Journal of Fluorescence, Vol. 14, No. 2, March 2004 (©2004)

Cy3B™: Improving the Performance of Cyanine Dyes

Michael Cooper,[1,3] Andreas Ebner,[2] Mark Briggs,[1] Miles Burrows,[1] Nicholas Gardner,[1] Robert Richardson,[1] and Richard West[1]

Received October 3, 2003; revised November 4, 2003; accepted November 5, 2003

The spectral properties of a rigidified trimethine cyanine dye, Cy3B™ have been characterised. This probe has excellent fluorescent properties, good water solubility and can be bioconjugated. The emission properties of this fluorophore have also been investigated upon conjugation to an antibody. This study compared the conjugated emission properties of Cy3B with other commercially available fluorophores emitting at similar wavelengths.

KEY WORDS: CyDye; Cy3B; bioconjugation; fluorescence.

INTRODUCTION

The use of technologies that rely on fluorescence detection is increasing in both the fields of drug discovery and high-throughput screening [1]. Techniques with sensitivity approaching that of radiolabelling, but without the environmental concerns, are driving the need for robust and reproducible assays formatted in a fluorescence modality. Until recently, a limiting factor in the growth of this field lay also with the lack of suitable, commercially available instrumentation. This has been addressed with the evolution of turnkey, platform technologies formatted for both *in vitro* [2] and cellular screening [3]. Today, such available platforms are capable of screening up to 1 million compounds a day using a host of different fluorescence detection modalities [4]. Moreover, performing high volume screens also relies on the availability of robust and sensitive fluorescence probes that are compatible with the biological nature of the assay. The current trend towards assay miniaturisation [5] from 96 *via 384* to 1536 well plates requires highly soluble fluorophores with large extinction coefficients, good photostability and quantum yields approaching unity. Furthermore, assays are often performed in less than a 100 μL of solution and at concentrations that complement the nature of the bioassay thus, the fluorescent signal must be resolvable at sub-nanomolar concentrations in both a rapid and reproducible manner.

Here, we describe the use of a high performance fluorescent probe designed to meet the above criteria. For more than a decade, the cyanine dye [6] (Cydye™) range of fluorophores has been employed in a range of technologies involved with discovery science. Wessendorf *et al.* [7] described Cy3 as the "archetypal" bright fluorophore in comparative studies with other commercially available molecular probes including fluorescein, rhodamine derivatives and Texas red. More recently, Gruber *et al.* [8] have investigated the properties of Cy3 upon conjugation to macromolecules, such as immunogens, and the subsequent *anomalous* increase in emission relative to that of an equimolar solution of the free dye. Work described here, has therefore been undertaken to investigate the properties of a rigidified trimethine cyanine dye, Cy3B [9].

Nature, has a host of examples whereby trapping a chromophore within a restrained system has maximised the fluorescence output. Allophycocyanin [10] (APC) is one such fluorophore whereby the conjugated system is held within a proteinaceous matrix that maintains the chromophore in rigid state optimal for light absorption and subsequent emission. Indeed, APC is available in a

[1] Amersham Biosciences, Discovery Systems, The Maynard Centre, Whitchurch, Cardiff, CF14 7YT, United Kingdom.
[2] Institute of Biophysics, Johannes-Kepler University, Altenberger Strasse 69, A-4040 Linz, Austria.
[3] To whom correspondence should be addressed. E-mail: michael.cooper@amersham.com.

1053-0509/04/0300-0145/0

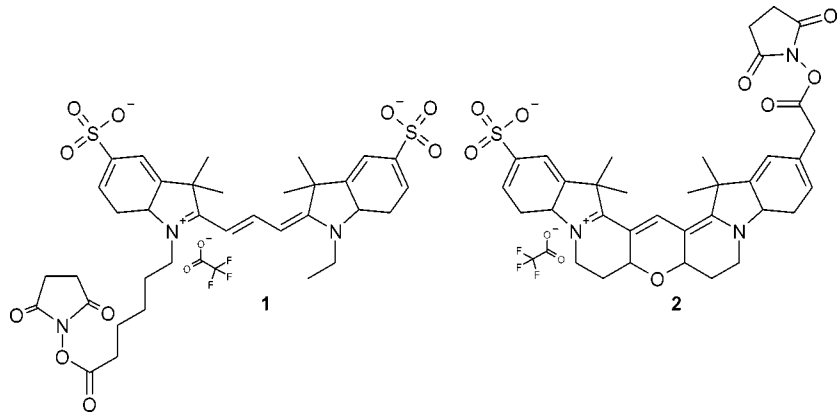

Fig. 1. The structures of Cy3 (1) and Cy3B (2).

conjugatable format (XL665) for screening purposes, although the high molecular weight of >100 kDa can be a hindrance in some assay formats [11] and, due to the proteinaceous nature of the probe it can suffer from long term stability issues.

A similar approach has been taken to optimise the spectral properties of Cy3B. This probe has the same common chromophore as Cy3 (Fig. 1). Open chain trimethine cyanine dyes e.g. Cy3 can exist in many conformations and are capable of undergoing numerous rotational and translational modes of vibration which result in the loss of energy by non-radiative processes and a resultant lowering of ϕ [12]. In Cy3B, a rigid backbone has therefore been introduced to provide a trimethine dye with a fixed conformation in both the relaxed and excited states. This is in a bid to increase the efficiency of the fluorescent output upon excitation. Within the design criteria, essential functionalities such as water solubility (via sulfonation), maintaining a low molecular weight and the ability to bioconjugate (via *N*-hydroxy succinimidyl ester) have been conserved.

EXPERIMENTAL

The synthetic procedures for the synthesis of Cy3B are described elsewhere [9]. PD10 gel filtration columns and Cy3B and Cy3 were obtained from Amersham Biosciences. 5-TAMRA, Texas Red, BODIPY-TMR-X and Alexa 555 were obtained from Molecular Probes, Eugene, OH, Innosense (now Cyanine Technologies) IRIS-3 and Innosense IRIS fuchsia were obtained from Web Scientific Ltd, Crewe, UK and Atto-Tec Atto 565 was obtained from Fluka, Dorset, UK. All dyes were purchased as *N*-hydroxy succinimidyl esters. Goat IgG was obtained from Sigma Aldrich, Dorset, UK.

All spectroscopic measurements were made via wavelength scans. UV-vis spectrophotometry was carried out using a Hitachi U-3310 with either a deuterium lamp (200–340 nm) or tungsten iodide lamp (340–800 nm) in absorbance mode. The scan speed was 120 or 300 nm/min with a slit width of 2 nm.

Fluorescence spectrophotometry was carried out using a Hitachi F-4500 with 150 W Xenon lamp in emission mode. The scan speed was 60 or 240 nm/min with an excitation slit width of 5 nm and an emission slit width of 5 nm. The PMT voltage was 700 V.

Buffer Solutions

Buffer 8.3 (coupling buffer): 100 mM NaCl, 35 mM H_3BO_3, pH 8.3 (with NaOH)
Buffer 7.5 (= buffer A): 100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, pH 7.5 (with NaOH)
Buffer 9: 100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, pH 9 (with NaOH)
Stop buffer (= stop solution): 100 mM $NaH_2PO_4 \cdot H_2O$

IgG Stock Solution

1 mg goat IgG/mL coupling buffer, pH 8.3.

Stock solutions were prepared freshly by dilution of goat IgG with buffer 8.3. Concentrations for exact dilution and final determination were measured by UV-vis spectroscopy: $[\text{IgG}] = (A_{280} - k_{\text{corr dye}} \times A_{\text{dye max}})/210.000$.

Dye Stock Solutions

The most concentrated stock solution of each dye "stock solution A" was prepared by dissolving a defined

mass of the solid dye in a defined volume of DMF (e.g. 2 portions of Cy5 sold as sufficient "to label 1 mg of protein" were diluted with 100 μL DMF), e.g. 50 μL dye stock A was diluted with 50 μL DMSO to give 100 μL stock solution B, and so on. All further dye stock solutions were prepared by serial dilution with DMSO to give a range of solutions of different dye concentration designed to facilitate labelling at different dye:antibody stoichiometries. The concentration of dye in the most concentrated dye stock solution (A) was calculated from the UV-vis spectrum using the following equation.

$$[\text{dye}] = (A_{\text{max dye}}/\varepsilon_{\text{ye}})$$

In particular, 8 μL of the DMF stock solutions were diluted with 1 mL of a 10 mM Na_2CO_3 solution to hydrolyse the dye. After 10 min this solution was diluted with 25 mL of buffer 7.5 and the spectrum was measured. The absorption at 280 nm was divided by the absorbance at the absorption maximum of the dye to give the 280 nm correction coefficient ($k_{\text{corr dye}}$) for each dye.

Standard Procedure for Goat IgG Labelling with Fluorescent Probe

10 μL of a particular dye stock solutions was added to 0.2 mL buffer 8.3 containing 0.2 mg goat-IgG while vortexing and subsequently left to react for 60 min in the dark. This was further vortexed every 10 min during this reaction period. The reaction was terminated with 300 μL of stop buffer.

Separation of labelled IgG from free dye was carried out by gel filtration on Amersham PD-10 columns. The column was pre-washed with 20 mL buffer 7.5, 500 μL of the labelling solution was loaded and allowed to drain. Then, 1 mL buffer 7.5 was added and allowed to drain (2x). For elution of the labelled IgG, 2 mL of water was added and the eluate collected. Finally, 10 mL water was added to elute the unbound dye.

UV-Vis Measurements

The number of bound dyes per antibody was measured by UV-vis spectroscopy. The antibody concentration was measured by absorption at 280 nm corrected for the dye absorption at 280 nm. $[\text{IgG}] = (A_{280} - k_{\text{corr dye}} \times A_{\text{dye max}})/210.000$.

The determination of the correction coefficient is as described above.

The dye concentration was also measured: $[\text{dye}] = (A_{\text{max dye}}/\varepsilon_{\text{dye}})$.

Table I. Spectral Properties of Cy3 and Cy3B

Compound	λ max.abs (nm)	λ max.em. (nm)	ε (L·mol^{-1} cm^{-1})	τ (ns)	ϕ	Relative brightness
Cy3	550	570	150,000	<0.3	0.04	1
Cy3B	558	572	130,000	2.8[a]	0.67[a]	7.9

[a]Non Radiative lifetime = 3.19 ns, $\phi_{\text{f}} = 0.88$.

Fluorescent Measurements

Each solution of labelled IgG, and free dye, was diluted to dye concentration of 50 nM with buffer pH 7.5. For each sample, the fluorescence spectra were recorded at different excitation wavelengths, at 5 nm intervals. The longest wavelength of excitation was close to the wavelength maximal absorption. Non radiative lifetime and quantum yield were calculated by spectral means [13] using trapezoidal numerical integration.

Spectral Properties of Cy3B

The spectral properties of Cy3B have some commonalities with Cy3. These are summarised in Table I. The rigidification has the effect of shifting the absorption spectrum of Cy3B by several nanometers (Fig. 2). Moreover, the pronounced effect of rigidification can be seen in the emission spectrum. An ~8 fold increase in fluorescence was observed with an equimolar solution of Cy3B when compared to Cy3. This is illustrated in Fig. 3. Using Rhodamine 6G as a standard ($\phi_{\text{f}} = 0.94$ [14]), the fluorescence quantum yields for both Cy3 and Cy3B were also measured and found to have a ϕ_{f} for Cy3B of ~0.67 compared to 0.04 for Cy3 in aqueous solution.

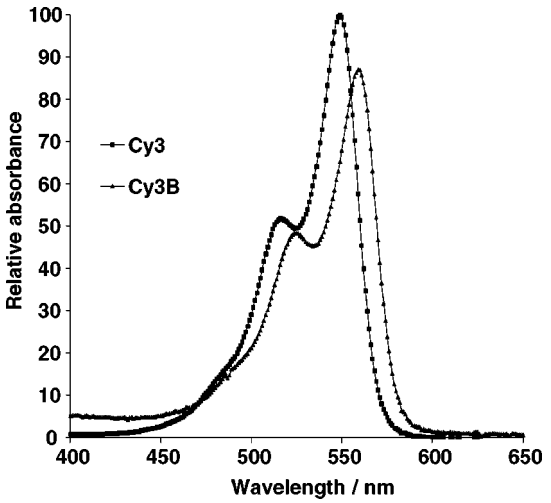

Fig. 2. Absorption spectra of Cy3 and Cy3B.

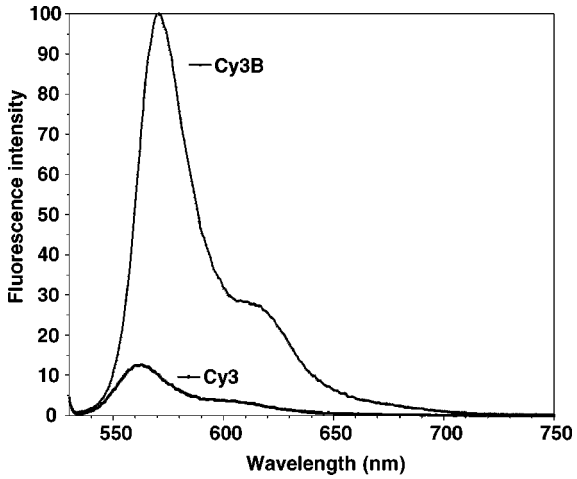

Fig. 3. Emission spectra of equal concentrations of Cy3 and Cy3B (excitation 550 nm).

The lifetime properties of Cy3B are also modified compared to Cy3. The rigid structure of Cy3B has the effect of increasing the mean fluorescent lifetime of the probe to ∼2.9 ns (non-radiative lifetime = ∼3.1 ns).

Bioconjugate Properties of Cy3B

The work of Gruber *et al.* [8] has recently illustrated the importance of measuring the effectiveness of fluorescent probes upon bioconjugation. Cy3B, like other Cydye reagents is, by design, a fluorescent probe for biological labelling and therefore any post-labelling change in the emission properties needs to be well understood. This is especially true when the bioconjugate is to be employed for the quantification of a biomolecular event.

A study was made of the emission properties for Cy3 and Cy3B, in comparison with a range of other spectrally matched, commercially available dyes. This was carried out after bio-conjugation of the probes to goat IgG and at a range of dye-to-antibody concentrations. A number of stock solutions of the different dyes were prepared that allowed a standard concentration of goat IgG to be labelled with different dye stoichiometries. After purification these solutions provided a range of known dye:antibodies ratios calculated from the UV-vis absorption spectra.

For the purpose of these experiments, relative brightness is defined as the fluorescent output of a range dye-labelled antibodies as compared using fluorescence spectroscopy. The level of dye labelling has been calculated using the absorption properties of the fluorescent probe in each case. An important assumption made during this study is that the molar absorptivity of the dyes does not change upon conjugation. The generation of absolute dye:protein data was considered to be beyond the scope of this study. Precedent [8] for this assumption is available and it has demonstrated that spectroscopic methods can provide important information regarding the trends of dye-labelled conjugates. Therefore, the findings of this publication demonstrate important methodologies for attaining relative comparable data. Such techniques offer an insight into the behaviour of these fluorescent probes upon bioconjugation.

RESULTS AND DISCUSSION

The data for the purified dye-antibody conjugates are shown in Fig. 4. A range of stoichiometries for each dye-antibody has been obtained and the emission characteristics of each solution have been measured. Figure 4 compares the emission characteristics of each dye at different IgG labelling ratios.

From these results it can be seen that the fluorescent performance of the different conjugated dyes is wide ranging. Many of the probes display emission quenched characteristics upon increased labelling. This observation is thought to be due to either Förster resonance energy transfer between like molecules, e.g. homotransfer [15] or alternatively, the formation of excimers. Homotransfer is a phenomenon made possible by the "anti-Stokes" overlap of the fluorescence emission spectrum and the absorption spectrum of *like* fluorophores located within a finite distance. This phenomenon becomes more prevalent where the donor concentration is suitably high, such as, at sufficiently high dye-antibody ratios. It is thought that such quenching can also expedited by the close association or aggregation of *like* dyes in solution, subsequently leading to proximal labelling events on the antibody or protein. Planar dyes or dyes with a propensity for π-stacking are more likely to exhibit such aggregation. Although, excimer formation may offer a more plausible explaination. Here quenching occurs due to the formation of excited state dimers. Homotransfer is more likely to occur in a more rigid protein system than the model IgG system chosen. To measure the degree of quenching occurring for Cy3B upon multiple labelling, the emission properties of the free dye were compared to the emission from the different dye:protein ratios obtained for Cy3B labelled IgG. This is illustrated in Fig. 5. Here it can be seen that as the dye:protein ratio increases, the subsequent quenching increases also. The absorption spectrum of the free dye compared to that of a biomolecule labelled with >4 Cy3B molecules also observes an increase in the 520 nm shoulder. This change in the spectral properties of the dye is most likely due to some excimeric formation. Changes in

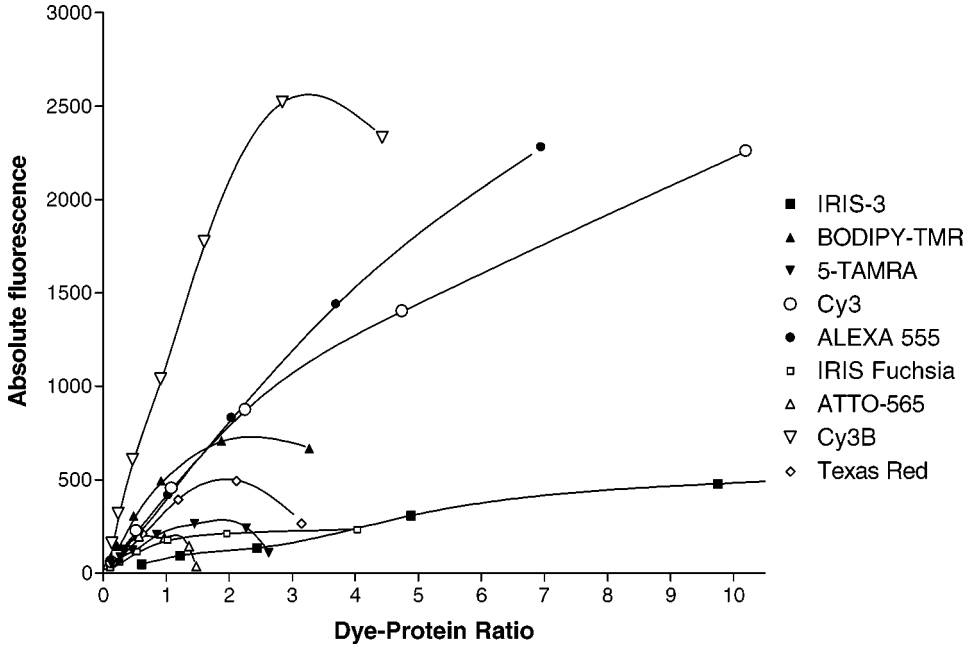

Fig. 4. Comparative emission properties of dye-labelled goat IgG conjugates obtained from a range of commercially available fluorophore NHS esters.

molar absorptivity may also arise due to charged residues prevalent upon the protein. These may have an effect upon the fluorescence quantum yield [19] for the labelled probes if the pKa for such probes is close to that of the buffer used in the experiment. In this instance, Cy3B (and Cy3) are known to be insensitive over the physiological pH range.[4]

Some dyes exhibit an increase in emission upon protein labelling. Gruber and co-workers have previously described this phenomenon for Cy3 [8]. This is often due to the dye being maintained in a more rigid dye conformation upon biomolecular labelling than in the free state in solution. The lipophilic nature of the biomolecule may also shield the dye from solvent quenching. It is apparent from the data presented here, that compared to Cy3B, Cy3 is brighter upon biomolecular labelling than it is in the free form. The ensemble experiments described earlier observed a ∼8 fold increase in brightness for Cy3B in free form when compared to Cy3. The IgG labelling data shows a difference in brightness of ∼2.0–2.5 for Cy3B compared to Cy3 at pertinent labelling concentrations. This is in agreement with a previous study [8], whereby it was demonstrated that the quantum yield of Cy3 increases upon biolabelling of antibodies. Due to the specificity of an antibody for an antigen, it is important that any modification of such immunogens with fluorescent dyes is performed in the least intrusive and non-destructive manner possible. This decreases the likelihood of reduced specificity of the dye-labelled adduct. As such, it can be seen that the maximum fluorescence, for Cy3B, can be obtained from a Cy3B-antibody adduct with approximately 3 dyes attached. Due to the high extinction coefficient and quantum yield of this probe, it can be seen that at all dye-to-antibody concentrations, this probe offers the

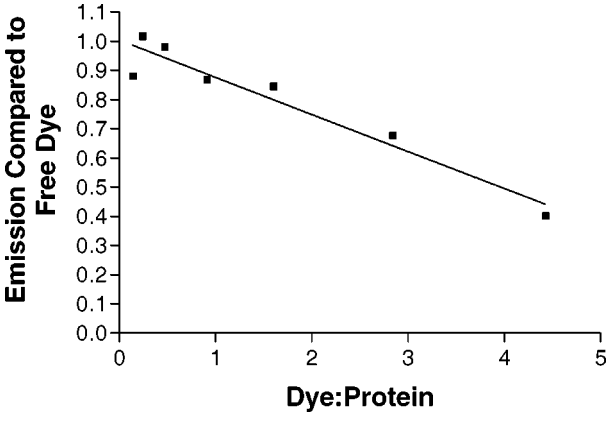

Fig. 5. Comparing the emission of the free Cy3B dye in solution with the emission from variable dye: Protein concentrations.

[4] Amersham Biosciences Unpublished data observe an increase in the shoulder at 520 nm upon the labelling streptavidin, for example with >4 molecules of Cy3B. This increase is indicative of excimeric dye formation.

most efficient emission properties. Upon labelling the antibody with 3 Cy3B dyes, the fluorescence characteristics are more than 2 fold that of Cy3 or Alexa 555 and greater than 3 fold for the remaining dyes employed in this study. Indeed, to obtain an equivalent fluorescence output from the other probes, up to 7 Alexa 555 dye labels are required or 10 Cy3 dye labels. At such high labelling concentrations, there is an increased probability that the specificity of the biological molecule will be affected [17]. It can be seen also that there is no increase in fluorescence for Cy3B upon employing more than 3 dyes per antibody. It is at this dye to protein concentration that some quenching can be observed.

The data in Fig. 4 show that at comparatively low labelling ratios, Cy3B offers the most efficient fluorescent output. This permits biomolecular labelling using Cy3B that is relatively unintrusive and with a low risk of damaging the specificity of the molecule.

Cy3B also has a fluorescent lifetime of 2.9 ns (Table I). This offers Cy3B as a promising dye for fluorescence polarisation. Recent work by Turconi *et al.* [18] described the use of Cy3B in a fluorescence polarisation screening assay whereby higher anisotropy measurements were obtained with Cy3B in comparison with other probes. Biological screening using fluorescence polarisation often requires sub-nanomolar concentrations of fluorophore in order to complement the biological interactions under scrutiny. It is here that brighter and more sensitive red-shifted probes such as Cy3B are beginning to realise potential [19].

ACKNOWLEDGMENTS

The authors would like to thank Dr Rudi Labarbe for assistance with theoretical calculations and the Institute of Biophysics, J. Kepler University, Linz for helpful discussion.

REFERENCES

1. A. J. Pope, H. M. Ulrich, and K. J. Moore (1999). Homogeneous fluorescence readouts for miniaturized high-throughput screening: Theory and practice. *Drug Discov. Today* **4**(8), 350–362.
2. P. Ramm (1999). Imaging systems in assay screening. *Drug Discov. Today* **4**(9), 401–410.
3. P. Ramm and N. Thomas (2003). Technological advances in high-throughput screening. Image-based screening of signal transduction assays. *Sci. STKE 2003*, p. 14.
4. J. Masino, S. G. Jones, and J. A. Alcock (2002). in *Poster Presented at IBC's 7th Annual Drug Discovery Technology*^{TM} *World Congress*, Boston, USA, 2002.
5. T. K. Garyantes (2002). 1536-well assay plates: When do they make sense? *Drug Discov. Today* **7**(9), 489–490.
6. R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, and A. S. Waggoner (1993). Cyanine dye labelling reagents: Sulfoindocyanine succinimidyl esters. *Bioconjug. Chem.* **4**, 105–111.
7. M. W. Wessendorf and T. C. Brelje (1992). Which fluor is the brightest. A comparison of staining obtained using fluorescein, TMR, rhodamine, Texas Red and Cy3. *Histochemistry* **98**, 81–85.
8. H. J. Gruber, C. D. Hahn, G. Kada, C. K. Riener, G. S. Harms, W. Ahrer, T. Dax, and G. H. G. Knaus (2000). Anomalous fluorescence enhancement of Cy3 and Cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalent linking to IgG and non-covalent binding to Avidin. *Bioconjug. Chem.* **11**(5), 696–704.
9. A. S. Waggoner and R. Mujumdar (2001). *Rigidized Trimethine Cyanine Dyes*, U.S. Patent 6133445A.
10. V. Oi, A. N. Glazer, and L. Stryer (1982). Fluorescent phycobiliprotein conjugates for analyses of cells and molecules. *J. Cell Biol.* **93**, 981–986.
11. E. Trinquet, F. Maurin, H. Bazin, and G. Mathis. (2003). in *Poster Presentation at MAF Conference*, Prague August 24th–27th, 2003.
12. D. F. O'Brien, T. M. Kelly, and L. F. Costa (1974). Excited state Properties of some Carbocyanine dyes and the energy transfer mechanism of spectral sensitisation. *Photogr. Sci. Eng.* **18**(1), 76–84.
13. J. R. Lakowicz (1999). *Principles of Fluorescence Spectroscopy*, 2nd ed., Kluwer Academic, New York, p. 10.
14. M. Fischer and J. Georges (1996). Fluorescence quantum yield of rhodamine 6G in ethanol as a function of concentration using thermal lens spectrometry. *Chem. Phys. Lett.* **260**, 115–118.
15. B. Wieb Van Der Meer, G. Coker III, and S.-Y. Simon Chen (1994). *Resonance Energy Transfer, Theory and Data*, VCH publishers, New York.
16. R. Sjöback, J. Nygren, and M. Kubista (1998). Characterization of fluorescein-oligonucleotide conjugates and measurement of local electrostatic potential. *Biopolymers* **46**, 445–453.
17. H. Karsilayan, I. Hemmila, H. Takalo, A. Toivonen, K. Pettersson, T. Lovgren, and V.-M. Mukkala (1997). Influence of coupling method on the luminescence properties, coupling efficiency, and binding affinity of antibodies labeled with Europium(III) chelates. *Bioconjug. Chem.* **8**(1), 71–75.
18. S. Turconi, K. Shea, S. Ashman, K. Fantom, D. L. Earnshaw, R. P. Bingham, U. M. Haupts, M. J. B. Brown, and A. J. Pope (2001). Real experiences of uHTS: A prototypic 1536-well fluorescence anisotropy-based uHTS screen and application of well-level quality control procedures. *J. Biol Screen.* **6**(5), 275–290.
19. T. C. Turek-Etienne, E. C. Small, S. C. Soh, T. A. Xin, P. V. Gaitonde, E. B. Barrabee, R. F. Hart, and R. W. Bryant (2003). Evaluation of fluorescent compound interference in 4 fluorescence polarization assays: 2 Kinases, 1 Protease, and 1 Phosphatase. *J. Biol. Screen.* **8**(2), 176–184.