TECHNICAL NOTE

#57

Understanding Fluorescence

Figure 1.

Images of fluorescently labeled samples. (a) Glial cells of a rabbit hippocampal brain slice were stained with anti-Glial Fibrillary Acidic Protein plus a fluorescently-labeled secondary antibody. The image was scanned on a Amersham Biosciences *confocal laser scanning microscope (CLSM). Glia were rendered with a Maximum Intensity projection (shown in green). Two individual glial cells were identified by seeding in three dimensions and displayed in red and blue overlays. (b) In a double-stain experiment, DNA samples were prestained with the intercalating dyes TOTO and TOTAB,[1,2] then mixed and separated by agarose gel electrophoresis. The wet gel was imaged using a Molecular Dynamics FluorImager™ system.*

Introduction

This technical note describes—

- Fluorescence excitation and emission.
- Spatial imaging and quantitation using fluorescent labeling.
- Effects of the sample environment on fluorescent dyes (fluorochromes).

The following section provides an introduction to fluorescence. The subsequent section gives more detailed information. A summary of the advantages of fluorescence and a glossary of useful terms appear at the end of the note. (For information on selecting fluorochromes and using optical filtration as well as a list of available fluorochromes, see the *Fluorescence Imaging Applications Guide* or related Amersham Biosciences Technical Notes.)

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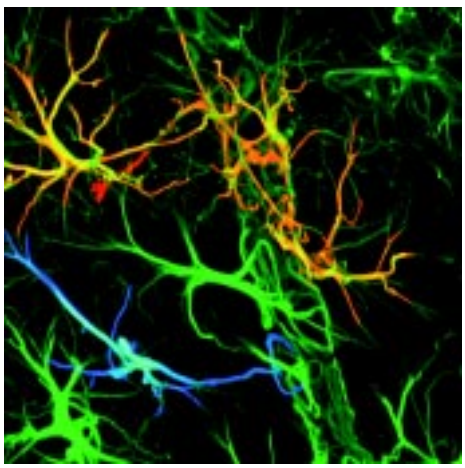

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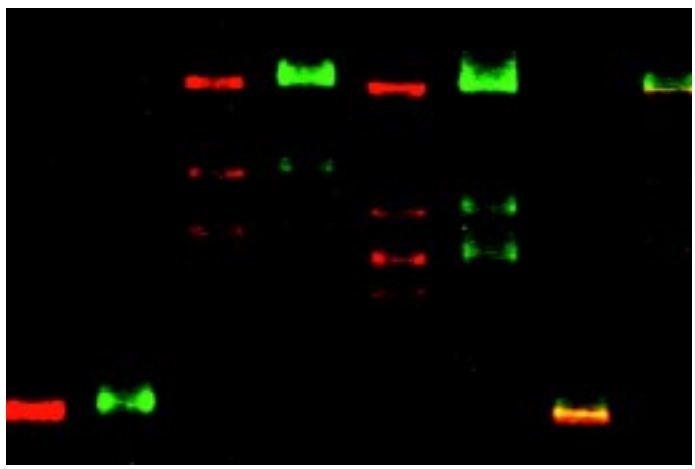
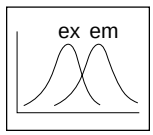


Sample courtesy of Dr. Steven Smith, Stanford University, Stanford, California.

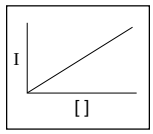
Fluorescence Basics

When a population of fluorochrome molecules is excited by light of an appropriate wavelength, fluorescent light is emitted. Using a scanning instrument, the light intensity can be measured pixel-by-pixel to create a digital image of the sample. Image analysis software, such as Amersham Biosciences ImageSpace™ and ImageQuaNT™, makes it possible to view, measure, render, and quantitate the resulting image (such as those shown in figure 1).

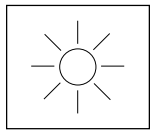
The following paragraphs explain the basic principles of fluorescence.



Excitation and Emission: Fluorochromes have characteristic light absorption and emission patterns (spectra). The absorption of light energy boosts an electron in the fluorochrome molecule to a higher energy shell (an unstable, excited state). When the excited electron falls back to the ground state, the fluorochrome emits light of specific *wavelengths*. The emission spectrum of the fluorochrome is always shifted toward longer wavelengths (lower energy) compared to the absorption spectrum. This shift makes it possible to separate excitation light from emission light with the use of optical filters. The emitted light can then be measured. The difference in wavelength between the apex of the absorption and emission spectra of a fluorochrome is called the *Stokes shift*.

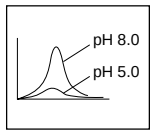


Linearity: The *intensity* of the emitted fluorescent light is a linear function of the amount of fluorochrome present when the illuminating light has a constant wavelength and intensity (for example, using a controlled laser light source). The signal becomes nonlinear at very high fluorochrome concentrations.



Brightness: Fluorochromes differ in how much intensity they are capable of producing. This is important because a dull fluorochrome is a less sensitive probe than a bright fluorochrome. The brightness depends on two properties of the fluorochrome—

- Its ability to absorb light (extinction coefficient).
- The efficiency with which it converts absorbed light into emitted fluorescent light (quantum efficiency).

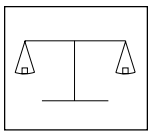


Environmental effects: For some fluorochromes, environmental conditions can affect the brightness or the wavelength of the absorption or emission peaks. Such fluorochromes are useful for analyzing changes in H^+, Mg^{2+}, or Ca^{2+} concentration, for detecting lipids or double-stranded DNA, and so on.

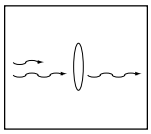
Another type of environmental effect is photodestruction (photobleaching), which is caused by intense light. The photobleaching occurs because the excited state of a molecule is generally much more chemically reactive than the ground state.

For photosensitive dyes (such as fluorescein), photobleaching can be significant in instruments that use high-intensity laser light (such

as the CLSM), but remains very low in instruments using lower-intensity laser light (such as the FluorImager system). If photobleaching is a problem in the high-intensity instruments, it can be reduced by using antifade agents or by lowering the laser power.



Quantitation: Measuring fluorescence is analogous to measuring radioactivity. In fact, scintillation methods of counting radioactive disintegrations have for many years used a cascade of energy transfers to create emitted light, which is then measured by the recording instrument. For both fluorescence and scintillation measurements, a light-sensitive device (usually a photodiode or a photomultiplier tube) converts the light energy into electric current. The amount of current is proportional to the intensity of the incident light.



Improving signal detection and measurement: A number of ways are available to improve detection and measurement of the emitted fluorescent signal. To begin with, the excitation light can be eliminated from the collection pathway by several methods, including—

- Orienting the excitation light path so that the light does not shine into the collection pathway.
- Inserting optical filters into the collection pathway to reject the excitation wavelength.
- Delaying collection until after a pulse of excitation light has disappeared.

The fluorescent signal can also be enhanced by increasing the dwell time or by scanning the sample multiple times and mathematically processing the signals to reduce random noise. Because of the high speed of scanning, such methods are useful and practical for increasing the sensitivity at the low end.

In addition, a band-pass optical filter can be used to reject broad-spectrum background emissions. This type of filter rejects wavelengths shorter and longer than the selected band, while allowing wavelengths in the selected wavelength range (centered around the fluorescent emissions of the sample) to pass through to the collection pathway.

In three-dimensional imaging systems (such as the CLSM, figure 1), confocal optics improve resolution in the third dimension. The system illuminates sequentially each point in three-dimensional space. The collection optics collect the signal from the illuminated point and reject any information that is out of focus.

Fluorescence in Depth

The following paragraphs provide additional detail about the topics discussed in the preceding section.

Excitation of the fluorochrome: During excitation, a fluorochrome molecule absorbs light. If the energy of the absorbed light is suffi-

cient to boost an electron from the ground-state energy level (S_0) to an excited-state energy level (S_n), excitation occurs (figure 2).

The probability of excitation of the fluorochrome by a given wavelength of incident light is shown in the excitation spectrum of the fluorochrome. The excitation spectrum is a plot of total emitted fluorescence versus excitation wavelength. It is the same as, or very similar to, the absorption spectrum (figure 3a) commonly provided by fluorochrome manufacturers.

The apex of the excitation peak is at a photon energy equal to the energy difference between the ground state of the fluorochrome and a favored vibrational level of the first excited state (S_1) of the molecule (figure 2b). In some cases, the excitation spectrum shows a second peak at a shorter wavelength (higher energy) that indicates boosting of an electron from the ground state to the second excited state (S_2).

The width of the excitation spectrum reflects the fact that an electron can start from any of several vibrational and rotational energy levels within the ground state and can end up in any of several vibrational and rotational energy levels within the excited state. In practice, a fluorochrome is most effectively excited by wavelengths near the apex of the excitation peak. The three fluorochromes in figure 3a are excited with different efficiencies by 488-nm laser light, as indicated by the relative height of each excitation curve at 488 nm compared to the height at maximum absorption.

Emission of light from the fluorochrome: Following pulse excitation of a population of fluorochrome molecules, fluorescent emissions begin instantaneously and decay rapidly. For the fluorochromes commonly used in fluorescence imaging, the half-life of the excited state (and, therefore, of the emissions) is usually a few nanoseconds.

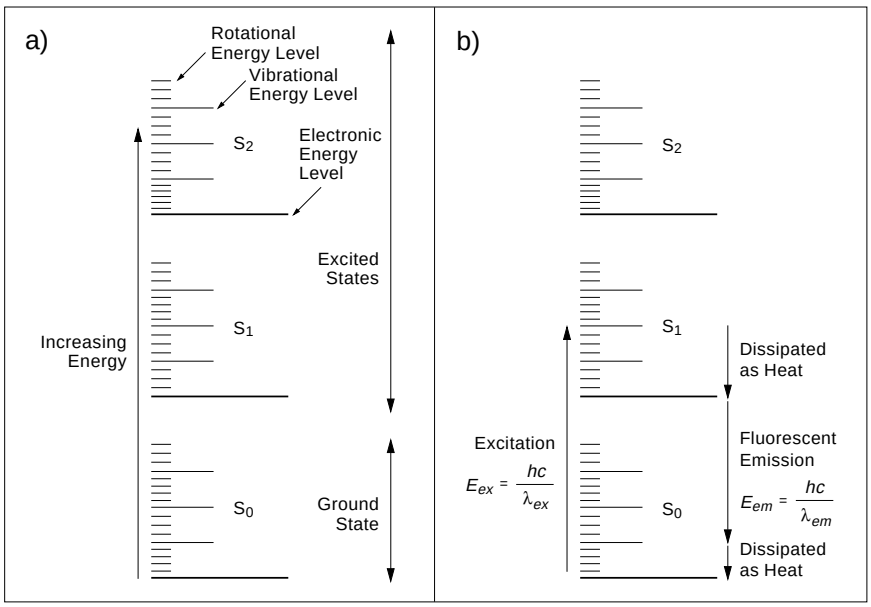
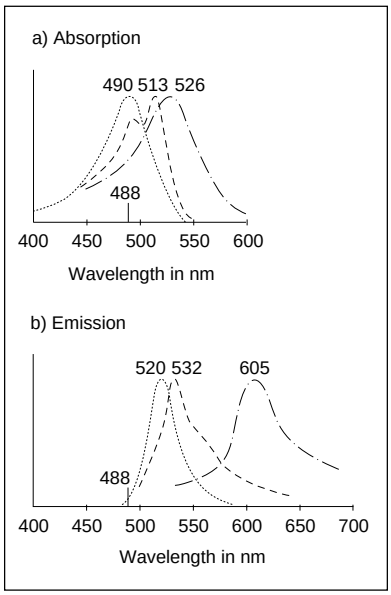


Figure 2.

Diagram of singlet energy levels of a molecule, including superimposed vibrational and rotational energy levels (a) and an example of excitation and fluorescent emission (b). E = the energy difference between the origin and destination energy levels during excitation (ex) and emission (em) of light; h = Planck's constant; c = the speed of light; λ = the wavelength of the absorbed or emitted light. The energy of light is inversely proportional to the wavelength of the light ($E = hc/\lambda$), that is, shorter-wavelength light is higher energy than longer-wavelength light.

Figure 3.

Absorption (a) and emission (b) spectra of fluorescein (·····), DNA-bound TOTO (– – – –), and DNA-bound ethidium bromide (— · —). The curves are normalized to the same peak height. The wavelength with maximum absorption (a) or at maximum emission (b) is shown above each curve. (The curves are approximations based on data collected at Amersham Biosciences or presented in Rye, et al. or Haugland.)[1,3]



During the brief lifetime of the excited state, the excited electron generally decays toward the lowest vibrational energy level within the electronic excited state (figure 2b). The energy lost in this decay is dissipated as heat. When the electron falls from the excited state to the ground state, light is often emitted. The energy of an emitted photon equals the difference between the energy of the electron in the excited state and the energy level in the ground state to which it falls (figure 2b). That energy difference determines the wavelength ($\lambda_{em} = hc/E_{em}$) of the emitted light.

The probability that light will be emitted is the *quantum efficiency* (quantum yield, ϕ) of the fluorochrome.

$$\phi = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

Values for ϕ range from 0 (for nonfluorescent compounds) to 1 (for 100% efficiency). Two example fluorochromes that demonstrate the range of values obtained are fluorescein ($\phi \approx 0.9$) and Cy5™ ($\phi \approx 0.3$). In practice, the ϕ is usually listed as the quantum efficiency at the wavelength of maximum absorption.

The probability that the emitted photon will have a particular wavelength is described in the emission spectrum (figure 3b). The emission spectrum of a fluorochrome is a plot of the relative intensity of emitted light as a function of the emission wavelength. (In practice, the emission spectrum is obtained by exciting the fluorochrome at a constant intensity with a fixed wavelength of light.) The apex of the emission peak is at the wavelength with energy equal to the energy difference between the base level of the excited state and a favored vibrational level in the ground state (figure 2b).

The emission spectrum is always shifted toward a longer wavelength (lower energy) relative to the excitation spectrum. The difference in wavelength between the apex of the emission peak and the apex of the excitation peak is known as the Stokes shift. This shift in wavelength (energy) represents the energy dissipated during the lifetime of the excited state before the fluorescent light is emitted. Compare the excitation and emission curves of the three fluorochromes in figure 3. For a copy of the excitation and emission spectra of your fluorochrome, contact the fluorochrome supplier.

Brightness of the fluorochrome: Fluorochromes differ in the intensity ("brightness") of light they emit. The brightness of a fluorochrome is proportional to the product of the extinction coefficient (ε) and the quantum efficiency (ϕ) of the fluorochrome.

$$\text{Brightness} \propto \varepsilon\phi$$

The *extinction coefficient* of the fluorochrome is the amount of light of a given wavelength that is absorbed by the fluorochrome. The molar extinction coefficient is defined as the optical density of a

one-molar solution of the fluorochrome through a one-cm light path. Useful fluorochromes have a molar extinction coefficient at peak absorption that is in the tens of thousands.

Two examples of very bright fluorochromes are fluorescein ($\varepsilon \approx 70{,}000$, $\phi \approx 0.9$) and Cy5 ($\varepsilon \approx 200{,}000$, $\phi \approx 0.3$). Note that these fluorochromes are quite different in quantum efficiencies and extinction coefficients, but are similar in brightness. Thus, both extinction coefficient and quantum efficiency should be considered when evaluating new fluorochromes.

Quantitation of fluorescence: As discussed previously, the **energy** (wavelength) of the emitted fluorescent light is a statistical function of the available energy levels in the fluorochrome and is independent of the intensity of the incident light. In contrast, the **intensity** of the emitted fluorescent light is a function of the intensity and wavelength of incident light, the brightness of the fluorochrome, and the amount of fluorochrome present.

When more intense light is used to illuminate a sample, more of the fluorochrome molecules are excited. The number of photons emitted (the number of electrons falling to the ground state) rises with increasing illumination intensity. If the illumination is very intense, all the fluorochrome molecules are in the excited state most of the time (saturation).

When the illumination wavelength and intensity are held constant (for example, using a controlled laser light source), the number of photons emitted is a linear function of the number of fluorochrome molecules present (figure 4). At very high fluorochrome concentrations, the signal becomes nonlinear because the density of the fluorochrome molecules is so high that excitation occurs only at or near the surface of the volume and because some of the emitted light is reabsorbed by other fluorochrome molecules (self-absorption).

The amount of light emitted by a given number of fluorochrome molecules can be increased by repeated excitation and emission cycles. In practice, at a constant excitation light intensity and fluorochrome concentration, the total emitted light is a function of how long the excitation beam continues to illuminate those fluorochrome molecules (dwell time). If the dwell time is long relative to the lifetime of the excited state, each fluorochrome molecule can undergo many excitation and emission cycles.

Measuring fluorescent light intensity (emitted photons) is analogous to counting radioactive disintegrations. Light intensity can be measured by any photosensitive device. For detection of low-intensity light, a photoelectric cell with a built-in amplifier (photomultiplier tube, PMT) is useful. In a PMT, when light of sufficient energy hits a photocathode, electrons are emitted and the resulting current is amplified. The strength of the current is proportional to the intensity of the incident light. The light intensity is usually reported in arbitrary units (such as relative fluorescence units, rfu).

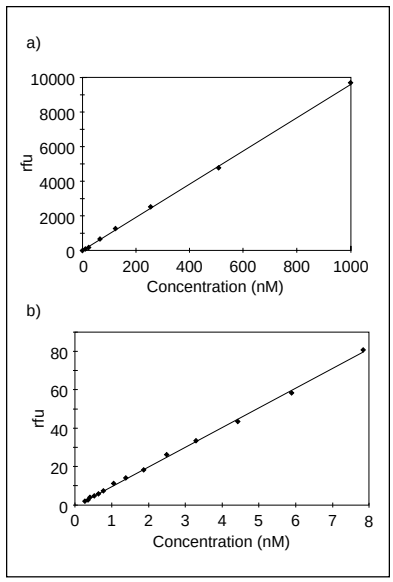


Figure 4.

Linearity of fluorescent signal. Fluorescein concentrations from 0.2 nM to 1 mM in 40 nM TAE buffer were placed in a 96-well microplate and scanned in a FluorImager system using 488-nm laser light. A background signal of 7.5 relative fluorescence units (rfu) was subtracted from each value. Graph b is an expanded view of the lower values in graph a.

Collecting spatial information: Amersham Biosciences instruments determine spatial information in one of two ways—*point excitation* or *point collection of emissions*. For point excitation, the sample is illuminated one pixel at a time. This is accomplished by moving the sample and by reflecting light off mirrors controlled by galvanometers and other motors. A converging lens or fiber-optic bundle collects the emitted light and directs it to the PMT. The light intensity is converted to electric current in the PMT. The current is then converted to digital information and stored along with the location of the illuminated pixel. The image file contains the intensity recorded for each pixel in the image.

For point collection of emissions, an entire strip of the sample is illuminated, and the emissions from different points along the strip are collected separately (for example, by a photodiode array). The intensity collected at each point is stored along with the location of the collection point.

In three-dimensional imaging systems (such as the CLSM), confocal optics improve resolution in the z direction. The illumination light is sequentially focused on each three-dimensional point in the scan. The confocal detection optics collect the signal from the illuminated point and effectively reject information that is out of focus in the z-axis. (The compromise you make for the improved resolution is a somewhat slower scanning speed.)

After the information has been collected, Amersham Biosciences image analysis software—such as ImageSpace, ImageQuaNT, or application-specific software—allows you to view, measure, render, and quantitate the resulting image.

Improving signal detection and measurement: Detection and measurement of the fluorescent signal can be enhanced in several ways, including the following:

- Directing the excitation laser light away from the data collection pathway.

  Amersham Biosciences fluorescence scanning instruments are designed so that the laser light illuminating the sample is directed away from the data collection pathway.

- Adding optical filters to eliminate excitation light from the collection pathway.

  Any laser light that is reflected or scattered by the sample is rejected from the collection pathway by a series of optical filters (long-pass filters) that reject light with wavelengths shorter than a specified wavelength (cutoff point) and allow light of longer wavelengths to pass through. Because the instruments use laser light of one or a few wavelengths (monochromatic excitation), appropriate long-pass filters can reject the illuminating wavelengths while allowing the longer-wavelength emission signals to pass through. (For more information on filters and filtering

fluorochrome emissions, see *Technical Note #58, Using Dyes and Filters in the FluorImager System* or related Amersham Biosciences Technical Notes.)

- Introducing a time delay between excitation and collection of the emitted signal.

  Excitation and emitted light can be separated by introducing a time delay, following pulse illumination, before the emitted light is collected. This approach takes advantage of the 1 to 10 nanosecond half-life of the decay of the fluorescent signal.

- Adding optical filters to reduce background fluorescence from the sample matrix.

  When the background signal from the sample matrix (for example, some gels, TLC plates, and membranes) has a broad, flat spectrum, a band-pass optical filter can be used to remove the background signal that is of longer or shorter wavelengths than the fluorochrome emissions. The filter allows a band of emitted wavelengths to pass through to the collection system.

- Increasing the dwell time or accumulating multiple scans for mathematical processing.

  Detection of weak fluorescent signals can be improved by increasing the dwell time or by accumulating multiple scans of the sample and then applying mathematical processing (for example, averaging, summing, or other accumulation methods). Averaged results, for example, represent the average of the constant signal and a reduction of random background effects (averaged noise). Figure 5a shows a gel scanned once and figure 5b

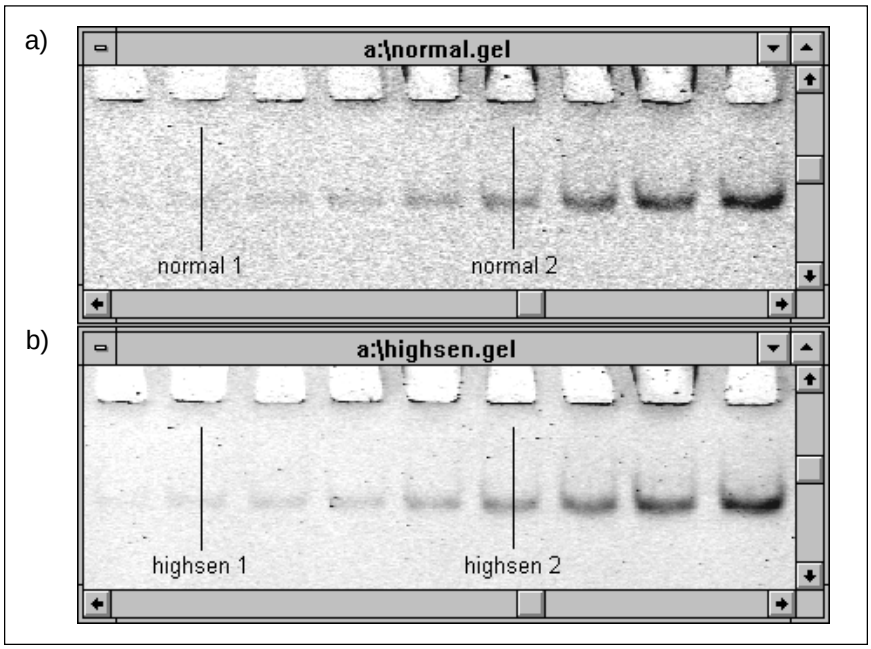
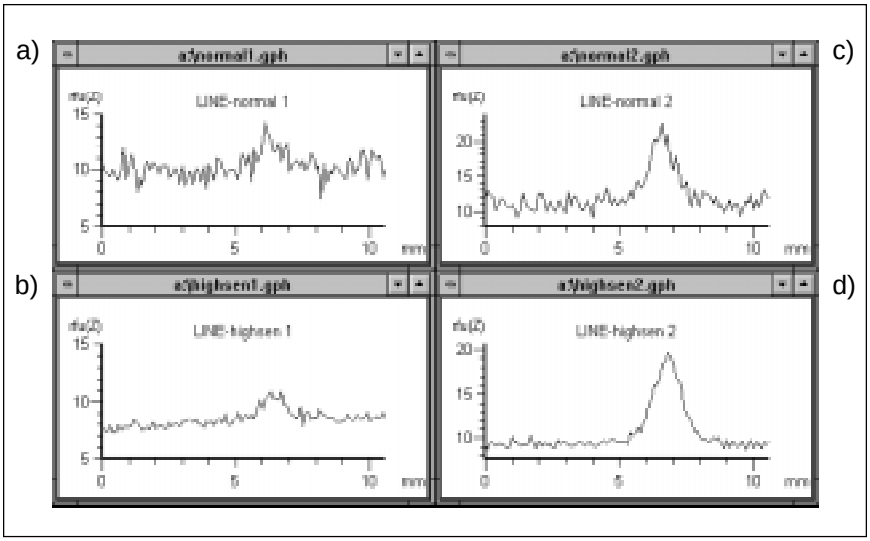


Figure 5.

Images generated from a single scan (a) or from the average of multiple scans (b). Serial dilutions of a fluorescein-labeled oligonucleotide (24-mer) were run in a 10% polyacrylamide gel and scanned on a FluorImager system one time (a) or eight times with averaging (b). The dwell time on each pixel per single scan was approximately 10 microseconds. Lines 1 and 2 were drawn through the lanes in image a and copied to the same location on image b for analysis (figure 6).

Figure 6.

Signal intensity in single and multiple scans. Lines 1 and 2 (figure 5) were used to create line graphs of the signal intensity along the lanes. For graph creation, the lines were made 20 pixels wide (data from 10 pixels on either side of each line on the image were averaged). Graphs a and b represent line 1 in figures 5a and b, respectively. Graphs c and d represent line 2 in figures 5a and b, respectively.



shows the same gel scanned eight times with signal averaging. The background noise for line 1 decreases from approximately 10 rfu to 8 rfu, and the variation in the baseline decreases from approximately ±2.7 rfu to ±1.3 rfu. The band on line 1 is more clearly resolved in the averaged image than in the single-scan image (figures 5a,b and 6a,b). A more intense band, on line 2, is easily resolved in both scans (figures 5a,b and 6c,d).

Methods for removing background signals, whether due to residual laser light or sample matrix fluorescence, enhance the dynamic range of an assay. For example, if the collection instrument has a dynamic range of 10^5 arbitrary fluorescence units (such as rfu), but your support material has a background of 100 rfu, the effective dynamic range of the assay is only 10^3 rfu. By selecting low fluorescence sample-support material and using the various methods described above to lower the background to 10 rfu or less, the effective dynamic range increases to 10^4 or more.

Environmental effects: The environment of a fluorochrome can affect its quantum efficiency and the excitation and emission spectra. Environmental influences may include temperature, ionic strength, pH, excitation light intensity, covalent coupling to a ligand, noncovalent interactions (for example, insertion into double-stranded DNA), and so on. Many suppliers provide information on the characteristics of their fluorescent reagents under various conditions.

Certain fluorochromes undergo specific, ion-induced structural changes that result in increased or decreased fluorescence intensity or in a shift of the location of the absorption or emission spectrum. These fluorochromes can be used as indicators of ion concentration. For example, fluorescein (figure 7), fluo-3, and Magnesium Green™ change fluorescence intensity in response to H^+, Ca^{2+}, or Mg^{2+} concentration, respectively.

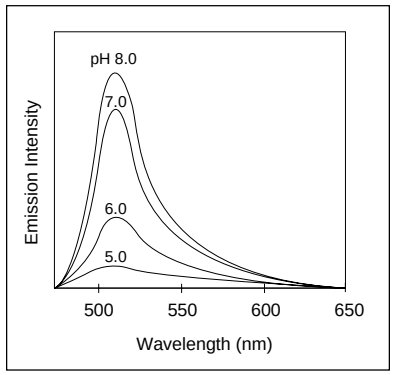
Phenol red, a common pH indicator in cell culture media, responds to H^+ concentration with a shift in the location of the absorption spectrum (that is, a shift in the color of light absorbed). This shift results in a change in the culture medium from red toward yellow orange as the solution becomes more acidic.

Another environmental effect, photodestruction of the fluorochrome (photobleaching), occurs because the excited state is generally much more chemically reactive than the ground state. A small fraction of the excited fluorochrome molecules participates in chemical reactions that alter the molecular structure of the fluorochrome and create a molecule with reduced fluorescence. The rate of these reactions depends on the sensitivity of the particular fluorochrome to bleaching, the chemical environment, the excitation light intensity, the dwell time of the excitation beam, and the number of repeat scans. For a sensitive dye, such as fluorescein, photobleaching can be significant in the CLSM microscope, which uses high-intensity laser light. In contrast, fluorescein photobleaching is very low in the FluorImager system, which uses lower laser intensity. (After 24 scans in the FluorImager 575 system, the signal from fluorescein at concentrations from 0.2 nM to 1000 nM decreased by 3% to 4%; data not shown.)

Photobleaching often results from the reaction of molecular oxygen with the triplet excited state of dyes, producing highly reactive singlet oxygen. If photobleaching is a problem (for example, in the CLSM), you can decrease or prevent photobleaching of many fluorochromes by using antifade agents, such as the antioxidants phenylalanine or azide, anoxic conditions, or low-light conditions for sample preparation and storage. In live cell studies, vitamin C (ascorbic acid) is often used to reduce photobleaching. A few dyes (for example, fluorescein) bleach even in the presence of anti-bleaching agents. Nevertheless, such dyes are very useful and are among the most widely used fluorochromes.

Figure 7.

The intensity of fluorescein emissions is a function of pH. The emission spectra show the relative fluorescence intensity emitted by a constant concentration of fluorescein excited at 488 nm in different pH environments. (The curves are approximations based on Haugland.[3])



Advantages of Fluorescence

Fluorescent labeling, in combination with the appropriate scanning instrument, is a sensitive and quantitative method with several advantages in experimental, analytical, and quality control applications. Some of the specific advantages are described below.

Wide variety: Fluorochromes with a wide variety of characteristics are available, including fluorochromes that—

- Respond to pH or ion concentrations.
- Localize based on hydrophobic and hydrophilic interactions.
- Can be cross-linked to proteins, nucleic acids, lipids, or polysaccharides.

Commercial availability: Fluorochromes are available cross-linked to many other molecules. For example, many monoclonal and polyclonal antibodies are available fluorescently labeled, sometimes with a choice of fluorochrome. Also on the market are several fluorescently labeled enzyme substrates, such as fluorescent chloramphenicol for chloramphenicol acetyl transferase (CAT) assays and fluorescein digalactoside for β-galactosidase assays (*lacZ* gene). For more information on available fluorochromes, see *Technical Note #58, Using Dyes and Filters in the FluorImager System* or related Amersham Biosciences Technical Notes.

Multiple-label possibility: A significant advantage of fluorescent labeling over other methods is the possibility of recording two or more fluorochromes separately using optical filters and a fluorochrome separation algorithm. Thus, components can be labeled specifically and identified separately in the same sample or lane (as in figure 1). For example, in PCR* reactions, standards and unknowns can be labeled with different fluorochromes to provide an internal standard for the assay.

Stability: The long shelf life of fluorescently labeled molecules compared to radiolabeled molecules offers significant advantages. For example, fluorescent monoclonal antibodies, oligonucleotide hybridization probes, and PCR primers can be stored for six months or more. In contrast, antibodies labeled with ^{125}I become unusable in about a month, and ^{32}P-labeled nucleotides and oligonucleotides decay significantly in about a week. Thus, fluorescent labeling eliminates frequent reagent preparation or purchase.

Reagent batches can be standardized and used for extended periods in antigen localization, ELISAs, enzyme assays (such as CAT and kinase), PCR-based genetic typing assays (such as STR analyses), DNA sizing and quantitation, DNA sequencing, protein sizing and quantitation, and so on.

Low hazard: Most fluorochromes are easy to handle, however, proper care should be observed with DNA and RNA stains. By their nature, such reagents are potentially mutagenic. Gloves provide adequate protection during use. In contrast, lead or acrylic shields may be required when handling radioactive materials.

In addition, fluorochromes can be broken down by incineration, so that there is little or no storage or disposal problem. In comparison, radioactive waste requires shielded storage, long-term decay, or regulated land-fill disposal.

Lower cost: The long shelf life and cheaper transportation and disposal costs for fluorochromes make fluorescent labeling, in many cases, less expensive than radiolabeling.

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* The polymerase chain reaction (PCR) process for amplifying DNA is covered by U.S. patent numbers 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche, Inc. and F. Hoffmann-LaRoche Ltd. Patents are pending or issued in other countries.

Glossary

absorption—energy transfer from a photon of light to a fluorochrome molecule.

absorption spectrum—a plot of absorption light wavelength versus the amount of light absorbed by the fluorochrome.

dwell time—the amount of time the excitation light illuminates a spot (pixel or voxel) in the sample.

emission—the release of light from a fluorochrome when an electron falls from an excited state to a lower energy state of the molecule.

emission spectrum—a plot of emitted light wavelength versus the relative intensity of emitted light. Essentially a plot of wavelength versus the probability that the emitted light will have the energy of that wavelength.

energy of a photon of light—$E = hc/\lambda$, where h is Planck's constant, c is the speed of light, and λ is the wavelength of the light.

excitation—the absorption of light energy by a fluorochrome, during which an electron in the fluorochrome molecule is boosted to a higher energy level.

excitation spectrum—a plot of incident-light wavelength versus the total fluorescence emitted. Essentially a plot of incident wavelength versus the probability of excitation.

extinction coefficient (ε)—the amount of light absorbed. The molar extinction coefficient is the optical density of a one-molar solution of the compound through a one-cm light path. The value usually quoted is the molar extinction coefficient at the wavelength of maximum absorption.

fluorescence—the emission of light (or other electromagnetic radiation of longer wavelength) by a substance as a result of absorption of other radiation. The emission continues only as long as the stimulus producing it continues and persists with a half-life of less than about 10^{-8} second.

fluorochrome—a fluorescent dye.

intensity of light—the flow of energy per unit area. Intensity is a function of the number of photons per unit area and their energy (energy of a photon is inversely proportional to wavelength).

photomultiplier tube (PMT)—a photoelectric cell that converts light into electric current and amplifies the current.

photon of light—a quantum of light. This concept is based on Planck's quantum theory of light, which says that the energy of an oscillating system can have only discrete (quantized) values. These values are proportional to Planck's constant, h.

quantum efficiency (quantum yield, ϕ)—the efficiency at which a fluorochrome converts absorbed light to emitted light (the ratio of the number of photons emitted to the number of photons absorbed).

relative fluorescence units (rfu)—the arbitrary units in which fluorescence intensity is reported by the FluorImager system.

Stokes shift—the difference in wavelength between the apex of the excitation spectrum (shorter wavelength, higher energy) and the apex of the emission spectrum (longer wavelength, lower energy).

voxel—a three-dimensional pixel (volume element).

wavelength of light (λ)—the distance in nanometers between nodes in a wave of light. The wavelength is inversely proportional to the energy of the light. $\lambda \propto 1/E$.

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