Optical Building Blocks Corp.
Technical Note: Time-Resolved Fluorescence (Fluorescence Lifetimes) is an Invaluable Compliment to Steady State Fluorescence (or Fluorescence Spectra)"

If you are currently doing any type of luminescence measurements, but have never thought about doing fluorescence lifetimes as being useful, I urge you to read this article. Intensity (steady state) and lifetime measurements are complementary. One must frequently combine results from the steady state and the lifetimes in order to obtain the most complete information about the studied object.

When I introduced the first modern day lifetime instrument some thirty years ago, while the fact of the complimentary nature of the fluorescence lifetime technique may have been well known, it was frankly irrelevant for most users. The cost, size and complexity of operation of the instrument discouraged all but a relatively few from using this technique. Although instrumentation cost have decreased quite dramatically, as well as the size and complexity of operation, prior to the introduction of the new EasyLife™, convincing someone that they needed fluorescence lifetime measurements was still a very hard sell. Hence I would not have even bothered to try.

The introduction of the EasyLife™ system has changed the playing field. I don't mean to be commercially crass here, but it is EasyLife™ being affordable, under $20,000 (prices may be slightly higher outside of North America), small and simple to use, that has changed my attitude towards fluorescence lifetimes as a technique that now everyone who is doing luminescence measurements can put to good use.

In the simplest case, you may want to fully characterize the excited state of an organic molecule, e.g. to find out what are the rate constants for the emission and for the nonradiative deactivation. This information is readily available by combining the lifetime from the time-resolved measurement with the quantum yield from the steady state.

Or you may want to characterize a molecule under study and its interactions with the surrounding environment. In the steady-state measurement alone, which can provide a fluorescence spectrum, fluorescence quantum yield or anisotropy value, most of this information is scrambled, as the measured parameters are time averages and the information about specific processes is lost. This lost information becomes especially important when fluorescent molecules are used as probes to study complex systems, such as proteins, nucleic acids, membranes, polymers, surfactants (micelles) etc. These systems frequently exhibit multiple structural domains and conformations. The fluorescence decay will reveal this information by exhibiting multiple lifetimes, while on the other hand this information will be totally obscured in the steady-state measurement alone.

For example, consider a simple case of a protein containing one Trp residue (e.g. human serum albumin HSA). Carry out a steady state measurement and you'll get a typical Trp spectrum reflecting no particular information about the protein, except that it contains Trp. However, if you measure its fluorescence decay, you'll find that this single Trp residue has 4 different lifetimes! You know immediately that the protein exists in at least 4 different conformational states.

A steady state experiment can reveal a binding between a fluorescent probe and a protein. Normally, the fluorescence intensity will change as a result of binding; it will either decrease or increase, depending on the nature of the probe. The information you get is very general: you detected some kind of binding and that is all.

Not so with the lifetimes: here the binding will affect the probe lifetime; it will either decrease or increase (e.g. see ANS binding to BSA), but at the same time you also detect two lifetimes, one for the bound and the other for the unbound probe, as well as their relative contributions (preexponential factors) to the overall decay. From the lifetime measurement you now know relative populations of bound and unbound probes (i.e. we know the efficiency of binding).

One of the major tools of fluorescence is studying quenching of fluorophores by added quencher molecules. For example, tryptophan residues in a protein can be quenched by acrylamide or iodide ions. A steady state experiment will just show the decrease of fluorescence intensity as the quencher is added. The lifetime experiment however will show more than one lifetime (due to different sites that Trp may occupy in the protein) and from the quencher effect on each lifetime, you can get information about localization of each type of the Trp residues (e.g. are they surface-exposed or buried inside the protein).

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The mechanism of fluorescence quenching in general cannot be revealed by the steady state experiment at all. There are 2 mechanisms that lead to quenching: 1) collisional (dynamic) quenching, where excited fluorophore and quencher collide and diffuse apart, 2) static quenching, where fluorophore in the ground state forms a nonfluorescent complex with quencher. In both cases the steady state experiment will show intensity decrease as more and more quencher is added. If you do lifetime measurements, in the 1st case you'll see the lifetime decrease as more quencher is added, while in the 2nd case there will be no change in the lifetime at all. Discerning between the two mechanism is especially very important when one uses FRET technique: it is critical to prove that a 'FRET-like' behavior is not caused by static quenching (mechanism 2). Only a lifetime experiment can rule it out.

Fluorescence anisotropy (polarization) is another example of the importance of lifetimes. A probe molecule in a buffer will show no or very little anisotropy. Attach it to a protein, DNA, membrane etc. and the anisotropy is increased. This is all that the steady state experiment can tell you: the probe is attached to a much bigger entity. However, if you measure the lifetime of the probe, you can estimate the rate of rotational diffusion and the size of the macromolecule your probe is attached to.

By somewhat trivializing, one can say that the main distinction between the information derived from the steady state and lifetime measurements is the following: the steady state tells you that something has happened, the lifetime measurement tells you what has happened.

An important advantage of the lifetime is that it is an "intrinsic" molecular parameter. It means that the lifetime value will not depend on the fluorescence intensity, e.g. intensity losses due to light scattering, local probe concentration etc. If the sample is diluted, the fluorescence intensity will decrease, while the lifetime will remain unchanged. It is difficult in a short article to tell you about all of the additional information that you can get with fluorescence lifetime measurements over simple intensity based measurements. This is not to say that one is better than the other just complementary. What is really nice from my point of view, is that this technique that I have invested thirty years of my life in, is now available for everyone to use and will take its place in the mainstream of luminescence measurements.

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