Covalent live-cell labeling of proteins using a photoreactive fluorogen

Tewoderos M. Ayele[†], Steve D. Knutson[†], Jennifer M. Heemstra[*]
Department of Chemistry, Emory University, Atlanta, GA, United States
*Corresponding author: e-mail address: jen.heemstra@emory.edu

Contents

Abstract

Fluorescence microscopy has dramatically advanced our understanding of the processes that drive biological systems by enabling the imaging and tracking of biomolecules of interest inside of living cells. In particular, proteins of interest can be genetically tagged with fluorescent proteins or labeled with small molecule fluorophore probes to enable visualization. However, both of these methods are generally limited in signal-to-background resolution and options are limited for achieving temporal control

[†] These authors contributed equally.

Methods in Enzymology
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2020.04.019

over labeling. Photoreactive "fluorogenic" dyes can overcome these limitations and enable user-defined crosslinking with low background fluorescence. In this chapter, we discuss current approaches for live cell protein labeling with particular emphasis on the novel use of photoreactive fluorogenic dyes for protein imaging. We further describe in detail the synthesis and characterization of a fluorogenic malachite green probe functionalized with a photoreactive diazirine crosslinker and illustrate how to apply this probe toward covalent photoaffinity labeling and imaging of target proteins in live cells.

1. Introduction

Fluorescence microscopy has significantly advanced our understanding of cell biology by enabling direct observation of the complex and dynamic activities of myriad biomolecules within the cell (Stephens & Allan, 2003). In particular, nucleic acids and proteins can be fluorescently tagged using a number of methods to enable visualization and tracking. The ability to observe the location and movement of biomolecules has in turn provided significant insights into the interactions of these molecules and their contributions to overall cellular structure and function (Giepmans, Adams, Ellisman, & Tsien, 2006).

To enable imaging of proteins in live cells, an early strategy was developed in which a protein of interest (POI) is genetically fused in frame to a fluorescent protein (FP) (Toseland, 2013). To obtain this gene fusion, the coding DNA sequence for the POI is amplified, isolated, and ligated to the gene of an appropriate FP, such as green fluorescent protein (GFP) (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994) or one of the many other color variants (Fig. 1A). This fusion construct is then inserted into an appropriate mammalian expression plasmid vector and introduced into cells, which subsequently express the fluorescent fusion product to enable visualization. This technique has been widely adopted and is quite useful, as all components needed for visualization are genetically encodable and produced inside of the target cells. Further, molecular cloning techniques are relatively cheap and straightforward, and various fusion protein designs and iterations can be quickly constructed and inserted into appropriate plasmid vectors using economical reagents (Giepmans et al., 2006). These constructs can also be easily introduced into most cell types using standard transient transfection techniques or, when required, stably integrated into cellular genomes using lentiviral or CRISPR-based strategies

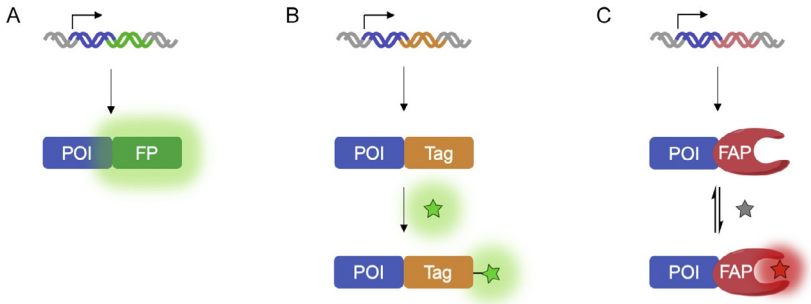


Fig. 1 Methods for genetic tagging and fluorescent visualization of target proteins. (A) The protein of interest (POI) can be genetically fused to an intrinsically fluorescent protein (FP), or (B) attached to a peptide or protein tag that is recognized by reactive fluorophores. (C) The POI can also be fused to a fluorogen activating protein (FAP), which enhances the fluorescent signal of specific fluorogen molecules. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

(Lackner et al., 2015). However, the constitutive fluorescence of these FP fusions presents a large limitation in signal-to-noise resolution. To achieve sufficient fluorescent signal inside this cell, strong expression promoters are typically required in mammalian plasmid vectors, including cytomegalovirus (CMV) and elongation factor-1a (EF-1a). While these promoters are often necessary to achieve sufficient protein levels for many POI fusions to enable robust visualization, this technique can also be problematic in that it results in the uncontrolled production of an unnaturally high copy number of the fusion protein. This can generate an overall diffuse signal throughout the cell and may not recapitulate natural expression levels or subcellular localization (Deer & Allison, 2004; Qin et al., 2010).

To overcome these limitations, a number of alternative protein labeling techniques have been developed that utilize chemical conjugation strategies to functionalize POI with fluorescent organic dyes. These approaches enhance the selective reaction of the fluorescent probe by expressing a POI fused with a genetically encodable peptide or protein motif that has high affinity to the fluorescent dye (Fig. 1B). An early example of this approach is the tetracysteine-biarsenical system (Griffin, Adams, & Tsien, 1998). The membrane-permeable fluorophore ligands used for this technique, ReAsH and FlAsH, contain dithioarsolane groups to enable strong non-covalent chelation with the Cys-Cys-X-X-Cys-Cys peptide motif fused to POI, where X can be any amino acid. This system is elegant in its simplicity but presents

limitations for broad applicability due to high background signal, potential arsenic toxicity, and elevated photobleaching properties of the fluorescent ligands (Marks & Nolan, 2006; Stroffekova, Proenza, & Beam, 2001).

This inspired the development of small-molecule labeling systems that instead take advantage of enzymatic recognition to drive selectivity. These approaches include the SNAP/Clip-tag (Gautier et al., 2008), LAP (Fernández-Suárez et al., 2007), Halo-tag (Los et al., 2008), and coiled-coil tag (Reinhardt, Lotze, Mörl, Beck-Sickinger, & Seitz, 2015). The advantages of these methods include substantially improved control over the intensity and timing of fluorescent labeling and the ability to harness the diverse palette of small-molecule fluorophores. However, covalent protein labeling techniques that target specific amino acids or short peptide sequences still lack high labeling selectivity in complex biological systems where other proteins or molecules are likely to have similar reactive groups. For example, protein labeling systems that rely on cysteine residue functionalization lose their selectivity in environments that contain high concentrations of glutathione or other biological molecules that have nucleophilic thiol groups. Additionally, these techniques still rely on intrinsically fluorescent molecules, which produce high background signal and hence require extensive washing steps to remove unreacted dye from the cytosol.

In order to address these challenges, several next-generation fluorophore molecules have been developed that are conditionally fluorescent and undergo a dramatic increase in emission when particular biochemical or physical conditions are met (Bruchez, 2015). One class of these probes are fluorogenic molecules, which can adopt somewhat similar planar architectures compared to traditional organic fluorophores yet display the important inclusion of a freely rotating bond, which prevents the molecule from spending a significant amount of time in the planar conformation. This results in low fluorescence in solution, with significant enhancement of signal upon rotational restriction imparted by temperature or viscosity changes. Alternatively, this signal enhancement can be achieved through binding to a nucleic acid strand or protein if the binding mode restricts the fluorogenic molecule in a planar conformation. In practice, visualization of POIs has been achieved by combining a fluorogenic small molecule and a "fluorogen activating protein" (FAP) that has been engineered to tightly bind to the dye molecule and restrict its overall rotational movement. Similar to previous genetic tagging methods, the POI can then be visualized by in-frame genetic fusion with the FAP domain (Fig. 1D), generating high signal-to-background ratio without the requirement of washing away unbound fluorogen (Chen et al., 2014; Telmer et al., 2015).

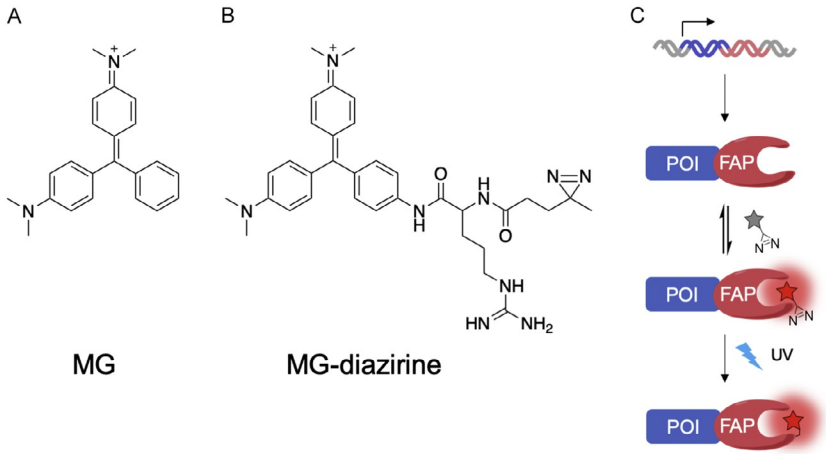


Fig. 2 Photoreactive malachite green enables covalent fluorogenic labeling of proteins. Chemical structures of (A) malachite green (Qin et al., 2010) and (B) malachite green diazirine (MG-diazirine), illustrating arginine spacer and diazirine photo-crosslinking group. (C) Genetic fusion of a POI with FAP combined with MG-diazirine enables fluorogenic localization and UV crosslinking of MG to target proteins. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

Malachite green (Qin et al., 2010) is a well-characterized and widely adopted small molecule fluorogenic dye, originally developed and used as an industrial pigment (Shukla & Mathur, 1995) and has since found extensive use in bioimaging applications. MG is weakly fluorescent in solution and displays low quantum yields due to free rotational movement of two of the aromatic rings in the triphenylmethane structure (Fig. 2A). However, when this rotation is restricted, fluorescence is greatly enhanced. This advantageous property was exploited for RNA imaging purposes, where researchers used directed evolution to evolve an RNA aptamer motif that can bind to MG and generate a fluorescent signal. By fusing this sequence to an RNA transcript of interest, this technique enables visualization of RNA molecules and real-time tracking of their movement within different cellular compartments (Babendure, Adams, & Tsien, 2003; Yerramilli & Kim, 2018). Similarly, researchers sought to apply this property toward protein labeling and imaging, and utilized yeast-display to select for single-chain antibody FAPs that exhibit strong binding affinity toward MG (Szent-Gyorgyi et al., 2008). While this approach circumvents many of the background resolution problems previously described, the fluorogen-FAP interaction is non-covalent, and thus the signal generated

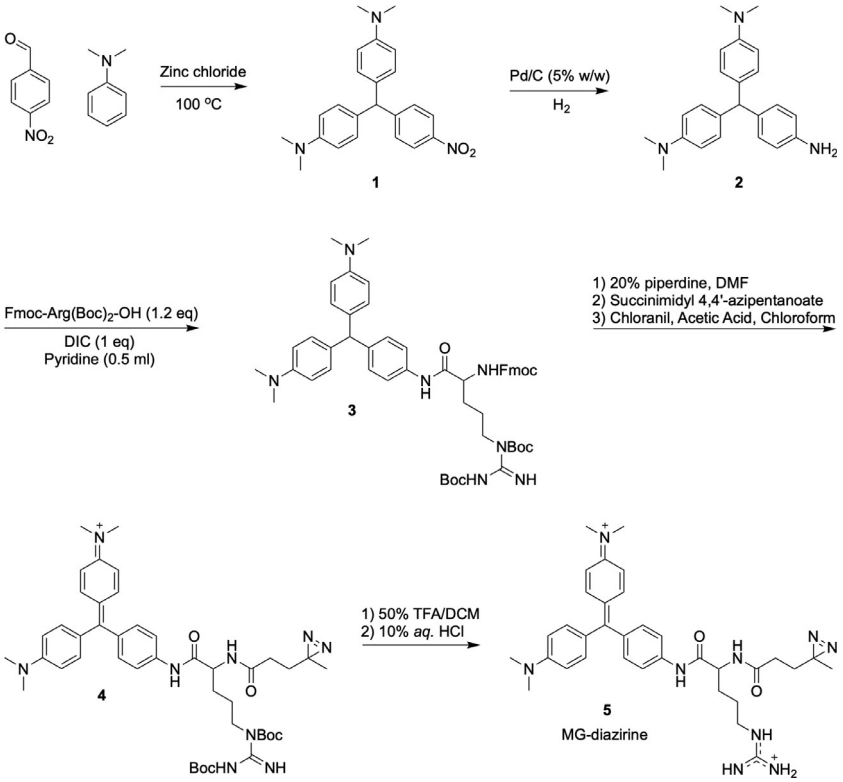
is directly limited by the strength of this binding interaction. Moreover, the MG dye can become unbound from the FAP and diffuse out of cells over time, resulting in shorter visualization windows and a lack of precise temporal control over when signal can be produced.

To address these limitations, we derivatized the MG scaffold with both an arginine spacer and a photoreactive diazirine group (Fig. 2B) (Ayele, Knutson, Ellipilli, Hwang, & Heemstra, 2019). The arginine moiety aids in solubility of the molecule by imparting charge, and appending this amino acid has also been shown to improve cell permeability and uptake of small molecule and peptide payloads (Brock, 2014; Rothbard et al., 2002). To provide a photoreactive group on the molecule, we attached a diazirine moiety proximal to the arginine linker. Diazirine forms a highly reactive carbene upon irradiation with ultraviolet light, which then reacts to form covalent attachments with neighboring biomolecules through insertion at C—H, N—H, or O—H bonds (Dubinsky, Krom, & Meijler, 2012). The derivatized MG-diazirine fluorogen retains the ability to bind with its cognate antibody-chain FAP (Szent-Gyorgyi et al., 2008) and produces significant fluorescence enhancement activity, and the appended diazirine enables permanent covalent attachment to the protein *via* UV irradiation (Fig. 2C). Together, this attachment results in stable fluorogenic labeling of target proteins and allows user-defined timing of this labeling event. In this chapter we describe in detail a protocol for the chemical synthesis, purification, and characterization of the MG-diazirine small molecule. We additionally illustrate how to introduce this compound into cells and visualize a POI-FAP fusion using photoaffinity labeling and fluorescence microscopy.

2. Chemical synthesis of malachite green diazirine

2.1 Rationale

Malachite green (Qin et al., 2010) is among the most widely used fluorogenic probes for biological applications, and binds to an engineered fluorogen activating protein (FAP) with high affinity (Szent-Gyorgyi et al., 2008). This FAP domain can be genetically fused to proteins of interest to enable fluorogenic live-cell labeling. However, this probe-protein interaction is non-covalent, and is thus limited by diffusion-based signal losses and a lack of temporal control in labeling. Using a procedure modified from previous synthetic routes (Xing et al., 2009), this protocol describes a route to synthesize and derivatize a malachite green fluorogen having a photoreactive diazirine crosslinker for covalent labeling of FAP-POI fusions (Scheme 1).



Scheme 1 Synthesis of MG-diazirine. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

2.2 Equipment

- **(a)** Rotary evaporator equipped with vacuum system
- **(b)** Magnetic stirrer with heating module
- **(c)** Stir bars
- **(d)** Glass funnel
- **(e)** #1 Whatman Paper
- **(f)** Latex balloons
- **(g)** Glass chromatography column
- **(h)** 5 mL Round bottom flask
- **(i)** 25 mL round bottom flask
- **(j)** 2 mL glass vial
- **(k)** 25 mL glass separatory funnel

(l) Preparative TLC plates (Sigma-Aldrich) and chamber
(m) Hamilton syringes
(n) NMR tubes

2.3 Chemicals

(a) *p*-Nitrobenzaldehyde (Sigma-Aldrich)
(b) Zinc chloride (Chem-Impex International, Inc.)
(c) *N,N*-Dimethylaniline (Sigma-Aldrich)
(d) Acetone (Fisher Scientific)
(e) Ethyl acetate (Fisher Scientific)
(f) Hexane (Fisher Scientific)
(g) Methanol (Fisher Scientific)
(h) Tetrahydrofuran (Fisher Scientific)
(i) Palladium on carbon (Pd/C 5%, w/w) (Sigma-Aldrich)
(j) Hydrogen gas (Nexair)
(k) Celite (Sigma-Aldrich)
(l) Pyridine (Chem-Impex International, Inc.)
(m) *N,N′*-Diisopropylcarbodiimide (Chem-Impex International, Inc.)
(n) Fmoc-Arg(Boc)$_2$-OH (Chem-Impex International, Inc.)
(o) Dichloromethane (Fisher Scientific)
(p) Water
(q) Anhydrous sodium sulfate (Sigma-Aldrich)
(r) Dimethylformamide (Sigma-Aldrich)
(s) Piperidine (Chem-Impex International, Inc.)
(t) SDA (NHS-Diazirine, or succinimidyl 4,4′-azipentanoate) (Thermo Fisher Scientific)
(u) Acetic acid (Fisher Scientific)
(v) Chloroform (Fisher Scientific)
(w) Trifluoroacetic acid (Sigma-Aldrich)
(x) Hydrochloric acid (Fisher Scientific)

2.4 Protocol—Chemical synthesis of MG-diazirine

2.4.1 p-*nitro-malachite green (compound 1)*

(a) Dissolve *p*-nitrobenzaldehyde (250 mg, 1.65 mmol) and zinc chloride (451 mg, 3.31 mmol) in *N,N*-dimethylaniline (0.503 mL, 3.97 mmol) in a 5 mL round bottom flask.
(b) Stir this solution while refluxing at 100 °C for 5 h.
(c) Cool the reaction mixture to room temperature and add 5 mL of acetone.

(d) Filter off the undissolved zinc salt using Whatman filter paper and a glass funnel. Repeat this washing step with acetone two more times to remove the maximum amount of zinc salt.

(e) Concentrate the solution under reduced pressure and purify the resulting crude oil by flash column chromatography (silica, 1:10 ethyl acetate/hexane). Note: To simplify the purification of the crude oil by flash chromatography, remove as much of the excess *N,N*-dimethylaniline as possible under reduced pressure before loading onto the silica column.

(f) Remove solvent from collected and pooled column fractions *in vacuo* to yield a yellow solid, *p*-nitro-leucomalachite green (compound **1**).

2.4.2 p-*amino-leucomalachite green (compound 2)*

(a) Dissolve *p*-nitro-leucomalachite green (**1**) (100 mg, 0.267 mmol) in 9 mL of methanol/tetrahydrofuran (1/2, v/v) in a 25 mL round bottom flask.

(b) To this solution add 10 mg of Pd/C (5%, w/w). Note: When working with Pd/C and hydrogen gas, proper precautions need to be taken to prevent ignition of flammable solvents.

(c) Cap the round bottom flask with a rubber septum and start stirring the mixture.

(d) With a needle and vacuum line, evacuate the reaction flask until bubbling is observed. Stop the vacuum and carefully insert the hydrogen balloon to backfill the flask. Remove the hydrogen balloon. Repeat this process three more times.

(e) Let the reaction stir at room temperature for 3 h.

(f) Filter the crude solution through a celite and wash the celite with 5 mL of methanol three times.

(g) Concentrate the pooled filtrate under reduced pressure to yield a light blue solid (**2**).

(h) To assess yield and purity of the collected intermediate product, perform ^{1}H NMR at this step. A representative spectrum is shown in Fig. 3.

2.4.3 *(9H-Fluoren-9-yl)methyl(1-((4-(bis(4-(dimethylamino)phenyl) methyl)phenyl)amino)-5-*N,N-*diboc-guanidino-1-oxopentan-2-yl) carbamate (compound 3)*

(a) To a 2 mL glass vial containing a micro stir bar, add **2** (50.0 mg, 0.148 mmol) to 500 μL of pyridine. To this solution, add *N,N′*-diisopropylcarbodiimide (70.0 μL, 0.445 mmol) and Fmoc-Arg(Boc)$_2$-OH (106 mg, 0.178 mmol). Stir at room temperature for 12 h.

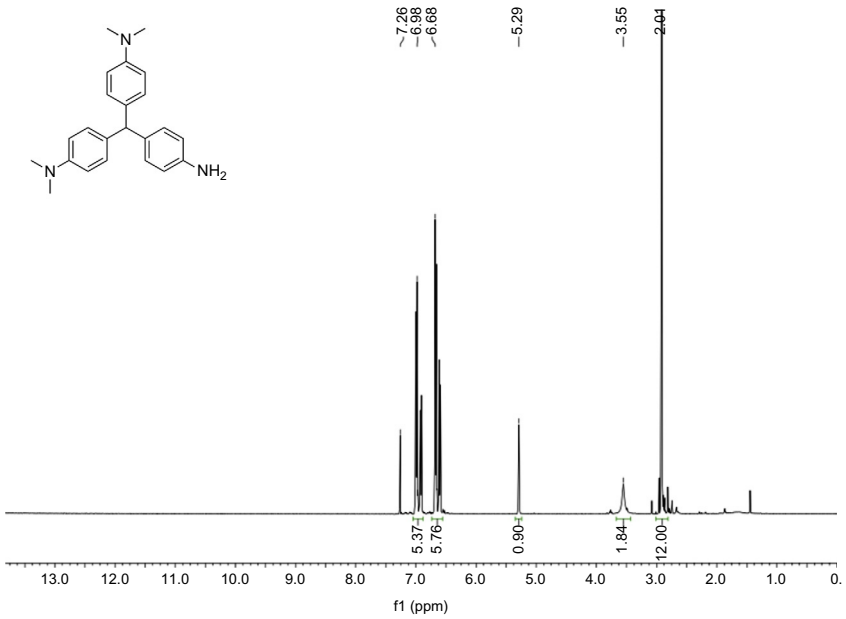


Fig. 3 Representative ^{1}H NMR spectrum of compound **2**. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

(b) Concentrate the reaction under reduced pressure, and dissolve the crude product in 5 mL of dichloromethane.

(c) Transfer the solution to a 25 mL separatory funnel containing water (10 mL) and shake vigorously.

(d) Allow the two layers to separate.

(e) Carefully drain the bottom organic layer into an Erlenmeyer flask, and collect the top aqueous layer into a separate suitable waste container.

(f) Transfer the collected organic layer from the previous step into a new 25 mL separatory funnel containing water (10 mL) and repeat steps (c)–(e) two additional times.

(g) Remove residual water from the combined organic layers by directly adding anhydrous sodium sulfate.

(h) Carefully decant organic layer into a new flask and concentrate under reduced pressure.

(i) Purify the crude product using flash column chromatography (silica). Load the crude product onto the silica with a minimal amount of

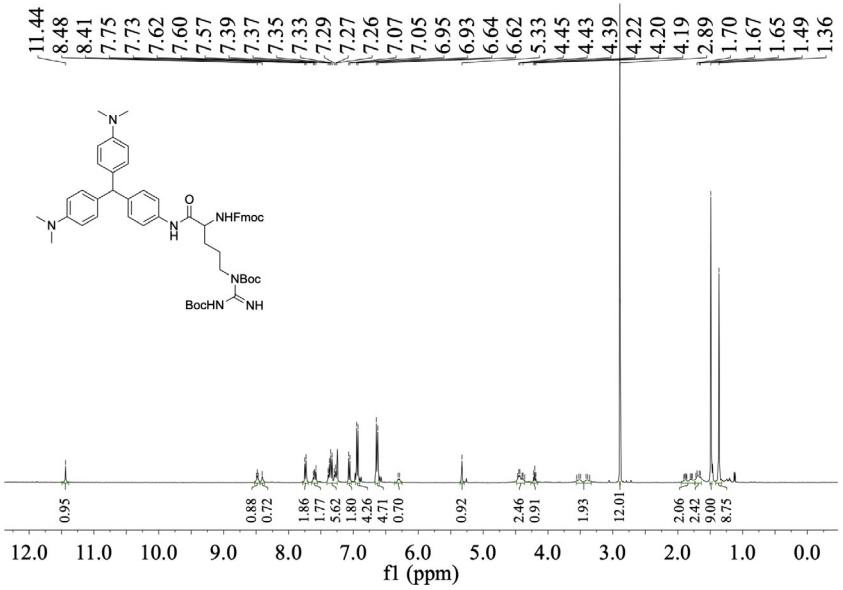


Fig. 4 Representative ^{1}H NMR spectrum of compound **3**. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

dichloromethane using a Pasteur pipette and elute with 5%, 10%, 15%, 35%, and 50% ethyl acetate in hexane to yield a dark green solid **3**.

(j) To assess yield and purity of the collected product, perform ^{1}H NMR at this step. A representative spectrum is shown in Fig. 4.

2.4.4 (Z)-N-(4-((4-(5-(2,3-Bis(tert-butoxycarbonyl)guanidino)-2-(3-(3-methyl-3H-diazirin-3-yl)propanamido)pentanamido)phenyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)-N-methylmethanaminium (compound 4)

(a) *Note*: Diazirine is photoreactive. Use proper precautions to minimize ultraviolet light exposure in all steps during this stage of the procedure.

(b) Dissolve compound **3** (30.0 mg, 0.0325 mmol) in 500 μL of dimethylformamide in a 2 mL glass vial.

(c) To this solution, add 500 μL of 20% piperidine in *N,N*-dimethylformamide and stir this solution for 1 h at room temperature.

(d) Purify this product using preparative TLC (silica, 2/100 methanol/dichloromethane) to yield a dark blue solid.

(e) Dissolve the obtained solid in 500 μL of *N,N*-dimethylformamide.

(f) To this solution, add succinimidyl 4,4′-azipentanoate (22.0 mg, 0.0975 mmol) and stir the solution at room temperature for 5 h.

(g) Concentrate the reaction mixture under reduced pressure to obtain a crude product of the diazirine functionalized intermediate.

(h) To this solid, add 100 μL of 30% acetic acid in chloroform, and reflux the solution at 60 °C for 4 h.

(i) Dry and concentrate the reaction under reduced pressure and purify compound **4** using preparative TLC (silica, 1:10 methanol/dichloromethane).

(j) To assess yield and purity of the collected product, perform ^{1}H NMR at this step. A representative spectrum is shown in Fig. 5.

2.4.5 N-*(4-((4-(Dimethylamino)phenyl)(4-(5-guanidino-2-(3-(3-methyl-3H-diazirin-3-yl)propanamido) pentanamido)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)*-N-*methylmethanaminium (compound 5, MG-diazirine)*

(a) *Note*: The diazirine functionalized intermediate at this stage is photoreactive. Use proper precautions to minimize ultraviolet light exposure.

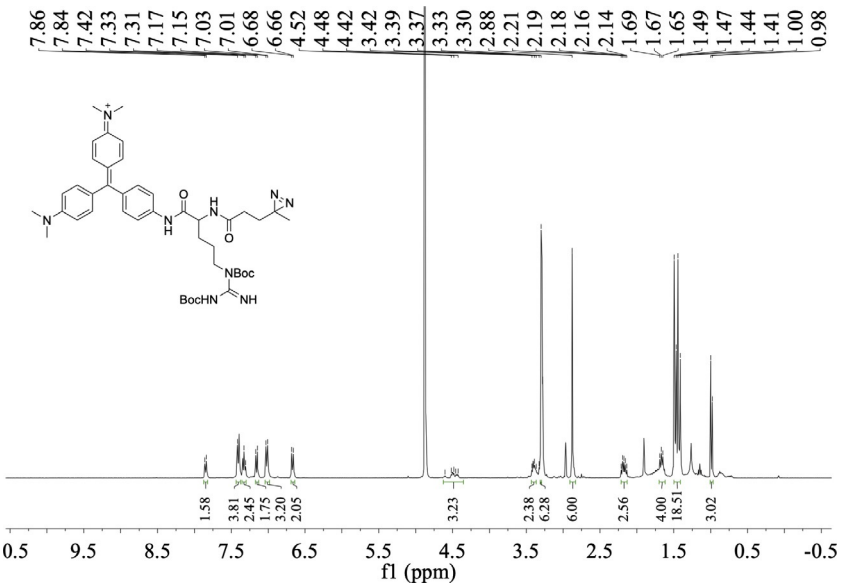


Fig. 5 Representative ^{1}H NMR spectrum of compound **4**. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

(b) In a 5 mL round bottom flask, dissolve compound **4** (20.0 mg, 0.0246 mmol) in 2 mL of 50% trifluoroacetic acid/dichloromethane.
(c) Stir the reaction for 4 h at room temperature to allow for Boc-deprotection. Remove the solvent *in vacuo*.
(d) To remove residual trifluoroacetic acid, redissolve the solid in 10% *aq.* hydrochloric acid and concentrate reaction mixture under reduced pressure to obtain product **5** as a dark blue solid (MG-diazirine.)
(e) To assess yield and purity of the collected final product, perform ^{1}H NMR at this step. A representative spectrum is shown in Fig. 6.
(f) Store final product as a solid at room temperature. Protect from light.

3. Preparation of FAP fusion vectors

3.1 Rationale

This procedure outlines how to prepare a plasmid for transfection into HeLa cells for subsequent live cell imaging with the MG-diazirine probe. Specifically, we utilized a commercially available pcDNA vector from Addgene (Fig. 7), which contains the FAP protein fused to a fluorescent

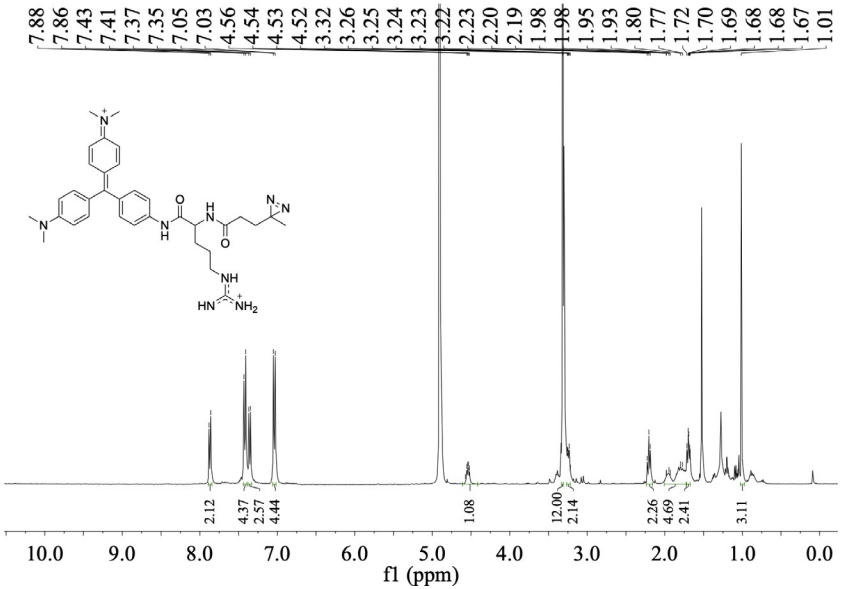


Fig. 6 Representative ^{1}H NMR spectrum of compound **5** (MG-diazirine). *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

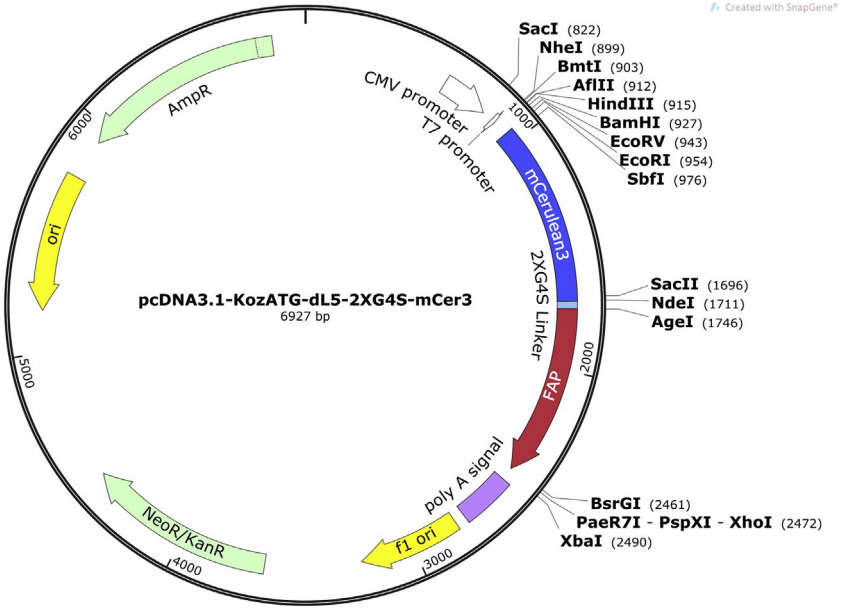


Fig. 7 Plasmid map of mCer3-FAP control expression vector highlighting important components for cloning and expression.

mCerulean3 (mCer3) reporter protein (Telmer et al., 2015). The fluorescence reporter protein served as a transfection control in the initial validation of this approach, but use of a fluorescent protein is not required for imaging applications. The vector also contains both bacterial replication features and appropriate promoters for mammalian expression. These vectors are typically supplied as agar stabs containing pre-transformed *E. coli* cells harboring the plasmid of interest. We outline how to replicate and extract this plasmid from bacteria for eventual transfection into HeLa cells for expression. This vector also contains numerous restriction enzyme sites surrounding the mCer3 gene (Fig. 7), allowing users to clone and insert virtually any POI into this backbone and fuse to FAP.

3.2 Equipment

(a) Autoclave
(b) 100 mm × 15 mm sterile petri dishes with lids (VWR)
(c) 5 mL culture tubes, plastic, with Caps (VWR)
(d) Sterile pipet tips/toothpicks
(e) Inoculating loop (optional)

(f) stationary incubator capable of reaching 37 °C
(g) 15 mL conical tubes with caps (VWR)
(h) shaker incubator capable of reaching 37 °C and 250 rpm
(i) centrifuge that can accommodate 15 mL conical tubes and reach >6800 x *g*
(j) microcentrifuge that can accommodate 1.5 mL tubes and reach >10,000 x *g*
(k) UV–Vis spectrophotometer

3.3 Chemicals

(a) Plasmid vector pcDNA3.1-KozATG-dL5-2XG4S-mCer3 (Addgene 73207)
(b) LB agar (Miller), granulated (EMD Millipore)
(c) LB broth (Miller), granulated (EMD Millipore)
(d) Ampicillin, sodium salt (Sigma Alrich)
(e) QIAprep Spin Miniprep Kit (Qiagen)
(f) Ultrapure water

3.4 Protocol—Preparation of mCer3-FAP plasmid vector

(a) Prepare luria broth (LB) agar (Miller) plates supplemented with ampicillin by first combining 37 g of the powdered media mixture for every 1 L of ultrapure water. Mix to dissolve completely, and sterilize in a suitable container by autoclaving.
(b) Separately, prepare liquid LB broth (Miller) media by combining 25 g of the powdered media mixture for every 1 L of ultrapure water. Mix to dissolve completely, and sterilize in a suitable container by autoclaving.
(c) Place hot LB broth and agar mixture in a water bath equilibrated to 50 °C. While media is equilibrating, prepare a 100 mg/mL ampicillin (amp) stock solution in ultrapure water.
(d) When LB broth and agar is cool to the touch, dilute amp stock 1000 × in the LB agar medium to a final concentration of 100 μg/mL.
(e) While LB agar is still a molten liquid, carefully but quickly pour ~20–25 mL into 100 mm × 15 mm sterile petri dishes. Cover loosely with lids and allow to solidify at room temperature.
(f) Addgene vectors typically arrive as agar stabs which contain *E. coli* cells transformed with the plasmid of interest. To grow more bacteria through replication, insert a sterile pipet tip, toothpick, or inoculating loop into the stab, and streak onto a LB + amp agar plate from step (e).

Use aseptic technique and serially streak while rotating the plate to obtain single colonies. Incubate plate(s) upside down at 37 °C overnight.

(g) Inspect plates. If successful, the streak plate(s) should display robust bacterial growth and many distinct colonies. To store plates long term, wrap plates in parafilm and store at 4 °C. Plates can be stored for at least 1 year if kept dry.

(h) Prepare an overnight starter culture by adding 5 mL of LB broth + amp to a desired number of culture tubes. Using a sterile pipet tip or toothpick, "pick" an individual bacterial colony by gently touching it. Drop the tip/toothpick directly into one culture tube with broth and cap. Repeat for desired number of tubes. Transfer all tubes to a shaking incubator in a suitable rack, and incubate overnight at 37 °C with shaking at 250 rpm.

(i) The next day, visually inspect culture tubes. Broth should be noticeably turbid, indicating successful bacterial growth.

(j) Extract plasmid using the QIAprep Spin Miniprep kit according the manufacturer's instructions. Several other similar kits are commercially available from a number of vendors. Quantify the concentration of purified plasmid by UV–Vis spectrophotometry, and store at 4 °C until further use.

4. Live cell imaging with malachite green diazirine

4.1 Rationale

Lastly, we describe how to transfect HeLa cells with these vectors and image the expressed fusion protein through introduction of MG-diazirine followed by photo-crosslinking and analysis by fluorescence microscopy.

4.2 Equipment

(a) Laminar flow hood
(b) CO_2 incubator
(c) Centrifuge
(d) Water bath (37 °C)
(e) Inverted brightfield microscope
(f) Fluorescent microscope
(g) Hemacytometer or automated cell counter
(h) T25 flasks

(i) T75 flasks
(j) Sterile 15 mL polypropylene conical centrifuge tubes
(k) Sterile 96-well tissue culture plates, with lids
(l) Disposable pipettes
(m) High intensity UV lamp, 100 W/ 365 nm (Analytik Jena)

4.3 Chemicals

(a) HeLa cells (ATCC CCL-2)
(b) Eagle's Minimum Essential Medium (EMEM) (Thermo Fisher Scientific)
(c) FBS (Fetal bovine serum) (Thermo Fisher Scientific)
(d) Penicillin-streptomycin (10,000 U/mL) (Thermo Fisher Scientific)
(e) Trypsin-EDTA (0.25%), phenol red (Thermo Fisher Scientific)
(f) 70% Ethanol
(g) Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific)
(h) Lipofectamine 3000 with P3000 reagent (Thermo Fisher Scientific)
(i) Dimethylsulfoxide (DMSO) (Fisher Scientific)

4.4 Protocol—Live cell imaging with MG-diazirine

4.4.1 HeLa cell culture and maintenance

Note: Mammalian cell culture requires use of appropriate biosafety control measures and may require institutional training and safety approval.

Note: All steps must be performed using sterile aseptic technique in a laminar flow hood. All media should be purchased sterile or filtered through 0.2 μm filters. All materials need to be autoclaved or sprayed down liberally with 70% ethanol prior to bringing inside the laminar flow hood.

Note: All media and liquids should be prewarmed to 37 °C before coming into contact with cells.

(a) Prepare "complete" EMEM media by combining the following. Mix well. Scale as needed.
  i. EMEM base media: 450 mL
  ii. FBS: 50 mL
  iii. Pen/Strep (10,000 U/mL): 5 mL
(b) HeLa cell stocks are stored in liquid nitrogen in cryovials. Begin a starter culture by immersing the frozen cryovial in a 37 °C water bath until fully thawed. Take care to only submerge the bottom half of the vial.
(c) When thawed, aseptically transfer contents of the vial (~1–2 mL) to a 15 mL conical tube containing 10 mL of EMEM complete media prewarmed to 37 °C.

(d) Pellet cells by spinning at 500 x *g* for at least 5 min. Carefully remove supernatant and resuspend gently in 10 mL of fresh EMEM complete media.
(e) Transfer to a T25 flask and incubate at 37 °C, 5% CO_2.
(f) The next day, use brightfield microscope to confirm adherence of cells and the absence of floating or dead cells.
(g) Continue to incubate flask at 37 °C, 5% CO_2. Replace media 2–3 times per week, or if media has begun to turn yellow-orange.
(h) When cells have reached >75% confluency, remove media in flask and rinse with ~2–3 mL of 0.25% Trypsin/EDTA. Rock back and forth gently and discard. Replace with 2 mL of fresh 0.25% Trypsin/EDTA and repeat. Remove 1.5 mL of this solution and return cells to 37 °C incubator for 5–20 min. Monitor cell detachment by visualizing under the microscope.
(i) When fully detached, add 9.5 mL fresh EMEM complete media and rinse cells from the bottom of the flask. Pipet up and down gently to fully resuspend and distribute cells.
(j) Split cells 1:2 into a larger T75 flask by combining 5 mL of the resuspended mixture from step (g) with 20 mL of fresh media. Mix gently by pipetting up and down and incubate flask horizontally at 37 °C, 5% CO_2. Replace media 2–3 times per week, or if media has begun to turn yellow-orange.
(k) If needed, subcultivate as outlined in steps (f)–(h) using splitting ratios between 1:2 and 1:10 where appropriate. Note: HeLa cells should be passaged 2–3 times after seeding a flask from cryostorage before they are suitable for transfection. Do not exceed confluency over ~95% in flasks. Do not culture cells beyond 25 rounds of passage.

4.4.2 Plasmid transfection

(a) At this stage, it is valuable to perform an optimization experiment to identify suitable conditions for transfection and confirm FAP-fusion expression inside cells. Additionally, this experiment further confirms successful synthesis of the MG-diazirine ligand.
(b) After a suitable number of passages, HeLa cells are ready for transfection at confluencies anywhere between 50% and 75% in a T75 flask. Begin plating by removing media in flask and rinsing with ~2–3 mL of prewarmed 0.25% Trypsin/EDTA. Rock back and forth gently and discard. Replace with 2 mL of fresh 0.25% Trypsin/EDTA and repeat.

Remove 1.5 mL of this solution and return cells to 37 °C incubator for 5–20 min. Monitor cell detachment by visualizing under the microscope.

(c) When fully detached, add 9.5 mL fresh EMEM complete media and rinse cells from the bottom of the flask. Pipet up and down gently to fully resuspend and distribute cells.

(d) Take a small sample of this suspension and count cells using a hemacytometer or automated cell counter.

(e) Dilute cells to approximately 1×10^5 cells/mL using EMEM complete media. Prepare at least 12 mL of this suspension for each full 96-well plate. This can be scaled up or down as needed. Mix solution gently but thoroughly using a pipet or inversion to evenly distribute cells.

(f) Transfer 100 μL of this suspension to each desired well of a 96-well plate. Add 100 μL EMEM media to any empty wells. Incubate plates at 37 °C, 5% CO_2 overnight.

(g) The next day, confirm adherence of cells and the absence of floating or dead cells.

(h) Monitor cells daily until they reach confluency of 70%–90%.

(i) Remove media from wells and replace with 100 μL pre-warmed Opti-MEM reduced serum medium.

(j) Prepare transfection solution A by combining the following. Amounts correspond to enough transfection material for 1 well. Scale up as needed.

- **i.** Opti-MEM reduced serum medium: 5 μL
- **ii.** Lipofectamine 3000: 0.15 μL

(k) Prepare transfection solution B by combining the following. Amounts correspond to enough transfection material for 1 well. Scale up as needed.

- **i.** Opti-MEM reduced serum medium: 10 μL
- **ii.** pcDNA FAP Plasmid Vector: 200 ng
- **iii.** P3000 Reagent: 0.4 μL

(l) Combine solutions by adding 5 μL each of A and B. Mix well by flicking the tube. Let incubate at room temperature for at least 30 min.

(m) Add 10 μL of this combined A/B mixture to each desired well of the 96-well plate. *Note*: It is strongly advised to also prepare control wells without DNA added as well as a "no transfection" control well.

(n) Incubate cells at 37 °C, 5% CO_2 for 12 h. After this period, remove Opti-MEM from each well and replace with 100 μL fresh EMEM complete media. Incubate cells at 37 °C, 5% CO_2 for an additional 6 h.

(o) Prepare a 50 mM MG-diazirine stock solution (~30.5 mg/mL) in DMSO.

(p) Prepare a 50 μM working solution of MG-diazirine in EMEM complete media by diluting the 50 mM stock 1:1000.

(q) Remove media from each well and add 100 μL of this working solution to each appropriate well. Let cells incubate for 15 min at 37 °C.

(r) Remove MG-diazirine solution from each well and briefly wash three times with 100 μL fresh EMEM complete media.

(s) Proceed to imaging. We previously used a Leica DMi8 confocal fluorescence microscope with a 10 × objective to obtain our images. However, most standard fluorescent microscopes should be suitable for imaging. The most important consideration is the available excitation lasers and emission filters in the microscope. If using the mCer3-FAP plasmid, the required spectral considerations for mCer3 are excitation maximum at 433 nm, emission at 475 nm and FAP/MG-diazirine excitation maximum at 640 nm and emission at 668 nm.

(t) Obtain images for each appropriate channel using the microscope. Images should show a clear distinction when comparing between cells that have been transfected with the reporter mCer3-FAP plasmid *versus* non-transfected controls. Representative images are shown in Fig. 8, illustrating signal from both mCer3 and FAP-MG diazirine. This experiment thus confirms successful synthesis of MG-diazirine and functional introduction into cells. Additionally, the images obtained from this experiment also provide a good overall measure of plasmid transfection efficiency in HeLa cells, allowing users to optimize conditions toward maximum percentage of expressing cells.

4.4.3 UV photo-crosslinking and imaging

(a) At this stage, it is valuable to perform a "wash out" experiment to further confirm proper experimental set up and establish successful photo-crosslinking conditions.

(b) UV light irradiation can produce large amounts of heat. To avoid thermal stress on cells, place the 96-well plate in a styrofoam container lined with ice while irradiating.

(c) Position the UV light source above the cells, and irradiate plate for 5 min. We have previously found this time to be sufficient for most cross-linking applications. However, light sources from different vendors can vary significantly in light output and efficiency, so it is advisable to optimize UV irradiation time before proceeding.

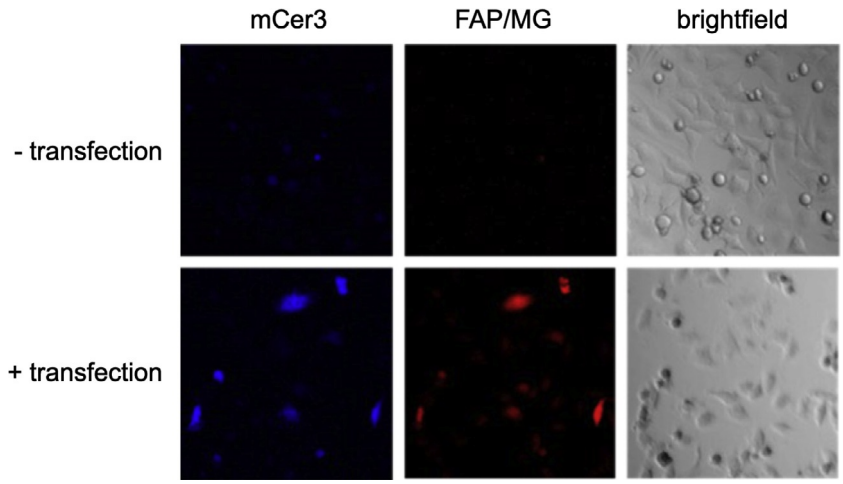


Fig. 8 Representative fluorescence microscopy images of transfected HeLa cells incubated with MG-diazirine. Transfection with the reporter mCer3-FAP plasmid should result in both mCer3 and MG-diazirine fluorescent signal to confirm successful transfection and expression of the construct, as well as functional MG-diazirine binding and fluorogenic activity. Cells not exposed to the vector should produce no detectable background fluorescence.

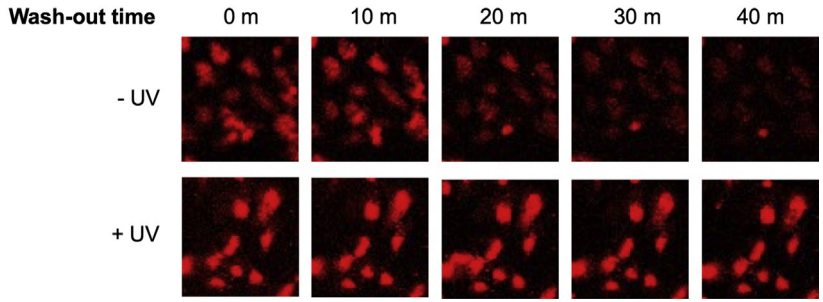


Fig. 9 Representative fluorescence microscopy images in transfected cells with or without UV irradiation. Increasing wash time should produce a steady loss of fluorescence in cells without UV treatment, while maintenance of signal is indicative of successful photo-crosslinking of MG-diazirine with FAP. *Adapted with permission from Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells.* Bioconjugate Chemistry, 30*(5), 1309–1313. Copyright (2019) American Chemical Society.*

(d) Proceed to fluorescence microscopy and obtain initial "time 0" images. At this point, cell samples that have and have not undergone UV irradiation should both produce robust MG/FAP signal.

(e) Remove media from wells and replace with 100 μL fresh prewarmed EMEM complete media. Let cells incubate at 37 °C for 10 min.

(f) Repeat step (d) and annotate images for "10 min."

(g) Repeat step (e) at 20, 30 and 40 min, obtaining images at each timepoint. Images should result in a retained signal in UV treated cells, but a predictable loss of signal in non-irradiated samples (Fig. 9).

5. Summary

Visualizing proteins inside of living cells is a powerful tool for understanding their dynamic biological roles and behavior. Genetic tagging of proteins with fluorescent protein domains or covalent labeling with small-molecule fluorophores are useful strategies for imaging and tracking of proteins by fluorescence microscopy. A promising alternative approach employs conditionally active fluorogenic dyes coupled with engineered fluorogen activating proteins to achieve significantly higher signal-to-background ratios and greater ease of use. We recognized that this approach could be further extended to enable time-resolved control of labeling through photocrosslinking of the fluorogen to the FAP receptor. In this chapter, we outline the basic techniques for synthesizing a malachite green fluorogen having a photo-reactive diazirine functional group, tagging proteins of interest with fluorogen activating proteins, and visualizing proteins inside of live cells using our covalent labeling approach.

References

Ayele, T. M., Knutson, S. D., Ellipilli, S., Hwang, H., & Heemstra, J. M. (2019). Fluorogenic photoaffinity labeling of proteins in living cells. *Bioconjugate Chemistry*, *30*(5), 1309–1313.

Babendure, J. R., Adams, S. R., & Tsien, R. Y. (2003). Aptamers switch on fluorescence of triphenylmethane dyes. *Journal of the American Chemical Society*, *125*(48), 14716–14717.

Brock, R. (2014). The uptake of arginine-rich cell-penetrating peptides: Putting the puzzle together. *Bioconjugate Chemistry*, *25*(5), 863–868.

Bruchez, M. P. (2015). Dark dyes–bright complexes: Fluorogenic protein labeling. *Current Opinion in Chemical Biology*, *27*, 18–23.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, *263*(5148), 802–805.

Chen, Y., Clouthier, C. M., Tsao, K., Strmiskova, M., Lachance, H., & Keillor, J. W. (2014). Coumarin-based fluorogenic probes for no-wash protein labeling. *Angewandte Chemie International Edition*, *53*(50), 13785–13788.

Deer, J. R., & Allison, D. S. (2004). High-level expression of proteins in mammalian cells using transcription regulatory sequences from the Chinese hamster EF-1α gene. *Biotechnology Progress*, *20*(3), 880–889.

Dubinsky, L., Krom, B. P., & Meijler, M. M. (2012). Diazirine based photoaffinity labeling. *Bioorganic & Medicinal Chemistry*, *20*(2), 554–570.

Fernández-Suárez, M., Baruah, H., Martínez-Hernández, L., Xie, K. T., Baskin, J. M., Bertozzi, C. R., et al. (2007). Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes. *Nature Biotechnology*, *25*(12), 1483.

Gautier, A., Juillerat, A., Heinis, C., Corrêa, I. R., Jr., Kindermann, M., Beaufils, F., et al. (2008). An engineered protein tag for multiprotein labeling in living cells. *Chemistry & Biology*, *15*(2), 128–136.

Giepmans, B. N., Adams, S. R., Ellisman, M. H., & Tsien, R. Y. (2006). The fluorescent toolbox for assessing protein location and function. *Science*, *312*(5771), 217–224.

Griffin, B. A., Adams, S. R., & Tsien, R. Y. (1998). Specific covalent labeling of recombinant protein molecules inside live cells. *Science*, *281*(5374), 269–272.

Lackner, D. H., Carré, A., Guzzardo, P. M., Banning, C., Mangena, R., Henley, T., et al. (2015). A generic strategy for CRISPR-Cas9-mediated gene tagging. *Nature Communications*, *6*, 10237.

Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., et al. (2008). HaloTag: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chemical Biology*, *3*(6), 373–382.

Marks, K. M., & Nolan, G. P. (2006). Chemical labeling strategies for cell biology. *Nature Methods*, *3*(8), 591.

Qin, J. Y., Zhang, L., Clift, K. L., Hulur, I., Xiang, A. P., Ren, B.-Z., et al. (2010). Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One*, *5*(5), e10611.

Reinhardt, U., Lotze, J., Mörl, K., Beck-Sickinger, A. G., & Seitz, O. (2015). Rapid covalent fluorescence labeling of membrane proteins on live cells via coiled-coil templated acyl transfer. *Bioconjugate Chemistry*, *26*(10), 2106–2117.

Rothbard, J. B., Kreider, E., VanDeusen, C. L., Wright, L., Wylie, B. L., & Wender, P. A. (2002). Arginine-rich molecular transporters for drug delivery: Role of backbone spacing in cellular uptake. *Journal of Medicinal Chemistry*, *45*(17), 3612–3618.

Shukla, S., & Mathur, M. R. (1995). Low-temperature ultrasonic dyeing of silk. *Journal of the Society of Dyers and Colourists*, *111*(11), 342–345.

Stephens, D. J., & Allan, V. J. (2003). Light microscopy techniques for live cell imaging. *Science*, *300*(5616), 82–86.

Stroffekova, K., Proenza, C., & Beam, K. G. (2001). The protein-labeling reagent FLASH-EDT 2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins. *Pflügers Archiv*, *442*(6), 859–866.

Szent-Gyorgyi, C., Schmidt, B. F., Creeger, Y., Fisher, G. W., Zakel, K. L., Adler, S., et al. (2008). Fluorogen-activating single-chain antibodies for imaging cell surface proteins. *Nature Biotechnology*, *26*(2), 235.

Telmer, C. A., Verma, R., Teng, H., Andreko, S., Law, L., & Bruchez, M. P. (2015). Rapid, specific, no-wash, far-red fluorogen activation in subcellular compartments by targeted fluorogen activating proteins. *ACS Chemical Biology*, *10*(5), 1239–1246.

Toseland, C. P. (2013). Fluorescent labeling and modification of proteins. *Journal of Chemical Biology*, *6*(3), 85–95.

Xing, W., He, L., Yang, H., Sun, C., Li, D., Yang, X., et al. (2009). Development of a sensitive and group-specific polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for detection of malachite green and leucomalachite green in water and fish samples. *Journal of the Science of Food and Agriculture*, *89*(13), 2165–2173.

Yerramilli, V. S., & Kim, K. H. (2018). Labeling RNAs in live cells using malachite green aptamer scaffolds as fluorescent probes. *ACS Synthetic Biology*, *7*(3), 758–766.