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TITLE:

Detection of Protein S-Acylation using Acyl-Resin Assisted Capture

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Biochemistry, immunology, cell biology, S-acylation, palmitoylation, post-translational modification, Acyl-RAC, splenocytes, lymphocytes.

SUMMARY:

Acyl-RAC (Acyl-Resin Assisted Capture) is a highly sensitive, reliable and easy to perform method to detect reversible lipid modification of cysteine residues (S-acylation) in a variety of biological samples.

ABSTRACT:

Protein S-acylation, also referred to as S-palmitoylation, is a reversible post-translational modification of cysteine residues with long-chain fatty acids via a labile thioester bond. S-acylation, which is emerging as a widespread regulatory mechanism, can modulate almost all aspects of the biological activity of proteins, from complex formation to protein trafficking and protein stability. The recent progress in understanding of the biological function of protein S-acylation was achieved largely due to the development of novel biochemical tools allowing robust and sensitive detection of protein S-acylation in a variety of biological samples. Here, we describe acyl resin-assisted capture (Acyl-RAC), a recently developed method based on selective capture of endogenously S-acylated proteins by thiol-reactive Sepharose beads. Compared to existing approaches, Acyl-RAC requires fewer steps and can yield more reliable results when coupled with mass spectrometry for identification of novel S-acylation targets. A major limitation in this technique is the lack of ability to discriminate between fatty acid species attached to cysteines via the same thioester bond.

INTRODUCTION:

S-acylation is a reversible post-translational modification involving addition of a fatty acyl chain to an internal cysteine residue on a target protein via a labile thioester bond[1]. It was first reported as a modification of proteins with palmitate, a saturated 16-carbon fatty acid[2], and therefore this modification is often referred to as S-palmitoylation. In addition to palmitate, proteins can be reversibly modified by a variety of longer and shorter saturated (myristate and stearate), monounsaturated (oleate) and polyunsaturated (arachidonate and eicosapentanoate) fatty acids[3–7]. In eukaryotic cells, S-acylation is catalyzed by a family of enzymes known as DHHC protein acyltransferases and the reverse reaction of cysteine deacylation is catalyzed by protein thioesterases, most of which still remain enigmatic[8].

The lability of the thioester bond makes this lipid modification reversible, allowing it to dynamically regulate protein clustering, plasma membrane localization, intracellular trafficking, protein-protein interactions and protein stability[9,10]. Consequently, S-acylation has been linked to several disorders including Huntington's disease, Alzheimer's disease and several types of cancer (prostate, gastric, bladder, lung, colorectal), which necessitates development of reliable methods to detect this post-translational protein modification[11].

Metabolic labeling with radioactive ([^{3}H], [^{14}C] or [^{125}I]) palmitate was one of the first approaches developed to assay protein S-acylation[12–14]. However, radiolabeling-based methods present health concerns, are not very sensitive, time consuming, and only detect lipidation of highly abundant proteins[15]. A faster and nonradioactive alternative to radiolabeling is metabolic labeling with bioorthogonal fatty acid probes, which is used routinely to assay dynamics of protein S-acylation[16]. In this method, a fatty acid with a chemical reporter (alkyne or azide group) is incorporated into the S-acylated protein by a protein acyltransferase. Azide-alkyne Huisgen cycloaddition reaction (click chemistry) can then be used to attach a functionalized group, such as a fluorophore or biotin, to the integrated fatty acid allowing for detection of the S-acylated protein[17–19].

Acyl-biotin exchange (ABE) is one of the extensively used biochemical methods for capture and identification of S-acylated proteins that bypasses some of the shortcomings of metabolic labeling such as unsuitability for tissue samples[15]. This method can be applied for analysis of S-acylation in a diverse range of biological samples, including tissues and frozen cell samples[20,21]. This method is based on selective cleavage of the thioester bond between the acyl group and the cysteine residue by neutral hydroxylamine. The liberated thiol groups are then captured with a thiol-reactive biotin derivative. The generated biotinylated proteins are then affinity-purified using streptavidin agarose and analyzed by immunoblotting.

An alternative approach termed acyl-resin assisted capture (Acyl-RAC) was later introduced to replace the biotinylation step with direct conjugation of free cysteines by a thiol-reactive resin[22,23]. This method has fewer steps compared to ABE and similarly can be used to detect protein S-acylation in a wide range of samples[1].

Acyl-RAC consists of 4 main steps (**Figure 1**),

1. Blocking of free thiol groups;
2. Selective cleavage of the cysteine-acyl thioester bond with neutral hydroxylamine (HAM) to expose cysteine thiol groups;
3. Capturing of the lipidated cysteines with a thiol-reactive resin;
4. Selective enrichment of the S-acylated proteins after elution with reducing buffer.

The captured proteins can then be analyzed by immunoblotting or subjected to mass spectrometry (MS) based proteomics to assess the S-acylated proteome in a varied range of species and tissues[22,24,25]. Individual S-acylation sites can also be identified by trypsin digestion of the captured proteins and analysis of the resulting peptides by LC-MS/MS[22]. Here, we demonstrate how acyl-RAC can be used for simultaneous detection of S-acylation of multiple proteins in both a cell line and a tissue sample.

PROTOCOL:

Mice used in this protocol were euthanized according to NIH guidelines. The Animal Welfare Committee at University of Texas Health Science Center in Houston approved all animal work.

1. Preparation of cell lysates

1.1. Prepare lysis buffer as described in **Table 1**. To 10 mL of PBS, add 0.1 g of n-dodecyl β-D-maltoside detergent (DDM) and rotate to dissolve. Add 100 µL of phosphatase inhibitor cocktail 2, ML211 (10 µM), PMSF (10 mM) and protease inhibitor cocktail (1x) and chill the buffer on ice before use.

1.2. Transfer required media containing cells from the incubator into 15 mL or 50 mL conical tubes and spin cells at 350 x *g* for 5 min and aspirate to get rid of any cell debris.

NOTE: We used 1 x 10^7 cells for each acyl-RAC reaction to be performed.

1.3. Wash the pellet by resuspending it in 5 mL of PBS and spinning at 350 x *g* for 5 min.

NOTE: Perform step 1.3 quickly to avoid cell lysis due to extended incubation in PBS.

1.4. Add 600 µL of lysis buffer prepared in step 1.1 to the pellet and lyse it by shaking at 1500 rpm in a thermal shaker for 30 min at 4 °C.

1.5. Clear lysates by centrifugation at 20,000 x *g* at 4 ˚C for 30 min to pellet detergent-insoluble materials. Collect cleared lysate in pre-cooled 1.5 mL microfuge tubes and keep them on ice.

1.6. Perform a Bradford/BCA assay to estimate protein concentration. It is critical to ensure the same amount of protein across different samples prior to performing the experiment. We recommend using at least 500 µg of protein per reaction.

NOTE: In the experiments described, we used cultured (Jurkat) cells and primary splenocytes from mice tissue. The lysis method described above can be adopted to other cell types as well. The average protein concentration obtained for the abovementioned cell types is approximately 500 µg per 1 x 10^7 cells. Jurkat cells were maintained in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4,500 mg/L glucose, and 1,500 mg/L sodium bicarbonate supplemented with 10% FBS at 37 °C with 5% CO_2. We used 1 x 10^7 Jurkat cells per reaction. Primary splenocytes were isolated from mouse spleen tissue as described[26]. Briefly, spleen tissue was macerated on ice, followed by lysis of erythrocytes in hypotonic solution and separation from the lymphocytes by centrifugation. We used 1 x 10^7 primary cells per reaction.

2. Acyl-RAC: Blocking of free thiol groups

NOTE: All subsequent steps can be performed at room temperature (RT).

2.1. Transfer lysate into a fresh 1.5 mL microfuge tube and perform chloroform-methanol (CM) precipitation as described below.

2.1.1. Add methanol (MeOH) and chloroform ($CHCl_3$) to the lysate at a final ratio of lysate: MeOH: $CHCl_3$ of 2:2:1 and shake vigorously to create a homogeneous suspension.

2.1.2. Spin at 10,000 x *g* for 5 min to form a pellet ("pancake") at the interphase between aqueous and organic phases.

2.1.3. Tilt the tube and aspirate as much solvent as possible using a needle or a gel loading tip.

2.1.4. Air dry the protein pellet for a few minutes and gently wash it by adding 600 µL of MeOH and mixing gently to avoid breaking up the pellet

2.1.5. Carefully remove the remaining MeOH and dry the protein pellet on a benchtop for approximately 5 min.

2.1.6. (Optional) Perform an additional centrifugation step to spin down any broken pellet after the MeOH wash to avoid loss of sample.

NOTE: The experiment can be stopped after the CM precipitation step. Once the pellet is obtained, it may be stored in 500 µL of MeOH in -20 °C up to a week.

2.1.6.1. Dissolve the protein pellet in 200 µL of 2SHB buffer by vortexing at 42 °C/1500 rpm in a thermal shaker until the pellet is dissolved.

2.1.7. (Optional) Incubate for an additional 5–10 min in a sonicating water bath to dissolve the pellet.

NOTE: The length of sonication varies depending on solubility of the material. However, prolonged sonication can cause protein degradation.

2.2. Prepare 0.2% methyl methanethiosulfonate (MMTS) (v/v) in 2SHB by adding 2 µL of MMTS to 998 µL of 2SHB buffer.

NOTE: Use freshly prepared 0.2% MMTS for each experiment.

2.3. Add 200 µL of 0.2% MMTS in 2SHB to each tube to a final concentration of 0.1% MMTS. Incubate for 15 min at 42 ˚C with shaking at 1500 rpm in a thermal shaker.

3. Acyl-RAC: Hydroxylamine (HAM) cleavage and capture of S-acylated proteins

3.1. Repeat 3-4x CM precipitations as described above to remove MMTS. Removal of MMTS can be estimated by the lack of distinct odor of MMTS. After each precipitation, dissolve the pellet in 100 µL of 2SHB buffer by vortexing at 42 ˚C/1500 rpm in a thermal shaker until the pellet dissolves, and then dilute with 300 µL of Buffer A.

3.2. After final precipitation, dissolve samples in 200 µL of 2SHB buffer as described above and dilute with 240 µL of Buffer A.

3.3. In case of comparing changes in S-acylation in response to several treatment conditions, measure protein concentration again and proceed with equal amount of protein for each condition.

3.4. Retain 40 µL from each sample as an input control.

3.5. Split samples into two equal parts of 200 µL and mark tubes as “+ HAM” and “- HAM”. Add 50 µL of freshly prepared neutral 2 M HAM (pH 7.0-7.5) to a final concentration of 400 mM to one of the tubes (+ HAM) and 50 µL of neutral 2 M NaCl to the second tube (- HAM), which will be used as a negative control. Proceed to addition of thiopropyl-Sepharose (TS) beads.

NOTE: The experiment can be stopped after any of the CM precipitation steps. Once the pellet is obtained, it may be stored in 500 µL of MeOH in -20 °C up to a week. Neutral pH of 2 M HAM ensures its selectivity for the acyl-cysteine thioester bond and should be carefully adjusted. Care should be taken when handling samples in 2SHB buffer to avoid loss of sample due to excessive foaming. All following steps are identical for - HAM and + HAM samples.

3.6. Add 30 µL of TS bead-slurry to each tube and rotate the tubes for 1-2 h at RT.

3.7. Wash the TS beads 4x with 1% SDS in Buffer A to remove residual HAM.

3.7.1. Gently spin down all bead samples using a microfuge for 1 min and carefully aspirate the supernatant.

3.7.2. Resuspend the beads in 500 µL of 1% SDS in Buffer A.

3.7.3. Repeat step 3.7.1–3.7.2 thrice.

NOTE: Activated thiopropyl-Sepharose (TS) is supplied freeze-dried in the presence of additives that must be washed away at neutral pH before coupling. Distilled water is recommended for swelling and washing. Weigh 0.1 g of beads and resuspend in 1 mL of distilled water and allow it to swell while rotating for 30 min-1 h at RT. Wash beads 1x with buffer A and prepare a slurry with buffer A, in a ratio of 50% settled medium to 50% buffer. Swollen TS may be stored at neutral pH in the presence of 20% ethanol at 4 °C up to a week. Do not use sodium azide as a bacteriostatic agent since azide ions react with the 2-pyridyl disulfide groups. For higher efficiency, prepare fresh beads. While handling the beads, the tip of a P200 pipette tip can be cut slightly so as to prevent any damage.

NOTE: The experiment can be stopped at any stage after CM precipitation. The pellet may be store in 500 µL of MeOH in -20 °C up to a week.

4. Elution and detection of S-acylated proteins

4.1. After the last wash, gently spin down the beads as described above and aspirate as much supernatant as possible without disturbing the beads.

4.2. Recover the proteins from beads with 4x SDS sample buffer with DTT.

4.2.1. Add 50 µL of 4x SDS sample buffer to the beads and incubate at 80 ˚C, 1500 rpm for 15 min in a thermal shaker. Let the tubes cool.

4.2.2. Centrifuge the beads at 5,000 x *g* for 3 min to completely pellet the beads and transfer the eluted proteins to a fresh 1.5 mL tube using a gel loading tip.

4.3. Run SDS-PAGE and analyze S-acylation of the protein(s) of interest by western blotting.

REPRESENTATIVE RESULTS:

Following the protocol described above, we first used acyl-RAC to simultaneously detect S-acylation of several proteins in Jurkat cells, an immortalized T cell line originally derived from the peripheral blood of a T cell leukemia patient[27]. Regulatory T cell proteins previously identified as S-acylated[9,28,29] were chosen to demonstrate the utility of this method. As shown in **Figure 2A**, tyrosine kinase Lck can be detected in lysates treated with neutral 2 M hydroxylamine to cleave the thioester bond between cysteine residues and a fatty acid moiety (+ HAM lane). The sample treated with 2 M NaCl (- HAM lane) represents an important negative control, indicating the selectivity of the assay for detection of the thioester bonds. The input lane further confirms the specificity of the signal and can serve as a positive control if the protein of interest is not S-acylated. The membrane was then stripped and reprobed with antibodies against proteins Fyn

and LAT to demonstrate that the acyl-RAC assay can be used to analyze S-acylation of multiple proteins at the same time (**Figure 2A**).

To demonstrate the utility of this method to detect protein S-acylation in tissue samples, we applied the acyl-RAC protocol to the mouse spleen. As shown in **Figure 2B,** S-acylation of Lck, Fyn and LAT can be readily detected in primary mouse splenocytes indicating that this modification is conserved between two species.

FIGURES AND TABLES

Figure 1. Schematic representation of acyl-RAC. The method consists of 4 main steps: (1) blocking free thiol groups with methyl methanethiosulfonate (MMTS), (2) selective cleavage of the cysteine-acyl thioester bond with neutral hydroxylamine (HAM) to expose cysteine thiol groups, (3) capture of proteins with newly exposed cysteine thiol by direct conjugation with a thiol-reactive resin and (4) recovery of captured proteins using a reducing elution buffer, followed by immunoblotting or MS analysis.

Figure 2. **Detection of S-acylated proteins**. An acyl-RAC assay was used to detect S-acylation of T cell signaling proteins in Jurkat T cells (**A**) and murine spleen tissue (**B**). Immunoblotting with Lck-, Fyn- and LAT-specific antibodies shows S-acylation of these proteins in the hydroxylamine treated sample (+ HAM lane). A sample treated with sodium chloride (- HAM lane) serves as negative control. Untreated lysates are shown in the input lane.

Table 1: Buffer composition of commonly used buffers required in the protocol. 2SHB buffer and Buffer A can be prepared in advance but their pH must be checked regularly. Lysis buffer and HAM must be prepared the day of experiment.

DISCUSSION:

Here, we successfully utilized the acyl-RAC assay to detect S-acylation of selected proteins in both cultured human cells and primary cells derived from mouse tissue. This method is simple, sensitive, and can be easily performed with minimal equipment requirements using standard biochemistry techniques. This method has been shown to successfully identify novel S-acylated proteins such as the β-subunit of the protein translocating system (Sec61b), ribosomal protein S11 (Rps11), and microsomal glutathione-*S*-transferase 3 (MGST3)[22]. The sensitivity and adaptability of acyl-RAC makes it suitable to study protein S-acylation in a variety of biological samples.

A significant fraction of S-acylated proteins can be associated with glycosphingolipid-enriched parts of the plasma membrane, also known as lipid rafts. Thus, some S-acylated proteins might not be fully extracted if mild detergents, such as Triton X-100, NP-40 or Brij58, are used for lysis[30]. Anionic detergents (such as SDS) or maltoside based non-ionic detergents (such as n-dodecyl β-D-maltoside (DDM)) can be used to ensure dissociation of lipid rafts and full recovery of the proteins of interest[30,31,32]. In this protocol, we used DDM, a mild, lipid-like non-ionic detergent that has been demonstrated to be effective in membrane protein extraction and maintaining their stable native state over prolonged periods[33].

The unregulated thioesterase activity during lysis of the sample can potentially result in reduced protein yield after the TS pull-down step and thus affect the detection of S-acylated proteins. A thioesterase inhibitor, such as ML211, a dual inhibitor for Acyl-Protein Thioesterase 1 (APT1, LYPLA1) and Acyl-Protein Thioesterase 2 (APT2, LYPLA2) can be added to prevent indiscriminate deacylation of proteins in the lysate[34,35].

An incomplete blockade of cysteine residues can cause binding of non-acylated proteins to TS, thus resulting in a high background evident by high signal in the –HAM samples. The background binding can be substantially reduced by an additional incubation with a thiol-crosslinking agent-2,2'-dithiodipyridine as described in a modified ABE protocol by Zhou et al.[36].

In contrast to metabolic labeling, acyl-RAC is not limited to live cells and can be performed to detect protein S-acylation in freshly isolated or frozen tissue samples. Since the acyl-RAC protocol avoids an immunoprecipitation step, it can be used to simultaneously detect lipidation of multiple proteins of interest in the same assay, thus potentially reducing the amount of biological material required in a single experiment. However, metabolic labeling-based assays are more suitable for experiments aimed to specifically detect de novo S-acylation or measure fatty acid turnover rates on a protein of interest. Another important limitation of acyl-RAC is that this technique is unable to distinguish between different fatty acids that can be covalently bound to the cysteine residues via the same thioester bond[3–7].

Both acyl-RAC and ABE assays are based on selective cleavage of the thioester bond between the cysteine residue and a fatty acid to reveal a free thiol group. Since acyl-RAC uses direct pull-down of S-acylated proteins by a thiol-reactive resin, this method requires fewer steps in comparison to ABE. Although similar, proteome-wide studies of S-acylation show some variation in the proteins detected using these two methods, most likely due to the technical differences. For example, in rat brain homogenate, ABE-based analysis identified 241 S-acylated proteins whereas acyl-RAC-based analysis identified 144[25]. 61 proteins in the rat brain proteome were commonly detected by both methods underscoring the need for use of multiple techniques to ascertain the S-acylation status of a protein.

Another potential drawback of acyl-RAC and ABE methods is the inability to reliably evaluate stoichiometry of S-acylation and determine the number of lipidated cysteines. A modification of these techniques, termed acyl-PEG exchange or PEG-shift assay, was developed to address these limitations[37,38]. In this method, pull-down step following hydroxylamine cleavage is substituted by incubation with a PEG-maleimide mass-tag. Resulting PEGylation of the exposed free cysteine residues can be observed as mass shift by SDS-PAGE.

In conclusion, acyl-RAC is a fast, sensitive and reliable method that can provide valuable insights into dynamics of protein S-acylation under both physiological and pathophysiological conditions in a great variety of biological samples. Considering limitations of the method discussed above, a combination of acyl-RAC with other techniques is recommended to fully characterize S-acylation of a protein of interest.

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DISCLOSURES:

No conflicts of interest declared.

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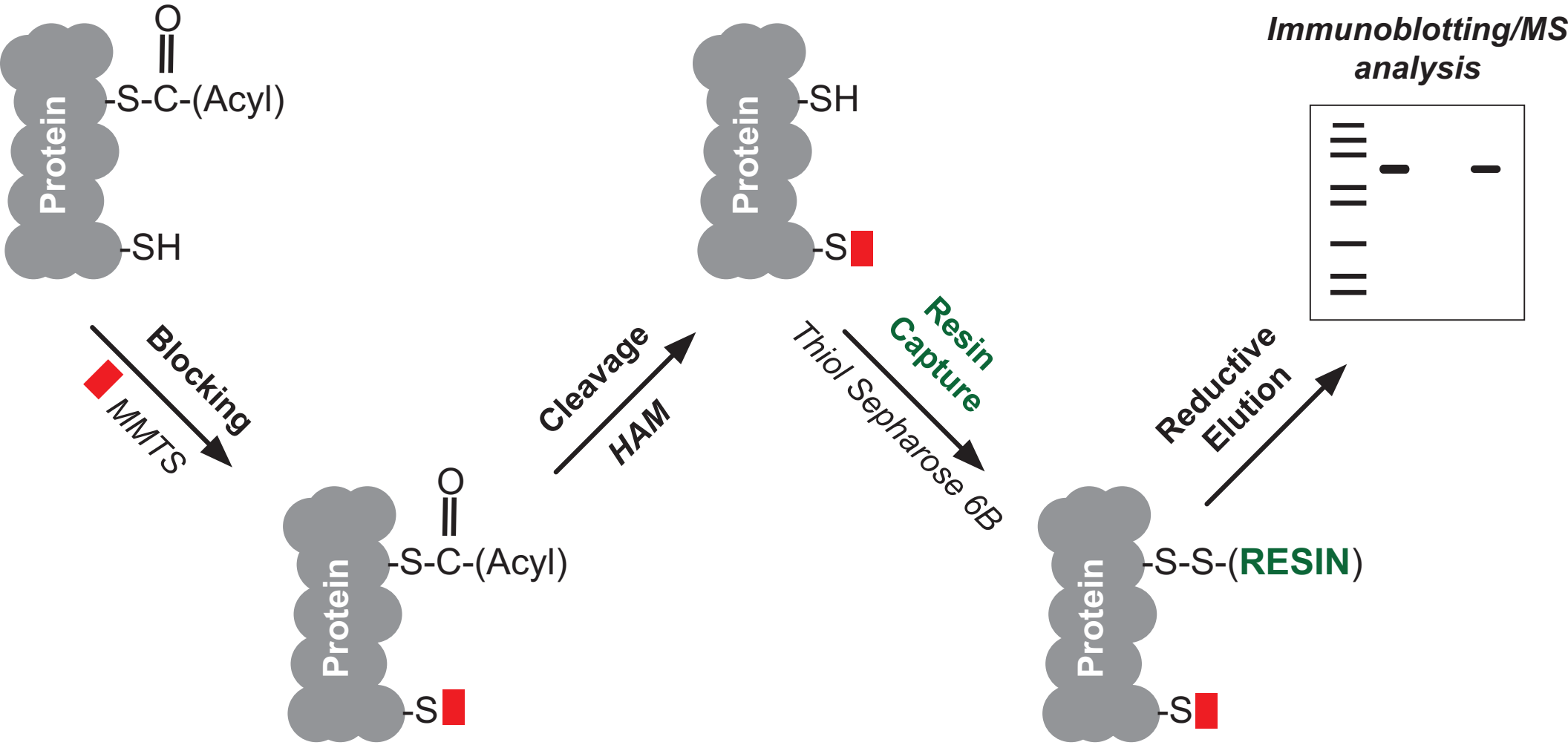
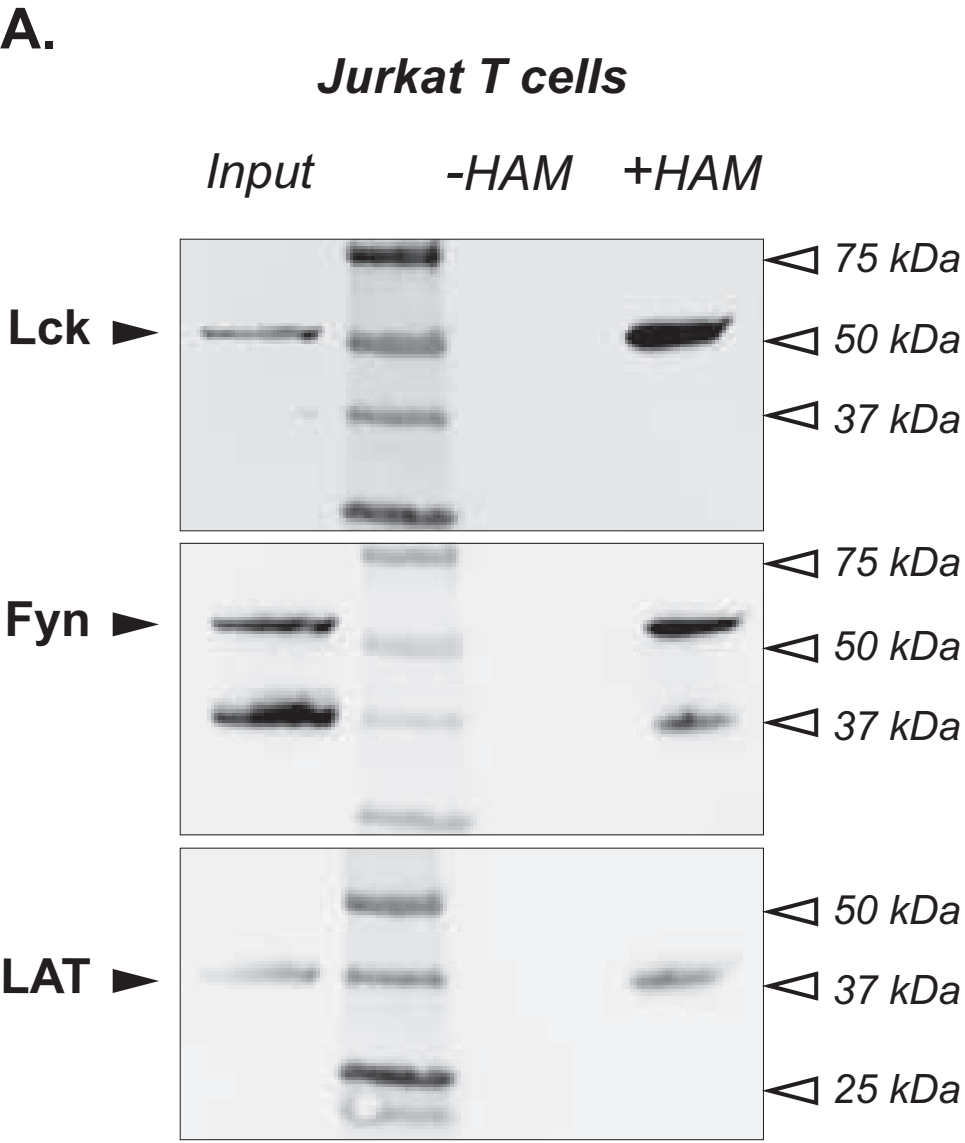
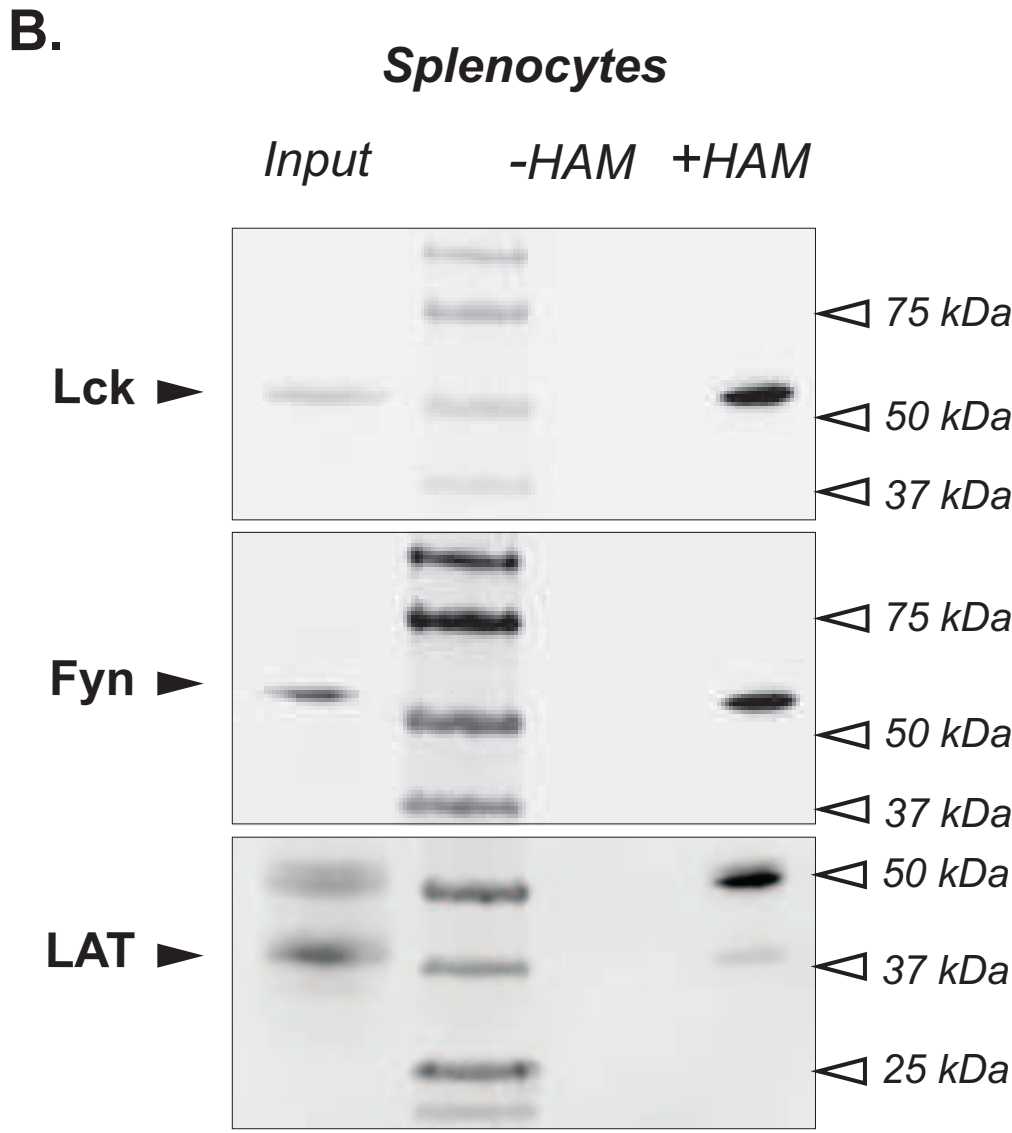
Figure







Buffer	Composition	Notes
Lysis Buffer (LB)	1% DDM in DPBS; 10 µM ML211; Phosphatase Inhibitor Cocktail 2 (1:100); Protease Inhibitor Cocktail (1X), PMSF (10 mM)	Prepare fresh, chill at 4 ˚C before use.
SDS Buffer (2SHB)	2% SDS; 5 mM EDTA; 100 mM HEPES; pH 7.4	
Buffer A	5 mM EDTA; 100 mM HEPES; pH 7.4	
Hydroxylamine (HAM)	2 M stock solution in distilled H_20	Prepare fresh. When first dissolved, HAM has a very low pH. Use 5 M NaOH to pH to 7-7.5.
4X SDS sample buffer	200 mM Tris-Cl (pH 6.8), 8% SDS (sodium dodecyl sulfate) 0.01% Bromophenol blue, 10% glycerol	Supplement with 5 mM DTT just before use.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
cOmplete Protease Inhibitor Cocktail t	Sigma	11836170001	
Eppendorf Centrifuge 5424	Eppendorf	22620444	
Hydroxylamine (HAM)	Sigma	159417	
Methyl methanethiosulfonate (MMTS)	Sigma	64306	
Mini tube rotator	LabForce		
ML211	Cayman	17630	
Multi-Therm Cool-Heat-Shake	Benchmark Scientific	H5000-HC	
n-Dodecyl β-D-maltoside (DDM)	Sigma	D641	
Phosphatase Inhibitor Cocktail 2	Sigma	P5726	
Thiopropyl-Sepharose 6B (TS)	Sigma	T8387	
Ultrasonics Quantrex Sonicator	L & R		

UTHealth™
The University of Texas Health Science Center at Houston

McGovern Medical School
Biochemistry & Molecular Biology

Askar M. Akimzhanov, PhD
Assistant Professor

December 18, 2019

Ronald Myers, PhD.
Senior Science Editor
JoVE
1 Alewife Center,
Suite 200,
Cambridge, MA 02140

Dear Dr. Myers,

Please find attached our revised manuscript entitled "**Detection of Protein S-Acylation using Acyl-Resin Assisted Capture**" for your consideration as a part of "Methods to detect protein S-palmitoylation" collection in Journal of Visualized Experiments (JoVE). The text is ~28,000 characters including spaces, with two figures and two tables.

We thank the reviewers for critically examining our manuscript and for their valuable suggestions. We have addressed the insightful editorial and reviewers' comments to the best of our knowledge. We have expanded the Discussion section to include critical steps in the protocol and troubleshooting. The reviewers' comments have been addressed in a point counterpoint format.

Additionally, an ethics statement has been added in the protocol and the issue with references has been resolved. The table of materials has been expanded to include all materials and equipment used in the protocol.

We look forward to seeing this manuscript in publication soon as well as future collaborations with JoVE.

Sincerely,
Askar Akimzhanov, Ph.D.

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Editorial comments:

We thank the editor for their time in providing insightful comments on our manuscript.

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for the suggestion. We have proofread the document thoroughly.

Protocol:
1. Please ensure all centrifuge speeds are expressed as centrifugal force (x g) instead of revolutions per minute (rpm).

We have replaced rpm with X g wherever applicable.

2. If necessary, please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement has now been added to the protocol in line 121.

3. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have followed the above guideline throughout the protocol.

Figures:
1. Please remove titles and legends from the Figures.

Figures have been modified accordingly.

Discussion:
1. Please include information on critical steps of the procedure, modifications, and troubleshooting in the Discussion.

Discussion section has been significantly expanded to include all reviewers’ suggestions.

References:
1. Please resolve the inverted parentheses about issue numbers.
2. Please do not abbreviate journal titles.

Issue with the references has been resolved.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Table of materials has been expanded to include all reagents and equipment used in the protocol.

Reviewers' comments:
Reviewer #1:

We thank the reviewer for carefully reading our manuscript and for their positive criticism. We have addressed the major and minor concerns as explained below.
Major Concerns:

* In line 50, can authors put references for isotopes 14C and 125I used for studying S-acylation?

Thank you for pointing out the missing references. We have added the pertinent references- Drahansky, M. et al, 2016 and Resh, M.D. et al., 2006.

* In line 132, after 2.1.4 authors can add step to carry out additional centrifugation to pellet down any broken pellet during MeOH wash in order to avoid loss of sample.

Thank you for the suggestion. We have added the pertinent centrifugation step: "(Optional) An additional centrifugation step may be performed to spin down any broken pellet after the MeOH wash to avoid loss of sample."

* In line 136, what conditions authors would like to recommend sonicate the pellet?

We used an Ultrasonics sonicator from L & R with input power average wattage- 117 Vac, 60 Hz 415 (815 w/heat), 3.547 amps, which has been added in table 2. The length of sonication varies depending on solubility of the material. We usually sonicate the sample for 5-10 minutes. An additional step and a note were added to the protocol in line 153.

* In line 141, 2SHB has 2% SDS which is a lot. Vigorous shaking of samples will generate lot of bubbles. Did authors consider that because of bubbles homogeneity of the samples is compromised and it might affect the overall efficiency of assay in some ways? What is the minimum concentration of SDS can be used to efficiently perform this experiment?

Thank you for a valid concern. However, in our experiments we have not observed excessive foaming or loss of sample upon addition of 2SHB. We do realize this might be a concern in some experiments, and thus have added a note of caution in the protocol in line 193: "NOTE 3: Care should be taken when handling samples in 2SHB buffer to avoid any loss of sample to excessive foaming."

* Though the whole protocol can be done in a single day, can authors comment on what stages a reader can stop if he/she wants to perform this experiment in multiple days due to any time crunch situations?

Thank you for pointing out the error in our part to omit reporting the several steps of respite in this protocol. We have added the option of stopping the experiment and storing the pellet in methanol in -20 ˚C in lines 147 and 214.

* In line 156, how long samples should be incubated with HAM before addition of beads?

The beads are added immediately after HAM addition. We have made the sentence clearer in lines 182-186: "Split samples into two equal parts of 200 µL and mark tubes as "+ HAM" and "- HAM". Add 50 µL of freshly prepared neutral 2 M HAM (pH 7.0-7.5) to a final concentration of 400 mM to one of the tubes (+ HAM) and 50 µL of neutral 2 M NaCl to second tube (- HAM) which will be used as a negative control. Proceed to addition of Thiopropyl-Sepharose (TS) beads."

* How long beads can be stored safely? Do their activity decrease over time?

The beads can be stored upto a week as added in line 209.

* For more clarity, authors might want to consider changing order of the statement in line 172-4 to, "Swollen TS should be stored at neutral pH in the presence of 20% ethanol at 4°C. Wash beads 1X with buffer A and prepare a slurry with buffer A, in a ratio of 50% settled medium to 50% buffer."

The paragraph has been re-arranged for more clarity.

* Statement in line 207-209 seems unnecessary in this manuscript.

The statement has been removed.

* For line 152, as multiple CM precipitations are being done in step before, taking input directly for analysis is okay for the experiment done by authors in this manuscript which is just to show existence of S-acylation. However, if one wants to compare changes in S-acylation upon various treatments, considering all samples equal after 3-4 CM precipitation is too simplistic. Authors are advised to add another step stating perform protein measurement and take equal amount of protein for following ±HAM treatments and input samples.

In our hands, we have not observed uneven loss of sample after CM precipitations when comparing changes in S-acylation in response to different treatments. However, we agree that this is a concern and thus we have added an additional step to measure protein concentration in line 178: "In case of comparing changes in S-acylation in response to several treatment

conditions, measure protein concentration again and proceed with equal amount of protein for each condition".

Minor Concerns:

* Ref 1 is repeated as Ref 24.

Ref 24 has been deleted

* In line 50, authors may also add that radiolabeling based method has health concerns.

The health hazard from radiolabeling has been highlighted in line 50.

* In fig. 1, full name for MMTS and HAM can be written in the legend.

Abbreviations have been expanded in the legend.

* A statement about what is the average protein concentration authors get with that many cells will be good in NOTE 1 in line 109.

Average protein concentration has been added in Note 1, line 109.

* May be for generality in 1.6 write "measurement of protein concentration performed standard method (Bradford/BCA)".

BCA assay has been added in 1.6

* In line 155, concentration of HAM written does not match according to the dilution of 2M HAM suggested by authors.

Thank you for thoroughly reading the protocol and pointing out our error in reporting the volumes used for dilution. We have corrected the text as follows: "After final precipitation, dissolve samples in 200 µL of 2SHB buffer as described above and dilute with 240 µL of Buffer A. In case of comparing changes in S-acylation in response to several treatment conditions, measure protein concentration again and proceed with equal amount of protein for each condition. Retain 40 µL from each sample as an input control. Split samples into two equal parts of 200 µL and mark tubes as "+ HAM" and "- HAM". Add 50 µL of freshly prepared neutral 2 M HAM (pH 7.0-7.5) to a final concentration of 400 mM to one of the tubes (+ HAM) and 50 µL of neutral 2 M NaCl to second tube (- HAM) which will be used as a negative control. Proceed to addition of Thiopropyl-Sepharose (TS) beads."

* In Line 163, does author really mean 0.5 x g? That seem too low speed.

Thank you for pointing this out. For simplicity, we have added in line 198: "Gently spin down all samples' beads using a microfuge for 1 min and carefully aspirate the supernatant."

* In Figure 2A&2B, rough size of the protein band/marker will be good to have.

Size of the protein markers have been added in Figures 2A & 2B.

Reviewer #2:

We thank the reviewer for their positive feedback. The minor issues have been addressed as explained below:

Acyl-RAC, which was originally published by the Casey group in 2010, is one of several common methods for the enrichment of S-acylated proteins. In the manuscript, Tewari et al. provided a detailed protocol for using Acyl-RAC to enrich S-acylated proteins from human Jurkat cells and mouse spleen. Overall, the manuscript was written well. However, some minor issues need to be addressed.

1. Throughout the manuscript, were 1×106 or 1×107 cells used for palmitoyl-protein enrichment? "10*106" is not a standard way of describing the number.

Thank you for pointing this error out. We have added the standard way of describing numbers in lines 92, 114 and 119.

2. For centrifugation, the unit of centrifuge rotor speed (PRM) needs to be converted into ×g.

Line 94: What is "1.1"?

1.1 refers to explanation of preparation of lysis buffer described in the first point of first step in the protocol.

4. Line 97: Detergent-insoluble pellets might contain lipid rafts. If this is the case, some lipid raft-resident palmitoyl-proteins may not be recovered by using the protocol. Please discuss this point in the Discussion section.

This is a valid concern. Consequently, we have added the rationale behind using DDM detergent in our Discussion section in lines 257-264" A significant fraction of S-acylated proteins can be associated with glycosphingolipid-enriched parts of the plasma membrane, also known as lipid rafts. Thus, some S-acylated proteins might not be fully extracted if mild detergents, such as Triton X-100, NP-40 or Brij58, are used for lysis[30]. Anionic detergents (such as SDS) or maltoside based non-ionic detergents (such as n-Dodecyl β-D-maltoside, DDM) can be used to ensure dissociation of lipid rafts and full recovery of the proteins of interest[31,30,32]. In this protocol, we used DDM, a

mild, lipid -like non-ionic detergent which has been demonstrated to be effective in membrane protein extraction and maintaining their stable native state over prolonged periods[33].”

5. Line 135: How was the 42°C, 1500 rpm condition achieved? Using a ThermoMixer?

Thank you for pointing out our error in omitting mentioning the Multi-Therm thermal shaker. We have added the same to the list of equipment in table 2.

6. Line 155: What was the pH of the 2 M NaCl solution?

We have added neutral pH in line 179.

7. Line 160: Please clarify whether 30 µL of beads or 30 µL of slurry was transferred to each tube.

30 µL of slurry was transferred and has been corrected in the text.

8. Line 183: How was the 80°C, 1500 rpm condition achieved?

Thank you for pointing out our error in omitting mentioning the Multi-Therm thermal shaker.

9. Line 185: Won't the beads be broken under such a high speed (16,000×g) for 3 min?

The high speed is mentioned only for the last step to completely pellet the beads and collect the eluted proteins. However, we agree such high speed is probably not required and have adjusted the speed to 5000 X g.

10. Line 228-229: The authors stated that Acyl-RAC "often produces significantly reduced background due to elimination of nonspecific binding of proteins to streptavidin". In fact, the ABE background is largely due to an incomplete blockage of residual free cysteines by a blocking reagent (e.g., N-ethylmaleimide) (Zhou et al., Anal Chem, 2019, 91: 9858). In contrast, non-specific binding of proteins to streptavidin is a negligible source of contamination in the ABE approach, because enriched proteins are specifically eluted using a reducing reagent (e.g., TCEP), rather than being non-specifically eluted using a harsh condition.

Thank you for critically reading our manuscript. We agree Acyl-RAC may not be any more effective in eliminating background noise compared to ABE and have thus deleted that statement. Additionally, we have also cited Zhou et al in our Discussion section while discussing troubleshooting.

11. In addition, it would be very helpful to palmitoylation researchers if the authors can describe the difference in the amount of palmitoyl-proteins that could be recovered using Acyl-RAC vs. ABE.

This was a great suggestion. We have expounded on the same in lines 283-289 in the Discussion section: "Although similar, proteome-wide studies of palmitoylation show some variation in the palmitoylated proteins detected using these two methods, most likely due to the technical differences. For example, in rat brain homogenate, analysis of ABE identified 241 palmitoylated proteins whereas analysis of Acyl-RAC identified 144[25]. 61 proteins in the rat brain proteome were commonly detected by both methods underscoring the need for use of multiple techniques to ascertain the S-acylation status of a protein."

Reviewer #3:

Thank you for critically examining our manuscript. Please find below our attempt to address your concerns.

Manuscript Summary:
This manuscript described an important protocol for detection of protein S-acylation using the Acyl-Resin Assisted Capture approach followed by immunoblotting, which should be of interest for the biomedical research community. The experimental protocols on all major steps have been described in details along with two examples of protein acylation detection. Overall, the manuscript is well-written and should be relatively easy to be implement.

Major Concerns:

1. It will be great for the authors to show at least one additional example to illustrate the detection of S-acylation changes between biological conditions. Simply comparing to negative controls seems to be insufficient.

We have used the described protocol successfully to observe changes in S-acylation of proteins in response to stimulation. Since that manuscript is in preparation, we are unable to share the data here. Additionally, since we had a very short window to submit the revisions on this manuscript, we were unable to do any new experiments to assay changes in S-acylation in proteins between biological conditions.

2. The authors should also briefly discuss some examples of discovery P-S-acylation from the authors' own work or others' work using this approach

This has been added in the Discussion section in lines 252-254: "This method has been shown to successfully identify novel S-acylated proteins such as the β-subunit of the protein translocating system (Sec61b), ribosomal protein S11 (Rps11), and microsomal glutathione-S-transferase 3 (MGST3)[22]." At this point, we are unable to share the screen performed in our lab to identify novel S-acylated proteins.

3. The authors briefly mentioned the coupling with MS for proteomics identification S-acylation sites. I suggest the authors to expand this point with a little more details for MS proteomics in

the introduction section.

We have expanded on the use of MS proteomics in the introduction section in lines 77-80: "The captured proteins can then be analyzed by immunoblotting or subjected to mass spectrometry (MS) based proteomics to assess the S-acylated proteome in a varied range of species and tissues[24,25,22]. Individual S-acylation sites can also be identified by trypsin digestion of the captured proteins and analysis of the resulting peptides by LC-MS/MS[22]."

4. The stability of S-acylation is often a concern during sample processing. It will be great if the authors can clarify the critical steps for preserving such modifications.

We have expanded the Discussion section to address this concern.

Minor Concerns:

1. The detailed protocol of resin-assisted capture for various protein thiol-modifications as well as for western blotting applications was previously published in Nature Protocols (PMID: 24336471), which should be cited.

We have now cited Guo et al in the introduction.

2. Line 78. proteomics references for S-acylation should be cited here again.

We have added the pertinent references-Forrester, M.T. et al., 2011; Zaballa, M.E. et al., 2018 and Edmonds, M.J. et al., 2017.