

Fluorogenic Dendrons with Multiple Donor Chromophores as Bright Genetically Targeted and Activated Probes

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Abstract: We have developed a class of dendron-based fluorogenic dyes (termed dyedrons) comprised of multiple cyanine (Cy3) donors coupled to a single malachite green (MG) acceptor that fluoresce only when the MG is noncovalently but specifically bound to a cognate single chain antibody (scFv). These cell-impermeant dyedrons exploit efficient intramolecular energy transfer from Cy3 donors to stoichiometrically amplify the fluorescence of MG chromophores that are activated by binding to the scFv. These chromophore enhancements, coupled with our optimized scFv, can significantly increase fluorescence emission generated by the dyedron/scFv complex to brightness levels several-fold greater than that for single fluorescent proteins and targeted small molecule fluorophores. Efficient intramolecular quenching of free dyedrons enables sensitive homogeneous (no wash) detection under typical tissue culture conditions, with undetectable nonspecific activation.

Introduction

Fluorescent detection with genetically targeted probes has fundamentally expanded the types of questions that can be addressed with biological microscopy and cytometry.¹ The ease of use and breadth of application of genetically expressed fluorescent protein chimeras has been truly revolutionary, especially for live-cell studies. However, the signal generated by a single fluorescent protein or organic dye fluorophore is intrinsically limited and typically falls well short of the requirements for robust single molecule detection.² Investigators often address this deficit of signal by overexpressing receptors and other rare proteins genetically fused to a single fluorescent protein; however, overexpression may lead to imbalances in protein regulation, biosynthesis, or assembly. An alternative signal amplification scheme is based on very large genetic fusions incorporating multiple fluorescent protein copies, but these may sterically interfere with biological function.³ Essentially the same limitations apply to any of several site-specific dye-targeting methods based on genetic fusions to peptide domains that can be specifically modified by an *in vivo* enzymatic activity.⁴

Highly fluorescent probes may be obtained by *in vitro* conjugation of antibodies and other proteins to quantum dots,^{5,6} phycobiliproteins,^{7,8} DNA multifluorophore assemblies,⁹ or

multiple small molecule fluorophores.¹⁰ These probes are often cumbersome to prepare and, when applied to live or fixed cells and tissues, generate nonspecific background fluorescence unless excess probe is removed. Because these fully exogenous probes are not genetically expressed by the biological system of interest, they are difficult to deliver to extracellular and especially intracellular targets in a way that allows real time monitoring and imaging of dynamic biological functions.¹¹

Recently we and others demonstrated the use of single-chain variable fragment (scFv) antibodies to noncovalently constrain chromophores of several nonrigid cyanine-family dyes, thereby activating fluorescence hundreds- to thousands-fold.^{12–14} These scFvs are examples of a broader but so far limited class of fluorogen activating proteins (FAPs).¹⁵ FAPs that bind derivatives of malachite green (MG) displayed the greatest fluorogenic activation, up to 20 000-fold,¹³ primarily because of an extremely low background signal. The cell impermeant M (MG diethyleneglycolamine, Chart 1) and cell-permeant MG-ester¹³

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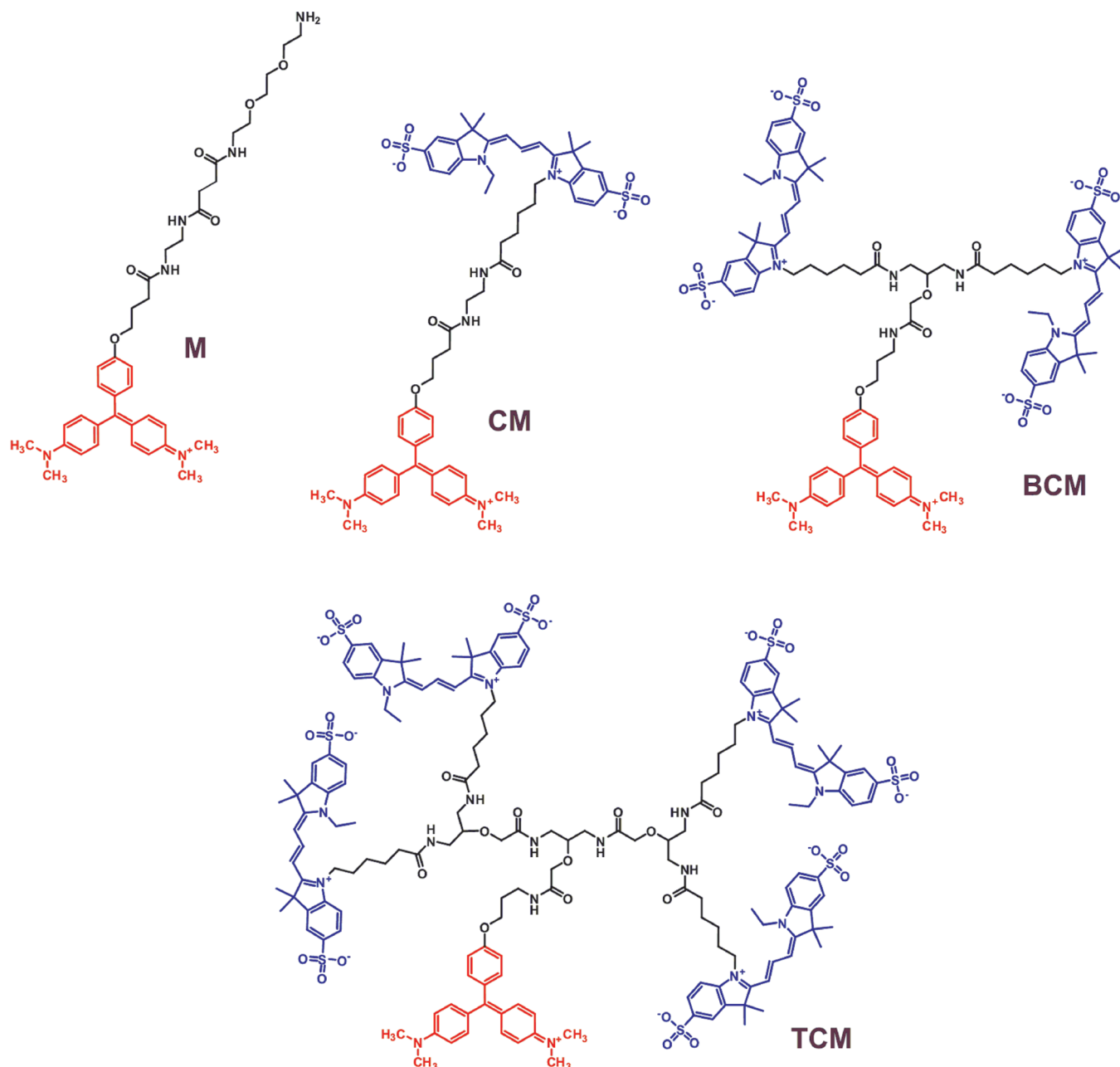
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Chart 1. Structure of Dyedrons^a

^a Donor groups are depicted in blue, and acceptor groups in red. M, malachite green diethyleneglycolamine; CM, Cy3.29 malachite green; BCM, Bis-Cy3.29 malachite green; TCM, Tetra-Cy3.29 malachite green. Malachite green (MG) is the chromophore shown in red.

are efficiently quenched in aqueous solution and remain so in complex biological milieus, including in cell lysates and on and within cells. This behavior contrasts with MG itself, which can become moderately fluorescent in these environments.¹⁶ Fusion proteins incorporating these FAPs can be labeled *in vivo* with extremely low background fluorescence by single-step addition of M or MG-ester, enabling new labeling applications. Employing confocal and stimulated emission depletion (STED) microscopy, we demonstrated intracellular targeting and labeling in live or permeabilized fixed cells using a disulfide-free

cytosolic FAP- β -actin fusion.¹⁷ FAP/fluorogen complexes (fluoromodules) are comparable to dyes and fluorescent proteins in brightness. Further improvements in brightness would result in better sensitivity and lower phototoxicity under typical imaging conditions.

Rationale

Because scFv-based fluoromodules rely on the intimate interaction between a nonfluorescent fluorogen and a specific

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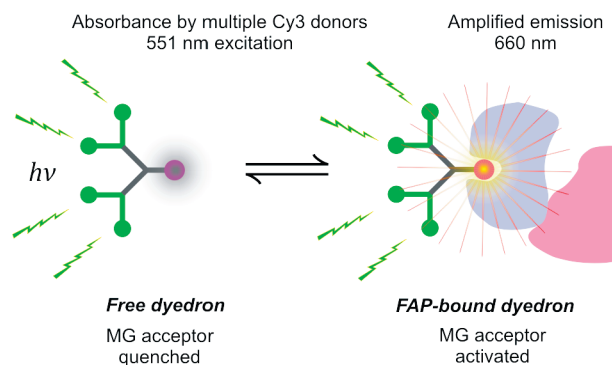


Figure 1. Binding activation of dyedrons.

binding polypeptide, additional molecules can be flexibly linked to the fluorogen to enhance the optical properties (such as brightness) without interfering with the fundamental binding-mediated activation. The brightness of fluorescent probes can be enhanced by improvements in the extinction coefficient and improvements in the fluorescent quantum yield; the product of these values reflects the molecular brightness. Using intramolecular energy transfer from Cy3 covalently coupled to MG, we have created compact multichromophore structures with large extinction coefficients at Cy3 excitation wavelengths while maintaining the MG fluorogenic properties. For dyedrons free in solution, the absorbance of light is increased in proportion to the number of Cy3 donor chromophores; the input energy is efficiently transferred to the MG acceptor chromophore, where it is nonradiatively dissipated (quenched) by unconstrained internal rotations. For dyedrons bound to a FAP, excitation is transferred in much the same way, except the MG acceptor chromophore is now rigidly constrained by the peptide binding pocket. Instead of dissipating, transferred energy is now emitted as donor-amplified fluorescence. Previous reports have shown that multichromophoric dendrimers have high absorbance and multiphoton cross sections¹⁸ as well as highly efficient intramolecular energy transfer,^{19–23} but such molecules are not conjugatable and do not have the inherent *in vivo* genetic targeting and activation capability of this new multichromophore class. This straightforward method for targeted activation is essential for the biological utility of these molecules (Figure 1).

Results

Compact multichromophore dyedrons in Chart 1 were prepared by a strategy similar to that of the convergent syntheses of Fréchet²⁴ and purified by reversed-phase liquid chromatography, yielding branched structures with one, two, and four Cy3 donor molecules covalently and stoichiometrically decorating the periphery of the molecule and a single MG quenching group at the base of the dyedron (Scheme S1, Supporting Information ‘Dyedron synthesis and characterization’, Figures S1–S3, Table

Table 1. Properties of L5-MG E52D Activated Dyedrons

Dyedron	M	CM	BCM	TCM
ϵ_{\max} [nm] ^a	642	552	551	552
ϵ_{\max} [M ⁻¹ cm ⁻¹] ^a	79 000	140 000	290 000	530 000
$\Phi_{\text{MG}}^{a,b}$	0.06	0.06	0.05	0.05
$\epsilon \times \Phi/10^3$ (brightness)	4.4	8.0	14	29 ^{c,d}
$F_{532}/F_{635}^{a,e}$	0.05	1.54	2.86	4.95
FACS ₅₃₂ /FACS ₆₃₅ ^e	0.07	2.17	3.27	6.89
K_D [nM] ^f	<1	<1	4.0	15
$F_{\text{quenched}}/F_{\text{free}}^g$	N/A	0.0084	0.0036	0.0037

^a Determined for soluble dyedron/L5-MG E52D complex. ^b Quantum yield for MG excitation peak determined with 620 nm excitation. ^c For comparison, EGFP = 32, see ref 28. ^d For comparison, L5-MG E52D L91S = 160, calculated from data in Figure 5 as ratio of fluorescence of cell surface displayed L5-MG E52D L91S to L5-MG E52D at 30 nM TCM. ^e Numerator and divisor calculated from data in Figure 3 as (median of stained population – median of unstained control). ^f Determined for dyedrons binding to yeast cell surface displayed L5-MG E52D (Figure S6). ^g Calculated as total absorbance-normalized fluorescence (530–800 nm) of dyedron divided by total absorbance-normalized fluorescence of Cy3.29. The MG absorbance peak was used for dyedron normalization.

S1). For a detailed study of the dyedron properties, we employed a 110 amino acid scFv (L5-MG E52D) derived from the original L5-MG clone¹³ by directed evolution to increase affinity and brightness when bound to M; to demonstrate that directed evolution can generate dyedron/FAP fluoromolecules with a brightness significantly greater than that of dyes and fluorescent proteins, we subsequently characterized additional L5-MG mutants L91S and E52D L91S (Figure S4).

Spectral Characterization of Dyedrons Bound to L5-MG E52D. Free in solution, all dyedrons showed >99% quenching of Cy3 fluorescence when conjugated to MG (Table 1) and essentially undetectable fluorescence in the spectral range associated with MG (Figure S5), consistent with the extremely low quantum yield of MG in the absence of an activating polypeptide.²⁵ The fluorescence quantum yield of the unbound TCM dyedron was <0.0005, so direct emission of the donors is efficiently quenched and does not interfere with detection of a FAP-activated dyedron.

At a single concentration of dye molecule in the presence of excess L5-MG E52D, MG-probe normalized excitation spectra (710 nm detection) reveal that contributions of the Cy3 excitation increase in direct proportion to Cy3 number and show that these simple modifications substantially enhance the overall excitation cross section of the construct as compared to M alone (Figure 2a and Table 1). The magnitude of cross-sectional enhancement correlates well with the absorbance of Cy3 ($\epsilon = 150\,000\text{ M}^{-1}\text{ cm}^{-1}$).²⁶ Quantum yields of all dyedrons at the MG excitation peak were essentially constant. Corresponding fluorescence emission spectra show almost complete transfer (>99%) of the Cy3 excitation to far red emission from the bound MG dye and show substantial increases in the brightness of the probe constructs when used with increasing generations of the dyedron (Figure 2b). Relative quantum yields at the Cy3 excitation and the MG excitation indicate that little donor excitation is lost to competing radiative and nonradiative

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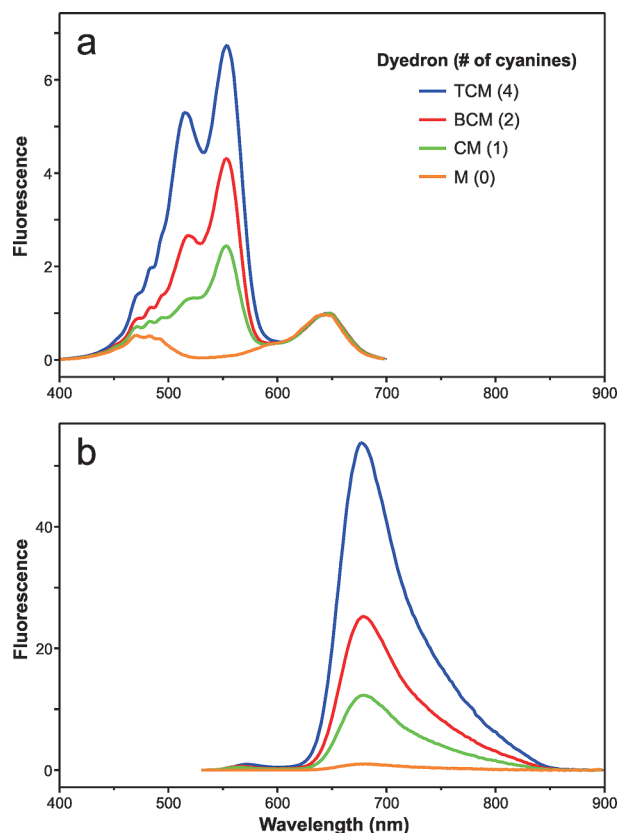


Figure 2. Fluorescence spectroscopy of dyedron/L5-MG E52D complex. (a) Relative fluorescence excitation of dyedron/L5-MG E52D complexes (710 nm emission). (b) Relative emission of dyedron/L5-MG E52D complexes (514 nm excitation). Spectra have been normalized to the MG excitation peak.

processes (Table S2). These observations support the concept that even inherently nonfluorescent or self-quenched donors in dyedrons could produce highly efficient sensitizing structures for bright fluorescence.^{10,27} Regardless of mechanism, dyedrons are efficiently quenched when unbound and efficiently activated for both direct and donor amplified fluorogen emission when bound.

When bound to the E52D FAP, the absorbance of M and all dyedron MG acceptors increase nearly 2-fold and their absorbance maxima red-shift and coalesce at ~ 642 nm, suggesting that acceptor photophysical properties are specifically modulated by the FAP binding pocket and are largely independent of the donors. In contrast, absorbance spectra at donor wavelengths of FAP-bound dyedrons and free Cy3 have similar features, suggesting that Cy3 photophysics are not greatly altered (Figure S7 and Figure S7 discussion), although some evidence of donor–donor interaction is evident in the BCM and TCM complexes.

Dyedron/L5-MG E52D Fluoromodules Expressed on Live Cell Surfaces. In vitro spectroscopic properties of L5-MG E52D fluoromodules are recapitulated when dyedrons (300 nM) are directly added to suspensions of live yeast cells expressing the fluorogen activating scFv as a fusion protein on the cell wall.¹³ Flow cytometry reveals step increases in brightness when excited at 532 nm and nearly constant brightness when excited at 635 nm (Figure 3), corresponding well to the differences seen in the excitation spectra and the consistent quantum yields measured at 620 nm excitation. Stained samples contain a subpopulation of nonfluorescent cells due to loss of an scFv-

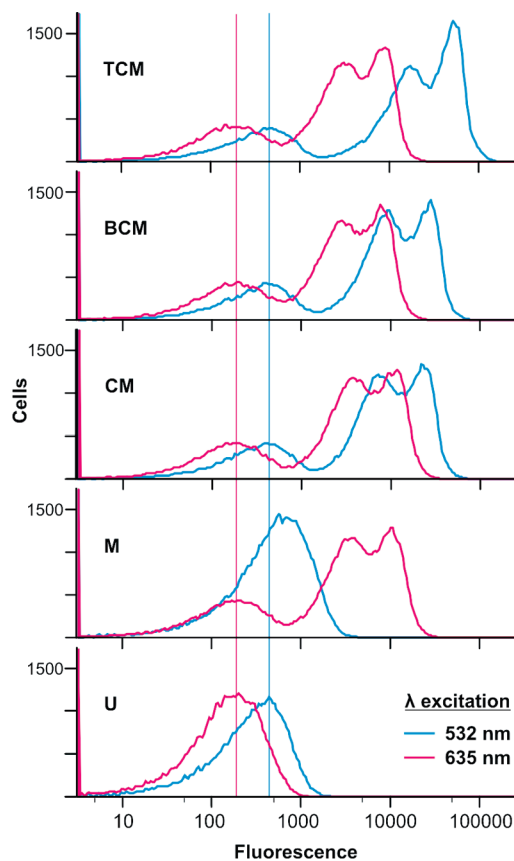


Figure 3. Flow cytometric analysis of dyedron-labeled yeast by exciting donor or acceptor. *Saccharomyces cerevisiae* cells expressing L5-MG E52D on their surface were analyzed as described.¹³ Two aliquots of each stained population and an unstained control (U) were respectively analyzed, with donor (532 nm) or acceptor (635 nm) excitation, and collecting emission through a 675/50 nm bandpass filter. Position of unstained cells is shown by thin lines. Each analysis comprised 100 000 cells.

encoding plasmid.¹³ Controls show virtually no fluorescence generated by dyedrons on cell surfaces in the absence of expressed FAPs (Table S3). Analysis of the staining ratio between yeast cells excited at 635 nm vs 532 nm reveals an increase in specific brightness by a factor of ~ 2 ($n = 1$), 3 ($n = 2$), and 7 ($n = 4$) in the (Cy3)_nMG construct (Tables 1 and S3). Hence, this approach increases molecular brightness in vitro and in vivo in direct correlation with the enhanced extinction provided by the donor array.

Dyedron-mediated signal amplification can also be applied to live cell fluorescence microscopy. Yeast cells expressing the L5-MG E52D fusion protein¹³ on their surface were imaged under a laser scanning confocal microscope (Figure 4). Yeast cells are specifically labeled on their surface. Virtually no fluorescence is detected from cells not expressing FAPs or from intercellular regions. The relative signal levels of M, CM, and TCM treated cells imaged at 561 and 633 nm excitation follow the same trends observed in solution fluorimetry and flow cytometry (Figures 2, 3).

Further Improving Dyedron Fluoromodules by Directed Evolution. The M/L5-MG E52D fluoromodule has a modest quantum yield, yet the TCM dyedron can amplify its signal to give a calculated molecular brightness similar to that of EGFP and most small molecule protein tags (Table 1). The L5-MG E52D FAP was created by applying directed evolution methods to the very low quantum yield progenitor L5-MG FAP.¹³ An error prone polymerase chain reaction using L5-MG DNA as a

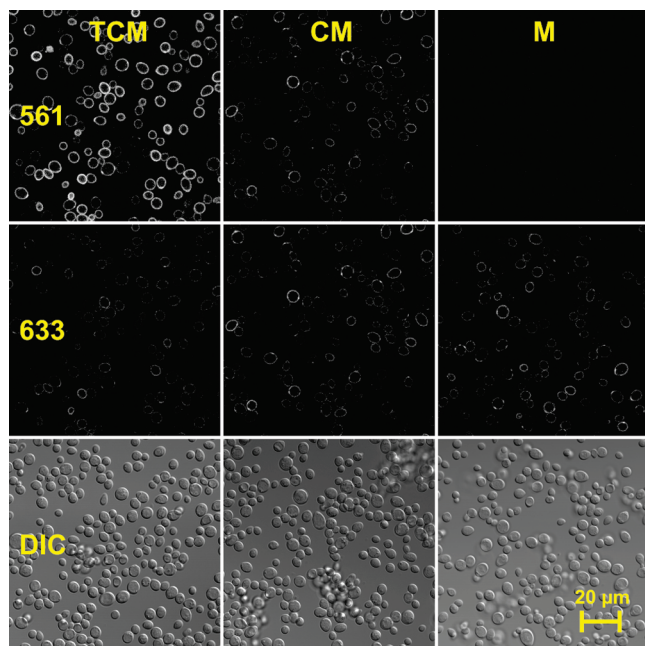


Figure 4. Fluorescence imaging of yeast cells surface displaying L5-MG E52D. Live yeast cells were imaged in PBS+ in the presence of 100 nM TCM, CM, or M on a Zeiss 510 MetaNLO confocal microscope using differential interference contrast (DIC) or fluorescence with donor (561 nm) or acceptor (633 nm) excitation and identical 650–710 nm BP emission settings (see Table S4 for other imaging parameters).

template was used to generate a population of plasmid encoded FAPs carrying random mutations, and FAP mutants displaying increased brightness and binding affinity when bound to M were selected from among this population by FACS screening of cell surface expressed FAPs.¹³

In addition to the L5-MG E52D FAP studied here, we characterized L5-MG L91S, which contains a single point mutation that increases the quantum yield of the M/L5-MG fluoromodule several-fold but does not increase the binding affinity to M (data not shown). We found that the L91S and E52D mutations in combination behave additively to create a FAP that binds TCM with similar affinity to L5-MG E52D and yields ~5-fold greater fluorescence when assayed on a microplate reader using live yeast (Figures 5 and S4). Yeast cells displaying L5-MG or evolved FAPs carrying the depicted point mutations were assayed in a 96-well microplate (554 nm excitation/660 nm emission) over a 1000-fold concentration range of TCM. Data were normalized to the number of yeast-displayed FAPs, independently determined by flow cytometric analysis of cells labeled with fluorescent antibody bound to the c-myc epitope present on each scFv (data not shown).¹³

The improved fluorescence properties of L5-MG E52D L91S are evident when imaging the surface of live yeast in PBS buffer using much lower concentrations of TCM (Figure 6). Yeast cells surface-displaying this FAP can also be readily imaged with TCM in yeast growth media (Figure S8), facilitating real time experiments on actively metabolizing cells.

Discussion

These dyedrons represent a new class of fluorescent detection reagent, where a genetically targetable acceptor chromophore is enhanced for efficient excitation by energy transfer from covalently attached donor molecules. Importantly, intrinsic donor chromophore emission is efficiently quenched in free solution,

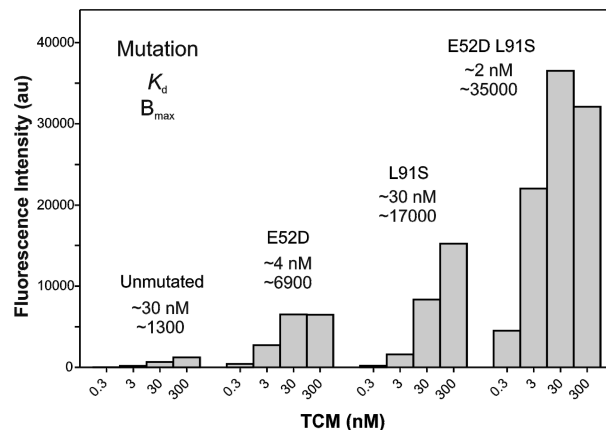


Figure 5. Improvement of TCM fluoromodule affinity and brightness by directed evolution. Live yeast cells expressing surface-displayed L5-MG FAPs carrying indicated point mutations were assayed on a fluorescence microplate reader. Values for binding affinity (K_d in nM) and relative brightness (B_{max} in arbitrary fluorescence units) are shown. The E52D and L91S mutations independently increase fluoromodule brightness, but only E52D confers tighter binding. The double mutant displays enhanced brightness and tighter binding.

so that only dyedrons bound to their biological target contribute significantly to the overall fluorescence. The molecular weight of these synthetic macromolecules remains small; dyedrons constitute a minor portion of fluoromodule mass, which is comparable to the size of GFP (~27 kDa), and are significantly smaller than fully exogenous conjugates currently in use as very bright fluorescent probes. Dyedrons greatly enhance the fluorescence of fluorogen/FAP complexes, as shown by the flow cytometry, microplate assays, and microscopy described here. The TCM/L5-MG E52D L91S fluoromodule has an estimated brightness value (160) that is nearly 5-fold higher than those of EGFP (Table 1) and the red fluorescent biarsenical complex (ReAsH = 34)²⁹ and ~10-fold brighter than the best available monomeric red fluorescent protein (mCherry = 16).³⁰ This improved dyedron fluoromodule has a long Stokes-shift and far-red emission (664 nm versus 610 nm for mCherry and ReAsH), ensuring that this probe provides substantial improvements in sensitivity.^{12,31} With dyedrons, binding of the fluorogen to the target peptide also brings the donor array into the binding site vicinity, reducing the overall peptide-fluorogen affinity (Table 1), but the stability of these complexes remains high (<20 nM for the E52D mutant). Such functional variation can be corrected or exploited by the directed evolution of scFvs or other recognition scaffolds.

Variation in donor chemistry can thus be combined with variation in fluorogen/peptide interaction to improve dyedron properties. One can select for improved fluorogen binding affinity and quantum yield in the context of a given donor array. The donor array can be designed to enhance the extinction coefficient but may also be designed to improve other optical properties, such as enhancing the multiphoton cross section of specific fluorogens, or providing targeted activation of photochemically or environmentally sensitive dyes.

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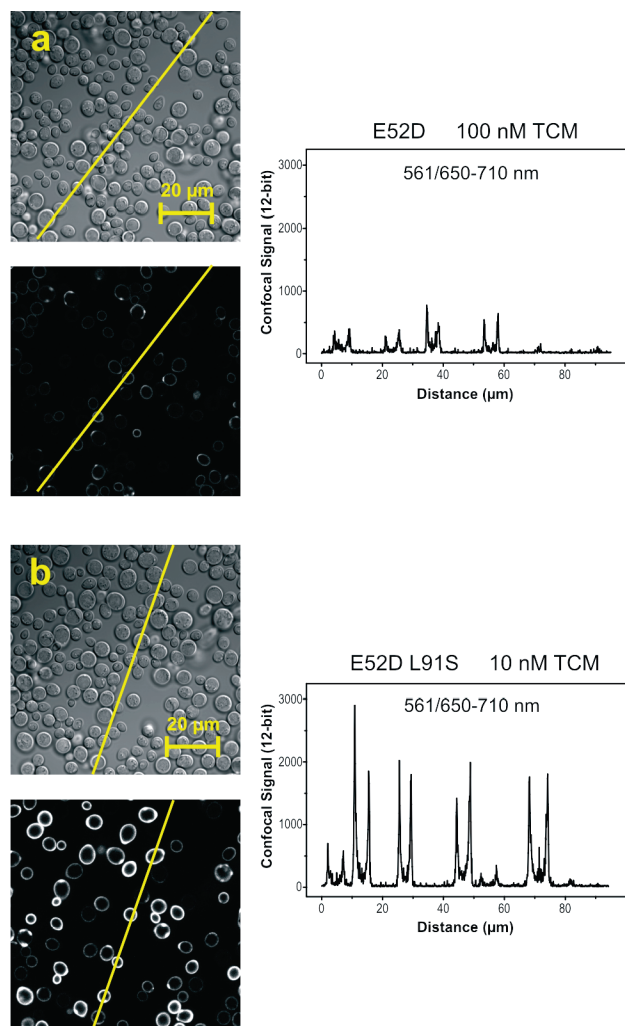


Figure 6. Live cell surface imaging with improved fluoromolecules. Yeast cells expressing L5-MG carrying E52D (a) or E52D L91S (b) mutations were imaged in PBS+ on a Zeiss 510 MetaNLO confocal microscope with identical donor (561 nm) excitation/650–710 nm BP emission settings using the indicated TCM concentrations. Scan profiles show that fluorescence of the E52D L91S double mutant at 10 nM TCM is ~5-fold greater than E52D fluorescence at 10-fold higher TCM concentration. The brighter signal seen at lower TCM concentration with the double mutant is consistent with its improved brightness and binding affinity.

The membrane impermeant nature of these dyedrons makes them ideal for studying a wide range of biological functions involving plasma membrane proteins that have exposed extracellular domains available for genetic fusion. Among these are receptors that mediate intracellular signaling such as G-protein coupled receptors³² and receptor tyrosine kinases, ion and metabolite transport channels, and cellular recognition and adhesion proteins. Exclusive labeling of extracellular domains confines detection of these proteins to the site of their biological function. In contrast, fusions of fluorescent proteins to these same extracellular domains would also be subject to detection during biosynthesis and intracellular transport, generating a background signal unrelated to function at the cell surface.^{13,33} M and its dyedron counterparts can be added to cell culture media directly

without washes or other treatments, distinguishing these fluorogens from other small molecule labeling methods that have been applied to membrane proteins.^{4,13,34–45} Enhanced sensitivity of expressible probes will reduce the need for high level overexpression in cell biological investigations³³ and may thus reduce artifacts related to expression level. Injection into transgenic animals is potentially feasible because these dyedrons are small enough to efficiently penetrate intercellular space in tissues.⁴⁶

The dyedrons described here currently provide optimal signal enhancement for applications such as flow cytometry, microplate assays, and imaging formats where individual fluoromolecules are subjected to moderate excitation flux. Future work will address characterizing and improving dyedron fluoromolecule photostability by addition of electron-withdrawing groups to Cy3 donors,¹⁴ by the use of other donor chromophores, and by directed evolution of the FAP to increase the on/off rate of dyedron binding, thereby continuously regenerating functional fluoromolecules.^{13,14}

Genetically expressed fluorescent protein fusions are widely used to study biological functions in the cytoplasm and other intracellular reducing environments. Current fluorogen-activating scFvs contain internal disulfide linkages that compromise function in these intracellular compartments, but directed evolution may be employed to remove such disulfides and adapt scFvs to intracellular function.^{17,47} It would then be desirable to introduce dyedrons into the cell for maximum utility. Several approaches to transmembrane delivery are available, including microinjection, pore formation, modifying dyedron physicochemical properties, addition of transport signals to dyedrons, or creation of dyedron-carrier vesicles or emulsions able to fuse with the plasma membrane. Microinjection of the TCM dyedron into live mammalian cells showed no detectable fluorescence up to 50× over the K_d (Figure S9). The low nonspecific activation of dyedrons indicates that the main barrier to intracellular use may be a problem of delivery, not one of specificity.

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Supporting Information Available: Scheme S1, Dyedron synthesis and characterization, Figures S1–S9, Tables S1–S4, Spectroscopy and microscopy methods, References. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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