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Corresponding Author:	Dale David Orr Martin, Ph.D University of Waterloo Waterloo, Ontario CANADA
Corresponding Author's Institution:	University of Waterloo
Corresponding Author E-Mail:	dale.martin@uwaterloo.ca
Order of Authors:	Dale David Orr Martin, Ph.D
	Lucia Liao
	Rachel Gray
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TITLE:

Optimized Incorporation of Alkynyl Fatty Acid Analogs for the Detection of Fatty Acylated Proteins using Click Chemistry

AUTHORS AND AFFILIATIONS:

Lucia M. Q. Liao[1], Rachel A. V. Gray[1], Dale D. O. Martin[1]

[1]Department of Biology, University of Waterloo, Waterloo, ON, Canada

Email addresses of co-authors:

Lucia Liao (lucia.liao@uwaterloo.ca)
Rachel Gray (20ravg@queensu.ca)
Dale Martin (dale.martin@uwaterloo.ca)

Corresponding author:

Dale Martin (dale.martin@uwaterloo.ca)

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click chemistry, bio-orthogonal labeling, fatty acylation, alkynyl fatty acid, biotin-azide, S-acylation, S-palmitoylation, palmitate, stearate, myristate

SUMMARY:

The study of fatty acylation has important implications in cellular protein interactions and diseases. Presented here is a modified protocol to improve click chemistry detection of fatty acylated proteins, which can be applied in various cell types and combined with other assays, including pulse-chase and mass spectrometry.

ABSTRACT:

Fatty acylation, the covalent addition of saturated fatty acids to protein substrates, is important in regulating a myriad of cellular functions in addition to its implications in cancer and neurodegenerative diseases. Recent developments in fatty acylation detection methods have enabled efficient and non-hazardous detection of fatty acylated proteins, particularly through the use of click chemistry with bio-orthogonal labeling. However, click chemistry detection can be limited by the poor solubility and potential toxic effects of adding long chain fatty acids to cell culture. Described here is a labeling approach with optimized delivery using saponified fatty acids in combination with fatty-acid free BSA, as well as delipidated media, which can improve detection of hard to detect fatty acylated proteins. This effect was most pronounced with the alkynyl-stearate analog, 17-ODYA, which has been the most commonly used fatty acid analog in click chemistry detection of acylated proteins. This modification will improve cellular incorporation and increase sensitivity to acylated protein detection. In addition, this approach can be applied in a variety of cell types and combined with other assays such as pulse-chase analysis, stable isotope labeling with amino acids in cell culture, and mass spectrometry for quantitative profiling of fatty acylated proteins.

INTRODUCTION:

Fatty acylation involves the covalent addition of fatty acids to proteins and is well known for its importance in promoting protein-membrane interactions but has also shown to promote protein-protein interactions, conformational changes, and regulate catalytic sites of enzymes[1–7]. Fatty acylation has emerged as a potential drug target in a myriad of diseases, including infection, cancer, inflammation, and neurodegeneration, where disruptions in palmitoylation have been documented[8–13]. This has been primarily spurred by the development of new chemical detection methods, which enabled large-scale identification of S-acylated protein targets.

Fatty acylation can include a variety of modifications involving the covalent addition of saturated and unsaturated fatty acids, but typically refers to N-myristoylation and S-acylation. N-myristoylation refers to the addition of myristic acid to N-terminal glycines either co-translationally on nascent polypeptides or post-translationally on newly exposed N-terminal glycines following proteolytic cleavage[2,14]. N-myristoylation occurs through an irreversible amide bond. On the other hand, S-acylation typically refers to the reversible addition of long chain fatty acids to cysteine residues via a thioester bond. The most common form of this modification includes the incorporation of palmitate and, therefore, is commonly referred to as S-palmitoylation, or simply palmitoylation[11,15]. In many ways, S-palmitoylation is similar to phosphorylation. It is dynamic, enzymatically regulated, and proving to be highly tractable.

Up until the last decade, studying fatty acylation was hindered by limited detection methods, which required radioactively labeled fatty acids. This had several disadvantages, including cost, safety issues and very long detection times. Typically, either tritiated or iodinated palmitate was used for the detection of S-acylation[16]. Tritiated palmitate required lengthy detection periods with autoradiography film, which can take weeks to months. While [^{125}I] iodo-fatty acid analogs shortened detection times, it presented a much higher safety risk and required close thyroid monitoring of experimenters. In addition, these methods were non-quantitative, therefore, limiting the ability to measure dynamic palmitoylation, and also time consuming to set-up and clean-up due to the extra personal protective equipment and radioactive monitoring. Finally, radioactive labels were not well suited for proteomic studies and typically limited to low throughput detection of specific proteins of interest. As more substrates were detected and, inevitably the enzymes that mediate each modification were identified, it was clear that new detection methods were required[17–21]. Almost simultaneously, several new methods arose for the detection of fatty acylated proteins. The first exploits the reversibility and reactivity of the thioester bond of S-acylation. The Acyl-biotin exchange (ABE) assay chemically replaces palmitate with biotin for subsequent pulldown of S-acylated proteins using avidin agarose beads, or direct detection using streptavidin on a western blot[22–24]. Next, bio-orthogonal labeling of fatty acids and chemoselective addition to tags or handles were developed that included the use of the Staudinger ligation and click chemistry[25–33]. Finally, similar to the ABE, Acyl-Resin assisted capture (RAC) essentially replaces S-acylated sites with thiol-reactive beads for capture and detection of S-acylated proteins[34,35]. Together, the exchange and click-chemistry-based assays have provided more efficient and sensitive methods of acylation detection and affinity purification for downstream analysis and have subsequently led to the discovery of thousands of S-acylated proteins[8,36].

The term click chemistry encompasses a group of chemical reactions, but most commonly refers to the Cu(I)-catalyzed azido-alkyne [3+2] cycloaddition reaction mechanism between an alkynyl group and an azido group[27,28,37]. Particularly, in the case of fatty acylation, click chemistry involves the detection of S-palmitoylation or N-myristylation by incorporating bio-orthogonal 16-carbon alkynyl-palmitate (15-hexadecynoic acid; 15-HDYA) or the 14-carbon alkynyl-myristate (13-tetradecynoic acid; 13-TDYA), respectively, into cells to label endogenously acylated proteins[28]. After cell lysis and immunoprecipitation of the protein of interest, a click chemistry reaction (covalent linkage between an alkyne and an azide) is performed to bind an affinity probe, typically biotin, for detection by western blot[28,37]. Alternatively, click chemistry can be performed on the total cell lysate and fatty acylated proteins can be affinity purified for identification by mass spectrometry. The initial click chemistry reaction with azido-biotin increased the selectivity and sensitivity of detection over a million times compared to radioactivity[2]. Another advantage of click chemistry is that it can be combined with other classical labeling methods, such as pulse-chase analysis of protein turnover using azido-homoalanine for quantitative analysis[38]. In addition, fluorescent probes can be used instead of biotin or other biochemical probes, such as FLAG or Myc tags, in order to examine protein localization[16,28,39].

Despite the relative ease of use of click chemistry, detection can be limited by the low solubility and potential toxicity of using long chain free fatty acids in cell culture[40]. In particular, despite the preference of palmitate during S-acylation for the majority of proteins, many studies have used the 18-carbon stearate (17-octadecynoic acid – 17-ODYA) rather than palmitate (15-HDYA) for detecting S-acylated proteins due to its commercial availability and relatively low cost. However, 17-ODYA is very insoluble and requires special attention when being used. In addition, click chemistry can require some nuanced preparation and storage of chemicals. Herein, the protocol describes a labeling approach, which optimizes delivery using saponification of fatty acids, delivery with fatty-acid free BSA, and delipidated FBS to increase solubility and bypass potential toxic effects of adding free fatty acids to cells[28]. This method works in a variety of cell types and has even been used in live animals[28].

PROTOCOL:

1. Cell culture

1.1. To supplement DMEM (Dulbecco's Modified Eagle Medium) for cell culture, add 10% Fetal Bovine Serum (FBS), 1x Penicillin-Streptomycin, 2 mM L-glutamine, and 100 mM sodium pyruvate (1% vol/vol).

1.2. Plate approximately 5 x 10^5 HEK293T cells/well of a 6-well tissue culture dish and grow for 18 h in a 37 °C humidified incubator with 5% CO_2 to reach 75%–80% confluency.

1.3. Fatty acid serum deprivation

1.3.1. To prepare labeling media, prepare DMEM as above (step 1.1) without 10% FBS. Replace

FBS with 5% dextran-charcoal coated FBS (DCC-FBS). Pre-warm to 37 °C before use.

1.3.2. Gently wash the cells with 1x phosphate buffered saline (PBS) at room temperature and replace with labeling media.

NOTE: HEK293T cells detach from tissue culture plates easily. Take care when washing cells and replacing media. Minimize agitation during transfer to and from the incubator as much as possible.

1.3.3. Return the cells to the 37 °C incubator with 5% CO_2 and incubate approximately 45 min (minimum 15 min is effective), up to 60 min, prior to proceeding with metabolic labeling with fatty acids.

2. Preparation and saponification of fatty acid analogs

2.1. Prepare the stock solutions of alkynyl fatty acids ahead of time by solubilizing in DMSO to achieve the following concentrations and store at -20 °C. Thaw at room temperature as needed. Store the preparations for their best performance under N_2 or Ar.
Alkynyl-myristate (13-tetradecynoic acid, 13-TDYA): 25 mM
Alkynyl-palmitate (15-hexadecynoic acid; 15-HDYA): 100 mM
Alkynyl-stearate (17-octadecynoic acid; 17-ODYA): 100 mM

2.2. To prepare 20% fatty acid free BSA (FAFBSA), weigh 2 g of FAFBSA in a 50 mL disposable tube.

2.2.1. Add 10 mL pre-warmed (37 °C) DMEM.

2.2.2. Mix by end-over-end revolutions or by vortexing, and place in a 37 °C water bath to dissolve FAFBSA completely.

2.2.3. Use 0.2 µm filter to filter sterilize the medium.

2.2.4. Aliquot in approximately 1 mL volumes and store at -20 °C. Thaw as needed and warm to 37 °C in a water bath prior to use.

2.3. To enhance the solubility of the fatty acid and fatty acid analogs, saponify by incubating with 20% molar excess of potassium hydroxide (KOH) in 3 mL conical reaction vials.

NOTE: These vials allow the salt to remain soluble while ensuring the FAFBSA does not congeal from the high heat. The use of glass also prevents fatty acids from sticking to the plastic.

2.3.1. Pipette out at least 2 µL of alkynyl fatty acid analog directly to the bottom of a 3 mL conical reaction vial. Prepare 2 µL of lipid per well of a 6-well plate used (see **Table 1**).

NOTE: Due to the hydrophobicity of fatty acids, it is best to coat the tip of the pipette by drawing up the desired volume several times before dispensing it into the reaction vial.

[Place **Table 1** here]

2.3.2. Dilute 1 M KOH to concentrations equal to 20% molar excess of the alkynyl fatty acid label (30 mM for 13-TDYA and 120 mM for 15-HDYA and 17-ODYA).

2.3.3. Pipette out an equal amount of diluted KOH (1 µL : 1 µL fatty acid : KOH) close to the bottom of the reaction vial on the edge of the glass, such that the dispensed volume of the KOH mixes with the fatty acid.

2.3.4. Close the lid of the vial and tap gently to mix the solutions.

NOTE: The mixture may solidify quickly, especially with increasing lengths of the hydrocarbon chain of the fatty acid. Be careful not to pipet the solid (i.e., do not mix by pipetting).

2.3.5. Heat the reaction vial at 65 °C for approximately 5 min, or as soon as the fatty acid is incorporated (solution becomes clear).

NOTE: Fatty acids with a higher number of carbons and decreased solubility, such as stearate (17-ODYA), may require longer incubation times to be fully incorporated into the KOH at 65 °C. Raise the temperature to 70 °C if needed. A water bath is best. Take care that the liquid does not evaporate too much.

2.3.6. Once the fatty acids have gone into solution and no visible solids remain, pipette prewarmed 20% FAFBSA such that the volume ratio of fatty acids: KOH: FAFBSA is 1: 1: 50 to achieve a final concentration of 20x BSA-bound alkynyl fatty acids.

2.3.7. Mix by pipetting up and down. The solution typically appears clear with no visible solids.

NOTE: Typically, any small solids will go into solution after incubation at 37 °C.

2.3.8. Incubate the fatty acid and FAFBSA for 15 min at 37 °C.

NOTE: The saponified label at this point is stable beyond 15 min.

2.4. As a control, repeat step 2.3. with fatty acids without an alkyne label.

2.5. Label the cells with 1/20 volume of the 20x fatty acid-BSA conjugate directly onto the starvation media (typically, 100 µL in 2 mL media/cells) to achieve a final concentration of 1% BSA and 25 µM for alkynyl-myristate, or 100 µM for alkynyl-palmitate and alkynyl-stearate.

NOTE: To minimize the amount of physical disturbance to the attached cells, form a droplet at

the tip of the pipette close to the surface of the media instead of pipetting directly into the media. If using acylation inhibitors, add at least 15 min prior to the labeled fatty acids. Times may vary depending on the cells or inhibitor used. It has been recommended to use the saponification for this step as well[28].

2.5.1. For comparison, add non-saponified lipids by pipetting 2 µL (or equivalent to volume saponified) of unlabeled fatty acid directly into the starvation media.

2.5.2. Place cells back into the incubator and incubate for 3–6 h.

NOTE: Optimal labeling timing may need to be determined for each cell type or experimental condition. Longer incubation times (more than 16 h) can potentially lead to the breakdown of the fatty acids by β-oxidation and/or incorporation into other lipid groups, such as phospholipids[28].

2.6. Gently wash the cells with 1x PBS at room temperature.

2.7. Harvest and lyse the cells with 500 µL ethylenediaminetetraacetic acid (EDTA)-free modified radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 50 mM N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES) pH 7.4, 150 mM NaCl, 1% non-denaturing detergent, 0.5% sodium deoxycholate, 2 mM $MgCl_2$ with freshly added 1 mM phenylmethylsulphonyl fluoride (PMSF), and 10 µg/µL Pepstatin A (or EDTA-free complete protease inhibitor cocktail)) by rocking the lysates for 15 min at 4 °C.

2.7.1. Centrifuge the lysates at 16,000 x *g* for 10 min at 4 °C.

2.7.2. Collect the supernatant in 1.7 mL microcentrifuge tubes and store at -20 °C until ready to proceed with click reaction.

NOTE: The protocol can be paused here. Lysates are stable at -20 °C for up to 1 month. However, it is recommended to proceed with the click reaction in a timely manner.

2.8. Quantify the protein concentrations using an appropriate assay as per the manufacturer's protocol, such as a detergent compatible (DC) assay.

3. Click reaction on cell lysates

3.1. Prepare the reagents for click chemistry.

3.1.1. Dissolve tris-(benzyltriazolylmethyl) amine (TBTA) in DMSO to 2 mM. Store in small aliquots with desiccant at -20 °C for up to 2–3 months. Best stored under N_2 or Ar.

3.1.2. Dissolve $CuSO_4$ in ddH_2O water to achieve 50 mM. Store at room temperature for up to 2 months.

3.1.3. Dissolve tris-carboxyethylphosphine (TCEP) in ddH_2O water to 250 mM. Store in darkness at 4 °C and make fresh 50 mM dilutions just prior to the click reaction.

3.1.4. Prepare 2 mM azide in DMSO. Store in small aliquots with desiccant at -20 °C for up to 6 months. Best stored under N_2 or Ar.

NOTE: It was observed that the products with three or more PEG groups worked best.

3.2. Bring 50–100 µg of protein lysates in 1.7 mL microcentrifuge tubes to the same volume using the same lysis buffer as above.

NOTE: Keep the reaction volume as small as possible (20–100 µL).

3.2.1. Add sodium dodecyl sulphate (SDS) to each sample to attain a final 1% concentration.

3.2.2. Prepare a master mix of click reagents so that the final concentrations after addition to the lysates are: 100 µM TBTA (2 mM stock), 1 mM $CuSO_4$, (50 mM stock) 1 mM TCEP (50 mM stock), and 100 µM azide probe (2 mM stock). Combine stock solutions accordingly (see **Table 2**).

NOTE: The order is important. Mix completely after addition of each component.

3.2.3. Add appropriate volumes of the master mix into the lysates. Mix by pipetting up and down.

[Place **Table 2** here]

3.2.4. Incubate for 30 min in the dark in a 37 °C water bath. Agitate/mix occasionally.

4. Click reaction on immunoprecipitated proteins

4.1. Alternatively, it is possible to perform the click reaction on immunoprecipitated (IP) proteins **on or off** the beads.

NOTE: Typically, performing click **off** the beads leads to the least background and is best when testing new proteins of interest.

4.1.1. Transfect cells for the protein of interest, in this case, wildtype myristoylated C-terminal huntingtin (HTT) fused to GFP (myr-ctHTT-GFP) and ctHTT-GFP with a G2A substitution, using calcium phosphate DNA coprecipitation, as previously described[41].

4.1.1.1. Plate 2.5 x 10^5 cells/well in 6-well tissue culture plates and grow overnight to ~40%–50% confluency.

4.1.1.2. Prepare the DNA mix by adding 2.5 µg DNA in 10 µL with 99.75 µL molecular grade H_2O to a 1.7 mL microcentrifuge tube. Then, add 15.25 µL $CaCl_2$ dropwise to the DNA mix.

4.1.1.3. Add DNA/$CaCl_2$ mixture to a separate tube containing 125 µL 2x HEPES buffered saline (HBS, pH 7.0) in a dropwise manner with mixing.

4.1.1.4. Slowly add the DNA/$CaCl_2$/HBS mix to cells. After 2–4 h, replace the media and incubate overnight. Proceed with labeling from steps 1.3–2.8.

4.1.2. Prepare equal volumes of 500 µg protein in lysis buffer.

4.1.3. Perform IP by incubating with rabbit anti-GFP for ctHTT-GFP rotating overnight at 4 °C.

4.1.4. Add 15–20 µL magnetic beads pre-equilibrated with 0.1% SDS-RIPA to each tube and allow it to react end-over-end at 4 °C for 3 h.

4.1.5. Wash the beads with lysis buffer and resuspend in 45 µL 1% SDS 50 mM HEPES buffer.

4.1.6. Heat the beads at 80 °C for 15 min and invert the tubes or agitate approximately every 5 min. Briefly spin the tubes and return to 80 °C.

4.1.7. Collect the supernatant containing the proteins while the samples are still warm.

NOTE: The protocol can be paused here and the immunoprecipitated samples can be stored at -20 °C or -80 °C, if not used for click chemistry right away.

4.1.8. Allow 43 µL of the supernatant to react with 7 µL master mix of click reagents.

4.1.9. Proceed with the reaction as stated in step 3.2.3.

5. SDS-PAGE and western blotting

5.1. Stop the reaction and denature by adding 1x sample loading buffer containing 25 mM dithiothreitol (DTT) and heat at 95 °C for 5 min.

NOTE: Up to 100 mM DTT can be used[28]. Do not use β-mercaptoethanol as it will hydrolyze the thioester bond and remove the clicked fatty acid analog.

5.2. Briefly spin down the samples.

5.3. Separate the proteins with SDS-PAGE on a polyacrylamide gel, in duplicates.

NOTE: Two gels are required for the alkaline treatment with KOH to confirm the presence of thioester bonds. If using a fluorescent azide probe, acylation can be detected in-gel or after

transfer using the indicated excitation channel.

5.4. Transfer to polyvinylidene fluoride (PVDF) membranes (activated in methanol and rinsed in ddH_2O – 5 min each) with semi-dry transfer apparatus at 25 V, 1.0 A (constant) for 30 min.

5.5. After briefly rinsing the membranes with ddH_2O, soak one replicate membrane in 0.1 M KOH in methanol/water (9:1 v/v) and the other in 0.1 M Tris-HCl pH 7.0 in methanol/water (9:1 v/v) as a non-alkaline control for 60 min at room temperature with gentle rocking.

5.6. Rinse the membranes briefly in ddH2O water, followed by 6 times of 5 min washes in PBS-T (1x PBS, 0.1% Tween 20).

NOTE: Wash thoroughly.

5.7. Block the membranes overnight in 5% skim milk blocking buffer (1x PBS, 0.1% Tween20).

6. Perform western blot

6.1. Where indicated, probe with fluorescently tagged Streptavidin (1:5000) and loading control, anti-GAPDH rhodamine (1:5000) in 5% BSA blocking buffer (0.01% SDS, 1x PBS, 0.1% Tween20) at room temperature for 45–60 min with gentle rocking, in darkness.

6.1.1. Probe the immunoprecipitated protein blot first with primary anti-GFP antibody in 5% skim milk blocking buffer, then with appropriate secondary antibodies in 5% BSA as in step 6.1.

6.2. Wash the membranes for 5 min in PBS-T (1x PBS, 0.1% Tween20) a total of four repeats and rinse with ddH_2O water before imaging.

REPRESENTATIVE RESULTS:

The difference in labeling efficiency between saponified and non-saponified (non-sap) alkynyl fatty acids for click chemistry detection can be visualized and compared by the signal intensity of fatty acylated proteins through western blot (**Figure 1**). A noticeable effect was observed with increasing length of the acyl chains. In cells labeled with alkynyl-stearate (alk-stear), saponification of the fatty acid and delivery with BSA for metabolic labeling drastically increased the detection of S-acylated protein signal through click chemistry and detection by a fluorescent azido probe (**Figure 1**, right), suggesting an overall increase in cellular incorporation of the alkynyl fatty acid label. Conversely, no noticeable difference was observed in cells treated with the shortest and most soluble fatty acid, alkynyl-myristate (alk-myr; 13-tetradecynoic acid or 13-TDYA). Cells labeled with alkynyl-palmitate (**Figure 1**, middle) showed an intermediate increase in label compared to alkynyl-myristate (13-TDYA), but less than alkynyl-stearate.

Importantly, treatment of PVDF membranes with 0.1 M KOH largely removed the fatty acid labels from cells incubated with alkynyl-palmitate and alkynyl-stearate, confirming that the majority of the signal was through an ester or thioester bond (**Figure 1**, middle and right, bottom panels). As

expected, the incorporation of alkynyl-myristate was mostly alkali-resistant (**Figure 1**, left, bottom panels), due to the attachment of myristate to proteins through an amide bond.

Figure 2 demonstrates the versatility and sensitivity of click chemistry to detect fatty acylation of immunoprecipitated proteins. HEK293T cells were transfected with myristoylated C-terminal huntingtin (HTT) fused to GFP (myr-ctHTT-GFP) and labeled with alkynyl-myristate, as previously described[18]. Following immunoprecipitation, ctHTT-GFP was released from the beads and subjected to click chemistry alongside the lysates. Not only was myristoylation of wildtype (WT) myr-ctHTT-GFP detected in the immunoprecipitates, but it was strongly detected in the lysates, whereas the G2A mutation completely blocked myristoylation of the ctHTT-GFP (**Figure 2**).

TABLE AND FIGURE LEGENDS:

Table 1: Saponified fatty acid labeling ratios. Experimental volumes of fatty acid, KOH, and FAFBSA for the saponification of fatty acid label according to the volume of media used.

Table 2: Click reagent and protein volume ratios. Experimental volumes of the click chemistry reagents and corresponding stock concentrations, in addition to the volumes of protein samples.

Figure 1: Detection of fatty acylated proteins using click chemistry. HEK293T cells were incubated with the indicated fatty acids directly (non-sap) or following saponification (sap) and incubation with the carrier protein BSA, as described in protocol 2.1–2.7. Alkynyl-fatty acids were linked to fluorescent azide by subjecting 100 µg of protein lysates to click chemistry, separated by SDS-PAGE, and transferred to PVDF membranes. After treatment with 0.1 M Tris pH 7.0 or 0.1 M KOH, to reverse thioester bonds, fatty acylation was detected using a fluorescent azide. GAPDH was used as a loading control; anti-GAPDH rhodamine (1:5,000). Alk-myr = 13-TDYA, alk-pal = 15-HDYA, alk-ste = 17-ODYA.

Figure 2: Detection of N-myristoylated ctHTT-GFP using click chemistry. HEK293T cells were transfected with C-terminally truncated (ct) and myristoylatable form of HTT (myr-ctHTT-GFP) and labeled with alkynyl-myristate. A non-myristoylatable form with the essential glycine replaced with an alanine was included (G2A). Following harvesting and lysis, ctHTT-GFP was immunoprecipitated using goat anti-GFP. Lysates were subjected to click chemistry reaction (left) as well as immunoprecipitates following release from the beads. Myristoylation was detected using Streptavidin Alexa 680 (SA680). GFP was detected using rabbit anti-GFP in combination with anti-rabbit Alexa 488.

Figure 3: Workflow scheme of experimental protocol. Workflow outline of the main experimental steps in the protocol. Several points are noted where the protocol may be paused.

DISCUSSION:

Direct addition of fatty acids to cells in culture can result in insolubility, precipitation of lipids, and lipotoxicity[40]. Consequently, adding fatty acids directly to cells may not only result in poor cellular uptake and low availability of the fatty acid label, but also a decreased number of viable

cells for downstream analysis, as well as activation of off-target pathways. However, many metabolic labeling protocols for click chemistry detection involve direct addition of fatty acids[25,28,42,43]. In addition, a large number of palmitoyl-proteome studies using click chemistry detection to date seldom saponify the fatty acids labels or incubate them with BSA[36]. It is important to consider the fact that the efficiency and sensitivity of click chemistry detection of fatty-acylated proteins are dependent on sufficient cellular uptake of the fatty acid analogs. Therefore, it is reasonable to speculate that many S-acylated proteins may have escaped detection in proteomic studies due to a low availability of the fatty acid labels from poor incorporation into the cells, especially when the longer chain fatty acid 17-ODYA was used. 17-ODYA, or alkynyl-stearate, has been the widely used label of choice for several studies due to its commercial availability and its early use[8,36]. However, the results of this protocol demonstrate that saponification of 17-ODYA results in the largest increase in the detection of S-acylated proteins, in comparison to shorter chain fatty acids such as palmitate or myristate. Therefore, repeating these experiments with saponified labels may yield additional substrates of S-acylation that may have been previously overlooked. Further, while most palmitoyl acyltransferases prefer palmitate for S-acylation, some do have preferences for other lengths of fatty acids such as stearate[15,38,44]. In addition, some proteins or even specific sites within proteins prefer one fatty acid over another[15,45]. Therefore, studies using 17-ODYA may have a bias towards proteins S-acylated with stearate, not palmitate, while also under-representing those proteins due to lower detection.

The improved metabolic labeling efficiency for click chemistry relies on the saponification of lipids and incubation with FAFBSA steps, as well as the delipidated FBS. All fatty acids must be completely saponified in KOH with no visible solids remaining before proceeding with incubation with FAFBSA. This can be a difficult step and timing is crucial. After the saponified fatty acids have gone into solution at 65 °C, add warm BSA immediately as further heating will cause evaporation of the DMSO from the fatty acids. In addition, the saponified label will begin to re-solidify as soon as it begins to cool. Therefore, the FAFBSA must be warm and added rapidly after the salt has become soluble. The glass reaction vials, and their shape are important for this step. They allow the saponified lipid to be warm enough to remain soluble, while cool enough to ensure that the FAFBSA does not congeal. Sufficient mixing via pipetting is also important at this step to ensure a homogenous solution for labeling.

The reagents for click chemistry need to be properly stored, typically with desiccants or under N_2 or Ar gas at -20 °C to -80 °C. Lack of acylation signal or weak signal may be due to unstable reagents, particularly older TBTA and azide stock solutions. Furthermore, care must be taken with fluorescent azide stock solutions, which need to be protected from light as much as possible. In addition, variables such as method of lipid deprivation and labeling time may need to be tested to determine the optimal conditions depending on the type of cell used. For example, neuronal cells may need longer labeling times because media changes and lipid deprivation are difficult (unpublished).

The benefit of this protocol is most dramatic when used for longer-chain fatty acids. For shorter chains, the increase in signal intensity becomes less dramatic, but is still likely protective to the

cells. While the suggested modifications will improve protein acylation detection in general, click chemistry is still considered a lipid-centric method[1] that is limited to detection of dynamically, not stably S-acylated proteins[1]. Other limitations to consider include the requirement of fatty acid deprivation to promote labeling, and its relatively limited range of compatible cell types when compared to the acyl-biotin exchange (ABE) detection of S-acylation[16]. Despite these limitations, click chemistry detection is quicker than most acyl-exchange assays and is better suited for detection of proteins, which do not tolerate the repeated protein precipitation steps required for acyl-exchange assays. In addition, this approach can be combined with simultaneous labeling using other click assays such as pulse-chase analysis[38].

The use of this modification for metabolic labeling for click chemistry increased overall detection of acylated proteins, particularly S-acylated, with a variety of proteomic techniques that are used in conjunction with click chemistry. As shown, fluorogenic detection can be used as an alternative to biotin (**Figure 1**)[28]. This is particularly useful because there are no endogenously fluorescent proteins in the cell lysates. In addition, fluorophores that are only activated after click chemistry can be used[46]. The saponified and FAFBSA-bound fatty acid for click chemistry labeling can help with difficulties detecting proteins of interest due to an overall increase in the amount of label available in the cell and by limiting toxic effects of adding fatty acids directly to the media. It can also be utilized in conjunction with mass spectrometry[27] to increase detection of low abundance proteins, especially when taken together with the recent advancement using machine-learning algorithms preventing redundant measurements to increase sensitivity to low abundance proteins, as opposed to existing data-dependent acquisition that favors detection of the most abundant proteins[47]. In addition, click chemistry can be combined with stable isotope labeling with amino acids in cell culture (SILAC) and pulse-chase methods to produce quantifiable data on dynamic protein S-acylation[27]. Finally, the Hannoush group has combined click chemistry with proximity ligation assay (PLA) to allow single-cell visualization and examinations into subcellular distribution of palmitoylated proteins[43,48].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Zaballa, M. E., Goot, F. G. van der. The molecular era of protein S-acylation: spotlight on structure, mechanisms, and dynamics. *Critical Reviews in Biochemistry and Molecular Biology*. **53** (4), 1–31 (2018).
2. Martin, D. D. O., Beauchamp, E., Berthiaume, L. G. Post-translational myristoylation: Fat matters in cellular life and death. *Biochimie*. **93** (1), 18–31 (2011).
3. Hallak, H. et al. Covalent binding of arachidonate to G protein alpha subunits of human platelets. *The Journal of Biological Chemistry*. **269** (7), 4713–4716 (1994).
4. O'Brien, P. J., Zatz, M. Acylation of bovine rhodopsin by [3H]palmitic acid. *The Journal of Biological Chemistry*. **259** (8), 5054–5057 (1984).
5. Liang, X. et al. Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction. *Journal of Biological Chemistry*. **276** (33), 30987–30994 (2001).
6. Thinon, E., Percher, A., Hang, H. C. Bioorthogonal chemical reporters for monitoring unsaturated fatty-acylated proteins. *ChemBioChem*. **17** (19), 1800–1803 (2016).
7. Veit, M., Reverey, H., Schmidt, M. F. G. Cytoplasmic tail length influences fatty acid selection for acylation of viral glycoproteins. *Biochemical Journal*. **318** (1), 163–172 (1996).
8. Sanders, S. S. et al. Curation of the mammalian palmitoylome indicates a pivotal role for palmitoylation in diseases and disorders of the nervous system and cancers. *PLOS Computational Biology*. **11** (8), e1004405 (2015).
9. Hannoush, R. N. Synthetic protein lipidation. *Current Opinion in Chemical Biology*. **28**, 39–46 (2015).
10. Martin, D. D. O., Hayden, M. R. Post-translational myristoylation at the cross roads of cell death, autophagy and neurodegeneration. *Biochemical Society Transactions*. **43** (2), 229–234 (2015).
11. Young, F. B., Butland, S. L., Sanders, S. S., Sutton, L. M., Hayden, M. R. Putting proteins in their place: Palmitoylation in Huntington disease and other neuropsychiatric diseases. *Progress in Neurobiology*. **97** (2), 220–238 (2012).
12. Hansen, A. L., Mukai, K., Schopfer, F. J., Taguchi, T., Holm, C. K. Sting palmitoylation as a therapeutic target. *Cellular & Molecular Immunology*. **16** (3), 236–241 (2019).
13. Veit, M. Palmitoylation of virus proteins. *Biology of the Cell*. **104** (9), 493–515 (2012).
14. Jiang, H. et al. Protein lipidation: Occurrence, mechanisms, biological functions, and enabling technologies. *Chemical Reviews*. **118** (3), 919–988 (2018).
15. Greaves, J. et al. Molecular basis of fatty acid selectivity in the zDHHC family of S-acyltransferases revealed by click chemistry. *Proceedings of the National Academy of Sciences of the United States of America*. **114** (8), E1365–E1374 (2017).
16. Gao, X., Hannoush, R. N. A decade of click chemistry in protein palmitoylation: Impact on discovery and new biology. *Cell Chemical Biology*. **25** (3), 236–246 (2018).
17. Tsutsumi, R., Fukata, Y., Fukata, M. Discovery of protein-palmitoylating enzymes. *Pflügers Archiv - European Journal of Physiology*. **456** (6), 1199–1206 (2008).
18. Roth, A. F., Feng, Y., Chen, L., Davis, N. G. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *The Journal of Cell Biology*. **159** (1), 23–28 (2002).
19. Lobo, S., Greentree, W. K., Linder, M. E., Deschenes, R. J. Identification of a Ras Palmitoyltransferase in Saccharomyces cerevisiae. *Journal of Biological Chemistry*. **277** (43), 41268–41273 (2002).

20. Ohno, Y., Kihara, A., Sano, T., Igarashi, Y. Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. **1761** (4), 474–483 (2006).
21. Huang, K. et al. Huntingtin-interacting protein HIP14 Is a palmitoyl transferase involved in palmitoylation and trafficking of multiple neuronal proteins. *Neuron*. **44** (6), 977–986 (2004).
22. Drisdel, R. C., Green, W. N. Labeling and quantifying sites of protein palmitoylation. *BioTechniques*. **36** (2), 276–285 (2004).
23. Roth, A. F., Wan, J., Green, W. N., Yates, J. R., Davis, N. G. Proteomic identification of palmitoylated proteins. *Methods*. **40** (2), 135–142 (2006).
24. Wan, J., Roth, A. F., Bailey, A. O., Davis, N. G. Palmitoylated proteins: purification and identification. *Nature Protocols*. **2** (7), 1573–1584 (2007).
25. Martin, D. D. O. et al. Rapid detection, discovery, and identification of post-translationally myristoylated proteins during apoptosis using a bio-orthogonal azidomyristate analog. *The FASEB Journal*. **22** (3), 797–806 (2007).
26. Kostiuk, M. A. et al. Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. *The FASEB Journal*. **22** (3), 721–732 (2008).
27. Martin, B. R., Wang, C., Adibekian, A., Tully, S. E., Cravatt, B. F. Global profiling of dynamic protein palmitoylation. *Nature Methods*. **9** (1), 84–9 (2012).
28. Yap, M. C. et al. Rapid and selective detection of fatty acylated proteins using ω-alkynyl-fatty acids and click chemistry. *Journal of Lipid Research*. **51** (6), 1566–1580 (2010).
29. Hang, H. C., Wilson, J. P., Charron, G. Bioorthogonal chemical reporters for analyzing protein lipidation and lipid trafficking. *Accounts of Chemical Research*. **44** (9), 699–708 (2011).
30. Thinon, E. et al. Global profiling of co- and post-translationally N-myristoylated proteomes in human cells. *Nature Communications*. **5** (1), 4919, (2014).
31. Charron, G., Wilson, J., Hang, H. C. Chemical tools for understanding protein lipidation in eukaryotes. *Current Opinion in Chemical Biology*. **13** (4), 382–391 (2009).
32. Hang, H. C. et al. Chemical probes for the rapid detection of fatty-acylated proteins in mammalian cells. *Journal of the American Chemical Society*. **129** (10), 2744–2745 (2007).
33. Heal, W. P. et al. Site-specific N-terminal labelling of proteins in vitro and in vivo using N-myristoyl transferase and bioorthogonal ligation chemistry. *Chemical Communications*. **0** (4), 480–482 (2007).
34. Forrester, M. T. et al. Site-specific analysis of protein S-acylation by resin-assisted capture. *Journal of Lipid Research*. **52** (2), 393–398 (2011).
35. Brigidi, G. S., Bamji, S. X. Detection of protein palmitoylation in cultured hippocampal neurons by immunoprecipitation and acyl-biotin exchange (ABE). *Journal of Visualized Experiments: JoVE*. **72**, 50031 (2013).
36. Blanc, M. et al. SwissPalm: Protein palmitoylation database. *F1000Research*. **4**, 261 (2015).
37. Heal, W. P., Wickramasinghe, S. R., Leatherbarrow, R. J., Tate, E. W. N -Myristoyl transferase-mediated protein labelling in vivo. *Organic & Biomolecular Chemistry*. **6** (13), 2308–2315 (2008).
38. Lin, D. T. S., Conibear, E. ABHD17 proteins are novel protein depalmitoylases that regulate N-Ras palmitate turnover and subcellular localization. *eLife*. **4**, e11306 (2015).
39. Charron, G. et al. Robust fluorescent detection of protein fatty-acylation with chemical

reporters. *Journal of the American Chemical Society*. **131** (13), 4967–4975 (2009).
40. Alsabeeh, N., Chausse, B., Kakimoto, P. A., Kowaltowski, A. J., Shirihai, O. Cell culture models of fatty acid overload: Problems and solutions. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. **1863** (2), 143–151 (2018).
41. Jordan, M., Schallhorn, A., Wurm, F. M. Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Research*. **24** (4), 596–601 (1996).
42. Martin, B. R., Cravatt, B. F. Large-scale profiling of protein palmitoylation in mammalian cells. *Nature Methods*. **6** (2), 135–138 (2009).
43. Gao, X., Hannoush, R. N. Single-cell in situ imaging of palmitoylation in fatty-acylated proteins. *Nature Protocols*. **9** (11), 2607–2623 (2014).
44. Muszbek, L., Haramura, G., Cluette-Brown, J. E., Cott, E. M. V., Laposata, M. The pool of fatty acids covalently bound to platelet proteins by thioester linkages can be altered by exogenously supplied fatty acids. *Lipids*. **34** (S1), S331–S337 (1999).
45. Brett, K. et al. Site-specific S-Acylation of influenza virus hemagglutinin. The location of the acylation site relative to the membrane border is the decisive factor for attachment of stearate. *Journal of Biological Chemistry*. **289** (50), 34978–34989 (2014).
46. Shieh, P. et al. CalFluors: A universal motif for fluorogenic azide probes across the visible spectrum. *Journal of the American Chemical Society*. **137** (22), 7145–7151 (2015).
47. Pelletier, A. R. et al. MealTime-MS: A machine learning-guided real-time mass spectrometry analysis for protein identification and efficient dynamic exclusion. *bioRxiv*. 110726 (2020).
48. Gao, X., Hannoush, R. N. Method for cellular imaging of palmitoylated proteins with clickable probes and proximity ligation applied to Hedgehog, tubulin, and Ras. *Journal of the American Chemical Society*. **136** (12), 4544–4550 (2014).

Figure 1

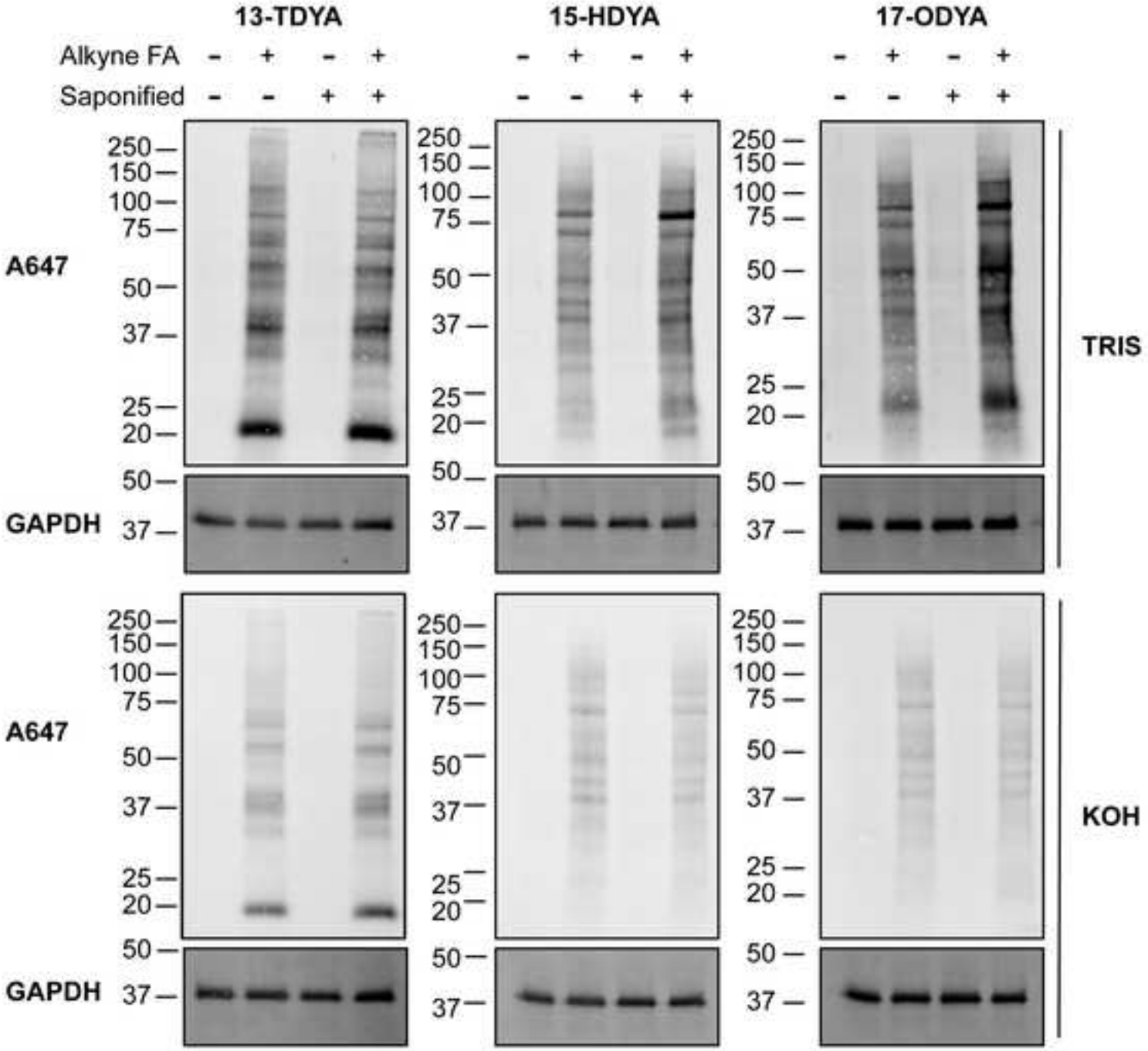

Figure 2

Click here to access/download;Figure;20210217 Figure 2 - Liao et al-01.tif

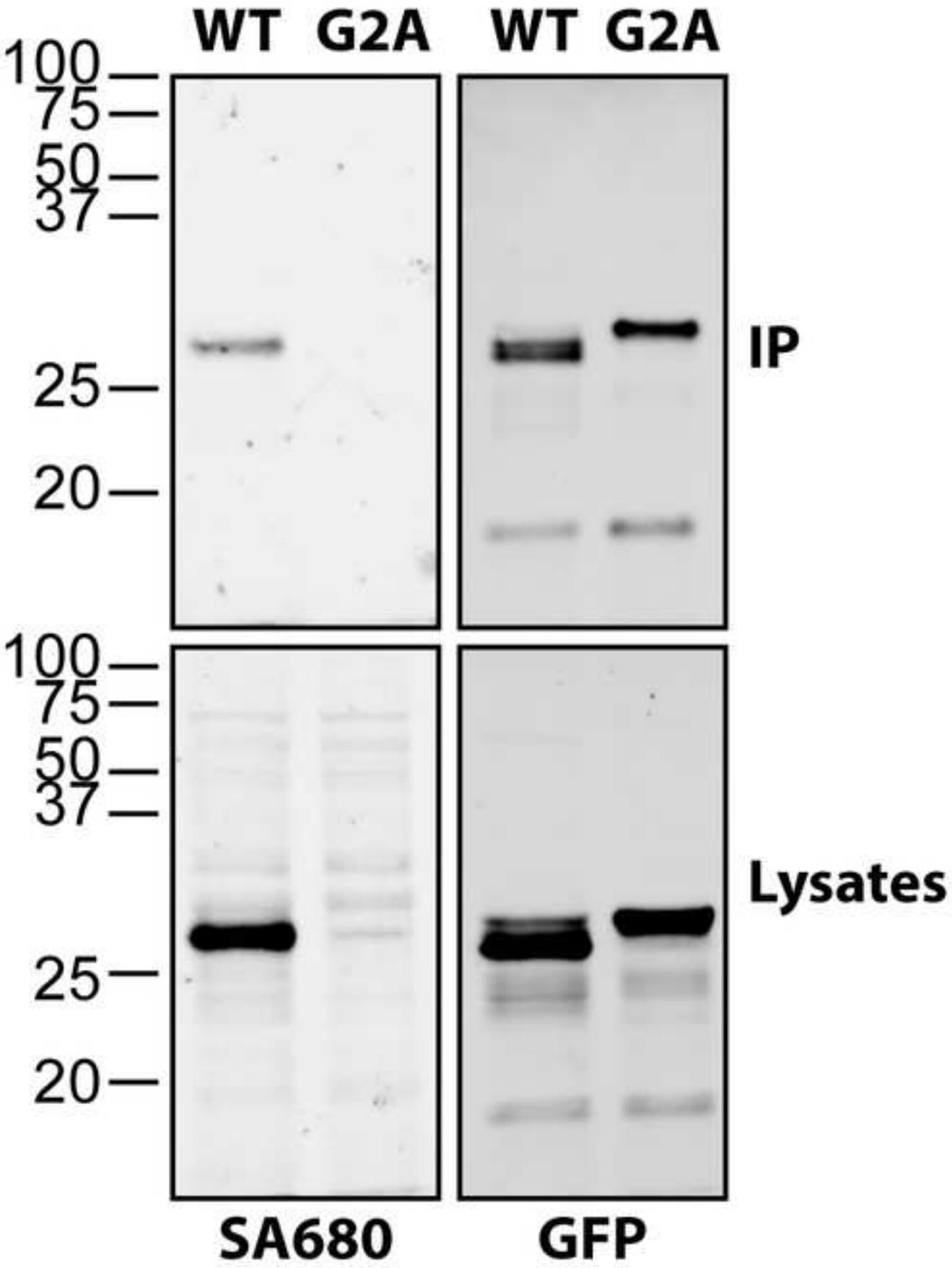

Figure 3

Click here to access/download;Figure;20210221 Figure 3 - Liao et al.pdf

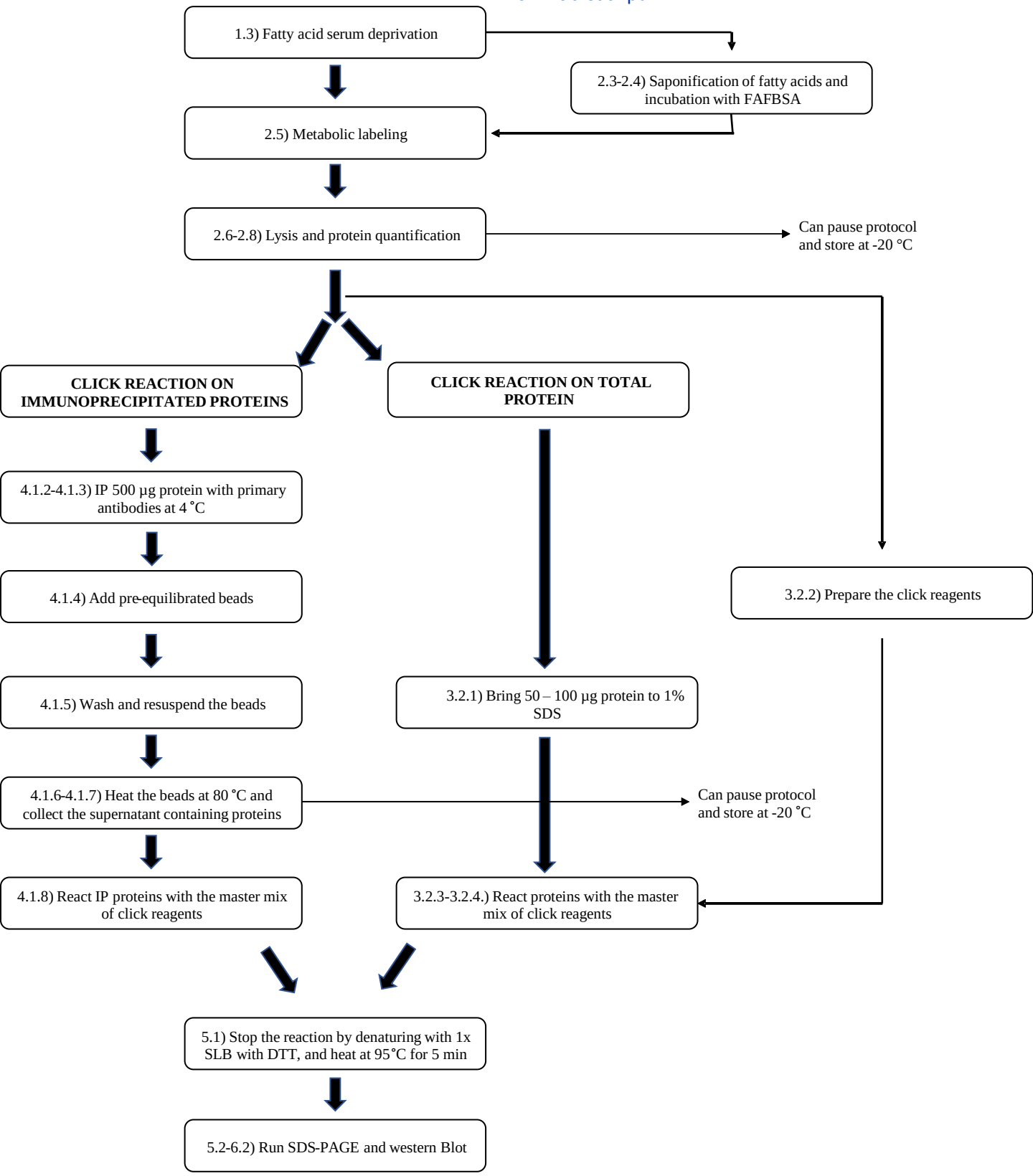

Table 1 Saponified fatty acid labeling ratios

Total media volume (mL)	Vol. fatty acid or fatty acid analog (µL)	Vol. KOH (µL)	Vol. 20% FAFBSA (µL)	Total vol. of BSA-conjugated saponified label (µL)
4	4	4	200	208
2	2	2	100	104

Table 2 Click reagent and protein volume ratios

Total reaction vol (µL)	Vol. protein (µL)	Vol. TBTA (2 mM) (µL)	Vol. CuSO4 (50 mM) (µL)	Vol. TCEP (50 mM) (µL)	Vol. azido probe (2 mM) (µL)
50	43	2.5	1	1	2.5
100	86	5	2	2	5

Table of Materials

Click here to access/download;Table of Materials;20210222 Updated Jove Materials - Liao et al.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
13-tetradecynoic acid (alkynyl myristic acid) (25mM)	Click Chemistry Tools	1164	
15-hexadecynoic acid (alkynyl palmitic acid) (100mM)	Click Chemistry Tools	1165	
17-octadecynoic acid (alkynyl stearic acid) (100 mM)	Cayman Chemical Company	90270	
30% acrylamide/bis solution 29:1	Biorad	1610156	
96-well plate reader	Biorad	N/A	
AFDye 647 azide plus	Click Chemistry Tools	1482	
Ammonium persulfate (APS)	Biorad	1610700	
Anti-GAPDH hFAB Rhodamine	Biorad	12004167	
Anti-rabbit Alexa 488	Invitrogen	A11034	
Anti-Tubulin hFAB Rhodamine	Biorad	12004166	
Biotin Azide	Click Chemistry Tools	1265	
Bis-tris, ultrapure	VWR	715	
Calcium chloride	J.T. Baker	1332-1	
Centrifuge 16,000xg, 4°C	Thermo Scientific	N/A	
Charcoal STRP FBS One Shot (DCC-FBS)	Life Technologies	A3382101	
ChemiDoc Imager	Biorad	N/A	
Copper sulfate (1 mM)	VWR	BDH9312	
Deoxycholic acid sodium salt monohydrate	MP Biomedicals	102906	
Detergent compatible (DC) assay	Biorad	N/A	
Dimethyl sulfoxide (DMSO)	VWR	0231-500 mL	
DMEM, 1x	Wisent Inc	319015CL	
Ethanol, anhydrous	N/A	N/A	
Fatty Acid Free BSA	MP Biomedicals	219989950	
Fast Blot Turbo Semi-dry transfer	Biorad	N/A	
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	12483-020	
FluoroTrans W PVDF (polyvinylidene fluoride) transfer membrane	Pall Life Sciences	BSP0161	
HEPES (4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic) acid	VWR	5011	
Humidified Incubator at 37°C and 5% CO2	VWR	N/A	
Igepal CA-630	Alfa Aesar	J61055	
Image Lab Software	Biorad	N/A	
L-glutamine supplement solution	Wisent Inc	609-065-EL	
Magnesium chloride	Fisher Scientific	BP214-500	
Methanol	VWR	BDH1135	
Myristic Acid (25 mM)	VWR	M0476-25G	
Palmitic acid (100 mM)	VWR	P0002-25G	
Penicillin-Streptomycin, 10x	Wisent Inc	450201EL	
Pepstatin A (synthetic)	Enzo Life Sciences	ALX-260-085-M005	
Phenylmethylsulfonyl fluoride	Enzo Life Sciences	ALX-270-184-G005	
Phosphate buffered saline, 10x, pH 7.4	VWR	75801-000	
Polyclonal Goat antibody to GFP (Affinity Purified)	Eusera	EU4	
FluoroTrans W PVDF (polyvinylidene fluoride) transfer membrane	Pall Life Sciences	BSP0161	
Potassium hydroxide	Ward's Science	470302-100	
Rabbit polyclonal antibody to GFP	Eusera	EU1	
Sodium chloride	VWR	0241-1KG	
Sodium Dodecyl Sulfate (SDS)	Fisher Scientific	BP166-500	
Sodium pyruvate	Wisent Inc	600-110-EL	
Streptavidin Alexa Fluor 680 conjugate	Thermo Fisher Scientific	S21378	
Tris-(benzyltriazolylmethyl)amine (TBTA) (100 uM)	Click Chemistry Tools	1061	
Tris-(2-carboxyethyl)phosphine HCl (TCEP) (1mM)	Soltec Ventures	M115	
Tris Base	Fisher Scientific	BP152-5	
Trypsin/EDTA	Wisent Inc	325-043-CL	
Tween 20 Reagent Grade 1L	VWR	97062-332	
WHEATON NextGen V Vials 3 mL	VWR	89085-424	

Editorial comments:

Changes to be made by the Author(s):

Responses are in italics:

We would like to thank the editors for the opportunity to resubmit and thank the reviewers for their time and help with the manuscript. We feel that the revisions have made this a stronger manuscript and we hope that it meets the needs of the journal.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript and corrected any issues to the best of our knowledge.

2. Please revise the following lines to avoid overlap with previously published work: 240 (To enhance…)-244, 260 (tracking…)-270 (…calculation)

Apologies, but we are not sure what this is referring to. There are no such phrases at the indicated lines or generally within the manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: NextGen V, AFDue 647 Azide Plus (Click Chem Tools) etc

We believe we have removed all commercial language from the main manuscript.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have edited the manuscript to be more direct in all aspects.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol

action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added additional details throughout. We hope this is satisfactory.

6. Please consider providing reaction set-ups and solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

Reaction set-ups are provided in Tables 1 and 2.

7. With a one line space between each protocol step, highlight only up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Completed.

8. Please include table legends (captions): title and description in the Figure and Table Legends section after the representative results section.

Completed.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes an optimized method to increase the detection of fatty acylated proteins. This method includes the use of saponified fatty acids in combination with fatty-acid free BSA, as well as delipidated media to overcome issues of poor solubility and potential toxic effects of adding long chain fatty acids to cell culture. This protocol will be of great use to researchers studying palmitoylation of proteins both in vitro and in vivo and is particularly useful due to its ability to be used with other methods including mass spectrometry. The protocol is outlined in depth with clear instructions and a list of materials to ensure greatest success.

Major Concerns:

None

Minor Concerns:

It may be useful to provide a list of common issues that may occur and means to troubleshoot these common issues.

Thank you for your time and suggestions. We have added more "Notes" to areas that require special care. We have also included a section in the Discussion (lines 450-466) that include more details.

Reviewer #2:

Manuscript Summary:

This study developed a method for efficient delivery of several alkynyl fatty acid analogs into the cultured cells with the aid of saponification and FAFBSA, followed by conjugation with several fluorescent tag via click chemistry. The overall enhancement of the analytical sensitivity has been obtained, demonstrating a practical value of this method. The method is interesting and could conceivably be very useful for specific biochemical projects that follow protein acylation. The manuscript would benefit substantially from additional experiments to strengthen the statements and potential uses of the method.

We would like to thank the reviewer for taking the time to consider the manuscript and for the helpful feedback. We would like to clarify that this is not a new method. This is meant to provide a visual guide for researchers to follow the methods previously described in Yap et al., JLR, 2010 using commercially available products. Dr. Martin helped develop this method, which was a modification of one of the first papers describing bio-orthogonal detection of fatty acylation (Martin et al., FASEBJ, 2008). Many of the valid concerns raised by the reviewer have been addressed in Yap et al. and in detail by several other labs when click chemistry for acylation was first described (Ploegh, Cravatt, Berthiaume, and Tate) and in the past decade (Reviews from the labs of L. Berthiaume, R. Hannoush, and G. van der Goot). The Azido-fatty acid analogs (not

used in this study) have also been well characterized in Martin et al 2008 (FASEBJ), Hang et al 2008 (JACS) and Heal et al (Tate Lab).

As such, we have made changes throughout the manuscript to clarify that this manuscript is to help visualise the method from Yap et al (2010) and the following citations have been added:

H.C. Hang, E.J. Geutjes, G. Grotenbreg, A.M. Pollington, M.J. Bijlmakers, H.L. Ploegh, Chemical probes for the rapid detection of Fatty-acylated proteins in Mammalian cells, J. Am. Chem. Soc. 129 (2007) 2744e2745.

W.P. Heal, S.R. Wickramasinghe, P.W. Bowyer, A.A. Holder, D.F. Smith, R.J. Leatherbarrow, E.W. Tate, Site-specific N-terminal labelling of proteins in vitro and in vivo using N-myristoyl transferase and bioorthogonal ligation chemistry, Chem. Commun. (Camb.) (2008) 480e482.

W.P. Heal, S.R. Wickramasinghe, R.J. Leatherbarrow, E.W. Tate, N-Myristoyl transferase-mediated protein labelling in vivo, Org. Biomol. Chem. 6 (2008) 2308e2315.

G. Charron, M.M. Zhang, J.S. Yount, J. Wilson, A.S. Raghavan, E. Shamir, H.C. Hang, Robust fluorescent detection of protein fatty-acylation with chemical reporters, J. Am. Chem. Soc. 131 (2009) 4967e4975.

G. Charron, J. Wilson, H.C. Hang, Chemical tools for understanding protein lipidation in eukaryotes, Curr. Opin. Chem. Biol. 13 (2009) 382e391.

Major Concerns:
The saponified fatty acids used in this study are different from their intact fatty acids at their carboxyl groups with different dissociation states. Therefore I would ask whether or not this difference actually affects the acylation process catalyzed by the relevant enzymes?
The authors provided some representative data in the manuscript to show the improvement of the sensitivity. I'd like to know whether or not this improvement is dose/reagent/time dependent? It would be very informative if the data from additional experiments with different conditions are provided, such as different concentration of FAFBSA, different reaction time for saponification, etc.

These are valid concerns and have been addressed in earlier publications (described above). Many of these conditions were empirically determined for Yap et al 2010 JLR. However, we would like to clarify that it is not the carboxyl groups that are altered. The alkyne groups are at the omega site, or the distal end of the fatty acids. In addition, we found in Yap et al and indicate on Page 5 line 232-233 that although 3-4 hours appears to be ideal across the literature, timing and concentration may need to be determined experimentally for new cell lines or proteins of interest. In particular, cells with slower metabolisms or proteins with slower S-acylation turnover may require longer labeling times. However, it is advised not to label too

long as the fatty acids will undergo beta oxidation or potentially elongation and could be incorporated into lipids or catabolites (lines 233-235) and cited in Yap et al.

In page 5, line 221, "For non-saponified controls, pipette 2 µL (or equivalent to volume saponified) of unlabeled fatty acid directly onto the starvation media." I wonder why non-saponified fatty acid is DIRECTLY added into the media without the use of FAFBSA? Even though the overall increase in sensitivity of detecting S-acylated proteins can be obtained by using the combination of saponification and FAFBSA, more solid experimental evidence should be provided to exploit which factor, saponification or FAFBSA, or both of them, contribute to the improvement of sensitivity.

We understand that referring to these as controls may have caused confusion. The purpose for adding this way was to demonstrate how less effective adding lipids directly to the media is, particularly with the longer fatty acids like stearate. This can be seen in Figure 1, which is considered a representative result for the method. Directly adding fatty acids is often the approach used by novices or researchers not familiar with lipid labeling, but this has been shown to be toxic, as referenced in Alsabeeh et al 2018*. More details about saponication can be found in* Yap et al (JLR)*. Consequently, we now refer to these as non-saponified comparison samples.*

Minor Concerns:
In page 2, line 106, the phrase "rather than 15-HDYA for detecting S-acylated cells due to the" should be modified. "S-acylated cells" should be better changed into "S-acylated proteins".

This has been corrected. Thank you for bringing this to our attention.

In page 4, line 197, the phrase "may require longer incubation to be incorporated into the KOH." is very confusing and should be modified.

*Beginning on line 192 the following has been added. "*NOTE: Fatty acids with a higher number of carbons and decreased solubility, such as stearate (17-ODYA), may require longer incubation times to be fully incorporated into the KOH at 65 °C. Raise the temperature to 70 °C if needed."

In Fig.1, I wonder why there is no sample loading of the negative controls (without addition of alkyne-FA) in experiments using Alkynyl-stearate (right, Figure 1)?

Unlabeled fatty acids were included only to demonstrate the levels of endogenously biotinylated proteins. Because stearate and palmitate undergo S-acylation through the same thioester bond, and the click chemistry is well established to be highly specific between azido and alkyne groups, the unlabeled palmitate was used as a control and comparison to unlabeled lysates for all S-acylation only to demonstrate how unlabeled lysates should appear. It is the same for the unlabeled myristate. That said, we have replaced Figure 1 with a clearer representation of the method using a fluorescent azide probe for detection and negative controls for all fatty acids. We have removed the inhibitors to simplify the interpretation and because, the field of S-acylation is moving away from the use of 2-bromopalmitate due to its lack of specificity.

In page 10, line 472, "The saponified and FAFBSA binding for fatty acid labeling for click chemistry can help with difficulties detecting" contains grammatical error and should be modified. Actually, the manuscript should be checked for these similar typos throughout the text.

Thank you. We hope we have addressed any and all typos.

Reviewer #3:
Manuscript Summary:
This manuscript by Liao, Gray, and MArtin describes an optimizer protocol for assaying fatty acylation of target proteins using click chemistry. This protocol is important to the field as some other methods to detect protein acylation have become challenging due to lack of availability of key reagents. The protocol is well-described and easy to read. Sufficient details are provided. Several steps in the protocol have proven difficult to follow based only on written descriptions, thus this protocol is particularly well suited for publication in JoVE, with the corresponding video. The complete description of reagent storage conditions and troublesome steps is excellent.

Thank you for taking the time to review our manuscript. We hope this will help others in the field.

Major Concerns:
None

Minor Concerns:
1. The cell density for HEK293 cells seems high (5x10^6 cells/6 well plates well). Should that be 10^5?

Corrected. Thank you for identifying this.

2. Western blot should be lower case, unless at the start of a sentence.

Thank you for highlighting this typo. It has been corrected throughout.

3. It would be useful to show more of the gel in Figure 2 to demonstrate the specificity of these blots. In addition, inclusion of more than one molecular weight marker would be useful.

The total gels are now shown.

Reviewer #4:
Manuscript Summary:

The authors have described a protocol for the saponification of bioorthoganol fatty acid analogues and the subsequent labeling and detection of these in endogenous and exogenously-expressed proteins in cells. This protocol is technically flawless. While several protocols for bioorthoganol fatty acid analogiue labeling and detection exist, this protocol is enhanced by the author's inclusion of the saponification process. Saponification of fatty acids is a topic that is very relevant and of high importance to researchers working in the lipidation field.

Major Concerns:
1. The major concern is in the author's conclusion in their abstract, that using saponified fatty acids in combination with fatty-acid free BSA, as well as delipidated media, "dramatically increased detection of fatty acylated proteins". The author's conclusion appears to be based on the data shown in Figure 1, which shows saponification appearing to cause a reduction in the detection of Alk-Myr, and a modest increase in the detection of Alk-Pal - which is also coincident with an increase in Tubulin protein levels. The most apparent increase in detection is seen with Alk-Stear, which the authors do acknowledge, however, this figure lacks the unlabeled fatty acid control which is shown in the other two panels. The authors should certainly include this important control, and even better would be to quantify the dramatic increases they state occurs. To do this, this authors could normalize the SA680 signal in each lane relative to a total protein stain and quantify the fold change in SA680 signal between non-saponified and saponfied methods across a minimum of 3 individual experiments. This would provide the authors the evidence that is needed to support their conclusions. The authors should also consider toning down their broad conclusion in the abstract - the description in the results is more appropriate.

Thank you for your comments and suggestions. As mentioned above, this manuscript will serve as a visual representation of the methods outlined in Yap et al 2010 (JLR), which was partly based on methods used in Martin et al 2008 (FASEBJ). Dr. Martin was heavily involved in developing both methods. We apologise for the confusion and have edited the manuscript accordingly.

We initially considered quantifying the results and did see an overall increase when doing so. However, we have found that saponification does not always increase 'total' S-acylation of all proteins, but predominantly of harder to detect proteins. Highly expressed and S-acylated proteins will typically be detected regardless of the method used. Low abundance proteins or proteins that do not undergo high levels or high turnover of S-acylation may not be detected when fatty acids are added directly to the media. Saponification is typically more consistent because it increases the solubility of the fatty acids and protects the cells from stress. When fatty acids are added directly to the media some likely precipitates and, thus, are not incorporated. In many cases, as shown in Figure 1, only specific bands increase, further indicating that not all proteins require this method for detection. While this may not lead to a statistically significant increase in overall fatty acylation, it is required for specific proteins of interest. Overall, the banding pattern will be different, or increased in specific bands, depending on the cell types, labeling times, etc. used.

Furthermore, as mentioned above, adding fatty acids directly can lead to increased stress, toxicity, and potentially cell death. Because both death receptors and caspases have been shown to be S-acylated, adding fatty acids directly could simply change the overall profile rather than total levels.

Initial and additional repetitions were quantified, and a signal increase was detected. However, because this can be vastly different among cell types, and for the reasons above, we felt that it would detract from the methods and did not include it. Testing multiple cell types is beyond the scope of this methods manuscript.

As described above, since stearate and palmitate undergo S-acylation through the same thioester bond, we did not include unlabeled stearate in addition to unlabeled palmitate within the same experiment. We felt that palmitate was sufficient for the negative control to delineate endogenously biotinylated proteins from labeled proteins. That said, we have added the control stearate and replaced Figure 1 with a fluorescent azide probe to further decrease any background signal. We also removed the inhibitors to simplify the figure.

As such, we have changed the language of the manuscript throughout to reflect these changes.

2. Figure 1. There is only a very small effect of Myr inhibitor with 13-TDYA. Why is this? The authors should discuss this. Have the authors looked at the effect of the inhibitor on 15-HDYA?

We have since repeated the experiment with more inhibitor and the effect was more robust. However, as described above, the inhibitors were removed from Figure 1 for simplification and using a fluorescent azide. As there are no endogenously fluorescent proteins compared to biotinylation, there is almost no background lysates using unlabeled fatty acids. We also added unlabeled stearate. The specificity of the inhibitors and the preference of the NMTs and PATs have been addressed in previously published papers cited within the manuscript. As noted, PATs have various preferences for different length fatty acids, but can also use all the fatty acids used in this study. NMT has a clear preference for myristate, but can modestly accommodate palmitate. To simplify the figure, we no longer include the inhibitors. In particular, 2BP is not a consistent or specific inhibitor and the S-acylation field is moving away from its use. The effects of the inhibitors on the published fatty acids is beyond the scope of this methods manuscript.

3. Lines 105-106. To avoid confusion, it would be helpful to expand here on the number of carbons 17ODYA and 15HDYA (and even 13-TDYA) have, and how they relate to palmitate, stearate etc. What does the number of carbon groups given in the name reflect? A figure and including the IUPAC nomenclature would be particularly useful here.

Thank you. The number of carbons have been added to lines 93-94 and 110. The number in the name indicate the carbon where the COOH group is attached. Because these are standard terms used in the field, PubChem and commercial terms were used to prevent any additional confusion for new users.

Minor Concerns:
1. Lines 27 and 44. The authors should add that fatty acylation includes the addition of shorter and longer chain, saturated and unsaturated fatty acids - not just saturated fatty acids on to proteins e.g. Hallak et al. JBC 269:4713, Liang et al JBC 276:30987, O'Brien et al JBC 259:5054, Thinon et al Chembiochem 17:1800, Veit et al Biochem J 318:163

The additional references have been added. The sentence has also been expanded to include a broader, more accurate definition of fatty acylation.

2. Line 47. "Fatty acylation has emerged as a potential drug target" This statement needs expanding for clarification and meaning. What aspect(s) of fatty acylation are potential drug targets?

Because this is beyond the scope of a methods paper and we did not want to detract from the methods aspect, we primarily added more references in which S-acylation has been targeted in diseases. The statement now reads 'Fatty acylation has emerged as a potential drug target in a myriad of diseases including infection, cancer, inflammation, and neurodegeneration, where disruptions in palmitoylation have been documented[8–13]. This has been primarily spurred by the development of new chemical detection methods, which enabled large-scale identification of S-acylated protein targets.'

3. Lines 48-49. This sentence needs expanding. How has the development of new chemical detection methods contributed to the potential to target fatty acylation therapeutically?

The sentence has been expanded in lines 47-51. Thank you.

4. Line 56. Consider rephrasing this, as short chain and unsaturated fatty acids can also be added.

The term 'typically' has been added in order to be more general.

5. Line 57. For clarity, replace 'use' with 'incorporation'.

This has been corrected. Thank you.

6. Lines 63-64. It would be worthwhile mentioning that another disadvantage of using radiolabeled palmitate is that this method is a non-quantitative, and so limits the possibility to measure dynamic changes in palmitoylation accurately and sensitively, and that it is also expensive.

Thank you for the suggestion. These points have been added to line 70.

7. Line 83. Remove 'hundreds and'.

This has been corrected. Thank you.

8. Lines 99-101. In addition to fluorescent probes, can use mPEG alkyne e.g. et al Methods Mol Biol. 2009:83.

This reference could not be found. Can you please provide the first author, DOI, or PMID?

9. Line 159. BSA should be fatty-acid free BSA.

Corrected. Thank you.

10. Line 338. Expand on the protocol of methanol-activation of PVDF membranes. How long do you activate in methanol?

Expanded. Thank you.

11. Line 342. An explanation is needed as to why you soak one membrane in 1M KOH.

Line 337, 383, and 409 have been revised to explain the treatment.

12. Figure 1. Include in figure that Alk-Myr is 13-TDYA, Alk-Pal is 15-HDYA and Alk-Stear is 17-ODYA.

Added to figure legend. Thank you.

13. Line 376. As KOH is active on ester bonds too, the authors should change this statement to "ester or thioester".

Revised. Thank you.

14. Line 434. The original reference should be included e.g. Brett et al. JBC 289:34978.

Included. Thank you.

15. Authors may wish to comment on different saponification protocols, for example do they see a benefit using KOH over NaOH?

While NaOH can be used, we have not assessed this since the first optimization of the assay in Yap et al 2010. As such, we did not include any references to the use of NaOH in the manuscript.

16. The following are not included in the Table of Materials: 13-tetradecynoic acid; 15-hexadecynoic acid; calcium chloride, potassium hydroxide, HEPES, Igepal, RIPA buffer, sodium chloride, sodium deoxycholate, magnesium chloride, PVDF membranes, anti-rabbit Alexa 488.

We have updated the Table Materials. Thank you.