Distance-Matched Tagging Sequence Optimizes Live-Cell Protein Labeling by a Biarsenical Fluorescent Reagent AsCy3_E

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Supporting Information

ABSTRACT: Cell permeable biarsenical fluorescent dyes built around a cyanine scaffold (AsCy3) create the ability to monitor the structural dynamics of tagged proteins in living cells. To extend the capability of this photo-stable and bright biarsenical probe to site-specifically label cellular proteins, we have compared the ability of AsCy3 to label two different tagging sequences (i.e., CCKAEACC and CCKAEAAKAEAAKCC), which were separately engineered onto enhanced green fluorescent proteins (EGFPs) and expressed in Escherichia coli. The cysteine pairs within the shorter protein tag (i.e., Cy3TAG) are designed to specifically match the 14.5 Å interarsenic atomic separation within AsCy3, whereas the longer protein tag (Cy3TAG+6) was identified using a peptide screening approach and reported to enhance the binding affinity and brightness. We report that AsCy3 binds both the tagged proteins with similar high affinities ($K_d < 1 \mu M$) under both in vivo labeling conditions and following isolation and labeling of the tagged EGFP protein. Greater experimental reproducibility and substantially larger AsCy3 labeling stoichiometries are observed under in vivo conditions using the shorter Cy3TAG in comparison to the Cy3TAG+6. These results suggest that the use of the distance-matched and conformationally restricted Cy3TAG avoids nonspecific protein interactions, thereby enabling routine measurements of protein localization and conformational dynamics in living cells.

INTRODUCTION

Small-molecule biarsenical fluorescent probes enable a chemistry-driven approach for site-specific labeling of recombinant proteins in living cells, avoiding the need for larger protein tags that can disrupt the structure or function of target proteins. The small size and tetracoordinate linkage between the biarsenical probe and the protein backbone facilitate the reliable measurement of protein conformational states and protein–protein interactions.

First-generation biarsenical probes have an interatomic distance of ~6 Å between the arsenic moieties, which is well-matched to allow FlAsH (green fluorescence), CrAsH (green fluorescence), and ReAsH (red fluorescence) to bind to a six-amino acid tagging sequence involving a pair of vicinal cysteines separated by two amino acids that chelate the respective arsenic moieties (i.e., CCXXCC or FlAsHTAG). Sequence differences between the vicinal cysteines permit the simultaneous use of ReAsH and FlAsH to label different tagging sequences (i.e., CCPGCC and CCKACC) within a protein complex. However, the competition for these very similar tagging sequences limits their applications to experiments involving target proteins with similar cellular abundances. Additional confidence in labeling orthogonal tagging sequences on different proteins was achieved through the introduction of a biarsenical fluorescent dye built around a cyanine scaffold (i.e., AsCy3) with a much larger interarsenic distance of ~14.5 Å that matches the spatial separation between the pairs of vicinal cysteines within a designed protein tagging sequence (CCKAEACC or Cy3TAG). Although originally synthesized as the sulfonate derivative AsCy3_S to increase water solubility, subsequent measurements demonstrated that the methoxyester-derivative AsCy3_E provides enhanced cellular permeability for live-cell imaging of the tagged proteins in prokaryotic and eukaryotic cells.

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In an effort to further optimize the affinity of the AsCy3 tagging sequence, the spatial separation between the vicinal cysteines was systematically varied within a family of peptides. Increasing the spatial separation between the vicinal cysteine pairs by six amino acids (i.e., CCKAEAAKAEAAKCC or Cy3TAG+6) was reported to enhance the binding affinity by 2 orders of magnitude and increase the brightness of the bound AsCy3. As previous results have also reported similar increases in fluorescence enhancements upon binding FLAsH to longer peptide tags (i.e., CCGGSGNDAGGCC instead of CCPGCC), it is of interest to understand how the length of the tagging sequence may affect the brightness and labeling stoichiometry of tagged cellular proteins by AsCy3.

To investigate how differences in the length of the AsCy3 tagging sequence affect the binding of AsCy3, we inserted either the original (i.e., CCKAEAAACC or Cy3TAG) or the recently suggested (i.e., CCKAEAAKAEAAKCC or Cy3TAG +6) AsCy3 tagging sequence into the His-tagged enhanced green fluorescent protein (EGFP*) and separately expressed each protein in *Escherichia coli*. Engineered tagging sequences are within an N-terminal sequence separated from the EGFP by a 47-amino acid linker sequence to minimize possible steric interactions between the tagging sequence and the EGFP. The construct design takes advantage of the long Förster critical distance ($R_0 = 60 \text{ Å}$) between the EGFP and the cyanine chromophore, which enables the detection of fluorescence resonance energy transfer (FRET) upon AsCy3 binding for both the construct designs.22,23 This latter consideration allows an assessment of how differences in the engineered tagging sequences may affect the binding affinities, labeling stoichiometries, and excited-state fluorescence lifetimes of AsCy3_E within living cells. Binding affinities were measured using the original sulfonate derivative AsCy3_S in lysates enriched in the EGFP or with the cell-permeable methoxyester variant AsCy3_E in living cells. We expect that these insights regarding the practical limitations and relative advantages of both short- and long-tagging sequences will facilitate the application of biarsenical fluorescent probes in live-cell measurements of protein dynamics.

**RESULTS AND DISCUSSION**

**AsCy3_S Binding Affinities.** AsCy3_S binding to engineered tagging sequences for Cy3TAG and Cy3TAG+6 located near the N-terminus of an engineered His-tagged EGFP (EGFP*) was examined following immobilized metal affinity chromatography (IMAC) purification. As a control, the EGFP was expressed with no AsCy3 tagging sequence. In all cases, the EGFP* represented the major protein following IMAC purification (Figure S1). Relative binding affinities of AsCy3_S to Cy3TAG or Cy3TAG+6 were compared with that of SlyD, a naturally occurring metallochaperone in *E. coli* that has previously been observed to bind FLAsH with high affinity and coelutes with the EGFP* during IMAC purification.

To assess AsCy3_S binding to endogenous proteins expressed in *E. coli*, we first examined lysates prepared from a control in which the EGFP* (no Cy3TAG or Cy3TAG+6 binding sequence) was expressed and isolated using IMAC affinity chromatography. Upon incubation of the IMAC-purified control lysate (5 μg/mL) with AsCy3_S (0.1 μM), SlyD is specifically labeled (Figure 1, inset). These results suggest that, as previously observed using FLAsH,22 the cysteine-rich binding sequence in SlyD (CCGGHGHDHGHEHGEGGCC) is also a target for biarsenical probe labeling using AsCy3. The presence of either Cy3TAG or Cy3TAG+6 in the EGFP construct results in reductions in SlyD labeling, with AsCy3 labeling of either the Cy3TAG or Cy3TAG+6 binding motif in the EGFP*.

A consideration of the concentration dependence of AsCy3_S labeling demonstrates that both tagging sequences have similar affinities, where $K_d = 0.9 \pm 0.2 \mu M$ (Cy3TAG) or 0.7 ± 0.1 μM (Cy3TAG+6) (Figure 1; Figure S2). Our measured binding affinity between AsCy3_S and Cy3TAG is similar to that previously reported for a synthetic peptide by Alexander and Schepartz, where $K_d$ was measured to be between 1.0 ± 0.1 and 2.4 ± 0.6 μM.20 However, we observe no significant increase in the binding affinity upon insertion of a six-amino acid linker in Cy3TAG+6, which is in contrast to the large (20-fold) increase in the binding affinity reported previously using peptide models.20 These results suggest that the positioning of the Cy3TAG+6 within the protein construct may alter the conformation or redox potential of the proximal cysteine pairs to modify the binding affinity between AsCy3_S and the tagging sequence. In this respect, we took care to introduce a 47-amino acid linker between the tagging sequence and the EGFP for both Cy3TAG and Cy3TAG+6 sequences to minimize the possible steric interactions that could modify the conformation of the Cy3TAG.22 However, relatively modest sequence differences between the pairs of cysteines can modify redox potentials,25 suggesting that cysteine oxidation within the Cy3TAG+6 tagging sequence may offset possible increases in affinity that result from the release of conformational constraints upon the elongation of the peptide linker between the pairs of vicinal cysteines. Regardless of the mechanism, the relative utility of the Cy3TAG and Cy3TAG+6 sequences requires a consideration of their usefulness for the site-specific modification of the tagged cellular proteins with AsCy3 in living cells.

**Live-Cell Labeling.** To better understand the utility of the Cy3TAG and Cy3TAG+6 sequences within a cellular context, we investigated the ability of the cell-permeable AsCy3_E biarsenical probe to label the tagged EGFP* expressed in the...
living E. coli cells. AsCy3_E has previously been shown to selectively label the Cy3TAG engineered near the C-terminus of the α-subunit of RNA polymerase expressed in living E. coli, permitting visualization of changes in cellular localization in response to metabolic conditions.4 These prior experiments directly excited the AsCy3_E chromophore, which non-selectively excited both AsCy3_E bound to RNA polymerase as well as any dyes remaining within the cell. In the current experiments, we seek to quantitatively assess possible differences in the relative affinities of AsCy3_E binding to the Cy3TAG- or Cy3TAG+6-EGFP* constructs, as well as possible differences in their fluorescence lifetimes, which are directly related to the brightness of the Cy dyes.56 To avoid any contribution from AsCy3_E chromophores not bound to the tagging sequences on the EGFP, we directly excited the EGFP near its absorption maximum at 488 nm and monitored the FRET to bound AsCy3_E (Figure 2).

Following the induction of the EGFP*, E. coli was resuspended in 20 mM N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES, pH 7.4) and 0.15 M NaCl and incubated with variable amounts of AsCy3_E for 1 h at 37 °C prior to repeated cell washes, essentially as previously described.5 Fluorescence emission spectra were normalized relative to the peak emission of the EGFP at 506 nm; the appearance of a peak at 580 nm is indicative of FRET to AsCy3_E upon EGFP* binding. For the Cy3TAG, we observe a significant and highly reproducible amount of FRET associated with AsCy3_E binding to the Cy3TAG (Figure 2C). In comparison, smaller and more variable (apparent from the very large error bars) levels of FRET are apparent upon the incubation of AsCy3_E with E. coli cells expressing the EGFP* engineered to contain the Cy3TAG+6. We note that these ratiometric measurements of FRET in living cells avoid potential artifacts associated with measurements of fluorescence intensities, which can vary considerably depending on the expression levels of the EGFP and the differences in the number of E. coli cells.

The fluorescence intensity of the EGFP is approximately two-fold larger than the maximal fluorescence for AsCy3_E bound to the Cy3TAG (Figure 2A). This result is consistent with the approximately two-fold higher quantum yield of the EGFP relative to cyanine dyes.18,27,28 In comparison, the fluorescence intensity of AsCy3_E bound to the Cy3TAG+6 is about 25% of that associated with AsCy3_E bound to the Cy3TAG. These results suggest a much higher level of in vivo protein labeling in applications using the shorter Cy3TAG. However, as AsCy3_E binding is associated with increases in fluorescence intensities,18,20 comparisons of the fluorescence intensities of AsCy3_E bound to either the Cy3TAG or the Cy3TAG+6 do not distinguish between the possible differences in either the quantum yield or binding stoichiometries. Both of these possibilities are consistent with the observed differences in AsCy3_E fluorescence intensities following incubation with E. coli expressing the EGFP* engineered to contain either the Cy3TAG or the Cy3TAG+6.

**Figure 2.** Live-cell labeling of EGFP* with AsCy3_E. Fluorescence emission spectra (panels A and B) and ratio of acceptor AsCy3_E (580 nm) over donor EGFP* (506 nm) fluorescence (panel C) for E. coli expressing the EGFP* engineered to contain the tagging sequences Cy3TAG (panel A; CCKAEAAACC) or Cy3TAG+6 (panel B; CCKAEAAKAEAKCC) in the absence (dashed curve) or presence of 0.2 μM AsCy3_E (dotted curve), 0.4 μM AsCy3_E (dot-dashed curve), or 0.7 μM AsCy3_E (solid curve). Symbols in panel C represent averages with indicated standard deviations, where Kd = 0.3 ± 0.1 μM (Cy3TAG) or Kd < 1.1 μM (Cy3TAG+6).

**Figure 3.** Live-cell FRET between EGFP and bound AsCy3_E. Frequency-domain fluorescence lifetime measurements for the EGFP* prior to (open circles) and following incubation with AsCy3_E (i.e., AsCy3_E binding (Figure 3B)) is very similar, suggesting that the tagging sequence does not significantly affect the overall protein fold. Upon AsCy3_E binding to the Cy3TAG+6 in the EGFP*, there is a shift in the frequency response toward higher frequencies that is indicative of a decrease in the fluorescence lifetime (Figure 3B). In comparison to that seen with Cy3TAG+6, there is a much larger alteration in the frequency response upon incubation of AsCy3_E with E. coli expressing Cy3TAG (panel A) or Cy3TAG+6 (panel B) engineered onto the N-terminal region of the EGFP*. Lines represent nonlinear least squares fits to a model requiring two lifetime components, which for EGFP* (no added AsCy3_E) is centered near 0.1 ns (61%) and 2.6 ns (39%). Measured FRET efficiencies (panel C) were calculated from decreases in the mean excited-state lifetime of EGFP upon AsCy3_E binding, which decreases from 1.1 ns prior to AsCy3_E binding (i.e., τ0) to 0.58 ns (Cy3TAG) or 0.79 ns (Cy3TAG+6) (i.e., τexc). λex = 488 nm; emitted light was collected subsequent to a Chroma HQ535/50 band-pass filter. FRET efficiencies were calculated from global fits to three independent data sets.

**Fluorescence Lifetime Measurements of FRET Efficiencies.** Measurements of decreases in the fluorescence lifetime of the EGFP* upon AsCy3_E binding to either Cy3TAG or Cy3TAG+6 tagging sequences provide a direct measurement of FRET efficiencies that are independent of possible differences in the quantum yields of AsCy3_E. We, therefore, used frequency-domain fluorescence spectroscopy to measure the fluorescence lifetime of the EGFP*. Using sinusoidally modulated light to excite EGFP, we measured the phase delay and loss of modulation as a function of the modulation frequency (Figure 3). Prior to AsCy3_E binding,
of the relative decrease in the mean fluorescence lifetime and the associated FRET, which varies from 28% for EGFP* containing the Cy3TAG+6 tagging sequence to 47% for EGFP* containing the Cy3TAG tagging sequence (Figure 3C). These latter results indicate that the amount of AsCy3_E bound to the shorter tagging sequence (i.e., Cy3TAG) is substantially larger than the amount of AsCy3_E bound to the longer tagging sequence (i.e., Cy3TAG+6). Thus, the Cy3TAG is a robust labeling sequence that permits facile in vivo labeling of tagged proteins using AsCy3_E.

**Fluorescence Lifetime of AsCy3_E.** Measurements of the fluorescence lifetime of AsCy3_E bound to either the Cy3TAG or Cy3TAG+6 in the EGFP* permit an assessment of possible changes in their relative brightness. In these experiments, AsCy3_E is indirectly excited through FRET from the EGFP* and the fluorescence emission of AsCy3_E is selectively measured using frequency-domain fluorescence spectroscopy (Figure 4). Similar frequency response curves are observed irrespective of the tagging sequence, indicating that there are minimal differences in average fluorescence lifetimes. In comparison to AsCy3_E bound to Cy3TAG+6, whose mean fluorescence lifetime is 1.0 ± 0.1 ns, there is a small shift toward higher frequency responses when AsCy3_E binds to the short Cy3TAG that is consistent with a small decrease in the mean lifetime, which is 0.9 ± 0.1 ns. In both cases, there is an approximately threefold increase in the fluorescence lifetime in comparison to unbound Cy3, which has an average lifetime of 0.3 ns. Thus, the measured fluorescence lifetimes of AsCy3_E bound to either the Cy3TAG or the Cy3TAG+6 are consistent with the reported increases in the fluorescence intensities associated with AsCy3 binding to these tagging sequences. However, small differences in the fluorescence lifetimes of AsCy3_E bound to the EGFP* engineered with either the Cy3TAG or Cy3TAG+6 are not the cause of the observed differences in the relative intensities of AsCy3_E observed upon excitation of EGFP* in Figure 2. Rather, these results collectively indicate that the labeling stoichiometry of AsCy3_E is substantially larger using the engineered Cy3TAG relative to that observed using the Cy3TAG+6 tagging sequence under in vivo conditions of live-cell labeling.

In summary, we have demonstrated that the cell permeable biarsenical probe AsCy3_E can be used to reproducibly label proteins tagged with the Cy3TAG (i.e., CCKAAEACC) in living cells. Under similar labeling conditions (i.e., 0.5 μM AsCy3_E), we previously demonstrated the ability to image changes in the localization of tagged proteins in response to metabolic conditions. Our current measurements demonstrate that the length, and associated conformational flexibility, of the AsCy3 tagging sequence does not significantly affect either the binding affinity or brightness of bound AsCy3_E (Figures 1 and 4). Rather, increases in the length and conformational flexibility of the tagging sequence result in large reductions in in vivo labeling stoichiometries (Figures 2 and 3), which may result from increases in the disulfide bond formation. Although not studied here, additional advantages associated with the use of the Cy3TAG binding sequence relate to the overall dimension between the pair of vicinal cysteines that matches the interresidual distance in AsCy3, which permits the simultaneous use of orthogonal tagging sequences for FlAsH (green fluorescence) and AsCy3 (red fluorescence) for two color experiments. In comparison, longer tagging sequences (e.g., CCGGSGNDAGGCC in SlyD) bind both FlAsH and AsCy3, as the spatial separation and peptide flexibility enable a substantially larger range of conformations.

**EXPERIMENTAL PROCEDURES**

**Expression Clone Construction.** Multisite Gateway Pro (Thermo Fisher Scientific) cloning was used to construct expression clones built around a previously used expression vector that, when appropriate, included an AsCy3 tagging sequence located within a linker region located between an N-terminal 17-amino acid sequence (MKTSAILVAVLATTAA) and the EGFP (~40 kDa) within the translated protein, as previously described in detail. For these experiments, three constructs were engineered in which the His-tagged EGFP (EGFP*) was expressed without an AsCy3 binding sequence (control) or containing the Cy3TAG (CCKAAEACC) or Cy3TAG+6 (CCKAEAAKAEAAKCC) tagging sequences. Polymerase chain reaction was performed using Pfu HotStart polymerase enzymes (Stratagene, La Jolla, CA). Constructs involved separately engineering gene fragments that, when appropriate, encode either Cy3TAG or Cy3TAG+6. These gene fragments were amplified, essentially as previously described. All gene fragments were amplified with the same forward primer:

GGGCAACAGTTTGTACAAAAAGCGAGGCTTCTTC-GATTAACTTACAAAGGAAGGTTCGTTATTGAAGGCATTTGCACCAGT, where the attB1 recombination site is underlined and the ε enhancer/ribosome binding site is in bold. The reverse primers used were as follows (attB5r restriction site is underlined, AsCy3 tagging sequences are in italics):

GGGCAACACCTTTTGTATACAAAGATTTCTTTC-CACTTCTTTCCATG, no Tag (control); GGGGACACTTTTTGTAACAAAGCAGCTCCACTTCCCTTG, no Tag (control); GGGGACACTTTTTGTAACAAAGCAGCTCCACTTCCCTTG, no Tag (control);
The EGFP gene was amplified using the following primers (attBS, attB2 sites underlined):

GGGGCAACACTTTGTATACAAAAAGTTTGTACAAACACT-TAGCCGGGCTCAGCCTTAAGCGCCATCAGCCTTACACAA,
Cy3TAG+6.

The MultiSite Gateway Pro system (Invitrogen, Waldham, MA) was used to combine gene fragments containing Cy3TAG or Cy3TAG+6 with the EGFP gene in the pEXP2-DEST plasmid to create three expression clones used in this work: EGFP*, EGFP* with the Cy3TAG, and EGFP* with the Cy3TAG+6. In all cases, the inserted tetracysteine labeling site is located near the N-terminus of the expressed protein construct and is within one-half of the 60 Å Förster critical distance relative to the EGFP chromophore, which results in near complete FRET upon AsCy3E binding.23

Protein Expression, Cell Lysis, and IMAC Protein Enrichment. All constructs were expressed in T7 Express lysY/Ip E. coli (NEB) and grown in the Luria Bertani (LB) medium supplemented with 100 μg/mL ampicillin at 37 °C. Growing cultures were induced at the exponential phase with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 °C for approximately 16 h. Induced cells were harvested at 6000 g for 10 min at 4 °C and the cell pellets were stored at −80 °C. Cells were thawed on ice and, unless otherwise indicated, lysed in 3.5 mL of lysis buffer [20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 2 M urea, 5% (v/v) glycerol, 2 mM MgCl2, 10 mM β-mercaptoethanol, 5 mg/mL egg white lysozyme (Amresco; Solon, OH), 1 μL/mL universal nuclease (Thermo Fisher Scientific; Waltham, MA), and protease inhibitor cocktail (Thermo Fisher Scientific; Waltham, MA)]. Alternatively, resuspended cells were incubated on ice for 60 min in the presence of 2% (v/v) sodium dodecyl sulfate (SDS) and sonicated at 80% amplitude for 1 min pulses with 30 s intervals on ice (Ultrasonic, GE®). In all cases, cell lysis was confirmed by visualization on a light microscope (Nikon, Labophot).

Lysates were separated from cell debris following centrifugation (9000g for 5 min at 4 °C) using a tabletop centrifuge (Eppendorf, 5415R). Purification was conducted using an ÄKTA start liquid chromatography system fitted with a 1 mL Hitrap FF crude column (GE Healthcare Life Sciences; Marlborough, MA). The column was first equilibrated using 10 mL of binding buffer [20 mM sodium phosphate, 500 mM sodium chloride, 2 M urea, 5% (v/v) glycerol, and 40 mM imidazole (pH 7.4)]. A cleared lysate was loaded through a 5 mL superloop at 0.5 mL/min. The column was washed using 15 mL of wash buffer [20 mM sodium phosphate, 500 mM sodium chloride, 2 M urea, 5% (v/v) glycerol, and 50 mM imidazole (pH 7.4)] at 1 mL/min. The EGFP* tagged with Cy3TAG+6 was eluted using a 10 mL linear gradient ranging from 100–500 mM imidazole. EGFP*, EGFP* tagged with Cy3TAG, and endogenous SlyD from untransformed E. coli were eluted using step gradients involving 5 mL each of 100, 200, 300, 400 mM imidazole, followed by a final 10 mL of 500 mM elution step. Eluted proteins were separated on a 4–20% Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) gel (Bio-Rad Laboratories; Hercules, CA), and proteins were visualized using an EZ-Run protein gel staining solution (Thermo Fisher Scientific; Waltham, MA). Buffer exchange was conducted on consolidated fractions using a 30 MWCO Amicon column into the storage buffer [that is, phosphate buffered saline (PBS) and 5% (v/v) glycerol] and concentrated approximately 10-fold. Protein aliquots were snap-frozen in liquid nitrogen and stored at −80 °C.

AsCy3_5 Labeling of Lysates Following IMAC Protein Enrichment. Concentrations of AsCy3_5-EDT1 were determined using an extinction coefficient of 180 000 cm⁻¹ M⁻¹, as previously described.18 Protein concentrations were determined using a bicinchoninic acid assay (Thermo Fisher Scientific; Waltham, MA). Variable amounts of AsCy3_5-EDT2 were added to protein lysates (5 μg/mL) in PBS, 5% glycerol (v/v), and 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich) for 30 min in the dark. Following labeling, proteins were denatured in the Laemmli sample buffer supplemented with 1 mM tributylphosphate (Bio-Rad Laboratories; Hercules, CA) and a protease inhibitor cocktail (Thermo Fisher Scientific; Waltham, MA) by boiling at 95 °C for 5 min prior to separation on a 4–20% Tris-glycine SDS-PAGE gel. Fluorescence intensities of AsCy3 bound to EGFP* (~45 kDa) or SlyD (~27 kDa) were captured using a UVP ChemStudio Imager (Analytik Jena USA, Upland, CA) prior to protein staining using an EZ-Run protein gel staining solution (Thermo Fisher Scientific; Waltham, MA). Relative fluorescence intensities were quantified using NIH ImageJ 1.49v.24 In all cases, a purified SlyD labeled with AsCy3 was used as a loading control. The binding isotherm associated with AsCy3 binding to the tagged EGFP (i.e., EGFP*) was fit to a Langmuir binding isotherm

\[ F_{\text{obs}} = F_{\text{max}} \frac{[\text{AsCy3}]}{K_{\text{app}} + [\text{AsCy3}]} \]

where \( F_{\text{obs}} \) is the observed fluorescence intensity, \( F_{\text{max}} \) is the maximum fluorescence intensity, [AsCy3] is the total amount of AsCy3 added to the reaction, and \( K_{\text{app}} \) is the apparent dissociation constant.

AsCy3_E Labeling of Live E. coli cells. E. coli (1 mL) transformed with the EGFP* alone or engineered to contain the Cy3TAG or Cy3TAG+6 tagging sequences was grown in a LB broth at 37 °C (300 rpm) for 5 h following the addition of β-1-thiogalactopyranoside * (IPTG) (1 mM) to induce EGFP* expression cells prior to the addition of AsCy3_3_E. After 1 h incubation with AsCy3_3_E-EDT2, the cells were washed multiple times to remove unbound AsCy3_3_E and resuspended in 20 mM HEPES (pH 7.5) and 0.15 M NaCl, essentially as previously described.6

Frequency-Domain Fluorescence Measurements. Fluorescence lifetimes were measured using an ISS K2 frequency-domain fluorometer (Champaign, IL), as described previously.22,32,33 Samples were excited using a 488 nm laser diode with emitted light detected subsequent to either an HQ535/50 band-pass filter for the EGFP (Chroma Technology Corporation, Bellow Falls, VT) or subsequent to an Omega 540 long-pass filter (AsCy3_5). All measurements were taken at 25 °C. Fluorescein was used as a lifetime standard (\( \tau_{\text{ref}} = 4.0 \) ns) (http://www.iss.com/resources/reference/data_tables/ StandardsLEDsLaserDiodes.html).

Analysis of Fluorescence Lifetime Intensity Decays. The frequency-domain fluorescence lifetime data were analyzed by fitting the time-dependent decay, \( I(t) \), of fluorescence to a sum of exponentials using nonlinear least squares, as previously described.54
\[ I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \]

where \( \alpha_i \) values represent the pre-exponential factors, \( \tau_i \) values represent the decay times, and \( n \) is the number of exponential components required to describe the decay. The intensity decay law is obtained from the frequency response of amplitude-modulated light and is characterized by the frequency-dependent values of the phase and the extent of demodulation. The values are compared with the calculated values from an assumed decay law until a minimum of the reduced squared error \( \chi^2 \) is obtained. After the measurement of the intensity decay, the mean lifetime was calculated

\[ \bar{\tau} = \sum_{i=1}^{n} \alpha_i \tau_i \]

Errors in the differential phase and modulated anisotropy were assumed to be 0.2° and 0.004, respectively. Weighted residuals \( \chi^2 \) were calculated as the difference between the measured and the fit data divided by the error of individual measurements (0.2° or 0.004 for phase shift and modulation data, respectively).

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