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Labelling biological molecules with fluorescent dyes

by Dr. Peter Roberts

Fluorescence technology has opened up huge possibilities in life science research. By attaching fluorophores to biomolecules a fluorescent probe is generated which can be used in a vast range of labelling applications. This article reviews some of the considerations which should be taken into account when selecting and optimising a fluorescent probe.

Fluorescence is one of the most important technologies used in life science research and has largely replaced the radiometric methods which were popular in the early 1990s. Fluorescence techniques offer enhanced performance and simplicity, while also being compatible with an increased focus on automation and miniaturisation. The technology has found applications in nucleic acid hybridisation and transcript imaging; detection and quantitation of specific analytes; and high throughput screening using miniaturised fluorescence-based assays. It has allowed the processes and structures of complex biomolecular assemblies, including live cells, to be studied with sensitivity and selectivity.

Fluorescent methodologies have been propelled by the continuous commercial development of high performance fluorochromes together with significant advances in detection techniques, microscopy and image analysis. Of the non-radioactive probe types, fluorescent probes have emerged as clear favourites.

Fluorophores

Fluorescent dyes, also known as fluorophores, are generally polyaromatic hydrocarbons or heterocycles. Fluorescent probes are designed to localise within a specific region of a biological specimen, or respond to a specific stimulus.

When atoms absorb one photon of light they are promoted to a higher energy state known as the excited electron singlet state (S_1'). This excited state generally lasts for 1-10 x 10^{-9} seconds. The atom undergoes conformational changes and is subject to many possible interactions within its molecular environment. As energy is partially dissipated, a relaxed singlet state is attained (S_1) from which fluorescent emission originates. This dissipation of energy means that the energy emitted on a return to ground state (S_0) is lower, and therefore of a longer wavelength, than the excitation photon [Figure 1]. This difference is known as Stokes' shift and is key to the sensitivity of fluorescence techniques. Fluorescence is detected and identified by changes in the emission and excitation of the spectra. Wavelength filters separate excitation photons from emission photons and an emission detector records output, usually as an electrical signal or as a photographic image.

Creating a bioconjugate

For fluorescent bio-labelling methods, a bioconjugate is required. This is formed by the coupling of a chemical or biochemical reagent with a natural biological molecule. It is important that the bioconjugate maintains a high fluorescence yield without changing the critical characteristics of the unlabelled biomolecule; it must retain selective binding to a receptor, activation or inhibition of a particular enzyme, or the ability to incorporate into a biological membrane. It may be necessary to compromise and select a bioconjugate of a lower fluorescence in order to preserve function or binding specificity.

The most common technique for labelling proteins is by chemical attachment of a fluorescent dye. This is normally achieved using the primary amino group of the lysine or the N-terminus. Virtually all proteins have lysine residues and most have a free amine at the N-terminus.

Amine-reactive probes

Amine-reactive probes are widely used to modify not only proteins, but also peptides, ligands, synthetic oligonucleotides and other biomolecules. These dyes are used most commonly to prepare bioconjugates for immunochemistry, fluorescence *in situ* hybridisation (FISH), cell tracing, receptor labelling and fluorescent analogue chemistry. In these applications the bioconjugate is often stored and re-used, therefore it is important that the bond between the dye and biomole-

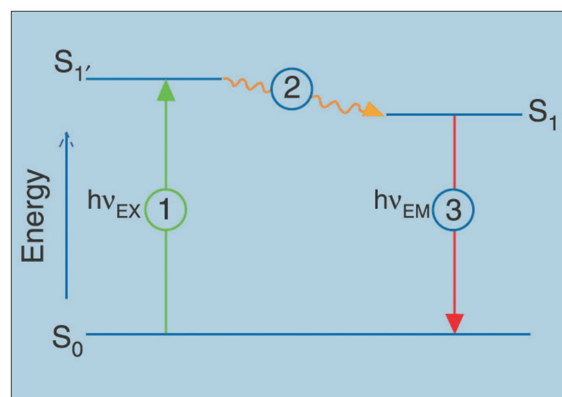

Figure 1. Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence.

cule is especially stable. This is also a concern as these bioconjugates may be subjected to rigorous hybridisation and washing steps. pH is another important factor in the labelling reaction - the amine group must not carry a charge or be deprotonated. Aqueous-based reactions require a basic pH in order to be efficient, which is due to the high ionisation point (pKa) of lysine's ε-amino group and the N-terminal amine of proteins and peptides.

Thiol-reactive probes

Proteins labelled using the thiol functional group are an alternative to amine-reactive probes. This is less common, occurring only in cysteine residues, and can therefore be labelled with high selectivity. Thus thiol-reactive reagents often provide a means of selectively modifying a protein at a defined site.

Thiol-reactive probes are ideal for analysing protein structure and function, for example, analysing the topography of proteins in biological membranes using polar thiol-reactive fluorescent reagents; or determining distances within a protein using fluorescence resonance energy transfer (FRET).

Labelling nucleic acids

An important application of fluorescence technology is the labelling of nucleic acids. Popular techniques such as microarrays and FISH are well-established molecular biology techniques using fluorophores attached to DNA or RNA. This was first achieved using fluorescein and became popular due to its speed, high spatial resolution and the opportunity to visualise several probes simultaneously on the same sample. Labelling of nucleic acids can be achieved by either direct or indirect labelling.

Direct labelling uses polymerases to incorporate fluorescence-tagged nucleotides into the DNA sequence of interest. This is ideal for use in FISH, DNA arrays, microarrays or other hybridisation techniques. It is often achieved by nick translation, but also by random priming and PCR reactions.

Chemical labelling is another option for the direct labelling of nucleic acids. One method is to use the ULYSIS kit from Molecular Probes Europe BV, Leiden, The Netherlands. This utilises a platinum-dye complex that forms a stable adduct with the N-7 position of guanine and to a lesser extent adenine bases in DNA, RNA, peptide nucleic acid (PNA) and oligonucleotides.
Indirect labelling is used for techniques such as FISH and involves using fluorescently labelled single stranded DNA complementary to the DNA sequence to be studied. The hybridisation of these probes to the sequence of interest is visualised using fluorescence.

Immunoassay

One of the most widespread applications of fluorescent bioconjugates is in immunoassays. Here antibodies are labelled with a fluorophore, for example, via amine modification. Antibodies are highly specific in their binding to an antigen. This makes them ideal reagents for the detection of target molecules. Fluorescent labelling enables the visual detection of antibody-antigen binding in an immunoassay.

Different detection technologies

In order to use fluorescent probes, one must also have a suitable method with which to detect them. Spectrofluorometers and microplate readers, fluorescence microscopes, fluorescence scanners and flow cytometers are all fluorescence instruments, but each produces distinctly different information and makes different demands on the fluorophore. For example, photobleaching is often a problem with microscopy, but not in flow cytometry where each cell spends just a short time in the excitation beam.

Choosing a probe for optimal results

The fluorescence output of a dye depends on the efficiency with which it absorbs and emits photons in addition to its ability to undergo repeated excitation and emission cycles. The selection of a fluorescent probe is greatly dependent on its application and the detection system used. For example, spectrofluorometers which are highly flexible, provide a continuous range of excitation and emission wavelengths. Flow cytometers are more demanding, requiring that probes are excitable at a single fixed wavelength, usually 488 nm, although often a second 633 nm excitation source is available. Separation of the fluorescence signal from the scattered excitation light is aided by a large Stokes' shift, however most biological samples labelled with fluorescent probes typically contain more than one fluorescent species, making signal isolation more complex.

Optimised fluorescent results

It is possible to optimise fluorescence technologies to yield more information from your experiment. In multicolour labelling experiments, for example, two or more probes are used to simultaneously monitor different biochemical functions. This relies on the ability to maximise the spectral separation of the multiple emissions. The ideal combination of probes for multicolour labelling will have narrow spectral bandwidths; strong absorption at coincident excitation wavelengths; and well separated emission spectra.

Fluorescence can be optimised further by minimising photobleaching. This is achieved by increasing the detection sensitivity, allowing for a reduction in the intensity of the excitation source. Antifade reagents may also help, or alternatively, a less photolabile fluorophore can be selected such as Alexa Fluor from Molecular Probes Europe BV, Leiden, The Netherlands.

Conclusion

Attaching a fluorophore to a biomolecule is a technique which allows researchers to take advantage of the huge versatility offered by fluorescence imaging. This technique can be used in a vast range of

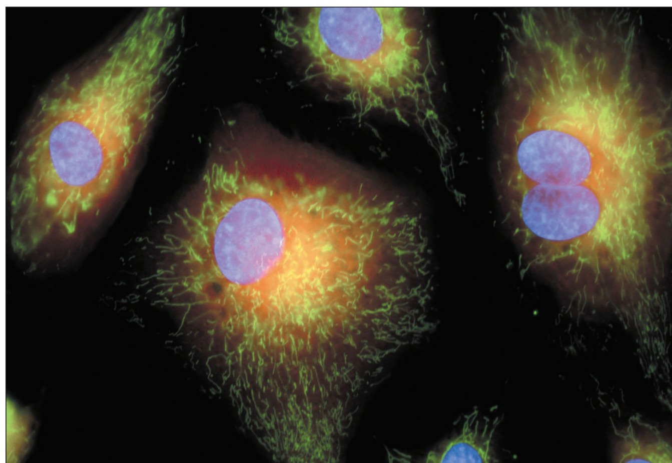

Figure 2. Live bovine pulmonary artery endothelial cells incubated simultaneously with CellTrace calcein red-orange AM and MitoTracker Green FM. The de-esterified calcein red-orange is present throughout the cell, whereas the green-fluorescent MitoTracker Green FM dye accumulated in the mitochondria. Nuclei were stained with blue-fluorescent Hoechst 33342.

applications, from immunoassays to FRET. In response to new developments in available detection and imaging devices, novel fluorescent probes and labelling mechanisms have been introduced. These continue to drive forward the scope of fluorescent technology and its achievements.

The author

Peter Roberts, Ph.D.
Marketing Manager
Molecular Probes Europe BV
Poortgebouw
Rijnsburgerweg 10
2333 AA Leiden
The Netherlands
Tel. +31 71 524 1894
Fax +31 71 524 1883
Email: peter.roberts@probes.nl
www.probes.com