Subcellular protein profiling with far-red light mediated proximal labeling

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Abstract

I have developed a far-red light mediated proximal labeling (LIMPLA) strategy for subcellular protein profiling in live cells. This strategy can facilitate the proteomic profiling of subcellular organelle, such as the mitochondria with no need of transgene. I modified the mitochondria-targeting dye rhodamine 123 with an alkynyl group (Alk-R123). These modifications do not change the mitochondria targeting property and can act as a bioorthogonal 'handle' for subsequently imaging or enrichment. After Alk-R123 are accumulated in the mitochondria, light illumination can activate the dye to label proximal proteins in situ. In order to enhance protein labeling efficiency, I have found that various mitochondria-targeting dyes such as Mitotracker deep red can mediate 2-Propynylamine (PA) to label proximal proteins under illumination. PA tagged proteins with alkynyl group are subsequently derivatized via click chemistry with azido fluorescent dye for imaging or with azido biotin for further enrichment and mass-spec identification.

Main text

Eukaryotic cells are elaborately subdivided into functionally distinct, membrane-enclosed compartments or organelles. Each organelle contains its own characteristic set of proteins, which underlie its characteristic structural and functional properties. For example, mitochondria, a double-membrane-bound organelle, are energy center and plays crucial roles in energy conversion and calcium ions storage. Understanding how organelles function appropriately and specifically requires identifications of proteomics in specific organelles.

Several protein proximity labeling (PL) methods combined with protein mass spectrometry have emerged for identification of organelle proteomics, in addition to traditional isolation of specific organelle method, which may contain contamination and result in artifacts. The first PL is based on genetic encoded promiscuous labeling enzyme, which target to specific organelle and initiate covalent tagging of endogenous proteins proximal to the enzyme. BirA*, a mutant form of the biotin ligase enzyme BirA (BioID method)[1-2] and APEX2, an engineered variant of soybean ascorbate peroxidase[3-5] are mostly common used enzymes. BirA* is capable of promiscuously biotinylating proximal proteins irrespective of whether these interact directly or indirectly with BirA*. This method has simple labeling protocol and only need additional biotin to initiate the labeling. Its disadvantages are long labeling time (18-24h) and background labeling signals before adding biotin due to endogenous biotin in living cells. The advantage of APEX2 is short labeling time (only 1 min or less). However, the APEX2 method requires expression of exogenous proteins and the use of high concentration of H_2O_2, which is toxic to cells.

The second PL is based on antibody conjugated enzyme, such as horse radish peroxidase (HRP), which target to specific protein and tag endogenous proteins proximal to the enzyme. The enzyme mediated activation of radical source (EMRS) method utilize HRP conjugated antibody to targeted protein on cell membrane, then HRP can mediate chemical reaction that converts aryl azide-biotin reagent to active radical species for proximal labeling[6]. Biotin labeled proteins can be high throughput analyzed. This method can only be used on cell membrane, due to antibody's impenetrability to cell membrane. Selective proteomic proximity labeling using tyramide (SPPLAT) method[7] is similar with EMRS and its substrate of HRP is changed to a cleavable biotin phenol. Biotinylation by antibody-recognition method[8] expend EMRS to intra-cell, in which fixed and permeabilized cells or tissues were treated with primary antibody for targeting protein of interest and then treated with a secondary HRP-conjugated antibody, H_2O_2 and phenol biotin for proximal protein biotinylation. However, this method need huge amount of expensive antibody, is only applicable to fixed cells and may introduce artifacts due to long diffusion distance of biotin free

radical without membrane.

Here, I describe a light mediated proximal labeling (LIMPLA) strategy for mitochondrial protein profiling, which is independent of exogenous gene transfection and compatible with living cells. The strategy utilized crosslink of proteins in mitochondria via photochemical reaction (**Figure.1 strategy 1**). Alk-R123, an analogue of rhodamine 123 (commercial mitochondrial fluorescent tracker), were accumulated to mitochondria via membrane potential. Upon illumination, Alk-R123 can label its proximal proteins via photochemical reaction. The labeled proteins were reacted with azide-biotin for further enrichment or mass spectrometry analysis. I started to synthesize Alk-R123 and Az-R123. Then these probes were incubated with bovine serum albumin (BSA) and illuminated with xenon light in vitro. With reaction with Azido-Cy5 or Alkyne-Cy5, respectively, via Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), labeling signals of BSA were detected using in-gel fluorescence scanning. (**Figure S1**). BSA treated with Alk-R123 or Az-R123 and light exhibit higher levels of signals than no light control group.

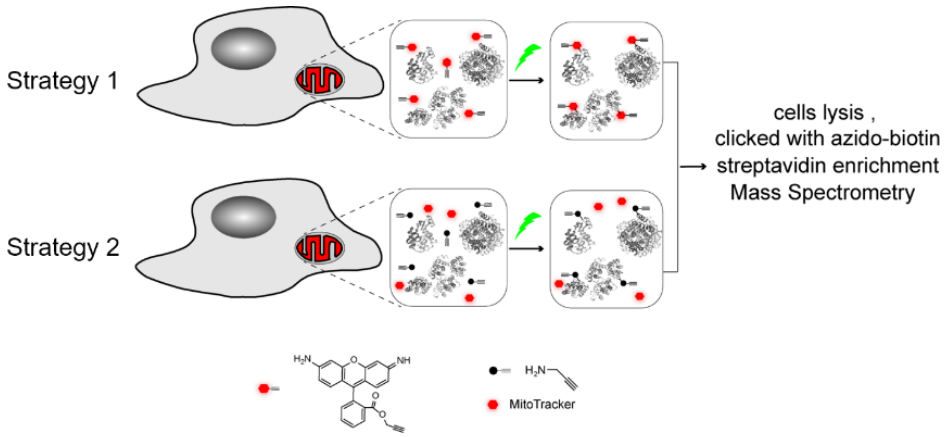

Figure 1. Schematic of light mediated proximal labeling. Strategy 1. Alk-R123, a derivative of mitochondria targeting dye Rhodamine 123, bearing alkyne group were accumulated in mitochondria, via mitochondria inner membrane potential. Upon illumination, these dyes can covalently label proximal proteins in situ. **Strategy 2.** MitoTracker dye, such as Rhodamine123 or MitoTracker deep red, accumulated in mitochondria can mediate alkynyl substrate PA to label proximal proteins with illumination. The labeled proteins were reacted with azido biotin for further enrichment and mass-spec identification.

To validate cellular permeability and mitochondria targeting of Alk-R123, HeLa cells were incubated with Alk-R123 and 0.5 μM Mito Tracker deep red, a commercial

mitochondria marker. Confocal laser scanning microscopy (CLSM) showed that signals of Alk-R123 were highly co-localized with signals of Mitotracker deep red (Pearson's correlation coefficient >0.95), which indicates that Alk-R123 selectively accumulated in mitochondria (**Figure 2B**). Mitochondrial localization was abolished by treatment with carbonyl cyanide m-chlorophenylhydrazone (CCCP)[9], a standard uncoupler of the mitochondrial membrane potential (**Figure S2**), which indicates the mitochondrial targeting of Alk-R123 was mediated with the high membrane potential of mitochondria.

To demonstrate the mitochondrial protein labeling with Alk-R123, HeLa cells treated with Alk-R123, were illuminated with xenon lamp. Cells were lyses and reacted with azido-Cy5 via CuAAC and analyzed with in-gel fluorescence scanning. The results showed that HeLa cells treated with Alk-R123 and illumination for 5 min exhibit labeling signals (**Figure. 2A lane4**). As a control, HeLa cells, only treated with Alk-R123 without illumination, exhibited level of labeling signals to the levels of background (**Figure. 2A lane2**). Labeling signals were almost abolished by CCCP (**Figure. 2A lane3**), as the membrane potential of mitochondria disappeared and Alk-R123 were not accumulated in mitochondria. Furthermore, the fluorescence images showed that green labeling signals of HeLa cells were well correlated with red anti-HSP60 signals (**Figure. 2B**). The Alk-R123 labeling signals were incubation time- and illumination time- dependent (**Figure S3-4**). These results were in accordance with the results of in gel fluorescence scanning, which indicates that the Alk-R123 accumulated in mitochondria can label proteins in situ with light. Similarly, Az-R123 can also label mitochondrial proteins, as shown in **Figure S5**.

What's the mechanism of LIMPLA? It was speculated that the photo-labeling was mediated by singlet oxygen generated by the process of dye photobleaching[10]. The sodium azide, a singlet oxygen quencher[11] ,can reduce the BSA labeling, while D_2O which increases singlet oxygen lifetime can enhance the BSA labeling (**Figure 2C**). Taken together, these data suggest that the singlet oxygen plays an important role in the photo-labeling.

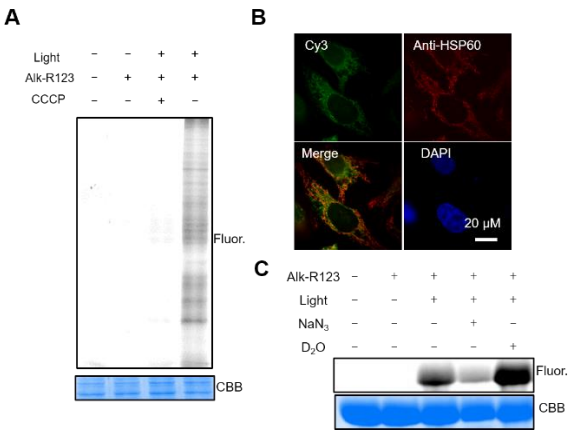

Figure 2. Mitochondrial protein labeling with LIMPLA. (A) HeLa Cells were stained with 10 μM Alk-R123 for 2h. Cells were then illuminated for 5 min. Cell lysate were reacted with azide-Cy5 for in gel fluorescence scanning. (B) After treatment of Alk-R123 and illumination, cells were fixed, permeabilized and clicked with azio-Cy3 via CuAAC. Cells were immunostained with Anti-HSP60. Green channel denotes azido-Cy3 signal. Red channel denotes HSP60 signal. Blue channel denotes DAPI signal. (C) 50 μM R123, 2 mg/mL BSA and 1mM PA (or + 5 mM NaN_3) in H_2O or D_2O were illuminated for 2 min. The labeled proteins were reacted with azido-Cy5 using CuAAC.

In order to further increase the photo-labeling signals, I was trying to increase the concentration of Alk-R123. But when the concentration of Alk-R123 is too high, Alk-R123 will nonspecifically bind non-mitochondrial part which will result in background signals. I speculated that Alk-R123 can be split to two parts—one is rhodamine 123 (R123) for mitochondrial targeting and the other is alkynyl substrate for enrichment handle and The R123 accumulated in mitochondria can mediate alkynyl substrate to label its proximal proteins with illumination (**Figure 1 strategy 2**). Then, I selected N-(4-aminophenethyl) pent-4-ynamide (AY) bearing benzenamine structure similar with rhodamine and a simple primary amine PA as the alkynyl substrates. In this strategy, cells were stained with R123, respectively incubated with AY or PA, and then illuminated with xenon lamp. Cell lysate were reacted with azido Cy5 via CuAAC and analyzed with in gel fluorescent scanning. As shown in **Figure 3A**, labeling signals in PA group are stronger than AA or Alk-R123 (AR) group. Treated cells were in situ reacted with azido Cy3, and the labeling signal were well correlated with

immunofluorescence signals of a mitochondrial protein HSP60 (**Figure. 3B**), which indicated that PA labeled proteins were located in mitochondria. In **Figure. 3C**, labeling signals in CCCP group were impaired and signals in no light or R123 group were completely disappeared. I also tested (o, m, p)-alkynyl benzenamine in HeLa cell labeling with R123 and under illumination (**Figure. S6**). All substrate can show certain signals and PA still shows the highest signals than other substrates. I termed this new version of light mediated proximal labeling as LIMPLA2. Compared with LIMPLA, LIMPLA2 has much higher labeling efficiency and has no need to do any organic synthesis, as compounds used in LIMPLA2 are all commercial available. Similar with the mechanism of LIMPLA, I find that the singlet oxygen also plays an important role in LIMPLA2, as the H_2O_2 can increase BSA labeling signals and sodium azide can decrease the signals (**Figure. S7**).

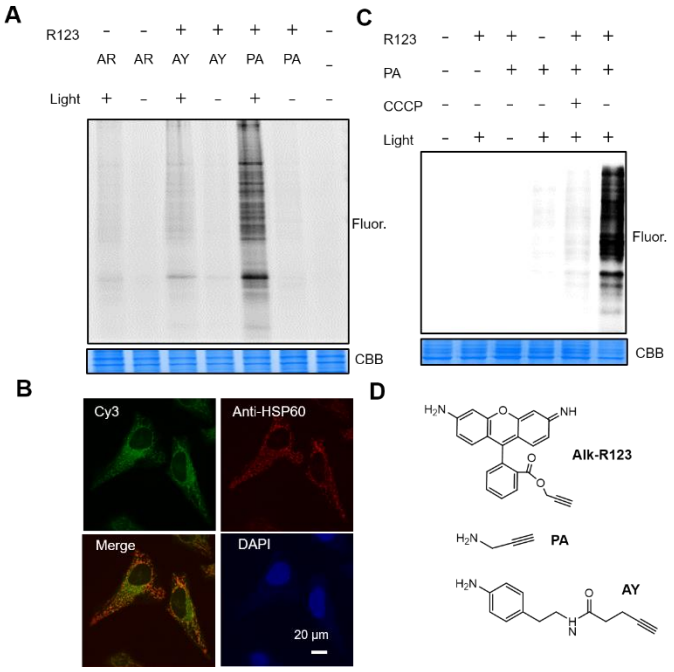

Figure 3. Mitochondrial protein labeled with LIMPLA2. (A) HeLa Cells were stained with 10 μM R123 or Alk-R123 for 3h. Cells stained with R123 were treated with 5 mM AY or PA for extra 5 min. Cells were then illuminated for 5 min. Cell lysate were reacted with azido Cy5 for in gel fluorescence scanning. (B) After treatment with R123, PA and illumination, cells were fixed, permeabilized and clicked with azio-Cy3 via CuAAC. Cells were immunostained with Anti-HSP60. Green channel denotes azido-Cy3 signal. Red channel denotes HSP60 signal. Blue channel denotes DAPI signal. (C) In gel fluorescence scanning analysis of protein labeling. Cells were pretreated with CCCP for 5 min, and then incubated with R123 and PA. (D) Chemical structure of Alk-R123 (AR), PA and AY.

I wonder if there is another mitochondrial tracker which can further increase the LIMPLA2's labeling efficiency. Three new mitochondrial trackers mitoView 405 (mito405), Tetramethylrhodamine (TMRM), MitoTracker deep red were tested. As shown in **Figure 4A**, all of new three trackers exhibit labeling signals, the labeling patterns are almost the same with R123 group's and mito405 have the strongest labeling signals. HeLa cells were treated with mito405, PA and light. Treated cells were then clicked with azido Cy3 via CuAAC and the results of imaging show that azido Cy3 signal were well correlated with immunofluorescence signals of a mitochondrial protein HSP60, which indicated that labeled proteins were located in mitochondria (**Figure 4B**). In **Figure 4C**, labeling signals in CCCP group were impaired and labeling signals in no light or R123 group were completely abolished. The labeling intensity is positively correlated with concentration of mito405 (**Figure S8**) and PA (**Figure S9**).

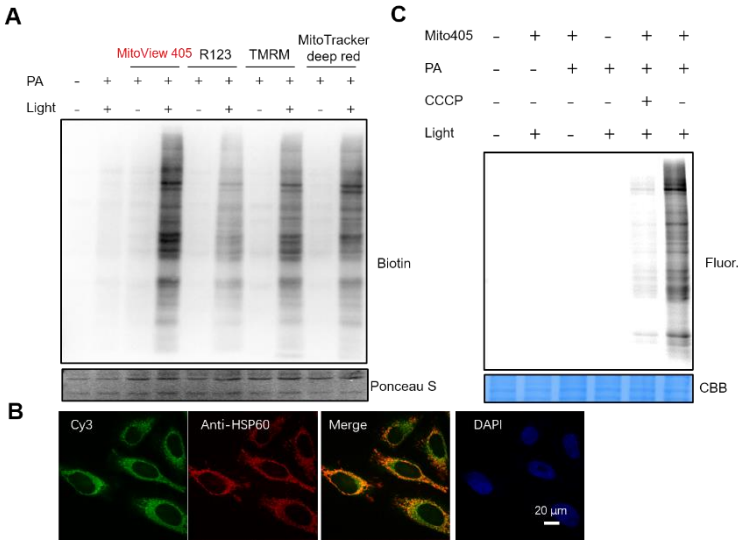

Figure 4. Evaluation of various MitoTrackers in LIMPLA2 (A) HeLa Cells were respectively stained with 10 μM R123 or 0.5μM other MitoTrackers for 2h. Cells were treated with 5 mM PA for 5 min and then illuminated for 5 min. Cell lysate were reacted with azido Cy5 for in gel fluorescence scanning. (B) After treatment with mito405, PA and illumination, cells were fixed, permeabilized and clicked with azido Cy3 via CuAAC. Cells were immunostained with Anti-HSP60. Green channel denotes azido Cy3 signal. Red channel denotes HSP60 signal. Blue channel denotes DAPI signal. (C) In gel fluorescence scanning analysis of protein labeling. Cells were pretreated with CCCP for 5 min, and then incubated with mito405 and PA.

I use mass spectrometry to identify the PA labeled proteins, I performed stable isotope dimethyl labeling based quantitative proteomics (**Figure 5B**). HeLa cells

treated with mito405, PA and illumination and cells treated with mito405, PA and illumination are respectively as experimental and negative control group (**Figure 5A**). Treated HeLa cells were lysed, reacted with Azido-biotin. Biotin labeled proteins were pull down with streptavidin beads. After reduced with DTT (DL-dithiothreitol) and alkylated with IAA (Iodoacetamide), the proteins on beads were digested with trypsin overnight and subsequently labeled with heavy formaldehyde $^{13}CD_2O$ for experimental group and light formaldehyde CH_2O for the control group in replicate 1 or labeled with light formaldehyde CH_2O for experimental group and heavy formaldehyde $^{13}CD_2O$ for the control group in replicate 2. Finally, samples in experimental group and control group were mixed in a 1:1 ratio and subjected to LC-MS/MS for identification and quantification**.** In replicate 1 and 2, I have respectively identified 283 and 201 proteins. About 60% proteins are mitochondrial proteins.

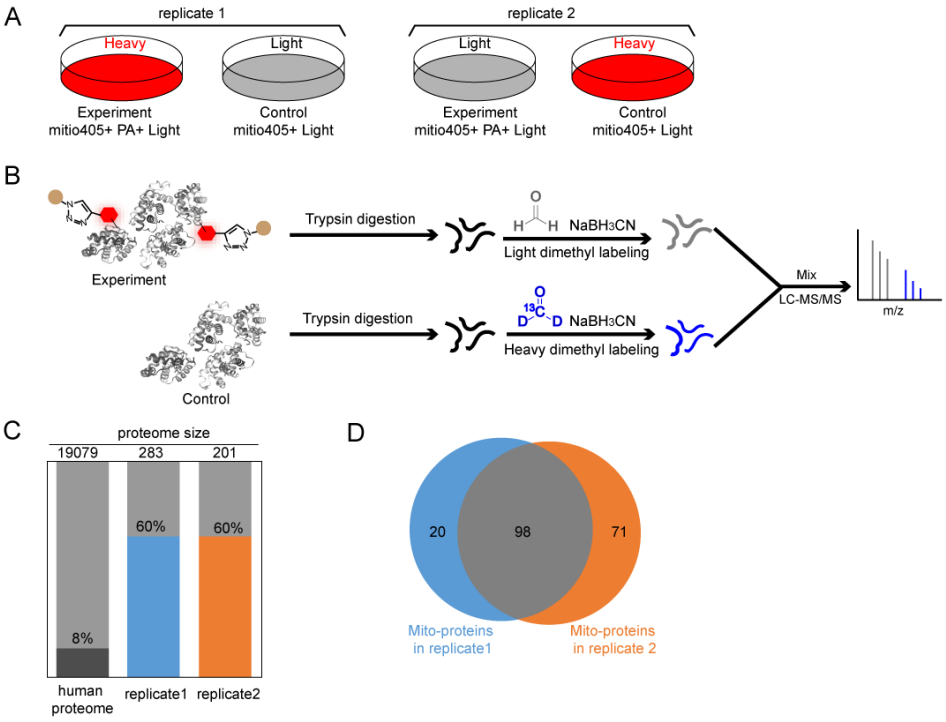

Figure 5. Identification of proteins labeled with LIMPLA2. (A) Experimental setup. HeLa cells treated with mito405, PA and illumination and cells treated with mito405, PA and illumination are respectively as experimental and negative control group. (B) The schematic of protein sample preparation for mass analysis. The cutoff of the H/L ratio in replicate 1 or L/H ratio in replicate 2 is 3. (C) Mitochondrial specificity analysis of identified proteins. (D) Overlap of identified mitochondrial proteins in two replicates. I have identified 189 mitochondrial proteins.

I applied LIMPLA2 to label mitochondrial proteins in primary neuron culture. Cortical neuron cells were stained with mito405, incubated with PA and illuminated for

xenon light. Cells were analyzed with in situ fluorescent imaging (**Figure 6A**) and in gel fluorescence scanning (**Figure 6B**), which shows that LIMPLA2 can be used to labeling mitochondrial proteins in primary neurons.

I tested if other cell subcellular tracker can be used for subcellular protein labeling with LIMPLA2. I selected DRAQ5, a cell nucleus tracker, as DRAQ5 can be excited to generate reactive oxygen species that catalyze DAB polymerization on chromatin in the nucleus for visualizing 3D chromatin structure[12]. HeLa Cells were stained with DRAQ5, treated with PA and then illuminated with xenon light. Cell lysate were reacted with azido Cy5 via CuAAC and the in gel fluorescence scanning results show that only DRAQ5 can mediated PA labeling (**Figure 6C**). Imaging results (**Figure 6D**) shows that DRAQ5 signals were well correlated with click signals, which indicated that the proteins labeling were specifically confined in cell nucleus.

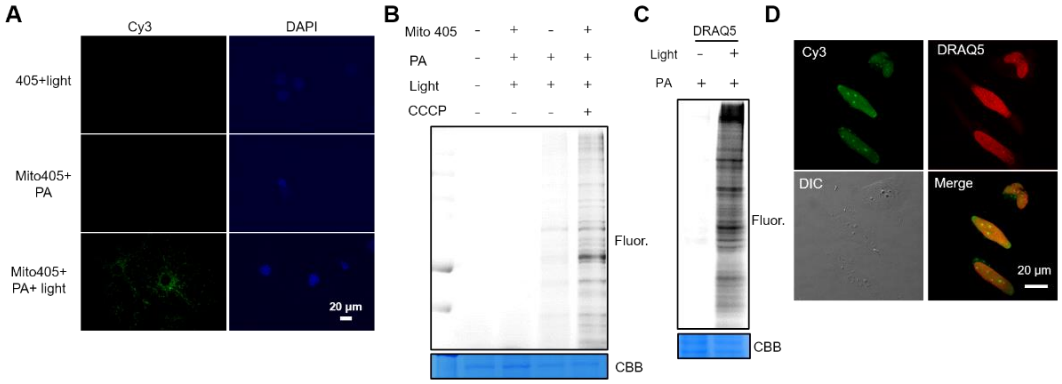

Figure 6. Cortical neuron cells mitochondrial protein and nucleus protein labeling with LIMPLA2. (A) Cortical neuron cells (DIV3) were stained with 0.2 μM mito405 for 2h, incubated with 5 mM PA for 5 min and illuminated for 5 min. Cells were clicked labeled with azido-Cy3 and analyzed with in situ fluorescent imaging (B) In gel fluorescence scanning analysis of labeling cortical neuron cells. (C) HeLa Cells were stained with 10 μM DRAQ5 for 2h. Cells were treated with 5 mM PA for 5 min and then illuminated for 5 min. Cell lysate were reacted with azide-Cy5 for in gel fluorescence scanning. (D) After treatment with DRAQ5, PA and illumination, cells were fixed, permeabilized and clicked with azido Cy3 via CuAAC. Green channel denotes azido-Cy3 signal. Red channel denotes DRAQ5 signal.

Further, I verified that far red light can mediate LIMPLA2 labeling (far red LIMPLA). It was reported that far red light with much longer wavelengths can result in decreased phototoxicity compared with blue or green light[13]. I chose far red dye Mitotracker deep red (spectrum shown in Figure 7A) for testing. BSA mixed with

Mitotracker deep red dye and PA were illuminated with xenon lamp or xenon lamp with various wavelength light filter. After protein precipitation, BSA were click labeled with azido Cy3 dye. In gel fluorescence scanning showed that far red group with 600-660nm band pass filter or 650 nm- long pass filter can show significant signals than negative group, but the signals were weaker than 400 nm- long pass filter or full length (xenon lamp only without filter) group. This result demonstrated far red light is compatible with LIMPLA2.

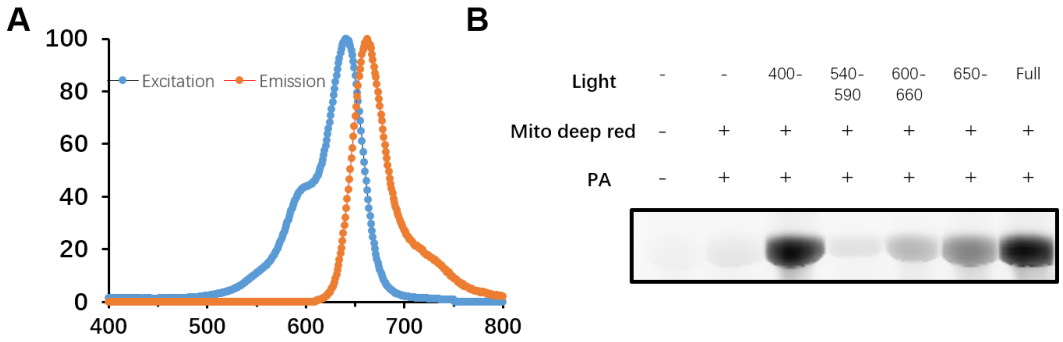

Figure 7. Protein labeling with far red LIMPLA. A. Excitation and emission spectrum of Mitotracker deep red. B. In gel fluorescence scanning analysis of protein labeling mediated with various wavelength light. 1mg/mL BSA mixed with 10 μM Mitotracker deep red and 1mM PA were illuminated with xenon lamp or xenon lamp with 400nm-, 540-590nm, 600-660nm filter for 2min. After protein precipitation, BSA were click labeled with azido Cy3.

I wonder that if the light mediated labeling strategy can be used for RNA labeling. HeLa cells were treated with R123 for 2h (negative group were treated with CCCP), cultured with 1 mM PA for 5min and then illuminated with xenon lamp for 5min. RNA were isolated from these cells and click labeled with azido biotin. The RNA labeling were analyzed by dot blot analysis. 10 and 50 μM R123 group showed significant signals, while the signals were abolished by CCCP which shows that the labeled RNA should be located in mitochondria. This result indicated that the light mediated labeling strategy can be extended to RNA labeling.

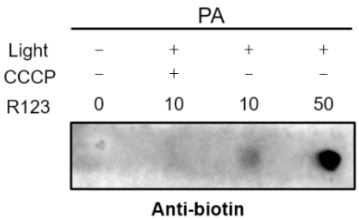

Figure 8. RNA labeling by LIMPLA2. HeLa cells were treated with 10 or 50 μM R123 (or supplemented 5 μM CCCP) for 2 h, cultured with 1 mM PA for 5 min. RNA were isolated from these cells and click labeled with azido biotin. Purified RNA were analyzed by dot blot analysis.

Discussions and conclusions

In this study, I developed 2 versions of light mediated proximal labeling strategy (LIMPLA). By combining with click chemistry and quantitative mass-spec technique, this strategy allowed the identification of 189 mitochondrial proteins, with a specificity of 60%.

The LIMPLA1 strategy is related to cell labelling via photobleaching (CLaP)[14], a method designed to specifically tagging individual cells based on photobleaching. In CLaP, a laser is used to crosslink fluorescent biotin conjugates to cell membrane of living cells via free radicals generated by photobleaching. Instead of cell membrane labeling, our method utilized crosslink of proteins in mitochondria via photobleaching. Mitochondria-localizable reactive molecules (MRMs)[15] were designed to label and profile mitochondrial proteins, which is no need of expression of exogenous proteins and H_2O_2. However, reactive molecules may react with highly abundant proteins while the molecules cross through the cytosol to mitochondria, which leads to artifacts and the MRMs labeling process requires starvation of serum for 6 h, which affects expression and phosphorylation of multiple proteins[16-17]. A fluorophore eosin mediated RNA and protein labeling method[18-21] is inspiring and powerful, in which eosin is spatially confined via conjugated with halo tag protein or other subcellular targeting moiety, and then eosin will activate PA for labeling proximal macromolecule catalyzed by visible light (LIMPLA2 is similar with this method). In this method, eosin derivatives are not commercial available which means much chemical synthesis work is indispensable; eosin may be relatively toxic to cultured cell even under natural light

compared with common dye as eosin is always used as a photosensitizer which also means eosin may have higher photo-labeling efficiency.

This strategy can be generalized to other organelle fluorescent trackers for subcellular proteins identification (nucleus tracker showed in **Figure. 6**) or to analyze proximal proteins of non-protein biomolecules, such as sugar, combined with metabolic labeling strategy. For example, similar with a method termed "protein oxidation of sialic acid environments" (POSE) to map the protein environment of sialic acids in situ[22], unnatural sialic acid bearing alkynly handle metabolically labeled on cell membrane were click labeled with azido dye and then sialic acid 's proximal protein can be labeled with LIMPLA. Further, LIMPLA also have potential to be used for labeling and identifying proximal RNA and DNA in subcellular areas (RNA labeling as shown in Figure 8).

Acknowledgments

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