Distinguishing GFP from Cellular Autofluorescence

by Andrew W. Knight and Nicholas Billinton

SUMMARY

Endogenous autofluorescence is a common nuisance that plagues many a researcher using green fluorescent protein (GFP) for biological investigations in cells and organisms. Yet an arsenal of photonic techniques is available to tackle the problem and to allow effective discrimination of GFP from autofluorescence.

The use of green fluorescent protein (GFP) from *Aequorea victoria* has evolved into one of the most important practical advances in cell biology in recent years. GFP genes can now be cloned and expressed in a diverse range of cells and organisms from bacteria and yeast to plants and animals. GFP possesses such favorable properties as low toxicity and high stability. Also, because it is inherently fluorescent, simple illumination with blue light determines its presence without the requirement of additional factors.

These characteristics make GFP a truly versatile marker for visualizing physiological processes, monitoring subcellular protein localization, distinguishing successful transfection or reporting on gene expression, and its use impinges on almost every area of biological research.[1,2] However, unless GFP is very highly expressed or densely localized, its fluorescence signal will invariably be contaminated with endogenous cellular or media autofluorescence.

Researchers often think they have failed in using GFP as a marker when they have succeeded. Their problem is simply detecting it amid the sea of other fluorescent species within biological material. This natural fluorescence, commonly called autofluorescence, is an ever-present annoyance for biophotonics researchers who wish to quantify or visualize specific fluorescent markers. Its presence often leads to low signal-to-noise ratios and loss of contrast and clarity in fluorescence microscope images.

Most researchers tackle the problem by trying to optimize the optical filters used for fluorescence excitation and emission. However, autofluorescence spectra are generally broad, extending over several hundred nanometers. Hence, its interference is often significant at the same emission wavelengths as GFP, making this approach ineffective.

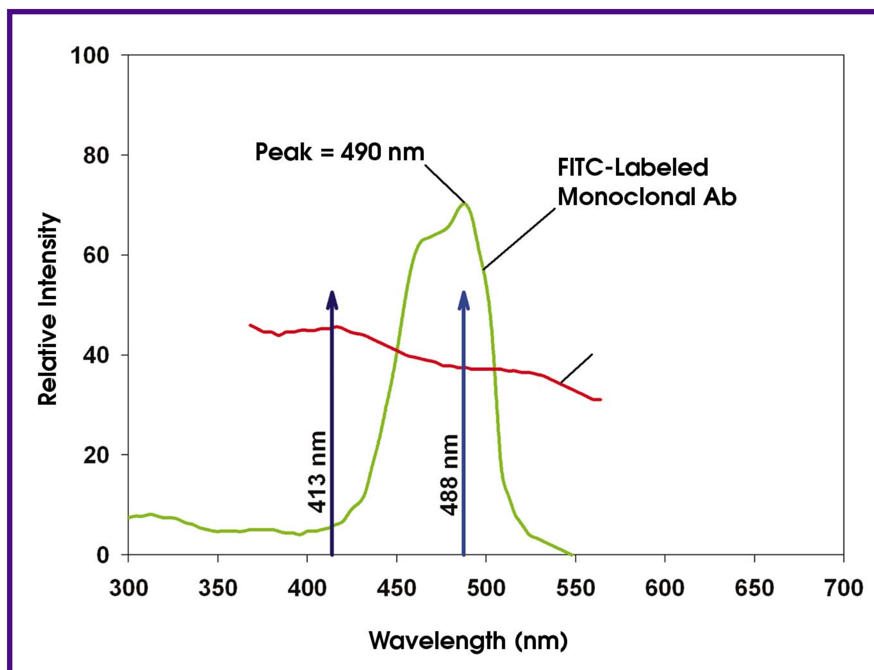

Figure 1. The excitation spectra of fluorescein isothiocyanate and autofluorescence show the principle of the dual-wavelength differential correction method. An argon-ion laser wavelength is used to excite the fluorescent marker and autofluorescence, while a krypton laser wavelength is used to excite autofluorescence alone.

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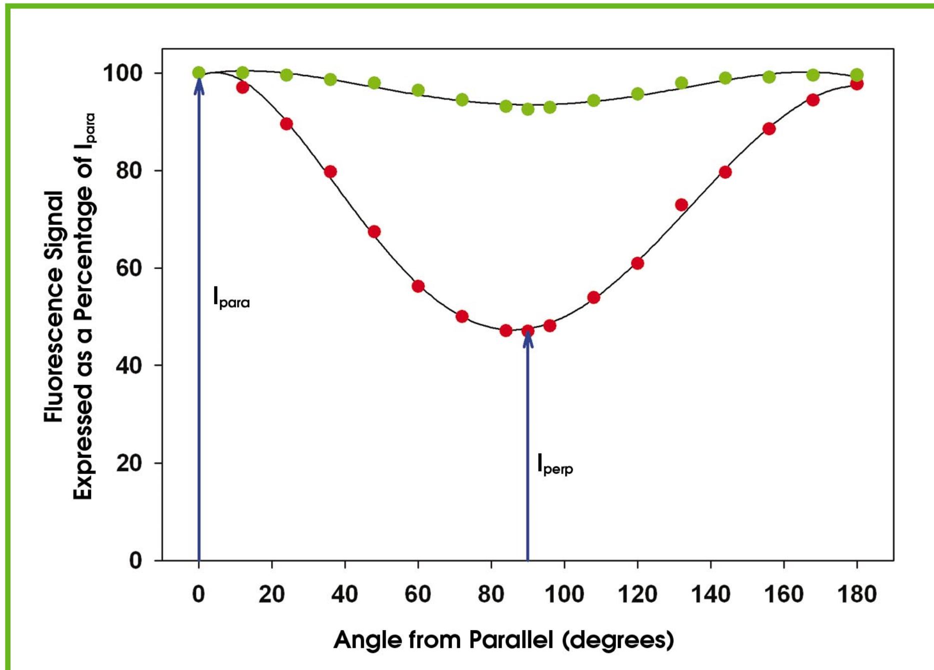


Figure 2. The fluorescence intensity varies with angle, with respect to the plane of polarization of the excitation light.

To tackle the problem of autofluorescence, it is first useful to have an idea of its source. Identification of the species likely responsible for autofluorescence can enable the researcher to optimize the experimental conditions to reduce its concentration or suppress its ability to fluoresce. Many cellular metabolites exhibit autofluorescence (Table 1). Because cellular extracts are often key components of culture media, such media can also be intensely autofluorescent, compounding the problem.

The most-cited source of autofluorescence is flavin, a ubiquitous coenzymatic oxidation reduction, or redox, carrier involved in the metabolism of most organisms and a photoreceptor in plants and fungi. Derivatives of riboflavin are the most intensely fluorescent examples. Other common species include nicotinamide adenine dinucleotide (NADH) and its derivatives, which are crucial to many biochemical reactions in most types of cell. Less-well-known sources include lipofuscin, a somewhat enigmatic substance found to positively stain for lipid, carbohydrate and protein.

Careful selection of optical filters is the most common approach to distinguishing GFP from autofluorescence. Because of the striking spectroscopic similarity between fluorescein and GFP, the commonly available filter set for the fluorescent probe fluorescein isothiocyanate (FITC) is most often employed in visualizing GFP, especially under the microscope. And UV filter sets are useful for viewing wild-type and blue-shifted GFP mutants. However, unless GFP expression is very high, these filters are not specific enough to adequately discriminate between the two.

Optimizing optical filters

Suppliers such as Chroma Technology Corp. and Omega Optical Inc. market more than 30 filter sets for GFP, each designed for a particular mutant or specific autofluorescent species. This diversity highlights the reasons why most researchers have chosen to optimize their own filter sets from a combination of suppliers: first, because autofluorescence arises from a disparate and often unknown range of molecules that can vary enormously among different cells and species; and second, because new genetically engineered GFPs with diverse spectroscopic properties are continually being developed.

In general, filters should be carefully selected to pick out areas of the spectrum where GFP can be excited, and its fluorescence transmitted, with greater efficiency than the autofluorescent species.

TABLE 1.
Common Sources of Autofluorescence

Autofluorescent Source	Typical Emission Wavelength (nm)	Typical Excitation Wavelength (nm)
Flavins	520 to 560	380 to 490
NADH and NADPH	440 to 470	360 to 390
Lipofuscins	430 to 670	360 to 490
Advanced glycation end-products (AGEs)	385 to 450	320 to 370
Elastin and collagen	470 to 520	440 to 480
Lignin	530	488
Chlorophyll	685 (740)	488

The choice of filter bandwidth will depend on the excitation source and detector characteristics. Wide bandwidths are used where color images are captured and the sharp, bright green of GFP can be distinguished from the broad-spectrum autofluorescence; narrower bandwidths are used only where quantitative fluorescence intensity measurements are made, such as in fluorimetric or flow cytometric methods. A suitable dichroic filter with a cutoff wavelength between the peak excitation and emission wavelengths completes the filter set and minimizes crosstalk interference.

Dual-wavelength correction

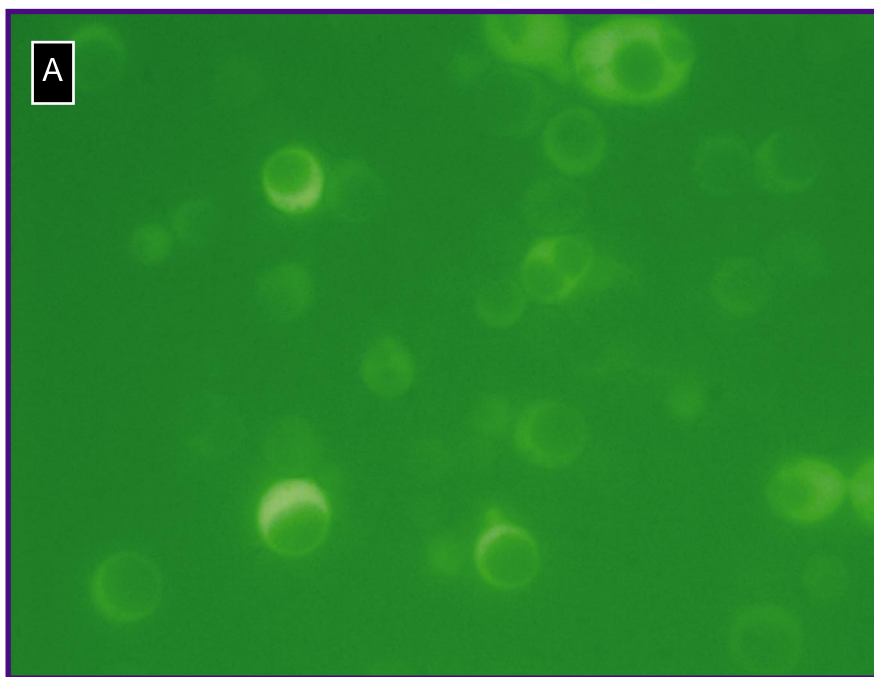
Dual-wavelength methods exploit the fact that autofluorescence excitation and emission spectra are generally broad and have few features, whereas the excitation and emission spectra of GFP are relatively narrow and well-defined. The sample is excited sequentially at two wavelengths: one to optimally excite the fluorophore, such as GFP — inevitably along with the autofluorescent components of the cell — and the other principally to excite only the autofluorescence. The difference between the two fluorescence measurements obtained from illumination at the respective wavelengths is then equal to the fluorescence of GFP alone. Steinkemp and Stewart used two laser wavelengths (Figure 1) and, although based on an FITC-labeled species, their work would be just as applicable to GFP.[3]

Conversely, it is possible to excite the sample at a single wavelength and measure at two. For example, GFP fluorescence intensity measured at 530 nm can be corrected for autofluorescence using the intensity measured at 600 nm, while illuminating only at 488 nm. Both of these methods assume that the autofluorescence intensity is uniform between the two wavelengths; however, if this is not the case, the ratio of the two fluorescent signals can be adjusted to zero by using unlabeled control cells or media.

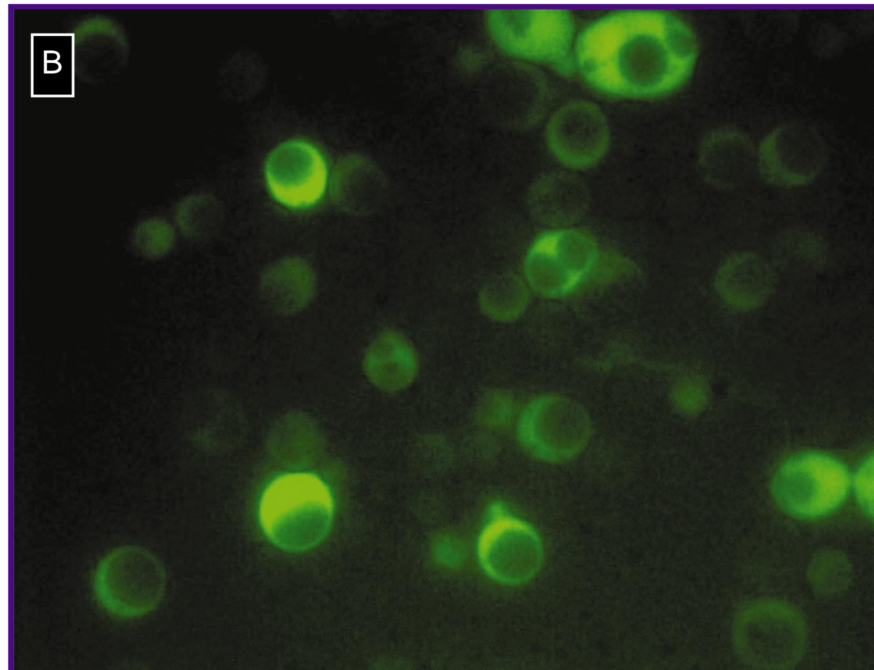
Because it is a relatively large fluorophore (27 kDa) and the actual fluorophore element is rigidly encapsulated within its cylindrical structure, GFP rotates in free solution at a slow rate compared with its fluorescence lifetime. This results in a large fluorescence anisotropy that can be exploited in a simple but effective discrimination method. If cells containing GFP are excited with plane-polarized light, the resulting fluorescence retains a significant degree of polarization (Figure 2). The intensity of fluorescence polarized perpendicular to the excitation light (I_{perp}) is approximately half that still polarized parallel (I_{para}) with the excitation light.

In contrast, the isotropic fluorescence of a smaller fluorophore — fluorescein, in this example — remains approximately constant with angle. Autofluorescence in yeast cells, for example, was recently reported as being largely unpolarized.[4] Thus the difference between these respective measurements ($I_{para} - I_{perp}$) leaves a large signal for GFP but a much diminished signal for the autofluorescence.

The beauty of this method is that, because it relies on a property of light other



Figures 3 A and B. A fluorescence microscope image of yeast cells expressing GFP in an autofluorescent medium (A) is corrected with software to remove autofluorescence to produce a clearer picture of the cells (B). Courtesy of M.G. Barker and J.A. Miyan, Manchester University Institute of Science and Technology.



than wavelength, it can be applied to the discrimination of GFP from autofluorescence with substantially the same spectral properties, which would be very difficult to achieve using conventional optical filters alone. The method also can be rapidly applied to many assay protocols simply by inserting inexpensive polarizing filters into existing laboratory equipment.

As the number of biochemical applications of GFP continues to increase, so the commercial drive exists to develop ever-brighter fluorescent proteins that fluoresce at longer wavelengths, outshining the autofluorescence.

Microscopy image correction

Digitally captured fluorescence microscope images of GFP-expressing cells or organisms can often be manipulated using software to enhance the visualization of GFP through the background autofluorescence. Many software packages allow simple adjustments of contrast, color balance, color transformation and sharpness to improve the image. In addition, automatic edge detection may allow GFP-labeled cell structures to be specifically highlighted. The simplest and crudest method is to convert the image to RGB (red-green-blue) format and select the green channel signal.

A more precise method uses a facility available in packages such as Corel PhotoPaint, whereby the image is analyzed pixel by pixel, and a histogram is produced showing the number of pixels of each brightness on a scale from 0 to 255. In Figure 3A, an image of GFP-expressing yeast cells in a medium shows intense autofluorescence. Analysis of the pixel brightness distribution pattern produced from this image indicates two clusters: one principally from the uniform autofluorescence, and one from the brighter cells (Figure 4). This analysis enables the selection of a threshold value between the clusters, and the software can remove from the image all pixels that fall below this value. The result is the removal of autofluorescence and a much clearer picture of the fluorescent cells (Figure 3B). With proper calibration, integration of the brightness distribution also can allow quantitative analysis of fluorescence captured in microscope images.

Autofluorescence is often unevenly distributed in samples; it is localized in specific subcellular regions or structures in cells and organelles, such as that arising from lignin in plants and collagen in animals. Targeted microscopy methods often allow the user to focus on just those areas where GFP is localized, while avoiding areas showing high autofluorescence. Such techniques include confocal and two-photon laser-scanning microscopy, established methods of obtaining high-resolution fluorescence images and three-dimensional reconstructions of biological specimens. The process involves scanning the sample with a laser beam focused into a small spot through a microscope objective. A computer then constructs the image by measuring fluorescence as a function of laser position, in a technique called optical sectioning.

An attractive property of GFP for microscopists is its tolerance of fixatives such as formaldehyde and glutaraldehyde, allowing its visualization in preserved tissues. However, formaldehyde enhances the autofluorescence of flavins and can diminish the fluorescence of some GFP fusion proteins, while others precipitate out, resulting in misleading bright spots throughout the cell. When using these fix-

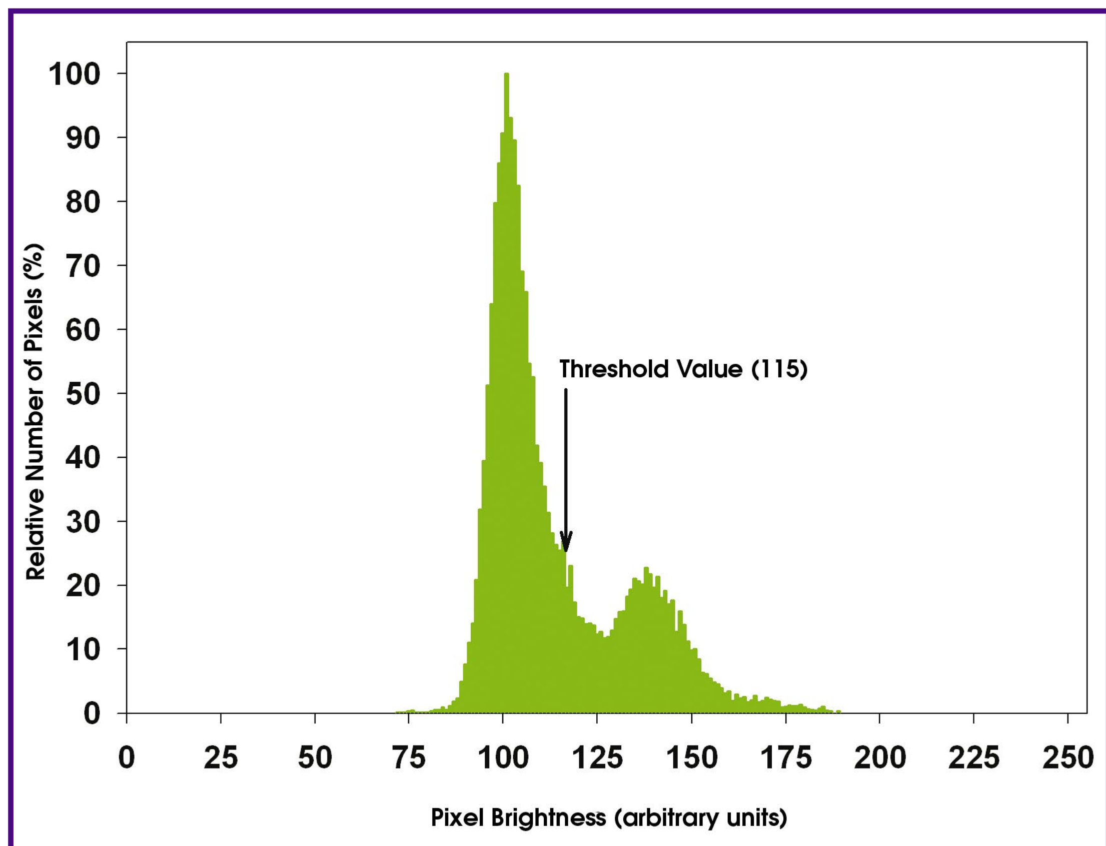


Figure 4. A distribution map was made of the pixel brightness of a section of the image showing GFP-expressing yeast cells against a background of media autofluorescence.

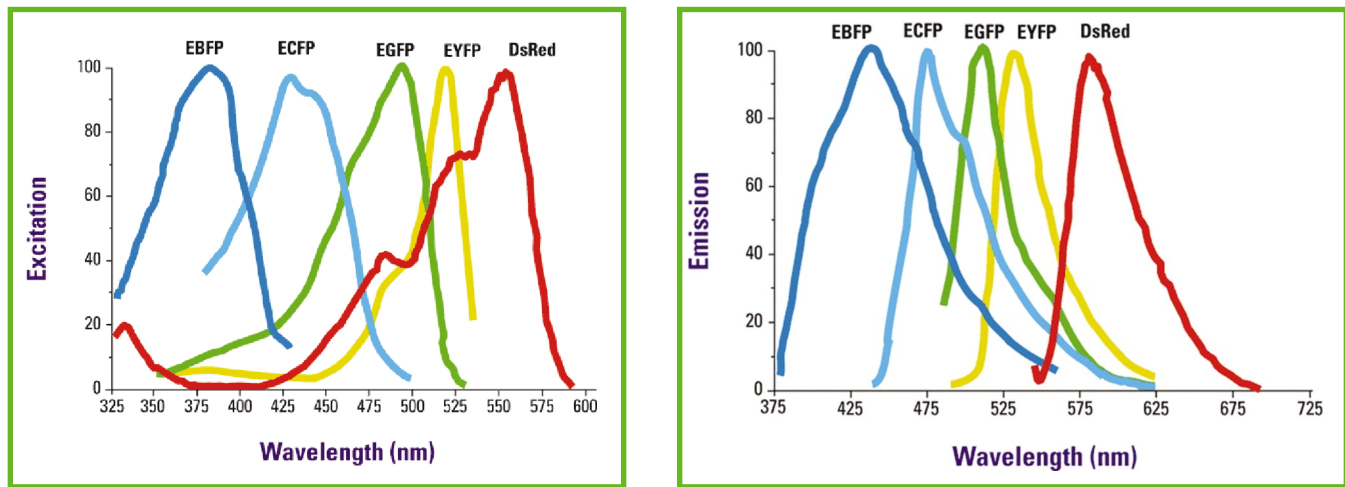


Figure 5. The excitation and emission spectra of enhanced blue, cyan, green, yellow and DsRed fluorescent proteins are widely diverse. Courtesy of Clontech Laboratories Inc., Palo Alto, Calif.

atives, it is best to thoroughly wash the sample prior to viewing to remove all traces of the chemical. GFP is very sensitive to some nail polishes used to seal microscope slide coverslips, so a molten agarose or rubber cement is recommended. GFP fluorescence disappears in the presence of absolute ethanol, making this an unsuitable fixative as well.

Mounting and embedding media used for sectioning work can be another source of autofluorescence. Those of natural origin, such as Canada balsam or glycerin-albumen, are among the worst culprits. Therefore, one of the many commercially available synthetic low-fluorescence mounting media should be used.

Time-resolved measurements

Time-resolved fluorescence spectroscopy is frequently used to quantify fluorescently labeled species in the presence of nonspecific autofluorescence. In its simplest form, the researcher would use a pulsed light source and, after a short interval during which the autofluorescence has decayed, measure the fluorescence of the labeled species. This method is best applied to species with a long fluorescence lifetime (>15 ns). GFP has a relatively short fluorescence lifetime, but significant differences exist among mutants. Lifetimes vary from 1.3 ns for cyan to 2.6 ns for green S65T and 3.7 ns for yellow fluorescent proteins, although these values are known to vary with temperature, pH and fusion to other proteins. This difference in lifetimes has, however, allowed the discrimination of three co-expressed GFPs using the complex technique of fluorescence lifetime imaging microscopy.[5]

Fluorescence quenching is a simple in situ method of reducing nonspecific autofluorescence without the need for additional optics or instrumentation. Tissue sections or cells are stained with a dye that quenches the autofluorescence while allowing visualization of the narrower-band GFP fluorescence. To be effective, the dye should have an absorbance spectrum that significantly overlaps the autofluorescence emission. The dye also should have a low molecular weight and high solubility so that it rapidly and evenly diffuses throughout the sample. And finally, it should have low cytotoxicity.

Finding a dye to satisfy all these criteria is difficult, and therein lies the limitation of this technique. Nevertheless, examples of dyes that have successfully quenched autofluorescence include toluidine blue, methylene blue and trypan blue, with peak absorbances of 626, 609/668 and 607 nm, respectively. Any fluorescence emission from these species is in the far-red region of the spectrum, which can easily be blocked with appropriate filters.

Photobleaching

When viewing GFP-labeled cells under the microscope, several workers have noticed that the broad-spectrum yellow-green cellular or media autofluorescence tends to fade more rapidly than GFP with prolonged illumination. This results in clearer resolution of the GFP, presumably caused by photobleaching of the autofluorescent species. Because of its barrel-like structure with the fluorophore element protected in the center, GFP is relatively resistant to photobleaching; it photobleaches at a reported rate of less than half that of fluorescein. Thus, improvements in contrast may be observed if a microscope image is captured several seconds after the illumination has been switched on. However, one must be careful if this approach is to be used for quantitative work, because GFP will also be photobleached to some extent, and the rate of photobleaching may vary, depending on the presence of modulating species in the surrounding media.

Photo conversion

Any method that enables excitation of, or emission from, GFP at an alternative wavelength may be useful in avoiding areas of the spectrum where autofluorescence occurs. Wild-type GFP, for example, shows an excitation peak at 395 nm with a shoulder at 475 nm. Prolonged irradiation with UV (280 to 395 nm) or blue light (up to 490 nm) causes a photoinduced isomerism reaction that produces a progressive decrease in the 395-nm absorption peak, with a corresponding increase in the 475-nm peak.

Fluorescence resonance energy transfer (FRET) is a phenomenon whereby one fluorescent molecule transfers excitation energy in a nonradiative way to a fluorophore in proximity. The energy transfer requires that the emission spectrum of the donor fluorophore overlap the excitation spectrum of the acceptor. Chromophore-mutated GFPs make excellent FRET pairs. Blue fluorescent protein emit-

ting at 466 nm is a good donor for S65T-GFP, which absorbs at 488 nm, while cyan fluorescent protein emitting at 505 nm is an excellent donor for yellow fluorescent protein, which absorbs at 514 nm.

The efficiency of the FRET process is highly dependent on the proximity of the respective fluorophores; thus, the two fluorescent proteins should be expressed as a fusion protein tethered by a short spacer of less than 20 residues, or with short tags that will strongly interact, bringing the proteins close together. This creates a fusion protein that has widely spaced fluorescence excitation and emission bands, making the optimization of optical filters for autofluorescence discrimination much easier and vastly reducing crosstalk. Unfortunately, there is a need for greater genetic modification, and any biochemical effects that change the orientation or distance between the fluorophores will significantly diminish the efficiency of the process.

Spectrally different mutants

The cloning of the wild-type GFP-encoding gene from *Aequorea victoria* in the early 1990s enabled the use of mutagenesis to create variant proteins with altered fluorescent properties and other biochemical characteristics. The availability of an expanding range of fluorescent proteins derived from GFP means that the researcher has the option of selecting a marker with fluorescent properties far removed from that of the interfering autofluorescence, although many demonstrate significantly lower fluorescence efficiencies (Figure 5).

As the number of biochemical applications of GFP continues to increase, so the commercial drive exists to develop ever-brighter fluorescent proteins that fluoresce at longer wavelengths, outshining the autofluorescence.

Until that happens, the above biophotonic techniques can be employed to distinguish GFP from endogenous autofluorescence. The most appealing methods are likely to be those that maintain the exquisite advantages of using GFP in the first place; namely, the ability for nondestructive in vivo expression and measurement. These are noninvasive methods such as dual-wavelength differential fluorescence correction, fluorescence polarization and time-resolved techniques. □

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