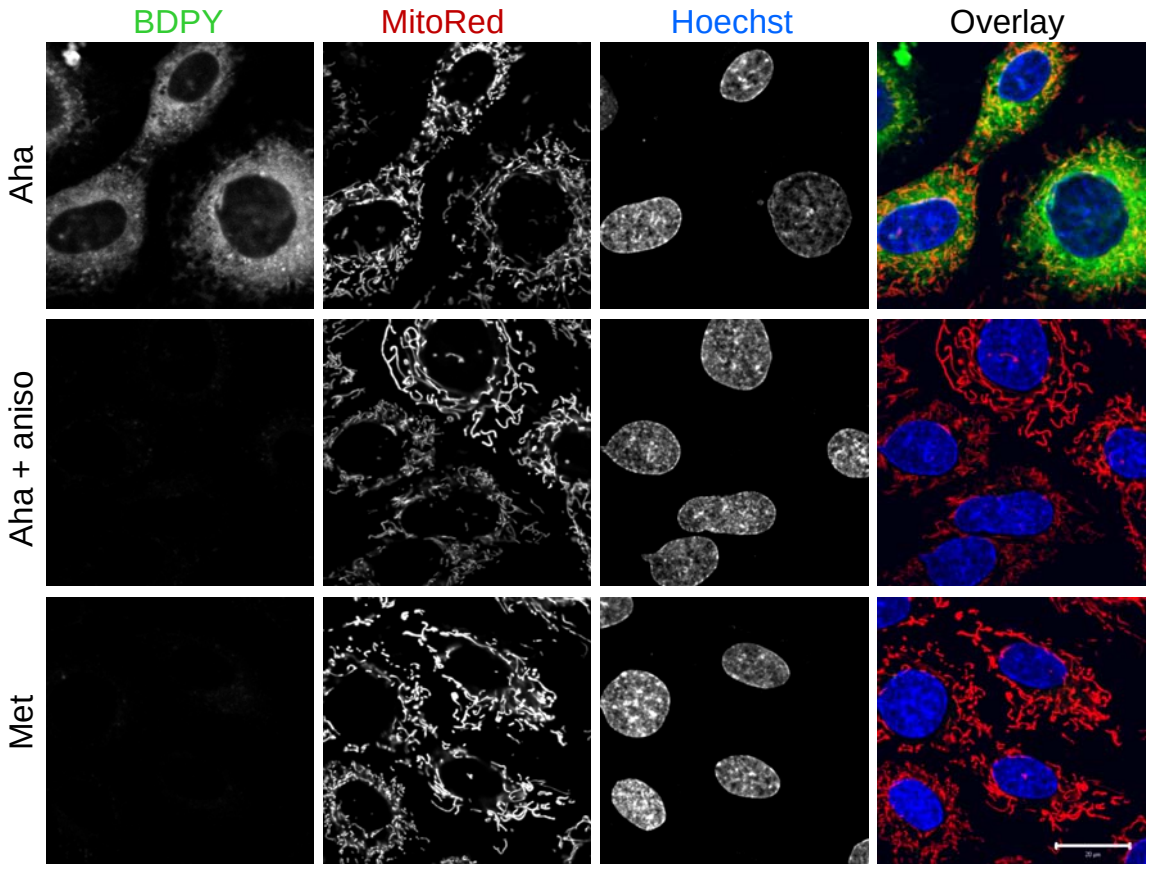


Figure 6.4. Fluorescence labeling of proteins with BDPY in Rat-1 fibroblasts. Cells were grown 4 h in media containing 1 mM Aha (top row), 1 mM Aha+aniso (middle row), or 1 mM Met (bottom row). After the pulse, cells were dye-labeled 10 min with 10 μM BDPY (first column). Cells were counterstained with MitoTracker Red (MitoRed) and Hoechst before imaging. The set of BDPY-fluorescent images were acquired using identical conditions. The overlay (last column) contains superimposed images of the BDPY (green), MitoRed (red), and Hoechst (blue) fluorescence. Scale bar represents 20 μm.

fluorinated coumarin-cyclooctyne described in Chapter 5. The fluorescence in cells treated with Met or [Aha+aniso] was very dim, indicating the most of the BDPY-labeled Aha was protein-associated. Notably, BDPY permits the concurrent imaging of two other fluorophores, as demonstrated by counterstaining with MitoTracker Red and Hoechst. The use of this dye with additional fluorescent probes to simultaneously follow

distinct cellular processes, such as trafficking or secretion, could be very powerful. In comparable research, Gaietta and coworkers used two temporally-defined fluorescent tags to elucidate the process of connexin trafficking in gap junctions[34]. This work enabled the temporal tagging of a pre-selected protein with short, genetically encoded tetracysteine motifs[35]. A more global analysis of trafficking may be possible using metabolic tagging of proteins[36].

Live-cell labeling with BDPY was further characterized using flow cytometry. After a 4 h pulse, cells were incubated with dye for 30 min with 0.5 to 100 μM BDPY (**Figure 6.5**). The largest fluorescence enhancement was observed at 50 μM. However,

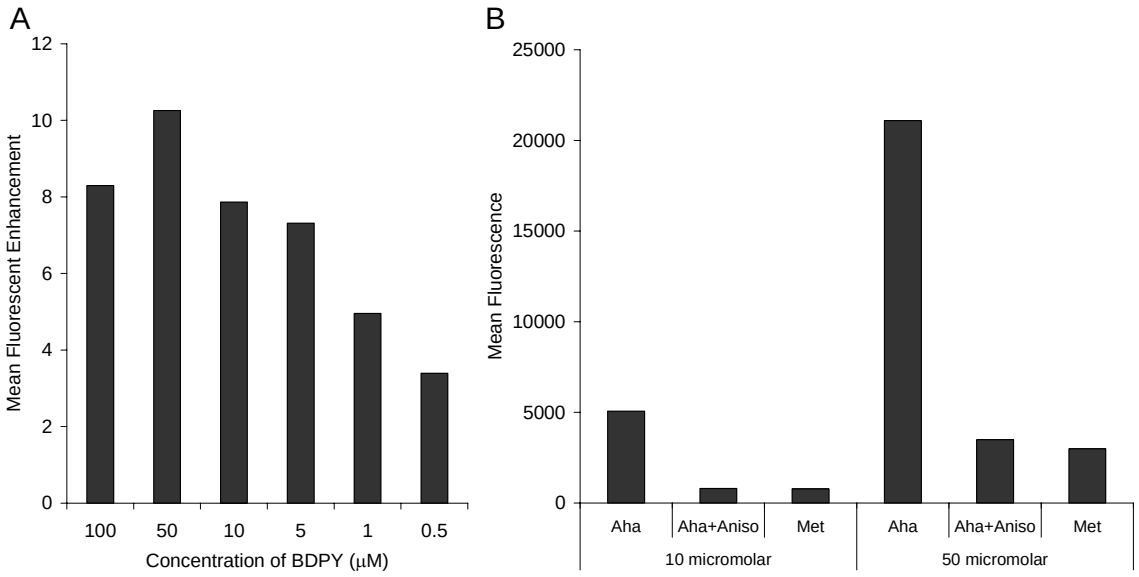


Figure 6.5. Flow cytometric analysis of BDPY fluorescence as a function of fluorophore concentration for live cells. **A.** Mean fluorescence enhancement for cells pulse-labeled 4 h and dye-labeled with different concentrations of BDPY for 30 min at 37 °C. For each sample, 30,000 events were collected. **B.** Mean fluorescence for cells pulse-labeled 4 h with 1 mM Aha, [1 mM Aha+aniso], or 1 mM Met. Cells were dye-labeled with 10 or 50 μM BDPY (30 min at 37 °C) before analysis. For each sample, 20,000 events were collected.

using 10 μM BDPY reduces the non-specific labeling observed for treatment of cells with 50 μM. Different pulse-labeling and dye-labeling conditions were also examined (**Figure 6.6**). Cells were pulsed with 1 mM Aha or Met for 10 min to 6 h. Following the pulse, cells were labeled with 50 μM BDPY for 30 min at 37 °C. After only 10 min of exposure to Aha, the mean fluorescence enhancement was 1.3-fold compared to Met-treated cells. Increasing the pulse-length resulted in further increases in mean fluorescence enhancement up to 10.2-fold at 6 h. Next, variations in the conditions

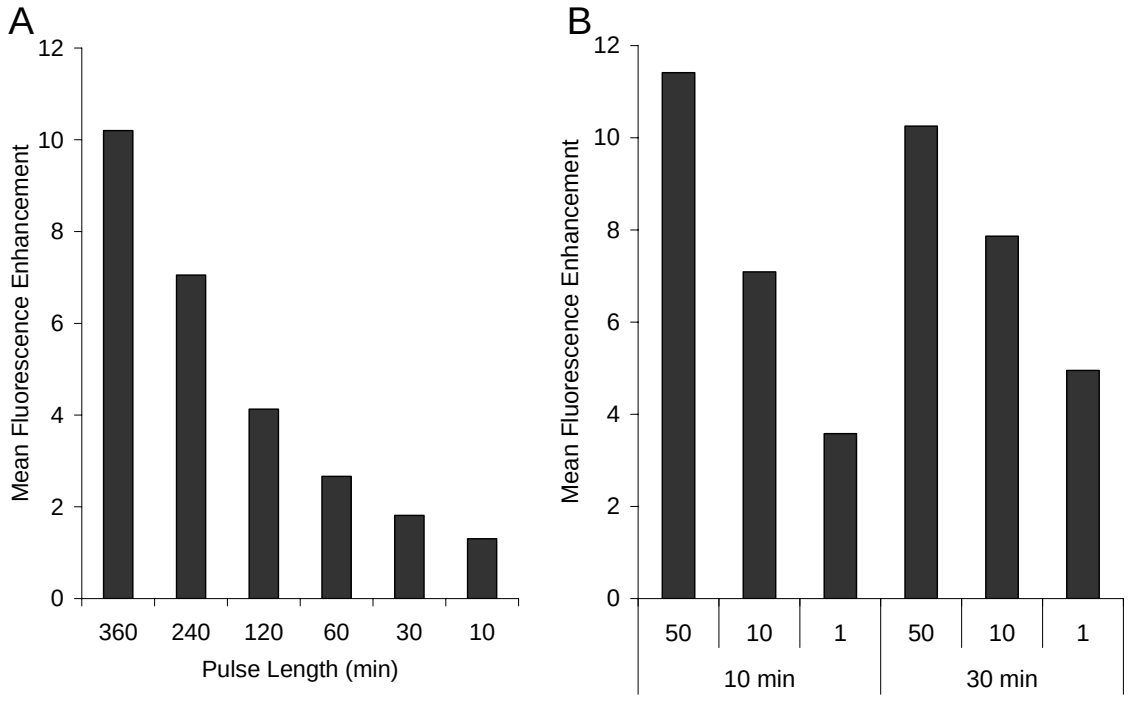


Figure 6.6. Flow cytometric analysis of the pulse-labeling and dye-labeling conditions for live Rat-1 fibroblasts. **A.** Cells were pulse-labeled for 10 to 360 min before dye-labeling with 50 μM BDPY for 30 min at 37 °C. For each sample, more than 15,000 events were collected. **B.** Cells were pulse-labeled for 4 h before dye-labeling with 50, 10, or 1 μM BDPY for 10 or 30 min at 37 °C. For each sample, 30,000 events were collected.

under which cells were dye-labeled were examined. Cells were pulse-labeled 4 h before incubation for 10 or 30 min with three different concentrations of BDPY (1, 10, and 50 μM). As shown in **Figure 6.6B**, a 10 min treatment with BDPY enabled selective dye-labeling under most conditions. However, lower concentrations of dye (e.g., 1 μM) required longer incubation with BDPY to enhance the fluorescence signal.

Our aim has been to provide a minimally perturbative method for imaging tagged proteins in living cells. Three distinct techniques confirmed that exposure of cells to BDPY and subsequent imaging did not compromise cell viability. First, MitoTracker Red was used to ensure that mitochondrial morphology remained normal during imaging (**Figure 6.4**). Second, cells were counterstained with propidium iodide and Hoechst to confirm that the cellular membrane remains intact after labeling with BDPY (**Figure 6.7**). Finally, phase contrast microscopy was used to verify that cells remained well-spread after imaging experiments.

6.4 Conclusion

We have evaluated an expanded set of reactive fluorophore-cyclooctynes for labeling newly synthesized proteins in living cells. Fluorescence micrographs of fixed cells indicated that BDPY and LR, but not FL, enable selective labeling of Aha-tagged proteins. Live cell imaging revealed that cells pulse-labeled with Aha and dye-labeled with LR or FL could not be distinguished from Met-treated cells. This can be attributed to the nearly complete exclusion of these fluorophores from the interior of cells. In contrast, BDPY enabled the rapid and selective labeling of newly synthesized proteins inside cells. The pulse- and dye-labeling conditions were further characterized by flow

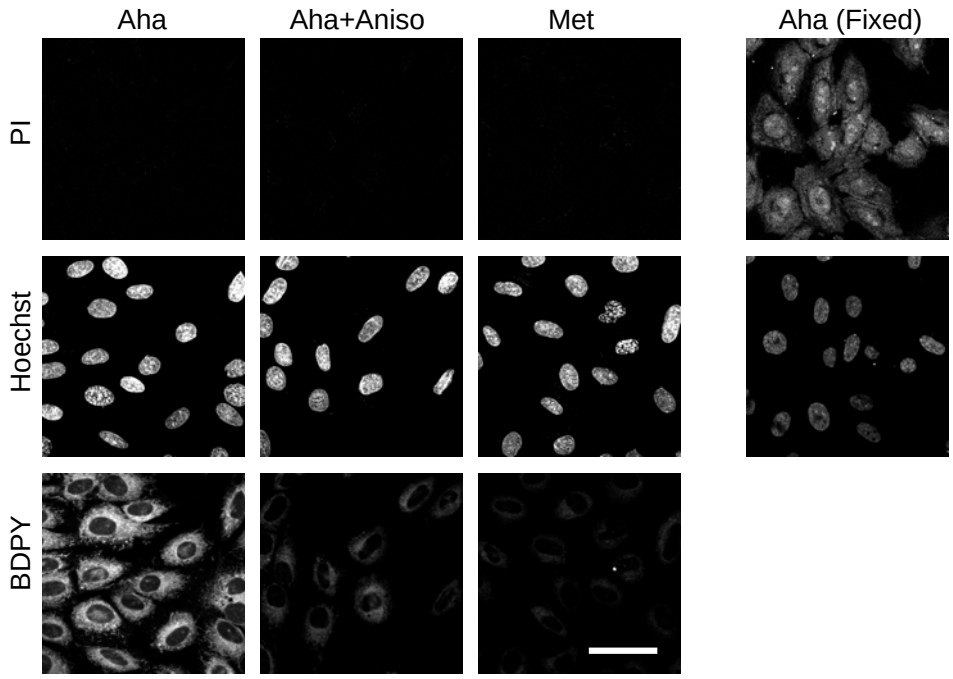


Figure 6.7. Viability of cells dye-labeled with BDPY. Cells were pulse-labeled 4 h in media containing Aha, [Aha+aniso], or Met. Cells were dye-labeled 10 min with 10 μM BDPY before 10 min of counterstaining with Hoechst and propidium iodide (PI). Fixed cells were pulse-labeled with Aha and then treated with 3.7% paraformaldehyde for 10 min. Fixed cells were not stained with BDPY. Scale bar represents 50 μm.

cytometry to evaluate the minimum requirements for specific and detectable protein labeling with BDPY.

6.5 Future Work

As stated above, we intend to evaluate this set of dyes for labeling several different azide-displaying metabolites. Since many metabolites have known cellular localization patterns, this will be a rigorous evaluation of the ability of each dye to selectively and accurately label tagged biomolecules in living cells. The fluorescence enhancement observed for LR in fixed cells indicates that LR could allow selective

labeling of metabolites found on the surface of cells, such as azido-glycans. FL will be evaluated, although the initial results with Aha-tagged proteins are not promising. Based on the results for Aha-tagged proteins, BDPY is the most likely dye to give selective labeling of a variety of reactive metabolites.

Interestingly, BDPY staining was found predominantly in the cytoplasm. This is similar to the labeling pattern we observed using the non-fluorinated and mono-fluorinated coumarin-cyclooctyne to dye-label proteins. In that case, the difluorinated coumarin-cyclooctyne gave more uniform labeling of the nucleus and cytoplasm (see Chapter 5). Synthesis and evaluation of a BDPY-conjugated difluorinated cyclooctyne would allow us to clarify the role of fluorination in accessing nuclear proteins.

In animals, tissue autofluorescence and imaging depth are important factors when selecting an imaging agent. Before reactive fluorophore-cyclooctynes can be applied to imaging *in vivo*, new infrared or near-infrared cyclooctyne probes will have to be synthesized and evaluated[31]. The set of procedures described in this thesis would be appropriate for examining any new reactive fluorophores.

6.6 Materials and Methods

6.6.1 Cell maintenance

Rat-1 fibroblasts (ATCC) were maintained in a 37 °C, 5% CO_2 humidified incubator chamber. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin (DMEM++). Near-confluent cells were passaged with 0.05% trypsin in 0.52 mM EDTA (Invitrogen).

6.6.2 *Preparation of Live Cells for Fluorescence Microscopy*

Near-confluent cells in 100 mm Petri dishes were rinsed twice with warm phosphate-buffered saline (PBS). Cells were detached with trypsin in EDTA and added to DMEM++. The cells were pelleted via centrifugation (200*g*, 3 min) and counted. Cells were added at a density of 1 x 10^4 cells per well to prepared slides and grown in DMEM++ overnight.

Lab-Tek chambered coverglass slides (8-well, Nalge Nunc International) were prepared by treatment with fibronectin solution (10 μg/mL). The wells were rinsed twice with PBS, blocked with a 2 mg/mL solution of heat-inactivated BSA at room temperature, and rinsed with PBS.

After growth overnight in DMEM++, each well was washed twice (200 μL) with warm PBS. Cells were incubated for 30 min in serum-free medium lacking Met [SFM: DMEM, with 1 mg/mL bovine serum albumin (BSA, fraction V, Sigma-Aldrich), with 2 mM Glutamax I (Invitrogen), without Met] to deplete intracellular Met stores. Anisomycin (40 μM, Sigma-Aldrich) was added to cells during this time to inhibit protein synthesis. After incubation, either 1 mM Met or 1 mM Aha was added to the medium. After 4 h, wells were rinsed twice with DMEM++ before adding the dye-labeling mixture.

Cells were exposed to the stated concentrations of reactive fluorophore-cyclooctynes in DMEM++. Labeling was allowed to proceed 10-30 min at 37 °C in the incubator chamber. After labeling, cells were washed twice with DMEM++ before counterstaining. Cells were counterstained with 160 μM Hoechst dye for 5 min. Cells were washed once before addition of 300 nM MitoTracker Red CMXRos (Invitrogen) for

10 min. After treatment, cells were washed thrice with DMEM-Imaging [DMEM lacking phenol red, with HEPES (Invitrogen), supplemented with 10% FBS and 1 mg/mL BSA], and then imaged in the same media. Cells were kept in an incubator until they could be imaged (up to 3 h).

For counterstaining with propidium iodide, cells were dye-labeled and washed twice with DMEM++ before the addition of Hoechst (160 μM) and a 1:1000 dilution of propidium iodide (1.0 mg/mL; Invitrogen) in DMEM++ for 5 min. Cells were washed thrice before imaging. Fixed (3.7% paraformaldehyde, 10 min) and permeabilized (0.1% Triton X-100 in PBS, 3 min) cells were also imaged as controls.

6.6.3 Preparation of Fixed Cells for Fluorescence Microscopy

Lab-Tek II Chamber Slides (8-well, Nalge Nunc International) were prepared as described above. After the 4 h pulse, cells were rinsed twice with warm PBS, fixed with 3.7% paraformaldehyde solution for 10 min, and rinsed thrice with PBS. Cells were treated with a blocking solution [10% (v/v) fetal bovine serum (Invitrogen), 50 mg/mL sucrose, 20 mg/mL BSA] overnight at 4 °C. Cells were exposed to reactive fluorophore-cyclooctyne (50 μM) in PBS (pH 7.4) for 30 min at 37 °C in the incubator. Cells were washed four times with PBS before the chamber walls were removed from the slide. Mounting medium was added, and a cover slip was attached before visualization.

6.6.4 Preparation of Live Cells for Flow Cytometry

Pulse-labeling was performed directly in the 6-well tissue culture dishes in which cells were grown. Each well was washed twice with warm PBS and incubated 30 min in

SFM to deplete intracellular Met stores. Cells were exposed to 1 mM Aha or 1 mM Met for pulse-labeling (10 min to 6 h) and then washed twice with PBS. Reactive cyclooctyne dye in DMEM++ was added to each well for fluorophore-labeling. Dye concentrations of 0.5 μM to 100 μM were examined. To examine alternative labeling times, 1, 10, or 50 μM dye was added for 10 or 30 min DMEM++. After labeling, cells were washed twice with warm PBS and detached using 250 μL of 0.05% trypsin in EDTA. Cells were added to 750 μL DMEM++, and 100 μL FBS was added to the bottom of the Eppendorf tube to minimize cell losses. Cells were pelleted by centrifugation (200*g*, 3 min) and washed once with 1 mL DMEM-Imaging, again with a cushion of FBS added to the bottom of the tube. Finally, cells were resuspended in 300 to 500 μL DMEM-Imaging before being filtered through a 50 μm Nytex nylon mesh screen (Sefar). Cells were stored on ice until analysis.

6.6.5 Preparation of Fixed Cells for Flow Cytometry

See Chapter 3 for protocols[28]. In brief, fixed cells were blocked overnight before reaction with 10 or 50 μM dye for 30 min at 37 °C in the incubator. Cells were washed twice with PBS and resuspended in PBS before filtering and analysis.

6.6.6 Fluorescence Microscopy

Live cells were imaged on a confocal microscope (Zeiss LSM 510 Meta NLO) at Caltech's Biological Imaging Center. A heated chamber was placed around the microscope to image the cells at ~37 °C. FL fluorescence was examined using excitation at 488 nm with emission collected between 500 and 550 nm. BDPY labeled cells were

excited at 488 nm with emission collected between 500 and 550 (live cells) or 535 and 590 (fixed cells). MitoTracker Red, propidium iodide, and LR fluorescence was examined by excitation at 543 nm with emission collected with a long pass filter (lp 560 nm, MitoTracker Red) or with a bandpass filter [565-615 nm, propidium iodide and LR]. Transmitted light images were also collected to differentiate individual cells. The set of images for each dye [i.e., Met, Aha, (Aha+aniso)] was obtained with identical conditions to capture each reactive fluorophore-cyclooctyne's fluorescence. Hoechst fluorescence was obtained by two-photon excitation at 750 nm (Ti:sapphire laser) with emission collected between 377 and 473.3 nm. Images were acquired with a Plan-Apochromat 63x/1.4 oil objective (Zeiss) and analyzed with Zeiss LSM and ImageJ software.

Fixed cells were imaged at room temperature using the same conditions.

6.6.7 Image Processing

The brightness and contrast were manually adjusted for the images corresponding to each dye using ImageJ Software. Then the minimum and maximum pixel values were applied to each image from samples treated with that dye.

6.6.8 Flow Cytometry

Cells were analyzed on a FACSAria flow cytometer (BD Biosciences Immunocytometry Systems) at Caltech's Flow Cytometry Facility. BDPY and FL fluorescence was excited by a 488 nm laser and detected after passage through a 530/30 bandpass filter. Forward- and side-scatter properties were used to exclude doublets, dead cells, and debris from analysis. For live cell labeling, 7-aminoactinomycin D (7-AAD;

Beckman Coulter) was used to exclude dead cells from analysis. 7-AAD was excited by a 488 nm laser and detected after passage through a 695/40 filter. Unlabeled cells, 7-AAD labeled cells, and reactive fluorophore-cyclooctyne singly-labeled cells were analyzed to ensure minimal cross-over fluorescence in each channel. If necessary, compensation was applied to reduce cross-over fluorescence.

LR labeling was analyzed at City of Hope's flow cytometry facility on a Moflo MLS (Dako). LR fluorescence was excited at 514 nm and emission was detected after passage through a HQ600/30 filter. After 50,000 total events were collected, forward- and side-scatter properties and pulse width were used to exclude debris, dead cells, and doublets from analysis. The excitation of LR is not maximal at 514 nm, but it was sufficient for analysis of fixed cells stained with this dye.

6.6.9 Synthesis of BDPY, LR, and FL

Synthesis of the fluorophore-cyclooctynes was done by J.D. Fisk and will be described elsewhere.

6.7 Acknowledgements

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6.8 References

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