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Tailoring Cyanine Dark States for Improved Optically Modulated Fluorescence Recovery

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Abstract

Cyanine dyes are well known for their bright fluorescence and utility in biological imaging. Yet, cyanines also readily photoisomerize to produce non-emissive dark states. Co-illumination with a secondary, red-shifted light source on-resonance with the longer wavelength absorbing dark state reverses the photoisomerization and returns the cyanine dye to the fluorescent manifold, increasing steady-state fluorescence intensity. Modulation of this secondary light source dynamically alters emission intensity, drastically improving detection sensitivity and facilitating fluorescence signals to be recovered from otherwise overwhelming background. Red and near-IR emitting cyanine derivatives have been synthesized with varying alkyl chain lengths and halogen substituents to alter dual-laser fluorescence enhancement. Photophysical properties and enhancement with dual laser modulation were coupled with density functional calculations to characterize substituent effects on dark state photophysics, potentially improving detection in high background biological environments.

Introduction

A valuable tool in biological imaging, near infrared (NIR) fluorescence benefits from low tissue autofluorescence and relatively deep tissue penetration.^{1–4} Stable, targetable, red and near IR probes are thus highly desirable for in vitro and in vivo imaging applications. Organic fluorophores have the additional advantage that their optical properties can be tailored to maximize sensitivity and compatibility through directed chemical synthesis.⁵ Since a usable fluorescence contrast agent needs to be hydrophilic, photostable, and strongly emissive, squaraine dyes, porphyrin derivatives, BODIPY (borondipyrromethane) analogs,⁶ and cyanines continue to find the most applications.^{7, 8}

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Supporting Information. Cyanine synthesis, additional ground state DFT calculations, excited state DFT calculations, SAFIRE experimental data for Cy5.5 compounds, FCS data for cyanine compounds. "This material is available free of charge via the Internet at <http://pubs.acs.org>."

Cyanines, in particular, have high extinction coefficients, moderate fluorescence quantum yields, and generally good biocompatibility coupled with straightforward labeling strategies.⁹ Further, structural modification enables tailoring of their photophysics for various biomedical applications. Varying alkyl chain length, for example, has been shown to affect molar absorptivity and fluorescence quantum yield¹⁰ and halogenation on the polymethine chain has been used to improve cyanine targeting to G-quadruplexes.¹¹ Also, halogenated pentamethine cyanines with quaternary ammonium side chains have proven useful as labels in biodegradable scaffolds.¹² Importantly, cyanine dyes can be reliably switched between fluorescent and dark states, which has proven them useful in optical switching applications such as data storage and super-resolution microscopy.^{13, 14}

The photophysics of Cy5 have been thoroughly studied using fluorescence correlation spectroscopy (FCS)¹⁵ and transient absorption,¹⁶ leading to the observation of multiple dark states, including photoisomers and triplet levels. Naturally in the all-trans ground state, cyanine π -electrons are well-modeled by a simple particle in a box or 1-D metal model.¹⁷ For Cy5, the cis-photoisomer absorption is red-shifted by ~45 nm, yielding a transient absorption at 690 nm with a lifetime of 150 μ s.¹⁶ The relatively long cis-Cy5 lifetime enables significant buildup of this dark state under even low steady-state excitation. Excitation of cis-Cy5 in its absorption band, however, photoreverts the Cy5 to the trans state and recovers fluorescence with enhancements of up to 50%.¹⁸ Although Cy5 excitation also shows triplet state dynamics for both trans- and cis-isomers, the T_1 levels have much shorter lifetimes of ~35 μ s and 6 μ s for the trans and cis isomers, respectively.¹⁶ Thus, our approach of fluorescence excitation combined with secondary laser co-illumination that is on-resonance with the transient absorption dynamically modulates the Cy5 fluorescence intensity via optical depopulation of primarily the photoisomerized dark state.

Using Synchronously Amplified Fluorescence Intensity Recovery (SAFIRE)¹⁹ we can modulate the long-wavelength secondary laser to modulate the cis vs. trans-Cy5 ground state populations, thereby modulating collected fluorescence and enhancing signal to noise. Such fluorescence recovery from high background was demonstrated for the commercially available parent Cy5 in solution¹⁸ and buried within tissue mimicking phantoms.²⁰ To understand and improve detection sensitivity, we synthesized cyanine structural variants and utilized optical modulation methods as a screening tool to assess how variations in cyanine structure affect modulatability and, therefore, signal recovery in high background fluorescence experiments. Our specific emphasis is on understanding the bright state and dark state photophysics to enhance and tailor optically recovered ground state populations.

Experimental

Sulfo-Cy5, Sulfo-Cy7, and Cy5.5 all in NHS ester form (Lumiprobe), and Merocyanine 540 (Sigma-Aldrich) were used as received. Cy5 derivatives were synthesized by reacting two equivalents of individual heterocyclic salts with either the commercially obtained malondialdehyde bisphenylimine salt or using our halogenated reagent (Figure S1, see supplementary information for synthetic details) in the presence of acetic anhydride with sodium acetate. This general cyanine structure is shown in Figure 1. Cyanine solutions were prepared by dissolution in DMSO followed by dilution to experimental concentrations. All

dual laser experiments were performed on an inverted microscope (Olympus IX71) using a 60 $\times$, 1.2 NA water immersion objective. All solution data were acquired by focusing $\sim$ 30 microns into solution. Signal was collected in a confocal arrangement with a 100- μ m multimode fiber serving as the pinhole and directing the emission to a photon-counting avalanche photodiode (APD, Perkin–Elmer). Intensity trajectories were recorded using a Becker-Hickl SPC-630 single photon counting board to time-stamp individual photon arrival times. Primary excitation was performed with a 514.5-nm Argon-ion laser (Coherent Innova 90C), 594 or 633 nm He-Ne lasers (JDS Uniphase and Melles-Griot), or a 730 nm diode (Thorlabs), and secondary co-illumination was done with a Ti:sapphire laser (Coherent Mira 900) with wavelengths ranging from 710 to 830 nm, operating in continuous wave mode. Primary and secondary excitation beams were spatially overlapped in the microscope after combining on a dichroic mirror. Appropriate band pass filters blocked the primary and secondary excitation wavelengths to only let the desired fluorescence signals reach the detector. When necessary to distinguish timescales comparable to those of diffusion, a defocusing lens was used to increase the laser spot size, shifting diffusion-based fluctuations to much longer timescales. An electro-optic modulator (Conoptics) was used to modulate the amplitude of the long-wavelength secondary laser beam, with recorded time traces being binned at least 2.2 times faster than the highest modulation frequency. Data were processed by Fourier transformation of each time correlated single photon counting fluorescence intensity time trace. The corresponding FFT peak amplitude at each modulation frequency was divided by the DC peak amplitude to calculate modulation depth.

DFT calculations were performed using Gaussian 09²¹ with Becke's three-parameter hybrid density function in combination with the Lee-Yang-Parr correlation functional (B3LYP) and the effective core potential (ECP) basis set Los Alamos ECP plus double zeta (LanL2DZ). Ground state geometries of Cy5 and its synthesized derivatives were optimized and the energy levels calculated, and then time-dependent density functional theory (TDDFT) was used to calculate transition energies for the geometry optimized ground states. The polarizable continuum model (PCM) was applied for ground state geometry optimizations and excited state absorption spectra calculations to determine the solvation effects of DMSO, as applied in experimental conditions.^{22–26}

Results

Cyanine photophysics and dark state recovery kinetics were investigated under single and dual laser excitation. Compounds (dissolved in DMSO) were co-excited with a 594 nm primary and 710 nm square wave-modulated secondary laser for Synchronously Amplified Fluorescence Intensity Recovery (SAFIRE).¹⁹ This dual laser method uses the primary excitation to produce fluorescence and populate the dark state, while the much lower energy secondary laser depopulates the non-fluorescent dark state, shifting population to the bright state faster than the dark state would naturally decay. By modulating this secondary laser, we directly modulate Cy5 fluorescence, shifting its signal to a unique, very-low-background detection frequency. At sufficiently high modulation frequencies, the system has insufficient time to establish steady-state populations, meaning that measurements of modulation depth, m , vs. modulation frequency, ν , report on the time to establish dark and bright manifold steady state populations, and is given by:^{27–29}

$$m = \frac{A}{\sqrt{1 + (2\pi\nu\tau_c)^2}} \quad (1)$$

When a specific timescale exists, fitting frequency-dependent modulation depth curves to equation 1 enables extraction of the characteristic time, τ_c . When a good fit to a single characteristic time, τ_c , is not possible, one can specify the time at which the modulation depth drops by half to extract a characteristic time.¹⁸ Changing with primary laser intensity, the inverse of τ_c is the characteristic frequency, or rate $k_c = k_{on} + k_{off}^o$, where

$k_{on} = \frac{\sigma_{abs} * I_{pri}}{h\nu} \Phi_D$ (σ_{abs} is the absorption cross section, I_{pri} is the primary laser intensity, and Φ_D is the dark state quantum yield) and k_{off}^o is the natural dark state decay rate constant.³⁰ The parameters k_{on} and k_{off}^o (or their inverses τ_{on} and τ_{off}^o) can be used to determine the rates at which molecules enter and exit the dark state, allowing one to determine fluorescence enhancement,³¹

$$Enh = \frac{\tau_{off}^o - \tau_{off}}{\tau_{on} + \tau_{off}} \quad (2)$$

in which τ_{off} is the dark state lifetime with the secondary laser on. The data in Figure 2A demonstrate this process, showing a diminishing modulation depth with increasing modulation frequency, and the fitted characteristic frequencies at varying primary power are used to extract on and off times (Figure 2B).

At low modulation depths (<15%), extracting accurate photophysical parameters can be difficult from modulation depth vs. frequency curves. Instead, by achieving steady state dark state population through extended primary illumination,³² thermal recovery from the dark state occurs upon rapidly turning the primary laser off. By varying the primary illumination off period, T_{off} , the fraction of molecules in the bright state, n_B/n_{tot} will recover to a degree that depends on the natural off time, τ_{off}^o according to equation 3:

$$\frac{n_B(t)}{n_{tot}} = 1 - \frac{\tau_{off}^o}{\tau_{on} + \tau_{off}^o} e^{-\frac{T_{off}}{\tau_{off}^o}} \quad (3)$$

By fitting the experimentally determined fraction of molecules in the bright state as a function of the laser off period we can determine the natural dark state decay rate. This process is illustrated in Figure 3 where Figure 3A shows a large intensity drop off with 300 μ s between laser pulses, Figure 3B demonstrates less drop off with 100 μ s between pulses, in Figure 3C there is even less decay with 50 μ s between pulses, and finally in Figure 3D the intensity drop is the least significant. This shows that the longer the laser off period, the greater the recovery from the dark state, which yields a higher initial fluorescence intensity when the primary laser is turned back on.

Even with the seemingly minor structural changes to Cy5 indicated in Figure 1, modulation depth and photophysical parameters are strongly affected by slight substituent changes. Using modulation frequency dependence and bright state recovery methods (Figures 2 & 3),

photophysical on and off times were extracted (Table 1). Overall, with the Cy5 derivatives, modulation depth increases with alkyl chain length and decreases with increasing halogen mass.

The ~100 μ s natural dark state lifetimes suggest that a ground state process such as photoisomerization leads to the modulatable metastable dark state, as suggested previously for commercially available Cy5.¹⁸ To probe the ground state energy differences between modulatable dark and bright states, thermally equilibrated samples were either kept in the dark or pre-illuminated with the secondary laser, followed by primary-only illumination to determine initial ground state population with and without prior secondary illumination (Figure 4). The relative initial fluorescence intensities with and without secondary pre-illumination then report on the energy difference between trans and photoreversible cis isomers involved in modulation. Assuming the dark state is completely depleted upon secondary laser pre-illumination, a Boltzmann distribution enables experimental trans-cis energy differences for all cyanine derivatives to be determined (Table 2). The relatively low energy difference between bright and dark states further supports the idea that a photoisomer, and not the much higher energy triplet level, is the experimentally modulatable dark state.

Although the timescales and pre-illumination experiments suggest that photoisomerization and back isomerization give rise to optical modulation, photoisomerization can potentially occur about any of the bonds along the cyanine polymethine chain (Figure 5A–D for Cy5, Cy5.5, Cy7 and merocyanine 540, respectively). To gain further insight into the isomerization mechanism, density functional calculations were employed to compare ground and excited state energies of the different possible isomers. The B3LYP functional and LanL2DZ basis set were used, taking into account DMSO solvation with the PCM, analogous to previous Cy5 calculations.²² Geometry optimizations and ground state energy calculations were performed on the all-trans and each of the various cis-photoisomers, and TDDFT was used to calculate vertical excitation energies (see SI). Experimental and calculated cis-trans energy differences are compared in Table 1. Arrhenius-extracted activation energies for cis to trans regeneration of the bright manifold are also given as calculated from the experimentally derived rate constants for dark state decay, k_{off}^0 .

Discussion

The fluorescence enhancement of Cy5 is due to optical depopulation of a dark state, and derivatives' modulation depth generally increases with increasing alkyl chain length and decreases with increasing halogen atomic mass at the central carbon (Table 2). Despite the added bulk to the polymethine chain, halogens seem to shorten the on and off times, while long alkyl chains appear to slow the forward and back-isomerization processes.

Comparing the DFT and experimental energy differences between cis and trans, the modulatable dark states of all cyanines other than Merocyanine 540 appear to result from isomerization around the 2–3 carbon-carbon bond. For Merocyanine, isomerization appears to occur about the 1–2 bond. The 2–3 cis isomer (and 1–2 cis for Merocyanine 540) and trans isomer energy difference is in fair agreement (~factor of 2) with the experimentally

obtained values, while the 1–2 isomer (and 2–3 for Merocyanine) is higher in energy, and much higher in the halogenated compounds. For calculated energies of the other photoisomers, please see the SI.

The ratio of off to on times is the most important criterion governing modulation depth. A fluorescent dye with high modulation depth quickly feeds into a long-lived dark state and is efficiently depopulated by a secondary laser. Experimental on and off times are shorter with halogens, and it appears that although the quantum yield of trans to 2–3 cis photoisomerization increases, the 2–3 cis ground state stability decreases with increased halogen size, or possibly that the triplet level may be longer lived and becomes the more populated dark state level in these molecules. Although these factors may combine to decrease modulation depth, the low energy difference between ground and modulatable dark state measured by secondary laser pre-illumination argues against the involvement of a triplet level, as this would presumably be significantly higher in energy than the ground S_0 level for either isomer. This argues in favor of steric effects that make dark state population increasingly difficult with increased halogen mass.

As mentioned above, the photophysical rates into and out of the dark state control the steady state dark state population. The larger the achievable steady-state dark state population, the larger the possible enhancement upon complete depopulation through secondary illumination. MHI97, for example, exhibits the highest enhancement and is the slowest to photoisomerize, but because the dark state is long-lived, a high modulation depth is recorded. On the other hand, halogenated E27 and E65 rapidly photoisomerize, but also back-photoisomerize very quickly, leading to low dark state buildup and low overall modulation depths. All of the Cy5 variants have similar secondary-induced τ_{off} values, minimizing its impact with these derivatives. Thus, within this family of Cy5-like compounds, structural modifications that increase the ratio of τ_{off}^0 to τ_{on} for a given primary excitation intensity have the greatest effect on overall enhancement. Through such optical control of emission with tailorable on and off times, applications in superresolution imaging may also become possible at faster timescales,^{13, 14, 34–37} possibly coupled with the improved sensitivity afforded by modulation-based SAFIRE.²⁷

Interestingly, Cy5.5 has photophysical on and off times similar to the best Cy5 derivatives, but shows much lower enhancement. While R1 and R2 substitutions showed the same trend as with Cy5, the lower modulation depths precluded full photophysical characterization, except for commercial Cy5.5. In contrast to Cy5, the low enhancement appears to arise from inefficient dark state depletion – an interpretation corroborated by FCS data which show a higher dark state population for Cy5.5 than for Cy5 when both primary and secondary lasers are on (Figure S2).

Considering the range of cyanines studied, increased conjugation length appears negatively correlated with dark state buildup. Merocyanine 540, for example, exhibits a relatively high modulation depth due to its long-lived dark state and fast forward photoisomerization rate, but its dark state is less efficiently depleted by the secondary laser, causing the modulation depth to not be as high as Cy5 and some of its related analogues. Cy7 exhibits a short-lived dark state, similar to the brominated Cy5 derivatives, and therefore does not offer as much

fluorescence recovery as the Cy5 compounds or Merocyanine 540. When Cy7 isomerization is chemically prevented around the central double bond as in Fig. 5, however, isomerization is forced to occur around other bonds, likely limiting accessible photoisomers to increase photoreversible dark state populations and modulation depth.

The combination of ground vs. dark state thermal populations, natural dark state lifetimes, and density functional theory calculations enable improved understandings of cis and trans photoisomer energy differences. DFT was used to calculate optimized ground state energies for the various isomers and vertical transition energies. Relative calculated energy differences are compared with experimental values to lend insight into which isomers are likely to give rise to the modulatable dark states for each cyanine derivative. It appears that the halogenated compounds have a larger energy difference between cis and trans isomers, a fact that could explain lower the activation barrier and observed higher reverse photoisomerization rates. On the contrary, DFT suggests that compounds with longer alkyl chains have smaller energy differences separating isomers, corroborating the observed larger activation energies and longer τ_{off}^0 values. Cy5.5 does have a higher than expected experimental cis-trans energy difference, but thermal reversion is also low, suggesting a higher barrier to ground state photoisomerization. For Cy5, its derivatives, and Cy5.5, the experimental values are closer in magnitude to the DFT energy differences between the Cis23 rather than the Cis12 isomer, suggesting that Cis23 is the photoisomer responsible for the dark state. In Merocyanine 540, however, the Cis12, rather than the Cis23, appears to be responsible for the photo-induced dark state, based on similarity of calculated isomer energy differences with experimental values. Time-dependent DFT excited state calculations further corroborate this interpretation as only the calculated Cis23 (and Cis12 for Merocyanine) vertical transition energies are slightly and appropriately³⁸ red-shifted from that of the all-trans isomer absorption. This data is included in the supporting information.

Conclusion

The enhancement and optical modulation of Cy5 fluorescence using red-shifted secondary co-illumination to depopulate photoaccessible dark states¹⁸ can be modified with conjugation length, and alkyl and halogen substitutions. A pentamethine chain is optimal for fluorescence enhancement; Cy3 has no enhancement and Cy7 has less than Cy5 and its related compounds, presumably in both cases related to shortened dark state residence time. Adding an extra phenyl ring to the indole structure also decreases modulation depth, as in the case of Cy5.5. Our experiments suggest this is due to inefficient dark state depletion. Longer alkyl chains on the nitrogen atoms in Cy5 derivatives increase modulation depth because they lengthen the dark state lifetime, while halogenation of the polymethine chain causes a reduction in modulation depth because it decreases the dark state lifetime.

Dark state depopulation relating to modulatable fluorescence is observable in fluorescence correlation spectroscopy (Fig. S2), but is convolved with the diffusion time giving rise to fluctuations. Thus, bulk experiments employing modulation depth vs. modulation frequency in larger spots have enabled us to probe the dark states giving rise to the enhanced sensitivity possible through optically modulated fluorescence. Common to many cyanines, modulatability through long-wavelength dark state illumination enables photophysics to be

elucidated and correlated with structural differences.²⁸ Combining these studies with DFT calculations generates mechanistic insight into the nature of the dark states giving rise to enhancement. Through further studies, such understandings are likely to ultimately improve fluorescence modulation depth through molecular design, facilitating the tailoring of modulation timescales to study specific molecular or intermolecular interactions. While substituents affect modulation depth, it is really the ratio between dark and bright state residence times that determines overall modulation depth. The magnitudes of the rates into and out of these states determine the characteristic frequencies and it is these rates that are individually altered through substituent effects – most likely arising from steric interactions destabilizing certain isomeric forms. Correlating DFT calculations and experimental rates and thermal populations lends further insight into the nature of the optically modulatable dark state for each cyanine dye.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

General structure of Cy5 analogs. Modifications were incorporated as alkyl substitutions at the heterocyclic nitrogens (R¹) and halogen incorporation on the *meso* position of the polymethine bridge (R²).

Figure 2.

A) Cy5 derivative MHI97 (See Table 1) modulation depth as a function of modulation frequency and fit to equation 1. Data were collected for 1 second at each modulation frequency. B) MHI97 characteristic frequency as a function of primary intensity and the line to which it fits.

Figure 3.

Fluorescence traces showing ground state recovery for MHI106 using different primary laser off periods, T_{off} . Using an electro-optic modulator, the primary laser is turned on for 500 μs and off for A) 300 μs , B) 100 μs , C) 50 μs , and D) 20 μs . Laser spot size was expanded 300 μm^2 , to increase diffusion timescales to $\sim 10\text{ms}$, such that it does not interfere with the modulation timescales. For each panel, data were collected for 20 seconds and modulation cycle averages are taken and ratios of initial to final intensities within the average primary illumination period, T_{on} , as a function of T_{off} are fit to equation 3.

Figure 4. Optical recovery of thermally populated dark states. Primary-only induced fluorescence from MHI97 with (black) and without (red) secondary laser pre-illumination. The increased fluorescence with secondary-only pre-illumination relative to no secondary pre-illumination generates higher initial fluorescence intensity by optically recovering molecules from the thermally populated dark state.

Figure 5.

All-trans isomers of A) Cy5, B) Cy5.5, C) Cy7, D) Merocyanine 540 numbered to show isomerization possibilities.

Table 1

Summary of experimental results including enhancement and on and off times for Cy5 derivatives. R^1 and R^2 positions are as shown in Figure 1. For τ_{on} and τ_{off} measurements, the primary and secondary laser intensities were 350 and 640 W/cm², respectively.

Dye	R^1	R^2	Mod depth	τ_{on}^0 (μ s)	τ_{off} (μ s)	τ_{on} (μ s)	ϕ_D
Cy5			50% (± 4)	73(± 7)	20(± 3)	88(± 9)	.011(± 0.002)
MHI84	CH ₃	H	40% (± 2)	87(± 4)	25(± 2)	130(± 8)	.0077(± 0.001)
LO4	CH ₂ CH ₃	H	50% (± 4)	130(± 3)	40(± 3)	140(± 7)	.0072(± 0.001)
MHI97	(CH ₂) ₃ CH ₃	H	58% (± 2)	210(± 8)	40(± 5)	250(± 11)	.004($\pm 5 \times 10^{-4}$)
MHI106	CH ₃	Cl	28% (± 3)	60(± 8)	45(± 7)	10(± 3)	.10(± 0.007)
E27	CH ₃	Br	13% (± 1)	25(± 3)	20(± 3)	7(± 2)	.14(± 0.01)
E63	CH ₂ CH ₂ OAc	Cl	20% (± 1)	50(± 5)	37(± 4)	25(± 3)	.040(± 0.003)
E65	CH ₂ CH ₂ OAc	Br	7% (± 2)	12(± 3)	10(± 2)	7(± 2)	.14(± 0.009)
Cy5.5			12% (± 2)	140(± 11)	118(± 10)	100(± 3)	.012(± 0.002)
Cy7			15% (± 3)	16(± 3)	13(± 3)	6(± 2)	.21(± 0.01)
Merocyanine 540			40% (± 3)	216(± 15)	123(± 10)	110(± 11)	.016(± 0.001)

Table 2

Summary of ground state DFT computational results: Cis23-Trans and Cis12-Trans refer to the energy differences between the ground states of the different cis-photoisomers and the all-trans ground states. (Note: Due to short lifetimes, E65 and Cy7 exhibited insufficient dark state buildup to determine experimental cis-trans values.) E_a is the activation energy obtained by Arrhenius calculations using the experimental k_{off}^0 and setting $A = 10^{12} \text{ s}^{-1.33}$

	Cis23-Trans (eV)	Cis12-Trans (eV)	Exp Cis-Trans (eV)	Ea (eV)
Cy5	.1345	.3091	.0757($\pm$.01)	.465($\pm$.04)
MHI84	.1366	.3087	.0592($\pm$.008)	.470($\pm$.03)
LO4	.1366	.3157	.0505($\pm$.007)	.480($\pm$.01)
MHI97	.09528	.2758	.0419($\pm$.007)	.492($\pm$.04)
MHI106	.1608	.7520	.0905($\pm$.02)	.460($\pm$.05)
E27	.1621	.8439	.0732($\pm$.008)	.438($\pm$.05)
E63	.1593	.9554	.0762($\pm$.009)	.455($\pm$.05)
E65	.1488	1.212		.419($\pm$.06)
Cy5.5	.1349	.3033	.110($\pm$.012)	.482($\pm$.04)
Cy7	.2086	.7661		.426($\pm$.07)
Merocyanine 540	.2985	.1628	.0540($\pm$.008)	.493($\pm$.04)