

A Rainbow of Fluoromodules: A Promiscuous scFv Protein Binds to and Activates a Diverse Set of Fluorogenic Cyanine Dyes

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Fluoromodules are specific combinations of fluorogenic dyes and cognate protein^{1–3} or nucleic acid^{4–6} partners. Separately, neither component is fluorescent, but when reconstituted, strong fluorescence is observed. Rational design and straightforward synthesis allow access to fluorogenic dyes spanning the visible and near-IR wavelengths, while powerful *in vitro* and *in vivo* selection methods facilitate isolation of dye-binding proteins and nucleic acids. Fluoromodules are gaining increasing use as labels and sensors for bioimaging and detection.

Previously we isolated and characterized several human single-chain antibody (scFv)-based Fluorogen Activating Protein (FAP) reporters that generate fluorescence from the fluorogenic dyes thiazole orange (TO) and malachite green (MG).⁷ These FAPs, isolated from a yeast surface display library of human scFv's,⁸ bind fluorogens with nanomolar affinity, increasing green or red fluorescence thousands-fold to brightness levels typical of fluorescent proteins. These reagents have already been useful in visualizing cell surface elements and certain membrane proteins within the secretory apparatus of mammalian cells.⁷ Some spectral variation was generated by combining a limited set of these scFv's and fluorogen derivatives. However, the spectral range of fluorescence emission is constrained by the chromophores of the fluorogenic dyes and the methods used to select the FAPs.

Dimethylindole Red (DIR, Chart 1) is a fluorogenic cyanine dye. It was designed to have low nonspecific binding to DNA and RNA by using the bulky dimethylindole heterocycle to suppress intercalation and the anionic propyl sulfonate group to introduce nonspecific electrostatic repulsions from nucleic acids.⁹ An RNA aptamer that was selected for binding to DIR exhibited $K_d = 86$ nM and enhanced the fluorescence of the dye ca. 60-fold.⁹ Given our earlier success in selecting scFv's for binding to the related unsymmetrical cyanine TO1-2p (Chart 1), we next subjected DIR to the two-step scFv selection procedure.

A biotin analogue of DIR⁹ was used to enrich the complex yeast surface display scFv library composed of ca. 10^9 clones of synthetically recombined human heavy and light chain variable regions.⁸ This enrichment was accomplished by two rounds of sequential selection using streptavidin magnetic microbeads followed by antibiotin magnetic microbeads.¹⁰ The resulting yeast sublibrary was further enriched by three rounds of fluorescence activated cell sorting, gating the cell sorter for cells that directly activated DIR fluorescence. Individuals from these enriched populations were automatically cloned by the cytometer onto both selective growth plates (for storage and archiving) and plates containing

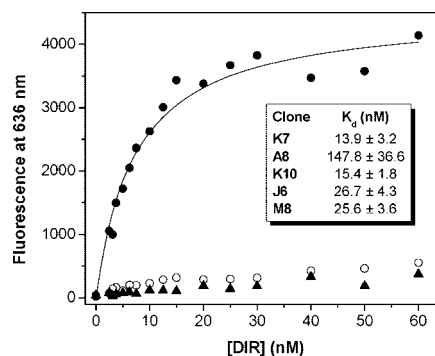
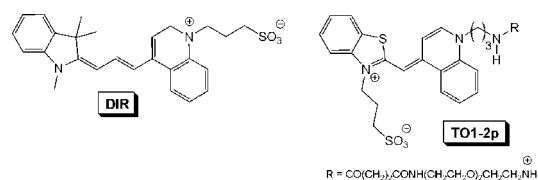


Figure 1. Fluorescence titration curve for binding of DIR to surface-displayed clone K7. Line represents fit to 1:1 binding model. Open circles and filled triangles: Titration of dye into cells not expressing the scFv and buffer, respectively. Inset shows calculated K_d values for five unique clones selected by flow cytometry.

Chart 1. Structures of Fluorogenic Cyanine Dyes



galactose in the media to induce the display of scFv's on the surface of the cells. These induction plates also contained 10 μ M DIR, and after several days of growth, inspection of these plates under excitation illumination allowed for the direct visual selection of clones that showed the greatest amount of DIR fluorescence. Five of these clones were chosen for further analysis.

Equilibrium binding constants for DIR with the five surface-displayed scFv's were determined by fluorescence titration of the dye into yeast cultures expressing an scFv. Expression of the scFv's required galactose, so a negative control experiment involving yeast that were not exposed to galactose was performed in each case. Figure 1 shows the results of the titration for the scFv named K7. DIR fluorescence is enhanced by more than 100-fold, and the titration data are well fit to a single site, 1:1 binding model that yields a $K_d = 13.9 \pm 3.2$ nM. Negligible fluorescence enhancement was observed when DIR was added to the control cells or to buffer. K_d values for the other four clones are also shown in the inset to Figure 1; even the weakest binding clone, A8, exhibits a respectable K_d (148 nM).

The high affinities and large fluorescence enhancements exhibited by these scFv's for DIR are well suited for fluorescence microscopy. Figure 2 shows an image obtained after mixing 100 nM DIR with

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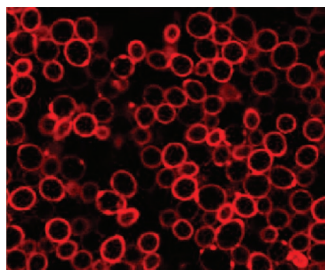


Figure 2. Scanning laser confocal fluorescence image of yeast expressing FAP K7 in the presence of 100 nM DIR.

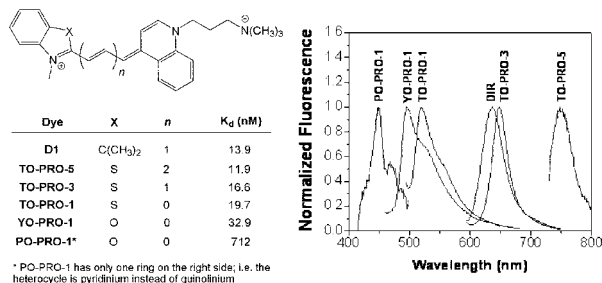


Figure 3. Dissociation constants and normalized fluorescence spectra for various unsymmetrical cyanines bound to scFv clone K7.

yeast expressing K7 on their surface. Note that because unbound dye has very low fluorescence, the cells need not be washed prior to imaging. Moreover, any dye that enters the yeast will be of similarly low fluorescence because of the weak affinity of DIR for cellular DNA and RNA. The fluorescence pattern in this confocal image indicates surface display of the DIR FAP K7 on virtually all of the cells.

To verify that the high affinity binding of DIR to K7 is not an artifact of the surface display of the protein, we next expressed a soluble version of K7 in *E. coli*. A fluorescence titration experiment with the soluble protein yielded $K_d = 10.3$ nM and confirmed the 1:1 stoichiometry of the complex (Figure S2).

The fluorescence quantum yield for DIR bound to the soluble K7 protein is 0.33, which is significantly higher than the quantum yield for the dye in a 90% glycerol solution ($\phi_f = 0.15$ at 24 °C) and in aqueous buffer ($\phi_f \approx 0.002$). Since the fluorescence enhancement of fluorogenic cyanines arises from conformational constraints, this result indicates a tight fit between the dye and its binding site on the protein and is consistent with the high affinity of the dye–protein complex. The quantum yield also compares favorably with that of the commonly used Cy5 ($\phi_f = 0.27$).

We next explored the cross-reactivity between DIR and the structurally related TO1-2p for binding to their respective scFv's. Binding of DIR to surface-displayed HL1-TO1, an scFv previously selected for binding to TO1-2p,⁷ was ca. 70-fold weaker than binding to K7 ($K_d = 442$ nM vs 13.9 nM). This is not surprising, given the larger size of the DIR chromophore relative to TO1-2p. Interestingly however, TO1-2p actually bound 3-fold stronger to K7 than to HL1-TO1, its cognate scFv: $K_d = 134$ nM (K7) vs 360 nM (HL1-TO1). The presumably larger binding site in K7 appears to readily accommodate the smaller TO dye.

The cross-reactivity of K7 for TO1-2p motivated us to investigate a number of other unsymmetrical cyanines as binding partners for this protein. As shown in Figure 3, K7 exhibits considerable promiscuity, binding with low nanomolar K_d 's to dyes having

methine bridge lengths ranging from 1 to 5 and with dimethylindole, benzothiazole, or benzoxazole heterocycles. (Note that D1 is the trimethylammonium analogue of DIR). Affinity only falls off when the quinoline ring is truncated to a pyridine (compare YO-PRO-1 to PO-PRO-1). Figure 3 also shows that the fluorescence spectra for these dye-scFv fluoromolecules cover most of the visible and near-IR region of the spectrum.

We next compared binding of DIR and TO1-2p to HL1.0.1-TO1, a TO1-2p-binding scFv that is a derivative of HL1-TO1 isolated for its improved binding of TO1-2p.⁷ As reported previously, the K_d of TO1-2p for the improved scFv is 3 nM compared to 360 nM for the original scFv.⁷ In contrast, the affinity of this improved clone for DIR remains relatively low ($K_d = 418$ nM). Thus, isolation of improved binding scFv-based FAPs may improve selectivity as well. This suggests such derivatives of a promiscuous DIR-binding scFv may have improved selectivity for a particular dye, possibly generating a family of scFv's that selectively bind different colored fluorogens; this will be the subject of future investigations.

In conclusion, the new fluoromolecules reported here add to the catalogue of fluorescence imaging tools, including inherently fluorescent proteins such as GFP¹¹ as well as proteins labeled by exogenous dyes, as in the biarsenical FIAsH/ReAsH technology² or the stilbene-binding antibodies,¹ but with added versatility. A single scFv protein “apomodule”, K7, provides access to emission wavelengths ranging from the blue (450 nm) to near-IR (750 nm) using dyes with submicromolar K_d values. Future work will focus on structural and biophysical characterization of these fluoromolecules.

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Supporting Information Available: Binding titration curves for yeast-displayed K7 with various cyanine dyes and for soluble K7 with DIR; sequence information for scFv's; experimental details for scFv selection, fluorescence microscopy, and binding titrations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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