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Journal:	*Journal of the American Society for Mass Spectrometry*
Manuscript ID	Draft
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
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Thiol-cleavable Biotin for Chemical and Enzymatic Biotinylation and its Application to Mitochondrial TurboID Proteomics

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ABSTRACT

Protein biotinylation via chemical or enzymatic reactions is often coupled with streptavidin-based enrichment and on-beads digestion in numerous biological applications. However, the popular on-beads digestion method faces major challenges of streptavidin contamination, the lost information of biotinylation sites, and limited sequence coverage of enriched proteins. Here, we explored thiol-cleavable biotin as an alternative approach to elute biotinylated proteins from streptavidin-coated beads for both chemical biotinylation and biotin ligase-based proximity labeling. All possible amino acid sites for biotinylation were thoroughly evaluated besides the primary lysine residue. We found that biotinylation at lysine residues notably reduces the trypsin digestion efficiency which can be mitigated by the thiol-cleavable biotinylation method. We then evaluated the applicability of thiol-cleavable biotin as a substrate for proximity labeling in living cells, where TurboID biotin ligase was engineered onto the mitochondrial inner membrane facing the mitochondrial matrix. As a proof-of-principle study, thiol-cleavable biotin-assisted TurboID proteomics achieved remarkable intra-organelle spatial resolution with significantly enriched proteins localized in the mitochondrial inner membrane and mitochondrial matrix.

KEYWORDS:

Cleavable biotin; Proximity labeling; TurboID; Mitochondrion; Streptavidin

INTRODUCTION

Biotin, also known as vitamin B7, is a cofactor for carboxylase enzymes required for fatty acid synthesis.[1] Biotin can be covalently linked to proteins, peptides, and nucleic acids through chemical and enzymatic biotinylation. Because of the exceptionally high affinity between biotin and streptavidin (K_d ~10^{-14} M), biotin-streptavidin system has been widely applied in biotechnology for affinity purification, immobilization, imaging and drug delivery.[2–5] However, the extremely stable and strong biotin-streptavidin interaction is a double-edged sword. Biotinylated proteins can only be eluted from streptavidin under harsh denaturing conditions (e.g., boiling in a denaturing buffer), on-beads digestion, or competitive elution with excess biotin in detergent.[4,6,7] Elution with detergent containing buffer (e.g., SDS or Triton) is not compatible with mass spectrometry (MS) analysis and on-bead digestion often leads to overwhelming contamination signals of streptavidin peptides.[7] The information of biotinylation sites is also lost from on-beads digestion since biotinylated peptides will remain attached to the beads. Alternative strategies have been developed in recent years that use structurally modified avidins with lower binding affinity (Kd $\approx 10^{-8}$ M), anti-biotin antibodies, or biotin derivatives with a cleavable moiety that can be selectively released under mild elution conditions (e.g., UV-radiation, chemical exposure, enzymes).[8–12]

A major class of applications of the biotin-streptavidin system involves attaching biotin to proteins of interest by chemical labeling or *in situ* enzymatic reactions. Recent advancements in enzymatic proximity labeling techniques have allowed both stable and transient protein networks and subcellular microenvironment to be captured in various living cells and organisms.[13–15] Engineered peroxidase or biotin ligase can be tagged to the bait protein to biotinylate neighboring prey proteins upon activation, which can then be enriched by streptavidin-coated beads for MS-

based proteomics.[13] However, proximity labeling studies face the same challenges of using the biotin-streptavidin system. To address these challenges, anti-biotin antibodies have been shown to sufficiently elute biotinylated peptides with excellent coverage of biotinylation sites.[9,10] But anti-biotin antibodies are much more expensive than streptavidin and have potential issues of antibody nonspecific bindings. The other alternative strategy is cleavable biotin but has rarely been used for proximity labeling studies.[16] Therefore, on-beads digestion is still the most popular approach despite the lost information of biotinylation sites, streptavidin contamination, and limited sequence coverage of enriched proteins.

Here, we sought to evaluate cleavable biotin for both chemical biotinylation and enzymatic proximity labeling in living cells. Commercially available thiol-cleavable biotin was used which contains a disulfide bridge that can be cleaved with the addition of a reducing agent after capturing biotinylated proteins on the streptavidin-coated magnetic beads. We first chemically labeled protein standards and complex human cell lysate with amine-reactive NHS-SS-biotin which primarily modifies the lysine side chains of proteins. The amino acid sites of SS-biotinylation and protein digestion efficiency were investigated in comparison to NHS-biotin labeled and unlabeled proteins. We then applied the thiol-cleavable biotin method to proximity labeling in living cells. Recently developed TurboID biotin ligase was selected because of its highly efficient labeling activity compared to the BioID method to reduce the diffusion of the reactive biotin cloud.[17] TurboID was genetically expressed onto the mitochondrial inner membrane, facing the mitochondrial matrix. Mitochondria are the powerhouses and the metabolic hubs of eukaryotic cells. The double membraned structure of mitochondrion created a unique compartment apart from the cytosolic environment. The inner mitochondrial membrane (IMM) is the major site for electron transport chain to produce ATP through aerobic respiration.[18] In the presence of ATP and thiol-

cleavable SS-biotin substrate, TurboID can catalyze the SS-biotinylation of nearby proteins within 10 nm labeling radius of the IMM, achieving remarkable intra-organelle spatial resolution for mitochondrial proteomics.

EXPERIMENTAL SECTION

In-vitro Protein Labeling with NHS-SS-Biotin and NHS-Biotin

Amine-reactive NHS-SS-biotin and NHS-biotin reagents (APExBIO) were used to label BSA protein standard (Fisher Scientific) and whole-cell human protein extract (Promega). BSA protein standard and human cell lysate were prepared in Phosphate-Buffer Saline (PBS) at 2 mg/mL concentrations. Urea and Tris buffers that contain primary amine groups cannot be used for NHS labeling. NHS-SS-biotin and NHS-biotin reagents are moisture-sensitive and were carefully prepared in DMSO (100 mM) to remain anhydrous in a desiccator in -30 °C. NHS reagent was mixed with each protein sample with a 50-fold molar excess for 30 min in a ThermoMixer at 25 °C. The labeling reaction was quenched with 10% hydroxylamine for 15 min and labeled samples were stored at -80 °C.

To evaluate the labeling reaction, an aliquot of each labeled protein samples (40 µg) was transferred to a new tube to react with Tris(2-carboxylethyl) phosphine (TCEP, 20 mM) in a ThermoMixer for 60 min at 37 °C to reduce/cleave the disulfide bonds. The free SH-group was alkylated with 50 mM iodoacetamide (IAA) for 30 min, followed by an additional 20 mM TCEP treatment for 10 min to get rid of excessive IAA. Trypsin enzyme (Promega) was added (enzyme: protein = 1:30, w/w) for an in-solution digestion at 37 °C for 18 hrs. For the comparison of Trypsin, LysC, and Tryspin/LysC mix enzymes (Promega), 0.4 µg of enzymes were used to digest 40 µg of SS-biotin labeled cell lysate in each replicate. Protein digestions were quenched with 10%

trifluoroacetic acid (TFA) until pH < 3. Peptide samples were desalted on a C18 96-well μElution plate (Waters), dried under SpeedVac, and stored at -30 ℃.

Cell Culture and Mitochondrial TurboID Stable Cell Line

HEK293T and HeLa cells were cultured in DMEM medium supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES, non-essential amino acids, and GlutaMAX. All reagents were purchased from Gibco. TurboID was genetically expressed onto a mitochondrial inner membrane protein, Stomatin-like protein 2 (STOML2), using the well-established TurboID method developed by the Ting group.[19] pLVX-puro-STOML2-TurboID-HA plasmid was created by PCR amplification and sub-cloning into the EcoRI sites of pLVX-puro vector (Clontech). The sequence coding TurboID was amplified from TurboID-V5-KDEL-pDisplay (kindly provided by Dr. Alice Ting) with a sequence coding HA-tag. To generate a stably transfected cell line expressing STOML2-TurboID-HA, lentiviruses were packaged in HEK293T cells, and HeLa cells were transduced with these viruses with 10 μg/ml Polybrene (Sigma) then optimized for protein expression via antibiotics selection. Wild-type HeLa cells without TurboID expression were used as the negative control group. Cells were cultured in 15 cm dishes until confluence. Both mitochondrial TurboID cells and control cells were incubated with 1 mM SS-biotin (Cayman) at 37 ℃ for 30 min, gently washed twice with PBS, and pelleted. Cell pellets were flash frozen in liquid nitrogen and stored at -80 ℃.

Sample Preparation of Mitochondrial TurboID Cells

Cell pellets were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1% SDS, 1% Triton-X, Ultra mini protease inhibitor). Lysis buffer needs to avoid adding reducing reagents such as TCEP or DTT. Cell lysates were homogenized, sonicated on ice for 15 minutes, and clarified by centrifugation at 16500 rpm for 15 minutes at 4 ℃. The total protein concentrations of cell

lysate samples were measured by a Detergent-Compatible Colorimetric Protein Assay (DCA, Bio-Rad). Streptavidin (SA) magnetic beads (Cytiva) were used to enrich biotinylated proteins with our previously developed enrichment and washing protocol.[7] Briefly, after a 16 hour incubation with the SA beads at 4 ℃, supernatants were removed and the beads were washed twice sequentially with each wash buffer (Buffer A: 2% SDS; Buffer B: 50 mM Tris−HCl, 500 mM NaCl, 2% Triton-X; Buffer C: 50 mM Tris−HCl, 250 mM NaCl, 0.5% SDS, 0.5% Triton-X, Buffer D: 2M Urea, 50 mM Tris−HCl). An additional two washes were performed with Buffer D to ensure complete removal of detergent. Then, SS-biotin labeled proteins were cleaved off beads with elution buffer (20 mM TCEP, 0.1% Rapigest (Waters), 30 mM NaCl, and 50 mM Tris-HCl) in a ThermoMixer for 60 min at 37 °C. The beads were eluted again with 1% formic acid (FA) in 50 mM Tris-HCl for another 30 min to ensure complete protein elution. The eluents were combined for alkylation with IAA and quenched with TCEP as described above. Trypsin/Lys-C mix was used for protein digestion (1:30 ratio) for 16 hrs at 37 ℃ in a ThermoMixer. Protein digestion was quenched with 10% TFA until pH < 3, incubated at 37 ℃ for 45 min, and clarified by centrifugation at 13000 rpm for 10 min to remove Rapigest. Peptides were desalted, dried, and stored at -30 ℃ until LC-MS/MS analysis.

LC-MS/MS analysis

Peptides samples were reconstituted in 2% acetonitrile (ACN), 0.1% FA in LC-MS grade water, briefly sonicated and clarified by centrifugation at 16500 rpm for 10 minutes at 4 ℃. A Dionex Ultimate 3000 RSLCnano system coupled with a Thermo Scientific Q-Exactive HFX mass spectrometer was used for LC-MS/MS analysis. The mobile phase A was 0.1% FA in water, and mobile phase B was 0.1% FA in ACN. Peptides were separated on an Easy-spray PepMap C18 column (2 μM, 100 Å, 75 μM × 75 cm) with a 2-hour LC gradient and 55 °C column temperature.

The flow rate was 0.2 μL/min. The MS scanned from *m/z* 380 to 2000 at 120 K resolution with a top 30 data-dependent acquisition with MS/MS resolution at 7.5K. Parent masses were isolated with a *m/z* 1.4 window and fragmented with higher-energy collision dissociation (HCD). The normalized collision energy was 30% and the dynamic exclusion time was 30 s. The maximum injection times were 30 ms for MS and 35 ms for MS/MS. The automatic gain control (AGC) was 1×10^6 for MS and 2×10^5 for MS/ MS.

Data Analysis

LC-MS/MS raw files were analyzed with Thermo Proteome Discoverer (2.4.1.15) software. The Uniprot BSA protein sequence was used to search labeled and unlabeled BSA sample. Swiss-Prot *Homo sapiens* database (reviewed) was used for human protein identification (1% false discovery rate cutoff). Common proteomics contamination database (from Max Planck Institute of Biochemistry) was included as the contamination marker. Trypsin was used as the enzyme with four maximum missed cleavages. Cysteine carbamidomethylation was included as a fixed modification. Methionine oxidation, and acetylation of protein N-terminus were included as variable modification. Biotinylation (+226.0776 Da) or thiol-cleavable biotinylation (+145.0198 Da) was added as an additional variable modification at lysine residue for biotin and SS-biotin labeled samples, respectively. Chromatographic alignment was conducted with a maximum retention time shift of 2 min and a minimum signal-to-noise ratio of 5. Precursor mass tolerance was 4 ppm. Fragment mass tolerance was 0.02 Da. Biotinylated proteins/peptides were also confirmed with Maxquant (1.6.17.0) software with the same parameters as the Proteome Discoverer software. For the analysis of mitochondrial TurboID samples, proteomics data was normalized to the endogenously biotinylated protein PCCA as described previously.[7] Statistical analysis was conducted with a *t-test* built in the Proteome Discoverer software. Protein Go-term

analysis was conducted by the Enrichr online software platform.[20] Raw LC-MS/MS data from this manuscript are available through the MassIVE repository (Identifier will be added here upon manuscript acceptance).

RESULTS AND DISCUSSIONS

Thiol-Cleavable Biotin for Chemical Labeling

In proximity labeling, biotin ligases (e.g. BioID[21], TurboID[17]) catalyze the biotinylation of neighboring proteins in the presence of ATP and biotin substrate within a 10 nm labeling radius (**Figure 1**). Thiol-cleavable biotin can be a promising alternative to traditional biotin substrate, allowing the efficient and complete elution of biotinylated proteins from streptavidin beads while preserving the information of biotinylation sites. To imitate biotin ligase-based proximity labeling in cells, we first investigated the thiol-cleavable biotin labeling *in vitro* with the succinimidyl 2-(biotinamido)-ethyl-1,3'-dithiopropinate (NHS-SS-biotin) reagent. NHS-SS-biotin is amine-reactive and labels the lysine side chain and N-terminus of proteins. To investigate chemical biotinylation, bovine serum albumin (BSA) standard and human whole cell lysate were labeled with NHS-SS-biotin reagent. Biotinylated proteins can be enriched with streptavidin-coated magnetic beads. Reducing agent can then be added to cleave the disulfide bond and release the proteins from the beads to the supernatant. Then the eluted proteins can be digested in-solution for subsequent proteomics analysis. TCEP was chosen as the reducing agent because it is more stable, effective, and odor-less compared to DTT. IAA was added to the eluent to carbomethylate the free thiol group and prevent the reformation of disulfide bonds (**Figure 1B**). The concentration of TCEP and IAA was optimized to achieve nearly complete (99.5%) cleavage and carbomethylation reaction for human cell lysate samples (**Table 1**). For NHS-SS-biotin labeled human cell lysate

proteins, we identified a total of over 3000 modified peptides (**Supplemental Table S1**). Additionally, to compare with the thiol-cleavable NHS-SS-biotin labeling, we also used the non-cleavable biotin derivative, N-hydroxysuccinimidobiotin (NHS-biotin) to labeled BSA and human whole cell lysate. The complete protein IDs and quantification in three groups is provided in **Supplemental Table S2**. As shown in **Figure 2** and **Figure S1**, peptides labeled with biotin at lysine residues have larger delta masses (+226.0776 Da) compared to the cleavable SS-biotin (+145.0198 Da). SS-biotin or biotin modification alters the molecular size and polarity and therefore caused the delayed LC-MS retention times. Peptide backbone fragmentations are comparable if not better in SS-biotin-labeled peptides vs. biotin-labeled and unlabeled peptides.

Thiol-Cleavable Biotin and Biotin Modify Proteins More Than the Lysine Residues

NHS-biotin reagents and biotin ligase-based proximity labeling primarily label proteins on the lysine side chain and protein N-terminus. NHS-biotin has also been shown to label serine and tyrosine residues that have hydroxyl groups.[22] However, a systematic evaluation of all the possible biotinylation sites is still lacking. Here, we examined all amino acid residues that have a primary amine (lysine, arginine, asparagine, glutamine) or hydroxyl residue (serine, threonine, tyrosine) that could react with NHS group. We also included histidine residue, which contains a secondary amine group. We found that both SS-biotin and biotin primarily modify the lysine residues as expected (**Figure 3**). However, ~10-14% of proteins were also modified at other amino acid residues (e.g., serine, threonine, histidine) with confident peptide identification (percolator q-values $<1 \times 10^{-3}$) (**Supplemental Table S3**). Interestingly, only a few protein N-terminuses were labeled with biotin. This is probably due to the existing modifications at the protein N-terminus and the low percentage of N-terminus unique peptide identifications. Protein N-terminus have been

shown to be co- or post-translational modified, such as acetylation, methylation, formylation, and methionine excision.[23] Since biotinylation modifies more than the lysine residue, other amino acids should also be considered as variable modifications in order to obtain a comprehensive coverage of biotinylation sites.

Biotinylation at Lysine Reduces Trypsin Digestion Efficiency

Trypsin is the most common enzyme for protein digestion that cleaves at the carboxyl side of lysine and arginine.[24] Since biotinylation primarily modifies the lysine residue, we hypothesized that such modification may hinder trypsin digestion efficiency. Therefore, we directly compared the trypsin digestion efficiency for SS-biotin-labeled, biotin-labeled, and unlabeled human cell lysate. A total of over 5000 proteins were identified and quantified. As expected, unlabeled samples showed excellent cleavage efficiency, and majority of unlabeled peptides have no miscleavages (**Figure 4A**). In contrast, more than 90% peptides modified with SS-biotin or biotin have at least one miscleavage. SS-biotin modified peptides mitigated the problem compared to biotin modified peptides with more percentage of peptides with $\leq$ 1 miscleavages and less percentage of peptides with $\geq$ 2 miscleavages. The improved digestion efficiency is probably due to the excision of the biotin group from the protein/peptide. Since digestion miscleavages result in longer peptides and probably higher peptide charges, we also compared the distribution of peptide charges and precursor masses to validate this finding. As illustrated in **Figure 4B** and **Figure S2**, SS-biotin and biotin labeled peptides have significantly higher peptide charges and larger precursor masses compared to unlabeled samples. Since the addition of Lys-C enzyme has been shown to improve digestion efficiency at the lysine site for trypsin digestion, we directly compared the digestion efficiency of SS-biotin labeled cell lysate using trypsin, LysC, and Trysin/LysC mix enzymes.[25] Although protein digestion with Trypsin/Lys-C mix provided the most protein/peptide

identifications compared to trypsin or Lys-C alone, the majority of SS-biotin modified peptides still have at least one miscleavages. (**Figure S3**). Therefore, we have proved that biotinylation at lysine reduces the protein digestion efficiency. This is critical for biotin ligase-based proximity labeling proteomics that routinely involves on-beads digestion to cleave the biotinylated proteins off the streptavidin beads. The miscleaved peptides will remain on the beads without sufficient elution and therefore sacrifices the protein/peptide identification and coverage. But peroxidase-based proximity labeling that biotinylates proteins at the electron-rich region (e.g., tyrosine residue) should not be influenced by this issue. In fact, we examined our previously developed LAMP1-APEX proximity labeling data and found that majority of peptides have no miscleavages (data not shown).[7] For biotin ligase-based proximity labeling or chemical biotinylation at the lysine residue, enzymes that cleave at other residues, such as Glu-C, Asp-N, and Arg-C may be used instead or in combination with trypsin digestion to improve protein coverage during on-beads digestion. Since our thiol-cleavable biotin method allows sufficient protein elution without the need for on-beads digestion, we can increase the maximum allowed miscleavages to three or four instead of routinely used two miscleavages for the proteomics data analysis.

Application of Thiol-cleavable Biotin to Mitochondrial TurboID Proximity Labeling

We have demonstrated the thiol-cleavable protein biotinylation by chemical labeling *in vitro*. Here, we aim to evaluate its applicability to the enzymatic proximity labeling reaction in living cells. Using the well-established TurboID proximity labeling method, we genetically engineered TurboID onto the Stomatin-like protein 2 (STOML2), a mitochondrial protein belonging to SPFH (stomatin/prohibitin/flotillin/HflKC) family. Members of SPFH family function as membrane organizers in various cellular membranes.[26] STOML2 is peripherally associated with the matrix-

side of the inner mitochondrial membrane, and is suggested to act as membrane scaffolds of the inner mitochondrial membrane (**Figure 5A**).[27] Thiol-cleavable biotin (SS-biotin) was added to the HeLa cells expressing STOML2-TurboID as well as the negative control group (N=3). Through the proteomics workflow illustrated in Figure 1A, we identified and quantified a total of 1566 proteins (**Supplemental Table S4)**. Statistical analysis revealed 173 significantly enriched proteins in the mitochondrial TurboID group vs. control (p-value < 0.05, fold change> 2), of which, 116 proteins were only identified and quantified in the TurboID group. These significantly enriched proteins are highlighted in red in the volcano plot, including the bait protein STOML2 and key mitochondrial proteins (**Figure 5B**). When compared to the routine on-beads digestion method, thiol-cleavable biotin method provided less nonspecific protein IDs (e.g., histones, actins) with greatly reduced streptavidin signals and enabled 305 biotinylate sites to be identified from ~200 modified peptides, mainly at K, but also at other amino acid residues like S, T, and H (**Supplemental Table S5**).

In order to confirm that the biotinylated proteins were completely cleaved and eluted from the streptavidin beads, we also conducted on-beads digestion of the leftover beads after elution. Only 156 proteins were identified including streptavidin, trypsin, keratins, actins, histones, and other nonspecific binding proteins, proving that the cleavage/elution was complete (**Supplemental Table S6**). Worth mentioning is that highly abundant biotin-dependent carboxylases were identified in the leftover beads digest. In our previous study, we found that these endogenously biotinylated carboxylases were highly abundant in both lysosomal and cytosolic APEX proximity labeling datasets. Our thiol-cleavable mitochondrial TurboID method reduced but not removed these carboxylases, including PC, PCCA, PCCB, MCC1, MCC2, ACACA, and ACACB (highlighted in green in **Figure 5B**).[28] These biotin-dependent carboxylases are probably partially

biotinylated endogenously and partially SS-biotinylated due to the addition of SS-biotin during proximity labeling in living cells. Although these carboxylases cannot be removed, they serve as a quality control check point as they should remain unchanged among different groups and negative control that were all incubated with biotin substrate, and therefore can be used to normalize the proximity labeling dataset to reduce variation as we demonstrated previously.

Protein enrichment analysis was conducted for the significantly enriched proteins in mitochondrial TurboID vs. control. Significantly enriched cellular location Go-terms include mitochondrion, mitochondrial matrix and mitochondrial inner membrane (IMM) (**Figure 5C**). TurboID labeling radius (~10 nm) is much smaller than the average diameter of the mitochondrion (~1 μm), achieving super spatial resolution of labeling activity inside the mitochondrion. Mitochondrial matrix and inner membrane proteins were much highly enriched than other locations of the mitochondrion. For instance, many mitochondrial ribosomal proteins were enriched (MRPLs and MRPSs), which are localized to the matrix attaching to the IMM and are responsible for mitochondrial protein synthesis.[29] Proteins related to the mitochondrial respiratory complexes localized at IMM were identified which are responsible for producing ATP.[18,30] The known STOML2 interactor, YME1L and PARL were also enriched in our dataset.[31] Mitochondrial enzymes in the matrix were enriched such as ACOT2, HSD17B10, CHPF, and PPA2. Whereas only few mitochondrial outer membrane proteins were enriched. Traditionally, such spatial resolution inside the organelle can only be achieved by super resolution microscopy and electron microscopy, but one protein at a time. Mitochondrial APEX proximity labeling, originally developed in the Ting group, achieved remarkable spatial resolution and high throughput mitochondrial proteomic identification.[32–34] As a proof-of-principle study, our thiol-cleavable

biotin-assisted mitochondrial TurboID also demonstrated excellent intra-organelle resolution without the need for on-beads digestion and mitochondrial fractionation.

CONCLUSION

To summarize, we have developed both chemical and enzymatic biotinylation methods using thiol-cleavable biotin as the substrate. Through a comprehensive biotinylation site analysis, we demonstrated that NHS-reagent and biotin ligase-based biotinylation modify proteins more than the lysine side chain. We found that biotinylation at lysine residues reduces the trypsin digestion efficiency, resulting in the increased miscleavages and longer peptides with higher peptide charges. Our thiol-cleavable biotin labeling mitigated this issue compared to the traditional biotinylation, in particularly with complete protein elution without the need for on-beads digestion. We also recommend setting the maximum allowed miscleavages to 3 or 4 for tryptic digested biotinylated proteins. Mitochondrial TurboID proteomics using the thiol-cleavable biotin method demonstrated intra-organelle spatial resolution of enriched mitochondrial proteins at mitochondrial inner membrane and matrix, providing a promising alternative method to study both chemical and enzymatic biotinylation of proteins in biological systems.

SUPPORTING INFORMATION

Figure S1. Example LC-MS chromatograms (A) and MS/MS spectra of peptide fragmentation (B) for SS-biotin labeled, biotin-labeled, and unlabeled peptide (PNDTQWITKPVHK) from ribosomal protein L15 (RPL15) in human cell lysate. **Figure S2.** Histogram distribution of precursor masses (M+H) of non-labeled, biotin-labeled, and SS-biotin-labeled peptides from human cell lysate samples. **Figure S3.** Comparison of the protein digestion efficiency using

Trypsin, LysC, and Trypsin/Lys-C mix for SS-biotin labeled human protein lysate. **Table S1**: Thiol cleavable SS-biotin modified peptide sequences in labeled human cell lysate. **Table S2**: Protein IDs and quantification in SS-biotin labeled vs. biotin labeled vs. unlabeled human cell lysate. **Table S3**: Average percolator *q*-values of modified peptides at various amino acid residues. **Table S4**: Protein IDs and quantification in STOML2-TurboID vs. control. **Table S5**: SS-biotinylated peptides in STOML2-TurboID Proteomics. **Table S6**: Leftover beads digestion showing that the elution was complete.

Notes: The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

This study is financially supported by the GW Faculty Startup Fund. L.H. acknowledges the ORAU Ralph E. Powe Junior Faculty Enhancement Award. A.M.F. and H.L. acknowledge the Bourdon F. Scribner Fellowship from GW Chemistry Department. We thank all the members in the Hao Lab for helpful discussions. S.S. is supported by the University of Pittsburgh, The Aging Institute Startup Seed, Samuel and Emma Winters Foundation and UPMC Health System Competitive Medical Research Fund.

Author contributions: H.L. and A.M.F. contributed equally to this work.

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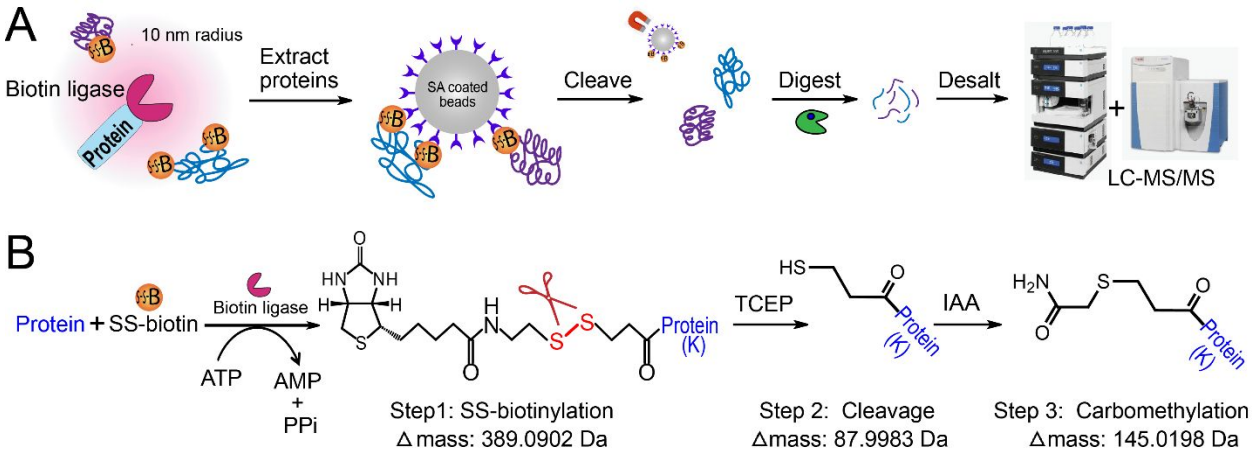


Figure 1. Development of thiol-cleavable biotin-enabled proximity labeling. (A) General workflow for thiol-cleavable biotinylation and protein sample preparation. (B) Schematic of enzymatic reaction of SS-biotinylation, chemical cleavage of disulfide bonds to elute proteins off the streptavidin beads, and carbomethylation of the free thiol group to avoid reformation of disulfide bonds.

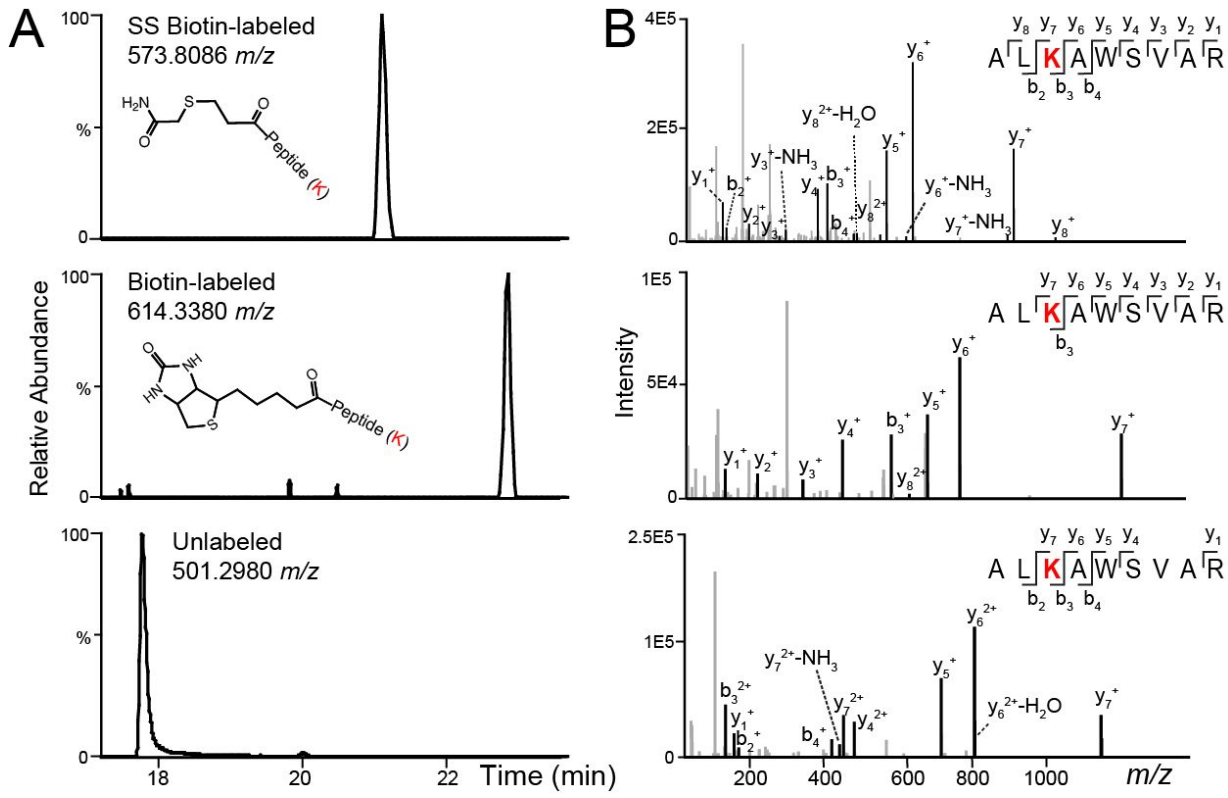


Figure 2. Example LC-MS chromatograms (A) and MS/MS spectra of peptide fragmentation (B) for SS-biotin-labeled, biotin-labeled, and unlabeled peptide (ALKAWSVAR) from bovine serum albumin (BSA) protein.

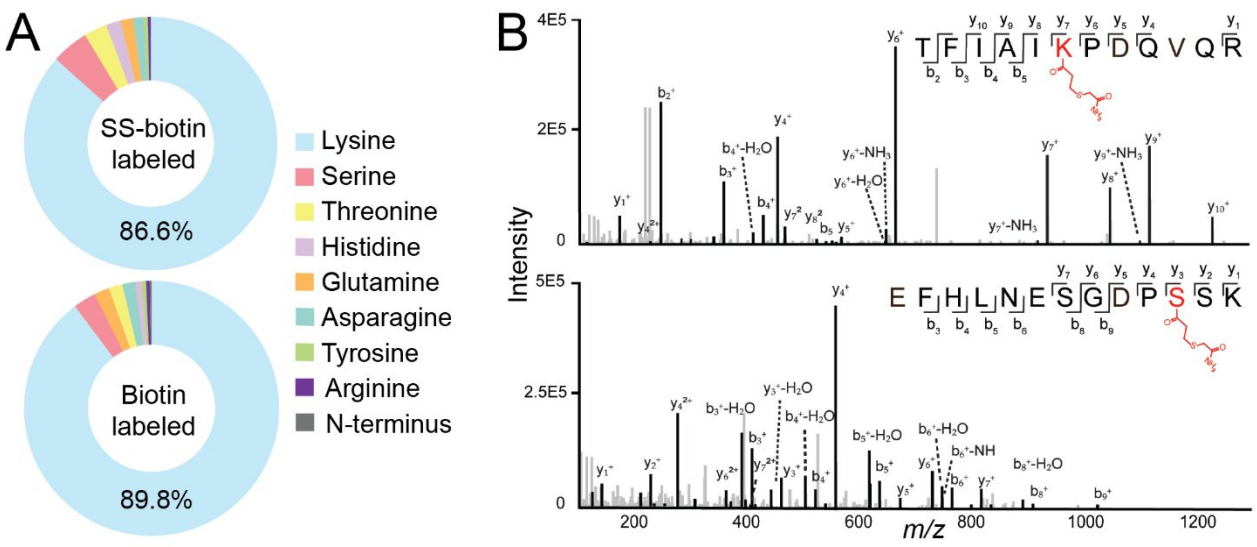


Figure 3. Evaluation of protein biotinylation sites. (A) Percentage distribution of biotinylation sites from labeled human cell lysate. (B) Example fragmentation spectra of SS-biotin modified peptides.

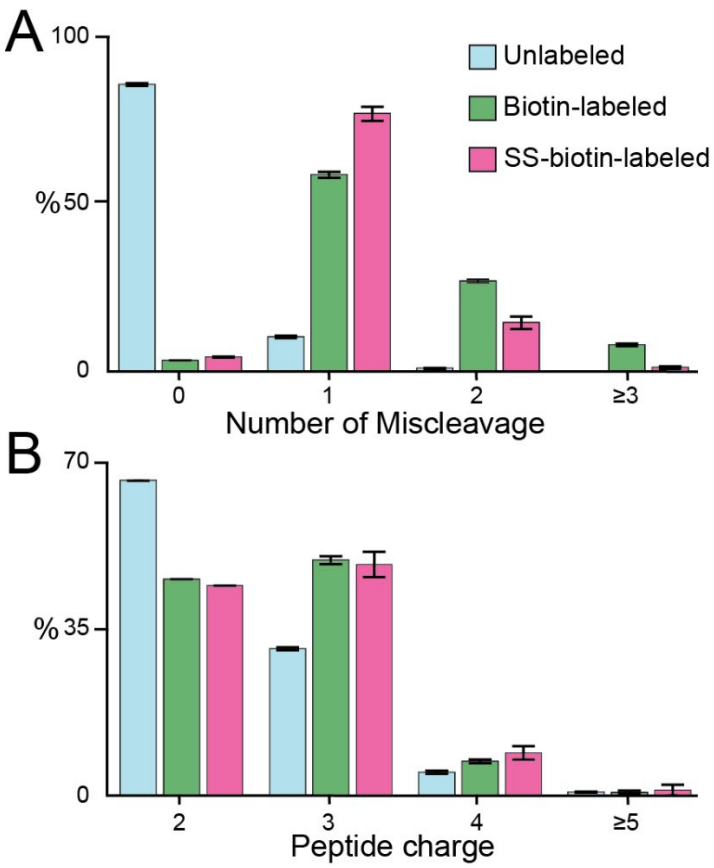


Figure 4. Evaluation of the trypsin digestion efficiency for unlabeled, biotin-labeled (K), and SS-biotin-labeled (K) peptides from human cell lysate samples. Histogram distributions of the number of miscleavages (A) and peptide charges (B).

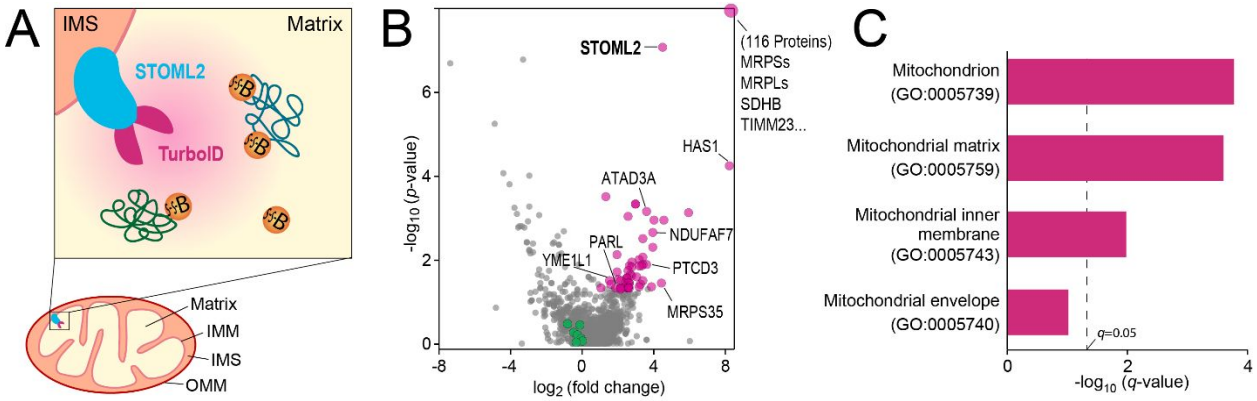


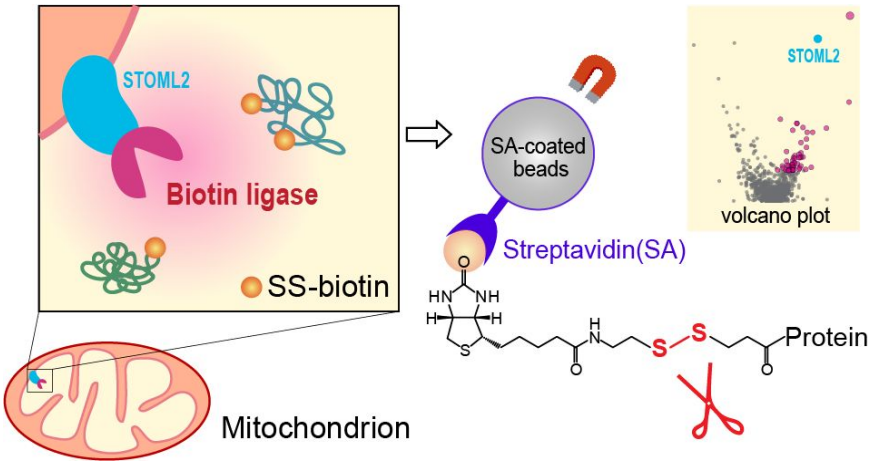
Figure 5. Mitochondrial TurboID Proteomics with thiol-cleavable biotin. (A) Schematic of STOML2-TurboID inside the mitochondrion using thiol-cleavable SS-biotin as the substrate. (B) Volcano plot of STOML2-TurboID proteomics vs. negative control (N=3). Significantly enriched proteins are highlighted in red. Endogenously biotinylated carboxylases were highlighted in green. (C) Go-term analysis of significantly enriched proteins (Top 4 ranked Go-terms for cellular location). Mitochondrial structure: outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), and mitochondrial matrix.

Table 1: Reaction efficiency of SS cleavage and carbomethylation from SS-biotin-labeled (K) human cell lysate proteins.

Modification	Delta Mass	Percentage of Biotinylated Peptides	Average Percolator q-Value	Average Percolator PEP
Cleaved, carbomethylated [a]	+145.0198	99.5%	8.90E-04	1.47E-02
Cleaved, not carbomethylated [b]	+87.9983	0.31%	5.50E-03	1.22E-01
SS-biotinylated, not cleaved [b]	+389.0902	0.20%	2.50E-04	8.46E-04

[a] Complete reaction of thiol-cleavable biotinylation

[b] Incomplete reaction of thiol-cleavable biotinylation



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