Detection of Heterogeneous Protein S-acylation in Cells

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ii. Summary/Abstract

The use of synthetically-synthesized azide and alkyne fatty acid analogues coupled with bioorthogonal Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction-based detection methods to study protein S-acylation reactions has replaced the traditional method of using *in vivo* metabolic radiolabelling with tritiated palmitic acid and has greatly facilitated our understanding of this essential cellular process. Here, we describe the chemical synthesis of myristic (C:14), palmitic (C16:0) and stearic (C18:0) acid-azide probes and detail how they may be utilized as chemical reporters for the analysis of S-acylation of exogenously expressed proteins in cells.

iii. Key Words

Click chemistry; fatty acid azide; fatty acylation; palmitoylation; S-acylation.

1. Introduction

Protein S-acylation (palmitoylation) describes the reversible post-translational attachment of fatty acids on to cysteine amino acids. Although palmitic acid (C16:0) is the most frequent fatty acid to modify proteins in this way, other shorter and longer chain saturated and unsaturated fatty acids may be added on to S-acylated proteins [1-6]. Historically, the only

method to detect protein S-acylation was by *in vivo* metabolic radiolabelling using tritiated palmitate followed by fluorographic detection [7-9]. Despite being the primary method used for decades, this approach has several limitations, including a low signal intensity that requires long exposure times (up to three months) in the order for S-acylation to be detected. Over the past decade, however, there has been a significant advance over this traditional methodology through the use of synthetically-synthesized azide and alkyne fatty acid analogues as alternatives to tritiated palmitic acid [10-17]. These fatty acid analogues mimic endogenous fatty acids and can be utilised as reporters of cellular processes involving fatty acids, such as protein S-acylation [6]. Coupled with bioorthogonal Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition ('Click') reaction-based detection methods, the use of azide and alkyne fatty acid analogues is a highly sensitive, quantitative and practical method for studying protein S-acylation reactions that can be adapted for high-throughput analysis [18]. Since fatty acyl chain diversity can also be incorporated during the synthesis of these chemical probes, these reporters facilitate our understanding of how acyl chain heterogeneity contributes to protein S-acylation [6].

This method describes the detection of S-acylation of EGFP-tagged synaptosomal-associated protein of 25 kDa (SNAP25), expressed in HEK293T cells along with hemagglutinin (HA)-tagged zDHHC3 as a catalyst, using chemically-synthesized myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acid azide probes. Detection of S-acylation of other substrate proteins may not necessarily require the co-expression of a zDHHC enzyme [19]. HEK293 cells are a well-established mammalian cell line used for heterologous protein expression due to their efficient transfection rate and subsequent high level expression of proteins from plasmid DNA [20]. In this method, HEK293T cells are transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) with a plasmid encoding the S-acylated protein of interest. Following high-level expression, cells are labelled with the synthetically

synthesized fatty acid-azide probes, which can become metabolically incorporated into S-acylated and other lipidated cellular proteins. The azido-fatty acid modification on cellular proteins is then conjugated to an alkyne infrared fluorophore by performing a Cu(I)-catalyzed 'Click' reaction. Isolated cellular proteins are then resolved electrophoretically in vertical discontinuous sodium dodecyl sulphate polyacrylamide gels according to the Laemmli method [21] and expression of exogenous proteins is confirmed by western blotting.

2. Materials

2.1 Reduction of carboxylic acids

1. Hotplate stirrer
2. Cryogenic bath for cooling
3. Spatula
4. 500 mL B24 round bottom flask
5. B24 suba seal
6. Schlenk line
7. 250 mL measuring cylinder
8. Sintered filter funnel with side-arm
9. B24 water condenser
10. Tubing for water condenser
11. Silicone oil bath
12. Magnetic stirrer bar
13. Pressure tubing for filtration
14. 1 L separating funnel with stopper
15. 2 x 500 mL conical flask
16. Rotary evaporator

2.2 Bromination of diols

1. Hotplate stirrer
2. Spatula
3. 2 neck 250 mL B24 round bottom flask
4. B24 glass stopper
5. B24 100 ml pressure equalising dropping funnel
6. Silicone oil bath for heating
7. 250 mL measuring cylinder
8. Sintered filter funnel with side-arm
9. B24 water condenser
10. Tubing for water condenser
11. Magnetic stirrer bar
12. 500 mL separating funnel with stopper
13. Pressure tubing for filtration
14. 3 x 250 mL conical flask
15. 80 x 3 cm glass column with ball joint for chromatography
16. Rotary evaporator

2.3 Oxidation of primary alcohols

1. Hotplate stirrer
2. Spatula
3. 100 mL B24 round bottom flask
4. B24 glass stopper
5. 100 mL measuring cylinder

6. Sintered filter funnel with side-arm
7. Magnetic stirrer bar
8. Pressure tubing for filtration
9. 250 mL separating funnel with stopper
10. 3 x 250 mL conical flask
11. 80 x 3 cm column with ball joint for flash chromatography
12. 100 mL conical flask
13. Rotary evaporator

2.4 Azide addition

1. Hotplate stirrer
2. Schlenk line
3. Spatula
4. 50 mL B24 round bottom flask
5. B24 suba seal
6. Schlenk line
7. 50 mL measuring cylinder
8. Sintered filter funnel with side-arm
9. B24 water condenser
10. Tubing for water condenser
11. Silicone oil bath
12. Magnetic stirrer bar
13. Pressure tubing for filtration
14. 100 mL separating funnel with stopper
15. 3 x 100 mL conical flask

16. Rotary evaporator

2.5 Cell Culture Reagents and Equipment

1. Human Embryonic Kidney 293T (HEK293T) cell culture media: DMEM containing L-Glutamine and 10 % Foetal Bovine Serum, store at 4 °C.
2. Trypsin-EDTA (0.05%).
3. Lipofectamine 2000 (Invitrogen) transfection reagent, store at 4 °C.
4. Fatty-acid free cell culture media: DMEM containing L-Glutamine and supplemented with 1 mg/ml of fatty-acid free Bovine Serum Albumin.
5. Fatty-acid azides: Saturated fatty acid azides are stored at -20 °C as 100 mM stocks made up in DMSO (See **Note 1).**
6. Fatty-acid azide labelling cell culture media: Fatty-acid free cell culture media containing100 µM fatty-acid azide.
7. Poly-D-lysine coated 24-well cell culture plates.
8. T75 flasks
9. Pipettes
10. Pipette Gun
11. Falcon tubes
12. Sterile 1.5 ml tubes
13. Laminar Flow tissue culture hood
14. Neubauer Chamber
15. Light microscope

2.6 Click chemistry

1. Phosphate-buffered Saline (PBS; 10X): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4. (See Note 2.)
2. Lysis Buffer: 50 mM Tris, pH 8.0, 0.5% SDS. Store at 4 °C. Protease inhibitors should be added immediately before use.
3. LI-COR IRDye® 800CW Alkyne Infrared Dye (LI-COR Biotechnology UK Ltd, Catalogue number 929-60002): Resuspend in Dimethyl Sulfoxide (DMSO) at a concentration of 4mM and store stock solutions in aliquots at - 20 °C for short –term or - 80 °C for long-term storage (See Note 3)**.**
4. Copper Sulphate: 40 mM solution.
5. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): Resuspend in DMSO at a concentration of 100 mM and store stock solutions in small aliquots at - 20 °C.
6. Ascorbic acid: 4 mM solution (See Note 4).
7. Acetone: 100 % and 70 % stock solutions stored at - 20 °C (See Note 5).
8. End-over-end rotator.

2.7 SDS-Polyacrylamide Gel Electrophoresis

1. SDS Lysis buffer (4X): 200mM Tris-HCl, pH 6.8, 40% Glycerol, 8% Sodium Dodecyl Sulphate (SDS) (See Note 6), 0.4% Bromophenol Blue, supplemented with 25 mM DTT (See Note 7).
2. Dithiothreitol (DTT): 1M stock made up in dH_20 and stored in 1 ml aliquots at –20°C.
3. Resolving gel buffer (2 X): 750 mM Tris-HCl, pH 8.9, 4 mM Ethylenediaminetetraacetic acid (EDTA), 0.2% SDS.
4. Stacking gel buffer (2 X): 250 mM Tris-HCl, pH 6.8, 4 mM EDTA, 0.2 % SDS.

5. 30 % acrylamide-bisacrylamide solution, stored at 4 °C.
6. N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED), stored at 4 °C.
7. Ammonium persulphate: 10 % stock made up in H_20 and stored at 4 °C (See Note 8).
8. Tris-Glycine electrophoresis buffer (10 X): 250 mM Tris-HCl, 2.5 M glycine, 1 % SDS (See Note 9).
9. Prestained molecular weight markers.
10. Bio-Rad Mini-PROTEAN Tetra Cell system.
11. Bio-Rad PowerPac Universal Power Supply.
12. Heat block.

2.8 Western Blotting

1. Nitrocellulose membrane with 0.45 μM pore size.
2. Whatman 3mm blotting paper
3. Bio-Rad Trans-Blot Cell.
4. Bio-Rad PowerPac Universal Power Supply.
5. Transfer buffer (10 X): 480 mM Tris-Base, 390 mM Glycine, 13 mM SDS (See Note 10).
6. PBS-T: PBS containing 0.02 % Tween 20 (See Note 11).

2.9 Detection of S-acylated proteins.

1. Western blotting membrane incubation box
2. Orbital rotator
3. PBS-T: PBS containing 0.02 % Tween 20 (See Note 11).
4. Blocking Buffer: 5 % non-fat skimmed milk powder in PBS-T (See Note 12).

5. Appropriate primary antibody (Living Colors® Av Monoclonal anti GFP Antibody (JL-8), Clontech).
6. Secondary antibody diluent: 1 % non-fat skimmed milk powder in PBS-T (See Note 12).
7. LI-COR IRDye® 680RD Secondary Antibody raised against the appropriate species of primary antibody (See Note 13). For use with the anti-GFP JL8 antibody, we use LI-COR IRDye® 680RD Goat anti-Mouse IgG (H + L), catalogue number 925-680700.
8. LI-COR Odyssey Imaging System (See Note 14).

3. Methods.

Unless otherwise stated, all commercially available reagents were used as supplied without any further purification. All procedures should be carried out at room temperature unless stated otherwise. Prepare all solutions using ultrapure Milli-Q water and store at room temperature unless stated otherwise. Culture Human Embryonic Kidney (HEK)-293T cells in 75 cm^2 flasks in DMEM containing L-Glutamine supplemented with 10 % (v/v) FCS at 37 ºC in a humidified atmosphere containing 95 % (v/v) air and 5 % (v/v) CO_2 according to standard procedures [21]. Allow 24 hours between seeding and transfecting the HEK293T cells, and 24 hours between transfection and cell harvesting. Harvest and re-seed HEK293T cells every 7 days. All HEK293T media should be pre-warmed to 37 ºC before use.

General details for fatty acid azide synthesis: Dry THF was used directly from a PureSolv MD 5 Solvent Purification System by Innovative Technology Inc., and handled under inert atmosphere. Flash chromatography was carried out using Merck Kieselgel 60 H silica. Analytical thin layer chromatography was carried out using aluminium-backed plates coated with Merck Kieselgel 60 GF254 that were visualised using p-anisaldehyde. Nuclear magnetic

resonance (NMR) spectra were recorded on a 400 MHz Ultrashield Magnet, Prodigy liquid nitrogen cryoprobe, AVIII console and a Z420 HP workstation running TopSpin 3.X running at 400 MHz (1H NMR) and 101 MHz (13C NMR); an 500 MHz Ascend magnet, BBO multi nuc' Smart probe, AVIIIHD500 console and Z420 HP workstation running TopSpin 3.X running at 500 MHz (1H NMR) and 126 MHz (13C NMR) or a 600 MHz Ultrashield magnet, BBO multi nuc' probe, AVII+ console and a Z420 HP workstation running TopSpin 3.X running at 600 MHz (1H NMR) or 151 MHz (13C NMR). Chemical shifts are reported in parts per million (ppm) in the scale relative to CDCl3, 7.26 ppm for 1H NMR and 77.16 for 13C NMR; DMSO-d6, 2.50 ppm for 1H NMR and 39.52 for 13C NMR. Coupling constants are measured in Hertz (Hz). Low resolution mass spectra (LRMS) were recorded on an Agilent 6130 single quadrupole with APCI/ESI dual source, on a ThermoQuest Finnigan LCQ DUO electrospray, or on an Agilent 7890A GC system, equipped with a 30 m DB5MS column connected to a 5975C inert XL CI MSD with Triple-Axis Detector. MALDI were performed on an Axima-CFR from Kratos-Shimadzu. High resolution mass spectra (HRMS) were obtained courtesy of the EPSRC National Mass Spectrometry Facility at Swansea University, UK. Infrared spectra were recorded on an Agilent 5500a FTIR equipped with ATR (Attenuated Total Reflectance) and were reported in cm–1. In vacuo refers to evaporation under reduced pressure using a rotary evaporator connected to a diaphragm pump, followed by the removal of trace volatiles using a high vacuum (oil) pump. Melting points were determined with a Gallenkamp SG92 melting point apparatus and are uncorrected.

3.1 Preparation of fatty acid azides.

Fatty acid azides followed the synthesis outlined in Figure 1.

1. Preparation of 1,14-tetradecanediol **4** (See Note 15). Add solid $LiAlH_4$ (1.47 g, 38.7 mmol) to a solution of 1,14-tetradecanedioic acid (5.00 g, 19.35 mmol) in THF (194 mL) at 0 °C. Allow the reaction to warm to room temperature and stir for 20 h. Upon completion, add wet $NaSO_4$ portion-wise until the grey suspension turns white. Stir the suspension at room temperature until the white solid is free-flowing, and add solid $MgSO_4$ (5 g). Filter the reaction and wash the filter cake with 5 × 50 mL Et_2O. Evaporate the solvent *in vacuo* to give the product (3.88 g, 87%) as a white solid.

   δ_H (600 MHz, DMSO-d_6) 4.29 (t, 2H, J 5.0 Hz, 2 × OH), 3.35–3.38 (m, 4H, 2 × CH_2OH), 1.36–1.42 (m, 4H, 2 × CH_2CH_2OH), 1.22–1.29 (m, 20H, 10 × CH_2).

   δ_C (151 MHz, DMSO-d_6) 60.7, 32.5, 29.1, 29.0, 29.0, 28.9, 25.5.

   LR-MS (MALDI-TOF) 253.2 ($[M+Na]^+$).

   HR-MS calcd for $C_{14}H_{31}O_2$ ($[M+H]^+$) 231.2318, found 231.2318.

   υ_{max} (thin film, cm^{-1}) 3410, 3351, 2921, 2891, 2850.

   Mp 88–90 °C.

2. Preparation of 14-bromotetradecan-1-ol **7**. Add HBr (48% in H_2O, 41 mL) to a suspension of diol **4** (3.57 g, 15.5 mmol) in cyclohexane (41 mL). Heat the biphasic mixture to reflux for 10 h and then cool to room temperature. Separate the layers and extract the aqueous phase with CH_2Cl_2 (4 × 30 mL). Wash the combined organics with $NaHCO_3$ (4 × 20 mL of a saturated aqueous solution), brine (20 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 95:5 petrol/EtOAc then 70:30 petrol/EtOAc, to afford the product (2.85 g, 63%) as a pale yellow solid (See Note 16).

   δ_H (400 MHz, $CDCl_3$) 3.64 (t, 2H, J 6.6 Hz, CH_2OH), 3.40 (t, 2H, J 6.9 Hz, CH_2Br), 1.80–1.90 (m, 2H, CH_2CH_2Br), 1.53–1.60 (m, 2H, CH_2CH_2OH), 1.25–1.46 (m, 20H, 10 × CH_2).

δ_C (101 MHz, $CDCl_3$) 63.2, 34.2, 33.0, 33.0, 29.7, 29.7, 29.6, 28.3, 28.9.

LR-MS (EI+) 294.9 ([M(^{81}Br)]$^+$, 0.5%), 292.9 ([M(^{79}Br)]$^+$, 1%), 276.9 ([M(^{81}Br)-H_2O]$^+$, 3.5%), 292.9 ([M(^{79}Br)-H_2O]$^+$, 3%), 213.9 ([M-Br]$^+$, 2.5%).

HR-MS calcd for $C_{14}H_{33}ON^{79}Br$+ ([M+NH_4]$^+$) 310.1740, found 310.1744.

υ_{max} (thin film, cm^{-1}) 3274, 2919, 2850.

Mp 46–48 °C.

3. Preparation of 14-bromotetradecanoic acid **10**. Dissolve CrO_3 (3.89 g, 38.92 mmol) in concentrated H_2SO_4 (7.2 mL). Add cold H_2O (16.2 mL) slowly and stir the solution for 10 min. Add the resulting solution drop-wise to a solution of alcohol **7** (2.85 g, 9.73 mmol) in acetone (243 mL). Stir the reaction mixture for 20 h before adding H_2O (100 mL) and CH_2Cl_2 (40 mL). Separate the layers and extract the aqueous phase with CH_2Cl_2 (4 × 30 mL). Wash the combined organics with brine (30 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 90:10 petrol/EtOAc (+0.1% AcOH), to afford the product (2.63 g, 88%) as a white solid.

   δ_H (400 MHz, $CDCl_3$) 3.41 (t, 2H, J 6.9 Hz, CH_2Br), 2.35 (t, 2H, J 7.5 Hz, CH_2CO_2H), 1.81–1.89 (m, 2H, CH_2CH_2Br), 1.59–1.68 (m, 2H, $CH_2CH_2CO_2H$), 1.24–1.45 (m, 18H, 9 × CH_2).

   δ_C (101 MHz, $CDCl_3$) 179.0, 34.2, 34.0, 33.0, 29.7, 29.7, 29.6, 29.4, 29.2, 28.9, 28.3, 24.8.

   LR-MS (EI$^+$) 309.0 ([M(^{81}Br)]$^+$, 16%), 307.0 ([M(^{79}Br)]$^+$, 20%), 291.0 ([M(^{81}Br)-H_2O]$^+$, 13%), 289.0 ([M(^{79}Br)-H_2O]$^+$, 17%), 227.1 ([M-Br]$^+$, 24%).

   HR-MS calcd for $C_{14}H_{26}O^{79}Br$ ([M-H]$^-$) 305.1122, found 305.1123.

   υ_{max} (thin film, cm^{-1}) 3036, 2917, 2852, 1696.

Mp 63–66 °C.

4. Preparation of 14-azidotetradecanoic acid **13** (See Note 15). Add NaN_3 (636 mg, 9.78 mmol) to a solution of bromide **10** (500 mg, 1.63 mmol) in DMF (6.5 mL). Stir the reaction at 80 °C for 40 h and then cool to room temperature. Add a 1:1 mixture of EtOAc/H_2O (20 mL), separate the layers and extract the aqueous phase with EtOAc (3 × 10 mL). Wash the combined organics with brine (10 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 90:10 petrol/EtOAc (+0.1% AcOH), to afford the product (403 mg, 92%) as a white solid (See Note 17).

   δ_H (400 MHz, $CDCl_3$) 3.25 (t, 2H, J 7.0 Hz, CH_2N_3), 2.34 (t, 2H, J 7.5 Hz, CH_2CO_2H), 1.55–1.68 (m, 4H, $CH_2CH_2N_3$, $CH_2CH_2CO_2H$), 1.23–1.38 (m, 18H, 9 × CH_2).

   δ_C (101 MHz, $CDCl_3$) 180.1, 51.6, 34.2, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 26.9, 24.8.

   LR-MS (ES^-) 268.1 ($[M-H]^-$, 100%), 240.1 ($[M-N_2-H]^-$, 34%).

   HR-MS calcd for $C_{14}H_{26}N_3O_2$ ($[M-H]^-$) 268.2031, found 268.2028.

   υ_{max} (thin film, cm^{-1}) 3016, 2915, 2848, 2101, 1701.

   Mp 38–40 °C.

5. Preparation of 1,16-hexadecanediol **5** (See Note 15). Add solid $LiAlH_4$ (1.33 g, 34.9 mmol) to a solution of 1,16-hexadecanedioic acid (5.00 g, 17.46 mmol) in THF (175 mL) at 0 °C. Allow the reaction to warm to room temperature and stir for 20 h. Upon completion, add wet $NaSO_4$ portion-wise until the grey suspension turns white. Stir the suspension until the white solid is free-flowing and add solid $MgSO_4$ (5 g). Filter the reaction and wash the filter cake with Et_2O (5 × 50 mL). Evaporate the solvent *in vacuo* to afford the product (3.68 g, 82%) as a white solid.

δ_H (600 MHz, DMSO-d_6) 4.29 (t, 2H, J 4.9 Hz, 2 × OH), 3.34–3.39 (m, 4H, 2 × CH_2OH), 1.36–1.42 (m, 4H, 2 × CH_2CH_2OH), 1.22–1.29 (m, 24H, 12 × CH_2).

δ_C (151 MHz, DMSO-d_6) 60.7, 32.5, 29.1, 29.0, 29.0, 29.0, 28.9, 25.5.

LR-MS (MALDI-TOF) 281.3 ($[M+Na]^+$), 297.3 ($[M+K]^+$).

HR-MS calcd for $C_{16}H_{35}O_2$ ($[M+H]^+$) 259.2632, found 259.2632.

υ_{max} (thin film, cm^{-1}) 3414, 3353, 2919, 2891, 2848.

Mp 91-94 °C.

6. Preparation of 16-bromohexadecan-1-ol **8**. Add HBr (48% in H_2O, 36 mL) to a suspension of diol **5** (3.48 g, 13.5 mmol) in cyclohexane (36 mL). Heat the biphasic mixture to reflux for 10 h before cooling to room temperature. Separate the layers and extract the aqueous phase with CH_2Cl_2 (4 × 30 mL). Wash the combined organics with $NaHCO_3$ (4 × 20 mL of a saturated aqueous solution), brine (20 mL), dry over $MgSO_4$, filter and concentrate in vacuo. Purify the crude residue by column chromatography, eluting with 95:5 petrol/EtOAc then 70:30 petrol/EtOAc, to afford the product (2.40 g, 55%) as a pale yellow solid See Note 16).

   δ_H (400 MHz, $CDCl_3$) 3.64 (t, 2H, J 6.6 Hz, CH_2OH), 3.41 (t, 2H, J 6.9 Hz, CH_2Br), 1.80–1.91 (m, 2H, CH_2CH_2Br), 1.52–1.62 (m, 2H, CH_2CH_2OH), 1.25–1.46 (m, 24H, 12 × CH_2).

   δ_C (101 MHz, $CDCl_3$) 63.3, 34.2, 33.0, 33.0, 29.8, 29.8, 29.7, 29.6, 28.9, 28.3, 25.9.

   LR-MS (EI+) 320.9 ($[M(^{81}Br)]^+$, 0.5%), 318.9 ($[M(^{79}Br)]^+$, 0.5%), 304.9 ($[M(^{81}Br)-H_2O]^+$, 1%), 302.9 ($[M(^{79}Br)-H_2O]^+$, 1%), 241.8 ($[M-Br]^+$, 1.5%).

   HR-MS calcd for $C_{16}H_{37}{}^{79}BrON$ ($[M+NH_4]^+$) 338.2053, found 338.2056.

   υ_{max} (thin film, cm^{-1}) 3274, 2917, 2850.

   Mp 54-56 °C.

7. Preparation of 16-bromohexadecanoic acid **11**. Dissolve CrO_3 (3.00 g, 30.0 mmol) in concentrated H_2SO_4 (5.5 mL). Add cold H_2O (12.5 mL) slowly and stir the solution at room temperature for 10 min. Add the resulting solution drop-wise to a solution of alcohol **8** (2.40 g, 7.50 mmol) in acetone (188 mL). Stir the reaction mixture for 20 h before adding H_2O (100 mL) and CH_2Cl_2 (40 mL). Separate the layers and extract the aqueous phase with CH_2Cl_2 (4 × 30 mL). Wash the combined organics with brine (30 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 90:10 petrol/EtOAc (+0.1% AcOH), to afford the product (2.30 g, 92%) as a white solid.

   δ_H (400 MHz, $CDCl_3$) 3.41 (t, 2H, J 6.9 Hz, CH_2Br), 2.35 (t, 2H, J 7.5 Hz, CH_2CO_2H), 1.80–1.90 (m, 2H, CH_2CH_2Br), 1.59–1.68 (m, 2H, $CH_2CH_2CO_2H$), 1.25–1.48 (m, 22H, 11 × CH_2).

   δ_C (101 MHz, $CDCl_3$) 178.6, 34.2, 33.9, 33.0, 29.8, 29.7, 29.6, 29.4, 29.2, 28.9, 28.3, 24.8.

   LR-MS (EI^+) 337.0 ($[M(^{81}Br)]^+$, 5%), 335.0 ($[M(^{79}Br)]^+$, 7%), 317.0 ($[M(^{81}Br)\text{-}H_2O]^+$, 3%), 315.0 ($[M(^{79}Br)\text{-}H_2O]^+$, 5%), 257.1 ($[M\text{-}Br]^+$, 8.5%), 237.1 ($[M\text{-}Br\text{-}H_2O]^+$, 22%).

   HR-MS calcd for $C_{16}H_{30}{}^{79}BrO$ ($[M\text{-}H]^-$) 333.1435, found 333.1430.

   υ_{max} (thin film, cm^{-1}) 3034, 2917, 2850, 1696

   Mp 72-74 °C

8. Preparation of 16-azidohexadecanoic acid **14** (See Note 15). Add NaN_3 (582 mg, 8.96 mmol) to a solution of bromide **11** (500 mg, 1.49 mmol) in DMF (6 mL). Stir the reaction at 80 °C for 40 h before cooling to room temperature. Add a 1:1 mixture of EtOAc/H_2O (20 mL), separate the layers and extract the aqueous phase with EtOAc (3 × 10 mL). Wash the combined organics with brine (10 mL), dry over $MgSO_4$, filter

and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 90:10 petrol/EtOAc (+0.1% AcOH), to afford the product (402 mg, 91%) as a white solid (See Note 17).

δ_H (400 MHz, $CDCl_3$) 3.25 (t, 2H, J 7.0 Hz, CH_2N_3), 2.34 (t, 2H, J 7.5 Hz, CH_2CO_2H), 1.55–1.67 (m, 4H, $CH_2CH_2N_3$, $CH_2CH_2CO_2H$), 1.24–1.40 (m, 22H, 11 × CH_2).

δ_C (101 MHz, $CDCl_3$) 180.0, 51.7, 34.2, 29.8, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 29.3, 29.2, 29.2, 29.0, 26.9, 24.8.

LR-MS (ES-) 268.2 ($[M\text{-}N_2\text{-}H]^-$, 26%), 296.2 ($[M\text{-}H]^-$, 100%).

HR-MS calcd for $C_{16}H_{32}NO_2$ ($[M\text{-}N_2+H]^+$) 270.2440, found 270.2433.

υ_{max} (thin film, cm^{-1}) 3014, 2915, 2848, 2103, 1701.

Mp 48–50 °C.

9. Preparation of 1,18-octadecanediol **6** (See Note 15). Add solid $LiAlH_4$ (1.44 g, 37.96 mmol) to a vigorously stirred solution of dimethyl octadecanedioate (5.00 g, 14.9 mmol). Heat the reaction to reflux and stir at reflux for 20 h. Upon completion, cool the reaction to room temperature and add wet $NaSO_4$ portion-wise until the grey suspension turns white. Stir the suspension until the white solid is free-flowing, and solid $MgSO_4$ (5 g). Filter the reaction and wash the filter cake with Et_2O (5 × 50 mL). Evaporate the solvent *in vacuo* to afford the product (4.15 g, 99%) as a white solid.

δ_H (600 MHz, DMSO-d_6) 4.30 (t, 2H, J 5.1 Hz, 2 × OH), 3.34–3.39 (m, 4H, 2 × CH_2OH), 1.35–1.43 (m, 4H, 2 × CH_2CH_2OH), 1.23 (app. br. s., 28H, 14 × CH_2).

δ_C (151 MHz, DMSO-d_6) 60.6, 32.3, 28.8, 28.8, 28.7, 25.3.

LR-MS (MALDI-TOF) 293.3 ($[M+Li]^+$), 309.3 ($[M+Na]^+$).

HR-MS calcd for $C_{18}H_{39}O_2$ ($[M+H]^+$) 287.2945, found 287.2946.

υ_{max} (thin film, cm^{-1}) 3416, 3353, 2919, 2891.

Mp 103–106 °C.

10. Preparation of 18-bromooctadecan-1-ol **9**. Add HBr (48% in H_2O, 21 mL) to a suspension of diol **6** (2.26 g, 7.9 mmol) in cyclohexane (21 mL). Heat the biphasic mixture to reflux for 10 h before cooling to room temperature. Separate the layers and extract the aqueous phase with CH_2Cl_2 (4 × 20 mL). Wash the combined organics with $NaHCO_3$ (4 × 20 mL of a saturated aqueous solution), brine (20 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 95:5 petrol/EtOAc then 70:30 petrol/EtOAc, to afford the product (1.65 g, 60%) as a pale yellow solid (See Note 16).

    δ_H (400 MHz, $CDCl_3$) 3.64 (t, 2H, J 6.6 Hz, CH_2OH), 3.40 (t, 2H, J 6.9 Hz, CH_2Br), 1.81–1.89 (m, 2H, CH_2CH_2Br), 1.53–1.60 (m, 2H, CH_2CH_2OH), 1.25–1.46 (m, 28H, 14 × CH_2).

    δ_C (101 MHz, $CDCl_3$) 63.3, 34.2, 33.0, 33.0, 29.8, 29.8, 29.7, 29.6, 28.9, 28.3, 25.9.

    LR-MS (ES+) 332.5 (M(^{81}Br)-H_2O, 0.5%), 330.5 (M(^{79}Br)-H_2O, 0.5%).

    HR-MS calcd for $C_{18}H_{41}{}^{79}BrON$ ($[M+NH_4]^+$) 366.2366, found 336.2368.

    υ_{max} (thin film, cm^{-1}) 3274, 2917, 2850.

    Mp 59–61 °C.

11. Preparation of 18-bromooctadecanoic acid **12**. Dissolve CrO_3 (1.42 g, 14.2 mmol) in concentrated H_2SO_4 (3.5 mL). Add cold H_2O (7.9 mL) slowly and stir the solution for 10 min. Add the resulting solution drop-wise to a solution of alcohol **9** (1.65 g, 4.73 mmol) in acetone (120 mL). Stir the reaction for 20 h before adding H_2O (60 mL) and CH_2Cl_2 (40 mL). Separate the layers and extract the aqueous phase with CH_2Cl_2 (4 × 20 mL). Wash the combined organics with brine (20 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting

with 90:10 petrol/EtOAc (+0.1% AcOH), to afford the product (1.43 g, 83%) as a white solid (See Note 18).

δ_H (400 MHz, $CDCl_3$) 3.41 (t, 2H, J 6.9 Hz, CH_2Br), 2.35 (t, 2H, J 7.5 Hz, CH_2CO_2H), 1.81–1.90 (m, 2H, CH_2CH_2Br), 1.59–1.68 (m, 2H, $CH_2CH_2CO_2H$), 1.25–1.46 (m, 26H, 13 × CH_2).

δ_C (101 MHz, CDCl3) 179.6, 34.2, 34.1, 33.0, 29.8, 29.7, 29.7, 29.6, 29.4, 29.2, 28.9, 28.3, 24.8.

LR-MS (EI+) 365.0 ([M(^{81}Br)]+, 9%), 363.0 ([M(^{79}Br)]+, 12%), 347.0 ([M(^{81}Br)-H_2O]$^+$, 5%), 345.0 ([M(^{79}Br)-H_2O]$^+$, 7%), 284.0 ([M-Br]$^+$, 12%), 265.1 ([M-Br-H_2O]$^+$, 29%).

HR-MS calcd for $C_{18}H_{34}{}^{79}BrO_2$ ([M-H]$^-$) 361.1748, found 361.1741.

υ_{max} (thin film, cm^{-1}) 3034, 2915, 2850, 1696.

Mp 77–80 °C.

12. Preparation of 18-azidooctadecanoic acid **15** (See Note 15). Add NaN_3 (537 mg, 8.3 mmol) to a solution of bromide **12** (500 mg, 1.38 mmol) in DMF (5.5 mL). Stir the reaction at 80 °C for 40 h before cooling to room temperature. Add a 1:1 mixture of EtOAc/H_2O (30 mL), separate the layers and extract the aqueous phase with EtOAc (3 × 10 mL). Wash the combined organics with brine (10 mL), dry over $MgSO_4$, filter and concentrate *in vacuo*. Purify the crude residue by column chromatography, eluting with 90:10 petrol/EtOAc (+0.1% AcOH), to afford the product (403 mg, 90%) as a white solid (See Note 16).

δ_H (400 MHz, $CDCl_3$) 3.25 (t, 2H, J 7.0 Hz, CH_2N_3), 2.34 (t, 2H, J 7.5 Hz, CH_2CO_2H), 1.55–1.67 (m, 4H, $CH_2CH_2N_3$, $CH_2CH_2CO_2H$), 1.25–1.39 (m, 26H, 13 × CH_2).

δ_C (101 MHz, $CDCl_3$) 180.1, 51.6, 34.2, 29.8, 29.7, 29.7, 29.6, 29.6, 29.4, 29.4, 29.3, 29.2, 29.0, 26.7, 24.8.

LR-MS (ES-) 296.3 ([M-N_2-H]$^-$, 24%), 324.1 ([M-H]$^-$, 100%).

HR-MS calcd for $C_{18}H_{34}N_3O_2$ ([M-H]$^-$) 324.2657, found 324.2649.

υ_{max} (thin film, cm^{-1}) 3040, 2915, 2850, 2098, 1696.

Mp 56–58 °C.

3.2 HEK293T cell culture and seeding.

1. Warm Trypsin-EDTA and HEK293T Culture Media to 37 ºC in a pre-warmed water bath.
2. In a Laminar flow tissue culture cabinet, remove the media from HEK293T cells cultured in a 75 cm^2 flask and discard.
3. Wash cells briefly with 3ml of pre-warmed Trypsin-EDTA and immediately discard.
4. Add 3ml of pre-warmed Trypsin-EDTA to the cell monolayer and incubate at 37 ºC for 5 minutes.
5. Detach cell monolayer by gently tapping the side of the flask and wash the remaining cells off the flask surface by pipetting up and down 5-10 times. Care should be taken to ensure all cells are harvested.
6. Transfer the contents of the flask to a sterile falcon tube and add 7ml of pre-warmed HEK293T Culture Media. Pipette up and down five times to mix.
7. To reseed cells into a new 75 cm^2 flask, dilute cells 1:10 by mixing 1 ml of the cells with 11ml of HEK293T Culture Media.
8. Determine cell density using a Neubauer Chamber. Resuspend cells in HEK293T Culture Media at a density of approximately 10^6 cells/ml and seed 500 µl/well into Poly-D-lysine coated 24-well cell culture plates.

9. Incubate cells overnight at 37 ºC in a humidified atmosphere containing 95 % air and 5 % CO_2.

3.2 HEK293T cell transfection.

1. HEK293T should be transfected approximately 24 hours after seeding.
2. For each well of HEK293T cells to be transfected, set up 2 tubes each containing 50 uL of Serum Free DMEM. In Tube 1 add up to 3ug plasmid DNA (we used 0.8 ug EGFP-SNAP25B + 1.6 ug HA-ZDHHC3). In Tube 2 add two volumes of Lipofectamine 2000 reagent per ug of plasmid DNA used (we used 4.8 ul of Lipofectamine 2000 reagent). Incubate for 5 minutes at room temperature (See Note 19).
3. Mix the contents of the two tubes and incubate the DMEM:DNA:Lipofectamine 2000 mixture for 20 minutes at room temperature.
4. Add the transfection complex to one well of the 24-well plate of HEK293T cells.
5. Incubate cells overnight at 37 ºC in a humidified atmosphere containing 95 % air and 5 % CO_2.

3.3 Metabolic Labelling of Transfected Cells with azido-fatty acid.

1. Serum-starve transfected HEK293T cells by removing and discarding the culture media and replacing it with 500 µl/well of DMEM containing L-Glutamine supplemented with 1% fatty-acid free BSA (See Note 20). Incubate cells at 37 ºC in a humidified atmosphere containing 95 % air and 5 % CO_2 for 30 minutes.
2. Add 500 µl/well of DMEM containing L-Glutamine supplemented with 1% fatty-acid free BSA and 100µM fatty acid azide and incubate cells for 4 hours cells at 37 ºC in a humidified atmosphere containing 95 % air and 5 % CO_2 (See Note 21).

3.4 Cell lysis.

1. Wash cells twice on ice by gently aspirating off the media and replacing it with 1ml ice-cold PBS.
2. Remove the PBS and add 100µl of Lysis Buffer (containing protease inhibitors) to each well. Incubate cells on ice for 10 minutes with gentle agitation (See Note 22).

3.5 Click Chemistry and Protein Precipitation

1. Remove aliquots of 4 mM Alkyne Infrared Dye and 100 mM TBTA from the freezer and bring up to room temperature.
2. Make up 40mM Ascorbic Acid solution by dissolving 0.07g of ascorbic acid in 10 ml of H_20 and mixing with a magnetic stirrer (See Note 23).
3. In a 1.5 ml tube, mix 0.12 µl 4 mM Alkyne IR800 dye, 10 µl 40 mM $CuSO_4$, 0.4 µl 100 mM TBTA and 69.48 µl dH_2O (See Note 24).
4. Add the cell lysate to the click reaction cocktail and vortex to mix.
5. Add 20 µl of 40mM ascorbic acid to the lysate (See Note 25).
6. Vortex and incubate for 1 hour at room temperature with end-over-end rotation.
7. Precipitate proteins using ice-cold acetone. Add 3 volumes (600µl) of ice-cold acetone to the tubes and vortex. Incubate at -20 ºC for a minimum of 20 minutes (See Note 26).
8. Centrifuge at 20, 000 x *g* for 5 minutes at 4 ºC to pellet proteins.
9. Remove and discard supernatant. Add 1 ml ice-cold 70 % acetone and vortex tube to wash pellet.
10. Centrifuge at 20, 000 x *g* for 5 minutes at 4 ºC to pellet proteins.
11. Remove and discard supernatant and allow pellet to air-dry for 5 minutes.

12. Resuspend pellet in 100 µL of 1 x SDS Lysis Buffer containing 25 mM DTT (See Notes 26 and 27).

3.6 SDS-Polyacrylamide Gel electrophoresis and in-gel analysis.

1. Clean and dry two 1 mm spacer plates and two short plates by washing with 70 % ethanol and rinsing in H_20. Allow to dry.
2. Assemble the gel cassette by placing the casting frame upright with the pressure cams in the open position and facing forward on a flat surface. With the labeling on the spacer plate up, place a short plate on top and slide the two glass plates into the casting frame with the short plate facing the front (See Note 28). Secure the glass cassette sandwich in the casting frame by engaging the pressure cams. Place the casting frame onto the casting gasket, pressure cams facing outwards, and lock onto the casting stand by engaging the spring-loaded lever of the casting stand onto the spacer plate (See Note 29).
3. Prepare the resolving gel monomer. For two 12 % gels, mix 5 mL of Resolving gel buffer, 4 mL of 30 % acrylamide-bisacrylamide solution and 1 mL H_20. Add 200 µL of 10 % ammonium persulphate and 8 µL of TEMED and mix. Carefully pipette 4.5 mL of the resolving gel monomer solution between the glass plates and immediately overlay with 500 µL of propan-1-ol. Allow the gel to polymerize for 45 min. Pour off the propan-1-ol and rinse the gel surface completely with distilled water. Dry the top of the resolving gel with filter paper.
4. Prepare the stacking gel monomer. Mix 4 mL of Stacking gel buffer, 1.3 mL of 30 % acrylamide-bisacrylamide solution and 2.7 mL H_20. Add 200 µL of 10 % ammonium persulphate and 10 µL of TEMED and mix. Carefully pour on top of the resolving gel monomer until the top of the short plate is reached and insert a 15-well comb in the

gel cassette, aligning the ridge with the top of the short plate. Allow the gel to polymerize for 45 min (See Note 30).

5. Place the gel cassettes into the clamping frame with the short plates pointed inwards and clamp into place. Place the clamping frame in the tank and fill the inner and outer chamber with 1 L of Tris-Glycine electrophoresis buffer.
6. Heat the samples in a heat block for 5 minutes at 100 ºC to denature proteins. Centrifuge the samples briefly to bring down the condensate.
7. Load 5-20 μL/well of each sample into the wells with a Hamilton syringe or a pipette using gel loading tips along with 5 μL/well of prestained protein standards.
8. Place the lid on the Mini-PROTEAN Tetra tank ensuring to align the colour-coded banana plugs and jacks. Insert the electrical leads into the suitable power supply and electrophorese at 180 V constant until the dye front reaches the bottom of the gel.
9. Turn off the power supply, disconnect the electrical leads and remove the tank lid. Pour off and discard the running buffer.
10. Remove the gel cassettes from the assembly and separate the glass plates to remove the gels from the gel cassette. Place the gels in an incubation box containing H_20.
11. To visualize S-acylation of your expression protein via in-gel analysis, place the gel on the scanning surface of a LI-COR Odyssey Infrared Imagining system taking care not to trap bubbles underneath.
12. Scan the gel in the 800 nm channel using the Image Studio Software Acquire ribbon (See Note 31).

3.7 Electrophoretic transfer of proteins to nitrocellulose membranes

1. For each gel to be transferred, cut one nitrocellulose membrane and two Whatman filter paper to the dimensions of the gel (See Notes 32 and 33).

2. Soak the gel, cut nitrocellulose membranes and filter paper in transfer buffer.
3. Prepare the gel sandwich: Open the gel holder cassette from the Bio-Rad Trans-Blot Electrophoretic Transfer Cell with the grey side down. Place a pre-wetted fiber pad on the grey side of the cassette. Place the wet filter paper on the fiber pad and place the gel on top. Place the nitrocellulose membrane on top of the gel and the final piece of filter paper on top of the membrane (See Note 34).
4. Add a fiber pad on top and close and lock the gel holder cassette with the white latch.
5. Place the gel holder cassette in the tank, ensuring the grey side is towards the plate cathode, and completely fill the tank with transfer buffer.
6. Place the lid on the Transfer Cell and insert the electrical leads into the suitable power supply and run at 120 mA overnight.
7. Upon completion, disassemble the blotting sandwich and place the membrane in an incubation box containing PBS-T (See Note 35).

3.8 Western blotting

1. Wash membranes once in PBS-T.
2. Place the membranes in an incubation box and block the membranes in Blocking buffer for 45 minutes with gentle agitation.
3. Prepare the primary antibody by diluting in PBST using the manufacturer's recommended dilution for Western blot applications (See Note 36). Use enough antibody solution to completely cover the membrane. Incubate the membrane in diluted primary antibody for between 1 hour at room temperature to overnight at 4ºC, with gentle agitation (See Note 37).
4. Wash the membranes by pouring off the primary antibody solution and rinsing the membranes 5 X in PBS-T for 5 minutes each, with vigorous agitation (See Note 38).

5. Prepare the secondary antibody by diluting the IRDye secondary antibody 1:10,000 in secondary antibody diluent. Use enough antibody solution to completely cover the membrane. Incubate the membrane in diluted secondary antibody for 45 minutes at room temperature with gentle agitation.
6. Wash the membranes by pouring off and discarding the secondary antibody solution. Rinse the membranes 5 X in PBS-T for 5 minutes each, with vigorous agitation.
7. Rinse membranes in PBS to remove residual Tween 20 (See Note 39).
8. Image the blots using a LI-COR Odyssey Infrared Imagining system by placing the membranes on the scanning surface and taking care to ensure no bubbles are trapped underneath. Scan the membranes simultaneously in the 700 nm and 800 nm channels using the Image Studio Software Acquire ribbon.

4. Notes

1. Longer chain saturated fatty acid azide stocks (C18:0 and longer) may need warming until the solution becomes clear before use.
2. Add 100 mL of 10 X PBS to 900 mL of water for use.
3. IRDyes should be protected from light and repeated freeze-thaw cycles should be avoided.
4. Prepare immediately before use.
5. Acetone is flammable and should be stored in a spark-free freezer.
6. SDS precipitates at low temperatures; if this occurs, warm buffer in a water bath before use.
7. Add 250 µL of 4 X SDS Lysis buffer to 725 µL of water and add 25 µL of 1 M DTT.
8. The ammonium persulphate solution can be stored at 4 °C for 7 days.
9. Add 100 mL of 10 X Tris-Glycine electrophoresis buffer to 900 mL of water.

10. Add 250 mL of 10 X Transfer buffer to 1750 mL of water and add 500 mL of methanol.
11. Add 2 mL of 10 % Tween 20 to 1 L of PBS.
12. This should be made up fresh each time.
13. Store the IRDye secondary antibody in darkness and minimize exposure to light.
14. For dual imaging the system requires two infrared channels with 685 and 785 nm lasers.
15. Perform reaction under a N_2 atmosphere.
16. For the mono-bromination, reaction time and vigorous stirring were crucial for success to prevent bis-bromination.
17. For azide addition the reaction should be monitored carefully by TLC to ensure the reaction has reached completion. Addition of additional azide was found to be problematic with this process.
18. For the reduction of the bis-carboxylic acids the reaction generally proceeded efficiently at room temperature, however, this was not reliable with 1,18-octadecanedioic acid, therefore for reproducibility we used 1,18-dimethyl octadecanedioate as the substrate, performing the reduction at reflux in THF.
19. It is possible to scale up the transfection by multiplying by the number of wells to be transfected.
20. For incubation times of up to 8 hours, it is not necessary to use aseptic techniques.
21. Depending on the expression level and extent of S-acylation of the protein of interest, it may be necessary to increase the incubation time beyond 4 hours. In this case, aseptic techniques should be used especially in the case of overnight incubations. Prepare the DMEM containing L-Glutamine supplemented with 1% fatty-acid free

BSA and 100µM fatty acid azide and in a laminar flow hood use a syringe to pass the media through a 0.22 µm sterile filter.

22. Cell lysates may be frozen by directly transferring the plates to -20 ºC allowing the procedure to be continued at a later date.
23. This should be made up fresh each time. The ascorbic acid may take several minutes to dissolve.
24. The total volume per lysate should equal 80µl. If performing multiple click reactions, scale the reaction volume up accordingly and aliquot 80 µl of the click reaction mix per experimental replicate into separate 1.5 ml tubes.
25. Final concentrations are 2.5 µM Azide Dye, 2mM $CuSO_4$, 0.2mM TBTA and 4mM Ascorbic Acid. Total volume including lysate is 200µl.
26. Samples may be stored at -20 ºC allowing the procedure to be continued at a later date.
27. Vigorous vortexing may be required to ensure pellet is fully resuspended in the SDS Lysis Buffer.
28. Ensure that the plates are flush on a level surface to minimize the risk of leaking.
29. Ensure the grey casting stand gaskets are clean and dry before use.
30. Gels in a cassette sandwich may be made ahead and stored at 4 °C for a few days. Ensure that the gels remain hydrated by wrapping the glass plates first in tissue paper soaked in water and then in cling film.
31. If the signal is too strong, the saturated pixels will appear as cyan in the image. In this case, re-scan using a lower intensity setting.
32. We cut membranes to 6 x 9cm.
33. Always wear gloves when handling membranes to prevent contamination.

34. Remove any air bubbles which may have formed by using a glass tube to roll out any air bubbles.
35. The membranes can be stored in PBS-T for up to 48 hours in the dark at 4 °C.
36. For the anti-GFP JL8 antibody, we dilute 1:3000.
37. Optimal incubation times will vary depending on the primary antibody.
38. The diluted primary antibody solution may be stored at – 20 ºC and reused.
39. If the blot is prepared in advance, air-dry the blot and store in the dark until ready to image.

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