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Authors
Li, Ying
Aggarwal, Mahima B
Ke, Ke
et al.

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Improved Analysis of RNA Localization by Spatially-Restricted Oxidation of RNA-Protein Complexes

Ying Li[1], **Mahima B. Aggarwal**[1], **Ke Ke**[1], **Kim Nguyen**[1], and **Robert C. Spitale**[1,2]

[1]Department of Pharmaceutical Sciences University of California, Irvine. Irvine, California. 92697

[2]Department of Chemistry. University of California, Irvine. Irvine, California. 92697

Abstract

Recent analysis of transcriptomes has revealed that RNAs perform a myriad of functions beyond coding for proteins. Critical to RNA function is its transport to unique subcellular locations. Despite the importance of RNA localization it is still very challenging to study in an unbiased manner. We recently described the ability to tag RNA molecules within subcellular locations through spatially-restricted nucleobase oxidation. Herein we describe a dramatic improvement of this protocol through the localized oxidation and tagging of proteins. Isolation of RNA-protein complexes enabled the enrichment of challenging RNA targets on chromatin and presented a considerably optimized protocol for the analysis of RNA subcellular localization within living cells.

Graphical Abstract

Authors are required to submit a graphic entry for the Table of Contents (TOC) that, in conjunction with the manuscript title, should give the reader a representative idea of one of the following: A key structure, reaction, equation, concept, or theorem, etc., that is discussed in the manuscript. Consult the journal's Instructions for Authors for TOC graphic specifications.
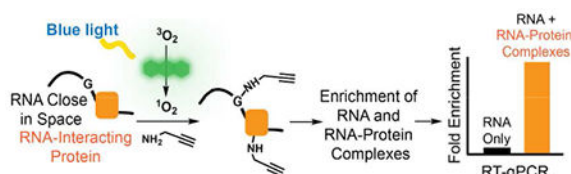
RNA molecules perform key functions at the heart of many normal biological pathways and are key drivers in the onset of many diseases.[1–3] The localization or sequestration of RNA molecules to specific subcellular compartments is critical to the regulation of their functions.

[4–6] Despite the importance of RNA localization, there is a real lack of methods to systematically analyze RNA location inside cells in an unbiased manner[7–10].

Our laboratory recently developed a novel method to assay RNA localization within intact living cells through spatially-restricted nucleobase oxidation.[11] Using Halo-tag fusion proteins, we localized the oxygen photosensitizer dibromofluorescein (DBF) within subcellular compartments. Blue light exposure generated short-lived singlet oxygen, resulting in guanosine oxidation in RNAs nearby in space. Oxidized guanosines can react with nucleophiles in solution and as such we utilized propargyl amine (PA) to append alkyne handles onto these RNAs for downstream study. RT-qPCR data demonstrated that this oxidative approach provides subcellular, and even sub-organellar resolution for RNA localization studies (Scheme 1A).

Despite our exciting results, two observations convinced us to seek an improved RNA tagging approach. First, the overall yield of guanosine labeling is far from satisfactory (~10% on a free oligonucleotide in solution [11]). Considering the various singlet oxygen quenchers and competitive nucleophiles in cells, we anticipated even lower reactivity. Second, several positive-control chromatin-associated RNAs were not tagged and enriched, even when localizing DBF to chromatin, through Histone 2B (H2B)-Halo tethering. This result was especially surprising as confocal fluorescence imaging data indicated robust and localized PA adduct formation in the nucleus. These observations challenged us to interrogate the molecular details of our approach. Within this Communication we report a dramatic improvement in our approach, through the isolation of RNA-protein complexes, which have been tagged through singlet oxygen-mediated tagging (Scheme 1B). We anticipate that our improved method provides a streamlined and experimentally superior approach to increase the yield of subcellular RNA tagging in an unbiased manner.

To systematically identify the biomolecules which form adducts with PA, cells were processed as previously described, lightly fixed, permeabilized, and incubated with RNase, DNase, or proteinase, respectively (Supporting Information, SI). PA adducts were visualized in fluorescence confocal microscopy through Cy5 signals after copper-catalyzed azide-alkyne cycloaddition (CuAAC) treatment. Imaging results (Figure 1A) revealed that RNase and DNase treatment didn't cause significant Cy5 signal reduction. In contrast, Cy5 signal was undetectable after proteinase treatment, while Hoechst stain remained. These results suggested that the large majority of PA adducts in cells, and the corresponding signal in imaging experiments, stem from PA-protein adducts.

Many examples in the literature have demonstrated that electron-rich side chains (phenylalanine, tryptophan, histidine, cysteine) can be oxidized in presence of singlet oxygen.[12–16] Oxidized amino acids can then be attacked by nucleophiles to form adducts. [16, 17] These observations, coupled with our results above, prompted us to explore whether our approach was capable of forming PA adducts with proteins *in vitro.*

We utilized bovine serum albumin (BSA) as a model protein. We incubated BSA with tetrabromofluorescien (TBF; Figure 1B) and PA in the presence of blue light. After incubation, PA adducts were labeled by Cu-AAC with Cy5 azide and imaged using

denaturing gel electrophoresis. As shown in Figure 1C, Cy5 signal was only observed in the presence of TBF, PA, and light. We also performed a time-course analysis of adduct formation and observed increasing Cy5 signals over time (Figure 1D). In contrast, increasing concentrations of sodium azide (singlet oxygen quencher[18], NaN$_3$)) resulted in decreasing adduct formation (Figure 1E). The same concentrations of sodium chloride (NaCl) didn't affect the Cy5 signals, suggesting that the protein modification was indeed driven by singlet oxygen. These results confirmed that proteins are also subjected to singlet-oxygen induced oxidation and nucleophilic addition under the conditions previously used for imaging, RNA tagging and enrichment.

Our observations suggest that proteins are robustly tagged with PA *in vitro* and they contribute most to the PA adduct formation in cells. These results may explain the inability to tag certain RNAs that are tightly associated with protein complexes and have limited solvent accessibility. Recent data demonstrated that the large majority of RNA sequences are covered or interacting with protein molecules.[19–21] RNAs might have even more hampered solvent accessibility within densely packed heterochromatin regions[22–24], thus were not detected by our previous labeling method. We were enticed to explore if protein tagging could also take place in a spatially-restricted manner, providing a roadmap for the possibility of utilizing such characteristics for assaying RNA localization.

To ensure that protein labeling still affords tight spatial resolution, we performed protein analysis in H2B-Halo expressing cells (Figure 2A). We first confirmed the adduct formation with adjacent histone protein H3 via histone protein extraction after light treatment (SI, Figure S1). To systematically test the spatial resolution of proteins in detail, we picked six proteins to compare: H3, HDAC1, and hnRNPH (chromatin associated)[25]; Nucleolin (nucleolus)[26]; Gapdh and Alpha Tubulin (cytoplasm)[27, 28]. After treatment, the cells were lysed and the lysate were submitted to biotinylation through CuAAC with biotin-azide, enrichment via streptavidin-biotin interaction, gel electrophoresis and western blot (**SI**). As shown in Figure 2B, the chromatin associated proteins, H3, HDAC1 and hnRNPH, were highly enriched (~10% of the input). Nucleolin, Gapdh, and alpha Tubulin, despite being highly abundant in the cells were not enriched.

To further test our resolution, we compared these results to a nuclear localization sequence (NLS)-Halo fusion, which has diffuse nuclear and nucleolus localization (Figure S2).[29] As shown in Figure S3, the nuclear and particularly nucleolus proteins were enriched, albeit to a much lower percent, compared to the 10% input loading. These results further suggest that tagging is tightly controlled by the spatial proximity of DBF to the protein of interest, which could lay the foundation for us to assay RNA localization through RNA-protein complexes (Scheme 1B). We foresee our protein tagging approach may complement other existing technologies such as APEX[30] and Bio-ID[31] in a light controllable manner. Very recently, APEX has been applied to assay cellular RNA spatial organization with short-lived phenoxyl radicals (< 1 ms[32]).[33] We anticipate that reactive singlet oxygen (< 3.5 μs[34, 35]) will provide a complementary approach.

To take advantage of spatially-restricted protein labeling for the analysis of RNA localization we utilized mild crosslinking (0.1% PFA), which has been shown to trap *in*

cellulo RNA-protein complexes.[25, 33] We developed an optimized protocol for enrichment of protein-RNA complexes that had been modified with PA (SI, Figure S4). We profiled the RNA isolated from these ribonucleoprotein complexes by dot blot (Figure S5) and RT-qPCR. We utilized the positive-control chromatin-associated RNA 7SK as our benchmark and also focused on other chromatin-associated RNAs (XIST, KCNQ1OT1, HOTAIR)[25], which were barely detectable in our previous study. RNAs from other sites in the cell (U13 and U3 – nucleolus[36], GAPDH and ACTIN – cytoplasm[37, 38]) were chosen as negative controls. As shown in Figure 3A, our new protocol based on RNA isolation after enrichment (IAE, shown in green) effectively enriched chromatin-associated RNAs. The fold enrichment was dramatically increased compared to our previous method (RNA isolation before enrichment (IBE); shown in black). Meanwhile, RNAs that localized primarily in the nucleolus and cytoplasm (shown in red) were not enriched by the RNP approach. These data jointly demonstrated that the enrichment of protein-RNA crosslinks improved the yield of RNAs without sacrificing the spatial resolution.

Our RT-qPCR result is consistent with literature reports, which rely on more complicated and laborious fractionation protocols.[39] Ruthenburg and coworkers assayed the distribution of RNAs in the nucleus by isolating RNAs separately from the soluble nuclear fraction and the insoluble chromatin fraction through multiple rounds of laborious cellular fractionation and centrifugation. For their calculation of enrichment they compared the Chromatin Pellet Extract (CPE) with the Soluble-Nuclear Extract (SNE) ratios. We compared our fold-enrichment analyses with the chromatin/nuclear ratios of the RNAs we interrogated. High enrichment of XIST and KCNQ1OT1 is consistent with their tight association with chromatin (high CPE/SNE); HOTAIR is more diffuse in the soluble nuclear fraction (low CPE/SNE ratio) and were less enriched.[39] Lastly, consistent between our approaches cytoplasmic RNAs GAPDH and ACTB had very low CPE/SNE ratios and extremely low enrichment by our approach. These results strongly suggest our experimental method can enrich RNAs within tightly associated subsections of cells with high resolution.

Finally, RNA enrichment with the NLS-Halo construct (diffuse nuclear localization) showed that chromatin-associated RNAs have much lower fold-enrichment and RNAs localized in the nucleolus are now enriched at a similar level if not higher to those on chromatin (Figure 3C). These results further demonstrated that the proximity of singlet oxygen determines the efficiency of PA-adduct formation (Figure 3D) and the new RNP protocol affords tight spatial resolution.

The results described here extend the possibilities of using spatially restricted singlet-oxygen formation to assay cellular localization of various biomolecules. We have confirmed the tight spatial resolution for protein labeling. In addition, we have reported an improved protocol to assay RNA localization by enriching RNA-protein complexes. We envision our optimized protocol will be readily applied to many other locations in cells as Halo-fusion proteins are now commercially available for many human proteins. The critical investigation herein lays an experimentally optimized roadmap for wide-scale and systematic analysis of cellular RNA localization in parallel to protein localization. Such studies are currently underway in our lab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

ABBREVIATIONS

PA,	propargyl amine
DBF,	dibromofluorescein
TBF,	tetrabromofluorescein
ORF,	open reading frame
CuAAC,	coppercatalyzed azide-alkyne cycloaddition

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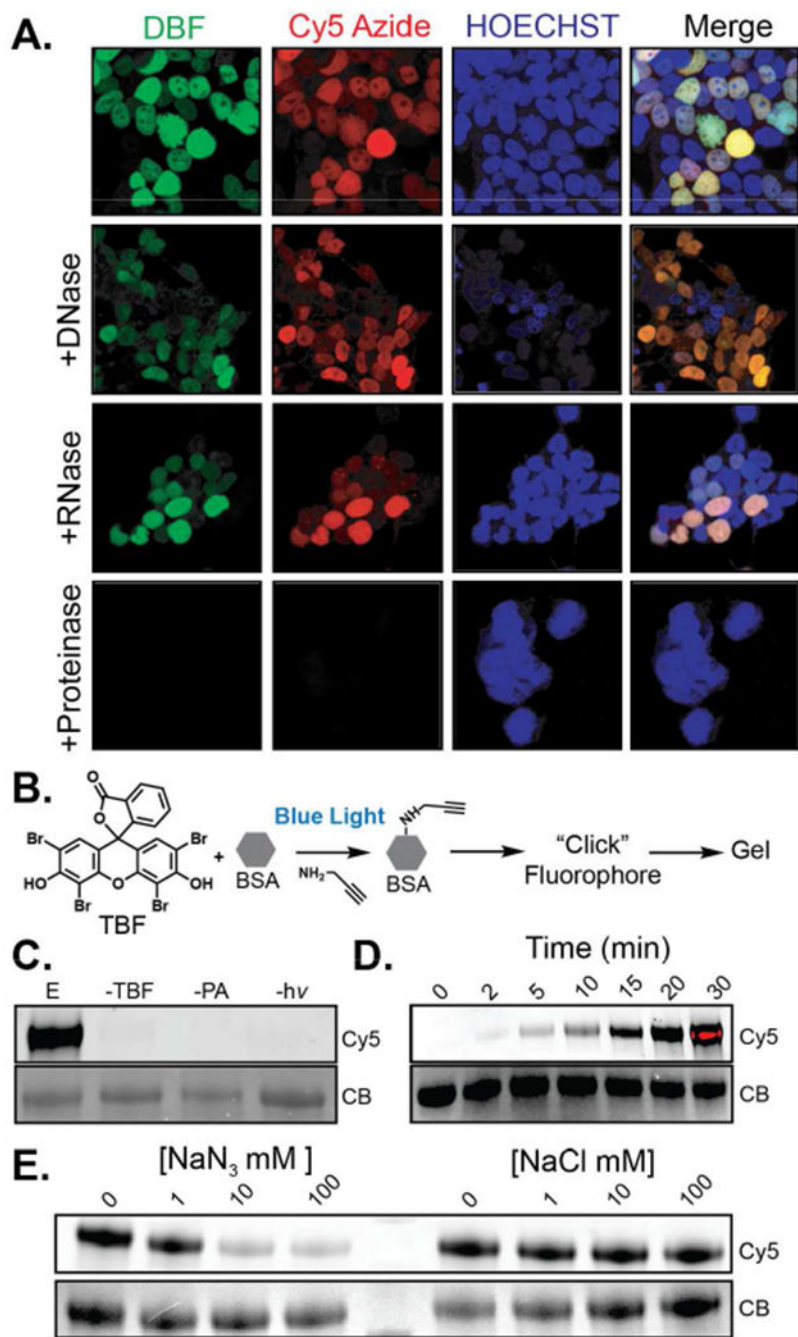
Figure 1. Singlet oxygen-based protein tagging.
A. Imaging results after cells being treated with nothing, DNase, RNase and proteinase, respectively. DBF denoted in green, PA adducts denoted in red, and nuclear stained with Hoechst and shown in blue.B. Schematic of protein tagging with singlet oxygen.C. Denaturing gel-imaging with Cy5; E represents "Experimental", which has TBF, PA and hv, while three other headers represent different negative control conditions.D. Time course of adduct formation (red: over-saturated signal).E. Quenching of protein tagging with NaN_3. CB: Coomassie blue.
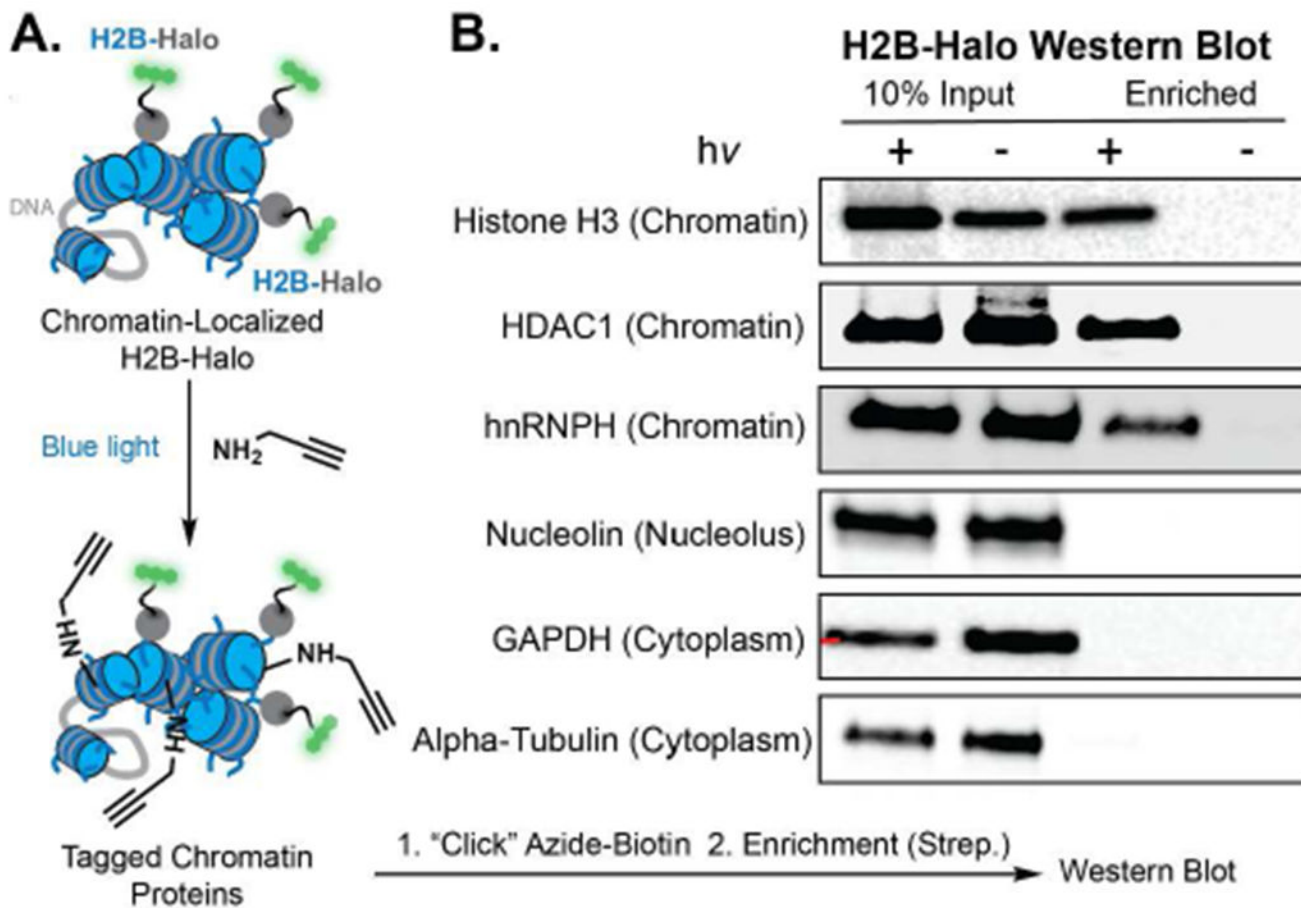
Figure 2. Spatially-restricted singlet oxygen-based protein tagging.
A. Schematic of spatially-restricted chromatin tagging with singlet oxygen.B. Biotin-Streptavidin enrichment-western blot analysis of tagged proteins with the H2B-Halo fusion construct.
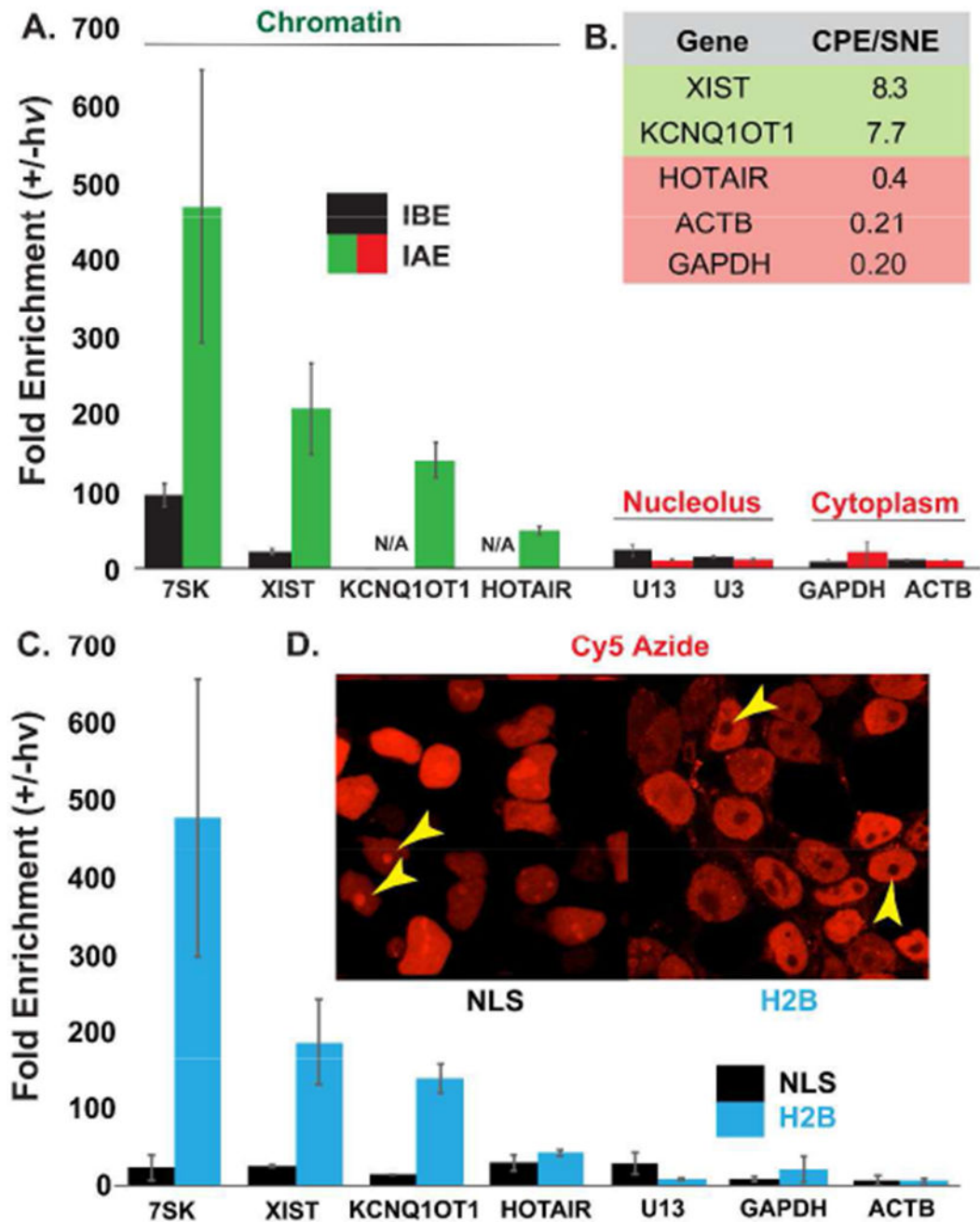
Figure 3. RT-qPCR analysis of RNAs tagged by enrichment of RNA-protein complexes.
A. RT-qPCR comparison of the previous method IBE and the current method IAE.B. Reported ratio of Chromatin Pellet Extract (CPE) to Soluble-Nuclear Extract (SNE)[39]. C. RT-qPCR comparison of H2B-Halo and NLS-Halo constructs by IAE approach. D. Fluorescence imaging signals of Cy5 azide (PA adducts denoted in red) for H2B-Halo and NLS-Halo with yellow arrows pointing at enhanced nucleolus signal in NLS but lack of signal in H2B. Enrichment was calculated against negative control with Ot method (SI), biological duplicates.
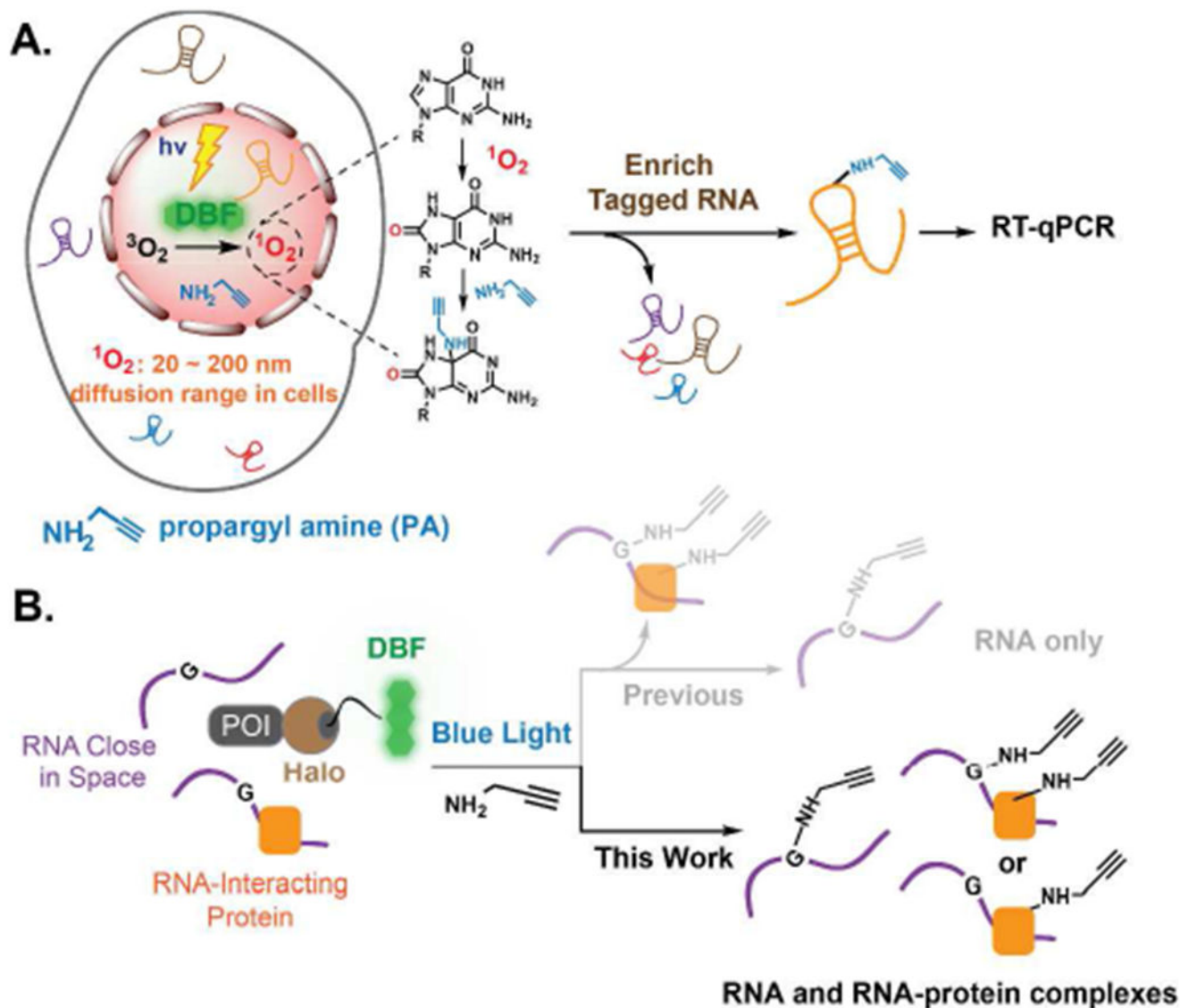
Scheme 1. Assaying RNA localization with spatially-restricted oxidation.
A. Schematic of RNA oxidation with fluorophores (DBF, shown in green) and subcellular RNA tagging with PA.B. Schematic of comparison between previous work and this work. In previous work, free tagged RNAs were isolated and profiled. In this work, pulldown of RNA-protein complexes increased the yield of RNA enrichment. POI: protein of interest; hv: light irradiation.