Fluorescence and Fluorescence Applications

Mark A. Behlke[1,2], Lingyan Huang[2], Lisa Bogh[3], Scott Rose[2], and Eric J. Devor[1]
[1]Molecular Genetics and Bioinformatics
[2]Molecular Genetics and Biophysics
[3]Analytical Services
Integrated DNA Technologies

Introduction

Since the introduction of the polymerase chain reaction in the early 1980s perhaps no single technology has had a greater impact on molecular biology than fluorescence. Fluorescence-labeled oligonucleotides and dideoxynucleotide DNA sequencing terminators have opened a seemingly limitless range of applications in PCR, DNA sequencing, microarrays, and *in situ* hybridization and have done so with vastly enhanced sensitivity and dramatically increased laboratory safety.

In this report we will present an overview of fluorescence and will discuss a number of issues related to applications of fluorescence and fluorescence-labeled oligonucleotides.

Principles of Fluorescence

To begin, let us first distinguish **fluorescence** from **luminescence**. Luminescence is the production of light through excitation by means other than increasing temperature. These include chemical means (chemiluminescence), electrical discharges (electro-luminescence), or crushing (triboluminescence). Fluorescence is a short-lived type of luminescence created by electromagnetic excitation. That is, fluorescence is generated when a substance absorbs light energy at a short (higher energy) wavelength and then emits light energy at a longer (lower energy) wavelength. The length of time between absorption and emission is usually relatively brief, often on the order of 10^{-9} to 10^{-8} seconds. The history of a single fluorescence event can be shown by means of a Jablonski Diagram, named for the Ukranian born physicist Aleksander Jablonski (Fig.1). As shown, in Stage 1 a photon of given energy $h\nu_{ex}$ is supplied from an outside source such as a laser or a lamp. The fluorescent molecule, lying in its **ground energy state** S_o, absorbs the energy creating an **excited electronic singlet state** S_1'. This excited state will last for a finite time, usually one to ten nanoseconds (sec^{-9}), during which time the fluorescent molecule (aka, **fluorophore**) undergoes conformational changes and can be subject to myriad potential interactions with its molecular environment. The first phase of Stage 2 is characterized by the fluorophore partially dissipating some of the absorbed energy creating a **relaxed singlet state** S_1. It is from this state that the fluorophore will enter the second phase, the emission of energy, $h\nu_{em}$. Finally, in Stage 3, the fluorophore will return to its ground state, S_o.

The term fluorescence comes from the mineral fluorspar (calcium fluoride) when Sir George G. Stokes observed in 1852 that fluorspar would give off visible light (fluoresce)

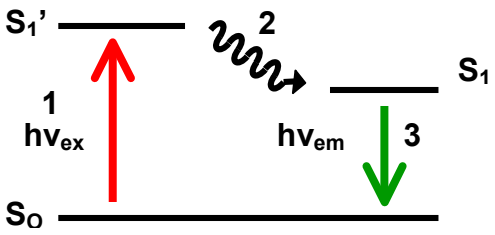

Fig. 1. Jablonski Diagram of a fluorescence event. The fluorescent molecule begins in its ground energy state, S_0, and is converted to an excited singlet state, S_1', by absorbing energy in a specific wavelength. The molecule will transition to the relaxed singlet state, S_1, by releasing some of the absorbed energy. Finally, the molecule will return to its ground energy state by releasing the remaining energy. The duration of a single fluorescence event is a few nanoseconds.

when exposed to electromagnetic radiation in the ultraviolet wavelength. Stokes' studies of fluorescent substances led to the formulation of Stokes' Law, which states that the wavelength of fluorescent light is always greater than that of the exciting radiation. Thus, for any fluorescent molecule the wavelength of emission is always longer than the wavelength of absorption.

Fluorescence Spectra and FRET

As noted, molecules that display fluorescence are called **fluorophores** or **fluorochromes**. One group of fluorophores routinely used in molecular biology consists of planar, heterocyclic molecules exemplified by fluorescein (aka FAM), Coumarin, and Cy3 (figure 2). Each of these molecules has a characteristic absorbance spectrum and a characteristic emission spectrum. The specific wavelength at which one of these

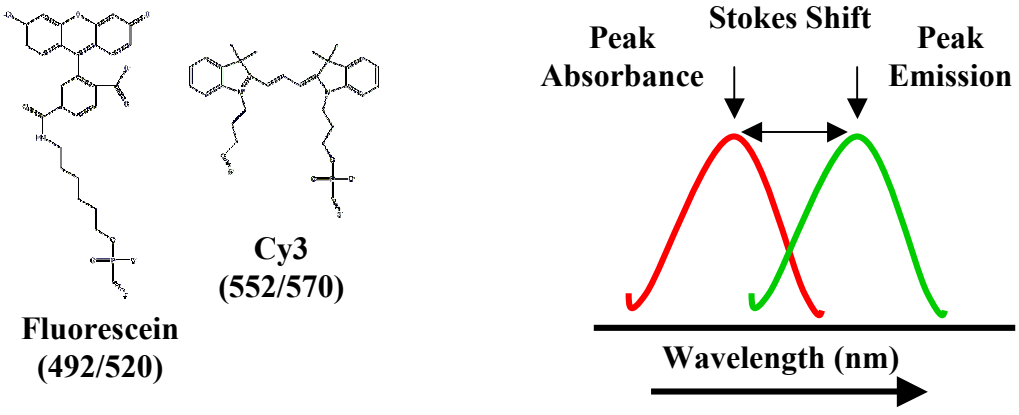

Fig. 2. On the left are examples of the ring structures characteristic of fluorescent molecules. The peak absorbance and peak emission (in nanometers) of each fluorophore is shown. On the right a generalized representation of the absorbance and emission spectra of a fluorophore is shown.

molecules will most efficiently absorb energy is called the **peak absorbance** and the wavelength at which it will most efficiently emit energy is called the **peak emission**. A

generalized representation of these characteristic spectra is also shown in figure 2. The difference between peak absorbance and peak emission is known as the **Stokes Shift** after Sir George Stokes. Peak absorbance and peak emission wavelengths for most of the fluorophores used in molecular applications are shown in Table 1.

Table 1

Peak absorbance and peak emission wavelength, Stokes shift, and Extinction Coefficient, ε, for 43 Common Fluorophores[&]

Dye	Ab(nM)	Em(nM)	SS(nM)	Extinction Coef[#]
Acridine	362	462	100	11,000
AMCA	353	442	89	19,000
BODIPY FL-Br2	531	545	14	75,000
BODIPY 530/550	534	545	10	77,000
BODIPY TMR	544	570	26	56,000
BODIPY 558/568	558	559	11	97,000
BODIPY 564/570	563	569	6	142,000
BODIPY 576/589	575	588	13	83,000
BODIPY 581/591	581	591	10	136,000
BODIPY TR	588	616	28	68,000
BODIPY 630/650*	625	640	15	101,000
BODIPY 650/665*	646	660	14	102,000
Cascade Blue	396	410	14	29,000
Cy2	489	506	17	150,000
Cy3*	552	570	18	150,000
Cy3.5	581	596	15	150,000
Cy5*	643	667	24	250,000
Cy5.5*	675	694	19	250,000
Cy7	743	767	24	250,000
Dabcyl*	453	none	0	32,000
Edans	335	493	158	5,900
Eosin	521	544	23	95,000
Erythrosin	529	553	24	90,000
Fluorescein*	492	520	28	78,000
6-Fam*	494	518	24	83,000
TET*	521	536	15	-
Joe*	520	548	28	71,000
HEX	535	556	21	-
LightCycler 640	625	640	15	110,000
LightCylcer 705	685	705	20	-
NBD	465	535	70	22,000
Oregon Green 488*	492	517	25	88,000
Oregon Green 500	499	519	20	78,000
Oregon Green 514*	506	526	20	85,000
Rhodamine 6G	524	550	26	102,000
Rhodamine Green*	504	532	28	78,000
Rhodamine Red*	560	580	20	129,000
Rhodol Green	496	523	27	63,000
TAMRA*	565	580	15	91,000
ROX*	585	605	20	82,000
Texas Red*	595	615	20	80,000
NED	546	575	29	-
VIC	538	554	26	-

*Routinely Offered by IDT [#]Energy capture efficiency

[&]Figures are given for an activated NHS-ester with a linker arm.

Fluorescence Resonance Energy Transfer (FRET)

Energy emitted from a fluorophore is characteristically in the form of light. However, energy emission from some fluorophores can be in the form of heat dissipation. Molecules that dissipate absorbed energy as heat are a special class known as **quenchers**. Quenchers have the useful properties that they will absorb energy over a wide range of wavelengths and because they dissipate their absorbed energy as heat they remain dark. As a result of these properties, quenchers have become very useful as energy acceptors in **fluorescent resonance energy transfer** (FRET) pairs. The FRET phenomenon involves the direct excitation of an acceptor fluorophore by a donor fluorophore following excitation of the donor by electromagnetic radiation in the proper wavelength (figure 3).

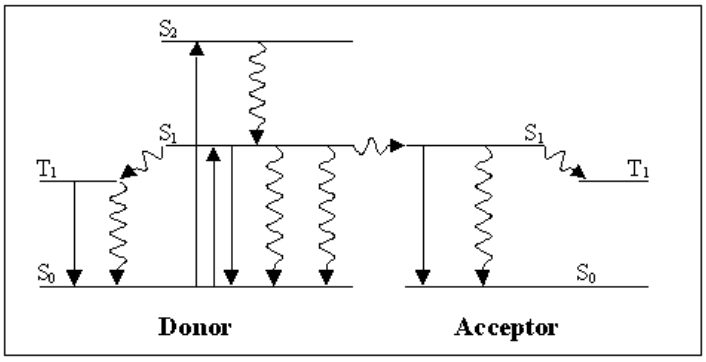

Fig. 3. Jablonski Diagram of fluorescence resonance energy transfer, FRET. Excitation and emission of energy in the donor molecule conforms to the model shown in figure 1. The fate of the emitted energy in a FRET pair is excitation of the acceptor molecule which is modeled on the right. Here, resonance energy is emitted as in figure 1 but at a substantially longer wavelength than would be emitted by the donor molecule.

Acceptance of donor energy by a FRET acceptor requires that two criteria must simultaneously be satisfied. One of these criteria is **compatibility** and the other criterion is **proximity**. Compatibility is precisely defined. A compatible acceptor is a molecule whose absorbance spectrum overlaps the emission spectrum of the donor molecule (figure 4). If the absorbance spectrum of a molecule does not overlap the emission spectrum of the donor, the emitted energy will not be able to excite the potential acceptor. If the absorbance spectrum of the acceptor does overlap the emission spectrum of the donor, energy from the donor will excite the acceptor molecule provided that the proximity criterion is met.

Proximity is less precisely defined in operational terms. Proximity means that a compatible acceptor molecule is “close enough” to the donor for the energy to excite it. In practical terms, it is assumed that the mechanism for excitation energy transfer between a compatible donor-acceptor fluorophore pair is the Förster mechanism in which the singlet energy transfer rate $\kappa(R)$ is,

$$\kappa(R) = \kappa_F \, (1 / (1 + (R / R_F)^6) \qquad (1)$$

where R is the distance between the two molecules, R_F is the **Förster radius** and κ_F is the rate of transfer between donor and acceptor when the distance between them is small; i.e., $R / R_F \rightarrow 0$ (Förster, 1948). From (1) it can be seen that, when $R = R_F$, $\kappa(R) = ½$. Thus, for convenience, we may define the Förster radius as the distance at which resonance energy transfer between compatible FRET pairs drops to 50%. What this means in molecular biology terms is that there is a maximum length of an oligonucleotide, with one member of a FRET pair tethered at each end, beyond which FRET will not be sufficiently efficient for reliable assays (figure 4). In practice, this maximum length is greater than 60 – 70 nucleotides (nt) for many FRET pairs.

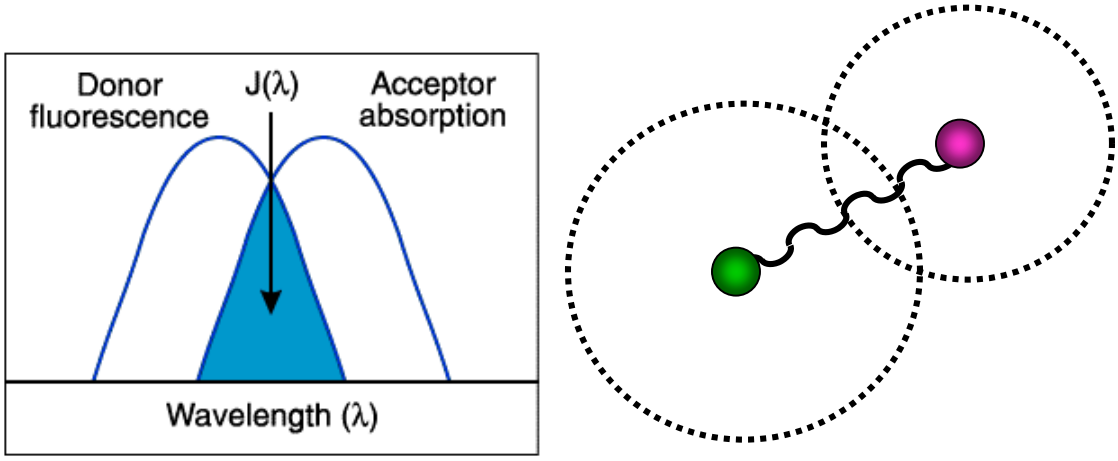

Fig. 4. Representation of compatibility and proximity in a FRET donor and acceptor fluorophore pair. On the left, the relationship between the absorbance and emission spectra of the FRET pair is shown. On the right is a representation of acceptable proximity for a FRET pair in terms of their Förster radii. The tether between the two fluorescent molecules is an oligonucleotide.

In terms of fluorescence assays using FRET pairs, consider the example of the classic FRET pair of FAM and TAMRA. Peak absorbance wavelength for FAM is 494 nanometers (nm) with a peak emission wavelength at 518nm. If FAM and TAMRA are tethered at the 5' and 3' ends respectively of a 35-mer oligonucleotide and this construct is excited at 494nm, so long as the oligonucleotide remains intact emission will be at 580nm and not at 518nm due to FAM transferring its energy to TAMRA. Once the oligonucleotide is disrupted by, say, an exonucleolytic reaction, excitation at 494nm will result in emission at 518nm. This is due to the fact that the pair is no longer tethered and, even though they are compatible, they are no longer proximate.

Dark Quenchers

In recent years TAMRA, as well as other fluorescent acceptor molecules, has been replaced with one or another of the growing family of dark quencher molecules. Quenchers are chemically related to fluorophores but instead of emitting absorbed fluorescence resonance energy as light they have the useful property of transforming the light energy to heat. Heat dissipation of fluorescence energy means that replacing a fluorescent acceptor like TAMRA with a quencher such as Iowa Black™ FQ will result in an oligonucleotide construct that has no measurable fluorescence so long as the oligonucleotide tether remains intact. Such constructs can greatly simplify many fluorescence assays since there will be no background fluorescence. For this reason, fluorophore-quencher dual-labeled probes have become a standard in kinetic (real-time) PCR. A compilation of recommended fluorophore/quencher FRET pairs is provided in Table 2.

Table 2
Reporter/Quencher Combinations

Dabcyl	BHQ™-1	BHQ™-2	Iowa Black™ FQ/RQ
Oregon Green™ 488-X 6-FAM™ TET™ JOE HEX™ Cy3™ (TAMRA™) (ROX™) (Texas Red®)	Oregon Green™ 488-X 6-FAM™ Rhodamine Green™-X Oregon Green™ 514 TET™ JOE HEX™ Cy3™ Rhodamine Red®-X TAMRA™	HEX™ Cy3™ Rhodamine Red™-X TAMRA™ ROX™ Texas Red™-X Bodipy 630/650™-X Bodipy 650/665™-X (Cy5™)	6-FAM™ Rhodamine Green™-X Oregon Green™ 514 TET™ JOE HEX™ Cy3™ Rhodamine Red™-X ROX™ Texas Red™-X TAMRA™ Bodipy 630/650™-X Bodipy 650/665™-X Cy5™

As can be seen, quenchers absorb fluorophore emission energies over a wide range of wavelengths. This expanded dynamic range greatly adds to the utility of fluorescence quenchers, particularly in the case of multiplexing assays with different fluorophores. A graphical representation of the dynamic range of several fluorescence quenchers is shown in figure 5.

Much of what has been discussed here with respect to FRET applies to all oligonucleotide constructs in which a fluorescence donor and a fluorescence acceptor are paired. It is particularly appropriate for dual-labeled probes used in real-time PCR applications. There is one type of dual labeled oligonucleotide construct that does deserve special mention due to additional design demands associated with it. This, of course, is the **molecular beacon**. While most dual-labeled oligonucleotide probe applications fall under the general heading of **hydrolysis probes** (cf., Bustin, 2000, 2002), molecular

beacons rely upon uni-molecular/bi-molecular thermodynamic relationships for their action. A separate discussion of molecular beacons is presented as **Supplemental Material** to this report.

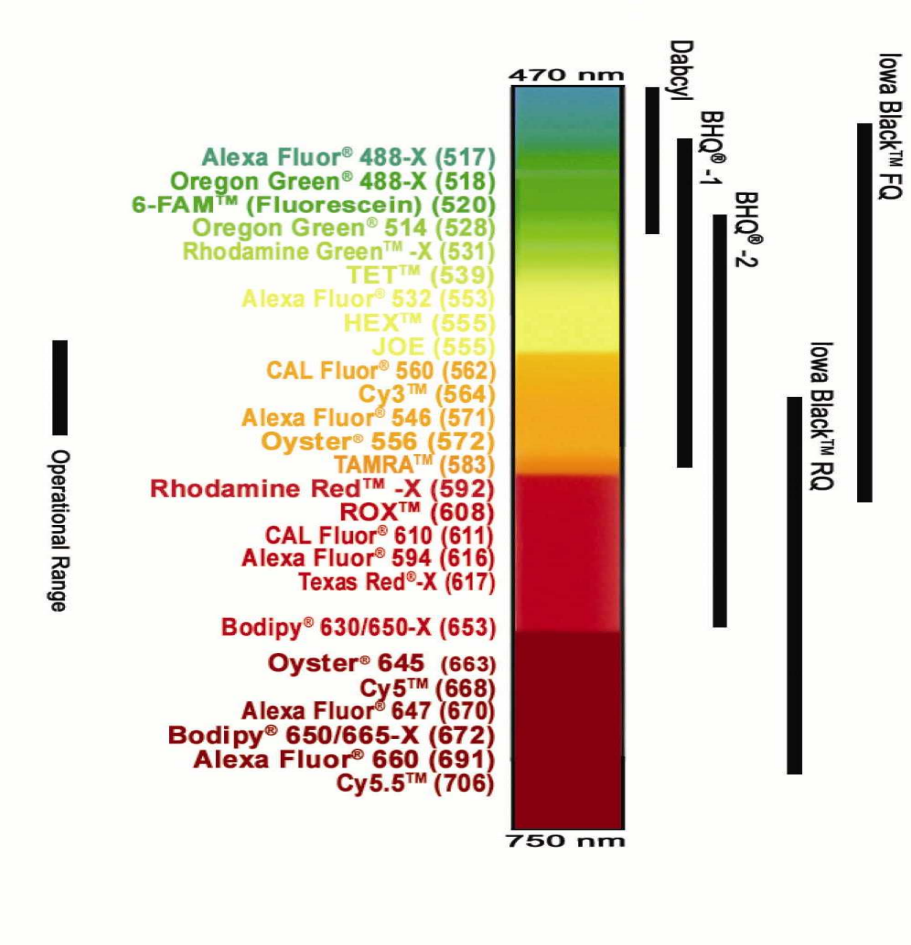

Fig. 5. Dynamic ranges of a number of fluorescence quenchers. The number of wavelengths over which one of these quenchers will absorb fluorescence energy provides flexibility in choosing fluorophores for multiplex assays.

Caveat: Proximal G-base Quenching

Detection of dye-labeled nucleic acids via fluorescence reporting has become a routine technique in molecular biology laboratories. Given this, it is important to note that the quantum yield of fluorophores attached to nucleic acids is dependent upon a number of factors. One of these is the choice of the base that lies adjacent to the fluorescent molecule. Fluorescence quenching by an adjacent guanosine nucleotide is an under-appreciated phenomenon that can significantly effect quantum yield. Depending upon the fluorophore, this effect can be as much as 40%.

The mechanism of fluorophore quenching has been explained by electron sharing/donor properties of the adjacent base (Nazarenko et al., 2002). Quenching of 2-aminopurine fluorescence in DNA is dominated by distance-dependent electron transfer

from 2-aminopurine to guanosine (Kelly and Barton, 1999). Seidel et al. (1996) found that photo-induced electron transfer plays an important role in this type of quenching. The order of quenching efficiency is G<A<C<T if the nucleobase is reduced but it is the reverse, G>A>C>T, if the nucleobase is oxidized (Seidel et al., 1996). Nazarenko et al. (2002) also report that quenching by adjacent nucleobases is dependent upon the location of the fluorophore within the oligonucleotide.

We have investigated some of the practical aspects of fluorescence quenching by an adjacent guanosine nucleotide. A series of fluorescence-labeled oligonucleotides sharing the same core sequence was synthesized such that one of three commonly used fluorophores and each of the four possible adjacent nucleotides was present in each construct (Table 3).

Table 3.
Fluorescent Test Oligonucleotides Studied

5'-Dye	DNA Sequence	3'-Dye
	GGAAACAGCTATGACCATA	-Fluorescein
	GGAAACAGCTATGACCATG	-Fluorescein
	GGAAACAGCTATGACCATC	-Fluorescein
	GGAAACAGCTATGACCATT	-Fluorescein
	GGAAACAGCTATGACCATA	-Cy3™
	GGAAACAGCTATGACCATG	-Cy3™
	GGAAACAGCTATGACCATC	-Cy3™
	GGAAACAGCTATGACCATT	-Cy3™
Hex™-T	GGAAACAGCTATGACCAT	
Hex™-G	GGAAACAGCTATGACCAT	
Hex™-C	GGAAACAGCTATGACCAT	
Hex™-A	GGAAACAGCTATGACCAT	

The concentration of each oligonucleotide was normalized by OD_{260} in buffer (10mM Tris HCl (pH 8.3), 50mM KCl, 5mM $MgCl_2$). Fluorescence measurements were made for a 200nM solution of each oligonucleotide on a PTI (Photon Technologies International) scanning fluorometer. Results for each of the three dyes are presented in figure 6. As can be seen both 3' fluorescein and 5' HEX™ (hexachlorofluorescein) displayed significant quenching when the adjacent nucleotide was guanosine. In contrast, the 3' Cy3™ was little affected by the choice of adjacent nucleotide.

Fluorescence intensities at the emission maximum for each dye were normalized relative to the value obtained when the adjacent base is adenine. These data are shown in figure 6. Here, it is clear that an adjacent guanosine has the greatest affect on all three fluorophores even though it is minimal for Cy3™. These results suggest that adjacent guanosine nucleotides should be avoided when designing oligonucleotides that contain a fluorescent reporter molecule.

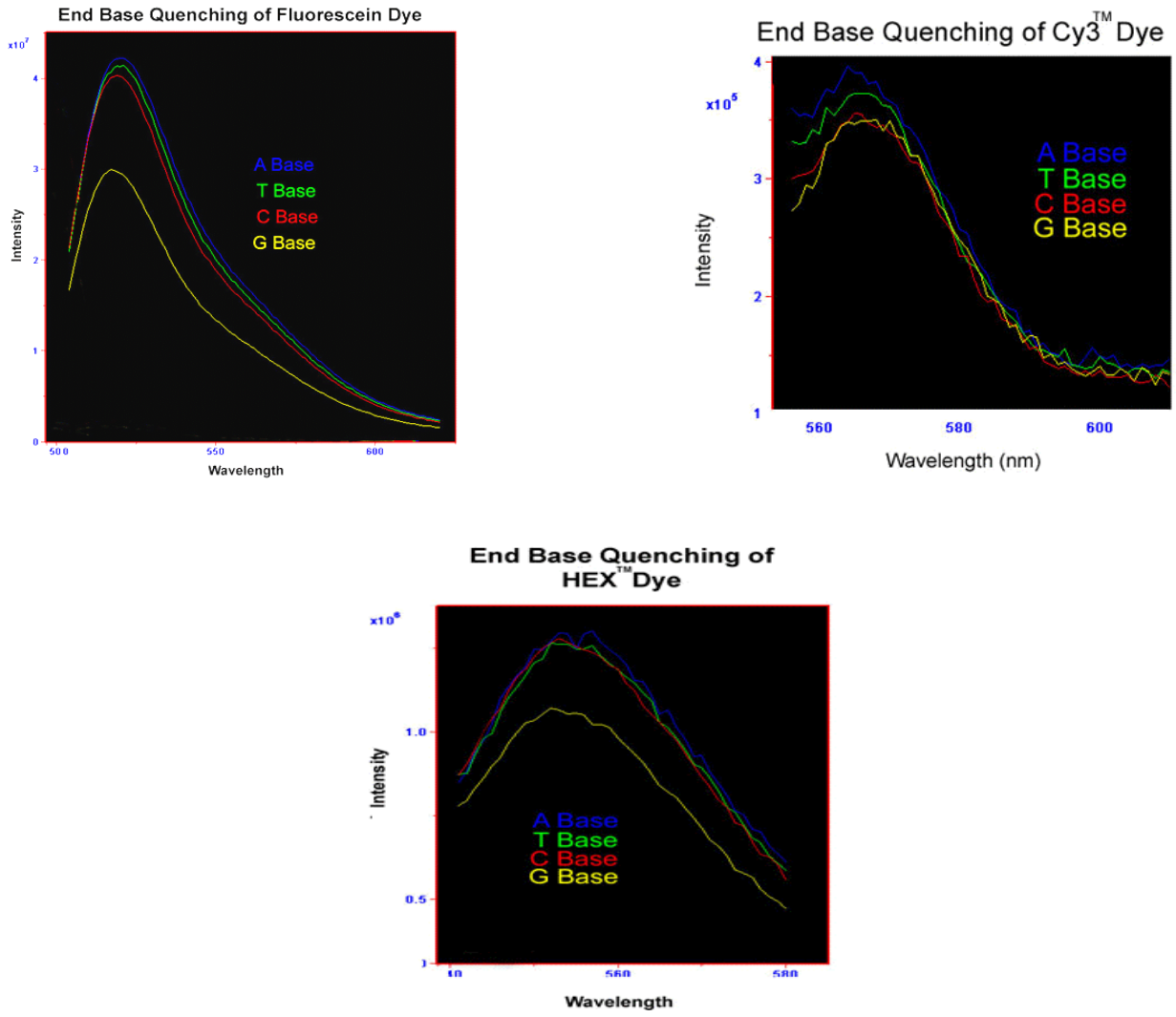

Fig. 5. Scanning fluorometer results obtained with the oligonucleotide constructs shown in Table 3.

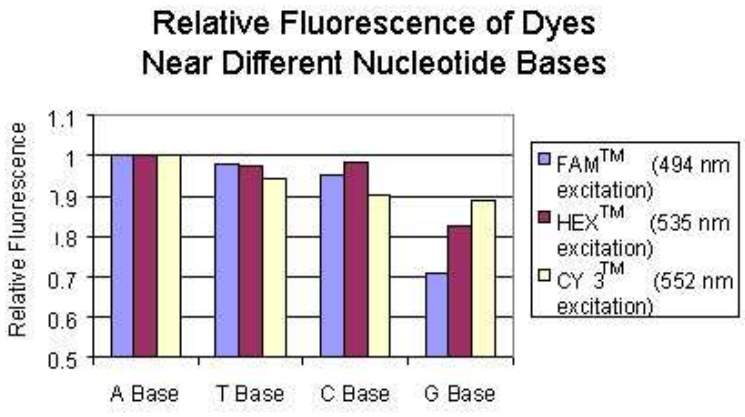

Fig. 6. Relative fluorescence intensities of FAM™, HEX™, and Cy3™ as a function of the nucleotide adjacent to the fluorophore.

References
Bustin SA 2000 Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction. *J Mol Endocrinol* **25**: 169-193.

Bustin SA 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* **29**: 23-39.

Förster T 1948 Zwischenmolekulare energiewnaderung und fluoreszenz. *Ann Phys* **2**: 55-75.

Kelley, S.O. and Barton, J.K. (1999) Electron Transfer between bases in double helical DNA. *Science* **283**:375-381.

Nazarenko, I., Pires, R., Lowe, B., Obaidy, M., and Rashtchian, A. (2002) "Effect of Primary and Secondary Structure of oligodeoxyribonucleotides on the fluorescent properties of Conjugated dyes. *Nuc. Acids. Res.* **30**:2089-2195.

Seidel, C.A.M., Schulz, A., and Sauer, M.H.M.(1996) Nucleobase-Specific Quenching of Fluorescent Dyes. 1. Nucleobase One-Electron Redox Potentials and Their Correlation with Static and Dynamic Quenching Efficiencies. *J. Phys. Chem.* **100**:5541-5553.

Supplemental Material: Molecular Beacons

Annealing of an oligonucleotide to its complement is a highly specific molecular recognition event. Under appropriate conditions, a single-stranded oligonucleotide of sufficient size can find a complementary sequence even in the presence of a large excess of other nucleic acids. However, detectable changes that accompany the formation of the double-stranded duplex are relatively few. Therefore, the hybridizing probe molecule must be labeled in a way that permits unambiguous detection of the duplex state. Dual-labeled fluorogenic molecular beacons are proving to be superior probes for detecting oligonucleotide hybridization. Unlike traditionally labeled oligonucleotide probes, molecular beacons enable dynamic, real-time detection of nucleic acid hybridization events both *in vitro* and *in vivo* (Tyagi and Kramer, 1996; Kostrikis *et al*., 1998; Tyagi *et al*., 1998). What is more, since molecular beacons can be used to discriminate between targets that have a single base-pair change, they are ideal for hybridization-based investigations of single nucleotide polymorphisms (SNPs).

Uses of Molecular Beacons

One of the primary advantages of molecular beacons is that they can discriminate between targets that differ by as little as a single base pair change, making them ideal for

investigating single nucleotide polymorphisms (SNPs). Bonnet and colleagues (1999) undertook a thermodynamic analysis of the molecular beacons and concluded that enhanced specificity is a feature of conformationally constrained probes in general. Although the perfectly matched probe:target duplex is more stable than the single-stranded hairpin structure of the molecular beacon, the mismatched probe:target duplex is not, and this thermodynamic feature is the key to the exquisite specificity displayed by molecular beacons. Tyagi and colleagues (1998) found that mismatched hairpin-probe duplexes were less stable than mismatched linear probe duplexes at all target concentrations. In addition, they found that the molecular beacons could discriminate a perfectly matched and a mismatched target, regardless of the base pair combination of the mismatch. Smit and colleagues (2001) found that unlike conventional methods, molecular beacon-based genotyping assays were compatible with automated methods, making them ideal for high-throughput screening of heritable diseases.

Molecular beacons have been used in PCR assays to detect rifampin resistance in Mycobacterium tuberculosis (El-Hajj *et al*., 2001; Piatek *et al*., 1998) and to detect virus replication in HIV type1-infected individuals (Lewin *et al*., 1999). A molecular beacon that contained a G-rich 18-mer was used to investigate the thermodynamics of triplex DNA formation (Anthony *et al*., 2001). In addition, molecular beacons have been used to identify RNA transcripts in living cells (Sokol *et al*., 1998) and to detect DNA-binding proteins (Heyduk and Heyduk, 2002). Stojanovic and colleagues (2001) have also constructed a catalytic molecular beacon by sandwiching a hammerhead-type deoxy-ribozyme between the beacon's self-complementary ends.

Design Considerations

Molecular beacons are designed so the probe sequence is sandwiched between two complementary sequences that form the hairpin stem (figure 1). Molecular beacons must be designed so that the transition between two conformational states -the hairpin and the probe:target duplex - is thermodymically favorable. The temperature and the buffer used will influence probe specificity and must be carefully controlled. As a general rule, the melting temperature of both the hairpin structure and the probe:target duplex should be 7-10^{o}C higher than the temperature used for detection or for primer annealing.

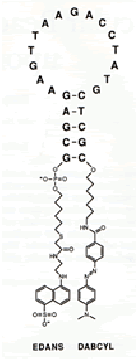

Fig.1. The classic model of a molecular beacon as first presented by Kramer and colleagues.

A fundamental feature of a molecular beacon is that probe-target hybrids cannot co- exist with stem hybrids due to the rigidity of DNA helices. A perfect match probe-target hybrid will be energetically more stable than the stem-loop structure whereas a mismatched probe-target hybrid will be energetically less stable than the stem-loop structure. This characteristic is the basis of the extraordinary specificity offered by molecular beacons. If it is desirable to tolerate mismatches in the assay, specificity can be relaxed by making the probe sequence in the loop and the probe-target hybrid more

stable. In practice, stems of 5-6 bases and probe-loop sequences of 16-22 bases are most commonly used. These averages assume that the molecular beacon targets a genome having an average G/C content. For more G/C-rich target sequences, the probe length can be reduced to as few as 16 nucleotides and still retain high specificity. Similarly, for A/T-rich target sequences, the probe length can be increased to as many as 25 nucleotides.

Another consideration in molecular beacon design is the choice of fluorophore and quencher. Dabcyl (4-(4'-dimethylaminophenylazo)benzoic acid) has been found to be the optimal choice for the quencher. Dabcyl is a neutral, hydrophobic molecule that makes it ideal for pairing with a variety of fluorophores. Further, dabcyl must be close to or directly in contact with the fluorophore for energy-transfer quenching to be efficient. Thus, dabcyl has an operational range for quenching that is small compared to the total length of a beacon oligonucleotide. Thus a stem-loop beacon is quenched while a probe-target hybrid is not quenched.

Table 1
Molecular Beacons Synthesized by IDT

5' Reporter	Quencher
5' 6-FAM™	3' Dabcyl
5' TET™	
5' HEX™	
5' 6-FAM™	3' BHQ™-1
5' TET™	
5' HEX™	3' BHQ™-2
5' Cy3™	
5' Cy5™	
5' Cy5.5™	
5' Oregon Green® 488-X NHS Ester	3' Dabcyl
5' Texas Red® NHS Ester	
5' TAMRA™ NHS Ester	
5' ROX™ NHS Ester	
5' JOE™ NHS Ester	

Applications

The versatile features of Molecular Beacons permit their use in many different quantitative and qualitative target detection assays. As a tool to detect amplified targets, Molecular Beacons have been adapted to both real-time and end-point PCR and RT-PCR assays. They have also been used in the detection of RNA species in a homogenous, real-time NASBA assay (Leone *et al.*, 1998). Historically, the first use of a molecular beacon was in real-time monitoring of DNA amplification during PCR (see Tyagi and Kramer,

1996). Exploiting the option to employ different dyes, molecular beacon assays can be multiplexed and have been used for real-time fluorescent genotyping (Kostrikis *et al.*, 1998; Tyagi *et al.*, 1998) and in the simultaneous detection of four different pathogenic retroviruses in clinical samples (Vet *et al.*, 1999).

The specificity of Molecular Beacons allows for use in single nucleotide polymorphism (SNP) detection (Marras *et al.*, 1999). Their simplicity and sensitivity enables use in thermodynamic studies of the state transitions of the probes themselves (Bonnet *et al.*, 1999). Finally, the non-toxic, homogenous nature of the probes allows for their use *in vivo*. Molecular Beacons have been used to detect transcripts in tissue culture cells following microinjection (Sokol *et al.*, 1998). Applications to FISH, chromosome painting, and even real-time visualization of mRNA migration are envisioned. Many other applications are sure to appear in the scientific literature as the full potential of this exciting new technology emerges.

Bonnet, G., Tyagi, S., Libchaber, A., and Kramer, F.R. (1999) "Thermodynamic basis of the chemical specificity of structured DNA probes." *Proc. Natl. Acad. Sci. U.S.A.*, **96**:6171-6176.

Fang, X., Liu, X., Schuster, S., and Tan, W. (1999) "Designing a novel molecular beacon for surface-immobilized DNA hybridization studies." *J. Am. Chem. Soc.*,**121**:2921-2922.

Kostrikis, L.G., Tyagi, S., Mhlanga, M.M., Ho, D.D., and Kramer, F.R. (1998) "Molecular beacons: spectral genotyping of human alleles." *Science*, **279**:1228-1229.

Leone, G., van Schijndel, H., van Gemen, B., Kramer, F.R., and Schoen, C.D. (1995) "Molecular beacon probes combined with amplification by NASBA enable homogenous real-time detection of RNA." *Nucleic Acids Res.*, **26**:2150-2155.

Marras, S.A.E., Kramer, F.R., and Tyagi, S. (1999) "Multiplex detection of single-nucleotide variation using molecular beacons." *Genet. Anal. Biomol. Eng.*, **14**:151-156.

Sokol, D.L., Zhang, X., Lu, P., and Gewirtz, A.M. (1998) "Real time detection of DNA:RNA hybridization in living cells." *Proc. Natl. Acad. Sci. U.S.A.*, **95**:11538-11543.

Tyagi, S., and Kramer, F.R. (1996) "Molecular beacons: probes that fluoresce upon hybridization." *Nature Biotechnology*, **14**:303-308.

Tyagi, S., Bratu, D.P., and Kramer, F.R. (1998) "Multicolor molecular beacons for allele discrimination." *Nature Biotechnology*, **16**:49-53.

Vet, J.A.M., Majithia, A.R., Marras, S.A.E., Tyagi, S., Dube, S., Poiesz, B.J., and Kramer, F.R. (1999) "Multiplex detection of four pathogenic retroviruses using molecular beacons." *Proc. Natl. Acad. Sci. U.S.A.*, **96**:6394-6399.

