

SELF-QUENCHING IN FRET ASSAYS

Self-quenching occurs when the apparent fluorescence of a dye is reduced due to the presence in its vicinity of other molecules of the same dye. Several biophysical processes can lead to this phenomenon, particularly where multiple dye molecules are bound to a localized substrate. Here, however, we discuss only the effects of reabsorption of emitted photons by dyes in solution as they apply to homogeneous FRET assays.

Self-quenching is evidenced by a non-linear relationship between the concentration of fluorescent reagent and the resulting fluorescence signal, where all other aspects of the analytical system would predict linear response. In extreme cases, the addition of fluorescent reagent can even result in decreased response.

Self-quenching occurs primarily in fluorescent dyes with small Stokes' shifts, due to overlap between their absorbance and emission spectra (Figure 1). Because they absorb photons at some of the same wavelengths at which they emit, photons emitted by a given dye molecule may be absorbed by others in the reaction mixture before they reach the fluorescence detector (Figure 2).

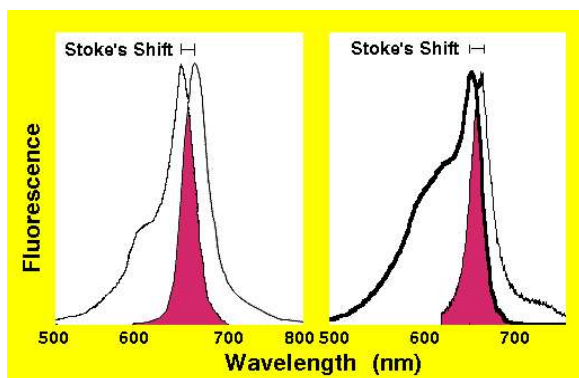


Figure 1 - Overlap (shaded area) between absorbance and emission spectra of Cy5 (left) and Allophycocyanin (right).

NOTE: fluorescence scale is relative.

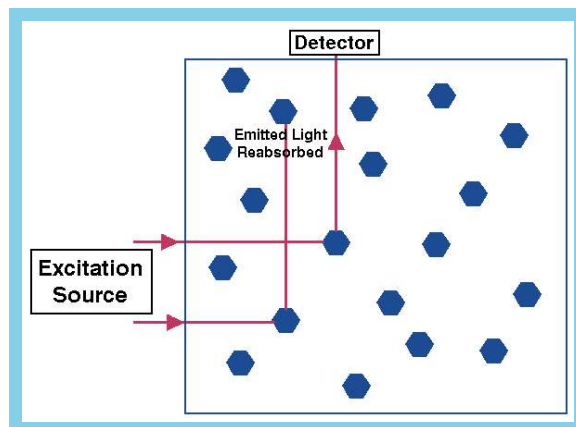


Figure 2 - Reabsorption of fluorescence in a dye solution

At low dye concentrations reabsorption is minimal. As the dye concentration increases, it can lead to significant nonlinearity in the relationship between dye concentration and fluorescence (Figure 3). In the extreme, measured fluorescence decreases with increases in dye concentrations.

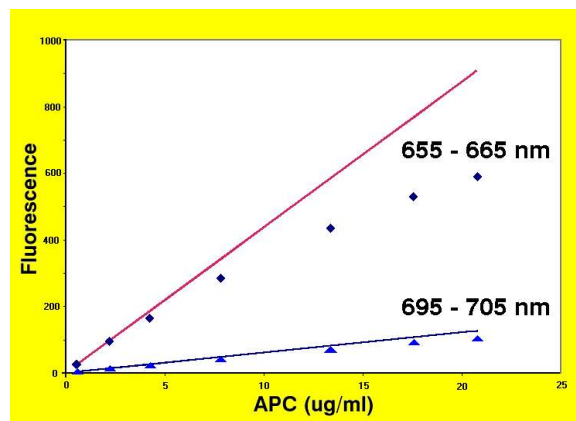


Figure 3 - Nonlinearity of Allophycocyanin (APC) fluorescence as a function of concentration for two different detection windows.

The amount of self quenching observed will depend on the wavelength window used for detection, and can be minimized by the selection of an off-peak detection window, but the loss of signal that results can more than offset the benefits obtained.

Self-quenching is increased by any process that decreases the randomness of the distribution of molecules in solution. If the nature of the reagents (Table 1) or that of the assay system (Table 2) tends to bring dye molecules closer to one another than would occur if they were randomly distributed in

solution, self quenching will be greater than predicted based on measured dye absorbance.

Some of the factors that lead to self-quenching can be controlled in the process of assay design through careful selection of dyes, wavelengths, and physical conditions, while others may be unavoidable for specific assays. Investigators should be cognizant of the potential for self-quenching in fluorescent assays, avoid it through design when possible, and take it into account in data interpretation when it is not.

TABLE 1 - Characteristics of reagents that affect self-quenching

- **Extent of overlap between excitation and emission curves** - broad emission curves and small Stokes shifts lead to the greatest amount of self-quenching.
- **Quantum efficiency** - dyes with high quantum efficiency are less subject to self-quenching because they can be used at lower concentrations to achieve a desired level of fluorescence, thus minimizing the absorbance of the solution.
- **Formation of molecular associations** - many dyes (including phycobiliproteins and cyanine dyes) occur in solution in equilibrium between single molecules and associations of two or more molecules. Since these associations reduce the randomness of the molecules in solution, they increase self-quenching.

TABLE 2 - Characteristics of assay systems that affect self-quenching

- **Cell-based systems** - where multiple dye molecules are attached to the surface of a cell, the average distance between molecules is reduced relative to molecules in solution and self-quenching increases.
- **Immobilized reactants** - when the dye is attached to a surface (bead, plate, fiber, etc) randomness is reduced and self-quenching increases.
- **Incomplete mixing** - if the reaction mixture is not uniform, dye molecules will be localized and self-quenching increases.
- **Multiple tagging** - when more than one dye molecule is attached to a reactant, self-quenching increases. NOTE: while this reduces fluorescence yield from the labeled reagent somewhat, it will not in itself lead to nonlinearity of the analyte-fluorescence relationship, provided the reagent is properly standardized.

Use of Excess Energy Donor

Normally, it is better to have an excess of the energy donor dye (*i.e.*, the dye that is initially excited by the source). Specifically, if one component of the assay system must be added in excess, it should be the donor; the amount of acceptor in the system should be minimized.

Figure 4 illustrates this principle. At low concentrations of donor (BPE), improved fluorescence response cannot be achieved with additions of acceptor (APC). The use of higher B-PE concentrations leads to a significantly enhanced signal. At high concentrations of acceptor, significant self-quenching of acceptor fluorescence occurs due to the presence of excess acceptor in solution.

Summary

Self-quenching is a fact of life in the use of fluorescent dyes. To obtain the most accurate results, assays must be designed and standardized to minimize its impact. Simple maximization of the fluorescence signal by adding more acceptor is almost never indicated and, thanks to the exquisite sensitivity of the detection systems that are available, is seldom necessary.

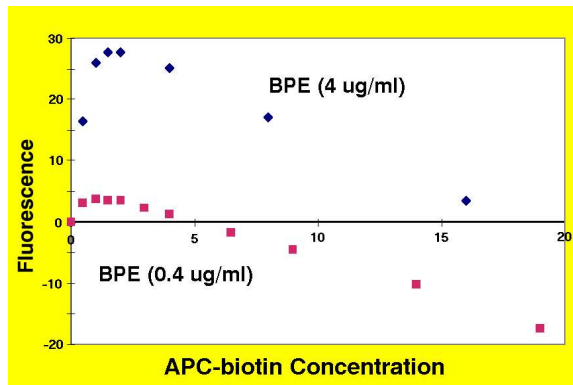


Figure 4 - Net fluorescence effect (background corrected) of serial additions of biotinylated Allophycocyanin (APC) to two different concentrations of Streptavidin-B-Phycoerythrin conjugate (SA-BPE).

TECHNICAL SERVICE

This and other TechNotes are available on PROZYME's webpage located at:

<http://www.prozyme.com/technical/index.html#technotes>

PROZYME customers are an important source of information regarding advanced or specialized uses of our products. We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.



1933 Davis Street, Suite 207
San Leandro, CA 94577-1258

TOLL FREE (800) 457-9444
PHONE (510) 638-6900
FAX (510) 638-6919

E-MAIL info@prozyme.com
WEB www.prozyme.com