Abstract

Protein post-translational modifications (PTMs) serve to give proteins new cellular functions and can influence spatial distribution and enzymatic activity, greatly enriching the complexity of the proteome. Lipidation is a PTM that regulates protein stability, function, and subcellular localization. To complement advances in proteomic identification of lipidated proteins, we have developed a method to image the spatial distribution of proteins that have been co- and post-translationally modified via the addition of myristic acid (Myr) to the N terminus. In this work, we use a Myr analog, 12-azidododecanoic acid (12-ADA), to facilitate fluorescent detection of myristoylated proteins in vitro and in vivo. The azide moiety of 12-ADA does not react to natural biological chemistries, but is selectively reactive with alkyne functionalized fluorescent dyes. We find that the spatial distribution of myristoylated proteins varies dramatically between undifferentiated and differentiated muscle cells in vitro. Further, we demonstrate that our methodology can visualize the distribution of myristoylated proteins in zebrafish muscle in vivo. Selective protein labeling with noncanonical fatty acids, such as 12-ADA, can be used to determine the biological function of myristoylation and other lipid-based PTMs and can be extended to study deregulated protein lipidation in disease states.

Key words: fatty acid/transferase • muscle • fluorescence microscopy • cells and tissues • lipidation • click chemistry • cellular imaging • chemical biology

Recent advancements in chemical biology techniques utilize click chemistry-functionalized biomolecules to study protein regulation in situ. Noncanonical amino acids, sugars, and fatty acids that carry click chemistry functionality can be metabolically incorporated into and onto proteins and have been used to study various aspects of protein biology, including synthesis, turnover, cellular localization, glycosylation, and acylation (1–6). These studies take advantage of the bio-orthogonality of azide or alkyne groups (click chemistry functional groups) that are selectively reactive with each other, but are inert to natural biological chemistries. For example, the methionine analog, azidohomoalanine, has been shown to be readily incorporated into newly synthesized proteins in vitro in mammalian cell lines (7, 8) and primary cells (9), and in vivo in a variety of model organisms including Escherichia coli (10), Arabidopsis (11), Caenorhabditis elegans (12), zebrafish (13), and mouse (14). The azide reactive group of azidohomoalanine and other noncanonical biomolecules can be covalently linked to alkenes [known as copper-catalyzed azide-alkyne cycloaddition (CuAAC) or cycloalkynes [known as strain-promoted azide-alkyne cycloaddition (SPAC)] to form a stable triazole ring (15–18). In this way, fluorophores or affinity reagents (e.g. biotin) can be specifically attached to noncanonical biomolecules within complex mixtures in vitro and in vivo for proteomic and imaging analysis. Recent examples of using noncanonical sugars or fatty acids to study post-translational modifications (PTMs) include live-cell imaging of cell membrane fucosylated glycans in developing zebrafish embryos (19) and imaging of engineered proteins with lipoic acid ligase in mammalian cells (20). We add to this growing body of work by demonstrating that protein myristoylation can be imaged in situ as a function of time and cellular differentiation using an azide-functionalized myristic acid (Myr) analog, 12-azidododecanoic acid (12-ADA) (outlined in Fig. 1).

Supplementary key words: fatty acid/transferase • muscle • fluorescence microscopy • cells and tissues • lipidation • click chemistry • cellular imaging • chemical biology

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Fluorescent imaging of protein myristoylation during cellular differentiation and development

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Methods

Abbreviations: 12-ADA, 12-azidododecanoic acid; CuAAC, copper-catalyzed azide-alkyne cycloaddition; DBCO, dibenzocyclooctyne; DM, differentiation medium; GM, growth medium; hpf, hours post fertilization; Myr, myristic acid; NMT, N-myristoyltransferase; PTM, posttranslational modification; SPAAC, strain-promoted azide-alkyne cycloaddition TAMRA, tetramethylrhodamine.

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Myristoylation is the PTM of proteins via the covalent attachment of Myr to the N terminus by the enzyme, N-myristoyltransferase (NMT). Myristoylation plays an essential role in a variety of eukaryotic biological processes, such as targeting proteins to membranes, increasing protein stability, promoting protein-protein interactions, and regulating apoptosis (21, 22). Fundamental work by Gordon and colleagues showed, in vitro, that the yeast NMT enzyme can successfully label proteins with noncanonical Myr analogs that contain handles, such as azides, alkynes, and ketones, generating critical click chemistry-based tools that can be utilized to study protein myristoylation (21, 23). Azide- and alkyne-functionalized Myr analogs are incorporated onto naturally myristoylated proteins and proteins engineered to display NMT peptide recognition sequences in recombinant systems coexpressing NMT with high selectivity (6, 24–27). These Myr analogs have also been shown to be metabolically incorporated into mammalian cells in vitro and zebrafish embryos in vivo (27); making them useful probes for global profiling of the myristoylated proteome. Here, we show that the expression and subcellular localization of 12-ADA-labeled proteins varies dramatically in undifferentiated and differentiated C2C12 skeletal muscle cells. The utility of this method is extended by labeling and visualization of myristoylated proteins within the developing zebrafish embryo using 12-ADA. When combined with proteomic profiling, selective protein labeling using 12-ADA can be used to determine the biological function of myristoylation in different cell states or in response to treatment within discrete time windows; for example, the dysregulation of protein myristoylation in cancer and other diseases (28, 29). Additionally, we expect this method to be readily translatable to visualize lipid-based PTMs in other cellular and in vivo systems.

### MATERIALS AND METHODS

#### Synthesis of 12-ADA

The 12-ADA was synthesized as previously described (26, 30) with minor modifications (see structure in Fig. 1A). Briefly, a mixture of 12-bromododecanoic acid (1.8 g, 0.0064 mol), sodium azide (1.2 g, 0.019 mol), and 18-crown-6 (0.5 g, 0.0019 mol) were stirred in 25 ml N,N-dimethylformamide under an argon blanket at room temperature overnight. N,N-dimethylformamide was then removed under vacuum. The residue was diluted with 25 ml dichloromethane, followed by the addition of hydrochloric acid (1 M, 25 ml) to quench unreacted sodium azide. The organic layer was rinsed three times with 25 ml water, dried with Na2SO4, filtered, and concentrated under vacuum yielding the desired product (1.5 g, 97% yield) at 95% purity as a pale-yellow liquid. Characterization data for the final product (1H NMR) yielded data that matched published results (30) 1H NMR 3.19 (t. 2H, J = 6.9 Hz, CH2); 2.28 (t. 2H, J = 7.5 Hz, CH2); 1.53 (m. 4H, 2xCH2); 1.21 (m. 14H) plus 18-crown-6 as a residual impurity 1H NMR 3.51(s. 2H, CH2).
C2C12 cell culture and incubation with 12-ADA

C2C12 mouse myoblast cells were obtained from ATCC (catalog number CRL-1772). C2C12 cells were expanded in in growth medium (GM) [DMEM (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS (Corning, Corning, NY), 1% penicillin/streptomycin (Corning), and 1% Glutagro (Corning)] at 37°C and 5% CO2. The medium was changed every 48–72 h and cells were passaged at 80% confluence. For all 12-ADA incorporation experiments, cells between passages five and ten were plated at 1.0 × 106 cells/cm2. To induce myogenic differentiation, confluent cell cultures were incubated with differentiation medium (DM) [DMEM containing 1% penicillin/streptomycin, 1% Glutagro, and 1% FBS]. Differentiation was confirmed by visual inspection (after 1–3 days). Confluent myotubes or myoblasts were then incubated with medium containing 100 μM 12-ADA, Myr (Sigma-Aldrich, St. Louis, MO), or vehicle alone (2.8 mM DMSO; Sigma-Aldrich) for 9 h, unless otherwise specified.

Harvesting and lysing C2C12 cells

After incubation with 12-ADA, Myr, or vehicle alone, medium was aspirated and cells were rinsed with 1× PBS. Undifferentiated myoblast cells were harvested with 0.25% (w/v) trypsin and 1 mM EDTA. Cell suspensions were centrifuged at 200 g at 4°C and washed with 1× PBS to remove excess medium. The resultant cell pellets were either stored at −80°C for further use or directly lysed. For differentiated cell cultures, myocytes were isolated using mild trypsinization (0.025% trypsin diluted with 1× PBS) for 5 min, following (31). The mild trypsinization detaches myotubes leaving only mononuclear myoblasts. This was followed by the normal trypsinization method described above to remove the remaining cells, which were categorized as mid-differentiated myoblasts. Cells were washed and pelleted as described above. Harvested cells were lysed with Mem-PER™ mammalian protein extraction kit (Thermo Fisher Scientific) as per the manufacturer’s instructions. Protein concentrations of the soluble cytosolic and membrane fractions were quantified with Pierce 660 nm protein quantitation assay with the Ionic Detergent Compatibility reagent (Thermo Fisher Scientific).

The 12-ADA incorporation validation via SDS-PAGE

The 12-ADA-labeled proteins within the cell protein lysates were selectively labeled with tetramethylrhodamine (TAMRA) alkylene (Click Chemistry Tools, Scottsdale, AZ) (Fig. 1D) using CuAAC (15). A ClickiT protein reaction buffer kit (Thermo Fisher Scientific) was used per the manufacturer’s instructions. A maximum of 60 μg of total protein lysate was added to each click reaction. Free TAMRA-alkylene dye was removed by methanol-chloroform precipitation. Reacted proteins were solubilized in 1× Laemmli sample buffer with 5% β-mercaptoethanol and then boiled at 95°C for 5 min. Protein concentration was quantified with the Pierce 660 nm assay to ensure equal gel loading, and was then resolved by SDS-PAGE on 4–20% polyacrylamide gels (Bio-Rad, Hercules, CA). Gels were scanned for fluorescence on an Azure Biosystems c400 gel imager on the Cy3 channel (526 nm excitation/565 emission) to detect proteins labeled with the TAMRA-alkylene.

To quantify the rate of 12-ADA incorporation onto myristoylated proteins, in-gel fluorescence was quantitated using the line tool in ImageJ (National Institutes of Health) and the mean fluorescence intensities for each lane were normalized to the minimum (background) and maximum intensities in each image and plotted as a function of time. Loading consistency was confirmed on the same gel with Coomassie Blue-based GelCode Blue protein stain (Pierce; Thermo Fisher Scientific) and scanned with an Azure Biosystems c400 gel imager. Data were plotted and analyzed using Prism software (GraphPad, La Jolla, CA). Data were fit to a pseudo-first order association curve: \[ y = (\text{max} - \text{min}) \times (1 - e^{-kt}) \]

where for normalized values \( \text{max} = 1 \) and \( \text{min} = 0 \), \( t \) is the time of incubation with 12-ADA, and \( k \) is the pseudo-first order rate constant (\( k = 0.22 \, \text{h}^{-1} \)).

Proliferation assay

C2C12 cells were incubated with 12-ADA, Myr, or DMSO for 6 h, as described above, and then incubated for another 3 h with 12-ADA, Myr, or DMSO media containing 40 μM Edu (Thermo Fisher Scientific). Cells were fixed, permeabilized, and blocked as described above. After blocking, cells were incubated with the following click reaction cocktail for 30 min at room temperature: 2 M Tris (pH 8.5), 50 mM CuSO4, AlexaFluor 488 azide (0.5 mg/ml) (Thermo Fisher Scientific) diluted 1:500, and 0.5 M ascorbic acid, following (32). Each well was rinsed three times with 1× PBS, incubated in blocking buffer, and then stained with DAPI (to label nuclei, 1:1,000; Sigma-Aldrich) for 10 min at room temperature with slight rocking. Cells were imaged at 5× magnification with a Leica DMI6000 microscope; the number of Edu+/ nuclei were normalized by the total number of DAPI-stained nuclei in each frame to determine the percentage of proliferating cells, and averaged over three images per treatment.

Protein synthesis inhibition by anisomycin

C2C12 cells were grown to 80% confluence and fed with GM supplemented with 0–40 μM anisomycin (Sigma-Aldrich) for 30 min. Then, 100 μM of 12-ADA were added and cells were incubated for 6 h. Cells were then rinsed with 1× PBS and harvested and lysed with Mem-PER™ mammalian protein extraction kit. The cytosolic lysates were analyzed for 12-ADA incorporation using SDS-PAGE as described above.

Hydroxylamine treatment to assess S-acylation

C2C12 cells were incubated with 100 μM of 12-ADA or Myr for 9 h and were harvested and lysed as described above. Cytosolic proteins containing 12-ADA were labeled with TAMRA-alkylene or AlexaFluor 647-alkylene following (14). Briefly, 60–100 μl of cytosolic protein lysate (50–80 μg total protein, normalized within each experiment) were combined with 10 μl of 400 mM sodium ascorbate for 5 min and then 20 μl of 0.5 M iodoacetamide (VWR, Radnor, PA) were added and incubated for an additional 5 min. Dye-alkylene (0.5 μl 0.8 mM), CuSO4 (16 μl 25 mM) (Sigma-Aldrich), and tris(3-hydroxypropyltriazolylmethyl)amine (40 μl 50 mM) (Click Chemistry Tools) were combined and then added to the protein lysate mixture. After vortexing, 40 μl 100 mM aminoquinidine (pH 7) (Sigma-Aldrich) were added and the final mixture was rotated end-over-end for 15 min at room temperature and protected from light. Unreacted dye was removed using methanol-chloroform precipitation and the protein pellets were air dried for at least 30 min. S-acylation was disrupted using hydroxyamine following (33). Pellets from the methanol-chloroform precipitation were resolubilized in 1× Laemmli sample buffer containing 0, 0.3, 0.7, or 1 M aqueous hydroxyamine (Alfa Aesar, Ward Hill, MA) with 5% β-mercaptoethanol. Samples were boiled at 95°C for 5 min and analyzed using SDS-PAGE. In-gel fluorescence was quantitated as described in section “12-ADA incorporation validation via SDS-PAGE.”

To investigate the effects of hydroxyamine on fluorophore stability, 800 nM AlexaFluor 647-alkylene or TAMRA-alkylene were mixed with 0.01–2 M aqueous hydroxyamine. Samples were analyzed in a 96-well plate for fluorescence on an Azure Biosystems c400 gel imager. Fluorescence was quantified using the line tool in ImageJ (National Institutes of Health) to obtain the mean fluorescence intensities for each well.
Competition with Myr and inhibition of NMT

To assess whether 12-ADA was specifically incorporated at sites of myristoylation, C2C12 cells were incubated with 12-ADA as described above, with the following modifications. For competition with Myr, cells were incubated with 10 μM 12-ADA in combination with increasing concentrations of Myr (1, 10, or 100 μM) for 9 h. To specifically inhibit NMT, cells were preincubated for 30 min with the NMT inhibitor, DDD85646 (Cayman Chemical, Ann Arbor, MI), and then labeled with 10 μM 12-ADA or Myr in the presence of DDD85646 for 6 h. Cells were harvested and the cytosolic fractions were labeled with alkyne fluorophore, as described for the hydroxylamine experiments, except that 12-ADA incorporation was visualized using AlexaFluor 647-alkyne. In-gel fluorescence was quantitated as described in section “12-ADA incorporation validation via SDS-PAGE,” with the following modifications. Gels were scanned for fluorescence on an Azure Biosystems c400 gel imager on the Cy5 channel (628 nm excitation/676 nm emission).

Visualization of 12-ADA incorporation by C2C12 cells

To visualize 12-ADA incorporation, IBIDI μ-slide angiogenesis 15-well plates (IBIDI, Fitchburg, WI) were coated with 0.1 mg/ml Matrigel (Corning) diluted in PBS and incubated for 30 min at 37°C incubator. Excess Matrigel medium was removed via aspiration. Cells were seeded between passages five and ten at 5–20 × 10⁴ cells/cm² and incubator. Excess Matrigel medium was removed via aspiration. Cells were then incubated with 12-ADA, Myr, and DMSO alone as described above. Cells were washed with 1× PBS before fixing with 4% paraformaldehyde (diluted in 1× PBS) for 10 min at room temperature. Fixed cells were rinsed with 1× PBS and permeabilized with 0.2% Triton X-100 in 1% donkey serum (diluted in PBS; Lampire Biological Laboratories, Everett, PA) for 15 min at room temperature. Cells were then washed with 1× PBS and blocked with blocking buffer (10% donkey serum in PBS) for 30 min at room temperature and then rinsed with 1× PBS.

The 12-ADA-labeled proteins were tagged selectively with TAMRA-alkyne using CuAAC, as described in section “12-ADA incorporation validation via SDS-PAGE” or with TAMRA-dibenzocyclooctyne (DBCO) (Click Chemistry Tools; Fig. 1D) using SPAAC (34–36), described as follows. Fixed and permeabilized cells were incubated with 20 mM of iodoacetamide for 30 min to react with free thiols, reducing overall background staining (37). The iodoacetamide solution was removed and 10 μM of TAMRA-DBCO were added to each well for 10 min at room temperature and protected from light. Each well was rinsed three times with 1× PBS and then cells were blocked with 10% donkey serum for 5 min before immunocytochemistry.

To identify differentiated cells, cultures were incubated with an antibody against skeletal muscle myosin, MY32 (1:100 dilution, antibody against skeletal muscle myosin, MY32 (1:100 dilution, ab7784; Abcam, Cambridge, MA) overnight at 4°C. After rinsing with 1× PBS, cells were incubated with AF633 goat anti-mouse IgG2b (1:500, A-21050; Thermo Fisher Scientific) and DAPI (to label nuclei, 1:1,000; Sigma-Aldrich) for 1 h at room temperature. Antibodies were diluted in blocking buffer and all staining steps were protected from light. Labeled cells were kept hydrated in 1× PBS and imaged on a Leica DMi6000 microscope. Images were processed and analyzed using Fiji 2.0v software (National Institutes of Health).

Visualization of 12-ADA incorporation in zebrafish embryos

Zebrafish, including adult fish and embryos, were maintained according to the Purdue Animal Care and Use Committee protocol (#1501001180) at Purdue University. To ensure that 12-ADA would be taken up by the embryos, 18 h post fertilization (hpf) zebrafish were dechoronated using pronase (Sigma) diluted in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄ in milliQ water) and incubated overnight at 27°C. At 24 hpf, embryos were rinsed with 1× PBS and transferred to a clean specimen dish filled with 20 μM 12-ADA diluted in E3. The larvae were incubated at 27°C for 12 h. After incubation, larvae were rinsed in 0.1% PBST (1× PBS + 0.1% Tween-20) and fixed with 4% paraformaldehyde in 1× PBS for 2 h at room temperature. Embryos were either stained immediately or dehydrated in methanol and stored at −20°C. After fixation or dehydration, the zebrafish larvae were washed twice in a PBDT solution (PBST + 1% DMSO). They were then washed briefly in PBS and permeabilized for 20 min in 10 μg/ml proteinase K (Roche) in PBST. Larvae were washed four times for 5 min with PBST. To prevent nonspecific binding of staining reagents, larvae were incubated in a blocking solution made of 5% BSA (Sigma-Aldrich) and 10% goat serum (Lampire) in PBST for a minimum of 3 h at 4°C or 2 h at room temperature. Larvae were then rinsed briefly in PBS.

To identify 12-ADA-labeled proteins, larvae were incubated (when indicated) in the following CuAAC reaction cocktail [as described in (13) with minor modifications]: 990 μl 1× PBST, 4 μl 50 mM tris(3-hydroxypropyltriazolymethyl)amine (Click Chemistry Tools), 1 μl 500 mM tris(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich), 1 μl 1 mM AlexaFluor 488 alkyne (Thermo Fisher Scientific), and 4 μl 50 mM CuSO₄. Each component was added in the order noted and vortexed after addition of each reagent. Larvae were incubated overnight at room temperature with gentle agitation and protected from light. The reaction cocktail was then removed and the larvae were washed four times for 10 min with 0.5 mM EDTA and 0.1% PBST. Embryos were stained to visualize differentiated muscle and nuclei, as described above for C2C12 cells; however, the secondary antibody incubation was overnight.

Statistical analysis

To determine whether variations in fluorescence intensity and cell proliferation were statistically significant, the data were analyzed by a one-way ANOVA with a 95% confidence level (P < 0.05), using MiniTab (State College, PA).

RESULTS

The 12-ADA incorporation by mammalian cells

To validate that 12-ADA could be readily incorporated into the murine myoblast proteome, C2C12 cells were incubated with 100 μM 12-ADA, Myr, or vehicle alone (DMSO) for 9 h. Cytosolic and membrane fractions were reacted with TAMRA-alkyne dye using CuAAC. Only 12-ADA-incubated samples were labeled with TAMRA-alkyne, demonstrating the specificity of the CuAAC reaction (Fig. 2). Cytosolic and membrane fractions, resolved using SDS-PAGE, had differential fluorescent banding patterns, indicating variations in the distribution of 12-ADA-modified proteins between these two cellular compartments (Fig. 2A). Total protein banding patterns between 12-ADA, Myr, and DMSO samples were consistent (Fig. 2A), suggesting that treatment with 12-ADA does not overtly alter protein synthesis.

To image the spatial distribution of 12-ADA-labeled proteins, C2C12 cells fed with 100 μM 12-ADA or Myr were labeled with TAMRA-DBCO (SPAAC) or TAMRA-alkyne (CuAAC). TAMRA-DBCO fluorescence had a consistently
higher signal to background ratio for in situ imaging of cells relative to TAMRA-alkyne (Fig. 2B). The 12-ADA incubated myoblasts were specifically labeled with TAMRA-DBCO, whereas there was only minimal nonspecific background staining in the Myr cultures.

To analyze the incorporation of 12-ADA onto C2C12 proteins as a function of time, undifferentiated myoblasts were harvested 0.5–24 h after the addition of 100 μM 12-ADA. Soluble lysates were then reacted with TAMRA-alkyne (CuAAC) and analyzed via SDS-PAGE. As expected, the fluorescence intensity of 12-ADA-labeled samples increased with time (Fig. 3A, top). Consistent protein loading across treatments was verified with Coomassie stain of the same gel (Fig. 3A, bottom), confirming the time-dependent increase of 12-ADA incorporation into C2C12 cells. Quantification of fluorescence intensity as a function of treatment time indicated that 12-ADA incorporation reached a steady state level after 12 h (Fig. 3B).

In order to confirm that 12-ADA and Myr did not disrupt cell proliferation, 40 μM of EdU were added to C2C12 cells cultured with 100 μM 12-ADA, Myr, or DMSO 3 h before harvest. There was no statistically significant difference in EdU incorporation between cells fed with 12-ADA, Myr, or DMSO-enriched medium (P = 0.565) (Fig. 3C).

To confirm that 12-ADA incorporation was occurring due to co- and post-translational modification, protein synthesis was inhibited using anisomycin. C2C12 cells were pretreated with increasing concentrations of anisomycin (0, 4, or 40 μM) for 30 min and then treated with both anisomycin (0, 4, or 40 μM) and 100 μM 12-ADA or Myr for 6 h. Cells were then harvested and soluble lysates were reacted with TAMRA-alkyne and analyzed via SDS-PAGE (Fig. 4A). Lysates from cells treated with 0 μM anisomycin showed similar 12-ADA incorporation as in Fig. 2A; while cells treated with 12-ADA and 4 or 40 μM anisomycin showed little to no fluorescent signal, similar to Myr control samples.

To investigate whether 12-ADA could be modified and appended to the proteins through S-acylation, which would indicate that 12-ADA was added at a palmitoylation (typically S-acylation at an internal cysteine) rather than a myristoylation (N-terminal acylation) site (33), 0.3–1 M hydroxylamine was added to TAMRA-alkyne-labeled 12-ADA cytosolic proteins in 1× Laemmli sample buffer and 5% β-mercaptoethanol. The difference in fluorescence intensity between lysates treated with increasing hydroxylamine concentrations and lysates without hydroxylamine was not significant (P = 0.838), indicating that proteins were not being modified by 12-ADA through S-acylation (Fig. 4B). Interestingly, we found that the fluorescence signal was highly dependent on the fluorophore that was used. The intensity of the TAMRA-alkyne signal was stable at the hydroxylamine concentrations used in the inhibition study; however, the AlexaFluor 647-alkyne signal became attenuated as hydroxylamine concentration increased (Fig. 4C, D).

To assess whether 12-ADA was specifically incorporated at sites of myristoylation, labeling was carried out in the presence of increasing concentrations of Myr. The 12-ADA labeling was significantly reduced in the presence of both 10 and 100 μM Myr compared with cells that were treated with 12-ADA alone (Fig. 4E, F; P < 0.01). In contrast, 1 μM Myr only had a minor effect on 12-ADA incorporation. Furthermore, the NMT inhibitor, DDD85646 (38), significantly
To visualize the spatial distribution of myristoylated proteins during cellular differentiation, undifferentiated and differentiated C2C12 cells were incubated with 100 μM 12-ADA or Myr for 9 h and stained with TAMRA-DBCO (SPAAC). The myoblasts and myotubes incubated with 12-ADA were efficiently labeled with TAMRA-DBCO, whereas the Myr-treated cells only showed nonspecific background staining (Fig. 5B). The majority of 12-ADA-labeled proteins in the undifferentiated cells were localized surrounding the nuclei and sparsely found in the periphery of the cell body (Figs. 2B, 5B). However, in the differentiated cells expressing myosin heavy chain (red), TAMRA staining was diffuse throughout the cell body, indicating that the spatial distribution of myristoylated proteins changes upon differentiation (Fig. 5B). In addition, it is interesting to note the distinct puncta in the mid-differentiated cells (i.e., mononuclear cells in differentiated culture that have not begun to express myosin heavy chain), which varies from the distribution of 12-ADA in the undifferentiated cells.

Based on successful tagging of myristoylated proteins in zebrafish embryos (27), we hypothesized that our in situ labeling technique would be translatable to imaging myristoylated proteins in vivo in developing zebrafish. Twenty-four hours postfertilization, zebrafish were incubated with 20 μM 12-ADA-supplemented medium for 12 h and then stained for incorporation using CuAAC (Fig. 6A, B). The 12-ADA, labeled with AF488-alkyne (green), was found throughout the developing zebrafish, with the highest signal in the musculature (red, Fig. 6A, B). Labeling specificity was confirmed by incubating embryos with AF488-alkyne without the CuAAC reaction components (Fig. 6C, D).

**DISCUSSION**

Using CuAAC and SPAAC, we have developed a method that can resolve the spatial distribution of myristoylated proteins in vitro and in vivo for the first time. As expected, we found that the Myr analog, 12-ADA, was readily taken up by the C2C12 mammalian cell line and covalently attached to myristoylated proteins by NMT (Fig. 2). It should be noted that we did not deplete the GM of exogenous Myr or inhibit endogenous synthesis of Myr. Thus, 12-ADA was incorporated into proteins at detectable levels despite competition with intracellular Myr. This process did not significantly hinder the proliferation of the cells (Fig. 3) and was blocked in the presence of a protein synthesis inhibitor, anisomycin (Fig. 4A). In addition, proteins were labeled by 12-ADA specifically through myristoylation; incorporation was attenuated by the NMT-specific inhibitor, DDD85646, and by the addition of excess Myr, and was not attenuated by reagents specific to cleavage of Sacylation (Fig. 4B–H). Furthermore, 12-ADA-modified proteins could be specifically labeled with alkyne-linked fluorophores in complex cellular lysates (Figs. 2–5). Changes in the spatial localization of myristoylated proteins as a function of myoblast differentiation could be visualized within C2C12 cells in situ by taking advantage of the azide functionality of 12-ADA to covalently attach a fluorescent-cyclooctyne via SPAAC.

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**Fig. 3.** Time-dependent incorporation of 12-ADA onto myristoylated proteins in C2C12 myoblasts. A: Myoblasts were incubated in 100 μM 12-ADA–enriched medium for 0.5–24 h. Cell lysates were labeled with TAMRA-alkyne, resolved using SDS-PAGE, fluorescently imaged, and stained for total protein with Coomassie Blue to confirm equal loading. Representative results from n = 3 independent experiments. B: Mean fluorescent intensities for each lane [of (A) and similar gels] were normalized and plotted to show the rate of 12-ADA incorporation. Line represents the fit to a pseudo-first order association curve as described in the Materials and Methods. C: Addition of 12-ADA and Myr to GM does not affect C2C12 cell proliferation (one-way ANOVA, P = 0.565) relative to DMSO carrier control. Results from n ≥ 3 independent experiments.
Fig. 4. The 12-ADA incorporation by C2C12 myoblasts is dependent on protein synthesis and is catalyzed by the NMT enzyme. A: Reduction of fluorescence intensity after anisomycin treatment indicates that 12-ADA is added to proteins co- and/or post-translationally. Undifferentiated C2C12 cells were fed with 0, 4, or 40 μM anisomycin for 30 min. Then, 100 μM of 12-ADA were added and cells were incubated for 6 h. B: Hydroxylamine treatment after CuAAC with TAMRA-alkyne did not significantly alter the banding pattern or intensity (one-way ANOVA, *P* = 0.838), indicating that proteins were not being modified by 12-ADA through S-acylation. C: Hydroxylamine treatment reduced the overall intensity of AlexaFluor 647-alkyne fluorescence in a concentration-dependent manner without changing the protein banding pattern. D: The fluorescence intensity of AlexaFluor 647-alkyne was markedly decreased at hydroxylamine concentrations greater than 0.3 M, while the TAMRA-alkyne was minimally affected (n = 3). E, F: Myr reduced 12-ADA labeling in a concentration-dependent manner; samples treated with 12-ADA (10 μM) alone showed significantly higher fluorescence intensities than samples treated in presence of 10 or 100 μM Myr (**P < 0.01, one-way ANOVA). G, H: Cells treated with DDD85646 displayed significantly lower levels of 12-ADA incorporation than cells treated with 12-ADA alone (**P < 0.01, one-way ANOVA). The dotted lines (F, H) represent background fluorescence for samples treated without 12-ADA. A–C, E, G: Representative results from n ≥ 5 independent experiments. D, F, H: Average and SEM results from n ≥ 3 independent experiments.
Finally, we demonstrated that 12-ADA labeling could be imaged in vivo using the zebrafish as a model system (Fig. 6).

The method described here represents a robust and reliable experimental platform with which proteomic changes in protein myristoylation can be studied in undifferentiated and differentiated cells. Previous work from the Tate and Hang groups have shown that myristoylated proteins could be labeled with alkyne and azide analogs of Myr and be enriched with affinity chromatography for identification with mass spectrometry (25, 27, 39, 40). Here, we utilize the azide Myr analog, 12-ADA, as it is readily synthesized and both CuAAC and SPAAC can be used for in vitro visualization (SDS-PAGE/blotting analysis) and in situ fluorescent imaging of proteins. Previous work has found that 12-ADA incorporation combined with biotinylation and streptavidin-fluorophore blotting was not optimal for visualization of myristoylated proteins; however, direct fluorescent labeling with alkyne-fluorophores did produce strong banding patterns for protein visualization (41). In our hands, visualization and fluorescence microscopy imaging of myristoylated proteins facilitated by 12-ADA incorporation and conjugation with alkyne and cyclooctyne fluorophores produced clear, robust, and repeatable results. Interestingly, we found that TAMRA-DBCO (SPAAC) gave consistently higher signal to background for in situ imaging of C2C12 cells relative to TAMRA-alkyne (CuAAC). We found that background fluorescence was lower when using SPAAC, which we attribute to the use of reduction and alkylation agents to inhibit the reaction of the fluorophore-cyclooctyne with free-thiols. Without this important step, there is a significant amount of background fluorescent labeling making it difficult to resolve the 12-ADA functionalized proteins with SPAAC (Fig. 2B).

Under standard, subconfluent growth conditions, C2C12 cells are mononuclear and proliferative and take on a more rounded phenotype; however, when plated at high densities or in serum-reduced media, C2C12 cells will begin to elongate, fuse into multinuclear myotubes, and upregulate components of the contractile machinery (e.g., myosin heavy chain) (42, 43). Therefore, this cell line is a beneficial model for researching various physiological states of muscle myogenesis, including proliferation, migration, fusion, myotube formation, and contraction. We found that 12-ADA-modified proteins were located in both the cytosolic and membrane fractions of both undifferentiated and differentiated C2C12 lysates (Fig. 5A). This is consistent with findings that myristoylation alone is not a strong enough hydrophobic interaction to drive myristoylated proteins to associate with membranes (44). Hydrophobic residues, polybasic peptides, and other lipid PTMs are often found in tandem with myristoylated proteins, leading to their association with cellular membranes (45, 46). Indeed, myristoylated proteins are found in a variety of...
cellular compartments, including the cytosol, nucleus, and endoplasmic reticulum, in addition to membranes (47, 48).

Upon imaging, we observed distinct differences in the spatial location of 12-ADA-modified protein between undifferentiated and differentiated cell states. In undifferentiated myoblasts, 12-ADA labeled proteins were localized to areas immediately surrounding the nuclei and at a lower density in the periphery of the cell body (Fig. 2B). In differentiated myocytes, we found that 12-ADA-modified proteins were more evenly distributed throughout the multinucleated cells (Fig. 5B). In addition, it is interesting to note the redistribution of 12-ADA modified proteins from relatively homogenous locations around the nuclei in undifferentiated myoblasts to the large distinct puncta observed in the cytoplasm of mid-differentiated cells in the differentiated cultures (cells that do not express myosin heavy chain, but are developing into mature myocytes). We interpret this as an indication of considerable cellular restructuring/reorganization during the differentiation process.

We expect that the method described here will be readily translated to in vivo fluorescence microscopy imaging of other lipid PTMs. Azide- and alkyn-functionalized analogs of Myr, palmitic acid, and isoprenoids have been used in proteomic profiling and visualization via SDS-PAGE or blotting analysis of lipid PTMs (27, 49–51). However, few studies have developed methods to image lipidated proteins in situ in cell culture and none have done so in vivo; notable exceptions are fluorescence imaging and quantification (via flow cytometry) of protein prenylation (51) and protein palmitoylation (41, 52) in mammalian cells. Furthermore, the method developed here can be extended to live cell imaging with the use of cell-permeable cyclooctyne dyes (6), and will be useful for imaging myristoylated protein translocation during cell division and differentiation and in response to chemical treatments.

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REFERENCES

1. Zhang, X., and Y. Zhang. 2013. Applications of azide-based bioorthogonal click chemistry in glycoanalysis. Molecules. 18: 7145–7159.
2. Yuet, K. P., and D. A. Tirrell. 2014. Chemical tools for temporally and spatially resolved mass spectrometry-based proteomics. Ann. Biomed. Eng. 42: 299–311.
3. Hang, H. C., C. Yu, D. L. Kato, and C. R. Bertozzi. 2003. A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. Proc. Natl. Acad. Sci. USA. 100: 14846–14851.
4. Spicer, C. D., and B. G. Davis. 2014. Selective chemical protein modification. Nat. Commun. 5: 4740.
5. Hannoush, R. N. 2012. Profiling cellular myristoylation and palmitoylation using omega-alkynyl fatty acids. Methods Mol. Biol. 800: 85–94.
6. Ho, S. H., and D. A. Tirrell. 2016. Chemoenzymatic labeling of proteins for imaging in bacterial cells. J. Am. Chem. Soc. 138: 15098–15101.
7. Beatty, K. E., J. C. Liu, F. Xie, D. C. Dieterich, E. M. Schuman, Q. Wang, and D. A. Tirrell. 2006. Fluorescence visualization of newly synthesized proteins in mammalian cells. Angew. Chem. Int. Ed. Engl. 45: 7364–7367.
8. Dieterich, D. C., A. J. Link, J. Graumann, D. A. Tirrell, and E. M. Schuman. 2006. Selective identification of newly synthesized protein in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). Proc. Natl. Acad. Sci. USA. 103: 9482–9487.
9. Dieterich, D. C., J. J. Hodas, G. Gouzer, I. Y. Shadrin, J. T. Ngo, A. Tytler, D. A. Tirrell, and E. M. Schuman. 2010. In situ visualization and dynamics of newly synthesized proteins in rat hippocampal neurons. Nat. Neurosci. 13: 897–905.
10. Link, A. J., and D. A. Tirrell. 2003. Cell surface labeling of Escherichia coli via copper(I)-catalyzed [3+2] cycloaddition. J. Am. Chem. Soc. 125: 11164–11165.
11. Glenn, W. S., S. E. Stone, S. H. Ho, M. J. Sweredoski, A. Moradian, S. Hess, J. Bailey-Serres, and D. A. Tirrell. 2017. Bioorthogonal non-canonical amino acid tagging (BONCAT) enables time-resolved analysis of protein synthesis in native plant tissue. Plant Physiol. 173: 1545–1553.
12. Yuet, K. P., M. K. Doma, J. T. Ngo, M. J. Sweredoski, R. L. Graham, A. Moradian, S. Hess, E. M. Schuman, P. W. Sternberg, and D. A. Tirrell. 2015. Cell-specific proteomic analysis in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA. 112: 2705–2710.
13. Hinz, F. I., D. C. Dieterich, D. A. Tirrell, and E. M. Schuman. 2012. Non-canonical amino acid labeling in vivo to visualize and affinity purify newly synthesized proteins in larval zebrafish. ACS Chem. Neurosci. 3: 40–49.
14. Calve, S. A. J. Witten, A. R. Ocken, and T. L. Kinzer-Ursem. 2016. Incorporation of non-canonical amino acids into the developing murine prostate. Sci. Rep. 6: 32577.
15. Tornøe, C. W., C. Christensen, and M. Meldal. 2002. Pepidiotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J. Org. Chem. 67: 3057–3064.
16. Rostovtsev, V. V., L. G. Green, V. V. Fokin, and K. B. Sharpless. 2002. A stepwise huisgen cycloaddition process: copper(I)-catalyzed...
modification of biomolecules in living systems. *J. Am. Chem. Soc.* **126**: 15046–15047.
35. Agard, N. J., J. M. Baskin, J. A. Prescher, A. Lo, and C. R. Bertozzi. 2006. A comparative study of bioorthogonal reactions with azides. *ACS Chem. Biol.* **1**: 643–648.
36. Yao, J. Z., C. Uttamapinant, A. Poloukhine, J. M. Baskin, J. A. Codelli, E. M. Sletten, C. R. Bertozzi, V. V. Popik, and A. Y. Ting. 2012. Fluorophore targeting to cellular proteins via enzyme-mediated azide ligation and strain-promoted cycloaddition. *J. Am. Chem. Soc.* **134**: 3720–3728.
37. van Geel, R., G. J. Puijn, F. L. van Delft, and W. C. Boeclens. 2012. Preventing thiol-yne addition improves the specificity of strain-promoted azide-alkyne cycloaddition. *Bioconjug. Chem.* **23**: 392–398.
38. Frearson, J. A., S. Brand, S. P. McElroy, L. A. Ceglhorn, O. Smid, L. Stojanowski, H. P. Price, M. L. Guther, L. S. Torrie, D. A. Robinson, et al. 2010. N-myristoyltransferase inhibitors as new leads to treat sleeping sickness. *Nature* **464**: 728–732.
39. Wilson, J. P., A. A. S. Raghovan, Y. Y. Yang, G. Charron, and H. C. Hang. 2011. Protonic analysis of fatty-acylated proteins in mammalian cells with chemical reporters reveals S-acetylation of histone H3 variants. *Mol. Cell Proteomics* **10**: M110.011198.
40. Yount, J. S., M. M. Zhang, and H. C. Hang. 2011. Visualization and identification of fatty acylated proteins using chemical reporters. *Curr. Protoc. Chem. Biol.* **3**: 65–79.
41. Charron, G., M. M. Zhang, J. S. Yount, J. Wilson, A. S. Raghavan, E. Shamir, and H. C. Hang. 2009. Robust fluorescent detection of protein fatty-acylation with chemical reporters. *J. Am. Chem. Soc.* **131**: 4967–4975.
42. Buratti, S., P. Ferri, M. Battistelli, R. Curci, F. Luchetti, and E. Falcieri. 2004. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *Eur. J. Histochem.* **48**: 223–233.
43. Gilbert, S. F. 2000. Myogenesis: the development of muscle. In Developmental Biology, 6th edition. Sinauer Associates, Sunderland, MA. Available from: http://www.ncbi.nlm.nih.gov/books/NBK10006/.
44. Peitsch, R. M., and S. McLaughlin. 1993. Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry.* **32**: 10436–10443.
45. Maurer-Stroh, S., M. Gouda, M. Novatchkova, A. Schleiffer, G. Schneider, F. L. Sirota, M. Wildpander, N. Hayashi, and F. Eisenhaber. 2004. MYRbase: analysis of genome-wide glycopeptide myristoylation enlarges the functional spectrum of eukaryotic myristoylated proteins. *Genome Biol.* **5**: R21.
46. Resh, M. D. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys. Acta.* **1451**: 1–16.
47. Olson, E. N., D. A. Towler, and L. Glaser. 1985. Specificity of fatty acid acylation of cellular proteins. *J. Biol. Chem.* **260**: 5784–5790.
48. Magee, A. L., and S. A. Courtneidge. 1985. Two classes of fatty acid acylated proteins exist in eukaryotic cells. *EMBO J.* **4**: 1137–1144.
49. Emmel, M., N. Sreeram, and G. Bennink. 2015. Percutaneous tricuspid valve replacement in childhood. *Ann. Pediatr. Cardiol.* **8**: 230–232.
50. Storck, E. M., R. A. Serwa, and E. W. Tate. 2013. Chemical proteomics: a powerful tool for exploring protein lipidation. *Biochem. Soc. Trans.* **41**: 56–61.
51. Palusulesai, C. C., J. D. Ochocki, M. K. Kuhns, Y. C. Wang, J. K. Warmka, D. S. Cherneck, E. V. Wattenberg, L. Li, E. A. Arriaga, and M. D. Distefano. 2016. Metabolic labeling with an alkynyl-modified isoprenoid analog facilitates imaging and quantification of the prenylome in cells. *ACS Chem. Biol.* **11**: 2829–2829.
52. Gao, X., and R. N. Hannoush. 2014. Single-cell in situ imaging of palmitoylation in fatty-acylated proteins. *Nat. Protoc.* **9**: 2607–2625.