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# A Bifunctional Converter: Fluorescein Quenching scFv/Fluorogen Activating Protein for Photostability and Improved Signal to Noise in Fluorescence Experiments

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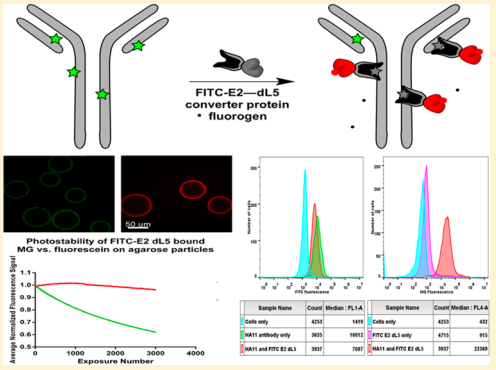
**ABSTRACT:** Monoclonal antibodies are one of the most useful and ubiquitous affinity reagents used in the biological sciences. Immunostaining of fixed and live cells for microscopy or cytometry measurements frequently employs fluorescently labeled antibodies, in particular fluorescein-labeled antibodies. This dye emits light at a wavelength overlapping with cellular autofluorescence, making it difficult to measure antibody binding to proteins of relatively low copy number or in cells of high green autofluorescence. A number of high affinity fluorescein binding antibodies and antibody domains have been developed that quench the dye's fluorescence. Using a fluorescein-binding recombinant antibody domain genetically fused to a fluorogen activating protein (FAP), we demonstrate a molecular converter capable of binding and quenching fluorescein, while binding and activating a fluorogenic triarylmethane dye. This reagent converts fluorescein conjugates to far-red fluorescent probes, where cellular autofluorescence is low, improving signal-to-background of cell-based antibody binding measurements by ~7-fold. Microscopy experiments show colocalization of both fluorescein and MG fluorescence. This dual affinity fluorescein-quenching-FAP can also be used to convert fluorescein to the red fluorescing MG fluorogen on biological molecules other than antibodies.




## INTRODUCTION

Fluorescent labeling of protein molecules is the cornerstone of modern biological detection and analysis. Proteins can be labeled fluorescently either through direct conjugation of small organic fluorophores to the protein of interest or genetic addition of fluorescent proteins to the protein of interest. Antibodies in particular are often labeled with small fluorophores instead of genetic tags due to the complexity of adding fluorescent proteins to the multichain immunoglobin molecule. Due to the specific and selective binding of antibodies to their antigens, they are extremely useful in biological research as labeling agents. One of the most commonly used and widely available fluorescent molecules conjugated to antibodies is fluorescein and/or the similar fluorescein isothiocyanate (FITC). Both are bright green dyes easily excited and detected by most commercial fluorescence measurement techniques and instruments such as microscopy and flow cytometry.

While fluorescein is bright, inexpensive, and relatively easy to conjugate to protein or other biological molecules, it suffers from poor photostability[1] and fluoresces in a region of high cellular autofluoresecence.[2] Fluorescein-conjugated antibodies, lipids, polymers, and proteins have been used in imaging and biological research for many years due to the availability of fluorescein conjugated probes and the fluorescein excitation and emission spectrum, which is compatible with most commercially available fluorescence measurement systems using the widely available 488 nm excitation laser. Antibodies and single chain variable fragment antibodies (scFvs) that bind and quench FITC fluorescence have been developed for a variety of uses including antibody and scFv crystal structure analysis,[3] mutational and folding analysis,[4,5] and as a protein targeting mechanism.[6] In particular, the FITC binding scFv FITC-E2 binds and quenches FITC and other fluorescein derivatives with a $K_d$ of 2.4 nM.[5]

Recently discovered and characterized fluorogen activating proteins (FAPs) are based on scFvs, selected for activation of otherwise nonfluorescent dyes from a yeast surface display library and subsequently affinity matured for tighter binding.[7] FAPs differ from most fluorescent dyes and proteins in that the fluorogen is exogenous, can be added at any time, and is virtually nonfluorescent until bound to the FAP. Malachite

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green derivatives are useful fluorogens, excited with a 633 nm excitation source and emitting at 670 nm.[8] Chemical modification to the base malachite green molecule, specifically addition of 2 or 11 polyethelyene glycol repeats to the fluorogen, lead to cell impermeant versions which bind FAPs with high affinity and exhibit low background binding or fluorescence.[7] FAPs have been applied in a variety of biological studies including membrane receptor internalization studies,[9] signal amplification studies,[10] CFTR trafficking studies,[11−13] targeted pH sensors,[14] incorporation into peptide-based membranes for use in animal studies,[15] STED nanoscopy studies,[16] and secondary labeling reagents.[17] The affinity matured light chain homodimer of L5 (E52D L91S in ref 8, L5** or L89S E50D in Kabat nomenclature, in ref 18), referred to as dL5 in this study, binds MG tightly with a $K_d$ of 18 pM.[18]

Secondary labeling of probes and proteins is a common factor in many fluorescence-based biological assays such as immunofluorescence, flow cytometry, ELISA, and live cell microscopy. Typically, secondary labeling is performed to boost the signal of primary labeling agents that directly bind the protein or biomolecule under investigation. A secondary reagent has affinity to the primary reagent, often a monoclonal antibody, and contains multiple brightly fluorescing molecules in order to aid in detection. Some primary antibodies are directly labeled themselves with fluorophores to eliminate this step. Most commercially available antibodies are available as a FITC or carboxyfluorescein-labeled conjugate in order to use primary labeling instead of secondary labeling. To overcome the poor photostability and high autofluorescence that limits sensitivity in the green fluorescence region, we have developed a straightforward FITC/fluorescein converter reagent which shifts the fluorescence excitation and emission into the red region of the visible spectrum to reduce cellular autofluorescence and to overcome rapid FITC/fluorescein photobleaching. The recombinant bifunctional protein, FITC-E2−dL5, binds FITC or fluorescein on target molecules and binds to the fluorogen MG-2p to shift the fluorescence spectra ∼150 nm to the red (Figure 1). It is important to note that this fluorescence shift is not based on fluorescence resonance energy transfer (FRET) as the FITC/fluorescein emission wavelength (peak maximum of 521 or 519) does not overlap with the MG-2p peak excitation region at 633 nm. The protein domains act independently of one another relying on the established fluorescein binding and quenching properties of FITC-E2 and the fluorogen activation of the dL5 FAP domain, respectively. Excitation of MG-2p is performed at different wavelengths than that of fluorescein in this system for optimal fluorescence emission, although MG itself can be directly partially excited at 488 nm, due to a small secondary excitation peak.[7] This reagent can be used to increase the signal-to-noise ratio of FITC-labeled probes, and can be used against many FITC or fluorescein-labeled molecules. FITC-E2−dL5 binds fluorescein-labeled molecules in a 1:1 ratio of fluorescein to MG-2p and binds in a non-cross-linking manner. (Figure 1)

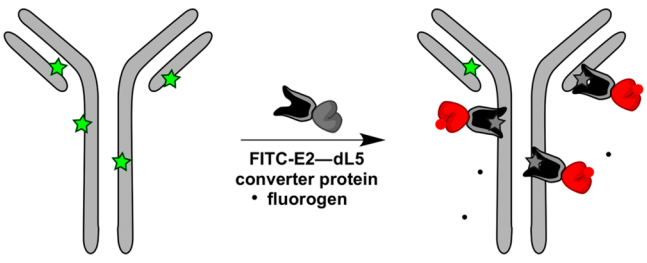


**Figure 1.** Conceptual schematic of the FITC-E2−dL5 protein binding to a fluorescein isothiocyanate (FITC)-labeled target, in this case an antibody. Green FITC fluorescence is quenched by binding of the FITC-E2 single chain fragment variable antibody (scFv) and replaced with red fluorescence via the dL5/MG-2p fluorogen activating protein (FAP) fluoromodule. The two protein domains (FITC-E2 and dL5) act independently of one another in binding their respective targets. FITC quenching is entirely achieved by the FITC-E2 domain (black) binding, and MG activation is achieved by binding to the fluorogen activating protein domain dL5 (red). No FRET signal is observed between the fluorophores, and independent excitation and emission of each fluorophore is measured.

## RESULTS

**In Vitro FITC-Labeled Antibody and Biotin−PEG−Fluorescein Binding.** To demonstrate that the FITC-E2−dL5 protein both binds and quenches FITC fluorescence while activating MG-2p fluorescence, experiments were performed in a fluorescence plate reader using a constant amount of fluorescein-labeled molecule and MG-2p fluorogen with an increasing titration of FITC-E2−dL5 protein. These experiments showed a concentration-dependent quenching of FITC fluorescence and activation of MG fluorescence (Figure 2A). For the FITC-labeled antibody, fluorescence decreases to approximately 35% of the starting value at a concentration of 1 $\mu$M FITC-E2−dL5, although higher quenching was seen with a different antibody conjugate (>90%, data not shown). Malachite green fluorescence emissions increased in a linear fashion with increasing FITC-E2−dL5 protein concentration as expected (Figure 2A inset). Titration of a fluorescein-conjugated PEG−biotin (200 nM) with increasing amounts of FITC-E2−dL5 demonstrated that the FITC-E2−dL5 is capable of binding carboxyfluorescein as well as FITC. Approximately 95% of the fluorescein fluorescence in this experiment was quenched at FITC-E2−dL5 concentrations above 200 nM (Figure 2B), while MG fluorescence increased in the same linear manner as in the FITC-conjugated antibody experiment (Figure 2B inset). The difference in quenching, approximately 65% on FITC-conjugated antibody vs 95% for fluorescein PEG−biotin is most likely due to accessibility of antibody conjugated FITC to FITC-E2−dL5 relative to soluble fluorescein in solution on the fluorescein PEG−biotin. The short conjugation linker on FITC ensures that the fluorophore remains close to the protein surface and potentially inaccessible to the larger FITC-E2−dL5 protein. These experiments show this protein is functional in binding both FITC or carboxyfluorescein on one end and MG-2p on the other end, leading to decreased green fluorescence and increased red fluorescence in a concentration-dependent manner.

**Flow Cytometry Analysis of Cells with Antibody-Bound FITC-E2−dL5.** Shifting the spectrum from the green region with high autofluorescence to the red, with low, is expected to improve the signal to background ratio. Comparisons of the signal-to-noise ratio of FITC-E2−dL5 bound to FITC-labeled antibodies and FITC-labeled antibodies alone for cell surface staining was performed by flow cytometry. A CHO cell line stably expressing a nine amino acid (sequence YPYDVPDYA) influenza hemaglutinin epitope (HA) tagged OPRM1 receptor was bound with a FITC-labeled monoclonal anti-HA antibody followed by FITC-E2−dL5. Flow cytometric analysis of these samples relative to unstained cells shows that the FITC-labeled anti-HA antibody yielded a median green


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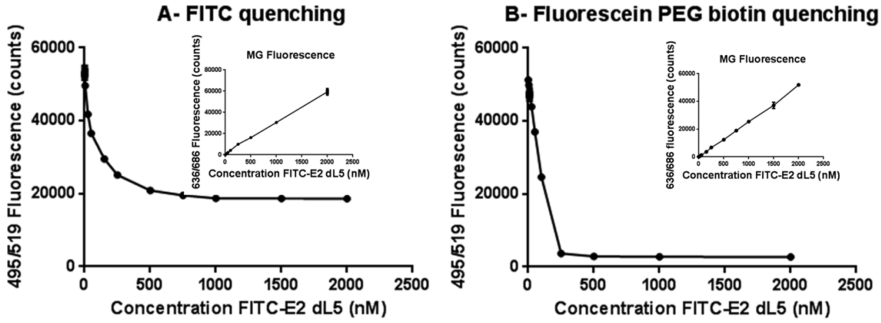
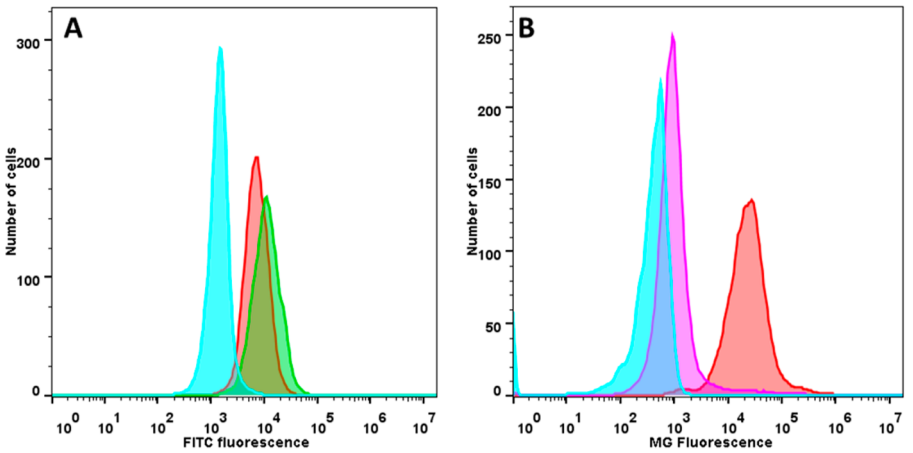



**Figure 2.** *In vitro* binding of FITC-E2−dL5 to 0.5 $\mu$g FITC-labeled CD11c monoclonal antibody or 200 nM biotin−PEG−fluorescein. Error bars are 1 standard deviation from three replicate samples. (A) FITC fluorescence measured using 495 nm excitation and 519 nm ±10 nm emission with an increasing concentration of FITC-E2−dL5 and 2.5 $\mu$M MG-2p. (B) 200 nM biotin−polyethelyne glycol (PEG)−carboxyfluorescein fluorescence measured the same as in A. Insets are of corresponding MG-2p fluorescence from the same samples.




| Sample Name | Count | Median : FL1-A |
|---|---|---|
| Cells only | 4253 | 1419 |
| HA11 antibody only | 3635 | 10812 |
| HA11 and FITC E2 dL5 | 3937 | 7087 |

| Sample Name | Count | Median : FL4-A |
|---|---|---|
| Cells only | 4253 | 432 |
| FITC E2 dL5 only | 4715 | 915 |
| HA11 and FITC E2 dL5 | 3937 | 22369 |

**Figure 3.** Flow cytometry fluorescence analysis of FITC-labeled HA11 antibody (antibody which binds to the HA epitope) bound to CHO cells expressing an HA-tagged OPRM1 receptor using FITC-E2−dL5 as a secondary labeling agent. (A) FL1 green channel fluorescence gives an antibody signal to cells only background signal ratio of 7.62 with 35% quenching of FITC fluorescence by FITC-E2−dL5. (B) FL4 far-red channel fluorescence gives MG-2p signal to cells only background signal ratio of 51.8 and approximately 2× background signal from nonspecific staining.

fluorescence signal to background ratio of 7.62 (Figure 3A). Binding FITC-E2−dL5 to cells with FITC-labeled HA antibodies and incubation with 250 nM MG in both samples gave a median signal to background ratio of 51.8 (Figure 3B). FITC fluorescence quenching upon addition of FITC-E2−dL5 to cells bound with FITC-labeled anti-HA antibody lowered the green fluorescence by approximately 35% of the starting FITC fluorescence (Figure 3A). Nonspecific binding of FITC-E2−dL5 to cells without FITC-labeled antibody gave approximately a doubling over the red autofluorescence signal, indicating relatively low levels of nonspecific binding, or sample fluid fluorescence associated with FITC-E2−dL5 around the cells (Figure 3B). These data show that FITC-E2−dL5 specifically binds FITC on anti-HA monoclonal antibodies bound to cells and generates fluorescent signal in the far-red region of the visible spectrum. The signal to background ratio of FITC-labeled antibody alone in the green region vs the signal to background ratio of MG fluorescence in the red region improves 6-fold using this protein as a secondary binding reagent.

**Microscopy Analysis Demonstrates Colocalization of FITC-Labeled HA11 Antibody and FITC-E2−dL5/MG Fluorescence.** FITC-labeled primary antibodies can be useful imaging targets on living cells. To determine whether the bifunctional FITC-E2−dL5 and MG-2p labeling system was practical for imaging live cells, HEK cells expressing a dopamine transporter with an N-terminal CFP tag and extracellular HA epitope[19] were visualized with primary anti-HA antibody and the converter construct. The dopamine transporter is trafficked to the plasma membrane, and addition of HA antibodies to the culture medium results in robust staining of plasma membrane localized transporter.[20] Addition of unlabeled monoclonal HA antibodies to living HEK cells followed by incubation with FITC-E2−dL5 and MG-2p resulted in low signals for both 488 nm channel green fluorescence (attributable to some crossover from CFP fluorescence) and for 640 nm channel red fluorescence (Figure 4A−C), indicating low nonspecific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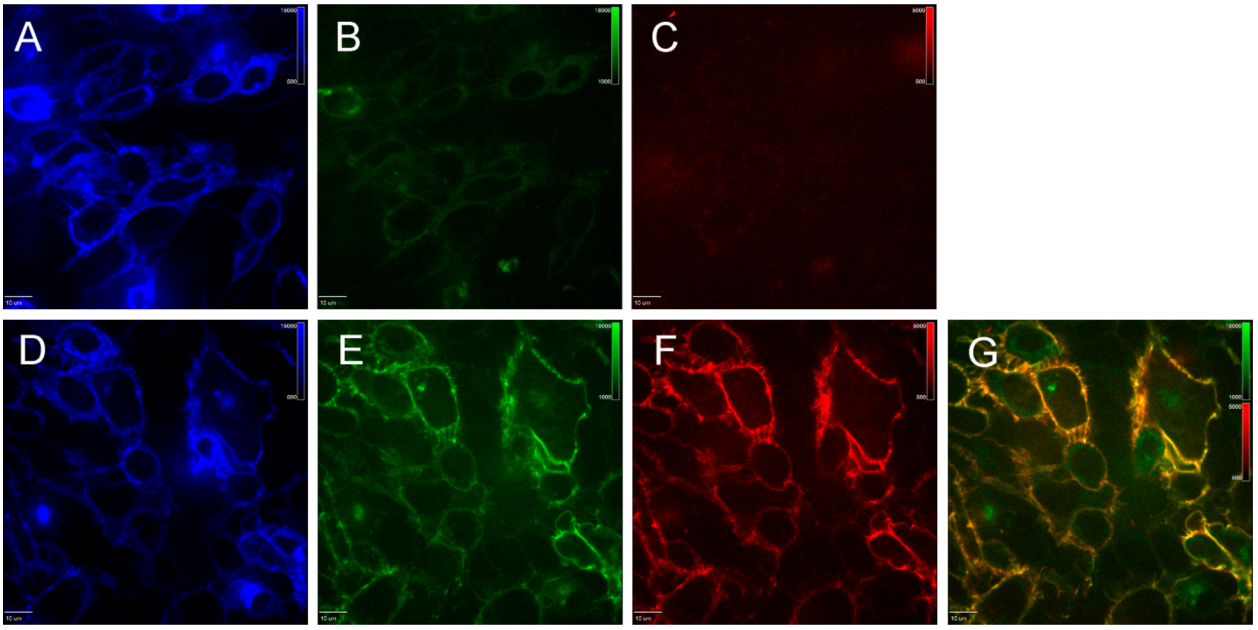


**Figure 4.** Live HEK cells expressing dopamine transporter with cyan fluorescent protein and HA epitope (CFP-HA-DAT) were incubated with unlabeled (A−C) or FITC-conjugated (D−G) anti-HA antibodies prior to incubation with FITC-E2−dL5 and MG-2p and imaging as described in Experimental Procedures. (A and D) CFP fluorescence representing total transporter. (B and E) FITC fluorescence, which includes background crossover from CFP. (C and F) Fluorescence from MG-2p. (G) Merged image from E and F showing colocalization of signals from anti-HA-FITC and MG-2p. Images for each channel were acquired and displayed using identical settings.

labeling with the FITC-E2−dL5 construct. Use of FITC-labeled anti-HA antibodies substantially increased plasma membrane 488 nm channel green fluorescence compared to samples incubated with unlabeled anti-HA (Figure 4D and E, note that crossover from CFP is still visible). Importantly, incubation of these FITC-anti-HA-labeled cells with the FITC-E2−dL5 and MG-2p produced robust far-red fluorescence of the plasma membrane that was highly colocalized with the FITC fluorescence from the HA antibodies and CFP fluorescence (Figure 4F and G). It is important to note that less than 10% of the FITC fluorescence was quenched in these experiments as subsaturating concentrations of FITC-E2−dL5 was used. The purpose of these experiments was to show colocalization of FITC and MG-2p fluorescence and demonstrate the low background of MG-2p fluorescence when FITC-conjugated antibody is not present. These data indicate that the FITC-E2−dL5 and MG-2p labeling system can be used to specifically label proteins of interest and analyze their dynamics on living cells.

**Fluorescence Correlation Spectroscopy of FITC-E2−dL5 with FITC-Labeled Dextran.** To demonstrate that FITC-E2−dL5 can bind FITC-labeled biomolecules other than antibodies, FITC-labeled dextran was bound by FITC-E2−dL5 in solution-based fluorescence correlation spectroscopy experiments. A relatively large FITC-labeled dextran with an average polymer MW of 150,000 with an average labeling rate of 0.004 FITC molecules per dextran residue was used to measure fluorescence correlation with FITC-E2−dL5. A ratio of 20.25 nM FITC dextran (effective FITC concentration of 75 nM) with 50 nM FITC-E2−dL5 was used to ensure most or all FITC-E2−dL5 protein would be bound to FITC dextran. MG-2p fluorescence was measured in these experiments rather than FITC fluorescence as the size change and diffusion rates are larger for FITC-E2−dL5 (54 kDa) alone vs FITC-E2−dL5 bound to FITC dextran (approximately 250−300 kDa, depending on how many protein molecules bind) than for FITC dextran alone (150 kDa) vs FAP-bound FITC dextran (250−300 kDa). FITC dextran size also varies considerably in the preparation with an average MW of 150,000, leading to a wider variety of FITC dextran diffusion times vs FITC-E2−dL5 diffusion times, as the latter is of a uniform size. Ten FCS spectra per experiment were averaged to give an averaged diffusion time, then converted to a diffusion rate as described in the Experimental Procedures for three experiments each for FITC-E2−dL5 alone and FITC-E2−dL5 with FITC dextran. The averaged diffusion rate for FITC-E2−dL5 alone is 100.3 $\mu m^2/s$ with a standard deviation of 2.1 $\mu m^2/s$, while the averaged diffusion rate of FITC dextran with FITC-E2−dL5 is 50.2 $\mu m^2/s$ with a standard deviation of 2.1 $\mu m^2/s$. The diffusion rate constant of FITC-E2−dL5 alone is nearly double that of the FITC dextran-bound sample, indicating that the protein is binding to and diffusing with the larger dextran molecule. These values are consistent with a simple model for differences in hydrodynamic diameter of the protein alone and complexed with the dextran based on molecular weight ($R_h \approx M^{1/3}$, and $D \approx 1/R_h$), which would predict a $D_{bound}/D_{free}$ ratio of ∼0.55 (using MW of 54,000 and 312,000 for free and bound fluorophore, respectively, assuming each 150,000 MW dextran binds three FITC-E2−dL5 molecules), compared to the observed $D_{bound}/D_{free}$ ratio of ∼0.50, measured.

**Photobleaching of Immobilized Fluorescein vs FAP-Bound MG.** Photostability experiments on avidin agarose particles to determine the relative photobleaching rates of immobilized biotin−PEG−fluorescein and FITC-E2−dL5/MG bound biotin−PEG−fluorescein were performed by taking 3000 continuous 200 ms exposure images using appropriate excitation sources and emission filters for each fluorophore. Laser output power was determined to be equivalent for both 488 and 640 nm lasers used in these experiments. Mean fluorescence analysis of these images were calculated,


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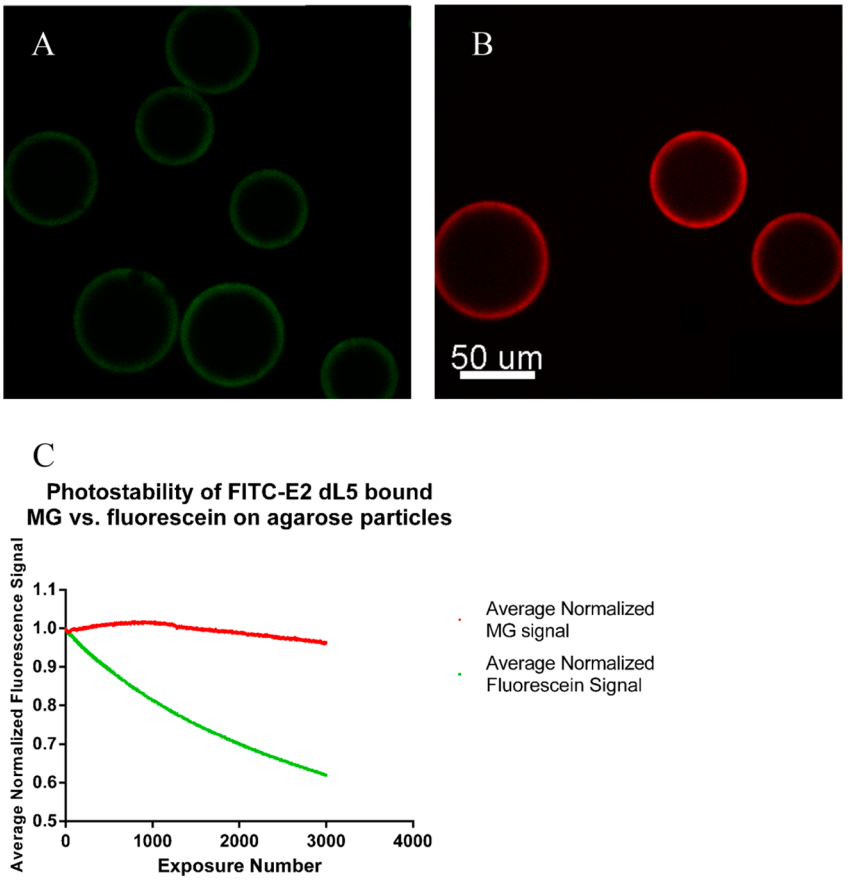


**Figure 5.** Photobleaching comparison of immobilized biotin−PEG−fluorescein on avidin agarose particles vs biotin−PEG−fluorescein with FITC-E2−dL5 and MG-2p bound afterward. (A) Green fluorescein fluorescence on representative paticles excited at 488 nm measuring fluorescein emission. (B) Red MG fluorescence on representative particles excited at 640 nm measuring MG emission. Scale bar is the same for both photos. (C) Normalized fluorescence from 3000 exposures of 200 ms at equivalent laser power output from three sample areas of both fluorescein bound particles and FITC-E2−dL5/MG-2p bound particles. Fluorescein was photobleached by approximately 38%, while MG photobleached by approximately 4.5% over the course of 3000 exposure frames.

normalized and averaged as described in the Experimental Procedures section. Background green fluorescence of control avidin agarose particles alone and red fluorescence background of particles with 500 nm MG-2p were negligent compared to signal from fluorescein or FITC-E2−dL5/MG bound particles (data not shown). This eliminates complications with background fluorescence seen in cellular experiments to better measure fluorophore photobleaching. Averaged normalized fluorescence from fluorescein was seen to decrease by 38% (±1.7% standard deviation) over the course of 3000 frames of 200 ms exposure (approximately 10.5 min) while FAP-bound MG fluorescence decreased by 4.5% (±3.5% standard deviation) (Figure 5). These experiments show that immobilized fluorescein is photobleached at a faster rate than MG is when bound to fluorescein by the FITC-E2−dL5 protein. This improved photostability could help with experiments where fast fluorescein/FITC photobleaching is a concern and demonstrates the improved photostability of using FITC-E2−dL5 as a secondary labeling reagent.

## DISCUSSION

We have shown that the bifunctional FITC-E2−dL5 converter protein specifically binds fluorescein on monoclonal antibodies and polymers, and simultaneously binds and activates malachite green fluorogens *in vitro* and on the surface of cells. The fact that FITC-labeled monoclonal antibodies are available against many protein targets allows rapid fluorogen-based visualization by microscopy or flow cytometry on cells with simple and straightforward antibody binding protocols. The pH sensitive nature of FITC, its high photobleaching, and the background autofluorescence associated with the blue excitation region often complicates these measurements using direct antibody conjugates. Use of the bifunctional FITC-E2−dL5 protein described here can convert any fluorescein-conjugated molecule to a readily detectable far-red (e.g., Cy5 channel) construct. This fluorescence conversion is not FRET based, and a different MG excitation source in the 630−640 nm range must be used than would be used to excite fluorescein or FITC in the 480−490 nm range. FAP-bound MG is also less pH sensitive and more photostable than solution-exposed FITC.[7] Continued work within the Carnegie Mellon University Molecular Biosensor and Imaging Center (MBIC) is focused heavily on use and development of additional fluorogen-based sensors which also bind the dL5 protein. Dyes that amplify signal have been developed and used[10] and could be used with FITC-E2−dL5 to amplify the signal from low copy proteins on the cell surface. Many of these applications can be adapted to work directly with the FITC-E2−dL5 system, using antibodies to target them and the dL5/fluorogen system as a reporter.

This approach also differs from direct genetic addition of GFP or other fluorescent proteins, as it does not change the nature, size, or sequence of the protein under study. Due to the


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relatively large size of fluorescent proteins and potential complications of protein folding, sorting, and function sometimes observed when adding a large protein tag to a target, genetic addition of fluorescent proteins is often not viable for studying protein biology in cells. Furthermore, genetic modifications must be made separately for each protein or variant under study, requiring time-consuming genetic manipulation and transfection steps for each protein studied. Use of FITC-labeled targeting agents for native proteins with the FITC-E2−dL5 protein alleviates these concerns.

Secondary labeling is commonly used in immunofluorescence and cytometry experiments (e.g., fluorescently labeled secondary antibodies) to bind the Fc region of primary antibodies. These secondary antibodies are specific to the source species of the primary antibody and do not cross-react with antibodies produced in different species. FITC-E2−dL5 will bind to FITC or fluorescein on any primary antibody, thus eliminating the need for species-specific secondary labeling antibodies when direct FITC conjugates are available. For living cells, the multivalency of secondary antibodies allows binding and bridging of multiple targets, potentially cross-linking primary antibodies, causing aggregation or stimulating biological responses.

Alternate approaches to secondary labeling systems involving fluorogens include genetic addition of fluorogen activating protein directly to protein G or streptavidin to label primary antibodies or biotinylated proteins with fluorogens.[17] Compared to these approaches, an advantage of the FITC-E2−dL5 converter is that FITC-labeled antibodies are typically conjugated with multiple FITC molecules per protein, allowing more monovalent FITC-E2−dL5 and fluorogen molecules to bind without promoting cross-linking or aggregation (e.g., streptavidin or bivalent antibodies). This is an approach to increase the signal beyond that seen with a one MG to one protein approach in a FAP-fused protein. Due to the incomplete quenching of FITC on primary antibodies when bound (Figures 2 and 3C), colocalization of primary antibodies and FITC-E2−dL5 can be shown by microscopy with both green and red fluorescence. The tight $K_d$ values of both FITC-E2 to fluorescein (2.4 nM)[5] and dL5 to MG (18 pM)[18] suggest that the bifunctional protein will stay bound to both target dyes for long periods of time at low concentrations of both target and fluorogen.

Use of FITC-E2−dL5 as a secondary reagent shifts the fluorescence to the far red and consequently enhances the signal-to-background ratio of stained cells relative to fluorescein-labeled antibodies alone (Figure 3). This signal to background increase is of particular advantage when a low copy number target protein is detected by a fluorescein-labeled antibody. Separating signal from noise, or finding rare cells expressing low-abundance antigens in such experiments may be difficult when a signal to background ratio of less than 10 is observed in cells expressing moderate receptor copy numbers as seen in these experiments (Figure 3A). By simple direct addition of FITC-E2−dL5 and the fluorogen MG-2p, a nearly 7-fold signal-to-background ratio improvement is seen (Figure 3B) Such improved signal-to-background may improve sensitivity for detection of low-abundance antigen on cells.

Fluorescein is a versatile, small organic fluorescent molecule and is conjugated to protein, lipid, and sugar molecules with relative ease. The photostability and photobleaching of FITC happens in a short time frame and is completely irreversible. One additional advantage of using this FITC-binding fluorogen activating protein is that the MG-2p−fluorogen FAP complex is highly photostable.[7] As described above, the use of this protein as a secondary labeling reagent for immunoassays eliminates the need for multiple secondary antibodies for different species-specific antibodies, and the cost and straightforward ease of growing and purifying a recombinant protein from *E. coli* may be lower than purchasing a secondary labeling agent. As shown in these studies, this labeling reagent can bind FITC or fluorescein (Figure 2B) on molecules other than protein, and therefore could be used as a labeling agent for a wide variety of biomolecules. The related dye Oregon Green is a carboxy-fluorescein derivative and has been previously shown to bind to FITC-E2.[3] As all of these fluorescein derivatives excite and emit in the same spectral region, the FITC-E2−dL5 protein can be used as a secondary reagent to increase the signal-to-noise ratio for biomolecules labeled with any of the three related fluorophores. This secondary FITC/fluorescein binding FAP also eliminates the need for any genetic manipulation of target proteins, making it a quick, easy to use, and cost-effective approach as a way to shift the fluorescence spectrum and reduce background for any FITC or fluorescein-labeled target.

## EXPERIMENTAL PROCEDURES

**Fluorogen and FITC-E2−dL5 production.** Malachite Green with an *O*-aminoethlydiethelyene glycol 2 PEG modification (MG-2p) was obtained from the Carnegie-Mellon Molecular Imaging and Biosensor Center. The molecule was prepared by previously published methods.[7] All restriction enzymes, Phusion DNA polymerase, and T4 ligase were obtained from New England Biolabs (Ipswich, MA). The FITC-E2 scFv encoding DNA was amplified by polymerase chain reaction (PCR) from the plasmid pPNL6 FITC-E2 (obtained from the laboratory of Dr. Peter Berget) with DNA primers containing *Hin*dIII and NcoI restriction sites, respectively, and complementary to the 5′ and 3′ ends of the FITC-E2 encoding DNA. Amplicon was isolated and treated with *Hin*dIII and NcoI restriction enzymes and ligated into a previously described modified pET21 expression plasmid digested with the same enzymes.[15] A previously described synthetic DNA insert encoding a glycine-serine linker region $(G_4S)_4$ was ligated into the NcoI/*Bam*HI cut plasmid.[15] DNA encoding the fluorogen-activating protein (FAP) dL5**[18] was amplified with PCR primers containing the restriction sites *Bam*HI and NotI and ligated in frame with the FITC-E2 coding region into the FITC-E2-$(G_4S)_4$ plasmid digested with the same enzymes. DNA sequencing was performed at each subcloning step to ensure accurate in-frame insertion of scFv, linker, and FAP. pET21 FITC-E2−dL5 plasmid was transformed into calcium-competent Rosetta-Gami 2 (DE3) *E. coli* cells (Novagen, Madison, WI). Bacteria were grown, induced, centrifuged and lysed, with scFv-FAP protein purified in a previously described manner.[15] Briefly described, 3 mL starter cultures of transformed *E. coli* were transferred to 500 mL of LB + (LB media (Difco, Detroit, MI) with 100 mM phosphate, 20 mM succinic acid, and 0.4% glycerol) and grown for 5−6 h at 37 °C until the OD600 of the culture was at 0.6. Cultures were then moved to 20 °C shaking incubators for 1 h and induced using 500 μM IPTG (Research Products International Corp., Mount Prospect, IL) and 0.4% glucose (Fisher Scientific, Hampton, NH) and grown overnight in 20 °C shaking incubators. Bacteria were lysed using an EmulsiFlex-C3 Homogenizer (Avestin, Ottawa, ON, Canada), and protein was purified on Ni-NTA agarose (Qiagen, Hilden, Germany)


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and eluted with HRV3C protease in a previously described manner.[15] Protein concentration was determined using absorbance at 280 nm and a calculated extinction coefficient of 87,300 $M^{-1}$ $cm^{-1}$ based on protein sequence. Typical protein yields were 0.5−1 mg/mL and could be stored stably at 4 °C for several months with no significant protein deterioration. For more concentrated protein preparations and long-term storage several 500 mL preparations were grown, purified, and concentrated to 2 mg/mL using a 10,000 Mw cutoff Amicon Ultra centrifugal filter (Millipore, Billerice, MA)

**In Vitro Fluorescence Measurements.** Fluorescence measurements were acquired in top-read mode on a Tecan Safire 2 fluorescence plate reader in a black Nunc round-bottom 96 well plate (U96 PP 0.5 mL, Sigma-Aldrich, St. Louis, MO). FITC-labeled hamster anti-mouse CD11c antibody (0.5 μg) (BD Biosciences, San Jose, CA) or 200 nM biotin−PEG−fluorescein (MW 3400, NANOCS, New York, NY) and 2.5 μM MG-2p fluorogen were used per well with increasing amounts of FITC-E2−dL5 protein in 200 μL volumes of PBS + 0.1% Pluronic F-127 (Invitrogen, Carlsbad, CA). Samples were incubated for 1 h prior to fluorescence measurements. FITC fluorescence measurements were taken using 495 ± 10 nm excitation and 519 ± 10 nm emission wavelengths with gain and Z position optimized for the well with no FITC-E2−dL5. Biotin-PEG-fluorescein measurements were taken using the same settings as FITC with a gain of 92. MG-2p fluorescence measurements were taken using 636 ± 10 nm excitation and 686 ± 10 nm emission wavelength. Instrument gain and Z positions were autocalculated by the TECAN Safire 2 instrument, usually on the highest intensity well. Each well was measured 25 times. Samples were set up independently in triplicate for measurements.

**Flow Cytometry of CHO Cells with HA Antibody Binding.** CHO cells expressing HA tagged OPRM1 receptor were created by transduction of CHO cells with the retroviral vector pBABEHL1.0.1 OPRM1 in a manner previously described.[9] pBABEHL1.0.1 OPRM1 was created by digestion of a previously described plasmid, pBABEHL1.0.1 ADBR2-Lac2,[9] with SfiI (New England Biolabs, Ipswich, MA) and ligation of DNA encoding the OPRM1 receptor with flanking SfiI restriction sites synthesized by Genscript (Piscataway Township, NJ). CHO cells stably expressing an HA tagged OPRM1 receptor were grown in F-12K media with L-glutamine (Life Technologies, Carlsbad, CA) and 10% Fetal Bovine Serum (FBS) (Life Technologies, Carlsbad, CA), passaged using Cellstripper (Life Technologies, Carlsbad, CA), and spun for 5 min at 804 rpm in an Eppendorf 5810R centrifuge. Cells were resuspended in 0.5 mL of F-12K media with FBS and FITC-labeled HA 11 antibody (Covance, Princeton, NJ) at a dilution of 1:250 at 37 °C for 30 min. Cells were recentrifuged as described above, media and antibody were removed, and cells were resuspended in 0.5 mL of media with 1 μM FITC-E2−dL5 and incubated for 30 min at 37 °C. Cells were then centrifuged and washed twice with PBSCM (PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) and finally suspended in 0.5 mL PBSCM. Control samples with HA11 antibody only and FITC-E2−dL5 protein only at the same concentrations or no added reagents were prepared alongside these samples. MG-2p was added at 250 nM to all samples and incubated for 15 min prior to flow cytometry analysis. An Accuri C6 flow cytometer was used to analyze cells with both 488 and 640 nm excitation and FL1 (533/30 nm) and FL4 (670 nm LP) emission filters. Ten thousand total events per sample were collected with cells gated by forward and side scatter. Fluorescence analysis was performed using FloJo flow cytometry analysis software.

**Microscopy of Cells with FITC-Labeled HA Antibodies and FITC-E2−dL5/MG.** Human HEK 293T cells were purchased from American Type Culture Collections, Inc. (Manassas, VA) and maintained according to ATCC instructions. A previously described N-terminally CFP-tagged dopamine transporter that contains an HA epitope in the second extracellular loop (CFP-HA-DAT)[19] was transfected at the time of seeding onto coverslips using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two days following transfection, cells were incubated with 4 μg/mL of either unlabeled or FITC-conjugated anti-HA antibodies (Covance, Princeton, NJ) for 1 h at 37 °C. Subsequently, cells were washed three times in ice-cold PBSCM (PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) and incubated for 1 h on ice with 1 μM of FITC-E2−dL5 in PBSCM. After 3 washes in ice-cold PBSCM, coverslips were moved to Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% FBS in the imaging cassette in an environmentally controlled microscope chamber. After equilibrating for 5 min MG-2p was added to a final concentration of 500 nM, and was not subsequently removed. Images were acquired at 37 °C in the presence of 5% $CO_2$ using a spinning disc confocal imaging system based on a Zeiss Axio Observer Z1 inverted fluorescence microscope (with 63× Plan Apo PH NA 1.4), equipped with a computer-controlled Spherical Aberration Correction unit, Yokogawa CSU-X1, Vector photomanipulation module, Photometrics Evolve 16-bit EMCCD camera, environmental chamber and piezo stage controller and lasers (405, 445, 488, 515, 561, and 640 nm) (Intelligent Imaging Innovations, Inc.), all controlled by SLIDEBOOK5 software (Intelligent Imaging Innovations, Inc.). Images of FITC and MG-2p fluorescence were acquired using 405/488/561/640 Quad dichroic with individual bandpass emitters for CSU-X (525/50 nm and 700/25 nm for FITC and MG-2p, respectively). CFP images were taken using 445/515/640 Triple Dichroic for CSU-X and a bandpass emitter 482/35 nm. All imaging acquisition settings were identical in each experiment.

**Fluorescence Correlation Spectroscopy of FITC Dextran with FITC-E2−dL5.** FITC DEAE dextran of an average MW of 150,000 and average FITC to dextran labeling rate of 0.004 (product number 75005, Sigma-Aldrich, St. Louis, MO) was mixed to an FITC dextran concentration of 20.25 nM (effective calculated FITC concentration of 75 nM) in phosphate-buffered saline (PBS) with 50 nM of FITC-E2−dL5 and 125 nM MG-2p and incubated at room temperature for 1 h. A control sample of 50 nM FITC-E2−dL5 and 125 nM MG-2p was also prepared. Samples were spun for 10 min at maximum speed in an Eppendorf 5417C centrifuge after 10 min of incubation at room temperature and transferred to a Tek II bottom-glass (No 1.5) 8-well dish (Mat-Tek, Ashland, MA). A Zeiss LSM 510 Meta NLO Confocor 3 microscope was used for fluorescence correlation spectroscopy (FCS). All measurements were acquired with an LD C-Apochromat 40×/1.1 NA water-immersion objective. A 633 nm HeNe laser with 3% laser power was reflected via a dichroic beam splitter to excite MG-2p. Fluorescent emission passes a 655−710 nm band-pass filter. Fluorescent signals that pass a 90-μm pinhole were collected with an avalanche photodiode detector (APD). Measurement time was set for 10 s, repeated 10 times to

produce a measurement series for an experiment run. Each sample was run 3 times. Using Zeiss ZEN-2008 software, the autocorrelation of the data was obtained, and the resulting curve was fitted with a model for one component 3D free translational diffusion. An average diffusion time ($\tau_D$) from each experiment run was used for calculation of the diffusion coefficient ($D$, $D = \omega_0^2/4\tau_D$), where $\omega_0$ is the waist radius of a laser beam obtained from instrument calibration (0.337 $\mu$m used).

**Photostability of Fluorescein vs FAP-Bound MG on Agarose Particles.** Biotinylated PEG−fluorescein (MW 3,400, NANOCS, New York, NY) was bound to monomeric avidin agarose (product # 20228 Thermo Scientific, Waltham, MA) by mixing 500 nM biotin−PEG−fluorescein with 5 $\mu$L avidin agarose in 500 $\mu$L of PBS + and incubation at 4 °C on a rotating device for 30 min. Bound agarose was then spun in an Eppendorf 5417C centrifuge (Eppendorf, Hamburg, Germany) at 16,100 rcf for 1 min. Liquid was removed from pelleted particles,and particles were resuspended in 500 $\mu$L of PBS +. This process was repeated twice to remove unbound biotin−PEG−fluorescein. A duplicate sample of biotin−PEG−FITC was then incubated with 500 nM FITC-E2−dL5 and 500 nM MG-2p for 30 min at 4 °C on a rotating device along with a control sample of avidin agarose particles and 500 nM MG-2p. Three centrifuge spin and wash steps were performed on these samples as described above. All samples along with an avidin agarose only control sample were transferred to 35 mm glass bottom culture dishes (MatTek, Ashland, MA) and allowed to settle for 15 min prior to imaging. Imaging and photobleaching were performed using an Andor Revolution XD spinning disc microscope (Andor, Belfast, Ireland) with a Nikon CFI Plan Apo VC 20X objective lens (Nikon, Tokyo Japan) Three regions of each sample preparation (biotin−PEG−fluorescein and biotin−PEG−fluorescein with FITC-E2−dL5/MG were measured for 200 ms for 3000 continuous frames (approximately 10.5 min) focused on settled agarose particles. Fluorescein was measured using 10% laser power on a 488 nm solid state laser and a 525/50 nm emission filter while FITC-E2−dL5/MG fluorescence was measured using 24.3% laser power on a 640 nm solid state laser and a 685/70 nm emission filter. Laser power was chosen to correspond to 0.00742 mW to have equal output power for both. Fluorescence quantification of images was performed using FIJI Bioformats import (ImageJ plugin) with freehand drawing of analysis regions around particles and mean fluorescence multimeasurement on all 3000 images for each measurement. Fluorescence was normalized for each measurement by dividing the mean fluorescence value for each frame by the starting (frame 1) mean fluorescence value, and triplicate measurements were averaged with standard deviations calculated after normalization for each frame.

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### Notes

The authors declare no competing financial interest.

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