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Synthesis of a Targeted Biarsenical Cy3-Cy5 Affinity Probe for Super-resolution Fluorescence Imaging

Na Fu, Yijia Xiong, and Thomas C. Squier*

Biological Sciences Division, Fundamental Sciences Directorate, Pacific Northwest National Laboratory, Richland, Washington 99352, United States

Supporting Information

ABSTRACT: Photoswitchable fluorescent probes capable of the targeted labeling of tagged proteins are of significant interest due to their ability to enable *in situ* imaging of protein complexes within native biomolecular assemblies. Here we describe the synthesis of a fluorescent probe (**AsCy3Cy5**) and demonstrate the targeted labeling and super-resolution imaging of a tagged protein within a supramolecular protein complex.

Optical microscopy is routinely used to visualize tagged proteins both within supramolecular complexes and inside cells.[1] Recent developments permit subdiffraction resolution of individual proteins,[2] which is essential to understand the structural arrangement and dynamics of subunits within macromolecular protein complexes. Key to this super-resolution imaging is the ability to accurately target fluorophores to proteins of interest, with subsequent identification of their locations by imaging the point-spread function (PSF) without interference by other proximal molecules.[2] This requirement is satisfied using autofluorescent proteins that undergo photoinduced spectral changes,[3] which permit the reconstruction of cellular structures.[4] However, the large size, limited brightness, and poor photostability limit the utility of autofluorescent proteins for super-resolution imaging and necessitate the development of fluorescent chemical probes with improved photoactivity that allow an easily controllable on/off photoswitchable capability.[4]

Existing chemical approaches to achieve super-resolution imaging have involved applications using synthetic photoswitchable fluorophores.[5] For example, using a Cy3-Cy5 emitter pair in close proximity it is possible to restore the photobleached emission of Cy5 through the brief optical pumping of the Cy3 molecule.[6] Following decoration of a secondary antibody with the Cy3-Cy5 emitter (through stochastic labeling with reactive derivatives of Cy3 and Cy5), super-resolution imaging of cellular structures is possible using fixed and permeabilized cells.[2a,5b] However, stochastic labeling methods commonly result in self-quenching through homotransfer mechanisms between proximal fluorescent dyes that complicate the underlying photophysics.[7] To achieve labeling specificity, we have designed a new imaging probe, **AsCy3Cy5** (Figure 1), which is derived from an existing biarsenical scaffold (i.e., AsCy3) previously shown to permit specific labeling of tagging sequences engineered in proteins of interest for live cell imaging of intracellular protein complexes.[8] Further, the covalent binding between the biarsenical scaffold and the tetracysteine tagging sequence permits the rigid attachment of the fluorophore to the protein backbone, thus allowing measurements of catalytically important protein domain movements.[9]

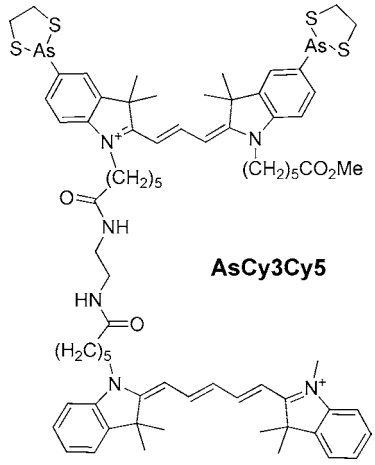

Figure 1. Structure of **AsCy3Cy5**.

AsCy3Cy5 was prepared by coupling unsymmetrical AsCy3 and Cy5 (see Scheme S1 in the Supporting Information (SI)). Considerations relating to the ease of synthesis dictated that the reaction was started with an unsymmetrical Cy5 with a monocarboxylic acid, which was coupled with *N*-Boc-ethylenediamine in DMF/EDC/HOBt at room temperature. After deprotection by the TFA/CH_2Cl_2, an amino-Cy5 was obtained with an overall yield of 76%. A monocarboxylic acid functionalized AsCy3 was prepared from a symmetrical Cy3, initially having two carboxylates, by postesterification of one of the COOH to a COOMe after the mercuration reaction. Similarly, the coupling reaction between the AsCy3 and amino-Cy5 was performed with the coupling reagent EDC/HOBt at room temperature; the isolated yield was 35%. The structure of **AsCy3Cy5** was confirmed by ESI-MS and NMR, as fully documented in the SI.

Absorption and fluorescence emission spectra of **AsCy3Cy5** show two distinct peaks in ethanol, which respectively correspond to the spectral features expected for Cy3 and Cy5 (Figure 2). An absorption peak ratio (i.e., $OD_{567\ nm}/OD_{645\ nm}$) of 0.7 is consistent with an equimolar stoichiometry of the

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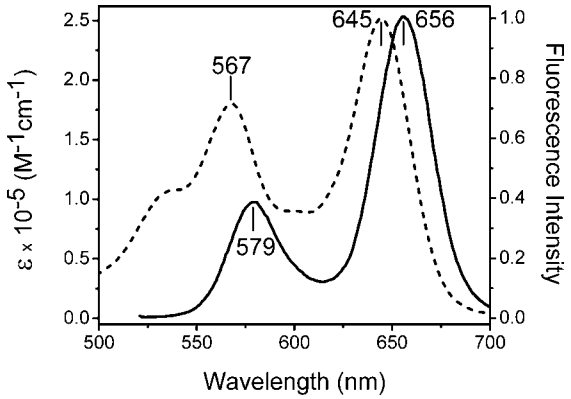

Figure 2. Absorbance (dashed line) and normalized fluorescence emission (solid line; λ_{ex} = 510 nm) spectra of AsCy3Cy5 (1 μM) in ethanol. Absorption maxima are 567 nm (Cy3) and 645 nm (Cy5). Fluorescence emission maxima are 579 nm (Cy3) and 656 nm (Cy5).

covalently bonded AsCy3 (λ_{max} = 567 nm) and Cy5 (λ_{max} = 645 nm), whose respective molar extinction coefficients are 180 000 and 250 000 M^{-1} cm^{-1}.[8,10] The presence of two fluorescence emission maxima at 579 and 656 nm upon excitation of Cy3 at 510 nm results from the close proximity of the Cy3 and Cy5 fluorophores, which are separated by less than 21 Å through a linker connected through their respective indolium nitrogens.[11] Such a close separation is designed to optimize fluorescence resonance energy transfer (FRET), as the Förster distance (R_0) is 53 Å.[12] A similar fluorescence emission spectrum was previously reported for covalent Cy3-Cy5 heterodimers separated by less than 30 Å, which resulted in a 41% FRET efficiency.[5b] It is apparent that the incomplete FRET efficiency arises due to restrictions in the relative orientations of the two fluorophores, as the spatial separation between these chromophores is within 0.5 R_0, where FRET efficiencies are expected to be complete for isotropically oriented dyes.[13]

To assess the utility of **AsCy3Cy5** for site-specific protein labeling, we inserted a tetracysteine tagging sequence (i.e., CCKAEAACC) engineered into the 42 kDa α-subunit of the RNA polymerase supramolecular complex (i.e., RpoA*). Specific labeling of RpoA* with **AsCy3Cy5** is evidenced by a single fluorescent band with an apparent molecular mass of 42 kDa (Figure 3). In comparison to control lysates not expressing tagged RpoA*, there is a substantial reduction of unbound **AsCy3Cy5** at the dye front of the gel. Minimal binding is observed between **AsCy3Cy5** and proteins within the lysate in the absence of an affinity tag. As expression of tagged RpoA* does not appreciably alter the overall cellular abundance (1%) of this protein[14] (Figure 3B), these results demonstrate the utility of **AsCy3Cy5** for site-specific labeling of individual proteins within native supramolecular complexes in biomolecular systems.

Upon **AsCy3Cy5** binding to RpoA* there is a fluorescence enhancement of the Cy3 peak at 579 nm that is consistent with prior observations that there is a 6-fold fluorescence enhancement of AsCy3 upon association with the tagging sequence (Figure 4);[8] observed increases in fluorescence intensity support a proposed mechanism whereby the rigid protein microenvironment stabilizes a highly fluorescent Cy3 conformer.[15] Concomitant with binding, there are increases in FRET efficiency between Cy3 and Cy5 that are apparent by the increase in the ratio of the fluorescence emission of Cy5 (acceptor) relative to Cy3 (donor), which increases from 0.35

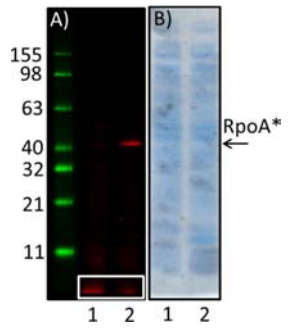

Figure 3. Specific labeling of tagged RpoA*. Fluorescence (panels A) and protein stain (panels B) following separation of cellular proteins by SDS-PAGE for *E. coli* lysates (2 μg/uL) with (lane 2) and without (lane 1) induction of RpoA*. The cell lysates were incubated with AsCy3Cy5 (0.1 μM) for 30 min. Images were collected using a FluorChem Q imager using Cy5 filters (AsCy3Cy5) or Cy2 filters (molecular mass standards). Unbound AsCy3Cy5 near the dye front is highlighted (white box).

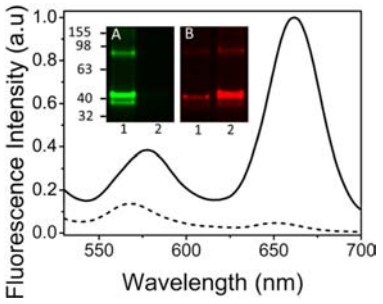

Figure 4. Emission spectra for AsCy3Cy5 (2 μM) in the absence (dashed line) or presence (solid line) of RpoA* (20 μM) in 25 mM HEPES (pH 7.6), 150 mM NaCl, and 200 μM TCEP. λ_{ex} = 510 nm. *Inset*: Following selective labeling of RpoA* with either AsCy3 (lane 1) or AsCy3Cy5 (lane 2); emission intensities centered near 580 nm (*panel A*, green) or 670 nm (*panel B*, red) (40 nm bandpass) were measured following separation by SDS-PAGE (λ_{ex} = 535 nm).

for the unbound AsCy3Cy5 to 2.6 for AsCy3Cy5 bound to RpoA*. The increase in FRET arises due to the disruption of aggregates of the unbound AsCy3Cy5 in solution (see Figure S3 in the SI). In total, there is a 20-fold increase in the fluorescence emission of Cy5 for AsCy3Cy5 bound to RpoA* in comparison to AsCyCy5 in aqueous solution, which is preserved on SDS-PAGE gels.

Distinct fluorescence signatures are apparent for **AsCy3Cy5**- and **AsCy3**-labeled RpoA* (see structure of AsCy3 in Figure S2 of the SI) within an enriched preparation of RNA polymerase (Figure 4, inset). The ability to simultaneously resolve fluorescence signatures of **AsCy3Cy5** and **AsCy3** binding to the same protein tagging sequence provides additional opportunities for multicolor measurements allowing, for example, pulse chase measurements of protein turnover using a single laser excitation near 532 nm to enable measurements of protein trafficking. Such multicolor measurements complement existing approaches that typically identify the colocalization of engineered fluorescent protein tags that absorb and emit at different wavelengths, which permit measurements of the organization of proteins within cellular structures.[18] Existing biarsenical probes have extended this capability by permitting measurements of protein trafficking and the resolution of synthesis and degradative mechanisms.[19] Such applications are

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further extended by the introduction of AsCy3Cy5, which is complementary to AsCy3 as both probes bind to the same tagging sequence and can be resolved by differences in their fluorescence emission spectra (Figure 4).

In order to evaluate the photoswitchability of **AsCy3Cy5**, time-dependent fluorescence trajectories were monitored for AsCy3Cy5-labeled RpoA* within an enriched RNA polymerase complex (see Figure S4 in the SI) immobilized within a chamber between a coverslip and microscope slide (Figure 5A).[16] Upon continuous photoexcitation by a red laser, Cy5 is driven into a long-lived dark state, which can be rapidly reactivated upon excitation of Cy3 using a green laser (Figure 5A). Reconstruction of the isolated image is facile following importing data into *ImageJ* that allows reconstruction of super-resolution images using an ImageJ plugin Quick Palm,[17] demonstrating a resolution of less than 100 nm (Figure 5B).

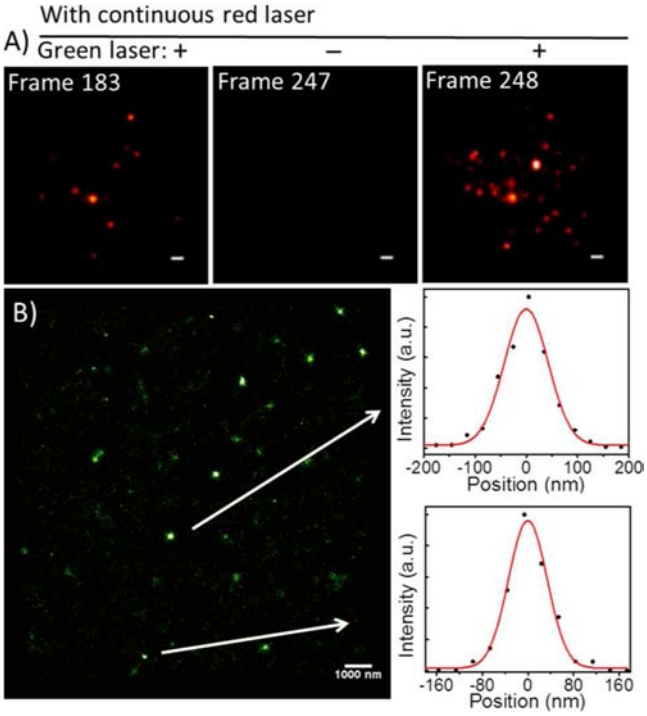

Figure 5. (A) Rapid turn-on fluorescence of individual single molecules for AsCy3Cy5-labeled RpoA* within the RNA polymerase supramolecular complex in response to excitation with a green laser (λ_{ex} = 532 nm; ~5 W/cm^2) is apparent at frames 247 and 248 monitored using a red laser (λ_{ex} = 633 nm; ~150 W/cm^2); 0.3 s per frame in video. (B) Reconstructed image showing positions of RpoA* (left); and Gaussian fits (right) of resolved intensity profile for two representative spots in the image. Scale bars represent 1 μm.

Critical to the design of AsCy3Cy5 is the functional linkage between switchability and subdiffraction (or super-resolution) imaging made possible using stoichastic optical resolution microscopy (STORM). In general, the resolution of individual molecules is diffraction limited (Figure 5A), with an average resolution of 414 nm (fwhm) for single molecule detection (see Figure S5 in the SI). The presence of Cy3 and Cy5 within the same molecule provides the ability to repeatedly turn the fluorescence on and off (photoswitching), permitting the identification of the positions of individual molecules with a resolution of 100 nm (Figure 5B). Resolution can be further enhanced (as low as 20 nm) using markers to enable drift correction.[20] While of general utility, such a resolution is essential to understand organizing principles within bacteria and other microbes whose overall dimensions limit the utility of light microscopy. Further, the high density of molecules within cells typically limits the ability to resolve individual molecules within cellular structures, as all fluorescent molecules are classically observed simultaneously. Key to super-resolution imaging is the ability to accurately target individual fluorophores to specifically label proteins of interest, with subsequent identification of the locations of an ensemble of labeled proteins by imaging individual proteins without interference by other proximal molecules that also are labeled, permitting reconstruction of an image with subdiffraction resolution.[2] In order to satisfy this prerequisite, fluorescent probes, such as AsCy3Cy5, are necessary to allow an easily controllable on/off photoswitchable capability.

In conclusion, we have synthesized a photoswitchable chemical probe capable of targeted protein labeling, thereby enabling the two critical requirements for single molecule super-resolution imaging. Upon **AsCy3Cy5** binding to a tetracysteine labeling sequence engineered into a protein of interest, there is a 20-fold increase in Cy5 fluorescence intensity related to (i) the rigid protein microenvironment that acts to restrict movement of the polymethine chain in the Cy3 dye and (ii) disruption of intra-/intermolecular contacts between Cy3 and Cy5. The close proximity of Cy3 and Cy5 permits rapid photoswitching, allowing super-resolution visualization of tagged proteins. Such subdiffraction imaging of the position of individual molecules will enable a range of applications regarding the position of an individual subunit within a supramolecular complex or the subcellular locations of proteins within individual membrane compartments. Future measurements using **AsCy3Cy5** will permit, for example, resolution of protein trafficking across cellular membranes in bacteria, whose small size commonly limits the ability to resolve the locations of proteins using light microscopy.[21]

■ ASSOCIATED CONTENT

Supporting Information

Procedures for the syntheses of AsCy3Cy5, structure of AsCy3, details of the protein separation, labeling and instrumental setup for super-resolution imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

thomas.squier@pnl.gov

Notes

The authors declare no competing financial interests.

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