Chapter 4

FLUOROPHORE LABELING OF TWO DISTINCT PROTEIN POPULATIONS IN MAMMALIAN CELLS

4.1 Abstract

The proteome can undergo complex dynamic changes in response to disease or environment. While there are many methods for identifying proteins, a more complete examination of the proteome would include complementary, time-resolved images of proteins in their natural environment. In Chapter 3, we demonstrated selective dye-labeling of a subset of the proteome in mammalian cells. Cells were pulse-labeled with a reactive methionine (Met) analogue, which permitted labeling of newly synthesized proteins with a fluorescent dye using an azide-alkyne ligation. The application of two reactive Met analogues, azidohomoalanine (Aha) and homopropargylglycine (Hpg), to dye-label two distinct protein populations inside fixed cells is now described, and will enable changes in the proteome to be tracked over time. Reactive lissamine rhodamine (LR), 7-dimethylaminocoumarin (DMAC), and Bodipy-630 (BDPY) dyes were synthesized and examined for selective dye-labeling of newly synthesized proteins. The LR and DMAC, but not BDPY, fluorophores were found to enable selective, efficient labeling of a subset of the proteome. Next, distinct and temporally defined protein populations were tagged by simultaneous or sequential pulse-labeling with Aha and Hpg. After pulse-labeling, cells were two-dye labeled using the reactive LR and DMAC dyes. Two-dye labeled cells were imaged using fluorescence microscopy and analyzed by flow cytometry, revealing that this
new method can be used to selectively dye-label and image two distinct protein populations inside cells.

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4.2 Introduction

In the most simplistic situation, each protein might be considered solely by its sequence of amino acid building blocks. Yet in a biological context, a vast amount of additional information is contained in the chemical, spatial, and temporal identity of each protein. Much of the chemical diversity occurs through post-translational modification of proteins[1]. Since the final identity of proteins is not determined exclusively by the DNA that encodes it, a more global analysis of proteins is not accessible by conventional genetic manipulations. Rather, more complete information about the proteome can be obtained using small, reactive metabolic analogues incorporated using the host cell’s machinery. Proteins within complex biological mixtures can be specifically tagged with ketones[2-5], azides[6-9], or alkynes[10-12]. During a subsequent bioorthogonal transformation, azides are reacted with either alkynes or triarylphosphines (Staudinger ligation) and ketones with hydrazide or aminooxy derivatives[2, 3, 7, 13-17]. This metabolic labeling strategy has enabled the identification and visualization of many different post-translational modifications, including glycosylation[18-20], phosphorylation[21, 22], farnesylation[23], and fatty acylation[24-26].

Similarly, reactive amino acid analogues can be incorporated into newly synthesized proteins to track spatial and temporal changes in the proteome[27-32]. In previous work, Met analogues Aha and Hpg were used to both identify[33] and visualize[34] a single, temporally defined subset of the proteome (Scheme 4.1). This tagging method is reminiscent of conventional pulse-labeling with radioactive amino acids, with the endogenous cellular machinery placing a reactive Met analogue at sites
Scheme 4.1. Structures of Met, Aha, and Hpg.

Scheme 4.2. Two-dye labeling of proteins using a simultaneous or sequential pulse of two reactive metabolic analogues.

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 the sensitive and selective copper-catalyzed azide-alkyne ligation[15, 16].

We envision that two-dye labeling of cells could be useful for processes reliant on spatial and temporal control of protein synthesis, such as protein translation in disease[32] (e.g., bacterial infection[35] or cancer[36]) or related to protein trafficking (e.g., secretion[37, 38]). The simultaneous or sequential addition of two reactive metabolites enables the tagging of two protein populations within cells (Scheme 4.2). This improvement in the method could be considered analogous to that obtained by using two or more fluorescent tags to observe distinct proteins within cells instead of using a single GFP-fusion; both types of two-fluorophore labeling facilitate the monitoring of dynamic
interactions[39-41]. As mentioned above, the azide, alkyne, and ketone functional groups can all be accessed for tagging proteins. Thus, in fixed cells, two populations can be examined by using two reactive groups for bioorthogonal ligations. The first demonstration of two-dye labeling of metabolically tagged proteins was described in 2007 by Chang and coworkers[42]. Flow cytometry analysis indicated that cells treated simultaneously with two reactive sugars could be two-dye labeled, although no information on the spatial distribution of glycosylated proteins was provided.

4.3 Results and Discussion

In this chapter, we demonstrate the selective fluorophore-labeling of two distinct sets of proteins inside mammalian cells. We synthesized three types of reactive fluorophores based on the rhodamine, coumarin, and bodipy dye scaffolds (Scheme 4.3). Each dye is spectrally distinct, facilitating the two-dye labeling of proteins. The reactive fluorophores were synthesized by coupling 3-azidopropylamine or propargylamine to commercially available amine-reactive dyes. With a set of azide and alkyne fluorophores in hand, 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 elucidate the relative 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 CuSO₄, 1 mM
Scheme 4.3. Structures of the reactive fluorophores.

triscarboxyethylphosphine (TCEP), 100 μM tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (triazole ligand)[9, 43], 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 (Figure 4.1). The dyes had access to both the nucleus and cytoplasm, and both types of fluorophore appeared to brightly stain the protein-rich nucleoli[44]. Additionally, these fluorophores did not have an inappropriate bias for any particular organelles (e.g., mitochondria or lysosomes.) Both of the DMAC dyes and the LR-azide dye gave uniform staining of cells, while the LR-alkyne gave more varied levels of labeling among different cells in a population. We recommend that the LR-azide, not the LR-alkyne, be used for this reason. Next, flow cytometry was used to quantify the fluorescence enhancement; cells treated with Aha or Hpg for 5 h were
**Figure 4.1.** Selective dye-labeling of newly synthesized proteins using azide or alkyne fluorophores. **A.** 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 to elucidate the relative contribution to labeling from free amino acid. 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. **B.** Differential interference contrast (DIC) images corresponding to images in A. Scale bars represent 20 μm.
Figure 4.2. Mean fluorescence of Rat-1 fibroblasts by flow cytometry. Cells were pulse-labeled 5 h in media supplemented with 1 mM Hpg, 1 mM Aha, or 1 mM Met. Control cells were pre-treated with the protein synthesis inhibitor anisomycin (aniso) 30 m prior to pulse-labeling to elucidate the relative contribution to labeling from free amino acid. Cells were fixed and blocked before dye-labeling. A. Cells were dye-labeled for 1 h at room temperature with 10 μM DMAC-alkyne, 50 μM LR-alkyne, 50 μM DMAC-azide, or 50 μM LR-azide. For each sample, 50,000 total events were collected. Dead cells and debris were excluded from analysis using forward-scatter and side-scatter properties. Black: LR fluorescence. Gray: DMAC fluorescence. B. Cells were dye-labeled for 1 h at room temperature with 10 μM BDPY-alkyne. Each bar represents two samples with 20,000 events collected for each sample. Error bars represent one standard deviation.

characterized by a mean fluorescence 3- to 7-fold higher than that of cells pulse-labeled with Met (Figure 4.2a). Addition of anisomycin to cells prior to the addition of the reactive analogue reduced the mean fluorescence to the level observed for the Met controls.

The BDPY-alkyne was also evaluated by flow cytometry. Flow cytometry analysis indicated that BDPY-alkyne provides sensitive and selective labeling of newly synthesized
proteins with Aha-treated cells being characterized by a 6.5-fold enhancement in mean fluorescence compared to Met-treated cells (Figure 4.2b). While BDPY-alkyne gave favorable labeling by flow cytometry, fluorescent images of individual cells treated with this dye revealed that BDPY-alkyne would be completely inappropriate for selective dye-labeling of cells. There was a large variation of the amount of fluorescence staining from cell to cell. A more substantial drawback is that the dye stained the same cytoplasmic structures in control cells treated with Met or [Aha+anisomycin] as it did in cells treated with Aha. This dye might be useful for other applications, such as labeling purified azide-tagged proteins, but we cannot recommend it for labeling azide-treated cells. We are unsure why BDPY-alkyne appears selective by flow cytometry compared to microscopy, but this observation does emphasize the necessity of evaluating fluorophore labeling by microscopy.

Although BDPY-alkyne was not conducive to proteomic labeling, the LR and DMAC fluorophores do enable selective labeling of newly synthesized proteins inside cells. Simultaneous pulse-labeling with two reactive amino acids provides the simplest means of introducing two distinct tags into cells[42]. Rat-1 fibroblasts were pulse-labeled 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. We found previously, using purified E. coli methionyl-tRNA synthetase, that $k_{cat}/K_M$ for Aha is approximately 25% larger than that for Hpg[45]. After the pulse, cells were fluorophore labeled for 1 h with 50 μM LR-azide, washed, and labeled 1 h with 10 μM DMAC-alkyne before imaging. Examination of individual cells by fluorescence microscopy revealed that it is possible to
Figure 4.3. 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 for 1 h with 50 μM LR-azide and 1 h with 10 μM DMAC-alkyne. Cells were delineated using differential interference contrast (DIC; top row) before fluorescent images were acquired for DMAC-alkyne and LR-azide. Images were false-colored in ImageJ (see calibration bar). Scale bar represents 20 μm.

selectively dye-label both Aha and Hpg (Figure 4.3). Due to the simultaneous addition of analogues, it was probable that the distribution of labeling would be identical for each fluorophore. The false-colored micrographs indicate that both dyes label the entire interior of the cell, although the LR appears to stain the nucleus slightly brighter than DMAC. The background labeling with LR is also slightly higher than that of DMAC. While ideally the dyes would behave identically, we are satisfied that we have identified two distinct fluorophore scaffolds that stain similarly.

Next, we sought to label two temporally distinct populations of proteins by the sequential addition of two amino acids. Rat-1 fibroblasts were pulse-labeled for 3 h in
media supplemented with one amino acid (1 mM 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 media was replaced with fresh media and supplemented with amino acid for the second pulse (2 h). As described above, fixed cells were dye-labeled for 1 h with LR-azide followed by 1 h with DMAC-alkyne. Individual cells were examined for two-dye labeling using confocal fluorescence microscopy (Figure 4.4). For cells treated with both reactive analogues, bright LR and DMAC fluorescence was observed. As expected, for cells treated with Met and Hpg or Met and Aha only LR fluorescence or DMAC fluorescence, respectively, was detected. For cells pulse-labeled twice with Met, only background fluorescence was observed.

![Figure 4.4](image)

Figure 4.4. Fluorophore labeling 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) fluorescence. Scale bar represents 20 μm.
observed. The microscope observations were validated by flow cytometry of cells sequentially pulsed with two analogues (Figure 4.5). Compared to cells pulse-labeled with Met, cells pulse-labeled with both Aha and Hpg showed substantial LR and DMAC labeling. Cells treated with a single reactive amino acid were only labeled by a single dye.

We have 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 (Figure 4.6) and flow cytometry (data not shown). As mentioned above, LR-azide is a more reliable reactive fluorophore than LR-alkyne for one-dye labeling, and this holds true

![Flow cytometry contour plot of two-dye labeled fibroblasts.](image)

**Figure 4.5.** Flow cytometry contour plot of two-dye labeled fibroblasts. Cell 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 50 μM LR-azide (1 h) and then 10 μM DMAC-alkyne (1 h). 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: [Grey: 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.
Figure 4.6. Fluorophore labeling 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. A. Cells were dye-labeled for 1 h with 10 μM DMAC-alkyne, washed, and then dye-labeled 1 h with 50 μM LR-azide [10 μM DMAC-alkyne; 50 μM LR-azide]. B. [50 μM DMAC-azide; 50 μM LR-alkyne]. C. [50 μM LR-alkyne; 50 μM DMAC-azide]. The overlay shows both the DMAC (green) and LR (red) fluorescence. Scale bar represents 20 μm.

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, presumably due to its higher rate of protein synthesis compared to Rat-1 fibroblasts (Figure 4.7).

4.4 Conclusion

We have introduced two reactive fluorophores, a LR and a DMAC, which enable two-dye labeling of the Met analogues Aha and Hpg. After the analogues were introduced simultaneously or sequentially, they could be selectively labeled by the reactive
Figure 4.7. Fluorophore labeling two distinct populations of proteins in pancreatic exocrine cells (AR42J). Cells were pulse-labeled for 1 h with an amino acid (Pulse A), washed, and then pulse-labeled for 1 h with a second amino acid (Pulse B). Cells were dye-labeled for 1 h with 50 μM LR-azide and then 1 h with 10 μM DMAC-alkyne before imaging. Scale bar represents 20 μm.

fluorophores. This labeling is easily observed by flow cytometry and fluorescence microscopy. The other scaffold examined, a bodipy dye, demonstrated the necessity of identifying fluorophores which can stain reactive metabolites in an unbiased fashion. Future work might focus on examining new fluorophores for multi-dye labeling of reactive metabolites, including amino acids or post-translational modifications.

4.5 Material and Methods

4.5.1 Cell Maintenance

Rat-1 fibroblasts (ATCC) and AR42J cells (ATCC) were maintained 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 (Sigma-Aldrich).

4.5.2 Preparation of Cells for Fluorescence Microscopy

Near-confluent cells in 100 mm Petri dishes were rinsed twice with 5 mL warm phosphate-buffered saline pH 7.4 (PBS, Invitrogen). 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 2 x 10⁴ cells (Rat-1 fibroblasts) or 2 x 10⁵ cells (AR42J) per well to prepared slides. Cells were grown in DMEM++ overnight.

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

After growth in DMEM++, each well was washed twice (200 μL) with warm PBS. Cells were incubated for 30 min in SFM [DMEM, with 1 mg/mL bovine serum albumin (BSA, fraction V, Sigma-Aldrich), with Glutamax (Invitrogen), without Met] to deplete intracellular Met stores. Anisomycin (40 μM; Sigma-Aldrich) was added to control cells during this time to inhibit protein synthesis. After incubation, either 1 mM Met, 1 mM Hpg, or 1 mM Aha was added to each well. After 1-5 h, cells were rinsed twice with warm PBS, fixed with a 3.7% paraformaldehyde solution for 10 min, and rinsed thrice with PBS. The cells were treated with a blocking solution [10% (v/v) fetal
bovine serum (Invitrogen), 50 mg/mL sucrose, 20 mg/mL BSA] for at least 30 min at room temperature or overnight at 4 °C.

For simultaneous pulse-labeling of cells with two amino acids, both amino acids were added at the same time. Cells were pulse-labeled for 5 hours, washed, fixed, and blocked.

For sequential pulse-labeling of Rat-1 fibroblasts with Aha and Hpg, the first analogue was added for 3 h. Cells were washed twice with warm PBS. SFM was added and cells were incubated for 15 min to deplete the first analogue. Then, fresh SFM and analogue were added for a second pulse of 2 h. Cells were washed, fixed, and blocked as described above. For AR42J, the first pulse was 1 h and the second pulse was also 1 h.

Cells were dye-labeled as previously described[9, 34]. Chamber wells were filled to the top with >1.2 mL of PBS (pH 7.5) containing 1 mM CuSO₄, 1 mM triscarboxyethylphosphine (TCEP), 100 μM tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (triazole ligand)[43], and 10 μM DMAC-alkyne, 50 μM LR-alkyne, 50 μM BDPY-alkyne, DMAC-azide, or 50 μM LR-azide. 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 1 h.

For two-dye labeling, the first dye mixture was added and reacted for 1 h before the slides were washed twice with PBS. Then, the second dye mixture was added and reacted 1 h. Longer reaction times did not improve the dye-labeling. After the second labeling reaction, cells were washed thrice with PBS containing 1% Tween 20 and 0.5 mM EDTA, and then twice with PBS. Slides were gently agitated for 1 min between
washes. Chamber walls were removed from the slide. Mounting medium (50% glycerol in PBS) was added, and a cover slip was attached before visualization. For two-dye labeling, we used 10 μM DMAC-alkyne, 50 μM LR-alkyne, 50 μM DMAC-azide, or 50 μM LR-azide.

4.5.3 Preparation of Cells for Flow Cytometry

As described above, pulse-labeling was performed directly in the 6-well tissue culture dishes in which cells were grown. After the pulse(s), cells were washed twice with PBS and detached using 0.05% trypsin in EDTA. Cells were centrifuged (200g, 3 min), lightly fixed in a 1% paraformaldehyde solution for 10 min at 37 °C, washed with PBS, and treated with a blocking solution for at least 30 min at room temperature or overnight at 4 °C. Cells were reacted at room temperature 1 h in PBS pH 7.5 supplemented with 1 mM CuSO₄, 1 mM TCEP, 100 μM triazole ligand, and fluorophore. For two-dye labeling, the first dye mixture was added for 1 h. Cells were washed once with 1 mL PBS before the second dye mixture was added. Cells were again reacted 1 h at room temperature. After the second labeling reaction, cells were washed once with 0.5 mL PBS (1% Tween 20, 0.5 mM EDTA), twice with 0.5 mL PBS, and lastly resuspended in 300 μL PBS before filtering through a 50 μm Nytex nylon mesh screen (Sefar). Concentrations of fluorophore used for one- or two-dye labeling were 10 μM DMAC-alkyne, 50 μM LR-alkyne, 50 μM DMAC-azide, or 50 μM LR-azide. During spins, a cushion of 100 μL serum was added to the bottom of the eppendorf tube to minimize cell losses.
4.5.4 Fluorescence Microscopy

Fixed cells were imaged on a confocal microscope (Zeiss LSM 510 Meta NLO) at Caltech’s Biological Imaging Center. Each set of images was obtained with identical conditions to capture either LR, DMAC, or BDpy fluorescence. To visualize LR, cells were excited at 543 nm and emission was passed through a bandpass filter (565-615 nm) before imaging. DMAC fluorescence was obtained by two-photon excitation at 800 nm (Ti:sapphire laser) with emission collected between 376–495 nm. BDpy fluorescence was obtained by excitation at 633 nm with emission collected after passage through a LP650 filter. All images were acquired with a Plan-Apochromat 63x/1.4 oil objective (Zeiss) and analyzed with Zeiss LSM or ImageJ software.

4.5.5 Flow Cytometry

Cells were analyzed on a Moflo MLS (Dako) at the City of Hope Flow Cytometry Facility. The excitation on the Moflo was not ideal for either dye, but this instrument did enable analysis of the two-dye labeling. DMAC fluorescence was excited by a 351 nm ultraviolet laser and detected after passage through a 450/40 bandpass filter. LR fluorescence was excited by a 514 nm laser and detected after passage through a 600/30 bandpass filter. Unlabeled and one-dye labeled (DMAC or LR) cells were analyzed to ensure minimal cross-over fluorescence. In total, 50,000 events were collected. Forward- and side-scatter properties and pulse-width were used to exclude doublets, dead cells, and debris from analysis.

Cells labeled with Bodipy633-alkyne were analyzed on a FACSaria flow cytometer (BD Biosciences Immunocytometry Systems) at Caltech’s Flow Cytometry
Facility. Bodipy fluorescence was excited by a 633 nm laser and detected after passage through a 660/20 bandpass filter. Forward- and side-scatter properties were used to exclude doublets, dead cells, and debris from analysis. After excluding these cells, 30,000 events were collected per sample.

Data were analyzed using FloJo7 software (Tree Star, Inc.).

4.5.6 Synthesis of Compounds

All solvents and compounds obtained from commercial suppliers were used as provided. 3-azidopropylamine was synthesized as described[46]. Hpg was purchased from Chiralex, and Aha was synthesized by Dr. Mandy K.S. Vink using the published protocol[47]. Mass spectrometry analysis was performed at the Caltech Mass Spectrometry Facility by Mona Shahgholi. Excitation and emission were examined on a PTI fluorimeter, using a 100 nm solution of each dye in PBS (pH 7.5). Structures of dyes are given in Scheme 4.3.

**BDPY-alkyne:** Methanol (100 μL) was added to a 5 mg (7.6 μmol) vial of Bodipy630/650-X SE (Invitrogen). Then, 7.8 μL (15 eq) of neat propargylamine (Sigma-Aldrich) and 15.9 μL (15 eq) of dry triethylamine were added, and the solution was stirred at room temperature overnight. The solution was evaporated, and the crude product was purified by silica column chromatography (230-400 mesh Silica Gel 60, EMD) in 2% ethanol in CH₂Cl₂. Thin layer chromatography was run on Baker-Flex silica gel IB-F. Fluorescent fractions were identified by UV light, combined, and evaporated to give 4.5 mg (~100% yield) of a blue solid. Ex 627 ± 3, Em 639 ± 2. MS calc for C₃₂H₃₁BF₂N₄O₃S (M+H) 601.2256, observed 601.2247.
**DMAC-alkyne:** Neat propargylamine [~470-fold excess, 33.9 mmol, 1.5 mL (Sigma-Aldrich)] was added to a 25 mg (72.6 μmol) vial of 7-dimethylamino coumarin-4-acetic acid succinimidyl ester (Anaspec). Then, 500 μL of pyridine was added. The mixture was stirred for several hours at room temperature. The product was precipitated by the dropwise addition to diethyl ether. The solid was isolated by centrifugation, dissolved in methanol, and re-precipitated in ether (3x) to obtain 11.9 mg of product (58% yield). Ex 388 ± 2, Em 474 ± 2. MS calc for C_{16}H_{16}N_{2}O_{3} (M+H) 285.1239, observed 285.1257.

**LR-alkyne:** LR-alkyne was synthesized as described[48]. Briefly, lissamine rhodamine B sulfonyl chloride [53 mg (92 μmol) of mixed isomers from Invitrogen] was dissolved in methylene chloride (500 μL). Next, neat propargylamine (~7-fold excess, 0.63 mmol, 28 μL) and 0.8 mL of dry pyridine were added to the solution. The mixture was stirred at room temperature overnight. The solvent was evaporated, and the crude product was purified by silica column chromatography in CH₂Cl₂ using a gradient of ethanol from 2-15%. Fluorescent fractions were identified, combined, and evaporated. The product was pump dried to give 7.8 mg of a pink solid (14% yield). Ex 569 ± 1, Em 583 ± 1. FAB MS calc for C_{30}H_{34}N_{3}O_{6}S_{2}⁺ (M+H) 596.1889, observed 596.1875.

**DMAC-azide:** Methylene chloride (500 μL) was added to a ~25 mg (~72.6 μmol) vial of 7-dimethylamino coumarin-4-acetic acid succinimidyl ester (Anaspec). Next, 3-azidopropylamine (5-fold excess, 0.36 mmol, ~39 μL) and triethylamine (5 eq, 50 μL) were added, and the solution was stirred at room temperature overnight. The solvent was evaporated, and the product was purified by silica column chromatography in 2% Ethanol in CH₂Cl₂. Fluorescent fractions were identified by UV light, combined, and evaporated to
give 30 mg (~100% yield) of product. Ex 390 ± 1, Em 476 ± 1. FAB MS calc for C_{16}H_{19}N_{5}O_{3} (M+H) 330.1566, observed 330.1557.

**LR-azide:** Methylene chloride (500 μL) was added to lissamine rhodamine B sulfonyl chloride [50 mg (86 μmol) of mixed isomers from Invitrogen]. Next, 3-azidopropylamine (5-fold excess, 0.43 mmol, ~46 μL) and triethylamine (5 eq, 50 μL) were added, and the solution was stirred at room temperature overnight. The solvent was evaporated, and the product was purified by silica column chromatography in 5% ethanol in ethyl acetate. Fluorescent fractions were identified by UV light, combined, and evaporated to give 39 mg (70% yield) of product. Ex 569 ± 2, Em 584 ± 1. FAB MS calc for C_{30}H_{37}N_{6}O_{6}S_{2}+ (M+H) 641.2216, observed 641.2204.

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4.7 References


44. This is consistent with the bright nucleolar staining previously observed. 35


