Selected Article

Biological and Biochemical Applications of the FluoroMax™-4

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The FluoroMax™-4, alone or when equipped with optional automated polarizers, phosphorimeter, and time-correlated single-photon counting accessory, can illuminate biochemical research into gene-expression using molecular beacons, Förster resonance energy-transfer between biomolecules, and anisotropy studies within biochemical environments.

Introduction

HORIBA Jobin Yvon's benchtop FluoroMax™-4 spectrofluorometer is ideal for laboratory studies of biochemical processes. The FluoroMax-4 can be equipped a variety of optional accessories, including automated polarizers, a phosphorimeter, and time-correlated single-photon counting apparatus, for research on local environments and reactions in and near biological molecules. This report provides several examples of experiments.

Molecular Beacons and the FluoroMax-4

Biological processes can be traced through study of gene-expression via a "molecular beacon" (single-stranded DNA, ssDNA), a hairpin-shaped oligonucleotide with a fluorophore (donor) and a quencher (acceptor). The hairpin's stem has two ends of complementary DNA (cDNA) that pair up. When hybridized, the fluorophore and quencher are close, producing little or no fluorescence. Molecular beacons are used to study enzyme interactions, cDNA sequencing, and biosensing[1]. Molecular beacons exhibit two forms of quenching (energy-transfer): direct and FRET (Förster resonance energy-transfer). Donor-quencher contact causes direct energy-transfer, dissipating heat energy. Over longer distances (2–10 nm, 20–100 Å), spectral overlap between the donor's emission and the quencher's absorption causes FRET[2].

When the ssDNA loop encounters cDNA, the hairpin spontaneously opens and the ssDNA hybridizes to this cDNA-separating fluorophore and quencher-increasing fluorescence (Figure 1). The amount of hybridization is related to fluorescence intensity. Heat also can open ssDNA. When heated, the ssDNA's arms separate, moving the donor and acceptor ends apart, causing fluorescence.

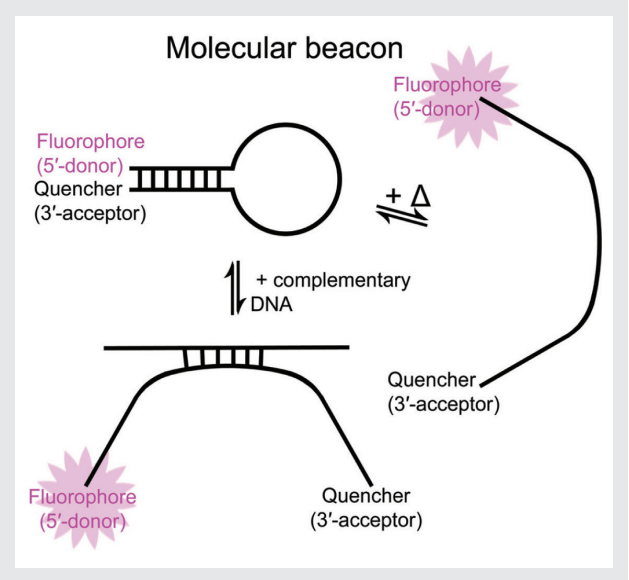

Figure 1 Two Processes that Open a Molecular Beacon, Enhancing Fluorescence
left: hybridization with cDNA right: heat input

As an example of the value molecular beacons have in the field of biochemistry, a fluorescent dye (tetrachloro-6-carboxyfluorescein, TET; λ_{em} = 447 nm) was attached to

a 5′- end of ssDNA, and a quencher (QSY) was bound to the 3′-end. With a FluoroMax-4 spectrofluorometer, the ssDNA was excited at 521 nm, and emission spectra were recorded from 525-675 nm between 20-95 °C. As the temperature increases-forcing the hairpin's arms apart-the TET and QSY separate, increasing fluorescence (Figure 2).

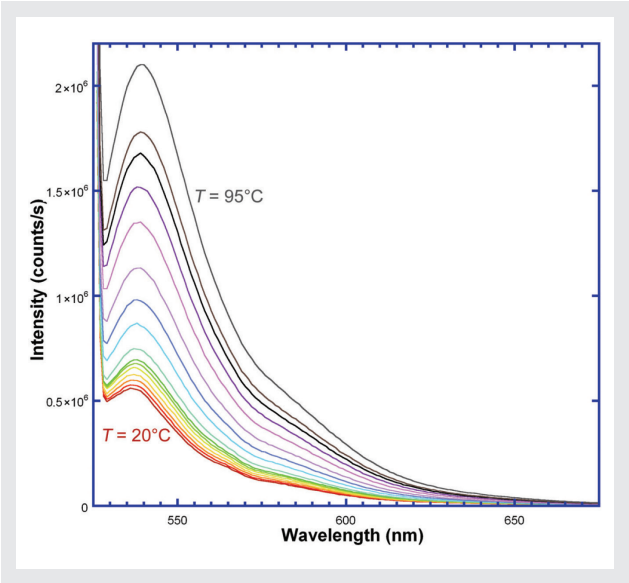

Figure 2 Emission Spectra from 20-95 °C of ssDNA with TET (fluorophore) and QSY (acceptor)
λ_{exc} = 521 nm. With rising temperature, fluorescence intensity rises, meaning greater distance between donor and quencher.

FRET with the FluoroMax-P

When a phosphorimeter accessory is added to a FluoroMax-4, the system is called a FluoroMax-P. Such a phosphorimeter integral to the FluoroMax-P spectrofluorometer can uncover important information on various systems of chemical and biochemical interest by revealing luminescence data normally masked by intense but rapid fluorescence. An example of this process shows Förster resonance energy-transfer (FRET) from a peptide-terbium-complex donor to a fluorescein acceptor. The peptide in the complex absorbs light at 280 nm and emits at 365 nm. The terbium absorbs near 365 nm, and phosphoresces at 485 nm, where fluorescein dye absorbs. The fluorescence of fluorescein at 520 nm can be observed when the sample is excited at 280 nm, using our phosphorimeter accessory.

Peptide-terbium complex was dissolved in aqueous solution, along with fluorescein in some samples. Phosphorescence measurements used the phosphorimeter's xenon flash lamp as the photon-source. An R928 photomultiplier tube was the detector. The phosphorimeter upgrade to the FluoroMax-4 includes all control electronics. A light-pulse excites the sample, and variable delays control when the detection window opens, and for how long. Sample excitation was at 280 nm, with 100 flashes measured. For luminescence spectra, the integration time was 0.2 s, except as noted. The scans were taken under ambient room conditions.

Experiments revealed that the the phosphorescent species is Tb. Figure 3 combines all three species (peptide, Tb, and fluorescein dye) in a plot of peptide-terbium complex with and without 0.67-μ*M* fluorescein, without and with a 50-μs delay between excitation with a xenon flash lamp. The spurious fluorescence at 363 nm is removed by delaying the sample window for 50 μs. The blue and green curves in Figure 3 directly compare fluorescein-containing and fluorescein-lacking solutions, showing fluorescein phosphorescence at 511 nm caused by energy-transfer from the complex to the fluorescein.

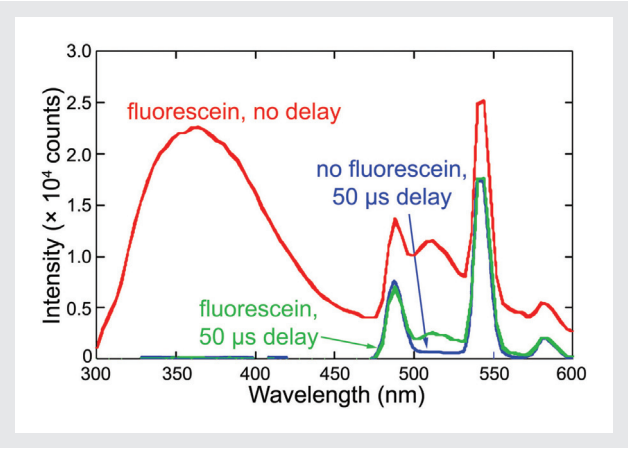

Figure 3 Luminescence Spectra of Complex of Peptide, Tb, and Fluorescein dye
(red) 0.67-μM fluorescein and no delay after excitation
(green)0.67-μM fluorescein and a 50-μs delay after excitation
(blue) no fluorescein and a 50-μs delay after excitation
Bandpass = 5 nm for excitation and emission. The 50 μs delay isolates the luminescence that persists from the energy-transfer to the fluorescein.

Using a time-correlated single-photon counting (TCSPC) accessory to determine lifetimes (Figure 4), a lifetime τ_D for the donor alone (not shown) was 1.77 ms and for the Tb-fluorescein (donor-acceptor) complex τ_{DA} was 1.41 ms. The shorter lifetime for the complex also indicates

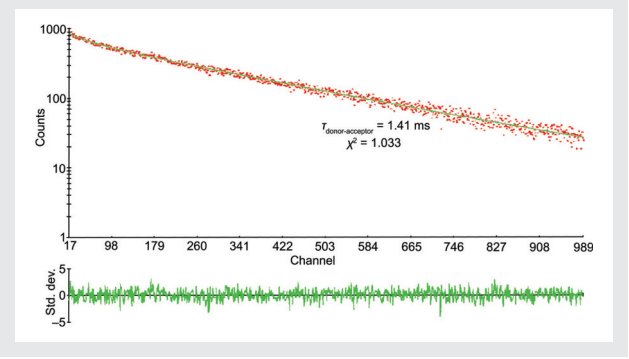

Figure 4 The Upper Trace is the Lifetime-decay Curve of the Tb-fluorescein Complex
Data are red dots; fit is the green line. From the fit, a lifetime t = 1.41 ms, with a χ^2 = 1.033. The lower trace is the residuals.

energy-transfer. The χ^2 and the low residuals indicate a good fit to the data.

Efficiency, E, of the FRET process was found to be

$$E = 1 \frac{\tau_{DA}}{\tau_D} \quad \cdots\cdots (1)$$
$$= 0.205$$

The Förster distance R_o was 43.4 Å. The distance between the donor and acceptor R_{DA} was found using the formula[3]

$$R_{DA}^6 = \frac{R_O^6 - E(R_O^6)}{E} \quad \cdots\cdots (2)$$
$$R_{DA} = 54.4 \text{ Å}$$

Fluorescence anisotropy and the FluoroMax-4

Polarized light striking a fluorescent molecule results in polarized fluorescence. This polarized emission gradually returns to unpolarized fluorescence depending on rotational diffusion and other factors. *Anisotropy* is directly related to the polarization, and is the ratio of the polarized-light component to the total light intensity. With optional polarizers installed in a spectrofluorometer, we define light intensities: I_{VV} is with excitation and emission polarizers mounted vertically; I_{HH} is for excitation and emission polarizers mounted horizontally. I_{HV} uses an excitation polarizer horizontal and the emission polarizer vertical; I_{VH} requires the excitation polarizer vertical and emission polarizer horizontal. The basic setup, called "L-format," is shown in Figure 5. The FluoroMax-4 spectrofluorometer with a polarizer accessory can do L-format polarization measurements.

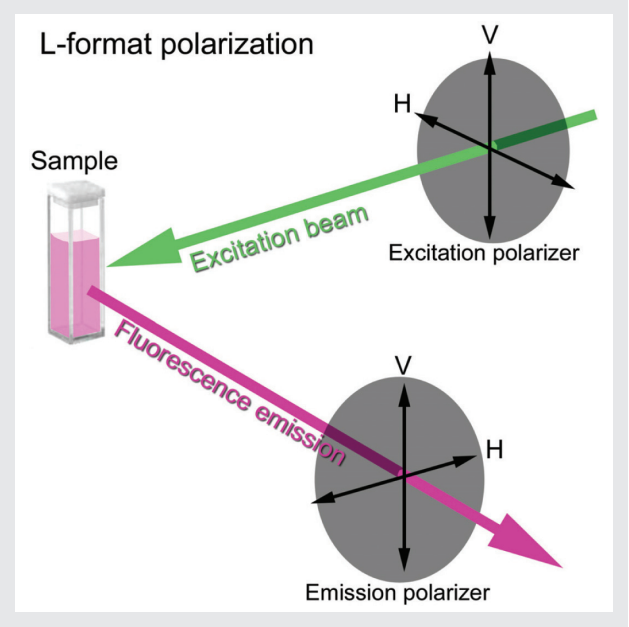

Figure 5 Diagram of L-format Fluorescence Polarization
Vertical (V) and horizontal (H) orientations of each polarizer are shown.

Anisotropy, $<r>$, is defined as[4]

$$<r> = \frac{I_{VV} - G * I_{VH}}{I_{VV} + 2 * G * I_{VH}} \quad \cdots\cdots (3)$$

where G, the "G factor," is

$$G = \frac{I_{HV}}{I_{HH}} \quad \cdots\cdots (4)$$

Conversion between $<r>$ and polarization, P, is shown in Formula 5:

$$P = \frac{3 <r>}{2 + <r>} \quad \cdots\cdots (5)$$

Four intensity measurements, corresponding to permutations of both polarizers' orientations, are needed to determine $<r>$ or P.

Anisotropy provides information on molecular size and shape, and local viscosities of a fluorophore's environment, as well as offering insight into changes in molecular sizes of polymers and other macromolecules. Protein-ligand interactions and binding assays can be investigated, and fluorophore lifetimes can be determined. A ribonuclease (RNase) is an enzyme that hydrolyzes

RNA into smaller molecules. Typical RNase probes present problems, because contamination can be difficult to identify. With sensitive fluoresence-polarization methods, results are easier to determine. Fluorescein-labeled RNA (F-RNA) was digested for ≥ 1 h with RNase A at 37 °C. The reaction was quenched with Tris-HCl at pH 8.0 in 0.125% sodium dodecyl sulfonate. The reaction is given below:

$$\underset{\text{high polarization}}{\text{F-RNA}} \xrightarrow{\text{R Nase}} \underset{\text{low polarization}}{\text{F-nucleotides + F-oligomers}}$$

The RNase is expected to lower the anisotropy as the RNA gets digested into smaller, more freely-rotating fragments. Figure 6 shows precisely this effect: as more RNase is added to the labeled RNA, the polarization falls, revealing the effects of digestion.

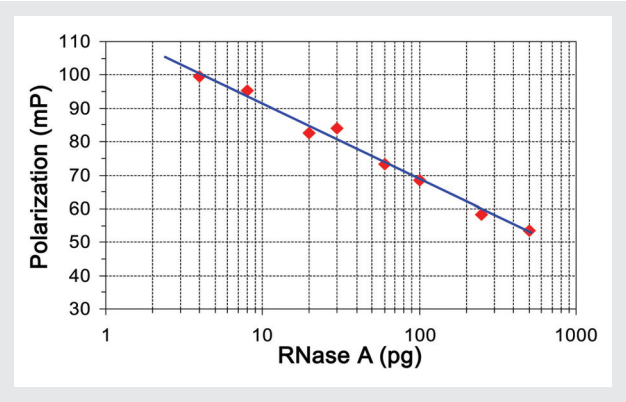

Figure 6 Polarization vs. Amount of RNase Added to 25 ng Fluorescein-labeled RNA
Data were taken after ≥ 1 h for complete hydrolysis. The anisotropy falls as more RNase is added, indicating increased fragmentation of the RNA.

Conclusion

Fluorescence measurements with the HORIBA Jobin Yvon FluoroMax-4 are a sensitive tool for probing biochemical interactions such as molecular beacons and DNA. Use of a FluoroMax-P, including gated delay of signal-acquisition, can reveal extra information, such as energy-transfer, about the physical and chemical properties of materials. Polarization measurements using the optional automated polarizers on a FluoroMax-4 are a sensitive tool for probing many biochemical interactions. Time-correlated single-photon counting using the TCSPC accessory is a rapid method for determining biochemical fluorescent lifetimes.

References

[1] X. *Liu, et al., Anal. Biochem*. 2000, **283**, 56–63; X. Fang, *et al., Anal. Chem*. 2000, **72**(14), 3280–3285.
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[3] Joseph R. Lackowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed., New York, Springer, 2006, p. 446.
[4] Joseph R. Lackowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed., New York, Springer, 2006, pp. 353–354, 361–364.

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