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Photoactivatable protein labeling by singlet oxygen mediated reactions

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ABSTRACT

Protein–protein interactions regulate many biological processes. Identification of interacting proteins is thus an important step toward molecular understanding of cell signaling. The aim of this study was to investigate the use of photo-generated singlet oxygen and a small molecule for proximity labeling of interacting proteins in cellular environment. The protein of interest (POI) was fused with a small singlet oxygen photosensitizer (miniSOG), which generates singlet oxygen ($^1O_2$) upon irradiation. The locally generated singlet oxygen then activated a biotin-conjugated thiol molecule to form a covalent bond with the proteins nearby. The labeled proteins can then be separated and subsequently identified by mass spectrometry. To demonstrate the applicability of this labeling technology, we fused the miniSOG to Skp2, an F-box protein of the SCF ubiquitin ligase, and expressed the fusion protein in mammalian cells and identified that the surface cysteine of its interacting partner Skp1 was labeled by the biotin–thiol molecule. This photoactivatable protein labeling method may find important applications including identification of weak and transient protein–protein interactions in the native cellular context, as well as spatial and temporal control of protein labeling.

Genome sequences of important model organisms are now available, owing to the technological advances in high throughput DNA sequencing. An important next step in understanding biology and disease is to identify protein interactome,[1–4] which however, unlike the genome, is highly dynamic and contains weakly associated components that are difficult to detect by affinity-based approaches.[5,6] In order to construct a complete human interactome,[7,8] development of new protein labeling techniques is necessary for mass spectrometric identification of weak and transient protein interactions.[9–11] Recently, an enzyme-based labeling technology has been described.[12] The next step is to enable spatial and temporal control of enzyme activity for protein labeling. Here we describe the concept and demonstration of a photoactivatable protein labeling technology.

This technology, dubbed SPRAY (**s**inglet oxygen activated **pr**otein l**a**beling, coupled to mass spectrometr**y**), uses a genetically encoded singlet oxygen ($^1O_2$) photosensitizer (SOG) and $^1O_2$-reactive molecules. The general concept is as follows: SOG is genetically fused to the protein of interest (POI), followed by addition of the labeling molecule that forms a reactive intermediate upon oxidation by $^1O_2$. The intermediate then reacts with specific residues (e.g. cysteine, lysine, histidine, and serine) of closely interacting proteins. After cell lysis, the labeled proteins can be isolated by streptavidin–biotin interaction. Mass spectrometry can be used for identification of the interacting proteins. Specific labeling of closely interacting proteins is likely achieved because of the short diffusion distance of $^1O_2$ in cells (~30 nm within half-life).[13] The SPRAY labeling technique can be tested on a variety of small molecules that are known to react with $^1O_2$. These molecules could potentially generate electrophilic species that further react with the side chains of cysteine, lysine, histidine, and serine, resulting in a diverse repertoire of chemical signatures for mass-spectrometric identification.

To realize the concept of SPRAY, first we made use of the genetically encoded small $^1O_2$ photosensitizer miniSOG (106-residue) that requires no exogenous cofactors.[14] MiniSOG is green fluorescent and generates $^1O_2$ when illuminated with blue light (450–490 nm). Next, we designed a labeling molecule that is small and likely cell permeable so that it is potentially applicable in intact cells. Most importantly, upon $^1O_2$ activation the molecule should have strong reactivity toward a protein residue. One candidate is a thiol-based molecule which, when oxidized by $^1O_2$, forms

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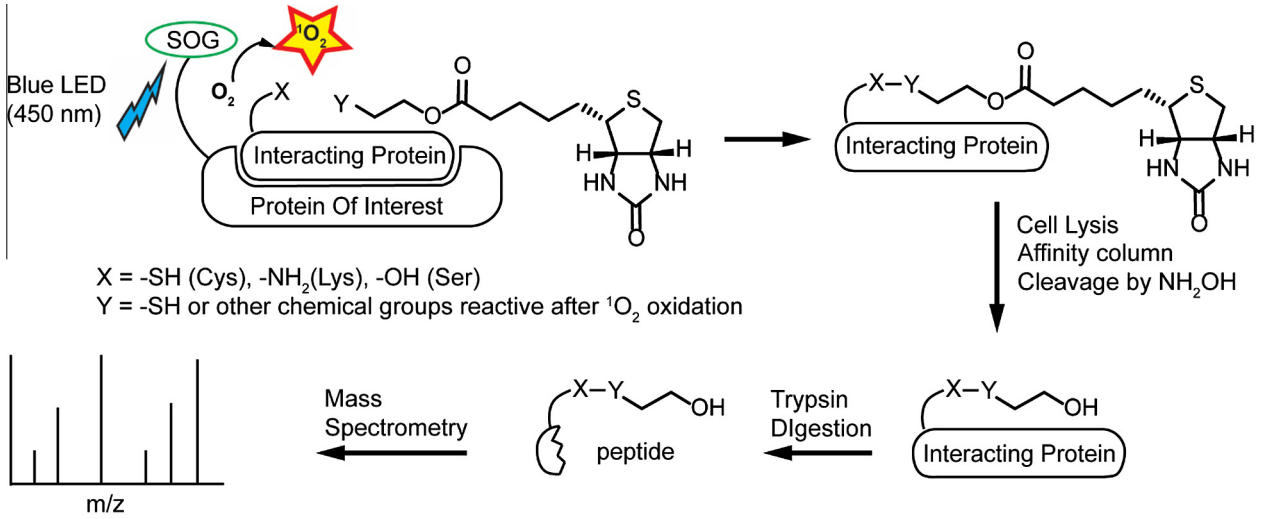
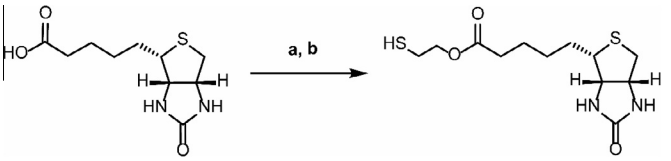


Figure 1. Design of photo-activatable protein labeling via singlet oxygen. The interacting protein in close proximity to the protein of interest (POI) is labeled. POI is fused to singlet oxygen generator (SOG) to generate the singlet oxygen upon irradiation. Then, the thiol-containing labeling molecule (Y = SH) forms a disulfide bond with a surface cysteine (X = SH) residue of interacting protein. The labeled protein can then be purified by affinity column and released by hydroxylamine followed by trypsin digestion and identified by mass spectrometry.



Scheme 1. Reagents and conditions: (a) 2-hydroxyethyl disulfide, EDC, HOBt, Et_3N, DMF,12 h, rt; (b) TCEP, CH_3CN–H_2O (2:1), 2 h, rt (53% over 2 steps).

a disulfide bond with the side chain of cysteine. In this oxidation process, it is believed that $^1O_2$ is the major reactive oxygen species generated by miniSOG and the contribution from other reactive oxygen species are insignificant as previously shown.[15,16] However we cannot completely rule out the partial contribution from other endogenous reactive oxygen species including hydrogen peroxide. Thus, we specifically designed a thiol-containing small molecule with a biotin group attached to it. The spacer arm of the molecule contains a cleavable ester site that may be broken with hydroxylamine to remove the biotin moiety.

The experimental workflow is as follows: we use blue light to generate $^1O_2$ from miniSOG (Fig. 1). $^1O_2$ then oxidizes the thiol group of the labeling molecule, which reacts with cysteine residues on the surface of nearby proteins, forming a disulfide bond.[17] The labeled proteins can then be separated from the unlabeled proteins through streptavidin–biotin interaction. The biotin labeled proteins can be eluted by biotin competition, for example, by desthiobiotin. Alternatively, the labeled proteins can be released from the streptavidin by hydroxylamine cleavage (Supplementary Fig. 1). The labeled proteins can also be digested with trypsin to enrich only the labeled peptides. Lastly, these labeled peptides can be fragmented and identified by tandem mass spectrometry.

As a proof of concept, we directly fused miniSOG to a phycobiliprotein, allophycocyanin (APC) that contains three cysteine residues, and tested the labeling method. We also synthesized a thiol molecule conjugated to biotin by ester formation and reduction (Scheme 1). This compound showed a low to moderate membrane permeability in PAMPA assay ($Pe = 1.7 \times 10^{-6}$ cm/s, PBS buffer (pH = 7.4)).

We then mixed 1 μM freshly purified miniSOG-APC protein with various concentrations of the biotin–thiol molecule, and performed labeling experiments with different illumination times. The reaction mixture was illuminated with a customized 450 ± 20 nm blue LED (Innovations in Optics, Woburn MA) at an intensity of ~200 mW/cm^2. After illumination, we first removed the free labeling molecules by centrifugal filtration. Then we purified the labeled proteins by streptavidin, and assayed the labeled proteins by LDS–PAGE (Fig. 2c, Supplementary Fig. 2). The data indicated that the amount of labeled proteins depends on both the illumination time and the concentration of labeling molecules. For example, with 10 μM biotin–thiol, the longer the illumination time was, the higher the labeling efficiency. Efficient labeling was achieved with 100 μM biotin–thiol and 30-min illumination time. Under this condition, about 67% of the fusion protein was recovered (Supplementary Fig. 3). On the other hand, when the illumination time was reduced to 5 min, negligible amount of proteins were labeled with 100 μM biotin–thiol.

To demonstrate the dependence of labeling on protein proximity, we compared the extent of labeling of miniSOG-APC fusion with that of free APC in the same mixture (Fig. 2c). The center-to-center distance between miniSOG and APC in the fusion is estimated to be around 5 nm, which is short compared to the average intermolecular distance of >50 nm between miniSOG-APC and APC in an equimolar mixture of 1 μM each. The LDS–PAGE data showed that the miniSOG-APC fusion featured a significantly stronger labeling efficiency (>5 fold) than free APC under all conditions tested, demonstrating that SPRAY preferentially labels proximal proteins.

To confirm the specific chemical modification, we digested the purified miniSOG-APC with trypsin and subjected the sample to LC/MS/MS analysis using an LTQ-Orbitrap XL mass spectrometer. While miniSOG does not contain cysteine, APC contains three cysteine residues (Fig. 2a): Cys52, Cys66 and Cys103. Furthermore, based on the modeled structure (Swiss-Model),[18] Cys103 (in red) resides on the protein surface (Fig. 2b), whereas Cys52 and Cys66 (in magenta) are buried inside the protein, with Cys52 linked to the cofactor phycocyanobilin (in blue). Therefore, we expected that the exposed cysteine Cys103 would be labeled by the biotin–thiol molecule. Indeed, the Cys103-containing tryptic peptide ^{103}CLKEASLTLLDEEDAKK^{119} was identified from its ion trap CID (collision induced dissociation) spectrum and Cys103 was assigned as the amino acid carrying the biotin–thiol modification (Fig. 2e).

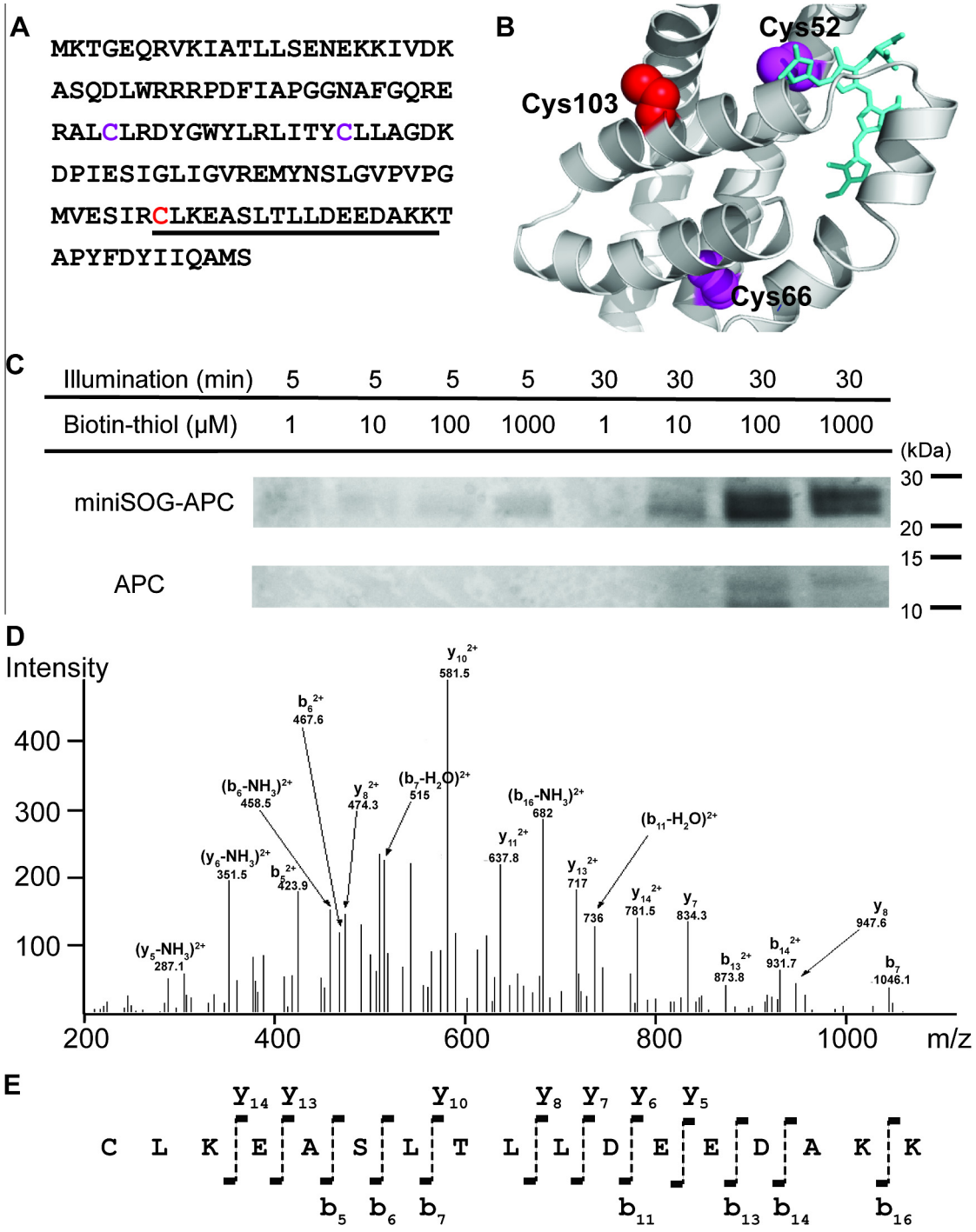


Figure 2. Demonstration of the photo-activatable protein labeling. (A) Primary sequence of allophycocyanin (APC) (cysteines are shown in Magenta and Red and the underlined residue represented the digested peptide fragment (B) Modeled structure of APC (Swiss-Model) The conserved Cys52 is likely linked to phycocyanobilin (in blue) (C) LDS–PAGE of a mixture of purified miniSOG-APC fusion protein (1 μM) and free APC (1 μM) (D) Ion trap CID of the identified tryptic peptide (E) Fragment ions detected are labeled according to the nomenclature.[20] The N-terminal Cys was identified as bearing the biotin-label.

On the other hand, the mass spectrometry results also suggested that the buried cysteines Cys52 and Cys66 were not labeled, presumably because they were not accessible to the labeling molecules.

To demonstrate application of the SPRAY method in protein labeling in living cells, we fused miniSOG to Skp2, an F-box protein of SCF (Skp1-cullin-F-Box) ubiquitin ligase. SCF is a multi-protein complex, composed of four subunits: Skp1 (S-phase kinase-associated protein 1), cullin1, a RING finger protein (RBX1/HRT1/ROC1) and a variable F-box protein. We expressed the Skp2-miniSOG fusion protein in cultured human cells (HeLa). The green fluorescence of miniSOG in the nucleus suggests correct localization of the fusion protein (Fig. 3a). Through the SPRAY labeling process (20 min irradiation on live cells and 10 min irradiation after lysis to minimize the possible reduction of disulfide bond), we identified one of the known interacting proteins, Skp1 (Fig. 3b). The Skp1 contains three cysteine residues (Fig. 3c): Cys62, Cys120 and Cys160. Based on the crystal structure of Skp1 and

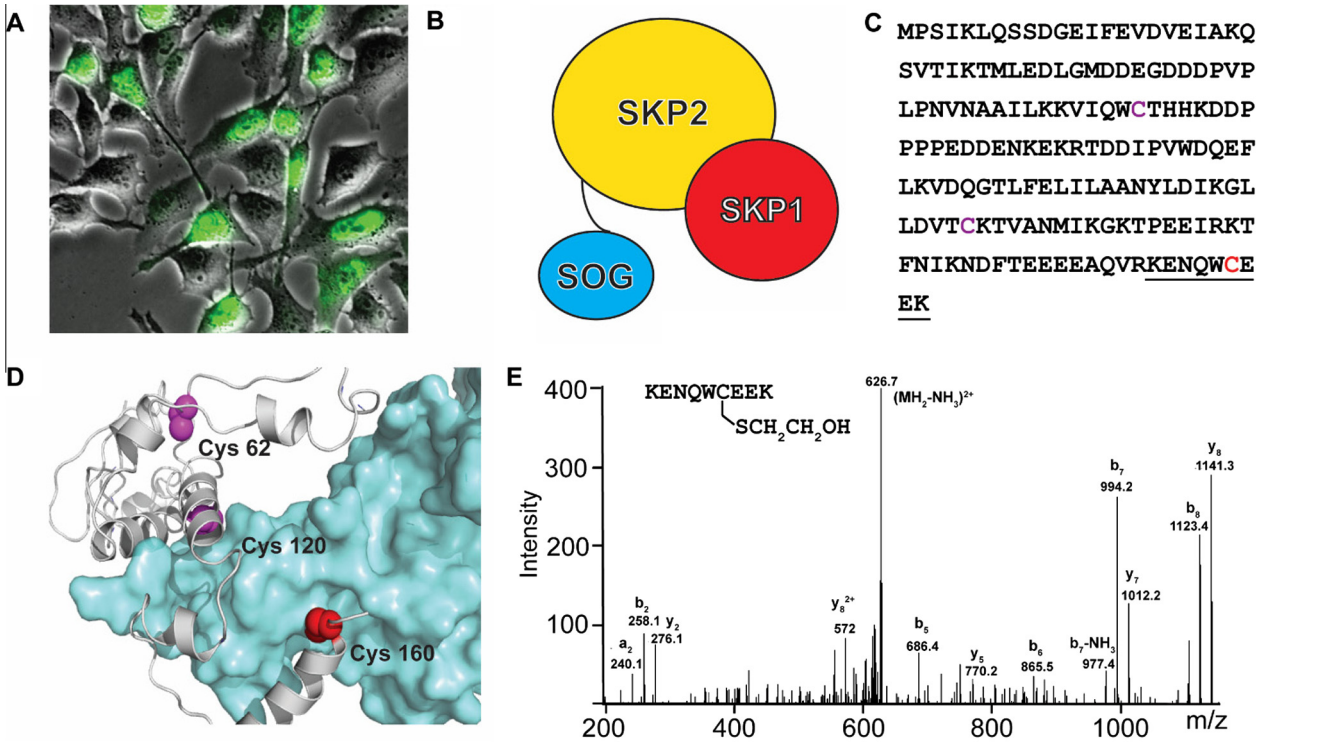


Figure 3. Photo-activatable protein labeling in mammalian cells. (A) Fluorescence images of miniSOG tagged Skp2, an F-box protein of SCF (Skp1-cullin-F-Box) complex. (B) Cartoon showing Skp2 and Skp1 (human S-phase kinase-associated protein 1) complex with Skp2 tagged by miniSOG. (C) Primary sequence of Skp1. Cysteines are shown in Magenta and Red and the underlined residues represented the digested peptide fragment (D) Modeled structure of miniSOG fused Skp1–Skp2 complex based on the crystal structure of Skp1–Skp2 complex (PDB ID 2ASS) using Swissmodel. (E) CID spectrum of *m/z* 635.2659(2+) representing tryptic peptide ^{155}KENQWC(DeStreak)EEK^{163} of Skp1. 'DeStreak' is the Unimod identification for cysteine mercaptoethanol (www.unimod.org). Detected fragment ions are labeled according to the nomenclature.[20]

Skp2 complex (PDB: 1FS1), Cys160 (in red) resides on the protein surface (Fig. 3d), whereas Cys62 (in magenta) is buried inside the protein, and Cys120 (in magenta) is buried within the interacting interface with Skp2. We used hydroxylamine to remove the biotin during elution and identified the labeled peptide containing surface cysteine Cys160 with the 'leftover' beta-mercapto-ethanol modification (Cys-S)-S-CH_2-CH_2-OH using mass spectrometry (Fig. 3e). Thus the SPRAY method is feasible to label interacting proteins in living cells.

However, we noted that Skp2 interacting partners other than Skp1 was not detected, suggesting that the current method requires further improvement. For instance, we may increase the number of cells used in the labeling step. In addition, development of highly cell-permeable probe molecule could increase the detection of the protein–protein interaction and this is the subject of future work. With further improvement, the SPRAY method might overcome the limitations of affinity-based approaches in identifying weak and transient protein–protein interactions.

It also needs to be pointed out that proteins are likely to be oxidized by singlet oxygen generated during the labeling procedure. For example, we detected oxidation of methionine and tyrosine (Supplementary Fig. 4). To minimize protein oxidation, in the future the labeling molecules should be more reactive toward singlet oxygen than the singlet oxygen-sensitive residues such as methionine so that the interacting proteins are labeled with low concentrations of singlet oxygen.

In summary, we have developed a singlet oxygen-mediated, photo-activatable protein labeling technology SPRAY. It uses a genetically encoded photosensitizer miniSOG to photogenerate $^1O_2$, which reacts with the biotin–thiol molecule. The oxidized/activated biotin–thiol then forms a disulfide bond with exposed cysteine residues of nearby proteins. This labeling produces a unique chemical signature that can be identified by mass spectrometry.

The light-inducible nature of this technology also allows spatial and temporal control of protein labeling. Future directions for technology development include application of this technology to identify interacting proteins in cellular context; design of new labeling molecules that label other amino acid residues such as lysine; and design of a new class of labeling molecules that label other biological molecules such as nucleic acids or glucose. For example, although each protein on average contains ~3 exposed cysteines,[19] some of them may be post-translationally modified. Thus, in the future a lysine residue may be a better target using a similar approach with an appropriate labeling molecule.

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Supplementary data

Supplementary data (methods and supplementary figures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.05.034.

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