

**Combining Biomolecules and Fluorogenic Cyanine Dyes into
Novel Fluorescent Labels**



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Abstract

Fluorescence technology is a pivotal element of modern science. Advances in a variety of fields from medical diagnostics to nanotechnology are highly interrelated to advances in new fluorescence detection systems, or fluorescent labels that will be employed. This dissertation describes our efforts to contribute to the progress in fluorescence technology by developing novel fluorescent labels. We developed two different types of fluorescent labels with different superiorities and thus different application areas. Despite being developed based on different rationales, the approach we took was common, that is the utilization of biomolecules and fluorogenic cyanine dyes and their unique binding interactions.

Chapter 2 describes the design, preparation and characterization of fluorescent DNA nanotags that are brighter than their competitors and have great potential for *in-vitro* labeling assays. DNA nanotags are intercalator dye arrays on a compact 3-dimensional DNA-Tetrahedron (TH) nanostructure. The template tolerates the structural distortions introduced by intercalation and allows concentration of multiple fluorophores within a small volume, resulting in brightly fluorescent nanotags with effective extinction coefficients in the order of $10^6 \text{ M}^{-1}\text{cm}^{-1}$. Förster resonance energy transfer from intercalated donor dyes to covalently attached acceptor dyes is efficient in the TH-nanotags leading to wavelength tunability. The compact nature of the TH also provides a protective medium for the fluorophores, leading to improved photostability and enhanced resistance to nuclease digestion, relative to one- or two-dimensional nanotags described previously.

Chapters 3 and 4 describe novel genetically encodable fluorescent labels that promise low signal-to-noise ratios, and spectral versatility for *in-vivo* applications.

Magnetic and fluorescence cell sorting was applied to isolate single chain variable fragment (scFv) proteins from a yeast-surface displayed library for their ability to activate the fluorescence of the fluorogenic dye Dimethyl Indole Red (DIR). Isolated scFvs exhibited strong binding affinities for DIR by enhancing its fluorescence more than 100-fold. One of the isolated scFvs (K7) demonstrated promiscuity, binding to several other fluorogenic cyanine dyes with strong affinity and high quantum yields. K7 was further characterized in terms of the origins for the promiscuity, employing fluorescence melting and intrinsic fluorescence studies. In an attempt to demonstrate the potential of these fluoromodules for being engineered according to various different needs, we employed directed molecular evolution on one of the DIR activating scFvs. We used error-prone PCR mutagenesis and fluorescence activated cell sorting to manipulate the affinity and ligand specificity of the parent scFv. The fluorescence spectroscopic analysis of selected scFvs revealed several fold increase in DIR affinity. Furthermore, the affinity of the scFvs for structurally similar fluorogenic cyanine dyes was suppressed which improved the DIR specificity of matured scFvs.

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Chapter 1: Fluorescent Labeling of Biomolecules

1.1 Labeling Biomolecules

The scientific desire in the post-genomic era is to understand the function of every gene product and how this function can be exploited for either fundamental knowledge or for therapeutic purposes. The ability to label and track those gene products opens up endless ways to fulfill this desire. Labeling biomolecules in combination with the right detection method can reveal important information about structural and chemical modes of action. Labeled biomolecules can be monitored in terms of how they are involved in certain biological events and how they interact with other biomolecules leading to invaluable information about dynamics, localization, and interactions both at nucleic acid and protein levels. Furthermore labeling is the key to all biosensors in the field of molecular diagnostics.

While labeling of biomolecules is crucial for fundamental knowledge about bioprocesses it is also an emerging tool in modern materials science at the nano- and microscale. Researchers often employ nucleic acids and proteins as materials of construction due to their unique and predictable recognition capabilities^{1, 2}. Several novel technologies including DNA microarrays, molecular lithography, nanobiosensors, and electronic nanodevices rely extensively on labeling methodologies.

Affinity labeling³, radioactive labeling, heavy metal labeling and chemical ligation are some of the methods being used to tag proteins and nucleic acids for various applications. Fluorescence labeling, on the other hand, is by far the most widely used technology and it will be the basis of this chapter.

1.2. Fluorescence technology

Fluorescence technology is very well suited for fast, sensitive, and reliable detection of biomolecules owing to its unique characteristics. Fluorescence refers to the emission of light from a fluorophore. The Jablonski diagram⁴ in Figure 1 depicts the process of fluorescence emission. An unstable excited electronic state is created when the fluorophore absorbs a photon of energy (1). The fluorophore loses some vibrational energy through internal conversion and relaxes to the lowest level vibrational energy (2). When the fluorophore rearranges from the semi-stable excited state back to the ground state, the excess energy is released and emitted as light (3).

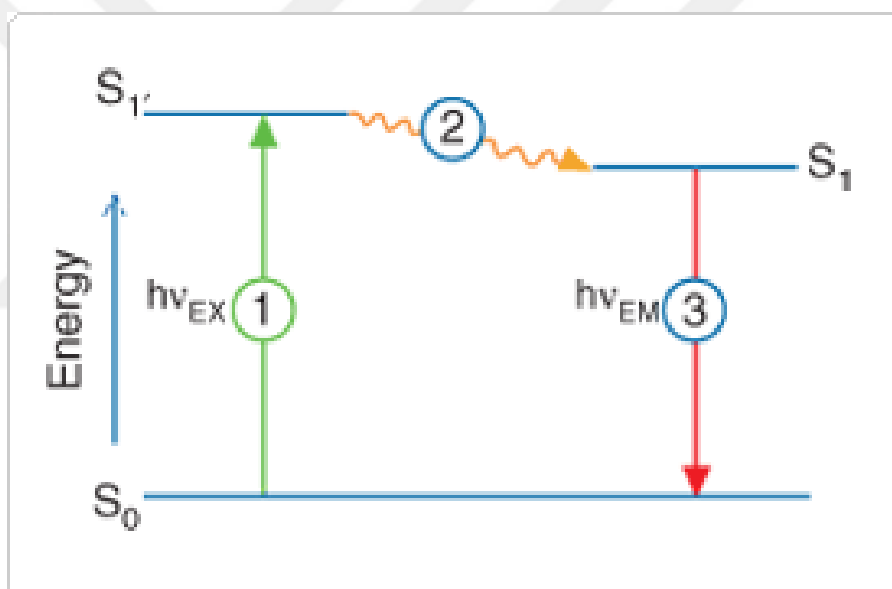


Figure 1 Jablonski Diagram depicting fluorescence process

The emitted light is of lower energy, and thus longer wavelength, than the absorbed light. The difference between the absorption and emission maxima refers to the Stokes Shift of a fluorophore which is mostly used as a measure of sensitivity of the fluorophore. Larger stokes shifts allow blocking of the excitation light from reaching the detector and enable low signal-to-noise ratios. The decay of the excited state can

also occur in a nonradiative fashion such as bond rotation or vibration, molecular collision, and photoinduced electron transfer (PET) leading to quenching of the fluorophore. Another important pathway for the decay of the singlet excited state involves Förster Resonance Energy transfer (FRET). A donor fluorophore in its electronic excited state may transfer energy to an acceptor fluorophore that is in proximity through nonradiative dipole–dipole coupling. It is a distance-dependent process and can be used as a “spectroscopic ruler” to measure the proximity of labeled entities⁴.

Fluorescent labeling has been more versatile than other labeling systems due to fast signal acquisition, suitability for parallel detection of multiple targets, high sensitivity, signal localization, and availability of labels that do not perturb the behavior of the labeled molecule⁵. Fluorescence based systems offer nano-scale resolution and sensitivity down to the single molecule level⁶.

Traditionally fluorescently labeled molecules are being detected by fluorescence spectrometers, and fluorescence microscopes. Also, several modern technologies have emerged that exploit the inherent strengths of the fluorescence process.

Fluorescence Activated Cell Sorting (FACS) facilitates sorting of labeled cells or particles in a heterogeneous mixture based on their fluorescence intensity and/or color and thus has been the basis of biomolecular detection and many molecular evolution methods.⁷

Fluorescence correlation spectroscopy (FCS) is a powerful single-molecule detection technique that measures and correlates fluorescence intensity fluctuations within a detection volume on the order of femtoliters. It is a widely used method for characterizing reaction dynamics, photophysical kinetics, molecular aggregation, conformational fluctuations, and translational motion in many chemical systems⁸.

Time Correlated Single Photon Counting (TCSPC) analyzes the relaxation of molecules from an excited state to a lower energy state and examines the energy states and dynamics of molecules⁸.

Fluorescence Recovery After Photobleaching (FRAP) exploits an inherent weakness of fluorophores i.e. their tendency to be photobleached. FRAP measures the rate of fluorescence recovery due to diffusion of fluorescently labeled molecules into an area containing the same molecule that has been quenched via an intense photobleaching pulse of laser light. This method has proven useful in biological studies of membrane diffusion and protein binding.⁹

Progress in new fluorescence technologies and how they are applied to new fields of science is strictly dependent on the availability of suitable fluorescent labels of different types. With brilliant discoveries based on fluorescence technologies so far and ongoing research and development of improved fluorescent labels, applications of fluorescence should continue to grow bigger.

1.2.1 Properties of fluorescent labels

The choice of suitable fluorescent labels is essential for fluorescence detection and imaging. Important characteristics to consider include chemical properties such as water solubility, aggregation behavior and suitability for chemical conjugation. The size of the label can limit utility as labels should have the appropriate size so that they do not interfere with the function of the target biomolecule. Biocompatibility is another requirement for the practical applicability of fluorescent labels to prevent any toxicity to the environments in which target biomolecules will be studied.

Brightness of a fluorophore refers to the intensity of the fluorescence signal and is defined as the product of the quantum yield and the extinction coefficient of the fluorophore in a certain environment. The fluorescence quantum yield (ϕ_f) is defined

as the ratio of the number of fluorescence photons emitted to the number of photons absorbed, whereas the extinction coefficient (ϵ) refers to the efficiency of a molecule to absorb light at a certain wavelength. Fluorescein, which is one of the brightest fluorophores has an extinction coefficient of $92,300 \text{ M}^{-1}\text{cm}^{-1}$ at 500.25 nm, and its quantum yield is 0.97¹⁰.

Among other photophysical characteristics brightness is the most important one since it determines the sensitivity of the fluorescence probe. The sensitivity of the detection can be compromised by background signals that are caused either endogenously by the sample as autofluorescence or by nonspecific binding of probes. While autofluorescence can be reduced by using labels that can be excited at longer wavelengths, prevention of nonspecific binding requires specific design criteria depending on the nature of labeling experiments.

Photostability refers to the resistance of fluorescent labels to photochemical destruction. Especially in fluorescence microscopy, the photostability is a crucial feature that determines the limits of sensitivity. Under high-intensity illumination conditions, photochemical destruction (i.e. photobleaching) becomes the primary factor determining detection limits. Photobleaching most often originates from the triplet excited state that is caused by the singlet state via intersystem crossing⁴.

While photostability of fluorescent labels can be manipulated by instrumental modifications such as low-light detection devices and wide bandpass emission filters, intrinsic photostabilities of fluorophores should be improved for high sensitivity experiments.

1.2.2 Environmentally Sensitive Fluorophores

While stability of photophysical properties within varying environments is desirable for many applications to obtain accurate data, there are cases where

environmentally sensitive probes are needed such as studying protein-protein interactions or protein conformational changes^{11, 12}.

In some cases environmentally sensitive fluorophores change their properties such as quantum yield, half-life, excitation or emission spectra, when the polarity of the environment changes. This behavior is mostly caused by a large change in the dipole moment within molecules due to electron donating and electron withdrawing groups at opposite ends. Besides intrinsically environmentally sensitive fluorophores such as coumarins, indole, and dansyl, there are fluorophores specifically modified to gain this property^{12, 13}.

Environmentally sensitive fluorophores for which quantum yields change depending on the viscosity of environment have attracted much attention as they can be used as probes for binding to biomolecules. In most cases these fluorogenic dyes exhibit quantum yield numbers less than 0.001 in fluid solutions. On the other hand, when they are in a viscous environment such as glycerol, their quantum yields increase several fold yielding bright sensors. Fluorophores with fluorogenic characteristics such as thiazole orange (TO) have been widely used for the detection of DNA as they can report DNA intercalation by a significant enhancement of the fluorescence intensity¹⁴.

The concept of fluorogenicity has been shown to originate from the restriction of rotation around the methine bridges connecting heterocycles on fluorophores¹⁵. Calculations indicated that there is a range of conformations over which these dyes will remain fluorescent, provided the molecule is rigidified below a critical angle through either solvent viscosity or a constraining binding pocket.

1.3 Conventional Fluorescence Labeling

Conventional fluorescent labels include those labels that are attached either covalently or non-covalently to biomolecules of interest *in vitro* or *ex vivo*.

1.3.1 Direct Fluorescence Labeling

1.3.1.1 Fluorescence Labeling with Organic Small Molecules

Organic small molecule dyes have been historically first examples of fluorescent labels. Their small sizes usually do not interfere with the biomolecule's function; their suitability for covalent attachment and variable spectral properties made them the focus of attention in the fluorescence science. Drawbacks such as short Stokes shifts, poor photochemical stability, susceptibility to photobleaching and decomposition are usually compensated by advantages of these dyes and can be fine tuned with synthetic efforts. Synthetic fluorophores with various core structures are commercially available and span the emission spectrum from blue to near-IR. Figure 2 illustrates most commonly used synthetic fluorophore core structures. Fluoresceins, rhodamines, BODIPY's, squaraines, cyanines, and Alexa dyes have been major dyes used in detection and visualization of biomolecules although more small molecules are available¹⁶.

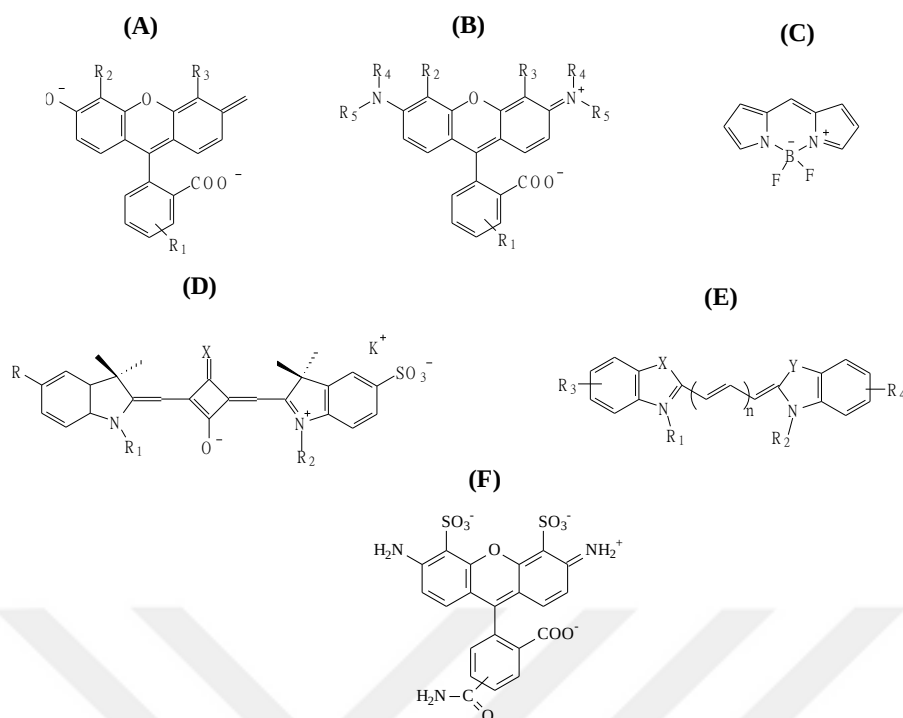


Figure 2 Generic structures of most commonly used organic fluorophores. (A) Fluorescein, (B) Rhodamine, (C) BODIPY, (D) Squaraine, (E) Cyanine (F) Alexa Fluor 488. R groups represent synthetic groups introduced to tune photophysical or chemical characteristics of fluorophores.

Fluoresceins and Rhodamines:

Fluoresceins and rhodamines are members of the xanthene family of dyes. They are widely used in sensing applications owing to their brightness, high quantum yields, low-energy excitation and emission wavelengths, and biocompatibility.

The amine-reactive fluorescein, Fluorescein-5-isothiocyanate, FITC, is among the most popular fluorescein dyes due to its high extinction coefficient, high fluorescence quantum yield and aqueous solubility. Its excitation maximum at 494 nm that matches well with the 488 nm spectral line of the argon-ion laser, makes this dye often the choice of label for confocal microscopy and flow cytometry. However fluoresceins are highly pH sensitive⁴ which is usually a drawback for the labeling experiment. Fluoresceins are also highly susceptible to photobleaching which limits

the use of these labels to short time illuminations, thus affecting the sensitivity¹⁷. There have been synthetic efforts to modify fluorescein dyes so that they have new protein conjugation characteristics^{18, 19}, improved photostability and pH-independent quantum yields²⁰. They also were incorporated into fluorescent amino acids for protein labeling²¹.

Rhodamine dyes, historically used for dyeing fabrics, have also been a popular choice for fluorescent labeling of biomolecules. With the excitation max. at 520 nm they can be excited with a 514 nm argon-ion laser. Red-fluorescing rhodamine dyes, such as the succinimidyl ester of 5-carboxyrhodamine 6G have better photostability relative to fluoresceins. Rhodamine 800²², Texas Red²³, Rhodamine 123²⁴ are some examples of rhodamine dyes that have found wide applications in bioanalysis.

BODIPY fluorophores:

BODIPY dyes are based on the 4,4-difluoro-4-bora-3a,4a-diazas-indacene molecule and its derivatives. BODIPY is a registered trademark of Molecular Probes, Inc. They exhibit optical properties that are superior to fluoresceins, and rhodamines including smaller Stokes shifts, higher quantum yields approaching 1, and sharp excitation and emission spectra contributing to higher sensitivity¹⁷. BODIPY dyes were shown to be versatile labels for various biological applications. There are many examples of studies where these dyes were linked to peptides, proteins, oligonucleotides, dextran, lipids, and polystyrene microspheres²⁵.

Squaraines:

Squaraines are a class of organic fluorophores with very intense fluorescence spectra in the near IR region. The advantages of fluorescence labeling at long-wavelength excitation are the decreased autofluorescence from cells and tissues and the ability to use inexpensive laser light sources such as diode lasers operating at 635,

645, and 650 nm. Squaraine dyes are one of the few dye families that can function in the near IR region. With their zwitterionic structure they are one of the most interesting classes of dyes. Owing to their high extinction coefficients (around $200,000 \text{ M}^{-1} \text{ cm}^{-1}$) and high photostabilities, which are due to the strong electron-accepting ability of squaric acid and their structural rigidity²⁶; they have been applied in many fields. They allow the introduction of reactive functional groups such as NHS ester which makes the covalent attachment to biomolecules possible. These dyes exhibit good quantum yields upon binding to proteins but they have inherently insufficient water solubility. Synthetic efforts resulted in squaraine dyes that have improved spectral and chemical characteristics²⁷.

Cyanines:

Since their discovery more than 150 years ago, cyanine dyes have been utilized as photographic sensitizers, nonlinear optical materials, and more recently as fluorescent probes for biomolecular labeling. Their straightforward syntheses, broad wavelength tunability, large molar extinction coefficients, and moderate-to-high fluorescence quantum yields make cyanine dyes very popular for labeling biomolecules. The literature in fluorescence science is full of examples of applications of cyanine dyes in genetic analysis, DNA sequencing, in vivo imaging, and proteomics²⁸. Cyanine dyes are generally classified into two subclasses: monomethine cyanines, which include the asymmetric cyanines thiazole orange (TO) and oxazole yellow (YO), and polymethine cyanines, which include commonly used dyes such as Cy3 and Cy5. TO and YO have been popular noncovalent nucleic acid labeling agents as they can intercalate into DNA and exhibit large fluorescence enhancements²⁹. Cy3 and Cy5 have also been routinely used in labeling of biological compounds such as antibodies, nucleic acids, lipids and other amino-group containing specimens^{30, 31} and

also as probes in microarray technology³². Drawbacks of cyanine dyes such as self-aggregation, and weak photostabilities were improved by synthetic efforts resulting in cyanine dyes with improvements in photophysical behavior^{33, 34}.

Alexa Fluorophores:

Alexa fluorophores are a series of fluorescent small molecules developed by Molecular Probes Inc. (Invitrogen) through sulfonating aminocoumarin, rhodamine or carbocyanine dyes. Due to the sulfonation, molecules become more hydrophilic and less prone to aggregation. Alexa fluorophores span the visible spectrum with derivatives emitting at different wavelengths, and demonstrate high photostability, pH insensitivity, and high extinction coefficients³⁵. They were shown to be superior to commonly used fluorophores such as fluorescein, Cy3 and Cy5 in bioapplications³⁶ and considered as competitive alternatives.

1.3.1.2 Fluorescence Labeling with Inorganic Molecules

1.3.1.2.1 Semiconductor Quantum Dots

Since their introduction into fluorescence labeling science a decade ago³⁷, semiconductor quantum dots such as CdSe, or CdTe became a useful but also controversial member of the fluorescence labels family. Their small sizes of only a few nanometers exert a quantum confinement effect leading to unique optical and electronic effects. They are advantageous over most organic fluorophores due to their broad absorption spectra at shorter wavelengths and narrow emission spectra that are easily tunable by the size of particles. The fact that quantum dots can be excited at any wavelength below their emission bands due to broad absorption spectra makes them very powerful probes for multiplexed detection³⁸. Quantum dots have been reported to be 20 times brighter, 100 times as stable against photobleaching, and one

third as wide in spectral line width compared to organic fluorophores such as rhodamine³⁹.

Due to their appealing properties quantum dots are being utilized for labeling biomolecules in various applications ranging from *in vitro* detection assays^{40, 41} to live-cell^{42, 43} and deep tissue imaging⁴⁴.

The controversy about quantum dots arises from the fact that they are poorly biocompatible without any surface modification^{45, 46}, and they can be toxic to live cells being detected or imaged. Furthermore the inherent optical blinking of quantum dots limits their use in quantitative assays⁴⁷. Efforts to eliminate the possibility of leaking of cytotoxic material and increase hydrophilicity focused on preparation of protective inorganic surface layers such as ZnS or ZnS/Si₂O shells modified with mercaptohydrocarbonic acids⁴⁸. Alternative approaches were also demonstrated such as employing polyethyleneimine or polyelectrolytes⁴⁹, or an additional amphiphilic inorganic shell like silica⁵⁰.

Quantum dots are especially attractive for biological applications that require multiplexing ability or near IR optical properties, i.e. high quantum yields in the 650-900 nm window with high stability, water solubility and low toxicity. Quantum dots have growing potential to fulfill these requirements whereas organic fluorophores lay behind.

1.3.1.2.2 Lanthanide Compounds

Lanthanide Chelates:

Lanthanide chelates that are being used as fluorescent labels are composed of an organic chromophore serving as an antenna for energy transfer and chelated lanthanide cations (Figure 3) that lead to a narrow emission spectrum. Different emission wavelengths are achieved by varying the lanthanide ion. Lanthanide chelates

have long fluorescence lifetimes if quenching by water and dissolved oxygen can be avoided, narrow emission spectra, large Stokes shifts (>150 nm), and high quantum yields⁵¹. Due to these properties they are being used in fluorescent labeling of biomolecules⁵².

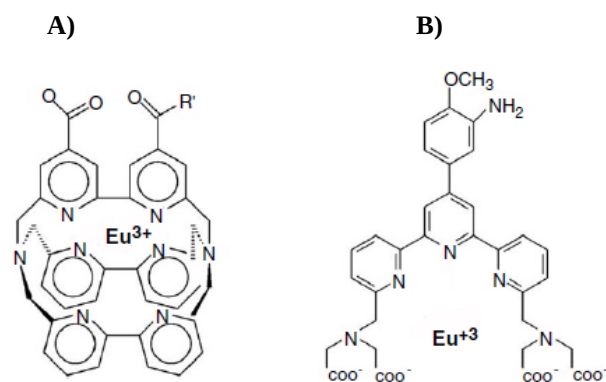


Figure 3 Representative structures of lanthanide chelates. A) cryptates, B) terpyridine. Structures were adapted from reference ⁵²

Lanthanides are also being used in the form of lanthanide doped inorganic nanoparticles as fluorescence labels. Lanthanide-doped nanoparticles offer advantages over lanthanide chelates such as better photostability and longer fluorescence lifetimes^{53, 54}.

Lanthanide-doped nanoparticles are mostly utilized due to their unique up-conversion properties, which is their ability to emit visible light by being excited with infrared light⁵⁵. While the study of up-converting materials is promising for the development of novel solid-state lasers, it also has proven useful for biolabeling. They have been applied as reporters of cell and tissue surface antigens⁵⁶, as probes in immunoassays⁵⁷, and labeling agents in DNA microarrays⁵⁸. Despite advantages of lanthanide doped nanoparticles, the surface modification and their conjugation to biomolecules is still a field that needs to be investigated for improvements.

1.3.2. Indirect Fluorescence Labeling

1.3.2.1. Immunofluorescence

Proteins in cells of tissues can be also localized by indirect labeling of these targets by immunohistochemistry. Immunohistochemical staining is often used in molecular diagnostics to localize biomarkers found in abnormal cells such as cancerous cells. The detection is accomplished by targeting the protein through an antibody which will be targeted by a secondary antibody that is tagged with a fluorophore. While antibodies were traditionally tagged with small organic fluorophores like fluorescein and rhodamine, the susceptibility of these dyes to photobleaching exerts a limit on applications of fluorophore tagged antibodies in immunofluorescence. Thus there is significant amount of research efforts to develop new fluorescent antibodies that are bright and photostable. As an alternative, antibodies are being tagged with specifically designed photostable fluorophores such as AlexaFluor (Invitrogen) and DyLight (Thermo Fisher Scientific) series of dyes. There are also efforts to develop antibody-quantum dot conjugates⁵⁹.

Another bright label for immunolabeling is phycoerythrin. Phycoerythrin is a member of phycobiliproteins that are found in photosynthetic cyanobacteria. Multiple bilin chromophores are encapsulated within a protein matrix that allows the right positioning for efficient light harvesting resulting in bright entities⁶⁰. Phycoerythrin exhibits brightness values that are 10-100-fold greater than other fluorescent proteins (e.g. GFP) and commonly used fluorescent dyes such as fluorescein or Cy3. Phycoerythrin conjugated antibodies are being widely used for immunolabeling.

1.3.2.2 Fluorescence in-situ hybridization

Fluorescence *in situ* hybridization (FISH) is a medical diagnostics tool to map and visualize the genetic material in patient cells. It allows the study of a variety of chromosomal abnormalities and genetic mutations leading to genetic diseases⁶¹. Fluorophore tagged single stranded short nucleic acid sequences are being targeted to genes of interest and the fluorescence is detected either by fluorescence microscopy or spectroscopy.

The power of *in situ* hybridization is extended by the simultaneous use of multiple fluorophores for multiplexed detection^{62, 63}. Multicolor FISH can be used to identify as many labeled features as there are different fluorophores used in the hybridization.

1.4 Genetically Encodable Fluorescent Labels

Conventional fluorescence labels discussed above are only applicable to either in vitro labeling of biomolecules or ex vivo labeling of biomolecules in fixed cells or tissues. While these labels are all indispensable for many bioanalytical detection applications they are limited when target biomolecules are needed to be studied in their local environments. In order to study biomolecules in vivo, researchers need fluorescent labels that can be genetically fused to the target molecule and coexpressed without affecting the function of the target. Genetically encodable fluorescent labels allow visualization and quantification of biomolecules with high spatial and temporal resolution in cellular environments.

1.4.1 Fluorescent Proteins

The discovery of Green Fluorescent Protein (GFP), originally isolated from the jellyfish *aequorea victoria*⁶⁴ along with cloning of the complementary DNA

sequence^{65, 66} has opened a new era in fluorescence labeling. GFP can emit light without any exogenously added proteins or co-factors, making it unique among other fluorescent systems of biological origin. The absorbance and fluorescence of GFP originate from a *p*-hydroxybenzylidene-imidazolidinone chromophore, which is generated by the cyclization and oxidation of the Ser–Tyr–Gly sequence at positions 65–67⁶⁵. GFP based probes can respond to a variety of signaling events in cellular environments, be targeted to subcellular compartments, and introduced into tissues and intact organisms without causing toxicity. The promising power of GFP led to the discovery of mutated analogs and analogous proteins from different marine species that have different spectral characteristics. Today the ‘GFP family’ of proteins covers at least 27 analogs that either emit light of different colors, or have improved brightness or photostabilities^{67, 68}. Among other GFP analogs, red fluorescent proteins⁶⁹ attracted the most attention due to their emission spectra extending to the near IR region of the spectrum and suitability of proteins’ spectral characteristics for FRET.

GFP and its analogs have been utilized in various applications including as biological labels to track and quantify multiple proteins, as probes to monitor protein-protein interactions, and as biosensors⁷⁰⁻⁷³. Major drawbacks to fluorescent proteins that limit certain applications are their relatively larger molecular mass (26 kDa) that might interfere with expression and folding of the target protein, their potential aggregation potentially leading to toxicity to cells, and short time blinking that exerts a challenge for single-molecule detection⁷⁴.

1.4.2 Tetracysteine Peptides

Tsien and colleagues were the first to design a system that uses advantageous small molecule dyes in a genetically encodable fluorescent label format. They utilized

the known affinity of arsenoxides for cysteine groups to develop a new labeling methodology⁷⁵. Fluorescein dye molecules were modified with arsenoxide groups (FlAsH) (Figure 4) to give non fluorescent species that would light up when dyes bind to tetracysteine peptide motifs containing the rare CCXXCC sequence.

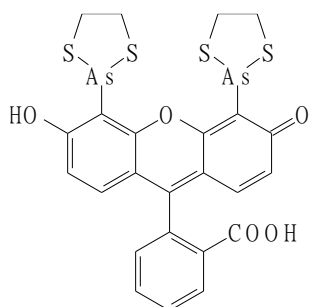


Figure 4 Structure of FlAsH

A host protein then can be genetically modified to coexpress a 6-20 amino acids peptide tag with the CCXXCC motif and the membrane-permeable FlAsH can be delivered externally to cells to bind to the target protein. Ethanedithiol is used as an antidote to the arsenic reducing the toxicity of the unbound dye to cells. The system provides almost no background fluorescence in the absence of the target protein leading to high sensitivities of detection. In order to expand the potential of this system to a wider range of applications various FlAsH analogs have been synthesized that span the visible spectrum, along with modifications on peptide sequences⁷⁶. Tetracysteine peptide labels have proven to be useful in applications such as the study of protein trafficking⁷⁷, or fluorophore-assisted light inactivation of recombinant proteins⁷⁸.

1.4.3 Fluoromodules

Fluoromodules are fluorescent entities composed of fluorogenic dyes, and biomolecules that can specifically activate the fluorescence of these dyes. Both

species are non fluorescent when they are separate, but when they bind each other, the fluorogenic dye lights up. The fluorescence enhancement is caused by the increase in the viscosity of the fluorogenic dye environment due to the binding of the dye to the biomolecule.

The biomolecule component that activates the fluorescence in fluoromodules can be either a nucleic acid or a protein. Nucleic acid fluoromodules are based on aptamers, and fluorogenic dyes that are being activated by these aptamers. Aptamers are short DNA or RNA sequences with a specific secondary structure that are engineered through repeated rounds of in vitro selections to gain a certain function. Aptamers also exist naturally in riboswitches. The RNA aptamer selected for its affinity for the dye Malachite Green (MG) constitutes a useful example of a fluoromodules that found various applications in fluorescent labeling⁷⁹. Binding of the aptamer to the normally non-fluorescent MG or its derivative enhances the fluorescence by >200-fold. MG aptamer fluoromodules along with other recently developed DNA or RNA aptamers⁸⁰⁻⁸³ have great potential as fluorescent labels for RNA labeling experiments.

Earlier examples of fluoromodules based on proteins include those composed of an antibody and stilbene molecule⁸⁴, and those composed of a Tetracysteine peptide and biarsenical dyes discussed in the previous section. More recently protein fluoromodules were developed that are composed of single chain variable fragment (scFv) antibodies and fluorogenic dyes including thiazole orange (TO) and MG⁸⁵. ScFvs that can activate fluorogenic dyes were isolated from a human naïve scFv library that was displayed on the surface of yeast cells, by Fluorescence Activated Cell Sorting. Biomolecules tagged with these fluoromodules can be visualized on the cell surface or within the secretory pathways of mammalian cells by membrane

permeant fluorogens. Fluoromodules developed by this approach can potentially be alternatives to other genetically encodable fluorescent labels such as tetracysteine peptide or GFP based labels.

1.5 Thesis Overview

The plethora of available fluorescent labels provides scientists with various options when designing experiments requiring detection and imaging of biomolecules. On the other hand, there is still a need for new labels that will overcome the deficiencies of existing ones, and be compatible with advances in fluorescence detection systems.

This dissertation covers our efforts to develop unique fluorescent labels by utilizing biomolecules and their inherent physicochemical characteristics. Unique design strategies that combine special small molecules with these biomolecules resulted in novel fluorescent labels that are in many ways superior to existing ones.

Chapter 2 focuses on the design, preparation and characterization of novel fluorescent labels that are based on a 3-dimensional DNA nanostructure and fluorogenic cyanine dyes that self assemble on the DNA template. This work is an example of utilizing DNA as a versatile construction material rather than a genetic entity. How the DNA template allows the self assembly of fluorophores and the impact of the interaction between DNA and fluorophores on the photophysical characteristics of resulting novel labels will be discussed.

Chapter 3 introduces our efforts to develop new fluoromodules that are based on an scFv protein and a specifically designed fluorogenic cyanine dye. Photophysical and thermodynamic characterization of these fluoromodules will be followed by a discussion of the promiscuity of the protein for fluorogenic dyes of various spectral properties both from a fundamental research and technological point of view.

Chapter 4 covers our efforts to engineer fluoromodules that we have developed to improve their brightness and specificity using directed evolution methodologies. Effects of directed evolution methods on photophysical characteristics of improved fluoromodules will be discussed along with the sequence-function relationship in scFv fluoromodules.



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Chapter 2: Fluorescent DNA Nanotags based on a Self-Assembled DNA Tetrahedron

2.1 Introduction

Deciphering complex biological events at the molecular level requires adequate tools to visualize and track interacting biomolecules. Fluorescent labeling of biomolecules is desirable due to the potential for fast signal acquisition, high sensitivity and suitability for multiplex assaying by using fluorophores that emit at different wavelengths⁸⁶. While commonly used small dye molecules offer tunability in spectral and physical characteristics, their relatively low brightness, defined as the product of the molar extinction coefficient (ϵ) and the fluorescence quantum yield (ϕ_f), complicates the use of such dyes in demanding applications such as single molecule detection.

Arrangement of multiple dye molecules into arrays, on the other hand, can dramatically improve the efficiency of light absorption and also provide efficient energy transfer pathways, allowing fine-tuning of the emission wavelength. The challenge in developing such multichromophore arrays is positioning the fluorophores at predefined spatial positions that prevent self-quenching but promote energy transfer. Nature has already solved this problem in the form of the phycobiliproteins found in photosynthetic cyanobacteria. The protein matrix of these structures encapsulates multiple bilin chromophores with sufficient spatial separation to prevent self-quenching, allowing efficient light harvesting and energy transfer to the reaction center protein⁸⁷. However, once decoupled from the reaction center, phycobiliproteins become intensely fluorescent with brightness values that are 10-100-fold greater than other fluorescent proteins (e.g. GFP) and commonly used fluorescent dyes such as fluorescein or Cy3.

Synthetic mimics of the phycobiliproteins have focused on dendrimers⁸⁸ or rigid π -conjugated spacers^{89, 90} as templates for covalent attachment of fluorophores at defined distances. While these systems have proven to be suitable for efficient energy transfer to create molecular wires, they have not found applications as fluorescent labels, possibly due to their large molecular weights and complex syntheses. Oligonucleotides have also been used as scaffolds for multiple covalent attachment of fluorescent dyes, where the phosphate backbone acts as a tunable spacer for the fluorophores, leading to energy transfer tags with multicolor labeling potential⁹¹⁻⁹³. As in the nonbiological scaffolds, considerable synthetic expertise is required for assembly of these covalent DNA-based assemblies.

Recently, we reported a novel noncovalent approach to create DNA-templated multichromophore arrays with much higher extinction coefficients and efficient energy transfer capabilities relative to systems involving covalently attached dyes.⁹⁴ The DNA template consisted of a branched 2-dimensional nanostructure assembled by hybridizing three or more partially complementary strands via Watson-Crick base pairing⁹⁵. These DNA nanostructures served as templates for the self-assembly of multiple intercalating dyes in a way which concentrated the fluorophores in a small, well-defined region. The inherent steric constraint on the DNA duplex kept the intercalating dyes at a unique distance far enough to prevent self quenching but close enough for efficient energy transfer, resulting in bright multichromophore assemblies we termed DNA nanotags. In the current study we exploited this approach further and explored a more compact 3-dimensional assembly based on a tetrahedron (TH) nanostructure^{96, 97}. The TH nanotag resulting from loading with fluorescent intercalating dyes exhibited several improvements over lower-dimensional analogues, including higher density of fluorophores without significantly compromising

brightness, improved photostability and good resistance to degradation by nuclease enzymes.

2.2 Experimental

2.2.1 Materials

DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (www.idtdna.com) as lyophilized powders. Sequences of the DNA oligonucleotides used to generate tetrahedron, 3WJ and linear nanostructures are provided in the supporting information. The DNA-intercalating dye YOYO-1 was purchased from Molecular Probes (now Invitrogen). Streptavidin-coated polystyrene beads (6.9 μm) were purchased from Spherotech, Inc. DNase I and Exo III were purchased from New England Biolabs. Calf thymus DNA was purchased from Sigma-Aldrich.

2.2.2 Assembly of Fluorescent DNA Tetrahedron (TH) Nanostructures.

Solutions of oligonucleotide strands were prepared in 10 mM aqueous sodium phosphate buffer (pH = 7.0) and stored at -4°C . Varian Cary 3 Bio UV-vis spectrophotometer was used to determine concentrations. A DNA-solution containing 1.0 μM of each strand in TM buffer (10 mM Tris, 5 mM MgCl_2) was prepared, heated to 95°C for 2 min, and cooled in ice bath. YOYO-1 concentrations were determined in DNA using a spectrophotometer with $\epsilon = 98,900 \text{ M}^{-1}\text{cm}^{-1}$. Assembled DNA TH nanostructures were incubated with saturating amounts of YOYO-1 (1 dye: 4 base pairs) for 30 min at RT. Fluorescent DNA-nanostructures were visualized on 1% agarose gel. Melting temperatures were determined by monitoring the absorbance of 50 nM DNA with and without 1.28 μM YOYO-1 at 260 nm while increasing

temperature at a rate of 1 °C/min and then calculating the first derivative of the resulting melting curve.

2.2.3 Fluorescence Measurements

All fluorescence studies were conducted on a Photon Technologies International fluorimeter. Titration experiments: 50 nM solutions of DNA TH nanostructures were titrated with 0.24 µM aliquots of YOYO-1. The spectral bandwidth for both excitation and emission monochromators were chosen to be 5 nm. *FRET experiments:* 50 nM solutions of YOYO-1 saturated, Cy3-labeled DNA TH nanostructures were prepared, where one or more strands had a 5'-conjugated Cy3. Samples were excited at 430 nm, and fluorescence was measured from 450-650 nm. FRET efficiency (E) was calculated according to the formula: $E = (1 - F_{DA}) / F_D$ where F_{DA} and F_D are the intensities of the donor (YOYO-1) at 509 nm in the presence and absence of acceptor (Cy3), respectively.

2.2.4 Photostability Experiments

Solutions of 50 nM DNA-TH and calf thymus DNA having the same base pair concentration (5.1 µM) saturated with YOYO-1 (4 base pairs per dye) were prepared. Samples in fluorescence cells were irradiated by a 150 watt Hg (Xe) lamp. A bandpass filter (Oriel, filter # 59830, $325 \text{ nm} < \lambda < 525 \text{ nm}$) and a neutral density filter that blocked 90% of the incident light were placed between the lamp and the sample for selective irradiation. Both absorbance and fluorescence spectra (excitation at 440 nm, emission at 509 nm) were acquired before and after every 10 min irradiation and cells were wrapped in aluminum foil between scans and irradiation to prevent unintentional photobleaching of the dyes.

2.2.5 Nuclease Digestion Experiments

50 nM DNA (linear, 3WJ or TH) solutions saturated with YOYO-1 were incubated with 6 μ L of the nuclease in 150 μ L final volume of the buffer supplied by the vendor for 1 hr at 37 °C. To determine the effect of the intercalator dye on the nuclease stability, nanostructures were also treated in the absence of YOYO-1. The digested DNA samples were then post-stained with the same concentration of YOYO-1. The nuclease activities were calculated as the percent decrease of the fluorescence intensity at 509 nm after being treated with the nuclease.

2.2.6 Microbead Labeling with Fluorescent DNA Nanostructures

DNA nanostructures prepared from biotin containing strands were saturated with YOYO-1. Streptavidin coated polystyrene beads were suspended in 50 μ L of calcium- and magnesium-free PBS so that there were 10^6 beads in the solution. The beads were then incubated with solutions containing 9.3 pmoles of DNA-TH (saturated with YOYO-1) for 30 min and washed 2 times with 150 μ L of PBS. Samples were resuspended in 0.6 mL PBS. Labeled beads were analyzed by fluorescence activated cell sorting (FACS) using a Coulter Epix Elite flow cytometer (Beckman-Coulter, Fullerton, CA) equipped with a 488 nm argon-ion laser. The following dichroic lenses (DL)/band pass (BP) filters were used: 550DL/525BP (PMT2), and 600DL/575 BP (PMT3). YOYO-1 was detected in PMT2 channel, and Cy3 was detected in PMT3 channel.

2.3 Results

2.3.1 Nanotag Design

TH nanotags were assembled by the incubation of intercalating dyes with tetrahedron nanostructures, which were prepared via a high-yield, single-step

synthesis originally reported by Turberfield and coworkers^{96, 97}. As shown in Figure 5, four oligonucleotide strands with partially complementary sequences can self-assemble into a tetrahedron consisting of 102 Watson-Crick base pairs. The six edges are 17 base pairs in length and are connected by two-nucleotide hinges which remain unhybridized and ensure sufficient flexibility to form the tetrahedron. The double-stranded edges of the tetrahedron provide the binding sites for the light-harvesting intercalating dyes. YOYO-1, the bisintercalating dimer of oxazole yellow,^{98, 99} was chosen as the intercalator dye because of its high affinity for double-stranded DNA, its high extinction coefficient of $98,900 \text{ M}^{-1}\text{cm}^{-1}$, and its fluorogenic properties¹⁰⁰.

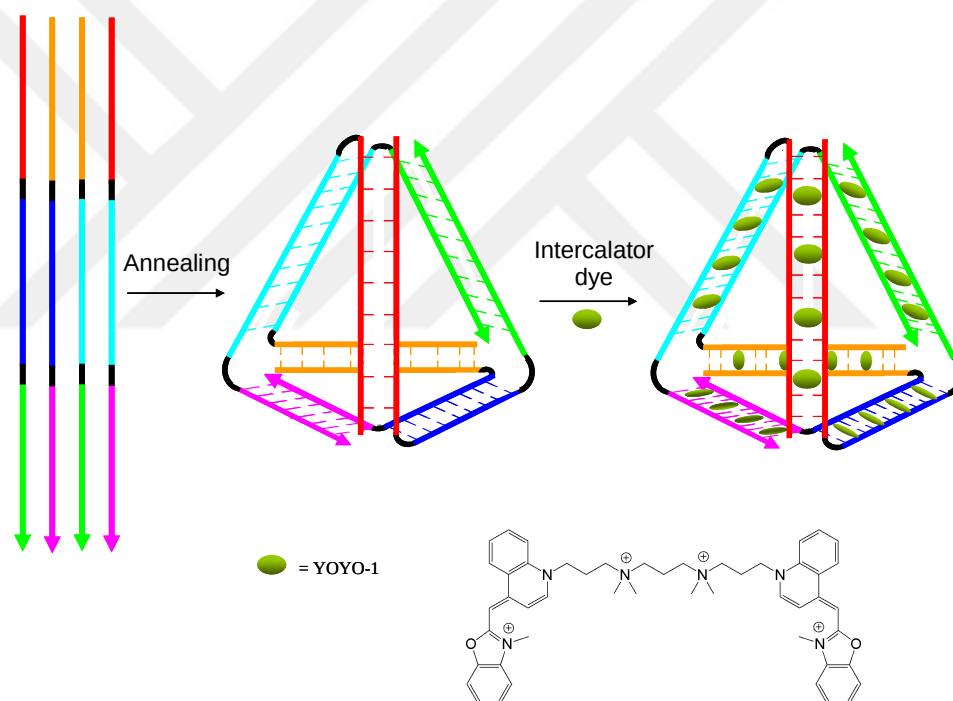


Figure 5 Assembly of DNA-Tetrahedron (TH) nanotags. Four strands with partially complementary sequences form the DNA-Tetrahedron nanostructure template for the self-assembly of intercalator dyes. Black sections represent two-nucleotide long, single-stranded hinges.

Figure 6 compares linear (1D), branched 3-way junction (2D) and tetrahedron (3D) DNA nanotags and illustrates the compact nature of the TH. The approximate

length of each edge of the TH is 8.5 nm, calculated by assuming that 8 intercalating chromophores bind to the 17 bp-long edge.

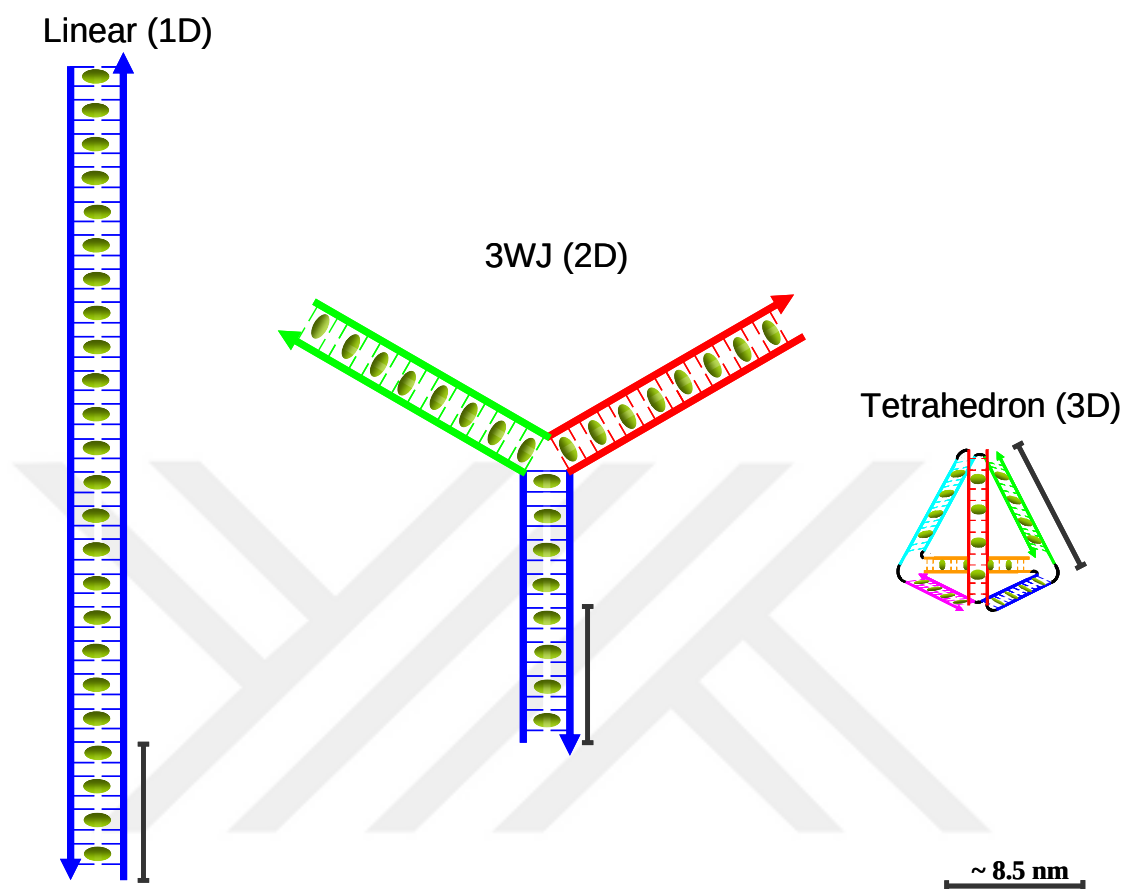


Figure 6 Fluorescent nanotags based on linear (1D), 3-Way-Junction (2D), and Tetrahedron (3D) DNA nanostructures and intercalating dyes (drawn to scale). Each nanostructure is composed of 102 Watson-Crick base pairs

2.3.2 Nanotag Assembly and Characterization

According to the neighbor exclusion principle¹⁰¹, intercalation density should saturate at one intercalator for every two base pairs and is determined by steric constraints imposed by the DNA, which must be stretched and unwound to create an intercalation site. Thus, full saturation of the DNA should occur when half of the binding sites are occupied. In theory, the 102 base pairs in the TH should accommodate up to 56 intercalator dyes, or 28 YOYO-1 molecules. However, given

the odd number of base pairs in each edge, it is likely that only 4 bisintercalators binds per edge, giving 48 total intercalated chromophores.

The DNA nanostructures were assembled by a simple thermal annealing process. Figure 7 illustrates UV melting curves recorded for the three structures with and without saturating amounts of the bisintercalating dye YOYO-1. While the branched 3WJ structure exhibits a broad low temperature transition in the absence of YOYO-1, all three nanostructures undergo cooperative melting transitions in the presence of the dye. Moreover, intercalation of YOYO-1 increases the thermal stability of the DNA structures by approximately the same amount, regardless of the dimensionality of the nanostructure. These results confirm that the DNA-dye nanostructures are fully assembled at room temperature.

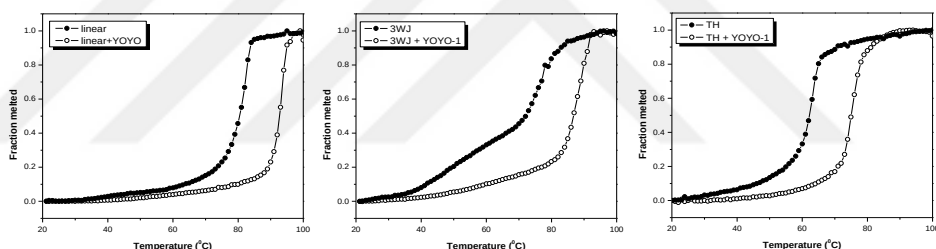


Figure 7 UV-melting curves recorded for tetrahedron (TH) DNA in the absence and presence of YOYO-1. [DNA] = 50 nM nanostructure, [YOYO-1] = 1.3 μ M (i.e. approx 1 bis-intercalator dye per 4 base pairs).

The impact of the intercalating dye on the TH nanostructure was also examined by nondenaturing gel electrophoresis. The TH migrates as a single band through the gel, even when the intercalator dye is added before electrophoresis (Figure 8). This indicates that the 3D nanostructure is preserved in the presence of the dye. Interestingly, the mobility of the TH is unchanged by intercalation of YOYO-1, whereas the linear and 3WJ DNA mobilities are retarded by the dye.

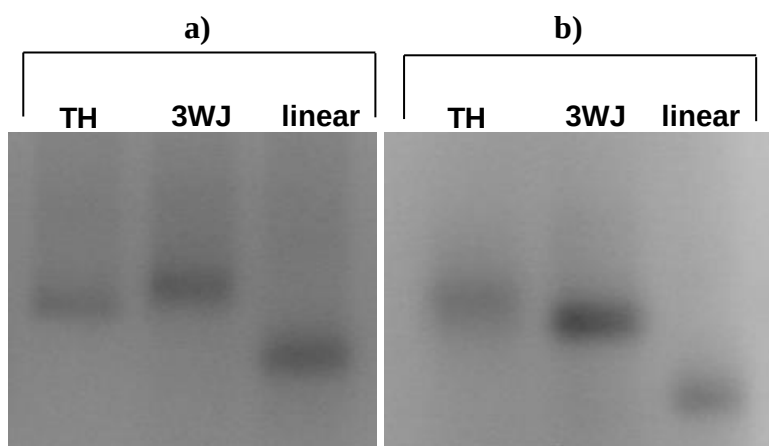


Figure 8 Visualization of YOYO-1 intercalation on agarose gel. Shown are TH, 3WJ, and linear nanostructures a) loaded with YOYO-1 and incubated for 1h prior to electrophoresis; and b) electrophoresed in the absence of YOYO-1 and then stained with ethidium bromide.

2.3.3 Optical Spectroscopy

Figure 9 illustrates the UV-vis absorbance spectra recorded for YOYO-1 in the presence of the linear, branched and TH nanostructures. The hypochromic effect is characteristic of intercalated dye and the similarity among the three spectra indicates similar amounts of bound dye, regardless of the dimensionality of the DNA template. While the binding constant of YOYO-1 for linear DNA has not been reported, we expect $K_b \geq 10^9 \text{ M}^{-1}$, based on the binding constant for the monointercalating YO-PRO-1¹⁰² and prior work on a bisintercalating ethidium dye¹⁰³. Thus, virtually all of the dye should be bound under these conditions. The nanostructure concentration was 50 nM and the absorbance was 0.09, giving an extinction coefficient of $\epsilon = 1.8 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ at 488 nm. This is comparable to phycobiliproteins and approximately 30-fold greater than enhanced GFP¹⁰⁴.

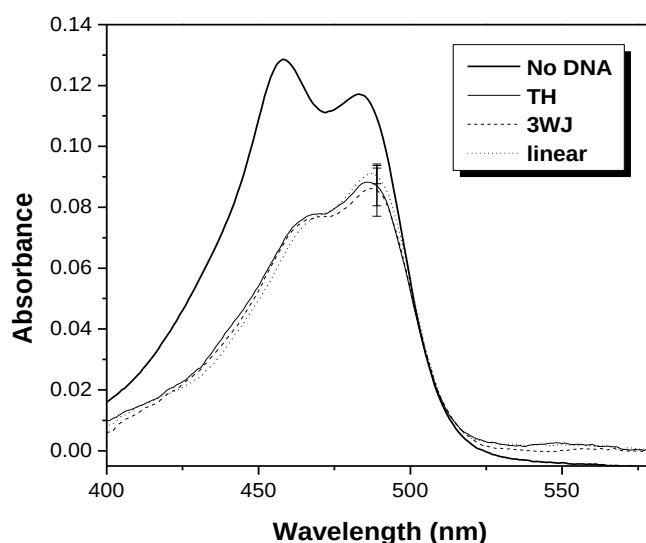


Figure 9 Comparison of absorbance spectra of YOYO-1 in presence of TH, 3WJ, and linear nanostructures, and free YOYO-1. 50 nM DNA nanostructure was mixed with 1.28 μ M YOYO-1 (i.e. one bisintercalator dye per 4 DNA bps); spectra were acquired after 1h incubation at room temperature.

Although the absorption spectra of the intercalator dye in the presence of the three nanostructures are similar, significant differences are observed in the respective fluorescence intensities (Figure 10). As the dye is titrated into a solution of the DNA nanostructure, the fluorescence is consistently weaker for the TH and levels off at a lower concentration. Intermediate behavior is observed for the 2D branched nanostructure. All three DNA templates have similar sequence composition (47-48% GC) so the difference in fluorescence intensity is unlikely to be due to the known sequence dependence of fluorescence quantum yield for this dye¹⁰⁵. An alternative possibility is that one or more of the chromophores insert into the junction regions linking different helices in the 2- and 3-D structures. Since these dyes will not be as constrained as a fully intercalated dye, the quantum yield would be expected to be lower. In addition, such dyes could serve as traps for energy migrating through the

assembly, further reducing the overall intensity. Regardless of the origin, the diminution in fluorescence is not that large. Taking the quantum yield for fluorescence in linear DNA to be approximately 0.5^{105} , the corresponding value for the TH is still 0.3, giving a brightness ($\epsilon \times \phi_f$) of $5.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. This is 16 times greater than enhanced GFP and within a factor of 3 of R-phycoerythrin.

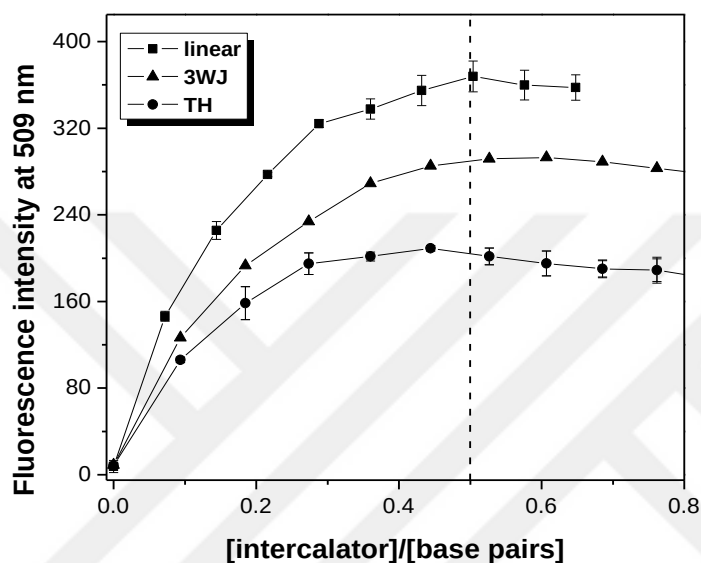


Figure 10 Change in the fluorescence intensity of the fluorophores intercalated to TH, 3WJ, and linear DNA nanostructures by increasing ratio of intercalator dyes per base pair. Samples containing $5.1 \mu\text{M}$ base pairs DNA (50 nM nanostructure) were titrated with $0.2 \mu\text{M}$ aliquots of YOYO-1.

2.3.4 FRET Experiments

In flow cytometry and fluorescence microscopy, it is useful to have labels of different colors, which can be excited at the same wavelength, so that different populations can be distinguished while using the same light source. Systems in which energy can be transferred from donor to acceptor molecules through Förster resonance energy transfer (FRET) are suitable candidates for such multicolor labeling¹⁰⁶. One of the most appealing features of the 2-D nanotags we reported previously was the

highly efficient FRET from intercalated YOYO-1 to terminal acceptor dyes such as Cy3 or WellRed-D2. We performed similar experiments with the TH nanotags by attaching 1-4 Cy3 acceptor dyes to the 5'-termini of the DNA strands, which positions them at the helix junction points on the tetrahedron (Figure 11). Fluorescence spectra demonstrate that the FRET efficiency increases monotonically with the number of Cy3 acceptors, reaching a maximum of 95% (Figure 12). However, even a single Cy3 is able to quench 52% of the donor emission.

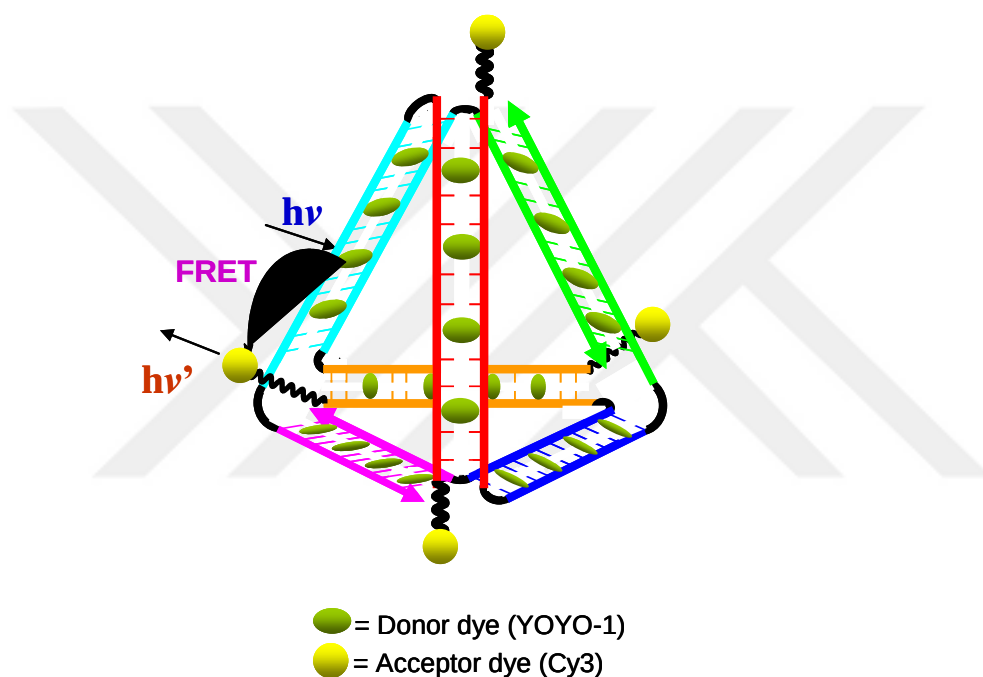


Figure 11 Schematic description of FRET in a Tetrahedron nanotag loaded with YOYO-1 intercalated dyes and covalently attached Cy3 acceptor dyes.

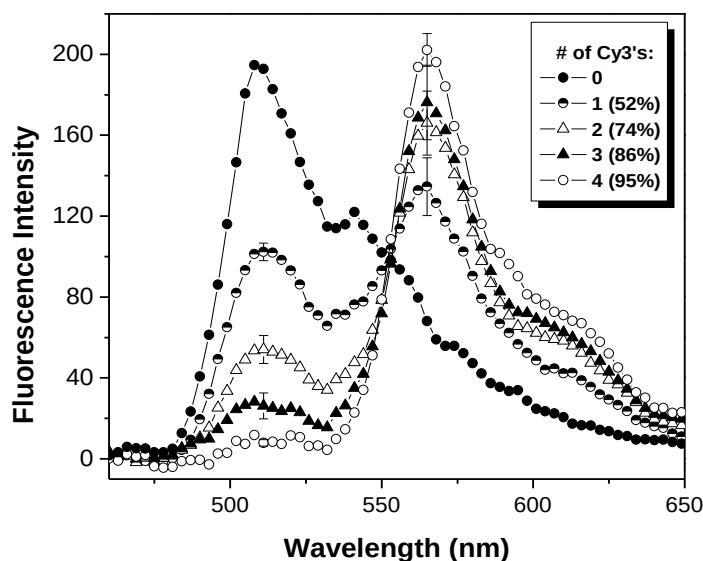


Figure 12 Fluorescence emission of TH nanotags with 0-4 covalently attached Cy3 molecules. Spectra acquired by excitation at 440 nm. Samples contained 50 nM DNA tetrahedron and 1.28 μ M YOYO-1. FRET efficiencies given in legend were determined by the % decrease in the YOYO-1 emission at 509 nm.

Such a high FRET efficiency was due to the compact nature of the TH, which localizes all intercalated dye molecules within a diameter of less than 10 nm, similar to the critical transfer distance of 7.3 nm for the YOYO-1/Cy3 pair. In addition, energy migration among the cointercalated chromophores is expected to be rapid¹⁰⁷, at least within a given edge of the TH. Thus, the TH acts as an antenna to harvest light with very strong efficiency and transfers that energy either to an adjacent intercalator dye or to a lower energy acceptor dye at a nearby helix junction. This indicates that TH nanotags with and without the Cy3 acceptor dyes can be used to label two different species of interest, while being excitable at the same wavelength, as we previously demonstrated for a 3WJ nanotag⁹⁴.

In order to demonstrate a potential application of FRET in these TH fluorescent nanotags, streptavidin-functionalized polystyrene beads were coated with

TH nanotags using a biotin molecule covalently attached to the 5'-terminus of one of the DNA strands. As shown in Figure 13, YOYO-1 saturated DNA-TH can act as a bright label. Beads were nonfluorescent when they were unmodified or when they were coated with biotinylated TH nanostructures without the intercalator dyes (curves A and B). Adding YOYO-1 to the beads in the absence of DNA led to significant fluorescence, presumably due to nonspecific interactions between the dye and either the streptavidin protein or the polystyrene bead (curve C). Interestingly, addition of nonbiotinylated DNA and YOYO-1 gave the same result as YOYO-1 alone, indicating better binding of the dye to the beads than to the free DNA (curve D). However, the beads became 10-fold brighter when they were coated with the biotinylated, YOYO-1 loaded TH-nanotags. The amount of dye and DNA present in experiments D and E are the same, yet the fluorescence is 10-fold higher in the latter. This suggests that immobilizing the DNA on the bead leads to higher effective concentrations, which competes more effectively with the bead for the dye than when the TH is diluted at nanomolar concentrations into the surrounding solution. The increase in brightness is indicative of a higher quantum yield for DNA-intercalated versus polymer-entrapped dye and shows that these noncovalent assemblies are sufficiently stable to allow labeling of polymer beads commonly used in flow cytometry-based diagnostics assays.

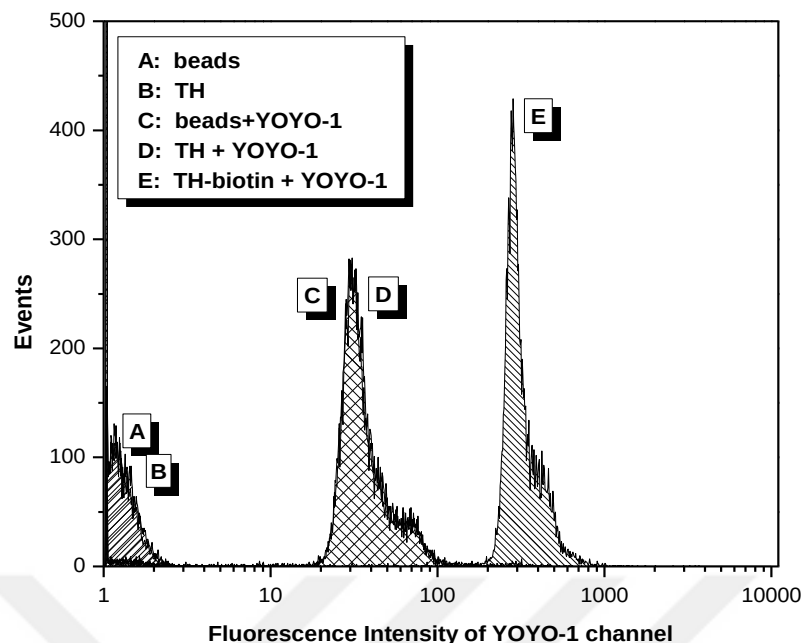


Figure 13 Flow cytometry analysis of TH-labeled streptavidin-coated polystyrene beads. Empty beads (A), and beads coated with biotinylated TH without YOYO-1 (B) exhibited background fluorescence/light scattering only. Non-specific interaction of YOYO-1 with the beads led to modest fluorescence, even in the presence of free DNA TH (C and D). When beads were coated with biotinylated TH nanotags intercalated with YOYO-1 (E), 10-fold greater intensity was obtained.

Figure 14 recapitulates the FRET spectra experiment shown in Figure 12. Addition of 3 Cy3 labels leads to a 73% decrease in the fluorescence intensity recorded in the YOYO-1 channel. This is only slightly lower than the FRET efficiency observed in solution (86%) and could be due to a small amount of dissociation and/or nonspecific binding to the beads in the cytometry experiment. Strong sensitized emission was visible in the Cy3 channel (Figure 8b) although the broad emission spectrum of YOYO-1 led to intense fluorescence in this channel even in the absence of FRET. For longer wavelength dyes such as Cy5, this “leakage” of the donor emission into the acceptor channel should be less significant.

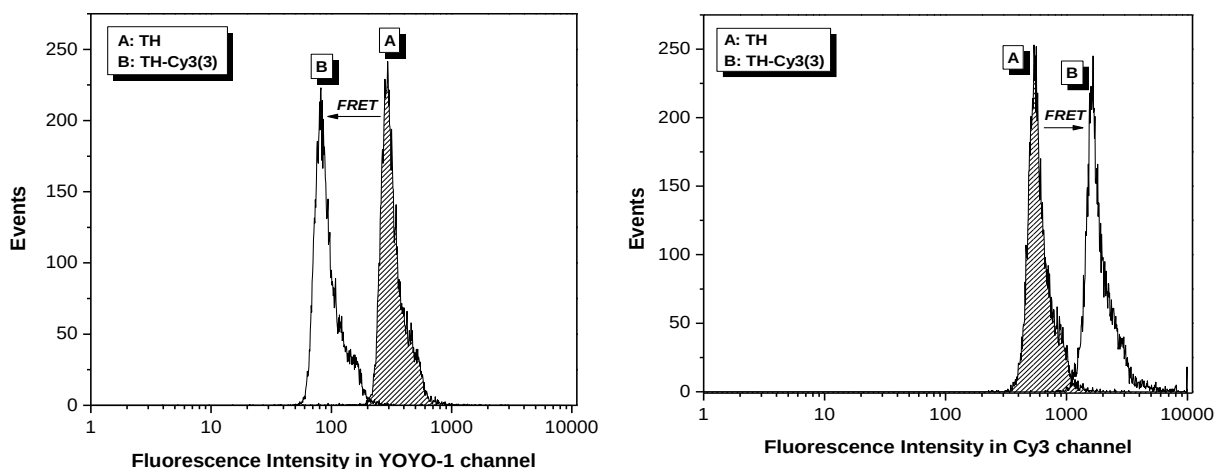


Figure 14 Flow cytometry results indicating FRET in TH nanotags. Fluorescence intensity was recorded in the YOYO-1 donor (left) or Cy3 acceptor channel (right) for different populations of beads labeled with either 0 or 3 Cy3 acceptor molecules.

2.3.5 Photostability Studies

One critical parameter for effective use of fluorescent labels in imaging applications is the photostability of the fluorophores. Stable fluorescence intensity is desired for applications requiring long irradiations such as single molecule spectroscopy or fluorescence imaging of low abundance species. Cyanine dyes are known to undergo photobleaching via reaction with singlet oxygen, which is produced by a small fraction of the dye excited state that intersystem crosses to the triplet manifold.¹⁰⁸⁻¹¹⁰ The TH nanotags were expected to minimize this reaction path by virtue of intercalation of the dyes into the DNA matrix, which should not only restrict access of molecular oxygen to the dye excited state, but also hinder reaction with any singlet oxygen that does get produced. Prior work by Kanony and co-workers showed that DNA-intercalated oxazole yellow dyes were less prone to reaction with singlet

oxygen, and were found to exhibit slower decay of fluorescence intensity under prolonged irradiation, in comparison to fluorophores which were free in solution.¹¹¹

We compared the photostabilities of YOYO-1 intercalated in linear (1D) and TH (3D) nanostructures. As shown in Figure 15, the TH-intercalated dye underwent less than 5% decrease in fluorescence intensity after 50 minutes of irradiation with an Hg lamp. In contrast, nearly 30% of the fluorescence was lost from dye that was intercalated into the linear DNA template. Interestingly, the dye absorption spectrum was not perturbed by the irradiation, even in the linear DNA experiment where a substantial decrease in fluorescence intensity was observed (data not shown). This indicates that the loss of fluorescence shown in Figure 15 is due to damage to the DNA template rather than to the dye. Kanony and coworkers made similar observations and attributed it to photo-induced oxidation of the DNA, specifically at guanine residues¹¹¹. The oxidized guanines can then serve as electron transfer quenchers of the dye, resulting in decreased fluorescence intensity without photobleaching of the dye. The ability of the TH nanostructure to resist photo-damage is a significant advantage relative to a linear DNA template and will be the subject of further study.

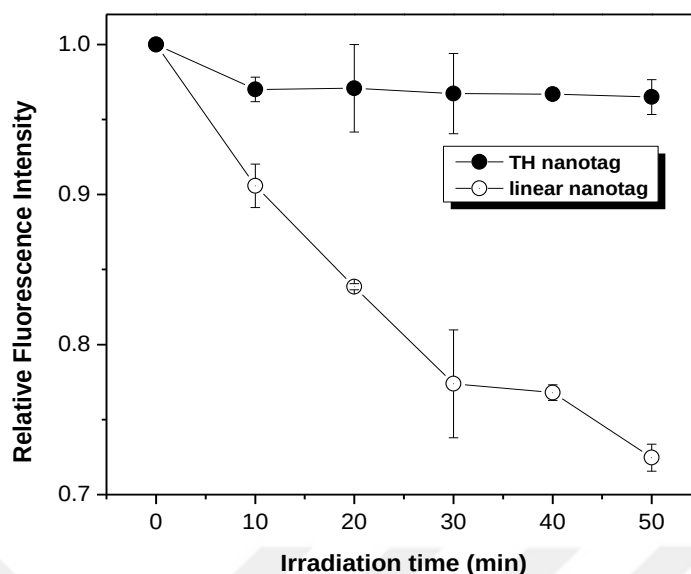


Figure 15 Comparison of photostability of YOYO-1 intercalated to TH and linear DNA templates. Fluorescence intensity at emission maximum of YOYO-1(509 nm) was recorded initially and after every ten minutes selective irradiation of YOYO-1.

2.3.6 Biochemical Stability

In order to more precisely define the range of conditions in which DNA nanotags can be used as fluorescent labels, we investigated the biochemical stability of these noncovalent assemblies. While the TH nanotags exhibit excellent photostability, it is also possible that the DNA backbone could be degraded by nuclease enzymes that are present in biological samples. This would release the dyes into solution and diminish the fluorescence brightness of whatever was labeled by the nanotag.

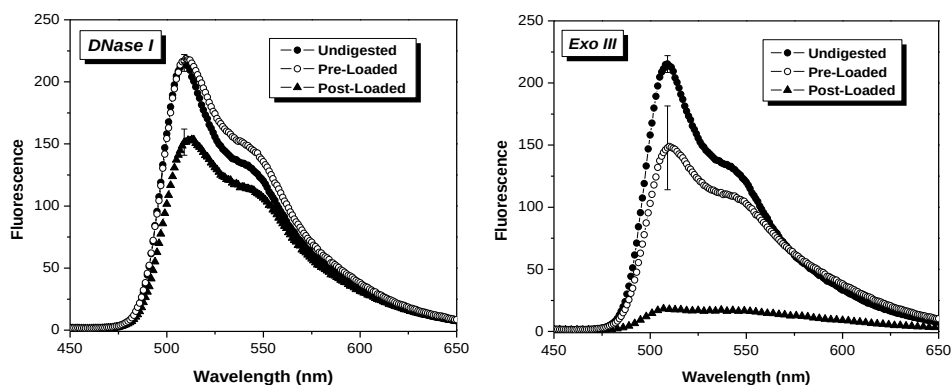


Figure 16 Fluorescence spectra of TH-nanostructures in the absence (“Pre-Digested”) and presence (“Post-Digested”) of YOYO-1 acquired after nuclease treatment, in comparison to nanotags which were not treated with nucleases. Greater fluorescence was retained by TH-nanostructures digested in the presence of the dye.

Previous studies have shown that bisintercalating dyes improve the stability of DNA duplex against endonuclease degradation presumably because of lengthening and unwinding of the duplex¹¹². Hence, TH nanotags were also expected to be less prone to hydrolysis by nuclease enzymes. This was verified by monitoring the fluorescence intensity of the nanotags treated with endo- (DNase I) or exonuclease (Exo III) before or after loading with intercalator dyes. As shown in Figure 16, TH nanostructures loaded with intercalators *prior* to exposure to the enzyme (i.e. “Pre-Loaded” samples) retained significantly higher fluorescence for both nucleases, consistent with a protective effect of the intercalator dye.

The susceptibility of the nanotag to enzymatic degradation was also compared for nanotags of different dimensionalities. As shown in Figure 17, the 3D TH nanotag was significantly more resistant than the other two against the endonuclease (DNase I), presumably because of the significantly shorter edges of the more compact TH nanostructure. Meanwhile Exo III, which attacks the 3’ end of the DNA strand and removes one nucleotide at a time, also exhibited lower activity against the TH than

the 3WJ and comparable activity with the linear nanostructure, in spite of the fact that there are more 3'-termini in the TH than the 3WJ and linear nanostructures (4 versus 3 and 2, respectively). Note that in practical applications, exonuclease activity can be further inhibited if the strands are designed in a way that allows ligation of adjacent termini⁹⁷ or by using terminal modifications such as phosphorothioates.

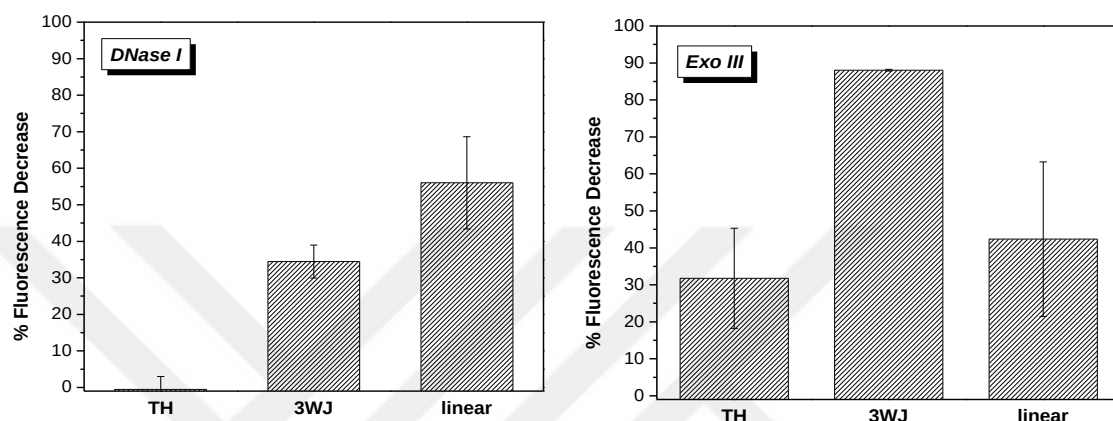


Figure 17 Loss of fluorescence due to pre-digestion with DNase I (left) or Exo III (right) for different DNA nanotags.

2.4 Discussion

Research in the last decade has redefined the fundamental biomolecule DNA as a unique nanoconstruction material^{96, 97, 113-117}. Various rational design approaches have led to DNA nanostructures of different dimensionalities and potential functions. We previously chose to induce fluorescent dye assembly on a two-dimensional 3WJ nanostructure to create bright fluorescent nanotags.⁹⁴ With our current research we extended this idea into three dimensions by using a DNA tetrahedron (TH) nanostructure^{96, 97} as a template for assembly of an intercalating cyanine dye array. In

addition to providing a more compact structure, the 3D TH nanotag exhibits substantial improvements in both photo- and biochemical stability.

In assembling the TH nanotag, some concern arose as to whether the constrained DNA nanostructure would be capable of accommodating a large number of intercalating dyes. The intercalation process imposes considerable strain on the DNA structure, which must extend and unwind to create a binding pocket for each dye.¹¹⁸ In the case of linear and branched templates, each arm of the DNA nanostructure can simply extend further from the core and binding up to the expected saturation point of 2 base pairs/intercalator is observed. It was not clear how the 3D tetrahedral template would respond to the bisintercalating dye, but as shown in Figures 5 and 6, a substantial amount of dye is bound by the DNA, and gel electrophoresis indicates that the tetrahedron remains intact after addition of the dye. Although fluorescence titrations indicate that the DNA saturates at approximately 3 base pairs/intercalator (Figure 10), the UV-vis absorption spectra (**Figure 9**) indicate that the amount of bound dye is similar for the linear, branched and tetrahedral templates. Therefore, the difference in fluorescence intensity observed in Figure 10 appears to be due to a lower average quantum yield for the dye bound to the TH compared with the linear and 3WJ templates, which could reflect binding of some oxazole yellow groups in the hinge regions at the TH vertices.

The minor loss of brightness on assembling the dyes on a 3D template compared with the 1D and 2D analogues is compensated not only by the more compact structure of the tetrahedron but also by improvements in both photo- and biochemical stability. For example, the TH nanotag retains >95% of its initial fluorescence intensity during prolonged irradiation, whereas the analogous linear nanotag loses nearly 30% of its intensity under identical conditions. Whether the

enhanced photostability of the TH nanotag is due to (i) less efficient singlet oxygen production, (ii) lower inherent reactivity toward singlet oxygen, or (iii) some other factor remains to be determined.

The 3D tetrahedral nanotag also exhibits impressive resistance to nuclease degradation; thus, the fluorophore binding sites on the DNA template stay intact and lead to high brightness even after extended incubation with endo- or exonuclease enzymes. These results indicate that the TH nanotags can be used in complex biological samples, provided that dissociation of the noncovalently bound dye from the DNA template does not cause problems, as we observed previously for 2D nanotags based on a 3WJ DNA template.⁹⁴ Efficient strategies for covalent attachment of dyes to DNA have been reported¹¹⁹ and will lead to even more stable nanotags that might even find use for intracellular imaging and detection experiments.

2.5 Conclusions and Outlook

Based on the evidence provided here with the tetrahedral DNA scaffold, this novel approach can be extended to other more compact DNA nanostructures. Rational design of DNA nano-architectures can result in templates of even higher densities of fluorophore binding sites in relatively small volumes of space, as in the examples of a 3D octahedron¹¹⁷ and 2D nanostructures consisting of 8- and 12-armed junctions¹²⁰ recently reported in the literature. The results obtained in this study demonstrate that a compact 3D nanostructure provides a protective environment for the fluorophores, which leads to intense and stable optical signals from the nanotags. As the ratio of binding sites per unit volume of the nanostructure increases, brighter and smaller nanotags can result and find various applications.

Due to the availability of well established methods to functionalize DNA for conjugation to antibodies, proteins, peptides, or other nucleic acids, these nanotags

can be utilized to label and track biomolecules. In that sense they can find similar applications as the fluorescent polymer¹²¹ and silica¹²² nanoparticles that have been developed for multiplex bioassays. In addition to ease of synthesis and conjugation to biomolecules, having the ability to control both the number and the proximity of fluorophores on a 3D DNA template as opposed to being embedded irregularly in a matrix will be advantageous. The DNA template allows rational manipulation of the energy transfer efficiency by varying the number and location of donor and acceptor fluorophores. Thus, nanotags constructed from an intercalating donor and terminal acceptor can yield more than two “colors” by tuning the FRET efficiency over the range from 0-100% and relying on ratiometric measurements.

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Chapter 3: Binding of Fluorogenic dyes to a Promiscuous Binding Site in an scFv Protein

3.1 Introduction

Non covalent interactions between organic fluorophores and biomolecules have been studied for decades, and exploited in several applications such as fluorescence labeling, biological detection and biosensing¹²³⁻¹²⁵. Biomolecules interact with fluorophores by a variety of physical mechanisms including hydrophobic interactions, electrostatic interactions and hydrogen bonding. Although the exact nature of these mechanisms are too complex and can only be revealed with structural data from NMR, or X-ray crystallography, usually evidence for binding is provided by a change in the absorption and emission spectra of the complex relative to the fluorophore alone. Noncovalent interactions between biomolecules and fluorophores attracted attention mainly due to the ease of preparation without elaborate conjugation reactions, and due to the fact that the biomolecule's function is potentially less affected by the fluorophore marker. Noncovalent fluorophore-biomolecule interactions through intercalation or groove binding constitute the fundamental basis for DNA quantification by gel electrophoresis or optical assays¹²⁶⁻¹²⁸. Similarly, protein-fluorophore interactions have also been utilized for protein detection and study of protein dynamics. Preceded by the environmentally sensitive 1,8-ANS dye that responds to different hydrophobic pockets by a change in quantum yield¹²⁹, other fluorogenic fluorophores were reported as probes providing information about protein conformation¹³⁰.

These interactions are mostly nonselective in nature; they occur due to the recognition between fluorophores and building blocks of biomolecules such as nucleic acid bases or amino acid side chains. For example, the fluorogenic dye thiazole

orange can intercalate to any DNA sequence by an increase in quantum yield and it cannot differentiate between different DNA sequences¹³¹. Similarly, most of the fluorescent probes used to study proteins such as prodan¹³² (6-propionyl-2-dimethylaminonaphthalene) and its derivatives do not exhibit selectivity towards certain amino acids¹³³. Selective noncovalent interactions, on the other hand, are desired to have more control over fluorescent probes and to utilize these interactions in more complex fluorescent labeling technologies. Biomolecule-fluorophore pairs that bind each other with strong affinity and by fluorescence enhancement are called fluoromodules.

Fluoromodules are usually developed by diversity-based systems. Pools of short DNA or RNA sequences, aptamers, were screened to isolate those that can fold into a secondary structure and activate the fluorescence of fluorogenic dyes¹³⁴⁻¹³⁸. The specific recognition between aptamers and fluorogens are being utilized as fluorescent probes and incorporated into many detection and sensing systems¹³⁹. An analogous specific recognition interaction was demonstrated by single chain variable fragment (scFv) antibodies that were isolated from a diverse library for their ability to activate fluorogenic dyes thiazole orange (TO) and MG¹⁴⁰. The library of scFvs that was screened for fluorogen affinity was displayed on yeast cell surface. This format allowed the screening of the library by Fluorescence Activated Cell Sorting (FACS) which is a powerful screening technology that allows high throughput screening of large libraries. Isolated scFvs that enhanced the fluorescence of fluorogenic dyes upon binding were shown to be successful in labeling of cell surface proteins.

Fluoromodules have the potential for being used as genetically encodable fluorescent labels and sensors. The aptamers or scFvs that activate the fluorogens can be genetically fused to targets of interest and coexpressed in cellular environments.

The exogenously delivered fluorogenic dyes bind to their cognate partners and probe the target molecule by fluorescence signal.

The suitability of fluoromodules for applications as biosensors depends on their sensitivity, which is usually determined by the signal to noise ratio of the detection. Higher sensitivity in cellular experiments is achieved by reducing the background signal that is caused by the nonspecific binding to genomic material and cellular compartments. For fluoromodules that are composed of scFvs and fluorogens, the complication for high background signal is that commonly used fluorogenic dyes such as TO intrinsically have strong affinity for DNA and RNA. Such competition for fluorogens would reduce the sensitivity of in vivo assays utilizing fluoromodules.

We developed new fluoromodules based on scFv antibodies and a specifically designed fluorogenic dye Dimethyl Indole Red (DIR) that has low affinity for nucleic acids. We screened a library of scFvs to isolate scFvs that can bind to and activate DIR. Resulting fluoromodules were characterized in terms of their spectral, photophysical, and thermodynamic characteristics. The analysis was performed to evaluate the potential of our fluoromodules as a novel technological platform for fluorescent probes, but also to understand fundamentals of recognition between scFvs and fluorogenic dyes in general.

3.2 Experimental

3.2.1 Selection of Yeast Surface Display Clones that Bind and Activate DIR

Yeast growth medium (pH 4.5):

20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto casamino acids, 14.7 g sodium citrate and 4.29 g citric acid monohydrate were dissolved in deionized H₂O to a volume of 1 liter and sterilized by filtration.

Yeast induction medium:

Prepared as for the yeast growth medium, but used 20 g galactose instead of dextrose.

Selections:

A yeast cell surface display library, composed of ca. 8×10^8 recombinant human scFvs derived from cDNA representing a naïve germline repertoire, was obtained from Dane Wittrup, MIT. This library is currently available from Pacific Northwest National Laboratory (PNNL, <http://www.sysbio.org/dataresources/singlechain.stm>). EBY100 was the host to the yeast display library. For studies of individual FAP genes, pPNL6 plasmids were isolated from yeast using Zymoprep I kit (Zymoresearch) and transferred to Mach1™-T1R or TOP10 *E. coli* (Invitrogen).

Enrichment and cloning of DIR-activating scFvs from the surface display library was essentially as described previously^{140, 141}. Number of cells that would represent 10fold excess of the size of the library was calculated, and the volume of the frozen stock that corresponds to this number was inoculated into 25 mL growth medium and incubated at 30°C for two days. OD of the cell culture was determined and cells were harvested by centrifugation. Induction culture was inoculated at 10^7 cells/mL in 25 mL induction medium. An uninduced control culture with the same concentration was also inoculated in growth medium. Cultures were incubated at 20°C for 2 days. After determining the OD, cells were harvested by centrifugation and resuspended in PBS with 2mM EDTA and 0.1 % pF127 pH7.4 (modified PBS).

Briefly 10^{10} induced yeast cells from the surface display library (ca. 10X oversampling) were incubated with 1 μ M biotinylated DIR¹³⁵ with gentle mixing for 60 minutes at room temperature, followed by a 10 minute incubation on ice. The cells were pelleted and washed once in modified PBS buffer. The cells were pelleted and

resuspended in 5 ml modified PBS to which was added 200 μ l streptavidin MACS microbeads (Miltenyi Biotec). The cells were held on ice for 30 minutes with gentle inversion every 2 minutes. The cells were pelleted and resuspended in 50 ml modified PBS for collection on a MidiMACS column as described by Chao¹⁴². The collected cells (ca. 3.5×10^6) were dispersed into low pH growth media and grown to saturation. The MACS isolation was repeated on these cells after induction except that anti-biotin MACS microbeads were used in the second isolation. 3.7×10^7 cells were isolated in the second selection and grown to saturation in low pH growth media. From this population, yeast cells that directly activate fluorescence from DIR after induction were isolated by two successive cycles of 2-color FACS enrichment based on enhanced fluorescence of the fluorogen and high surface expression of scFvs¹⁴⁰. Surface expression of scFvs was quantified by labeling the c-myc epitope tag present on the scFvs. This was done by incubating the cells with the anti anti-c-myc antibody (mouse) (Invitrogen) for 1h on ice. Rinsed cells were then incubated with Alexafluor goat anti-mouse 488nM secondary antibody (Invitrogen) for 30 min on ice. Labeled cells were then incubated with 1 μ M of DIR for the FACS analysis. FACS enrichment was carried out on a Becton Dickinson FACSVantage SE with FACSDiva option. In a final third FACS sort, cells were autocloned onto agar plates comprised of solidified induction media containing 10 μ M DIR. After 5 days of growth at 20°C, this plate was imaged under 635x20 nm band pass illumination from red LEDs, using an Andor DU434-BR-DD CCD camera fitted with a 680x25 nm band pass filter. Colonies showing the brightest DIR fluorescence signal were picked for further analysis.

Sequencing:

Colonies picked for sequencing were inoculated into 5ml YPD medium and grown overnight at 30°C. Yeast plasmid was extracted using Zymoprep™ Yeast

Plasmid Miniprep II (Zymo Research). Extracted DNA was electroporated into MACH1 cells and plated onto LB+amp plates. Colonies were inoculated into 5 mL LB+Amp medium and grown overnight, plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen). Extracted DNA was sequenced by GENEWIZ sequencing services (www.genewiz.com) using the following primers for pPNL6 vector.

pPNL6 forward primer: tag ata ccc ata cga cgt tc

pPNL6 reverse primer: gta cga gct aaa agt aca gtg

3.2.2 Determination of fluorogen binding affinity to yeast surface displayed FAP's

10^7 induced yeast cells in 150 μ L modified containing fluorogen over a concentration range were assayed in triplicate for fluorescence in 96 well microplates on a Tecan Safire2 reader. Fluorescence was corrected by subtracting the fluorescence of a dye only sample. As controls, uninduced EBY100 cells which do not express the scFvs were assayed with the same conditions. The following one site saturation binding algorithm equation was used to fit the resulting saturation binding curves and to calculate the binding affinities using Origin 6.1: $y = B_{max} * x / (K_d + x)$ where x is the concentration of the fluorogen.

3.2.3 Microscopy

The image in Figure 23 was acquired on an Olympus Fluoview 1000 scanning laser confocal microscope using a 100x oil immersion objective. Yeast cells were incubated with 100 nM DIR for 30 min on ice. The sample was excited with a 633 nm HeNe laser and emission was monitored in the Cy5 window.

For images in Figure 19, Figure 20 , electroporation slides were loaded with NIH3T3 mouse fibroblast cells and 6 μ M dye and loaded into the Epizap instrument

after rinsing. Slides were then observed and photographed using the Zeiss Apotome microscope (10X Ph 1 objective, N.A. 0.5) in the TRITC channel.

3.2.4 Expression and purification of soluble K7

K7-SfiI forward primer: 5'- g gcc cag ccg gcc atg gcg cag gtc cag ctt gtg cag tct ggg-3'

K7-SfiI reverse primer: 5'-ggc ccc cga ggc cgc gga tag gac ggt gag ctt gg-3'

In order to introduce SfiI sites into the scFv gene a PCR reaction was performed by using the scFv plasmid DNA as the template along with following reagents: 10µL 5X Phusion-HF buffer, 1ng template, 2.5µL 10 µM forward primer, 2.5 µL 10 µM reverse primer, 1.5 µL dNTPs, 0.5 µL Phusion polymerase (Finnzyme), and enough water to bring up the volume to 50 µL. The following conditions were used for the amplification:

98°C 30 sec initial denaturation

A) 98°C 30 sec. denaturation

B) 50°C 30 sec annealing

C) 72°C extension

30X cycles from A-C

72°C 4 min final extension

PCR product was analyzed on a 1% agarose gel and TOPO cloned without gel purification according to the instructions (Invitrogen). TOPO cloned DNA was transformed into Invitrogen Mach T1 cells, plated on LB/Sgal/IPTG/Kanamycin 50µg/mL plates. White colonies from plates were grown in LB+ KAN 50µg/mL medium and DNA was extracted for sequencing. Colonies were screened by restriction digest with SfiI (NEB). One of the colonies that has the scFv gene was subcloned into pAK400 vector. For that, plasmid DNA that was obtained from cells

containing pAK400 was digested overnight with SfiI along with the scFv insert obtained after TOPO cloning. Digestion products were gel purified, and ligated with T7 ligase with 1:6 vector to insert ratio in 20 μ L reaction mixture. 10 μ L of ligation mixture was transformed into Mach T1 cells. Cells were plated onto a LB+ Chloramphenicol (34 μ g/mL).

To express the soluble scFv, a single colony from LB+CAM plate was inoculated into LB+CAM medium, grown at 37⁰C for 4h. An overnight culture with $A_{600} < 0.05$ with 500 mL of 34 μ g/mL LB+CAM at 25⁰C was started. In the morning, cells were centrifuged, resuspended in fresh media and induced with 1mM IPTG for 5 h. Induced cells were harvested by centrifugation and resuspended in 10mM Tris (pH 8.0), 0.5 M NaCl, in a volume equal to weight of the pellet. Cells were lysed by a cell homogenizer. Lysed cells were centrifuged at 14K for 20 min, and the supernatant was incubated with Ni-NTA agarose beads (QIAGEN) and applied to a gravity column for the purification of the His-tagged scFv. After the flow through passed the column, the beads were washed with 1) 5mL of wash buffer 1 (20mM imidazole, 10mM Tris, 50 mM KH₂PO₄, 300 mM NaCl pH 8.0) 2) 5 mL of wash buffer 2 (20 mM imidazole, 10mM Tris, 50mM KH₂PO₄, 750 mM NaCl pH 8.0), and 5 mL of wash buffer 3 (50mM imidazole, 10mM Tris, 50 mM KH₂PO₄, 300mM NaCl pH 8.0). The scFv was eluted with the elution buffer (250 mM imidazole, 10mM Tris, 50mM NaH₂PO₄, 300 mM NaCl pH 8.0) in 6 X 0.5 mL aliquots. The purity of K7 was evaluated on 12.5 % PAGE. Concentrations were determined by absorbance at 280 nM using extinction coefficient of 37,200 M⁻¹ cm⁻¹.

3.2.5 Determination of DIR binding affinity to soluble K7

The DIR binding affinity to soluble FAP was determined using a Photon Technologies International fluorimeter. A saturation binding curve was generated by

titrating 2 nM aliquots of a DIR solution into a 10 nM K7 solution in 1 mL total volume of modified PBS. Titrations were performed in triplicate. Spectra were obtained by exciting the samples at 580 nm and measuring the fluorescence intensity from 600 to 750 nm. Fluorescence at 636 nm was monitored for the generation of the binding curve. The bandpass for both excitation and emission monochromators were 5 nm. Resulting binding curves were fitted with the one site binding algorithm as described above.

3.2.6 Determination of fluorogenic dye binding affinity to soluble K7

Saturation binding curves were generated by titrating 1 μ L aliquots of K7 into a 100 nM fluorogenic dye solution in 150 μ L total volume of modified PBS. Titrations were performed in triplicate. Excitation and emission wavelengths used in titration experiments are shown in Table 1.

Table 1 Excitation and emission wavelengths used in fluorescence titrations.

dye	Excitation wavelength (nm)	Emission wavelength (nm)
InQ3	580	634
D1	580	640
D3	580	642
D4	580	-
D5	580	642
TO-PRO-1	480	520
YO-PRO-1	430	497
PO-PRO-1	410	446
TO-PRO-3	600	652
YO-PRO-3	590	616
PO-PRO-3	490	562
TO-PRO-5	730	760

The bandpass widths for both excitation and emission monochromators were 5 nm. Resulting binding curves were fitted with the following equation (1:1 binding algorithm with a ligand depletion model):

$$\frac{F_i - F_0}{F_\infty - F_0} * 2 * [dye]_{total} = ([dye]_{total} + [scFv]_i + K_d) \pm \sqrt{([dye]_{total} + [scFv]_i + K_d)^2 - 4 * [dye]_{total} * [scFv]_i}$$

Only the titration curve obtained from PO-PRO-1/K7 titration was fitted with the simple 1:1 binding algorithm that was used to fit titration curves obtained from surface displayed scFvs since fitting with the ligand depletion model did not give a satisfying R² value.

3.2.7 Determination of Quantum Yields

Quantum yields of fluorogenic dyes bound to K7 were calculated by using the reference dyes shown in Table 2 and Table 3. Reference dyes were chosen because of the spectral overlap with dyes being studied. The relative quantum yield measurements were done with the dye in K7. For the reference dyes BO-PRO-1, TO-PRO-1, PO-PRO-1, and PO-PRO-3, molar excess of calf thymus DNA was added to the dye solutions. Fully corrected fluorescence spectra of dye solutions having absorbance ranging from 0.02 to 0.1 (at λ_{max}) were obtained on a Varian Cary Eclipse fluorimeter at 20°C. The fluorescence spectrum of each solution was integrated using Origin 6.1. The following equation was used to determine the quantum yield ϕ_x : (Numbers for absorbance and integrated fluorescence are averages obtained from 4 solutions)

$$\phi_x = \phi_{st} (I_x / I_{st}) * (OD_{st} / OD_x) * (\eta_x^2 / \eta_{st}^2)$$

where ϕ_{sd} is the quantum yield of the standard dye under the given conditions, I_x and OD_x are integrated fluorescence, and absorbance of the investigated dye, respectively, I_{st} and OD_{st} are integrated fluorescence and absorbance of the standard dye,

respectively , and η_{sd} and η_x are the refractive indices of the standard and dye solution/solvent.

Table 2 Reference dyes used for the determination of quantum yields, excitation and emission wavelengths used to obtain spectra

dyes	Quantum yield references	Excitation wavelength (nm)	Emission range (nm)
DIR, D1,D3,D5, TO-PRO-3, YO-PRO-3	Cresyl Violet, Cy 5.5	570	575-750
TO-PRO-1, YO-PRO-1, PO-PRO-3	Fluorescein, Lucifer yellow	450	460-660
PO-PRO-1	PO-PRO-1 in DNA, BO-PRO-1 in DNA	405	410-610
PO-PRO-3	PO-PRO-3 in DNA, TO-PRO-1 in DNA	480	500-700

Table 3 Quantum yields of the reference dyes. References: ⁴, www.invitrogen.com

Reference dye	Quantum yield	Solvent
Cresyl Violet	0.54	Methanol
Cy 5.5	0.27	PBS
Fluorescein	0.92	0.1 N NaOH
Lucifer Yellow	0.21	water
PO-PRO-1 in DNA	0.39	10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 7.4
BO-PRO-1 in DNA	0.16	10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 7.4
PO-PRO-3 in DNA	0.57	10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 7.4
TO-PRO-1 in DNA	0.25	10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 7.4

3.2.8 Sequences of DIR activating scFvs

K7

QVQLVQSGAEVKKPGASVKVSCKVSGYTLSESPMHWV
RQAPGKGLEWMGHFDPEDGEKIYAQKFQGRVTLTDDT
STDTVYLELSSLTSEDVAVYYCATETGWGPGLVTVSS
GILGSGGGGSGGGGSGGGGSGNFMLTQPRSVSESPGKT
TISCTRSSGSIASNYVQWYQQRPGSAPTTIIYEDHQRPS
GVPDRFSGSIDSSSNSASLTIAGLRTEDEADYFCQSYDA
NDVVFGGGTKLTVL

A8

QVQLVESGGRLVKPGGSLRLSCEASGFTFSDYYMGWIR
QAPGKGLEWVS YINGDSSYTNYTDSVKGRFSISRDN AK

NSLYLQLSRLRAEDTAVYYCVTSRGSSDSWYRVHWGQ
GTLVTVSSGILGSGG GGS GGGGGSGGGGSSYELTQSPSA
SGTPGQRTVISCSSSSNIGSQYVYWYQQLPGTAPKLLI
YKN NQRPSGVPDRFSGSGSKSGTSASLAISGLRSEDEADY
YCAAWDDSLSGPVFGGGGTQLTVL

K10

QVQLQQSGPGLAKPSQTLSTLCAISGDSVSSNTATWNW
IRQSPSSGLEWLGRITYYRSKWHNDYEVSVKSRITINPDT
SKNQFSLQLNSVTPEDTAVYYCARSGYFQEYLQHWGQ
GTLVTVSSGILGSGG GGS GGGGGSGGGGSSQPVL TQSPSV
SGTPGQKV TIFCSGSSSNVEDNSVYWYQQFP GTTPKVLI
YNDDRRSSGVPDRFSGSGSKSGTSASLAISGLRSEDEADY
YCLSWDDSLNGWVFGGGGTKVTVL

J6

QVQLVQSGAEVKKPGASVKVSCRVS GYRLSDLSIHWV
RQAPGKGLDWVGSFDPEAGETIYDQK FQGRVTLTEDT
STD TAYMELRRLTSDDTAIYYCATDQAAAWGQGTLVT
VSSGILGSGGGGGSGG GGS GGGGGSNFMLTQPHSVSESPG
KTVTISCTGSSGSIASNYVQWYQQRPGSAPT TVIYEDN
QRPS GVPDRFSGSIDSSSNSASLTVSGLKTEDEADYYC
QSYDSSNHAVFGGGGTQLTVL

M8

QVQLVESEGGLVQPGGSLRLSCAASGFTFSSYWMSWV
RQAPGKGLEGVATIKQDGSEKYYVDSVKGRITISRDN
KNSLNRQINSLRAEDTAVY YCARDRLVRETGGDYRGL
DLWGQGTTVT VSSASTKGPSGILGSGGGGGSGGGGGSGGG
GSQPVL TQSPSVSGTPGQKV TIFCSGSSSNVEDNSVYW
YQQFP GTTPKVLIYNDDRRSSGVPDRFSGSGSKSGTSASLA
ISGLRSEDEADYYCLSWDDSLNGWVFGGGGTKVTVL

3.2.9 Isothermal Titration Calorimetry

ITC titrations were performed on a Microcal VP-ITC instrument.¹⁰ μL aliquots of a $25\mu\text{M}$ K7 solution were injected (28 injections) into the cell containing $2.5\mu\text{M}$ fluorogen solution at 25°C . Solutions of dye and scFv were degassed before the start of the experiments. The spacing between injections was set to 300 sec. In order to avoid large heats of dilution the buffer composition in the syringe and the cell were kept the same. This required to equilibrate the imidazole (due to the His-tag elutions), and the DMSO (to keep fluorogenic dyes in solution) concentrations in PBS. Imidazole was added to the fluorogen solution so that the final imidazole

concentration in both fluorogen and scFv solutions was the same. Similarly, DMSO was added to the scFv solution so that the percentage of DMSO by volume became the same as in the fluorogen solution. ITC data were analyzed by using the Origin software for ITC.

3.3. Results

3.3.1 Design and Development of Fluoromodules

3.3.1.1 The fluorogenic dye component of Fluoromodules: DIR

The structure of DIR is depicted in Figure 18. It is a member of the asymmetric cyanine dye class that is known to exhibit fluorogenic behavior¹²³. The indole heterocycle is connected to a quinoline heterocycle through a trimethine bridge. DIR is specifically designed to have reduced affinity for nucleic acids. The indole ring has two bulky methyl groups that disturb the planarity of the molecule, thus preventing DNA intercalation. Furthermore, a negatively charged sulfonate

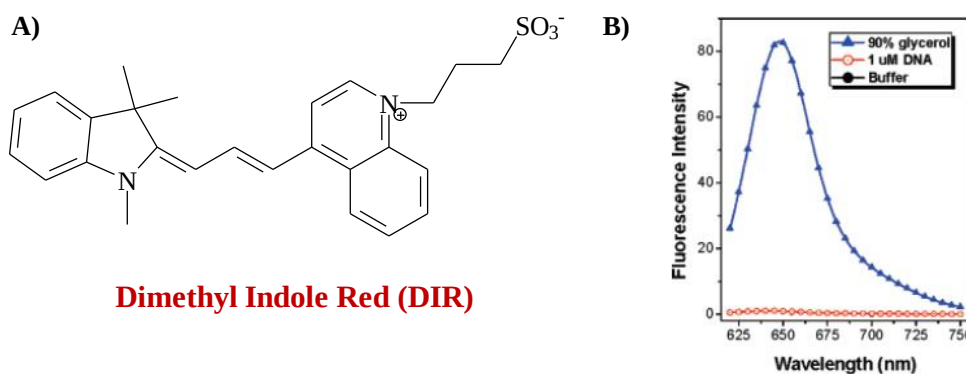


Figure 18 A)The chemical structure of Dimethyl Indole Red (DIR) B) Fluorescence emission spectra of DIR in aqueous buffer(closed circle), in 1 μM double stranded DNA(open circles), and in 90% glycerol(closed triangles). Spectra taken from reference 67.

group is introduced into the quinoline heterocycle so that interactions with nucleic acids are suppressed through electrostatic repulsion from the phosphate backbone.

With these modifications DIR is still a fluorogenic dye that is not fluorescent in an aqueous buffer and exhibits fluorescence enhancement in a viscous environment such as glycerol (Figure 18B). On the other hand, DIR does not interact with double stranded DNA as indicated by the lack of a fluorescence enhancement. It strongly absorbs red light ($\lambda_{\text{max}} = 602 \text{ nm}$) with an extinction coefficient of $134,000 \text{ M}^{-1}\text{cm}^{-1}$. To prove its potential as a fluorogenic dye, DIR was incorporated into fluoromodules based on RNA aptamers before¹³⁵. Using in-vitro evolution methods, high affinity RNA aptamers were selected that can activate DIR fluorescence upon binding. This study provided one of the few examples of a specific interaction between nucleic acids and cyanine dyes.

We demonstrated the potential of DIR for low background staining in cellular experiments by fluorescence microscopy. DIR was electroporated into mouse fibroblast cells and were imaged for fluorescence. Cells electroporated with TO that is a fluorogenic cyanine dye with no modifications to suppress nucleic acid affinity were also imaged as a control. Figure 19 demonstrates the significant difference in background cellular staining due to both dyes. While DIR did not stain cellular compartments significantly, TO stained both the RNA in the cytoplasm and the DNA and RNA in the nucleus. Although different exposure times used during images do not allow us to do a quantitative comparison, different staining patterns are obvious.

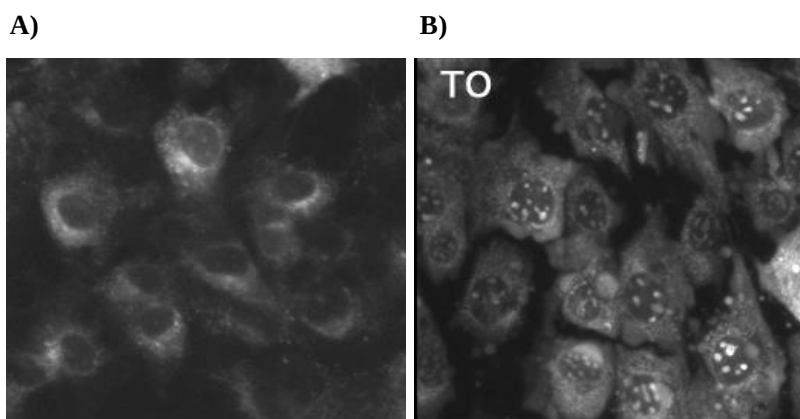
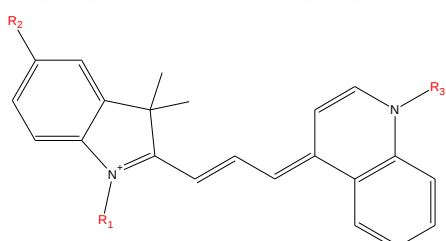


Figure 19 Fluorescence microscopy images of cells electroporated with DIR (A), and TO (B) The dye concentration was 6 μ M for both samples. Exposure times were 1 sec and 3sec for DIR, and TO1-2p, respectively

Synthetic analogs of DIR that have different substituents on the heterocycles have been reported before (Table 4)¹³⁵. These dyes have varying total charges that would potentially affect their nucleic acid affinities. The study of these analogs in

Table 4 Structures of the synthetic analogs of DIR



<i>Dye</i>	<i>R₁</i>	<i>R₂</i>	<i>R₃</i>	<i>Total Charge</i>
DIR	CH ₃	H	(CH ₂) ₃ SO ₃ ⁻	0
D1	CH ₃	H	(CH ₂) ₃ N ⁺ (CH ₃) ₃	+2
D3	(CH ₂) ₂ COO ⁻	H	(CH ₂) ₃ SO ₃ ⁻	-1
D4	(CH ₂) ₂ COO ⁻	SO ₃ ⁻	(CH ₂) ₃ SO ₃ ⁻	-2
D5	(CH ₂) ₃ SO ₃ ⁻	H	(CH ₂) ₃ N ⁺ (CH ₃) ₃	-1

terms of their fluorescence properties can allow us to elaborate on DIR binding interactions. Mouse fibroblasts were electroporated with the DIR analogs and imaged

under the same conditions (Figure 20). The exposure times, and dye concentrations were kept constant.

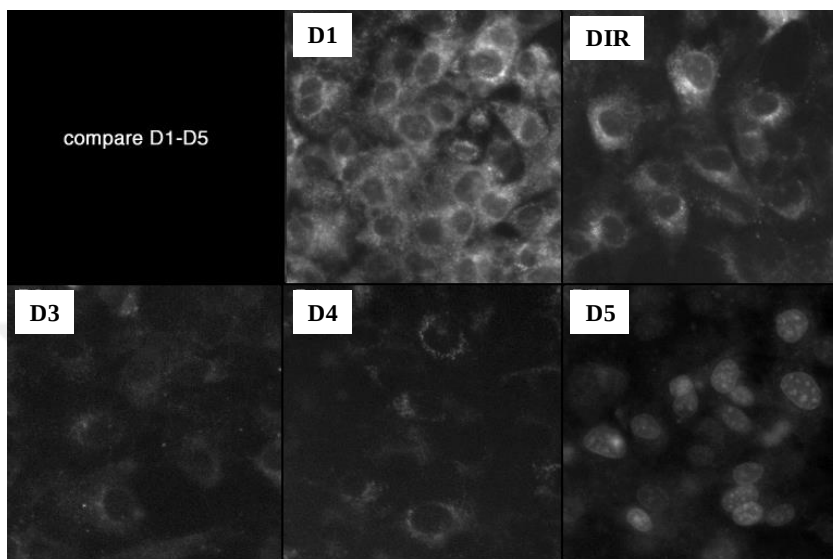


Figure 20 Fluorescence images of mouse fibroblast cells electroporated with 6 μ M fluorogen. Exposure times were 3 sec for each image.

Both the amount of staining and the staining pattern of the cells were different for the dyes. Dyes D4 and D3 with the total charges -1, and -2 respectively, demonstrated the least staining while cells were stained most when the dicationic D1 was electroporated. The dye D5 exhibited a unique pattern among other dyes by staining the nucleus most, indicating it has the strongest affinity for nucleic acids. The different staining patterns of D3 and D5 which both have a total charge of -1 reveal that the nucleic acid affinity is not only determined by the total charge of these dyes. Different electron distributions among the molecules caused by the presence of different substituents potentially affect interactions of the dyes with nucleic acids.

3.3.1.2 Protein Component of Fluoromodules: ScFvs

The protein component of our fluoromodules that provides the restricting binding environment for an increase in DIR quantum yield is an scFv antibody. ScFvs are one of the many engineered antibody fragments that were designed for incorporation into systems where they target biomarkers of diseases¹⁴³. They are composed of the heavy and light chains of antigen binding variable regions of immunoglobulins connected by an artificial peptide linker. Their smaller sizes (~25-27 kDa) relative to immunoglobulins (~150 kDa) while retaining antigen binding capabilities provide an advantage that broadens application areas of scFvs. Libraries of ScFvs have been constructed and expressed in yeast surface display¹⁴¹, phage display¹⁴⁴, ribosome display¹⁴⁵ or mRNA display¹⁴⁶ formats to allow isolation of molecules that can recognize antigens of interest.

ScFvs for our fluoromodules were isolated from a human naïve library expressed on yeast cell surface. The yeast display format, represented in Figure 21,

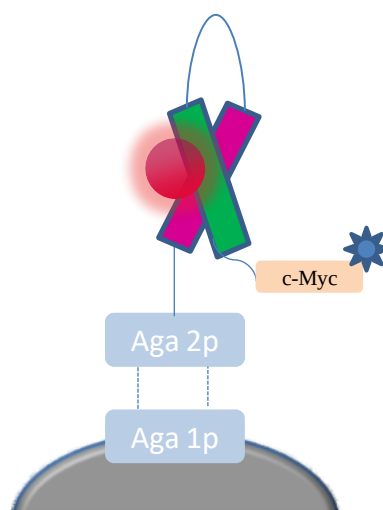


Figure 21 ScFvs expressed on yeast display format. The schematic representation is adapted from reference 115

exploits the agglutinin protein machinery of yeast cells to allow expression of the scFv as a fusion protein to Aga 2-2P that binds to the surface protein Aga 1p via a disulfide bridge¹⁴⁷. An epitope tag, c-Myc gene, is also genetically fused to the scFv gene to allow quantification of the expression on the yeast cell surface. Yeast surface display, in combination with FACS has been widely used for various applications including selections, epitope mapping, cDNA library screening, and cell adhesion engineering¹⁴⁸.

3.3.2 Isolation of DIR Activating ScFvs

A biotin analogue of DIR⁸⁰ 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 regions¹⁴¹. This enrichment was accomplished by two rounds of sequential selection using streptavidin magnetic microbeads followed by anti-biotin magnetic microbeads¹⁴². 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 galactose in the media to induce the display of scFvs 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.

3.3.3 Characterization of DIR Activating ScFvs on Yeast Cell Surface

3.3.3.1 DIR affinities

Equilibrium binding constants for DIR with the five surface displayed scFvs were determined by fluorescence titration of the dye into yeast cultures expressing an scFv. Expression of the scFvs required galactose, so a negative control experiment involving yeast cells that were not exposed to galactose was performed in each case.

Figure 22 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 of 13.9 ± 3.2 nM. Negligible fluorescence enhancement was observed when DIR was added to the control cells (open circles) or to buffer (triangles). K_d values for the other four clones are also shown in the inset to Figure 22; even the weakest binding clone, A8, exhibits a respectable K_d (148 nM).

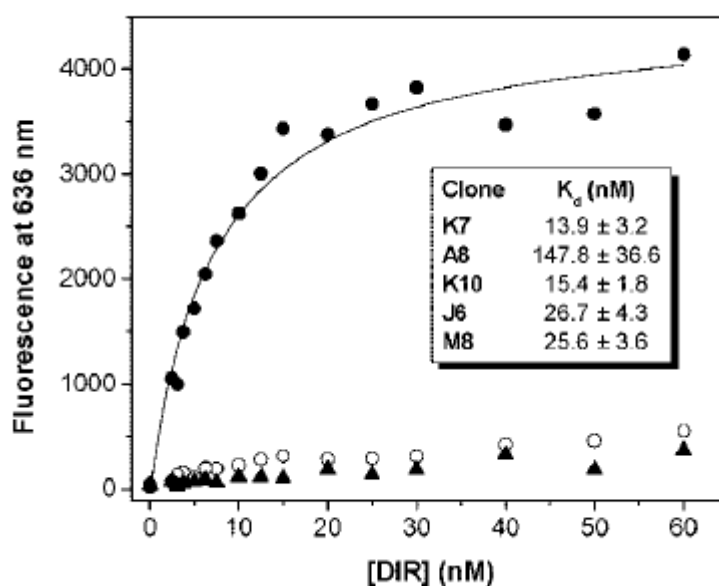


Figure 22 Fluorescence titration curve for binding of DIR to surface displayed clone K7. Line represents fit to 1:1 binding model. Open circles and filled triangles: Titration of dye into cells not expressing the scFv and buffer, respectively. Inset shows calculated K_D values for five unique clones selected by FACS

The high affinities and large fluorescence enhancements exhibited by these scFvs for DIR are well suited for fluorescence microscopy. Figure 23 shows an image obtained after mixing 100 nM DIR with 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 activating scFv K7 on virtually all of the cells.

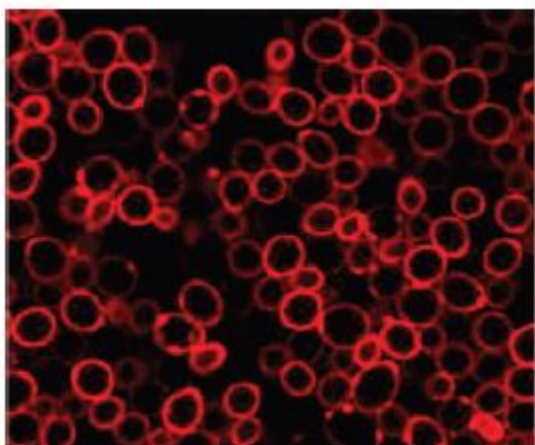


Figure 23 Scanning laser confocal fluorescence image of yeast expressing FAP K7 in the presence of 100 nM DIR.

3.3.3.2 Cross-reactivities of DIR and DIR activating scFv

We next explored the cross-reactivity between DIR and the structurally related TO1-2p for binding to their respective scFvs. TO1-2p is a fluorogenic TO analog modified with diethylene glycol diamine to improve aqueous solubility (structure shown in Figure 24).

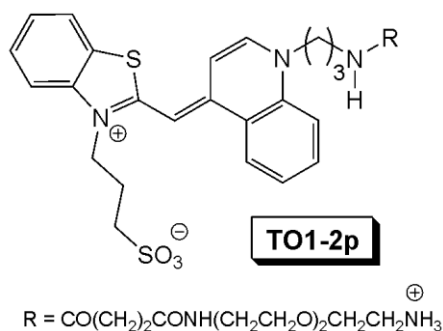


Figure 24 Structure of TO1-2p

Binding of DIR to surface-displayed HL1-TO1, an scFv previously selected for binding to TO1-2p⁸⁵ 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 25, 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 25 also shows that the fluorescence spectra for these dye-scFv fluoromolecules cover most of the visible and near-IR region of the spectrum.

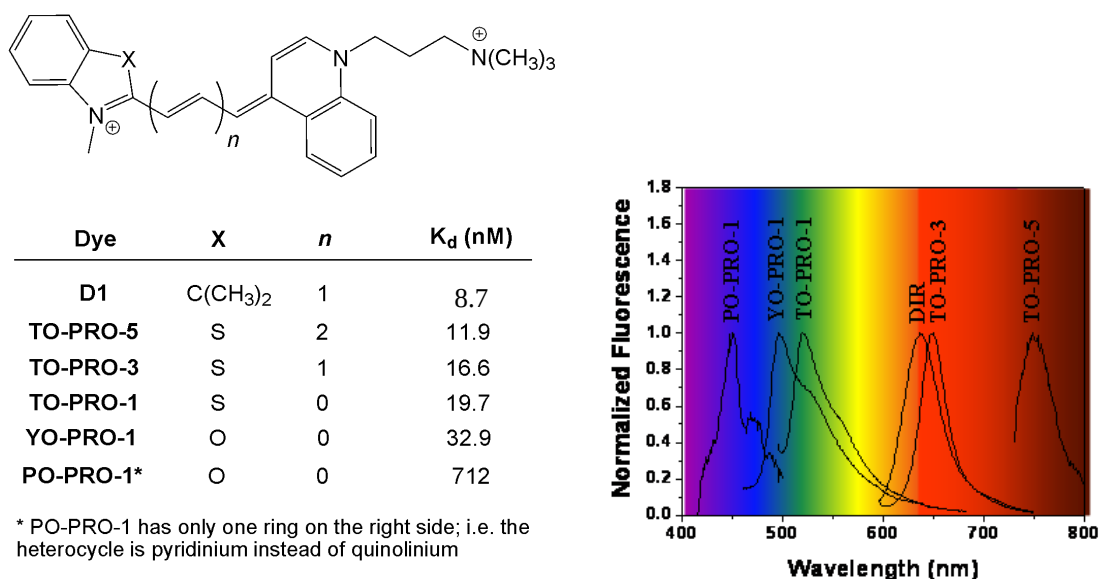


Figure 25 Dissociation constants and normalized fluorescence spectra for various unsymmetrical cyanine dyes bound to DIR activating scFv clone K7

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 TO1-2p¹⁴⁰. As reported previously, the K_d of TO1-2p for the improved scFv is 3 nM compared to 360 nM for the original scFv. 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 scFvs that selectively bind different colored fluorogens; this will be the subject of Chapter 4 of this dissertation.

3.3.4 Characterization of DIR Activating ScFv Clone K7 in Solution

While yeast surface display allows high throughput screening of a diverse library and isolation of target scFvs in a very effective way, it has limitations for a thorough characterization of isolated proteins. Although binding affinities between scFv-antigen pairs can be determined either by the flow cytometer, or a fluorescence

spectrophotometer, they might not reflect the actual affinities due to following reasons. (1) While on the cell surface, analogous to a solid support, scFvs can adopt different conformations than they would adopt in solutions. (2) They can form aggregates that would complicate the analysis of stoichiometries, or actual affinities. (3) Some methods including thermal methods are not suitable for the analysis of cell surface displayed proteins. In order to characterize the DIR activating scFv K7 without such limitations we cloned the gene for K7 into a vector that allows the expression of soluble scFv in E.coli. Data demonstrated and discussed in the rest of this chapter will be based on soluble K7.

3.3.4.1 Affinity and Brightness of K7

For non covalent binding interactions of biomolecules and fluorogenic dyes, the binding affinity and the quantum yield of the fluorogenic dye upon this binding interaction become crucial features that determine the brightness, thus the practical applicability of the system. We determined the affinity of K7 for DIR by fluorescence saturation titration experiments. Figure 26 represents the titration curve obtained when aliquots of DIR was added into a 10nM K7 solution. The dissociation constant was calculated by fitting the titration curve with a 1:1 binding algorithm. Fluorescence was not corrected for background signal, because DIR alone did not have any signal within this concentration range (0-40 nM).

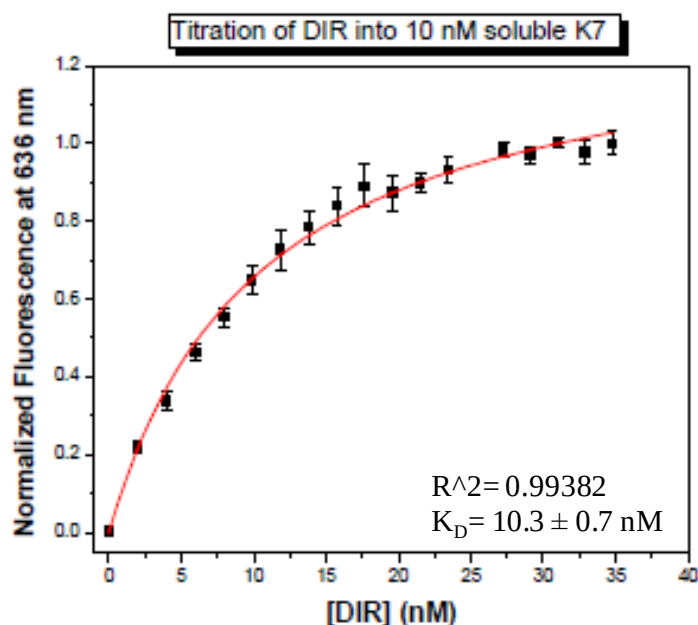


Figure 26 Fluorescence titration curve to determine the affinity of K7 for DIR

The strong affinity demonstrated on the yeast cell surface (13.9 nM) was confirmed with the soluble protein to prove that the recognition was a specific interaction between the scFv and the fluorogen, rather than being nonspecific due to any surface entities of the yeast cell.

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 ($\phi_f \sim 0.002$). 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 ($\phi_f = 0.27$) or the scFv that was isolated for its affinity for MG¹⁴⁰ ($\phi_f = 0.25$).

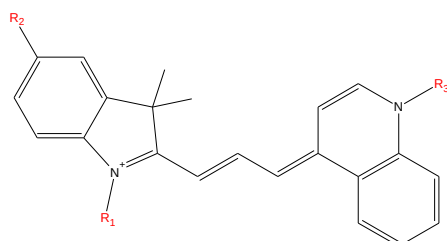
3.3.4.2 Promiscuity of K7

Based on preliminary data we obtained from yeast surface displayed K7 we investigated the potential promiscuity of this protein. We studied binding interactions between K7 and other structurally similar fluorogenic cyanine dyes. The comparison of binding interactions was based on binding affinities (K_{ds}) and quantum yields of these dyes bound to K7. In order to develop a systematic understanding of the basis of promiscuity we based our studies on two groups of fluorogenic cyanine dyes: 1) close structural analogs of DIR that emit fluorescent light of the same wavelength range as DIR, 2) fluorogenic cyanine dyes emitting light of different colors spanning the visible range.

Interactions of K7 with Close Structural Analogs of DIR:

We studied interactions of K7 with 5 structural analogs of DIR. These dyes are different from DIR by the ionic substituents attached to the indole or quinoline heterocycles which determine their bulkiness and overall charge (Table 5). The dye InQ3 which replaces the anionic propyl sulfonate group with a methyl but is the same as DIR otherwise, had similar affinity for K7, but demonstrated a very large quantum yield ($\phi_f = 0.64$) which was nearly twofold larger than the DIR quantum yield. This indicated that the binding pocket of K7 imposes a more constraining environment that holds the dye more rigid upon binding. When the anionic propyl sulfonate was replaced with a cationic tert-butylammonium substituent which changes the overall charge of 0 (DIR) to +2 (D1), the binding affinity dropped slightly, but the quantum yield increased almost twofold again ($\phi_f = 0.57$). Electrostatic interactions seemed to play an important role in the recognition, and the constraining environment of the binding pocket that determines the quantum yield. When the dyes are positively charged the quantum yield tended to be higher.

Table 5 Dissociation constants and quantum yields of DIR along with 5 structural analogs for binding to soluble K7. Dissociation constants were calculated by fitting fluorescence titration curves.



Dye	R ₁	R ₂	R ₃	K _D (nM)	Φ _f
DIR	CH ₃	H	(CH ₂) ₃ SO ₃ ⁻	10	0.34
InQ3	CH ₃	H	CH ₃	11	0.64
D1	CH ₃	H	(CH ₂) ₃ N ⁺ (CH ₃) ₃	45	0.57
D3	(CH ₂) ₅ COO ⁻	H	(CH ₂) ₃ SO ₃ ⁻	257	0.06
D4	(CH ₂) ₅ COO ⁻	SO ₃ ⁻	(CH ₂) ₃ SO ₃ ⁻	No activation	No activation
D5	(CH ₂) ₃ SO ₃ ⁻	H	(CH ₂) ₃ N ⁺ (CH ₃) ₃	897	0.06

While modifications on the quinoline heterocycle were well tolerated by the K7 binding pocket, this was not true when the indole ring was modified with substituents. The dye D4 with two bulky substituents on the indole heterocycle (giving an overall charge of -2) was not being activated by K7. Also, dyes D3 and D5 with only one substituent on the indole heterocycle with different overall charges (-1, and +1, respectively) exhibited very low binding affinities for K7 and extremely low quantum yields (Φ_f = 0.06).

These data might suggest that the DIR is located in the binding pocket of K7 in a way that holds the indole heterocycle more tightly, where the quinoline ring has more flexibility. Furthermore, the results indicate that amino acids within the binding pocket interact with DIR and its analogs at least in part through electrostatic

interactions since positively charged dyes are better tolerated than negatively charged dyes.

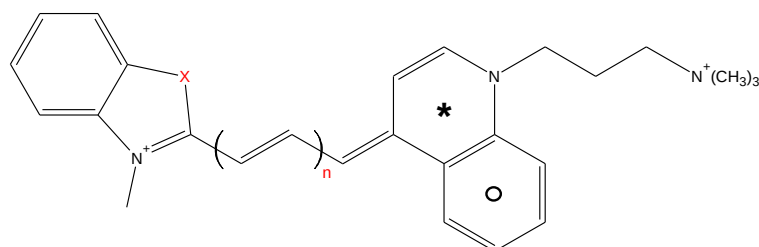
Interactions of K7 with Fluorogenic Cyanine Dyes of Different Spectral Characteristics:

We next studied binding interactions between K7 and dicationic fluorogenic cyanine dyes that are commercially available from Invitrogen Inc. They are mostly applied as nucleic acid stains for in-vitro studies or in flow cytometric, or microscopic studies in fixed cells. Since they are impermeable to live cells but permeable to compromised cell membranes, they also found applications as dead cell stains.

As demonstrated in Table 6, these fluorogenic dyes have varying heterocycles (benzoxazole, benzothiazole, quinoline, or pyridinium) and varying methine bridge lengths that connect the asymmetric heterocycles. Spectral properties, especially absorption and emission wavelengths are being tuned due to these structural changes. Fluorescence spectra of these fluorogenic cyanine dyes span the visible spectrum from violet to near infrared.

Dissociation constants were determined by fluorescence titration experiments where aliquots of K7 were titrated into a solution of the fluorogenic dye. This reverse titration approach eliminates the issue of the background signal caused by the dyes alone so that no signal corrections need to be done. To calculate dissociation constants, resulting titration curves were fitted with a 1:1 binding algorithm with a ligand depletion model¹⁴⁹. The use of this model is necessary if the concentration of the dye in the fluorescence cuvette is similar or higher than the dissociation constant of the binding interaction. Because under these conditions the scFv that is titrated depletes the dye solution due to the strong affinity, thus one should take it into account that the dye concentration does not stay the same throughout the titration.

Table 6 Dissociation constants and quantum yields of dicationic fluorogenic cyanine dyes in comparison to D1 that is the analog to DIR with +2 charge. Standard deviations for the quantum yields were ≤ 0.02



<i>Dye</i>	<i>X</i>	<i>n</i>	<i>heterocycle</i>	<i>K_D</i> (nM)	ϕ_f
D1	C(CH ₃) ₂	1	*○	45 ± 5	0.57
YO-PRO-3	O	1	*○	81 ± 9	0.50
TO-PRO-3	S	1	*○	50 ± 7	0.32
PO-PRO-3	O	1	*	60 ± 7	0.34
YO-PRO-1	O	0	*○	100 ± 12	0.44
TO-PRO-1	S	0	*○	20 ± 5	0.36
PO-PRO-1	O	0	*	221 ± 27	0.07
TO-PRO-5	S	2	*○	36 ± 8	N/A

Although the scFv K7 was isolated from the library for its ability to bind and activate DIR, it also demonstrated strong affinities for the dicationic fluorogenic cyanine dyes that were studied. For convenience, Table 6 represents the dissociation constants and quantum yields in a comparison to D1; the dicationic analog to DIR. YO-PRO-3 is structurally very close to D1 by having the same methine bridge length, and the same quinoline heterocycle. The only difference is that the quaternary carbon and two methyl groups are replaced by an oxygen atom. While this change led to a decrease in binding affinity (K_d increased from 45 nM to 81 nM), the quantum yield did not change significantly, indicating the dye was still being held very rigidly. Further analysis of interactions revealed that the affinity and quantum yields were

determined by a combination of structural features rather than being based on a single one. An example supporting this conclusion was the interaction of K7 with PO-PRO-3 vs. PO-PRO1. While PO-PRO-1 with its pyridinium ring demonstrated the lowest affinity and very low quantum yield ($K_d = 221$ nM, $\phi_f = 0.07$), PO-PRO-3 with the same methine bridge length as D1 but still with the pyridinium ring regained the affinity and the brightness ($K_d = 60$ nM, $\phi_f = 0.34$). Similarly, the significant difference between TO-PRO-1 and PO-PRO-1 in terms of binding affinity and quantum yield is not observed when the dyes TO-PRO-3 and PO-PRO-3 are compared. The longer length of the methine bridge can compensate for the loss in the affinity and brightness that was caused by the pyridinium heterocycle. It is also noteworthy that TO-PRO-5 that is a larger dye than DIR with a 5 carbons long methine bridge is also well tolerated by the K7 binding pocket as indicated by a strong affinity (36 nM).

Although the exact nature of the interactions between the fluorogenic dyes and K7 is difficult to reveal without structural data, our affinity and quantum yield studies demonstrated that the K7 has a flexible binding pocket that can fit dyes of different substituents, sizes and electrostatic charges well. The consequence of this promiscuity in terms of a technological point of view is that the scFv K7 isolated for its affinity for a certain dye can be used in combination with any of these dyes depending on the spectral requirements of an experiment. Thus, isolation of a single scFv equipped us with a rainbow of fluoromodules. Such versatility in the choice of the fluorogen makes K7 a powerful component of fluoromodules.

Investigation of promiscuity in protein ligand interactions is a relatively new field that attracts increasingly more attention. The old understanding of one protein having one function is changing slowly as more examples of promiscuous proteins in biological events are being revealed¹⁵⁰. Promiscuity in ligand interactions is mostly

related to structural flexibility. As opposed to the long believed theory of induced fit, and lock-and-key-models, now more studies demonstrate that protein-ligand interactions can be explained on a different basis. According to this model, there are preexisting protein conformations of varying energy minima; depending on the ligand one of these conformations becomes energetically more favored, and the complex is formed by shifting the equilibrium toward that conformation^{151, 152}. Such a model can also explain the basis of promiscuity of K7 that can interact with fluorogenic dyes of different structures.

Another potential explanation for the promiscuity of K7 can be the partial recognition of the fluorogenic dyes by the protein binding pocket. If only a certain region of the dyes is being recognized by the proteins, the structural changes on the dyes might be better tolerated, and thus lead to promiscuity. In the case of K7, strong affinities were mostly associated with large quantum yields. This indicates that the dyes were rigidified if they are within the binding pocket which renders partial recognition unlikely. On the other hand, the relationship between affinity and quantum yield is more complex since quantum yield is determined by the conformations of dyes within the binding pocket, rather than how tightly they are held.

In order to rule out the explanation based on multiple binding sites on K7 we performed an experiment to monitor the competition of DIR and TO-PRO-1 for a K7 binding site. Fluorescence titrations of K7/TO-PRO-1 were performed in the absence and presence of excess DNA (Figure 27). The affinity of K7 for TO-PRO-1 dropped when DIR was present since TO-PRO-1 had to compete with DIR for the binding site on K7. This result clearly indicated that there is one single binding site on K7 for the fluorogenic dye binding.

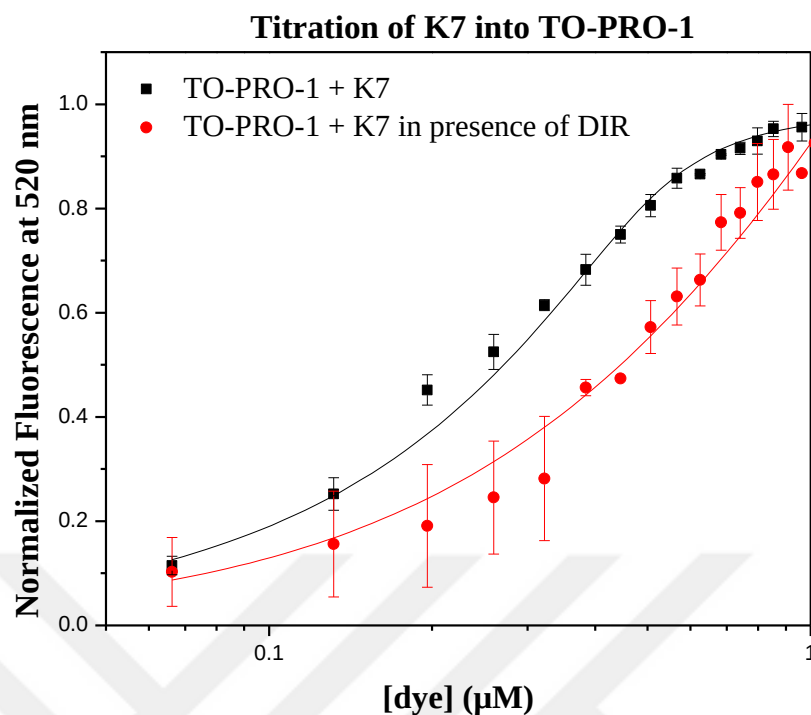


Figure 27 Fluorescence titration curves of TO-PRO-1 and soluble K7 in the absence and presence of 1μM K7. Solid lines represent the fits obtained from a 1:1 binding algorithm.

3.3.4.3 Thermal Stability of K7 based Fluoromodules

To further analyze the scFv K7 in terms of its features leading to promiscuity we studied the thermal stability of the protein w/ and w/o the fluorogenic dyes.

3.3.4.3.1 Stability based on intrinsic fluorescence

Changes in the intrinsic tryptophan fluorescence can be used to monitor structural changes in proteins. Quantum yield and the wavelength of emission maximum are sensitive to the tryptophan environment. The fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent decreases. Tryptophan residues which are buried in the

hydrophobic core of proteins can have spectra which are shifted by 10 to 20 nm compared to tryptophan residues on the surface of the protein⁴.

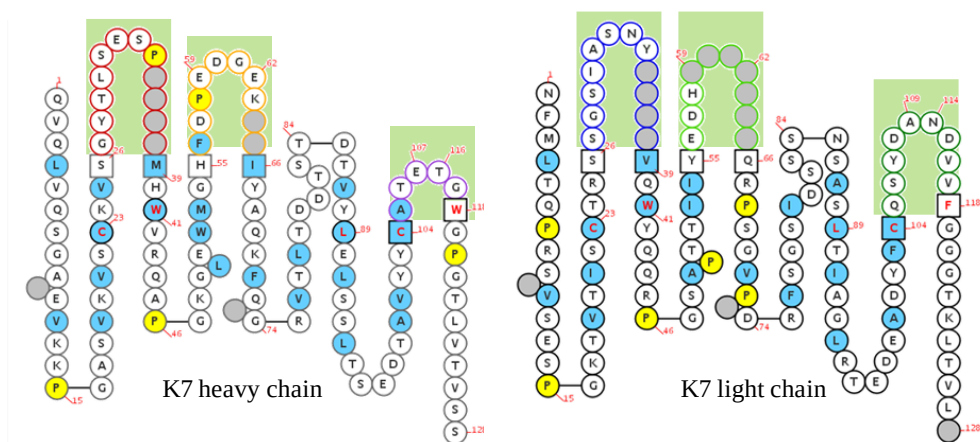


Figure 28 Collier de Perles representation of K7 amino acid sequences. Boxes indicate where the CDRs are.

K7 was analyzed on IMGT/V-QUEST¹⁵³ to determine the locations of the 4 tryptophan residues. IMGT/V-QUEST is online statistical analysis software that compares the protein sequence to other available immunoglobulins and determines the complementary determining regions (CDRs) that are expected to be in contact with the antigen. Figure 28 depicts the Collier de Perles demonstrations of the heavy and light chains of K7 which are 2-D graphical representations of immunoglobulin amino acid sequences. They allow easy visualization of CDR regions. Out of 4 tryptophan residues, only one is in the CDR of the light chain. The other 3 residues are located in the framework region of the protein.

We performed thermal studies to evaluate the stability of K7 in the absence and presence of bound DIR. The shift in the wavelength of emission maximum, and the fluorescence intensity at the emission maximum were monitored as the temperature was raised from 15⁰C to 65⁰C in 5⁰C intervals (Figure 29). While the fluorescence intensity of tryptophan residues in K7 decreased over a broad range

throughout heating without a significant transition temperature, the shift in the wavelength was rather cooperative, demonstrating a transition temperature at 45⁰C (Figure 29 A). At increasing temperatures more tryptophan residues were exposed to water leading to a quenching effect. The fact that the wavelength shift and fluorescence intensity profiles do not match might suggest that the thermal unfolding of K7 does not occur cooperatively. Some partially unstructured regions of K7 might cause such an effect and lead to unfolding over a broad temperature range. A significant red shift of the emission maximum occurred when the global protein unfolded.

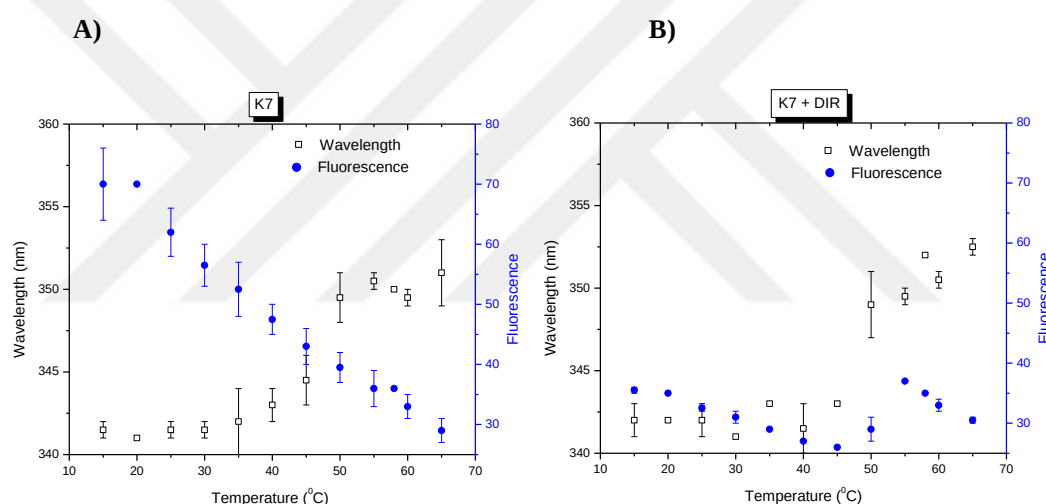


Figure 29 Thermal denaturation curves of tryptophan residues on A) K7, B) K7 bound to DIR. Emission spectra obtained at each temperature by exciting solutions at 280 nm. [K7]= 0.5 μ M, [DIR] = 1 μ M. Open squares demonstrate the wavelength at the emission maximum, closed circles represent fluorescence intensity.

On the other hand, when the K7 was bound to saturating amounts of DIR, changes in the fluorescence intensity due to heating demonstrated a different profile. The initial fluorescence intensity of the tryptophan residues which was lower than in K7 alone, stayed stable until 50⁰C and then increased with a transition temperature

that matched the transition in the wavelength shift. The presence of the DIR bound to the K7 exerted changes on the conformations of tryptophan residues or their environment, either by interacting with them, or by stabilizing the protein.

Figure 30 A demonstrates the emission spectra of a K7 solution alone, and a K7 solution saturated with DIR at 25°C. As mentioned previously, the tryptophan fluorescence was quenched when the protein was bound to DIR. The emission spectrum also broadened in the presence of DIR, indicating more tryptophan residues with different wavelength shifts are contributing to the emission spectrum.

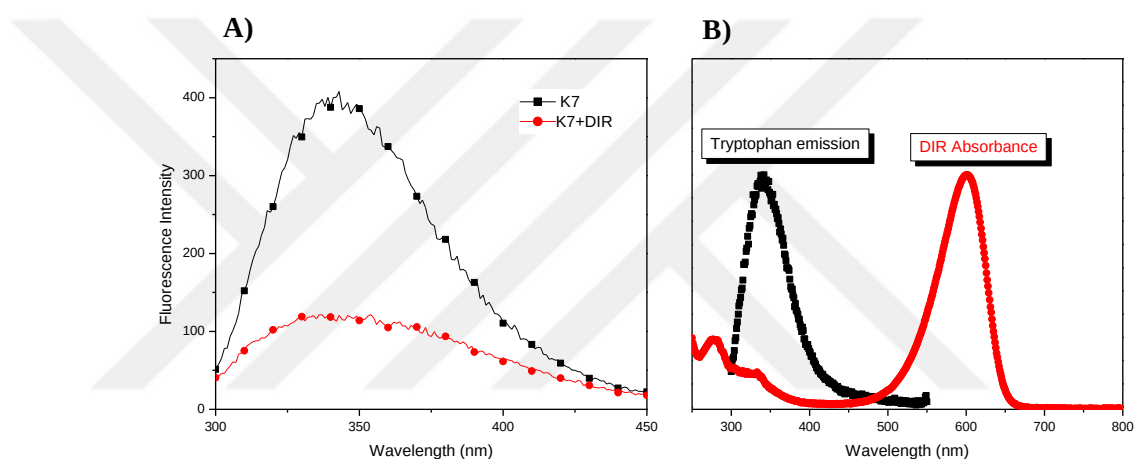


Figure 30 A) Fluorescence emission spectra of 0.5 μ M K7 (squares), and 0.5 μ M K7 bound to 1 μ M DIR. $\lambda_{ex.}$ = 280 nm. B) FRET overlap for tryptophan and DIR. Tryptophan solution was excited at 280 nm.

The quenching of tryptophan fluorescence upon binding to DIR might occur due to contributions from different processes. First of all, the binding of DIR to K7 might impose a conformational change to the protein that exposes more tryptophans to water which leads to a decrease in the overall intensity of tryptophans. The fact that there is only one tryptophan residue within the CDRs, but three tryptophan residues in the framework suggests that such a conformational change, if any, affects the whole protein secondary structure, rather than affecting only the antigen binding

site. Furthermore, the fluorescence can be quenched by the direct interaction of DIR with the tryptophans through excited state electron transfer. Tryptophans directly in contact with DIR might be contributing to quenching through this mechanism.

The second potential reason for the quenching is Förster Resonance Energy Transfer (FRET) from tryptophans to DIR. Figure 30 B demonstrates the spectral overlap between the tryptophan emission and DIR absorption spectra. Although there is minimal spectral overlap, the potentially close proximity between tryptophan residues and the DIR within the binding pocket can cause FRET, and thus quenching of tryptophan fluorescence. The 280 nm excitation wavelength also led to direct excitation of DIR which made it difficult to evaluate if FRET was responsible for the quenching. For a more thorough analysis of the possibility of FRET, excitation spectra of DIR in glycerol, and bound to K7 need to be obtained in order to evaluate any potential contribution of the tryptophan residues to the emission in the red region.

We utilized shifts of tryptophan emission wavelengths upon heating to compare the stabilities of K7 with fluorogenic dyes of different affinities for K7. Cooperative transition temperatures determine melting temperatures (T_m) of protein-dye complexes as indicators of thermal stabilities. When K7 was saturated with DIR there is not a significant increase in the T_m . On the other hand, when K7 was saturated with D1, the T_m for K7 alone (45⁰C) increased to 55⁰C (Figure 31).

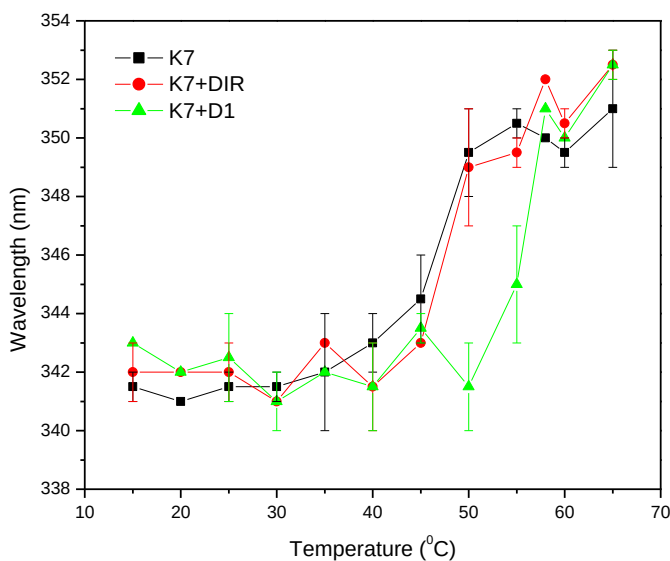


Figure 31 Melting curves of K7 (squares), K7+DIR (circles), and K7+D1 (triangles). Wavelength of emission maxima were monitored at each temperature by excitation at 280 nm. [K7]= 0.5 μ M, [DIR], [D1] = 1 μ M.

The fluorogenic dye D1 that is a dicationic analog of the uncharged DIR had a weaker binding affinity than DIR for K7 although its quantum yield was twofold higher. Apparently, the improved stability contributed to an environment that rigidifies D1 to a higher extent.

3.3.4.3.2 Stability based on extrinsic fluorescence

The fluorogenic nature of the dyes studied allowed us to investigate K7 stability bound to different dyes by fluorescence melting experiments. We monitored fluorescence intensities of fluorogenic dyes bound to K7 as we slowly raised the temperature (1 $^{\circ}$ C/min). Concentrations of fluorogenic dyes were at least 5 times higher than dissociation constants to ensure that K7 was saturated with the fluorogenic dye in each solution. Initial fluorescence intensities due to activation upon binding to K7 decreased as the K7 unfolded by the increasing thermal energy and the

dye dissociated from the binding site (Figure 32, Table 7). Fluorescence decay profiles for dyes D1, TO-PRO-3, and YO-PRO-3 demonstrated cooperative melting transitions. These dyes formed the most stable complexes with K7 with T_m 's 51.1 $^{\circ}\text{C}$ - 54.5 $^{\circ}\text{C}$. K7 complexes formed with other fluorogenic dyes investigated, including DIR, demonstrated lower T_m s below 50 $^{\circ}\text{C}$ with slightly different melting profiles. Instead of a cooperative transition in fluorescence intensity, the fluorescence intensity started decreasing at lower temperatures demonstrating a multi phase unfolding of the protein. For the dye PO-PRO-1 that had the lowest affinity for K7 (221nM), and the lowest quantum yield (0.07), a sharp transition was barely observed, instead it exhibited a noncooperative fluorescence decay over increasing temperatures.

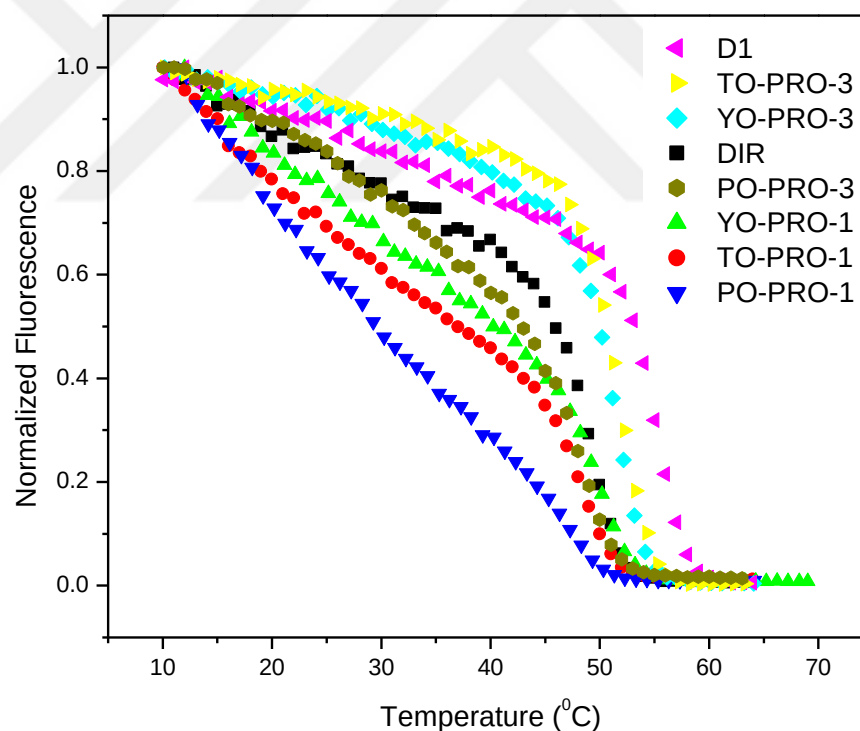


Figure 32 Fluorescence melting curves obtained by heating K7-fluorogenic dye solutions from 15 $^{\circ}\text{C}$ to 65 $^{\circ}\text{C}$ with 1 $^{\circ}\text{C}/\text{min}$. [K7] = 0.5 μM , [fluorogenic dye]= 1 μM . Excitation and emission wavelengths used are shown in Table 1.

Table 7 Melting temperatures (T_m s) of K7 bound to different dyes along with the corresponding binding affinities (K_D s) and quantum yields.

fluorogen	K_D (nM)	ϕ_f	T_m ($^{\circ}\text{C}$)
DIR	10 ± 0.7	0.34	48.9
D1	45 ± 5	0.57	54.5
TO-PRO3	50 ± 7	0.32	52
YO-PRO-3	81 ± 9	0.50	51.1
PO-PRO-3	60 ± 7	0.34	48
TO-PRO-1	20 ± 5	0.36	48.5
YO-PRO-1	100 ± 12	0.44	50.2
PO-PRO-1	221 ± 27	0.07	unstable

These differences in fluorescence melting profiles of fluorogenic dyes bound to the same protein suggest that K7 has unique features that need to be analyzed thoroughly. In order to understand whether these different melting profiles were caused by the temperature dependence of fluorogenic dye quantum yields, we performed the same experiment where we used solutions of the same dyes in glycerol. When dyes were in glycerol, the fluorescence intensity demonstrated a rapid decay at low temperatures as demonstrated by DIR, D1, and TO-PRO-3 (Figure 33). Melting curves did not exhibit any difference indicating that loss of quantum yield at increasing temperatures is not dependent on the dye. On the other hand, melting curves obtained from the same dyes bound to K7 exhibited transitions in fluorescence at different temperatures (Table 7). This result suggests that the different fluorescence melting patterns of K7/fluorogen complexes are caused by the different binding interactions rather than the differences in the quantum yields.

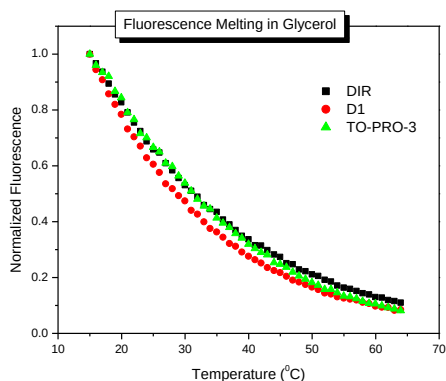


Figure 33 Fluorescence melting curves of DIR, D1, and TO-PRO-3 in glycerol.

The fluorescence decay of some fluorogenic dyes bound to K7 at lower temperatures, rather than exhibiting a cooperative melting transition supports data obtained from intrinsic fluorescence. Partially unstructured regions of K7 might lead to a multi phase transition of fluorescence. These partially unstructured regions are possibly stabilized to different degrees by different fluorogens. Such stabilization due to the fluorogen also relates to how tightly the fluorogen is held within the K7 binding pocket, as seen from the positive correlation of high quantum yields and T_{ms} .

3.3.4.4 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is a common technique for studying molecular recognition processes, and the technique of choice for determining the enthalpic and entropic contributions to the overall free energy of binding ΔG^0 ¹⁵⁴. It is a popular analytic method since it only requires a measurable enthalpy change upon complexation which is easily applicable to most molecular recognition systems.

We used ITC to gain a thermodynamic insight that would help us understand the promiscuous behavior of K7. Binding of K7 to DIR, D1 and TO-PRO-1 were studied by titrating aliquots of the K7 into a solution containing the fluorogenic dye.

A representative thermogram is demonstrated in Figure 34. Fitting of the titration curves with a 1:1 binding algorithm resulted in thermodynamic parameters shown in Table 8.

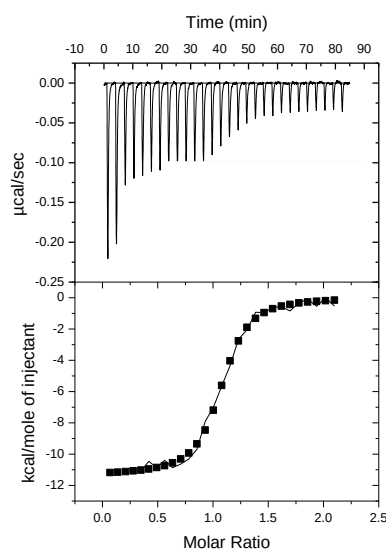


Figure 34 ITC thermogram obtained from DIR/K7 titration.

Table 8 Thermodynamic data obtained from isothermal titration calorimetry

Dye	K_D(nM) (calorimetry)	n	ΔH (kcal/mol)	ΔS (cal/mol/deg)
DIR	35	1.06	-11.34	-3.94
D1	14	1.08	-16.05	-18.05
TO-PRO-1	20	1.09	-12.92	-8.25

All three K7-fluorogen complexes demonstrated 1:1 stoichiometry, and dissociation constants similar to ones obtained from fluorescence titrations within an error range of ± 31 nM. The binding of fluorogen D1 to K7 led to a more negative enthalpy, and a significantly more negative entropy change than the binding of other two fluorogens. Although it is a complex process to explain just based on ITC data, D1 appeared to demonstrate a significantly different binding interaction with K7. Our

previous data on the higher quantum yield and thermal stability of D1 upon binding to K7 relative to DIR also agrees with the assumption of a different binding interaction.

The overall energy of binding of K7 to the fluorogen can be represented with the following equation:

$$\Delta G_{\text{bind}} = \Delta H_{\text{bind}} - T\Delta S_{\text{bind}} = \Delta H_{\text{bind}} - T(\Delta S_{\text{protein}} + \Delta S_{\text{fluorogen}} + \Delta S_{\text{solvent}})$$

The entropy change due to the protein can also be subdivided into conformational, rotational, and translational entropy changes¹⁵⁵. The conformational entropy change has been related to promiscuity of proteins before¹⁵⁶ as a linear relationship between the total conformational entropy and the number of alternative available contacts for various ligands. In order to investigate the promiscuity of K7 from an entropic view, NMR studies, and empirical calculations will be required.

3.4 Conclusions and Outlook

In this chapter we demonstrated the development and characterization of a novel fluoromodule that has strong potential as a genetically encodable fluorescent label. The protein component of the fluoromodules, the scFv K7 exhibited promiscuous behavior in that it can bind to and activate structurally similar fluorogenic cyanine dyes with different spectral properties. Analysis of interactions of K7 with these fluorogenic dyes with spectral and thermal methods suggested a flexible structure of K7 with partially unstructured regions that are being stabilized to different degrees with different ligands, as the basis of the promiscuity.

While the promiscuity of K7 provides versatility in terms of applications of K7 based fluoromodules, it also opens up the basis for improvement of K7

fluoromodules for cellular applications. The power of synthetic chemistry allows us to modify fluorogenic dyes to have improved features like higher photostability, lower aggregation tendency, or cell permeability. The promiscuity of K7 will allow us to incorporate new DIR analogs tailored according to the needs of the application along with the inherent fluorogenecity. Previously, a DIR analog that was modified with an α -CN group for improved photostability was shown to exhibit a strong affinity (63 nM) and a moderate quantum yield ($\phi_f = 0.18$) when bound to K7¹⁵⁷. The promiscuity of K7 provided a platform to analyze the viability of this new fluorogenic dye for future applications in fluoromodules. Affinity and brightness of fluoromodules composed of K7 and newly developed fluorogenic dyes can always be improved by employing directed molecular evolution.

In order to use DIR fluoromodules as genetically encodable fluorescent labels *in vivo*, DIR will be tailored with functional groups that allow its entry into cells without disturbing the viability of cells. Incorporation of a cell permeable DIR analog into fluoromodules will reinforce the power of DIR fluoromodules.

3.5 References

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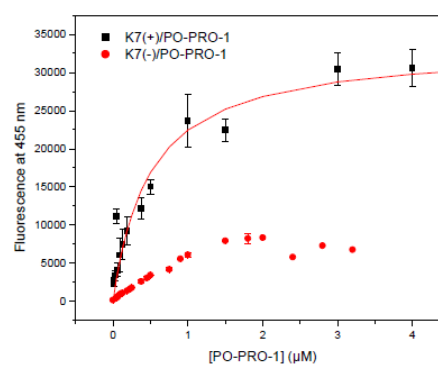
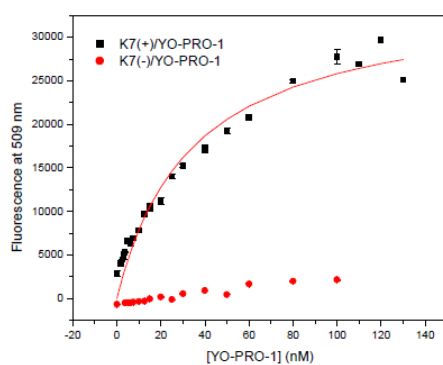
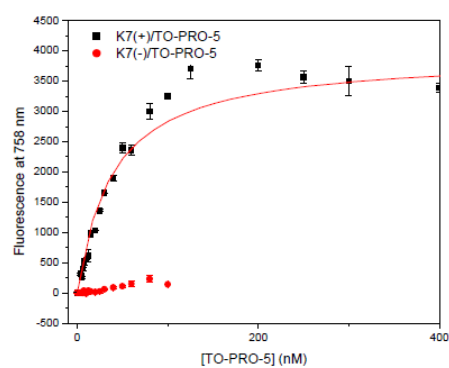
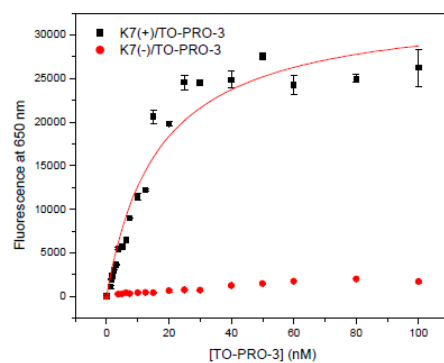
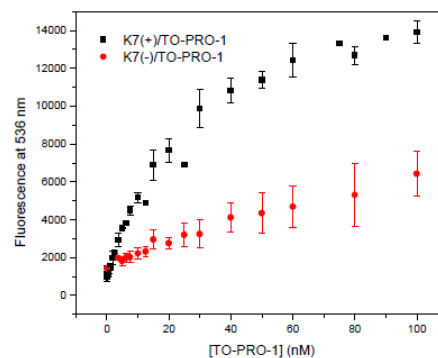
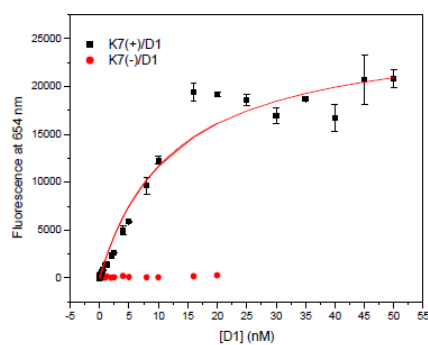
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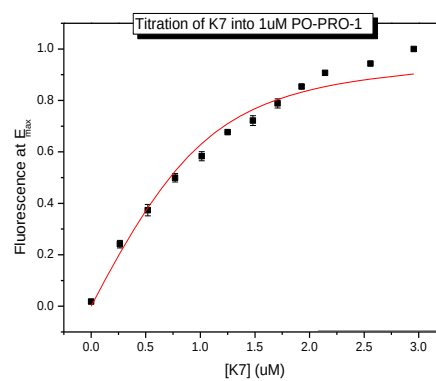
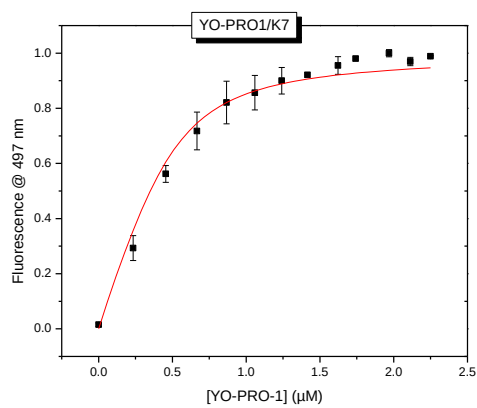
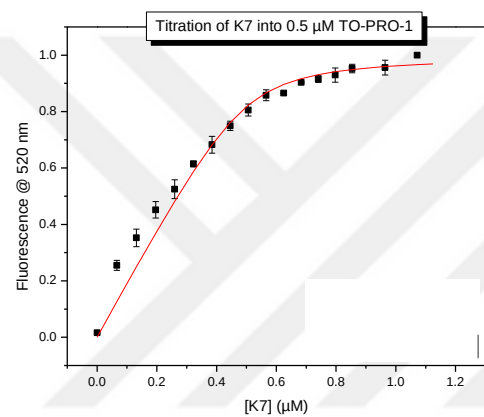
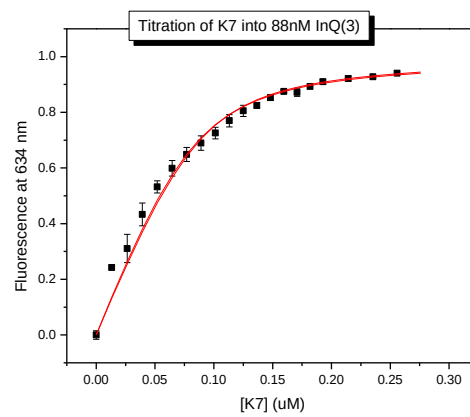
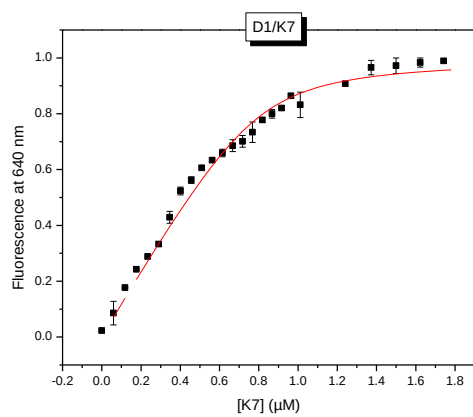


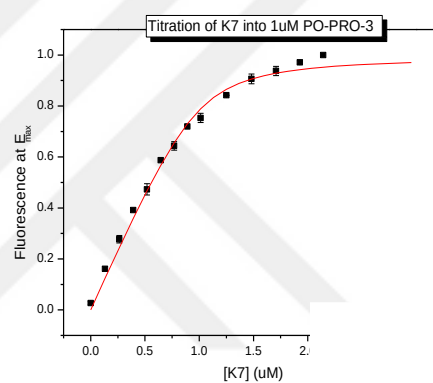
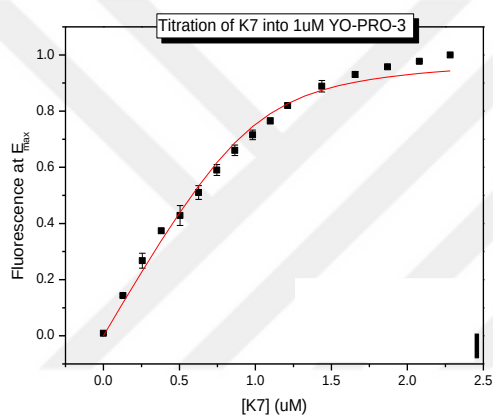
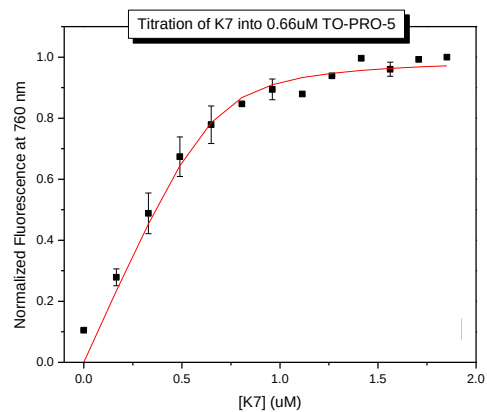
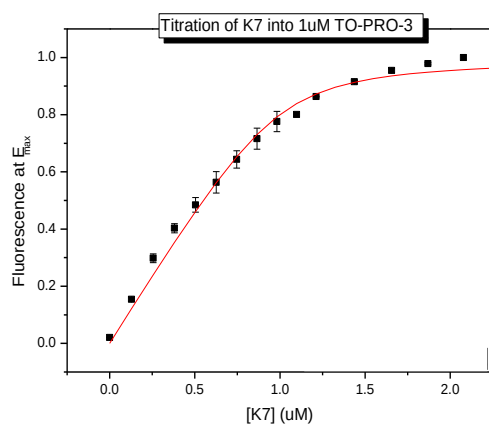
3.6 Appendix

3.6.1 Surface Displayed K7 Titration curves



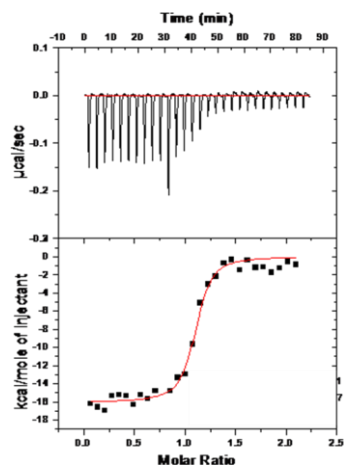
3.6.2 Soluble K7 Titration Curves



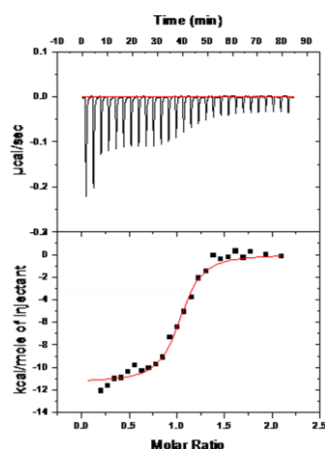


3.6.3 ITC Data

D1



TO-PRO-1



Chapter 4: Engineering Affinity and Selectivity in Fluorogen Activating scFvs

4.1 Introduction

Directed molecular evolution has become a versatile approach that allows scientists to design and develop proteins with desired functions. A protein of interest can be engineered to function differently in terms of its affinity, specificity, expression, stability, or catalytic activity¹⁵⁸⁻¹⁶⁰. While engineered proteins are being utilized in various biotechnological applications they also serve as crucial tools for the basic investigation of structure-function relationships of the proteome¹⁶¹. Directed evolution of proteins involves three steps¹⁶². (i) Creating diversity on the parent protein by employing DNA mutagenesis and recombination. (ii) Screening the mutagenized library by a suitable display technology and selecting for the function that is desired to be improved. (iii) Amplification of selected proteins for the next round of screening/selection.

Directed molecular evolution rapidly generates mutant proteins with tailored functions without the need for prior structural or functional knowledge. With advances in molecular biology tools, and sensitive high-throughput screening technologies, this field grows rapidly and impacts chemical, pharmaceutical and agricultural sciences.

Directed evolution has also been applied to aptamers and nucleic acid enzymes to develop new molecules with desired physical, or biochemical properties, and catalytic function. Aptamer studies mostly focused on altering the specificity by introducing mutations into the sequence space¹⁶³. Examples on the directed evolution on enzymes include RNA enzymes that catalyze the template-directed ligation of RNA, or DNA enzymes that catalyze the cleavage of RNA¹⁶⁴.

Despite the large numbers of studies reported on engineering of affinity and stability of proteins, there are only a few examples on successful engineering of ligand selectivity¹⁶⁵. Mostly, substrate selectivity and specificity of enzymes were altered using directed evolution in different display formats¹⁶⁶. Due to the presence of complex relationships between structure and substrate selectivity, progress in protein engineering for selectivity has been rather slow. An impressive achievement was engineering of a horseradish peroxidase enzyme that was not enantioselective into highly L- and D-enantioselective variants by a single amino acid mutation¹⁶⁷. This study along with other examples¹⁶⁸ employed negative selections where selection of proteins with the undesired function is suppressed. Negative selections within directed molecular evolution of proteins provide an opportunity to tune the specificity.

We employed directed molecular evolution to improve the affinity and the selectivity of fluorogen activating proteins. Fluorogen activating proteins are single chain variable fragment (scFv) antibodies that are isolated from a library for their ability to light up fluorogenic dyes when bound^{169, 170}. Dyes that are recognized by these proteins include specifically designed fluorogenic cyanine dyes that are not fluorescent in aqueous buffers. Only when they are in the restricting environment of a suitable binding pocket, or in a viscous solution, they release excitation energy through fluorescence emission¹⁷¹. Combining these fluorogenic dyes with proteins that are isolated from a pool of proteins for their ability to activate those dyes results in versatile fluorescent labels. While these proteins have already been proven successful as cell surface protein labeling probes, they have a large potential as genetically encodable fluorescent labels in cellular imaging applications. Thus they are strong alternatives for other genetically encodable labels such as fluorescent proteins and tetracysteine peptides.

We previously reported Dimethylindole Red (DIR) activating proteins that demonstrated varying degrees of low nanomolar affinities for DIR¹⁶⁹. While these proteins were isolated for their ability to activate DIR, they also exhibited strong affinities for other structurally similar cyanine dyes that emit light at a different wavelength. While this promiscuous behavior of the DIR activating proteins provides versatility, it is also desirable to develop proteins that can activate fluorogens selectively. Having a toolbox of selectively binding protein/fluorogenic dye pairs can further expand the application areas of these fluoromodules.

In this study, we aimed to engineer the affinity and the selectivity of DIR activating proteins using molecular evolution methods. We investigated how selection strategies affect binding characteristics of mutant proteins, and the relationship between mutations and functional changes in protein binding pocket.

4.2 Experimental

4.2.1 Sequences

Amino acid sequences of scFvs:

A8 (parent):

EVQLVESGGRLVKPGGSLRLSCEASGFTFSDYY**M**GWIR
QAPGKGLEWVS YINGDSSYTNYTDSVKGRFSISRDNK
NSLYLQLSRLRAEDTAVYYCVTSRGSSDSWYRVHWGQ
GTLVTVSSGILGSGGGGSGGGGSGGGGSSYELTQSPSA
SGTPGQRVTISCSGSSSNIGSQYVYWYQQLPGTAPKLLI
YKNNQRPSGVPDRFSGSKSGTSASLAISGLRSEDEADY
YCAAWDDSLSGPVFGGGTQLTVLS

Mutant (P):

EVQLVESGGRLVKPGGSLRLSCEASGFTFSDYY**Met**GWIR
RQAPGKGLEWVS YINGDSSYTNYTDSVKGRFSISRDNK
KNSLYLQLSRLRAEDTAVYYCVTSRDSSDSWYRVHWG
QGTLVTVSSGILGSGGGGSGGGGSGGGGSPYELTQSPS
ASGTPGQRVTISCSGSSSNIGSQYVYWYQQLPGTAPKL
LIYKDNQRPSGVPDRFSGSKSGTSASLAISGLRSEDEAD
YYCAAWDDSLSGPVFGGSTQLTVLS

Mutant (P+N):

EVQLVESGGRLVKPGGSLRLSCEASGFTFSDYYMGWIR
QAPGKGLEWVS YINGDSSYTNYTDSVKGRFSISRDN AK
NSLYLQLSRLRAEDTAVYYCVTSRDSSDSWYRVHWGQ
GTLVTVSSGILGSGGGGSGGGGSGGGGSSYELTQSPSA
SGTPGQRVTISCSGSSSNIGSQYVYWYQQLPGTAPKLLI
YKNNQRPSXVPDRFSGSKSGTSASLAISGLRSEDEADY
YCAAWDDSLSGPVFGGSTQLTVLS

Error-prone PCR primers:

Forward primer (nucleotides 1115-1168 of pPNL6 vector)

5'-gac aat agc tcg acg att gaa ggt aga tac cca tac gac gtt cca gac tac gct ctg cag-3'

Reverse primer:

5'-gat ctc gat gcg gcc gcc gag cta tta caa gtc ttc ttc aga aat aag ctt ttg ttc tag aat tcc g-3'

4.2.2 Media Recipes

Yeast growth medium (pH 4.5):

20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto casamino acids, 14.7 g sodium citrate and 4.29 g citric acid monohydrate were dissolved in deionized H₂O to a volume of 1 liter and sterilized by filtration. Uracil (20mg/L) was added since JAR200 strain requires it for growth.

Yeast induction medium:

Prepared as for the yeast growth medium, but used 20 g galactose instead of dextrose. Galactose induces the expression of scFvs which is under control of the GAL1-10 promoter.

4.2.3 Constructing the mutagenic library

Constructing the mutagenic PCR insert:

The plasmid of E.coli cells expressing A8 was extracted using QIAprep Spin Miniprep Kit (Qiagen). Extracted plasmid was diluted 100X and used as the template for the error-prone PCR reaction along with following reagents: 1X Taq buffer, 2mM MgCl₂, 0.5 μM forward primer, 0.5μM reverse primer, 200μM dNTPs, 1μL template,

2 μM 8-oxo0dGTP, 2 μM dPTP, 0.05 U μL^{-1} Taq DNA polymerase, and water in 50 μL final volume. DNA was amplified using the following conditions:

- 1) 94 $^{\circ}\text{C}$ initial denaturation for 3 min
- 2) 94 $^{\circ}\text{C}$ for 45 s
- 3) 60 $^{\circ}\text{C}$ for 30 s
- 4) 72 $^{\circ}\text{C}$ for 90 s
- 5) Repeat 2-4 10X
- 6) 72 $^{\circ}\text{C}$ for 10 min

The PCR product was gel-purified using a 1% agarose gel. The DNA band about 900 bp was cut and extracted using QIAquick gel extraction kit (Qiagen). The recovered mutagenic PCR insert was amplified to yield sufficient DNA for yeast transformation with the following reagents: 1X Taq buffer, 2mM MgCl_2 , 0.5 μM forward primer, 0.5 μM reverse primer, 200 μM dNTPs, 4 μL extracted PCR product, 0.05 U μL^{-1} Taq DNA polymerase and water in 50 μL final volume. The same conditions were used for the PCR as the mutagenic PCR except DNA was amplified 30X instead of 10X. PCR product was gel purified.

Vector preparation for homologous recombination:

pPNL6 vector was prepared for homologous recombination. 75 μg of vector was digested along with 40 μL 10X NEB buffer 2, 4 μL 100X BSA, 6 μL 20u/ μL Bam, 10 μL 10u/ μL Nhe1, and enough water to bring up to 400 μL . The DNA was digested for 3 hours and the following reagents were added: 5.25 μL 4M NaCl, 10 μL Sa1, 2.5 μL 10X NEB buffer 2, and enough water to bring up to 420 μL . The DNA was digested for 3 more hours. Digested vector DNA was precipitated using EtOH/Ammonium Acetate and resuspended in 50 μL water.

Yeast transformation:

Yeast cells were transformed with the mutagenic PCR insert and the digested vector. JAR200 yeast cell strain was inoculated into 5 mL of 2X YEPD medium and incubated overnight at 30 $^{\circ}\text{C}$. 2.5×10^8 cells were transferred into 50 mL fresh medium

so that the final concentration is 5×10^6 cells/ml and incubated at 30°C until the cell titer was at least 2×10^7 cells/mL. Cells were harvested by centrifugation, washed with 25 mL sterile water, and resuspended in 1 mL sterile water. The following reagents were mixed for each transformation:

- 1) 50 μL of boiled 2 mg/mL Deoxyribonucleic acid Sodium Salt Type III from Salmon Testes
- 2) 100 μL harvested yeast cells, centrifuged, supernatant removed
- 3) 240 μL 50% w/v PEG 3500
- 4) 36 μL 1M LiAc
- 5) 34 μL plasmid DNA+ water
- 6) 360 μL water

For the plasmid+water mixture we used 9 μg PCR insert and 1 μg digested vector. Transformation mixture containing the mutagenic PCR insert, along with control transformations were incubated at 42°C for 40 min. Cells were harvested by centrifugation and resuspended in 1.0 mL sterile water. Cells were diluted in yeast growth medium 1X, 10X, 100X and plated on selective yeast growth plates. Colonies were counted after 4 days to determine the size of the mutagenic library. The size of the library was determined to be 5.9×10^4 .

4.2.4 FACS experiments

The number of cells that would represent 10fold excess of the size of the library was calculated, and the volume of the frozen stock that corresponds to this number was inoculated into 25 mL growth medium, and then incubated at 30°C for two days. The OD of the cell culture was determined and cells were harvested by centrifugation. Induction culture was inoculated at 10^7 cells/mL in 25 mL induction medium. An uninduced control culture with the same concentration was also

inoculated in growth medium. Cultures were incubated 20°C for 2 days. After determining the OD, cells were harvested by centrifugation and resuspended in PBS with 2mM EDTA and 0.1% pF127 pH 7.4. (modified PBS) so that the final stock solution OD was 10^9 cells/mL. 10^7 cells in 500 μ L were incubated with 20 μ L of 1mg/mL anti-c-myc antibody(mouse) (Invitrogen) for 1h on ice, washed 2X with PBS and resuspended in 500 μ L modified PBS. 10 μ L Alexafluor goat anti-mouse 488nm secondary antibody was added to cells and incubated for 30 min on ice. Once cells were centrifuged and washed 2X with PBS, they were resuspended in 500 μ L modified PBS for subsequent dye labeling. Solutions containing uninduced cells, and cells that were not labeled with anti c-myc antibodies were prepared as controls.

Positive selections: Cells were labeled with 3 nM DIR and 1 μ M Propidium iodide (PI) (Invitrogen) as the dead cell indicator for 30 min and transferred into flow cytometer tubes for analysis and sorting. FACS enrichment was carried out on a Becton Dickinson FACSVantage SE with FACSDiva option. Cells were excited with a red laser, DIR fluorescence was monitored with 685/35 nm filter, PI fluorescence was monitored with 610/20 nm filter and AlexaFluor 488 fluorescence was monitored with 530/30 nm filter. Gated cells (positive in DIR, positive in alexa fluor488, negative for PI) were sorted into 25 mL growth medium for amplification. After 2-days growth, cells were induced and prepared for the next round as explained above. In the final FACS sort, cells were autocloned onto agar plates comprised of solidified induction media. After 5 days of growth at 20°C, this plate was stained with 1 μ M DIR and imaged under 635x20 nm band pass illumination from red LEDs, using an Andor DU434-BR-DD CCD camera fitted with a 680x25 nm band pass filter. Colonies showing brightest DIR fluorescence signal were picked for further analysis.

Negative selections: For negative selections anti-cmyc antibody labeling and dye labeling were not done simultaneously, but rather in separate solutions so that AlexaFluor 488 and TO-PRO-1/YO-PRO-1 signal do not interfere. Cells that will be sorted were labeled with 40 nM DIR, 100nM TO-PRO-1, and 100nM YO-PRO-1, and 1µM PI. TO-PRO-1 and YO-PRO-1 fluorescence was determined with 530/30 nm filter. In the last FACS sort, gated cells (positive for DIR, negative for YO-PRO-1 and TO-PRO-1) were autocloned onto induction plates, and randomly picked for further analysis.

4.2.5 Characterization of Mutants

Sequencing: Colonies picked for sequencing were inoculated into 5ml YPD medium and grown overnight at 30°C. Yeast plasmid was extracted using Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research). Extracted DNA was electroporated into MACH1 cells and plated onto LB+amp plates. Colonies were inoculated into 5 mL LB+Amp medium and grown overnight, plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen). Extracted DNA was sequenced by GENEWIZ sequencing services (www.genewiz.com) using the following primers for pPNL6 vector.

pPNL6 forward primer: tag ata ccc ata cga cgt tc

pPNL6 reverse primer: gta cga gct aaa agt aca gtg

Expressing scFvs on cell surface: Cells expressing mutant scFvs were stored in 1mL aliquots of freezing solution composed of 2% glycerol, 0.67% yeast nitrogen base. Cells were grown on growth plates, then a single colony was inoculated into 5 mL growth medium and incubated at 30°C for 2 days. Cells were harvested by centrifugation and an induction culture was inoculated at 10⁷cells/mL in induction

medium. Cells were induced for 2 days at 20°C, and harvested by centrifugation, stored in modified PBS at 10⁹ cell/mL concentration.

Constructing the scFv H1:

The gene for scFv H1 was constructed using restriction enzymes Nhe1 and PflF1 (NEB). DNA of parent protein was digested with these enzymes so that the heavy chain of the parent protein was extracted by gel purification. DNA of mutant (P) was digested with the same enzymes and the linearized vector with the light chain was gel purified. We ligated the heavy chain of the parent protein and the mutant (P) backbone to give the gene for H1 and transformed E.coli. Once the sequence was confirmed by DNA sequencing, yeast cells were transformed as explained before. Yeast cells displaying H1 were grown and induced as explained above.

Determination of fluorogen binding affinity to yeast surface displayed scFvs:

10⁷ induced yeast cells in 150 µL modified PBS containing fluorogen over a concentration range were assayed in triplicate for fluorescence in 96 well microplates on a Tecan Safire2 reader. As controls, uninduced JAR200 cells which do not express the scFvs, along with modified PBS solutions were assayed with the same dye concentration conditions. The following one site saturation binding algorithm equation was used to fit the resulting saturation binding curves and to calculate the binding affinities using Origin 6.1:

$$y = B_{\max} * x / (K_d + x)$$

x is the concentration of the fluorogen.

Cloning and expressing scFvs in pAK400 E.coli expression vector:

A8-SfiI forward primer: 5'-g gcc cag ccg gcc atg gcg gag gtg cag ctg gtg cag tct ggg-3'

A8-SfiI reverse primer: 5'-ggc ccc cga ggc cgc gga gag gac ggt cag ctg ggt-3'

In order to introduce SfiI sites into the scFv gene a PCR reaction was performed by using the scFv plasmid DNA as the template along with following reagents: 10µL 5X Phusion-HF buffer, 1ng template, 2.5µL 10 µM forward primer, 2.5 µL 10 µM reverse primer, 1.5 µL dNTPs, 0.5 µL Phusion polymerase (Finnzyme), enough water to bring up the volume to 50 µL. The following conditions were used for the amplification:

98°C 30 sec initial denaturation
D) 98°C 30 sec. denaturation
E) 50°C 30 sec annealing
F) 72°C extension
30X cycles from A-C
72°C 4 min final extension

PCR product was analyzed on a 1% agarose gel and TOPO cloned without gel purification according to manufacturer's instructions (Invitrogen). TOPO cloned DNA was transformed into Invitrogen Mach T1 cells, plated on LB/Sgal/IPTG/Kanamycin 50µg/mL plates. White colonies from plates were grown in LB+ KAN 50µg/mL medium and DNA was extracted for sequencing. Colonies were screened by restriction digest with SfiI (NEB). One of the colonies that have the scFv gene was subcloned into pAK400 vector. For that, plasmid DNA that was obtained from cells containing pAK400 was digested overnight with SfiI along with the scFv insert obtained after TOPO cloning. Digestion products were gel purified, and ligated with T7 ligase with 1:6 vector to insert ratio in 20 µL reaction mixture. 10 µL of ligation mixture was transformed into Mach T1 cells. Cells were plated onto a LB+ Chloramphenicol (34µg/mL) plate.

To express the soluble scFv, a single colony from LB+CAM plate was inoculated into LB+CAM medium, grown at 37°C for 4h. An overnight culture was started with $A_{600} < 0.05$ with 500 mL of 34 µg/mL LB+CAM at 25°C. In the morning

cells were centrifuged, resuspended in fresh media and induced with 1mM IPTG for 5 h. Induced cells were harvested by centrifugation and resuspended in 10mM Tris (pH 8.0), 0.5 M NaCl, in a volume equal to weight of the pellet. Cells were lysed by a cell homogenizer. Lysed cells centrifuged at 14K for 20 min, supernatant was incubated with Ni-NTA Agarose beads (QIAGEN) and applied to a gravity column for the purification of the His-tagged scFv. After the flow-through passed the column, the beads were washed with 1) 5mL of wash buffer1 (20mM imidazole, 10mM Tris, 50 mM KH₂PO₄, 300 mM NaCl pH 8.0) 2) 5 mL if wash buffer 2 (20 mM imidazole, 10mM Tris, 50mM KH₂PO₄, 750 mM NaCl pH 8.0), 3) 5 mL of wash buffer 3 (50mM imidazole, 10mM Tris, 50 mM KH₂PO₄, 300mM NaCl pH 8.0). The scFv was eluted with the elution buffer (250 mM imidazole, 10mM Tris, 50mM NaH₂PO₄, 300 mM NaCl pH 8.0) in 6 X 0.5 mL aliquots. The purity of scFvs was evaluated on 12.5 % PAGE, concentrations were determined by Absorbance at 280 nM using extinction coefficient of 50,200 M⁻¹ cm⁻¹ for the parent protein A8, mutant (P), and mutant (P+N).

Determination of dissociation constants of soluble scFvs:

Binding affinities were determined using a Photon Technologies International fluorimeter. A saturation binding curve was generated by titrating 1 µL aliquots of 3 µM scFv into a solution containing 100 nM fluorogenic dye in 150 µL modified PBS. Fluorescence spectra were obtained with 5 nm excitation and emission monochromator bandwidths using the following wavelengths:

Dye	Excitation wavelength (nm)	Emission max(nm)
DIR	580	642
TO-PRO-1	480	517
YO-PRO-1	430	501
YO-PRO-3	580	620

Resulting binding curves were fitted with the following equation (1:1 binding algorithm with a ligand depletion model):

$$\frac{F_i - F_0}{F_\infty - F_0} * 2 * [dye]_{total} = ([dye]_{total} + [scFv]_i + K_d) \pm \sqrt{([dye]_{total} + [scFv]_i + K_d)^2 - 4 * [dye]_{total} * [scFv]_i}$$

Only the titration curve obtained from DIR/A8 titration was fitted with the simple 1:1 binding algorithm that was used to fit titration curves obtained from surface displayed scFvs since fitting with the ligand depletion model did not give a satisfying R² value.

For dyes that were not activated by the fluorogenic dyes tested, we increased the concentration of the dye and the protein up to 5 fold, but still did not see an enhancement of dye fluorescence that is > 2 fold when protein is added.

Intrinsic Fluorescence Experiments:

Intrinsic Fluorescence Experiments were performed on a Cary Varian Eclipse Fluorescence Spectrophotometer. Tryptophan fluorescence spectra of 100 nM scFv solutions were obtained by exciting the solution at 280 nm and recording fluorescence intensity at 300-500 nm range. For spectra of DIR saturated scFvs, the final concentration of DIR was 200 nM.

Fluorescence Melting Experiments:

Fluorescence melting experiments were performed by using a Cary Varian Eclipse Fluorescence Spectrophotometer equipped with a Peltier Temperature Controller. Fluorescence of a solution containing 100 nM scFv and 200 nM DIR was monitored at emission max. as the temperature was raised with a rate of 1⁰C/min from 15⁰C to 65⁰C.

4.3 Results

4.3.1 Directed Molecular evolution of DIR Activating scFvs

4.3.1.1 Choice of DIR Activating scFv for Maturation

When the original human naïve scFv library displayed on yeast cells surface was screened for DIR brightness, 5 unique clones were isolated that were able to bind to and activate DIR fluorescence. These scFvs demonstrated strong DIR affinities in the low nanomolar region¹⁷² (Table 9).

Table 9 Dissociation constants of DIR Activating Proteins on the surface of yeast cells as determined from fluorescence titration experiments.

Clone	K _d (nM)
K7	13.9 ± 3.2
A8	147.8 ± 36.6
K10	15.4 ± 1.8
J6	26.7 ± 4.3
M8	25.6 ± 3.6

ScFv K7 has the strongest DIR affinity and has been shown to be a promiscuous protein that can activate other similar fluorogenic cyanine dyes¹⁷². ScFv A8, on the other hand, has the lowest DIR affinity among others, and has also been characterized to activate similar cyanine dyes when expressed on yeast cell surface. Figure 35 demonstrates the promiscuity of A8. Fluorogenic cyanine dyes incubated with yeast cells expressing A8 are brighter than uninduced yeast cells that do not

express A8 on their surface indicating A8s ability to activate the fluorescence of these fluorogenic dyes. Fluorescence titration experiments with TO-PRO-1 and YO-PRO-1 further confirmed the promiscuity of A8 by resulting in micromolar binding affinities (129 nM, and 835 nM, respectively).

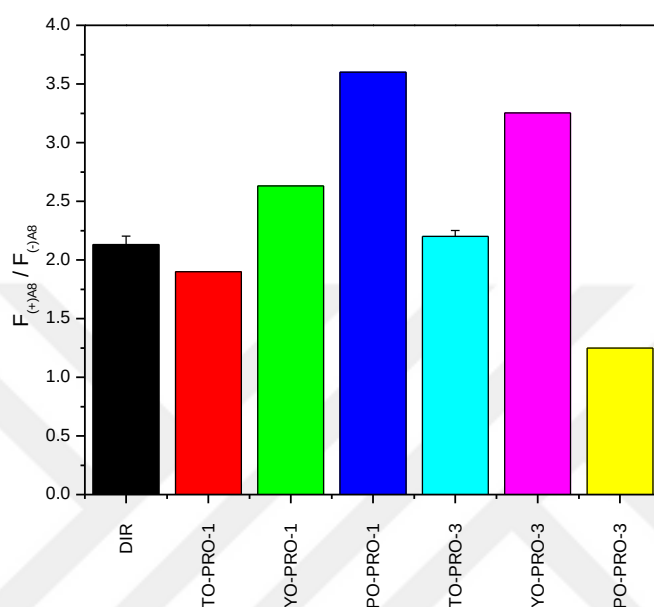


Figure 35 Promiscuity of the scFv A8. Bars represent the relative fluorescence of

In a previous study, Zahnd et al.¹⁷³ reported a rigorous directed evolution to improve the affinity of an scFv for a short peptide which already had a very strong affinity ($K_D=2.6$ nM). They characterized the structure of the evolved mutant with >500 fold improved affinity by crystallography. Data suggested that the improvement in affinity was caused by mutations in the framework sequence which were not in contact with the antigen. The conclusion of this study was that affinity optimization of very high affinity binders is achieved by modulating existing interactions via subtle changes in the framework rather than by introducing new contacts. This study suggests that starting from a parent protein that already has high affinity for a ligand;

it is more difficult to change the binding pocket of the protein that would tune the affinity for other ligands.

Since we were interested in improving the affinity and selectivity of DIR activating scFvs in parallel, we decided to choose the relatively low affinity scFv A8 as the parent for directed evolution rather than the strong affinity scFv K7. We assumed evolution of a weaker binding protein would result in more mutations in antigen binding regions rather than secondary mutations in the framework that would ease the tuning of ligand selectivity. In other words, there would be more room for parallel improvement in affinity and selectivity through more rapid responses to new selective pressures.

4.3.1.2 Creating Diversity on the Parent Sequence

The first step of directed molecular evolution of the parent protein A8 was creating sequence diversity from which mutants with improved functions can be selected. We used random mutagenesis through error-prone PCR. Mutation rates of the polymerase were enhanced by using nucleotide analogs 8-oxo-dGTP and dPTP that are known to lead to mismatch incorporation¹⁷⁴. Using this method the mutation frequency can be controlled by alteration of the number of PCR cycles. We mutagenized the parent DNA by running 10 PCR cycles which has previously been shown to result in 1 to 9 amino acid mutations per gene¹⁷⁵. In our case, sequencing of random clones from the library has been shown to give 0-5 amino acid mutations per gene. In order to select and isolate those scFvs that will bind to and activate DIR we transformed yeast with the mutagenic PCR product and linearized pNL6 vector by in vivo homologous recombination. The size of the mutagenized A8 library was determined as 5.9×10^4 by dilution plating.

4.3.1.3 Selection Strategies for Improved Affinity and Selectivity

In order to improve the affinity and the selectivity of the parent protein in parallel we followed two different selection strategies.

Positive Selections: For the positive selection of improved clones we focused on DIR affinity and aimed to investigate how selectivity will be indirectly affected by an improvement in affinity. The mutagenized library of the parent protein displayed on the surface of yeast cells was incubated with a solution of DIR of which the concentration is only 2% of the parent dissociation constant. Our goal was to study how the selectivity will evolve under the strong pressure of DIR affinity conditions. The cells were monitored with FACS for the DIR intensity and expression levels. Figure 36 demonstrates FACS data obtained from positive selections.

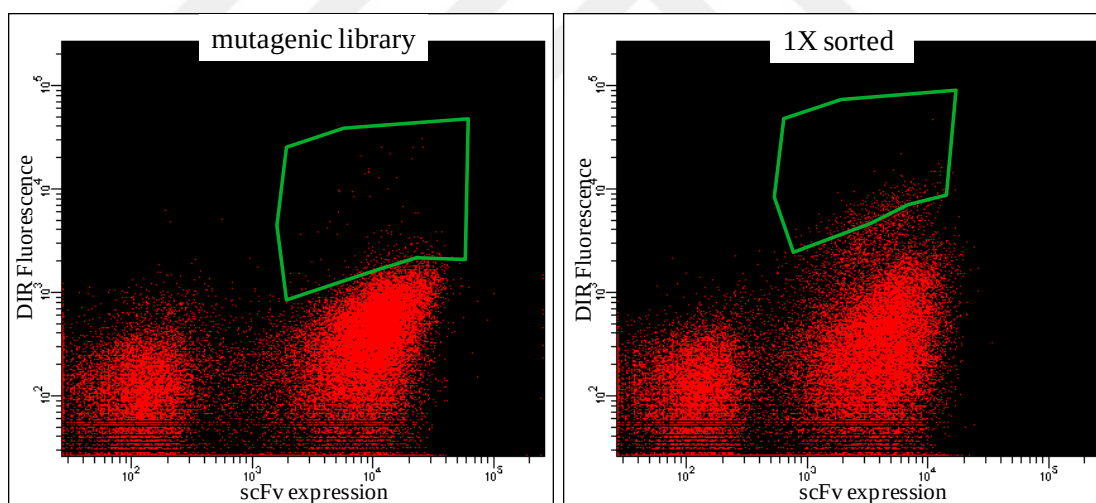


Figure 36 FACS data for the positive selection of clones. Yeast cells displaying scFvs were labeled with 4 nM DIR, and fluorescent anti c-myc antibody.

Cells in the green gate have high scFv expression levels and demonstrate large DIR intensities as well. The brightest cells in this gate were sorted into a flask containing liquid growth culture for amplification. In the next round the same DIR concentration

was used for the incubation of cells. A new population of cells with high scFv expression levels and large DIR intensities emerged in the dot plot from which the top 2% of the brightest clones were isolated. The isolated clones were autocloned onto an induction plate that has nutrients necessary for scFv expression. Once colonies were visible, the plate was stained with a solution of DIR and imaged to pick the brightest clones for further characterization (Figure 37 Induction plate for the positive selection stained 1 μ M

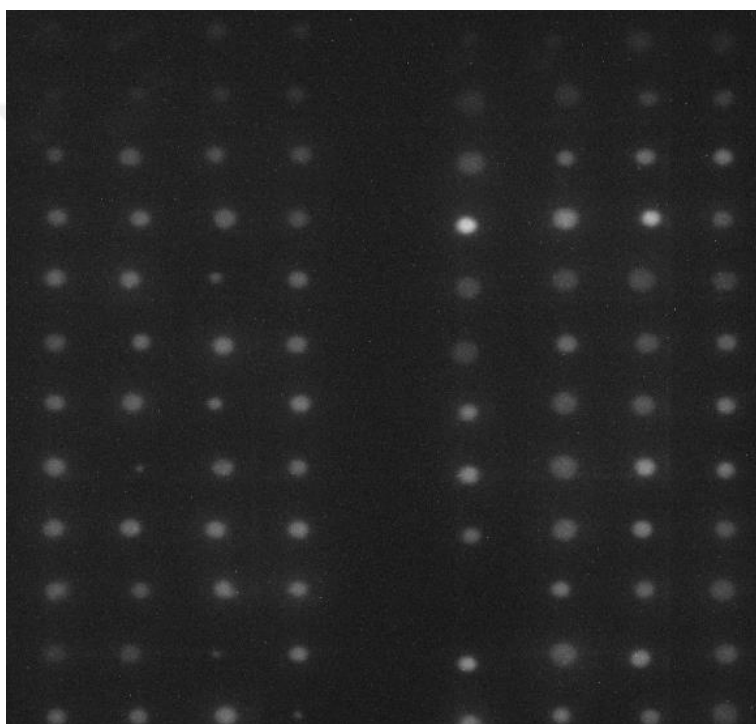


Figure 37 Induction plate for the positive selection stained 1 μ M

Out of the 5 brightest colonies that went through sequence analysis, 2 unique scFvs were obtained. For the rest of this chapter we will focus on the clone that is brighter in DIR intensity and name that clone “mutant (P)” as the mutant obtained from the positive selection.

Positive and Negative Selection: As the second approach for selecting scFvs with strong affinity and selectivity, we introduced a negative selection in parallel to a

positive selection. Yeast cells displaying the mutagenic library were incubated with 40 nM DIR and 100 nM of two competing cyanine dyes for which we wanted to knock down the affinity. Dyes chosen for the negative selections were the fluorogenic cyanine dyes TO-PRO-1 and YO-PRO-1 (structures shown in Figure 38).

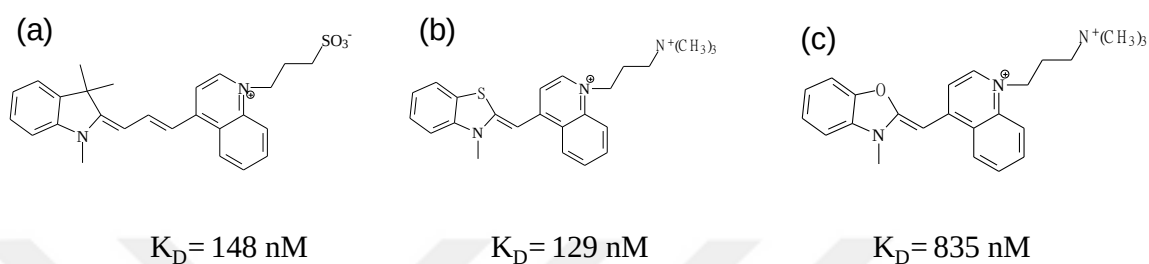


Figure 38 Structures of TO-PRO-1 (b), and YO-PRO-1 (c) in comparison to DIR (a), along with dissociation constants for A8 binding.

Parent scFv A8 can bind and activate these dyes when displayed on cell surface with low micromolar affinities. During FACS analysis, only those cells that have strong DIR intensity and weak or no TO-PRO-1 and YO-PRO-1 intensity (gate) were amplified (Figure 39A).

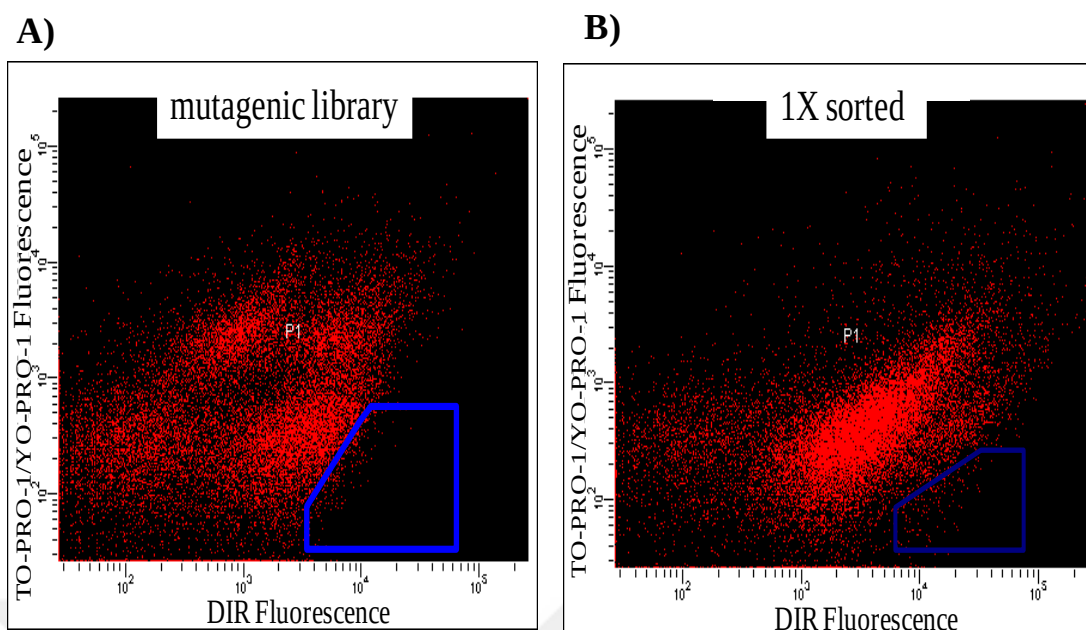


Figure 39 FACS data for the positive and negative selection of clones. Cells were incubated with 100 nM TO-PRO-1, YO-PRO-1, and 40 nM DIR. A) cells displaying the mutagenized library, B) cells that were isolated and amplified after the first round of sorting

In the second round of sorting the population of cells that were activating TO-PRO-1 and YO-PRO-1 disappeared, whereas a new population of cells emerged that were bright in the DIR channel and dark in the TO-PRO-1/YO-PRO-1 channel (Figure 39 B). As in the positive selection, the top 2% brightest cells were sorted onto an induction plate that was imaged for DIR brightness. 10 clones that were picked for sequencing turned out to have the same sequence. The only unique clone obtained from positive and negative selections was named “mutant (P+N)”.

4.3.2 Characterization of scFvs Obtained from Directed Molecular Evolution

4.3.2.1 Characterization of DIR Brightness on the Yeast Cell Surface

Parent protein, and mutants obtained from positive, and positive+negative selections displayed on the yeast cell surface were incubated with 100 nM DIR and

analyzed on a flow cytometer. The c-myc epitope tag was also labeled with the fluorescent secondary antibody to determine the expression levels of scFvs. The table in Figure 40 depicts DIR intensities for three proteins as normalized by the scFv expression levels. While mutant (P) is 2.1 times brighter than the parent protein, mutant (P+N) is 5.1 times brighter.

This experiment has proven that isolation and amplification steps have worked properly, so that brighter clones relative to the parent scFv were isolated. Relative intensities of DIR bound to scFvs expressed on cell surface do not directly reflect DIR quantum yields since scFvs might be bound to different amounts of DIR due to varying binding dissociation constants.

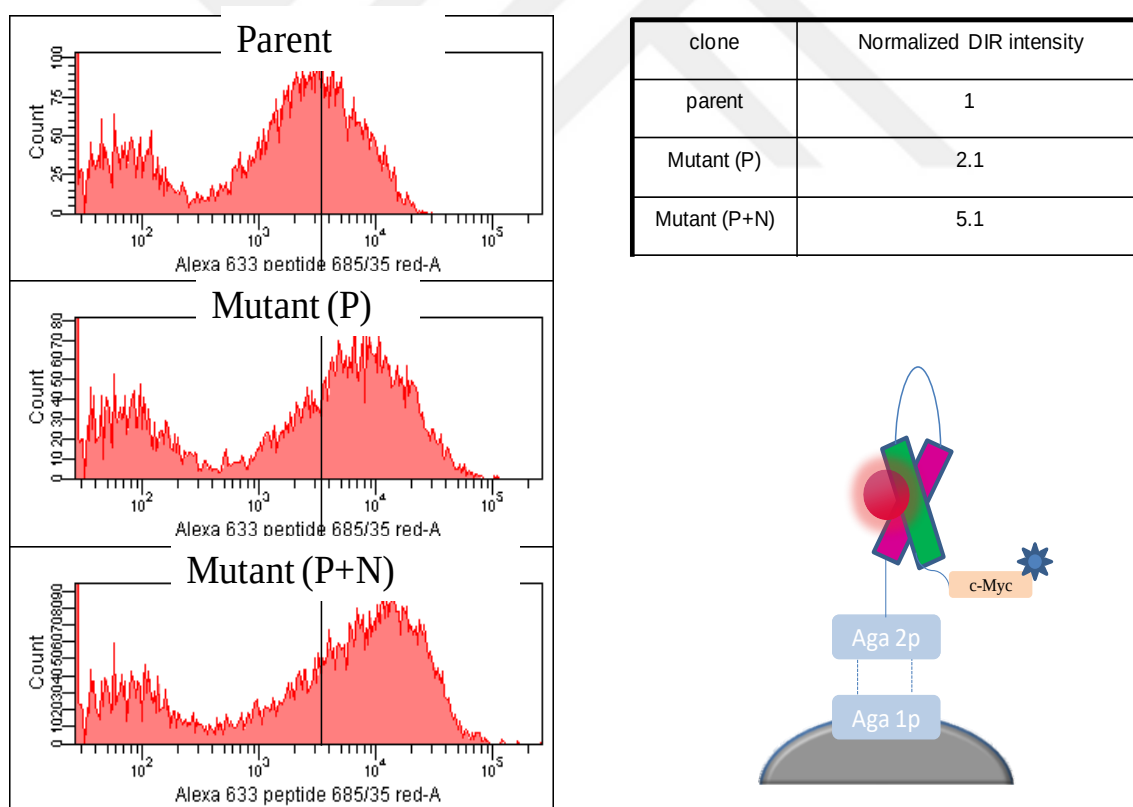


Figure 40 Characterization of DIR brightness of the parent, mutant (P), and mutant (P+N) on the yeast cell surface.

4.3.2.2 Characterization of Mutant (P+N) on Yeast Cell Surface

Mutant (P+N) was evolved during positive and negative selections to improve affinity and selectivity in parallel. We characterized dye binding interactions of this protein displayed on yeast cell surface as compared to the parent protein. Table 10 demonstrates dissociation constants of parent and mutant proteins for binding interactions with DIR, TO-PRO-1, and YO-PRO-1. Dissociation constants were determined by fitting fluorescence saturation titration curves with a one site binding algorithm. While the affinity of the parent improved four times following positive and negative selections, the affinity for TO-PRO-1 and YO-PRO-1 dropped three times.

Table 10 Binding affinities of parent protein, and mutant (P+N) evolved during positive and negative selections for DIR, TO-PRO-1, and YO-PRO-1. Values in parentheses represent the selectivity of the protein for DIR, calculated as the ratio of the TO-PRO-1 or YO-PRO-1 affinity to the DIR affinity

Dissociation constants on yeast surface (nM)		
	Parent	Mutant (P+N)
DIR	148	36
TO-PRO-1	129 (1X)	393(11X)
YO-PRO-1	835(6X)	2480(69X)

Thus, mutant (P+N) is more selective for DIR over the two dyes that were used in negative selections than the parent protein.

TO-PRO-1 and YO-PRO-1 are dicationic asymmetric cyanine dyes that are widely used as nucleic acid stains. They can intercalated double stranded DNA and stain genomic DNA if the cell membrane is compromised. Although they were not supposed to stain nucleic acids of yeast cells that were used in our screenings, they

can do so on the normally very small population of dead cells. In control experiments, uninduced yeast cells that do not express scFvs on the cell surface were labeled with TO-PRO-1 and YO-PRO-1 and enhanced fluorescence indicative of nonspecific binding (Figure 41).

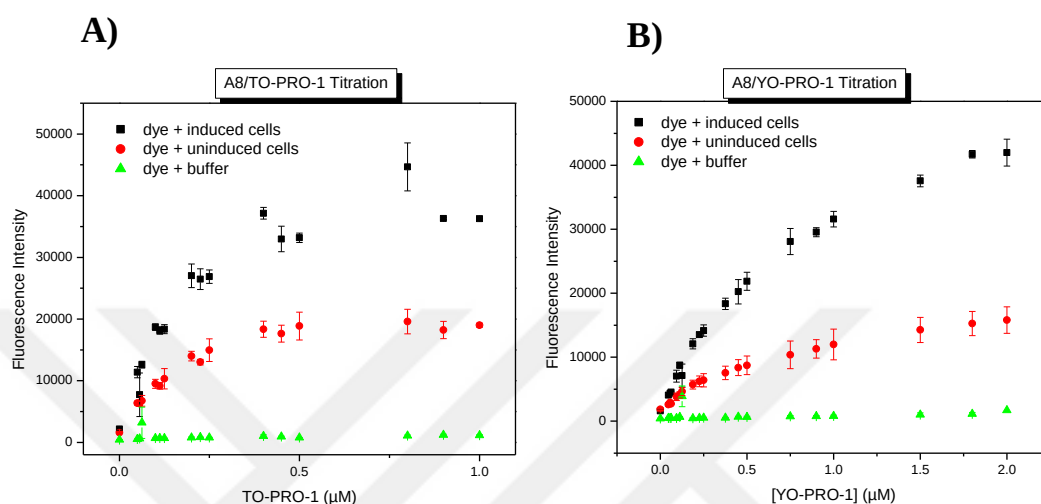


Figure 41 Fluorescence titration curves generated by monitoring fluorescence intensity at varying concentrations of TO-PRO-1 (A), and YO-PRO-1 (B) incubated with induced cells, uninduced cells, and buffer.

We assume that this nonspecific binding is a sum of effects due to (i) sticking of dyes to yeast cells nonspecifically (ii) binding of dyes to endogenous cells surface proteins on yeast cells (iii) staining of genomic DNA in compromised cells. Since this is a complex set of conditions that may or may not be limited to control cells only, we decided not to correct fluorescence intensity for the fluorescence of the control cells, rather we perceive these affinities as minimal affinities and are being aware that actual affinities might be stronger than these reported ones.

4.3.2.3 Sequence Analysis of Mutant scFvs

Mutant (P) that was obtained after positive selections for DIR brightness has 3 mutations in the amino acid sequence. Mutant (P+N), on the other hand, has only one mutation (Figure 42). The glycine to aspartic acid mutation is common in both scFvs and occurs in the heavy chain of the proteins. N-D and G-S mutations in the light chain of mutant (P) evolved when improvement in DIR brightness was the only parameter that was monitored, whereas they did not evolve when the negative selection pressure was present. This might indicate that these mutations lead to an improvement in interactions with TO-PRO-1 or YO-PRO-1. In order to further investigate the effect of these two mutations on DIR affinity, a new scFv was constructed that is composed of the parent heavy chain and mutant (P) light chain. The resulting scFv called H1 had a weaker affinity for DIR than both mutants suggesting that the G-D mutation that occurred in both selections is critical for the DIR affinity.

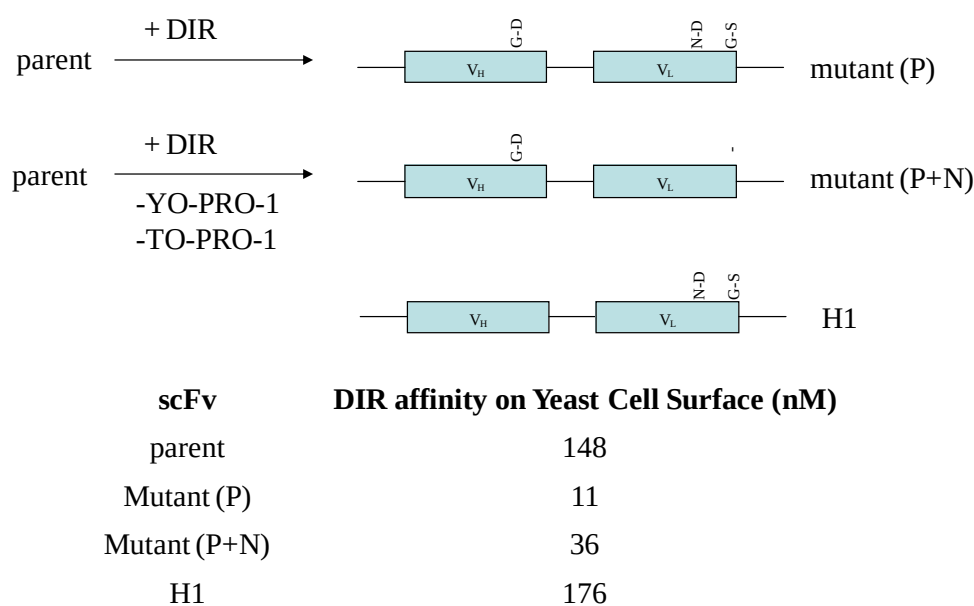


Figure 42 Sequence analysis of mutant scFvs evolved after directed molecular evolution

4.3.2.4 Characterization of DIR Brightness and Affinity of Soluble Mutant

The parent protein, along with the two mutants, were rescued from the yeast display format and expressed in soluble form from E.coli. Relative brightness and DIR affinities of proteins were determined by fluorescence saturation titration experiments. Aliquots of proteins were titrated into a solution containing DIR while fluorescence intensity at the emission maximum was monitored (Figure 44).

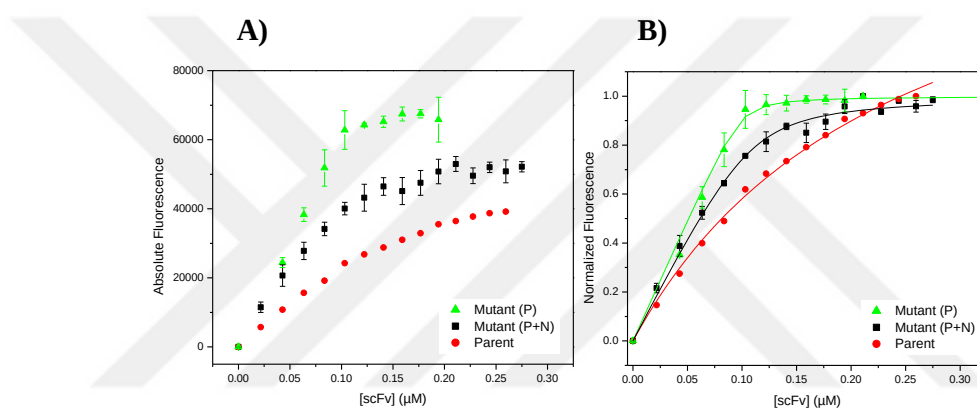


Figure 44 Fluorescence Saturation Titration Curves of parent, mutant (P), and mutant (P+N)

Table 11 Binding affinities of scFvs for DIR

scFv	DIR Dissociation Constant (nM)
parent	242 ± 17
Mutant (P)	1 ± 0.4
Mutant (P+N)	7 ± 0.7

At the protein concentration that is twice the concentration of DIR, all DIR is saturated with proteins, and thus relative fluorescence intensities reflect relative quantum yield numbers (Figure 44 A). DIR bound to both mutants had larger

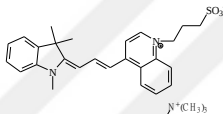
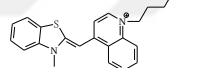
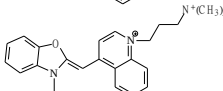
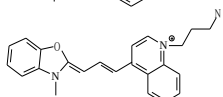
quantum yields than DIR bound to the parent protein. Mutant (P) that was evolved after positive selections was brighter than mutant (P+N) evolved after positive and negative selections. On the other hand, when proteins were displayed on yeast cell surface, mutant (P+N) was brighter. This difference is probably caused by the different natures of experiments. In the previous experiment we did not have conditions that would saturate the dye, rather we had excess dye. Whereas in this experiment the protein was in excess which is a more reliable condition to determine brightness. Furthermore, when displayed on the yeast cell surface, proteins might have different effective concentrations, and also various different conformations including aggregated forms that might lead to different levels of activation of the fluorogenic dye. More accurate data can be obtained when proteins are characterized in solutions with precise conditions.

DIR affinities of scFvs were calculated by fitting titration curves by non-linear regression and a ligand depletion algorithm¹⁷⁶. Figure 44 -B demonstrates normalized fluorescence intensities of scFvs, thus relative affinities for DIR. The dissociation constant for the parent protein that was 242 nM became 7 nM and 1nM for mutant (P+N) and mutant (P), respectively after directed molecular evolution (Table 11). The single point mutation in mutant (P+N) was able to cause a 35 fold improvement in DIR affinity. Apparently this mutation is powerful enough to impose a significant change in the DIR binding pocket of the protein either through electrostatic or thermodynamic stabilization. On the other hand, mutant (P) turned out to demonstrate stronger affinity for DIR. The selection pressure for DIR affinity was 10 fold higher for the positive selections. Apparently due to these stringent conditions, the protein with stronger affinity has evolved.

4.3.2.5 Characterization of DIR Specificity of Soluble Mutants

In order to understand how selection strategies employed for the directed molecular evolution affected the DIR specificity of mutant proteins, dissociation constants for protein/dye pairs were calculated by fluorescence titration experiments. Figure 9 depicts binding affinities of three proteins for dyes that were used during negative selections (TO-PRO-1 and YO-PRO-1) and for a dye that was part of neither positive, nor negative selections (YO-PRO-3).

Table 12 Dissociation constants of parent, mutant (P), mutant (P+N) for DIR, TO-PRO-1, YO-PRO-1, and YO-PRO-3

		Dissociation constants (nM)		
		Parent	P+N	P
	DIR	242	7	1
	TO-PRO-1	No activation	No activation	109
	YO-PRO-1	No activation	No activation	No activation
	YO-PRO-3	23	3	7

When the parent protein was expressed in the soluble form it lost its initial cell surface affinity for TO-PRO-1 and YO-PRO-1 and did not activate these dyes. We define the interaction of the protein and the dye as “no activation” when the fluorescence intensity of the dye is enhanced by < 2 fold under conditions at which the protein concentration is at least three times higher than the dye concentration. There may be multiple reasons for the difference in dye binding behavior of the parent protein in solution. The higher effective concentrations of the protein when expressed

on the surface display might result in a different apparent affinity for dyes. Furthermore, on the cell surface the protein can obtain different conformations that do not occur in solution. The other potential explanation for the lost affinity in solution is the poor folding of the protein, but this can be ruled out since the parent protein can still activate DIR in solution, although with a weaker affinity than on yeast cell surface.

While DIR affinity was improved for mutant (P), and mutant (P+N), TO-PRO-1 affinity did not behave the same. The parent protein that was not able to bind to and activate TO-PRO-1 evolved into a decent binder when only positive selections for DIR were employed whereas it remained as a non-activator when negative selections were also introduced. Thus, negative selections prevented the improvement of TO-PRO-1 affinity in parallel to DIR affinity. The second dye that was used in negative selections, YO-PRO-1 is different than TO-PRO-1 only by the oxygen atom that replaces the sulfur on the benzothiazole ring. This slight difference on the dye structure imposed a significant difference on the dye affinity. Mutant (P) that has a dissociation constant of 109 nM for TO-PRO-1 binding does not activate YO-PRO-1. Apparently the YO-PRO-1 with the oxygen atom does not fit the binding pocket of the protein as well as the TO-PRO-1 with the sulfur atom. The more electronegative oxygen atom presumably induces a negative effect on the interaction with the scFv indicating that the binding is dominated by electrostatics.

The parent protein had a dissociation constant of 23 nM for the dye YO-PRO-3. A comparison of structures of DIR and YO-PRO-3 reveals that they have the same methine bridge length, which was shorter by 2 carbons for TO-PRO-1 and YO-PRO-1. This similarity suggests that length of the methine bridge plays an important role on

the binding. Larger dyes DIR and YO-PRO-3 might induce a better fit to the binding site of the protein.

The YO-PRO-3 affinity was improved after directed evolution for both mutants. The absence or presence of negative selections did not lead to any changes in terms of evolving of YO-PRO-3 affinity. It is debatable if that behavior is caused by the initial strong affinity of the parent protein for YO-PRO-3 or by the lack of this dye in negative selections through evolution. A future molecular evolution experiment that will employ YO-PRO-3 in negative selections would shed more light on this discussion.

Overall, we can conclude that during directed molecular evolution positive selections with stringent DIR affinity conditions introduced partial specificity to a parent molecule, while negative selections along with positive selections resulted in a protein that can only activate DIR with strong affinity but not other dyes that were employed in negative selections.

4.3.3 Turning a DIR Activating scFv into a YO-PRO-1 Activating scFv by Directed Molecular Evolution

In order to evaluate the power of directed evolution methods for developing new specific dye/scFv pairs, we designed a new selection methodology. We aimed to mutate the DIR activating parent protein by directed molecular evolution so that it only activates YO-PRO-1 but not DIR. This was a challenging task since the parent protein intrinsically has a strong affinity for DIR but weak affinity for YO-PRO-1. Furthermore, we were limited to the diversity of the mutagenic library rather than a large pool.

We incubated cells displaying the mutagenic library with a mixture of DIR and YO-PRO-1 and monitored the fluorescence of each dye by FACS. We isolated

those cells that have minimal DIR fluorescence and strong YO-PRO-1 fluorescence and amplified these for the following round. Figure 10 demonstrates how DIR and YO-PRO-1 intensity of cells evolved during four rounds of isolation/amplification.

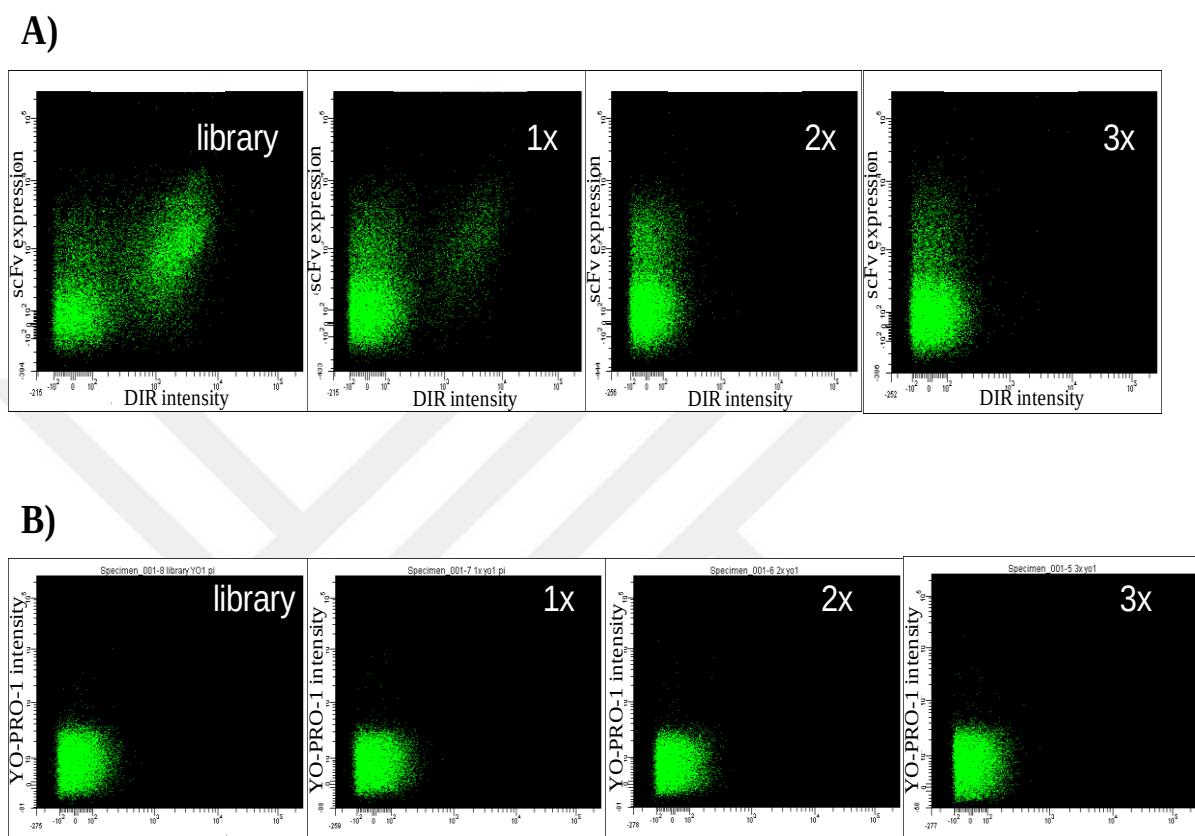


Figure 45 FACS data for YO-PRO-1 activating scFv selection. A) Cells labeled with Alexa488 secondary antibody targeting the c-myc epitope tag on the scFv and incubated with 200 nM DIR. B) cells labeled with 200 nM YO-PRO-1.

The population of cells that had high scFv expression levels and strong DIR intensity disappeared over four rounds of isolation/amplification (Figure 45 A). ScFvs remaining in the pool after four rounds were not able to activate DIR indicating the power of negative selections in directed molecular evolution. On the other hand, YO-PRO-1 affinity of these scFvs did not improve during these selections (Figure 45 B).

The cells displaying YO-PRO-1 activating scFvs were not amplified. Thus resulting scFvs were able to activate neither DIR, nor YO-PRO-1.

The mutagenic library of parent protein A8 is limited in terms of diversity since it contains only scFvs with amino acid sequences with only 1-10 mutations. Apparently this diversity is not enough to introduce a significant change in the binding pocket so that scFvs bind to and activate a different dye than the parent scFv. A different approach might be to start from an scFv library that did not go through any selection pressure previously so that there is larger diversity. Furthermore, the selection pressure applied might also have affected the outcome. Starting from the first round we only selected cells displaying no affinity for DIR. If we had instead, isolated cells with intermediate affinity with DIR and YO-PRO-1 and increased the pressure slowly over following rounds, we could have evolved different scFvs with desired binding affinities.

4.4 Discussion on the Improvement of Affinity and Selectivity of DIR Activating scFvs

4.4.1 Intrinsic Fluorescence

Changes in the intrinsic tryptophan fluorescence can be used to monitor structural changes in proteins. Quantum yield and the wavelength of emission maximum are sensitive to the tryptophan environment. The fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent decreases. Tryptophan residues which are buried in the hydrophobic core of proteins can have spectra which are shifted by 10 to 20 nm compared to tryptophan residues on the surface of the protein. Tryptophan

fluorescence can be quenched by neighboring protonated acidic groups such as aspartic acid or glutamic acid¹⁷⁷.

There are 6 tryptophan residues in the sequence of the parent protein A8, and none of these residues were mutated during molecular evolution. There is one tryptophan residue in CDRs of each chain, while the other four are located in the framework regions of proteins (Figure 43). Thus, tryptophan fluorescence spectra of the three proteins are expected to be the same. However, as Figure 46 A depicts; the spectra of the three proteins are different both in terms of fluorescence intensity and the shape of the spectrum. The average fluorescence intensity of the 6 tryptophan residues is highest for mutant (P) indicating that tryptophan residues are buried most in this protein. The mutant (P) spectrum also appears to be narrower as compared to the parent protein and mutant (P+N). This suggests that more tryptophan residues are exposed to water in the parent and mutant (P+N) than mutant (P). The extra two mutations in mutant (P) impose a conformational change to tryptophan residues. Although it is hard to reach any conclusions without structural data, intrinsic fluorescence spectra of proteins suggest that they have slightly different conformations which could lead to different binding affinities and quantum yields.

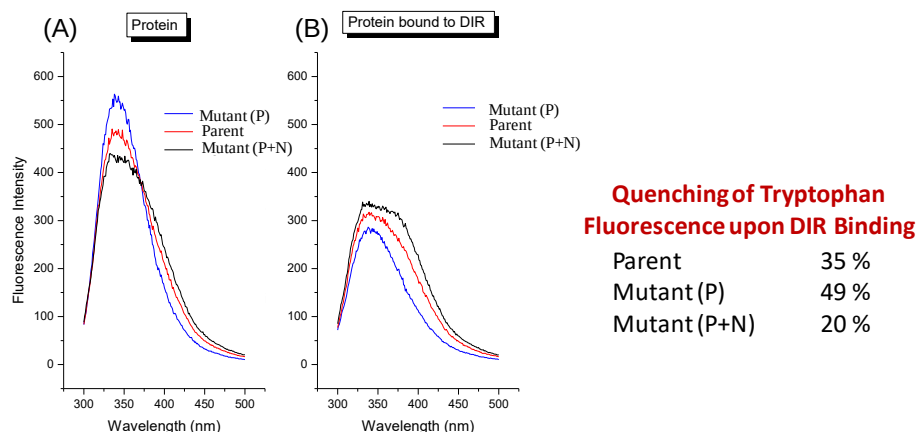


Figure 46 Intrinsic tryptophan fluorescence spectra of parent protein, mutant (P), and mutant (P+N) (A) without DIR, (B) with saturating amounts of DIR

When proteins were bound to saturating amounts of DIR, the fluorescence intensity of each protein was quenched to a different extent. The decrease in tryptophan intensity indicates that either more tryptophans are exposed to water due to DIR binding, or that the tryptophan fluorescence is being quenched due to direct interaction with DIR. In the experiment performed to study tryptophan quenching the fluorescence intensity of a tryptophan solution was not quenched when molar excess of DIR was added. Although we ruled out quenching of tryptophan fluorescence by DIR in solution, further experiments need to be done to understand whether the close proximity within the binding environment leads to FRET. We have previously demonstrated a minimal overlap between the emission spectrum of tryptophan and the absorption spectrum of DIR (Figure 30 B). The conformation of DIR within the binding pockets of proteins might put all or some of the tryptophan residues in close proximity with DIR and lead to energy transfer. Similarly tryptophan quenching might also occur through electron transfer if DIR and tryptophans are interacting.

Another point to make about these proteins based on intrinsic fluorescence experiments is the wavelength at which tryptophan residues emit. The emission maxima for all three proteins are within the range of 350-355 nm. This red shift

relative to buried tryptophans residues that emit within 320-330 nm range reveals that the majority of tryptophan residues are in an environment where they are exposed to water indicating a partially unfolded structure. Intrinsically unfolded proteins are increasingly attracting attention with their distinct properties^{178, 179}. They are proteins that lack global secondary and tertiary structure but still are able to maintain functionality. Coupled folding and binding of proteins allows a range of possible interactions, thus versatility in terms of ligand binding. The significant red shift in tryptophan fluorescence spectra indicates that the proteins we studied may also have unstructured regions. Mutant (P+N) and mutant (P) have 1 and 2 extra negative charges on the amino acid sequences, respectively, due to aspartic acid mutations. The fact that upon folding, a significant electrostatic repulsion would arise from charged residues might be one factor leading to unfolded regions on the proteins.

When proteins were saturated with DIR, fluorescence of each protein was quenched to a different degree. This result further strengthens the idea that these proteins have different conformations of binding pockets for DIR. Upon DIR binding, tryptophan fluorescence is quenched possibly due to conformational changes that expose more tryptophan residues to water. The shapes of spectra did not represent any significant conformational change except slight broadening of spectra (Figure 47).

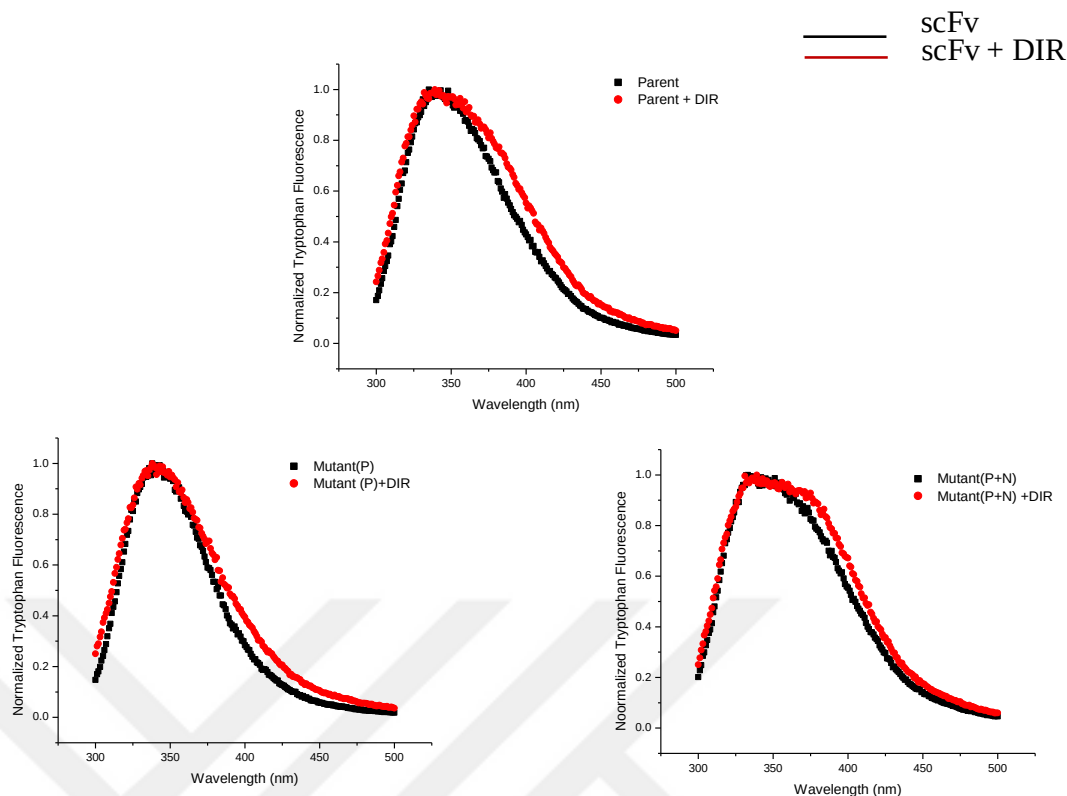


Figure 47 Normalized tryptophan fluorescence spectra of parent protein, mutant (P), and mutant (P+N) in the absence (circles) and presence (squares) of saturating amounts of DIR.

While the fluorescence intensity of the proteins was significantly quenched upon binding to DIR, the wavelength of maximum emission did not shift significantly. Apparently, DIR binding induces changes to expose more tryptophan residues to water by keeping these residues within the same secondary environment. Thus, DIR binding does not induce folding of the unstructured regions on the proteins. In order to evaluate this issue further, melting curves of proteins in the presence and absence of DIR need to be obtained by monitoring the wavelength shifts of the emission maximum, and fluorescence intensity.

4.4.2 Stability of Proteins

We studied the effects of DIR affinity improvement on the thermodynamic stability of the proteins by performing fluorescence melting experiments. We monitored the fluorescence of proteins incubated with saturating amounts of DIR as the temperature was slowly raised. The fluorescent intensity of DIR drops when the protein starts to unfold leading to dissociation of DIR-protein interaction. The temperature of the transition in fluorescence intensity determines the melting temperature (T_M) of the protein-DIR binding and relates to the thermodynamic stability.

Figure 48 demonstrates fluorescence melting curves of the parent protein along with mutant (P) and mutant (P+N) saturated with DIR. All proteins have melting temperatures of $45 \pm 1^\circ\text{C}$ indicated by a sharp transition in fluorescence. All three proteins also have a second minor transition at lower temperature possibly caused by a loosely folded region of proteins. There is no improvement in overall stabilities of mutants relative to the parent protein. Improvement in DIR binding affinity is not reflected as an increase in overall thermodynamic stabilities of proteins. Rather, binding interactions were improved due to local DIR interactions within the mutated regions of the proteins.

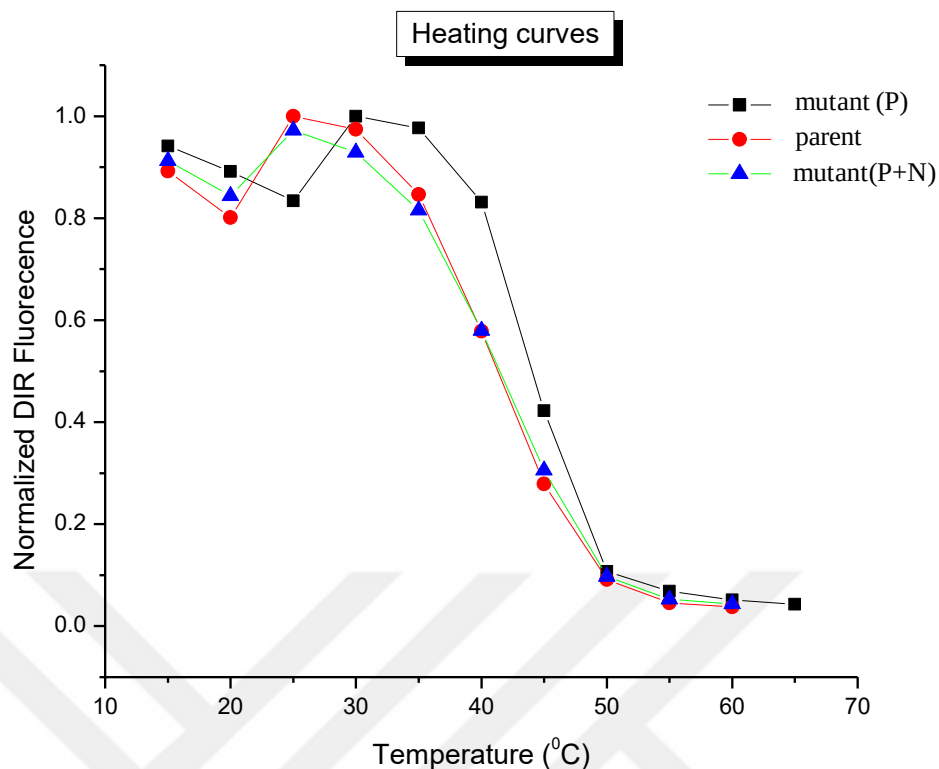


Figure 48 Fluorescence melting curves of parent protein, mutant (P), and mutant (P+N) with saturating amounts of DIR

During selections for improved affinity and selectivity only those mutations evolved that lead to changes in binding interactions. These mutations do not necessarily stabilize the mutant proteins. Furthermore, results of intrinsic fluorescence experiments point to the presence of unstructured regions within proteins which coincides with stability data. The first transition on heating curves of proteins may be caused by the unstable, unfolded region, whereas the main transition occurs due to the overall folding of the structured region of proteins. If most of the binding interactions take place in the unfolded region of proteins, the melting temperatures would not be affected by the strengthening of these interactions which was the case in our experiments.

4.5 Conclusions and Outlook

This study demonstrated a proof of concept methodology to control the affinity and selectivity of fluoromodules by applying directed molecular evolution. We showed that introducing different selection strategies can lead to the development of new proteins with desired binding characteristics. The parent protein that had a low initial affinity for a fluorogenic cyanine dye evolved into a protein that has improved affinity for this particular dye without gaining affinity for structurally similar dyes. Unlike a promiscuous protein like K7 discussed in Chapter 3, a fluoromodule composed of this protein can be used in protein labeling experiments without cross reactivity to dyes that can be used in the same labeling environment.

With our ability to utilize directed molecular evolution to control affinity and selectivity we can develop new specific fluorogenic dye/scFv pairs that not cross-react. These fluoromodules can be used in multi-color experiments where different species will be labeled with different fluoromodules of different colors and tracked simultaneously to reveal their interactions. Fluoromodules technology was already advantageous over other genetically encodable fluorescent labels due to our ability to incorporate any dye of desired spectral and photophysical characteristics. Now, as this study showed we can also tune the protein component of fluoromodules by controlling protein binding characteristics. Thus, options in developing new fluoromodules for various protein labeling applications are endless.

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