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Letters

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Light-Activated Proteomic Labeling *via* Photocaged Bioorthogonal Non-Canonical Amino Acids

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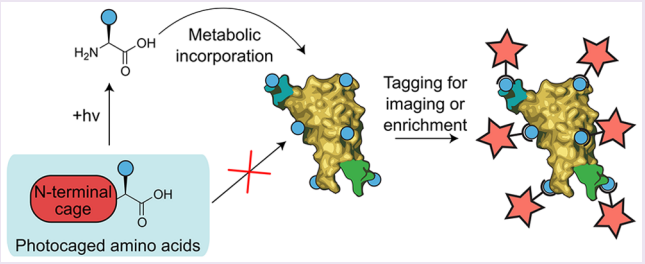
Supporting Information

ABSTRACT: This work introduces light-activated bioorthogonal noncanonical amino acid tagging (laBONCAT) as a method to selectively label, isolate, and identify proteins newly synthesized at user-defined regions in tissue culture. By photocaging L-azidohomoalanine (Aha), metabolic incorporation into proteins is prevented. The caged compound remains stable for many hours in culture, but can be photochemically liberated rapidly and on demand with spatial control. Upon directed light exposure, the uncaged amino acid is available for local translation, enabling downstream proteomic interrogation *via* bioorthogonal conjugation. Exploiting the reactive azide moiety present on Aha's amino acid side chain, we demonstrate that newly synthesized proteins can be purified for quantitative proteomics or visualized in synthetic tissues with a new level of spatiotemporal control. Shedding light on when and where proteins are translated within living samples, we anticipate that laBONCAT will aid in understanding the progression of complex protein-related disorders.



Biological processes are staggeringly dynamic and heterogeneous. Though all cells within an organism share a common genome, differential expression of genes into proteins regulates developmental processes, tissue morphogenesis and function, disease susceptibility and response, and a diverse array of signaling events governed by a wide variety of intra- and extracellular cues. While each protein is encoded by a gene, protein quantity and activity cannot be determined through genomic or transcriptomic analysis;[1,2] such techniques are blind to post-transcriptional phenomena (*e.g.*, translational regulation, modification, protein-biomolecular interactions), necessitating strategies to directly measure protein identity and abundance.[3]

Recent advances in mass spectrometry and genomic sequencing have enabled high-throughput proteomic analysis, whereby the abundance, turnover, modification, and interactions of thousands of proteins can be measured in minutes.[4,5] From this has emerged a growing appreciation of the exceptionally dynamic nature of the proteome, which undergoes large-scale biochemical shifts during cellular proliferation, migration, and differentiation. Efforts to quantify temporal variations of the proteome have focused on the pulsed labeling of cultures with specialty amino acids that can distinguish newly synthesized from pre-existing cellular proteins.

One particularly powerful method for interrogating proteomic fluctuations in culture is known as bioorthogonal noncanonical amino acid tagging (BONCAT). In BONCAT, pulsing cells with noncanonical amino acid (ncAA) analogs yields newly synthesized proteins that bear bioorthogonal reactive groups (*e.g.*, azides, alkynes).[6−8] Metabolically labeled proteins can be covalently modified with an affinity tag that is then exploited for purification,[9] enabling isolation of proteins synthesized over a short window of time and providing temporally resolved proteomics.

As protein expression, degradation, translocation, and post-translational modification occur at different rates depending on cellular and subcellular location within tissues,[10] we sought to control BONCAT within user-defined regions of culture. Recognizing light's unique ability to initiate chemical reactions at a time and place of interest, we developed a light-activated bioorthogonal noncanonical amino acid tagging (laBONCAT) approach (Figure 1). This strategy relies on the introduction of a molecular photocage onto the α-amine of an ncAA, preventing metabolic incorporation into proteins. Upon user-

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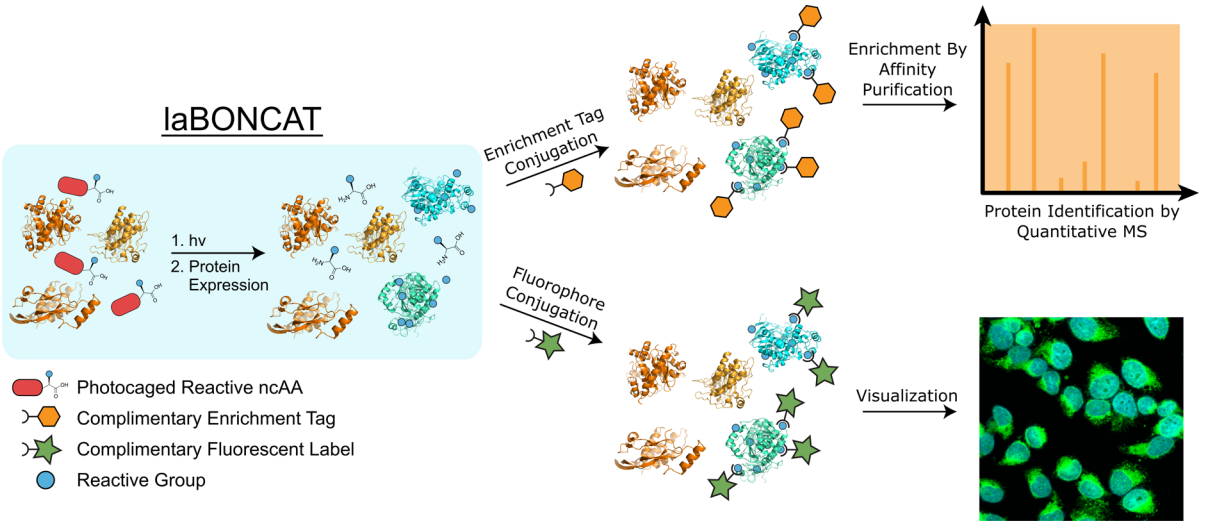

Figure 1. Light-activated bioorthogonal noncanonical amino acid tagging (laBONCAT). Photocaged noncanonical amino acids (ncAA) become available for stochastic incorporation into newly translated proteins following directed light exposure. Bioorthogonal handles installed by ncAAs can be exploited for protein purification prior to quantitative proteomics or fluorescent tagging for visualization.

directed light exposure, the ncAA is liberated and made available for incorporation into newly translated proteins. Subsequent labeling and enrichment of these proteins is exploited for proteomic analysis.

While laBONCAT methodologies can be theoretically applied to any amino acid (including stable isotopes of natural amino acids, ncAAs, and other variants useful for quantitative proteomics), we first sought to demonstrate its utility using L-azidohomoalanine (Aha). Aha is an azide-bearing ncAA that is metabolically incorporated by endogenous cellular machinery as a methionine (Met) surrogate[11−13] whose low-level incorporation does not significantly alter protein expression[14,15] (Figure 2a). Aha's azido functionality represents a useful bioorthogonal handle for subsequent labeling reactions, including the strain-promoted azide−alkyne cycloaddition[16] (SPAAC; Figure 2b). We synthesized a photocaged Aha (NPPOC-Aha, Figure 2a) through condensation of the α-amine of Aha[17] with the activated ester of 2,5-dioxopyrrolidin-1-yl (2-(2-nitrophenyl)propyl) carbonate[18] (NPPOC). As NPPOC-caged amines undergo irreversible β-elimination upon exposure to near-ultraviolet light[18] (λ = 365 nm; Figure 2c), Aha can be photochemically generated *in situ* in response to mild and cytocompatible light exposure.[18−22] We believe this to be the first example of an amino acid (canonical or otherwise) that has been photocaged at its N-terminus to prevent translation.

For this system to be effective, the kinetics of NPPOC uncaging should be rapid, such that the liberation of free Aha is not rate limiting compared with the biological processes under study. To determine its photolysis kinetics, NPPOC-Aha (dissolved in H_2O/CH_3CN, 50:50) was irradiated with collimated UV light[18] (λ = 365 nm, 10 mW cm^{-2}, 0−600 s exposure). Degradation products were quantitatively analyzed by HPLC, with elution fractions compositionally identified by mass spectrometry. A first-order decay constant of 0.0075 ± 0.0002 s^{-1} was observed for NPPOC photolysis (Figure 2d); 90% of the NPPOC cleaved after 5 min of mild irradiation (10 mW cm^{-2}), a time scale suitable for many biological applications.

To demonstrate photomediated incorporation of Aha, Met-depleted HeLa cells were incubated with NPPOC-Aha (250 μM). Subsequent irradiation with UV light (λ = 365 nm, 10

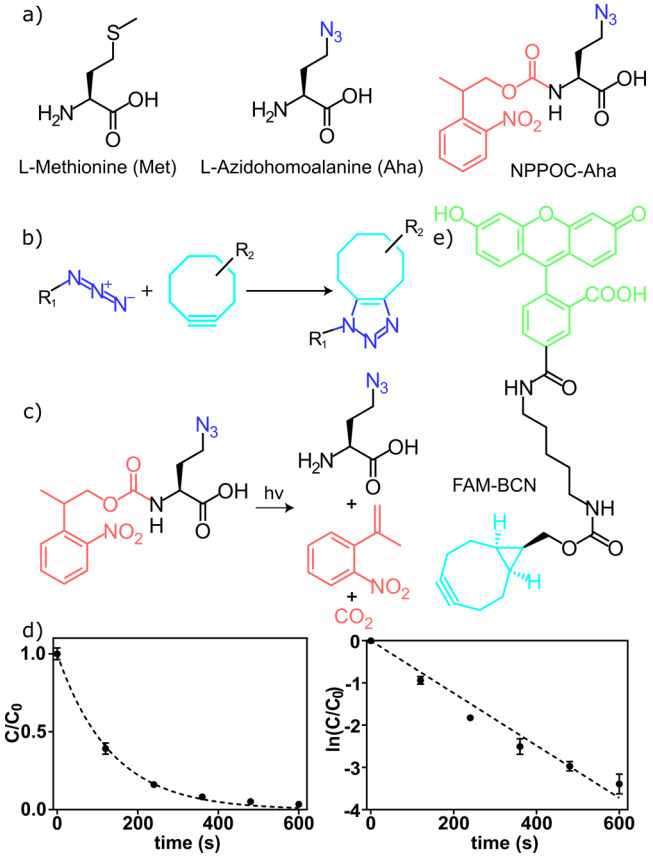

Figure 2. Chemical structures, reaction diagrams, and photokinetics. (a) Structures of L-methionine (Met), L-azidohomoalanine (Aha), and photocaged Aha (NPPOC-Aha). (b) SPAAC is a bioorthogonal reaction for labeling azides incorporated into proteins with strained cyclooctynes. (c) Upon light exposure, photolysis of NPPOC-Aha yields free Aha for incorporation into newly synthesized proteins. (d) Kinetic analysis of NPPOC-Aha photolysis demonstrates rapid uncaging suitable for biological sampling. (e) A carboxyfluorescein-labeled bicyclononyne (FAM-BCN) provides a fluorescent tag for labeling azide-functionalized proteins.

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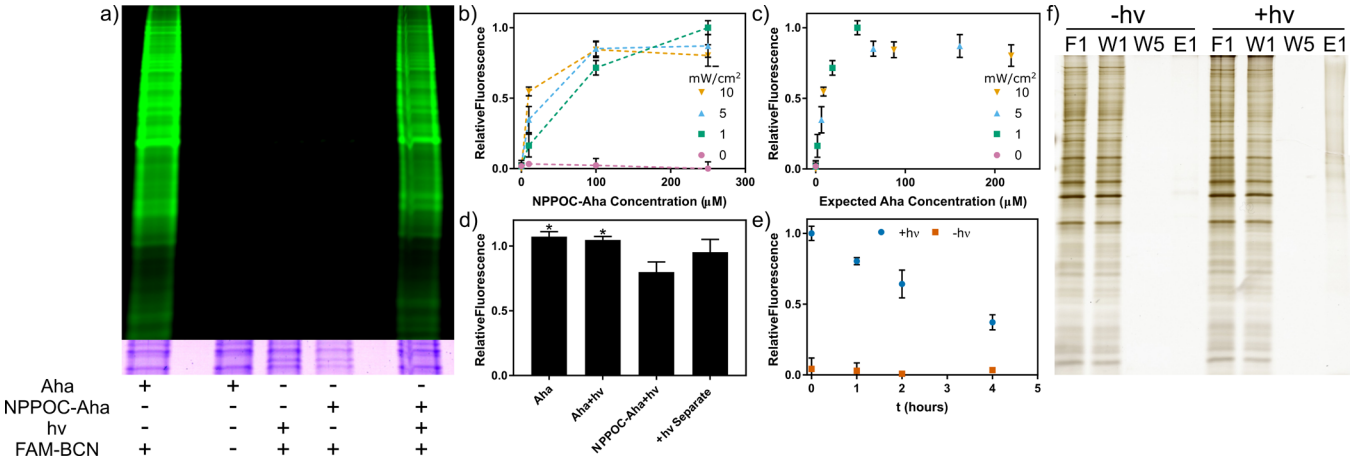

Figure 3. *In vitro* analysis of light-activated bioorthogonal noncanonical amino acid tagging (laBONCAT), stability, and isolation. (a) Analysis of fluorescently labeled protein lysate by SDS-PAGE. *In vitro* incorporation of free L-azidohomoalanine (Aha) and Aha after photoliberation provides azide-functionalized proteins for fluorescent tagging by carboxyfluorescein-labeled bicyclononyne (FAM-BCN). Only samples incubated with free Aha or light-treated photocaged Aha (NPPOC-Aha) are fluorescently labeled. Coomassie staining indicates near-uniform protein loading. (b) The effect of light intensity and NPPOC-Aha concentration on azide incorporation into newly synthesized proteins was investigated, based on protein fluorescence. (c) The degree of incorporation from part b normalized for expected free Aha concentration. (d) Light irradiation itself does not impact ncAA incorporation of Aha (*$p < 0.05$ by one-way ANOVA followed by Tukey's test). (e) A significant amount of NPPOC-Aha remains stable over several hours in media and in contact with live cells suitable for labeling new proteins in tissue culture. (f) Following photomediated Aha incorporation, newly synthesized proteins can be isolated by affinity purification for downstream proteomic analysis. Fractions from left to right: flow through 1 (F1), wash 1−5 (W1−5), and elution 1 (E1).

mW cm^{-2}, 5 min) yielded photoliberated Aha for metabolic incorporation. Two hours after light exposure, cells were lysed, and their proteins were treated with a bicyclononyne-modified fluorescein (FAM-BCN, 100 nM, Figure 2e) to introduce a fluorescent label by SPAAC. Protein fluorescence was then used to quantify the extent of Aha incorporation following protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Nonirradiated samples and the Met control exhibited a similar lack of fluorescence; the UV-treated NPPOC-Aha and Aha control displayed significant fluorescent enhancement (Figure 3a), indicating successful implementation of laBONCAT.

To control the extent of ncAA incorporation into newly synthesized proteins, we varied NPPOC-Aha concentration (0−250 μM) and the intensity of light irradiation (0−10 mW cm^{-2}) employed during photouncaging. As expected, Aha incorporation increased with NPPOC-Aha concentration for a given exposure condition; for low NPPOC-Aha concentrations, metabolic labeling increased with light intensity (Figure 3b). When the extent of incorporation was normalized for the expected concentration of liberated Aha, based on values predicted by the photokinetics and assuming no side reactions accompanying photolysis, the result was a smooth, continuous curve that plateaus above ∼50 μM free Aha (Figure 3c). To determine the potential effects of UV irradiation on metabolic incorporation, labeled protein fluorescence was compared for samples treated with Aha (50 μM) ± light (10 mW cm^{-2}, 5 min; Figure 3d). Finding no statistical difference in protein labeling following UV irradiation, we compared incorporation between Aha and irradiated NPPOC-Aha cultures (each at 100 μM). NPPOC-Aha + light gave rise to slightly less incorporation than Aha alone, which we attribute to incomplete photoconversion of NPPOC-Aha to Aha. This is supported by data that protein labeling does not depend on whether NPPOC-Aha is irradiated separately or during incubation with cells (Figure 3d).

To assess its *in vitro* stability, NPPOC-Aha (100 μM) was incubated in media with HeLa cells for 0−4 h prior to light exposure (10 mW cm^{-2}, 5 min) and subsequent metabolic labeling (2 h). Aha incorporation was observed for all irradiated samples, though its extent decreased over time. This was attributed to unknown cellular processing of NPPOC-Aha; simple hydrolysis yielding free Aha does not explain this behavior, as nonirradiated samples do not show increased incorporation over time. While the >4 h of working time is likely sufficient for many applications, we anticipate that different photocages and/or ncAAs may exhibit increased long-term stability. Such stability is useful in sampling biological systems, as it decouples media swaps from proteome labeling, allowing researchers to standardize their experimental conditions.

After demonstrating the ability to label newly synthesized proteins, we sought to extend the laBONCAT methodologies to their affinity purification. After NPPOC-Aha uncaging and metabolic incorporation of the ncAA, proteins were biotinylated *via* SPAAC with a dibenzocyclooctyne-modified biotin probe. Biotinylated proteins were captured on a streptavidin resin prior to protein elution by streptavidin denaturation. Eluents were subjected to SDS-PAGE and silver stained for visualization (Figure 3f). Results highlight the capability to selectively isolate newly synthesized proteins from irradiated samples.

Building on the capability to fluorescently tag cellular lysates as well as isolate species of interest via laBONCAT, we turned our efforts toward the technique's unique ability to label newly synthesized proteins *in vitro* with spatial control; we anticipate that this method could be applied to the isolation of proteins transcribed at user-specified times and locations from heterogeneous features in tissue culture, especially tissue slices. HeLa cells were treated with NPPOC-Aha (100 μM) and exposed to light (λ = 365 nm, 10 mW cm^{-2}, 5 min). Two hours after exposure, cells were fixed, permeabilized, and treated with

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FAM-BCN (100 nM) to introduce a fluorescent label *via* SPAAC. The extent of fluorescent labeling of cells treated in this method was similar to free Aha, while NPPOC-Aha in the absence of light exhibited low-level labeling similar to Met (Figure 4).

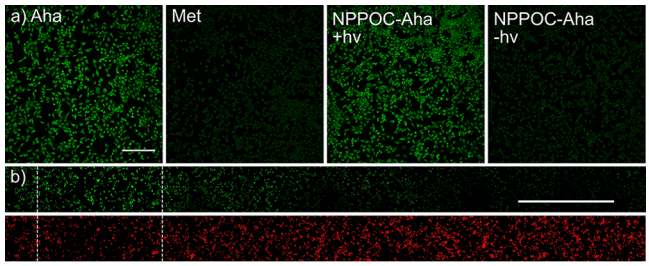

Figure 4. Irradiation of photocaged L-azidohomoalanine (NPPOC-Aha) yields free Aha for ncAA *in vitro* incorporation. (a) Fluorescent tagging of fixed cells demonstrates similar fluorescent labeling (green) of Aha and irradiated NPPOC-Aha, but not L-methionine (Met) and unexposed NPPOC-Aha (scale bar = 100 μM). (b) Metabolic labeling of cells in synthetic tissue is confined to regions near user-defined exposure (hashed area). Actin staining (red) remains uniform across sample (scale bar = 1 mm).

Next, we demonstrated the ability to control Aha incorporation spatially within synthetic tissues. Cells were encapsulated in oxime-based poly(ethylene glycol) hydrogels (7 wt %), treated with NPPOC-Aha and selectively irradiated through a slitted photomask. Cells were fixed and fluorescently labeled with FAM-BCN, phalloidin, and Hoechst. The observed cellular FAM signal was localized near exposed regions, corresponding to patterned Aha incorporation. FAM fluorescence decreased exponentially away from exposed regions in a diffusion-predicted manner. Actin and DNA staining lack substantial patterning (Figure 4, Supporting Information Figure 6).

This technique provides an inherent improvement to traditional BONCAT by allowing for precise timing in metabolic incorporation. In addition, the photochemical nature of the system provides a degree of spatial control for the investigation of heterogeneous biological systems. While not the focus of this manuscript, we anticipate that laBONCAT could be readily combined with strategies for pulsed stable isotope labeling by amino acids in cell culture[23] (pSILAC) to purify, identify, and quantify proteins expressed at user-defined regions in culture. This newfound ability is expected to prove particularly useful in the investigation of heterogeneous protein-related disease (*e.g.*, Alzheimer's), potentially yielding new diagnostic markers and therapeutic targets.

In this manuscript, we have demonstrated the ability to control protein translation spatiotemporally through laBONCAT. While the method has been initially validated using NPPOC-Aha responsive to near-UV light, facile extension of the technique to control incorporation of different amino acids using different photocage/light combinations is expected. We believe that this strategy shows unique promise toward the visualization and characterization of newly synthesized proteins at user-defined times and places of interest.

METHODS

Detailed methods and experimental conditions are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b01023.

Full experimental procedures, synthesis and characterization of all small molecules and functional polymers, control experiments for affinity purification of newly synthesized proteins, and full analysis of spatially labeled synthetic tissues (PDF)

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Author Contributions

For this manuscript, S.M.A and C.A.D. conceived and designed the experiments; S.M.A, E.R.R., P.E.F., and J.V.W. performed the experiments; S.M.A. and C.A.D. analyzed the data and prepared the figures; S.M.A. and C.A.D. wrote the paper.

Notes

The authors declare no competing financial interest.

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