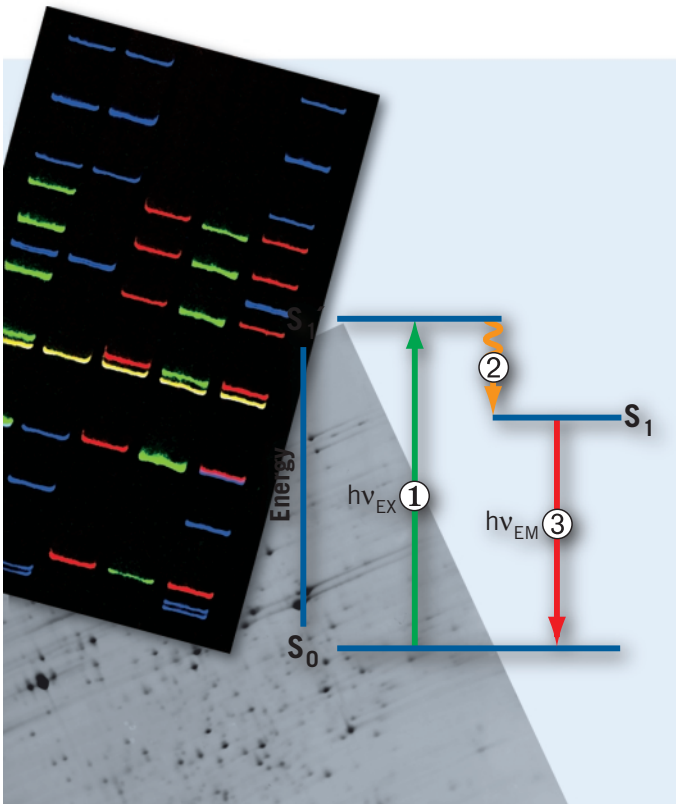
technical manual

Fluorescence Imaging

principles and methods



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Chapter 1

INTRODUCTION TO FLUORESCENCE

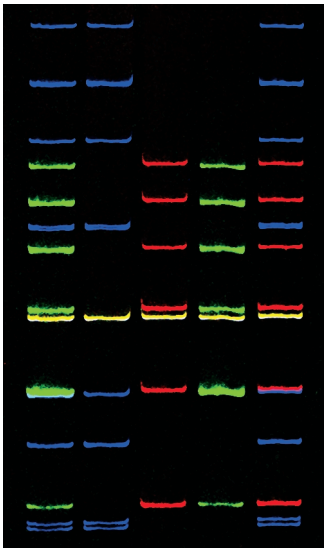

Fig 1. Fluorescently labelled DNA size ladders and PCR products loaded in the same lanes were electrophoretically separated in a polyacrylamide gel and imaged using Typhoon™ 8600 scanner. Fluorescein (green), Cy™3 (yellow), ROX™ (blue) and Cy5 (red) labels were used in amounts varying from 0.25 to 5 fmol per band.

Advantages of fluorescent detection

Fluorescent labelling and staining, when combined with an appropriate imaging instrument, is a sensitive and quantitative method that is widely used in molecular biology and biochemistry laboratories for a variety of experimental, analytical, and quality control applications. Commonly used techniques, including total nucleic acid and protein quantification, Western, Northern and Southern blotting, PCR✧ product analysis, and DNA sequencing, can all benefit from the application of fluorescence-based methods for detection. Fluorescent detection offers a number of important advantages over other methods, several of which are described below.

Sensitivity

Fluorescent probes permit sensitive detection of many biological molecules. Fluorescent stains and dyes are frequently the most sensitive option for detection of total DNA, RNA, and protein compared with traditional colourimetric methods. Many fluorescence applications approach the sensitivity afforded by radioisotopes.

Multiple-label possibility

With fluorescent labelling, two or more fluorochromes can be detected separately using optical filters and a fluorochrome separation algorithm. Therefore components can be labelled specifically and identified separately in the same sample or lane of a gel (Fig 1). For example, standards and unknowns used in PCR can be labelled with different fluorochromes to provide an internal standard for the assay.

Stability

Fluorescently labelled molecules offer several distinct advantages over radiolabelled molecules with respect to stability. Whereas fluorescent antibodies, oligonucleotide hybridization probes, and PCR primers can be stored for six months or longer, antibodies labelled with ^{125}I become unusable in about a month, and ^{32}P-labelled nucleotides and oligonucleotides decay significantly in about a week. Because of their long shelf-life, fluorescently labelled reagents can be prepared in large batches that can be standardized and used for extended periods.

✧ See licensing information on inside back cover.

This minimizes inter-assay reagent variability when used in applications such as DNA and protein sizing and quantification, enzyme assays, immunoassays, PCR-based genetic typing assays, and DNA sequencing. Additionally, the need for frequent reagent preparation or purchase is eliminated.

Low hazard

Most fluorochromes are easy to handle, and in the majority of cases, the simple use of gloves affords adequate protection. With radioactive materials, however, lead or acrylic shields may be required. In addition, since fluorochromes can be broken down by incineration, storage or disposal problems are minimal. Radioactive wastes, on the other hand, require shielded storage, long-term decay, or regulated landfill disposal.

Commercial availability

A variety of biologically important molecules are available cross-linked to fluorochromes, including monoclonal and polyclonal antibodies. Some suppliers even offer a choice of a specific fluorochrome as label on a given molecule. Other commercially available molecules include nucleotides and enzyme substrates, such as fluorescent chloramphenicol for chloramphenicol acetyl transferase (CAT) assays and fluorescein digalactoside for β-galactosidase assays *(lacZ* gene).

Lower cost

Long shelf-life and lower costs for transportation and disposal of fluorochromes make fluorescent labelling, in many cases, less expensive than radiolabelling.

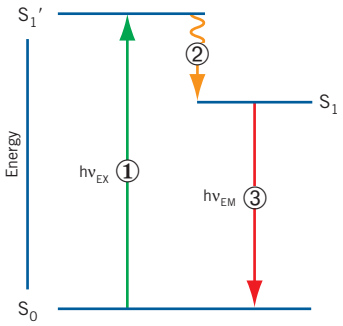

Fig 2. Jablonski diagram illustrating the processes involved in creating an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. ① Excitation; ② Vibrational relaxation; ③ Emission.

Fluorescence process

Fluorescence results from a process that occurs when certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores, fluorochromes, or fluorescent dyes absorb light. The absorption of light by a population of these molecules raises their energy level to a brief excited state. As they decay from this excited state, they emit fluorescent light. The process responsible for fluorescence is illustrated by a simple electronic state diagram (Fig 2).

Excitation

When a photon of energy, $h\nu_{EX}$, supplied by an external source such as a lamp or a laser, is absorbed by a fluorophore, it creates an excited, unstable, electronic state (S_1'). This process distinguishes fluorescence from chemiluminescence, in which the excited state is created by a chemical reaction.

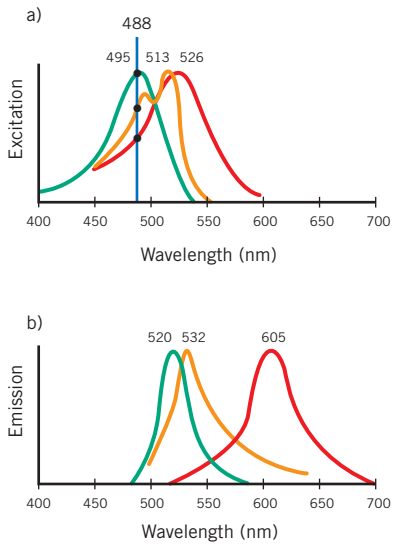

Fig 3. Excitation (a) and emission (b) spectra of fluorescein (green), DNA-bound TOTO (orange), and DNA-bound ethidium bromide (red). Curves are normalized to the same peak height. The wavelength at which maximum excitation (a) or maximum emission (b) occurs is shown above each curve. The position at which 488 nm laser light intersects with each of the three excitation spectra is indicated.

The curves are approximations based on data collected at Amersham Pharmacia Biotech or presented in references 1 and 2.

Excited state lifetime

The excited state of a fluorophore is characterized by a very short half-life, usually on the order of a few nanoseconds. During this brief period, the excited molecules generally relax toward the lowest vibrational energy level within the electronic excited state (Fig 2). The energy lost in this relaxation is dissipated as heat. It is from the resulting relaxed singlet excited state (S_1) that fluorescence emission originates.

Emission

When a fluorochrome molecule falls from the excited state to the ground state, light is often emitted at a characteristic wavelength. The energy of the emitted photon ($h\nu_{EM}$) is the difference between the energy levels of the two states (Fig 2), and that energy difference determines the wavelength of the emitted light (λ_{EM}).

$$\lambda_{EM} = hc/E_{EM}$$

where
E = the energy difference between the energy levels of the two states during emission (EM) of light;
h = Planck's constant;
c = the speed of light

A laser-scanning instrument or a CCD-camera can be used to measure the intensity of the fluorescent light and subsequently create a digital image of the sample. Image analysis makes it possible to view, measure, render, and quantify the resulting image.

Properties of fluorochromes

Excitation and emission spectra

A fluorescent molecule has two characteristic spectra—the excitation spectrum and the emission spectrum.

Excitation spectrum

The relative probability that a fluorochrome will be excited by a given wavelength of incident light is shown in its excitation spectrum. This spectrum is a plot of total emitted fluorescence versus excitation wavelength, and it is identical or very similar to the absorption spectrum (Fig 3a) commonly provided by fluorochrome manufacturers.

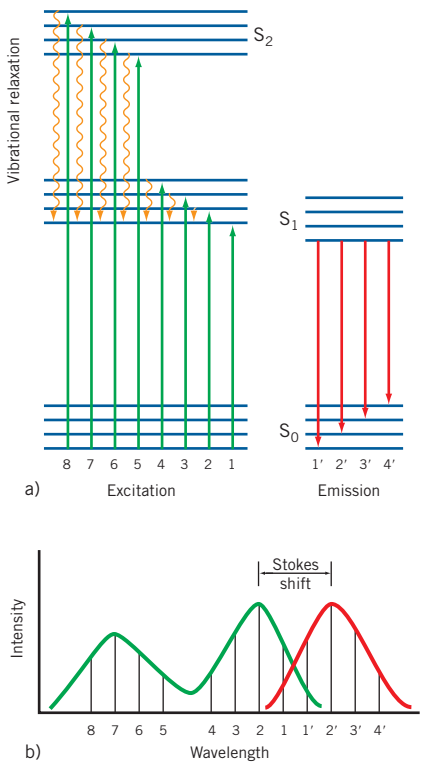

Fig 4. Diagram of the energy levels of a fluorochrome molecule, including superimposed vibrational energy levels (a) and an example of excitation and fluorescent spectra (b).

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The photon energy at the apex of the excitation peak equals the energy difference between the ground state of the fluorochrome (S_0) and a favored vibrational level of the first excited state (S_1) of the molecule (Fig 4a). In some cases, the excitation spectrum shows a second peak at a shorter wavelength (higher energy) that indicates transition of the molecule from the ground state to the second excited state (S_2).

The width of the excitation spectrum reflects the fact that the fluorochrome molecule can be in 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 its excitation peak. For example, as shown in Figure 3a, the efficiencies with which the three fluorochromes are excited by 488 nm laser light vary, as indicated by the relative height of each excitation curve at 488 nm compared with the height at maximum absorption.

Emission spectrum

The relative probability that the emitted photon will have a particular wavelength is described in the fluorochrome's emission spectrum (Fig 3b), a plot of the relative intensity of emitted light as a function of the emission wavelength. (In practice, the emission spectrum is generated by exciting the fluorochrome at a constant intensity with a fixed wavelength of light.) The apex of the emission peak occurs at the wavelength whose energy equals the difference between the energy of the base level of the excited state and that of a favored vibrational level in the ground state (Fig 4a).

The shape of the emission band is approximately a mirror image of the longest-wavelength absorption band (Fig 4b), providing that the vibronic structures of the excited and ground states are similar. In theory, the transition 1 in excitation and transition 1′ in emission (Fig 4a) should occur at the same wavelength. However, this is usually not the case in solution, mainly due to solvent relaxation (3).

The emission spectrum is always shifted toward a longer wavelength (lower energy) relative to the excitation spectrum, as shown for the spectra of the three fluorochromes in Figure 3. 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 as heat during the lifetime of the

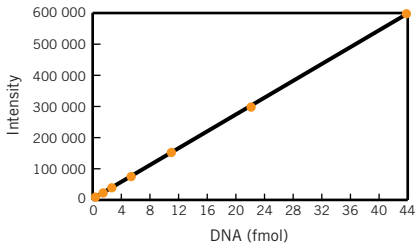

Fig 5. Fluorescence linearity. A 24-mer DNA oligonucleotide, 5' end-labelled with fluorescein (two-fold serial dilutions) was detected in denaturing polyacrylamide gel sandwich using Typhoon scanner with 532 nm excitation and 526SP emission filter. The plot shows signal linearity over a range of 100 amol to 44 fmol.

excited state before the fluorescent light is emitted. The Stokes shift is fundamental to the sensitivity of fluorescent techniques because it allows emission photons to be detected against a low background spectrally removed from excitation photons. See Appendix 2 for excitation and emission spectra of many commonly used fluorophores.

Signal linearity

The intensity of the emitted fluorescent light is a linear function of the amount of fluorochrome present when the wavelength and intensity of the illuminating light are constant (e.g. when using a controlled laser light source). Although the signal becomes non-linear at very high fluorochrome concentrations, linearity is maintained over a very wide range of concentrations. In fact, measurement down to 100 amol is not unusual, with linearity extending over several orders of magnitude (Fig 5).

Brightness

Fluorochromes differ in the level of intensity (brightness) they are capable of producing. This is important because a dull fluorochrome is a less sensitive probe than a bright fluorochrome. 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)

The brightness of a fluorochrome is proportional to the product of its extinction coefficient (ε) and its quantum efficiency (ϕ), as indicated in the following relationship:

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

The extinction coefficient of a fluorochrome is the amount of light that a fluorochrome absorbs at a particular wavelength. The molar extinction coefficient is defined as the optical density of a 1 M solution of the fluorochrome measured through a 1 cm light path. For fluorochromes that are useful molecular labels, the molar extinction coefficient at peak absorption is in the tens of thousands.

The probability that an excited fluorochrome will emit light is its quantum efficiency and is given by the following equation:

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

Values for ϕ range from 0 (for nonfluorescent compounds) to 1 (for 100% efficiency). For example, fluorescein has a ϕ of 0.9 and Cy™5 has a ϕ of 0.3. In practice, ϕ is usually listed as the quantum efficiency at the wavelength of maximum absorption.

Both fluorescein ($\varepsilon \approx 70\,000$, $\phi \approx 0.9$) and Cy5 ($\varepsilon \approx 200\,000$, $\phi \approx 0.3$) are very bright fluorochromes. Although their quantum efficiencies and extinction coefficients are quite different, they are similar in brightness. This illustrates the importance of considering both extinction coefficient and quantum efficiency when evaluating new fluorochromes.

Fluorescence intensity is also affected by the intensity of incident radiation. Although in theory, the more intense source will yield the greater fluorescence, in actual practice, photodestruction of the sample can occur when high intensity light is delivered over a prolonged period of time.

Susceptibility to environmental effects

The quantum efficiency and excitation and emission spectra of a fluorochrome can be affected by a number of environmental factors, including temperature, ionic strength, pH, excitation light intensity and duration, covalent coupling to another molecule, and noncovalent interactions (e.g. insertion into double-stranded DNA). Many suppliers provide information on the characteristics of their fluorescent reagents under various conditions.

A significant effect, known as photodestruction or photobleaching, results from the enhanced chemical reactivity of the fluorochrome when excited. Since the excited state is generally much more chemically reactive than the ground state, a small fraction of the excited fluorochrome molecules can participate 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.

Quantification 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, but it is independent of the intensity of the incident light. In contrast, the intensity of the emitted fluorescent light varies with the intensity and wavelength of incident light and the brightness and concentration of the fluorochrome.

When more intense light is used to illuminate a sample, more of the fluorochrome molecules are excited, and the number of photons emitted (i.e. the number of electrons falling to the ground state) increases. 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, as with the use of a controlled laser light source, the number of photons emitted is a linear function of the number of fluorochrome molecules present (Fig 5). At very high fluorochrome concentrations, the signal becomes non-linear because the fluorochrome molecules are so dense that excitation occurs only at or near the surface of the sample. Additionally, 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 cycles of excitation. In practice, however, if the excitation light intensity and fluorochrome concentration are held constant, the total emitted light becomes 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) can be accomplished with any photosensitive device. For example, for detection of low-intensity light, a photo multiplier tube or PMT can be used. This is simply a photoelectric cell with a built-in amplifier. When light of sufficient energy hits the photocathode in the PMT, 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).

For additional information, please see the General References section of this manual.

Chapter 2

FLUORESCENCE IMAGING SYSTEMS

Introduction

All fluorescence imaging systems require the following key elements:

- Excitation source
- Light delivery optics
- Light collection optics
- Filtration of the emitted light
- Detection, amplification and digitization

The design and components of a typical fluorescence detection system are illustrated in Figure 6. The following paragraphs provide additional details concerning the elements that comprise the system.

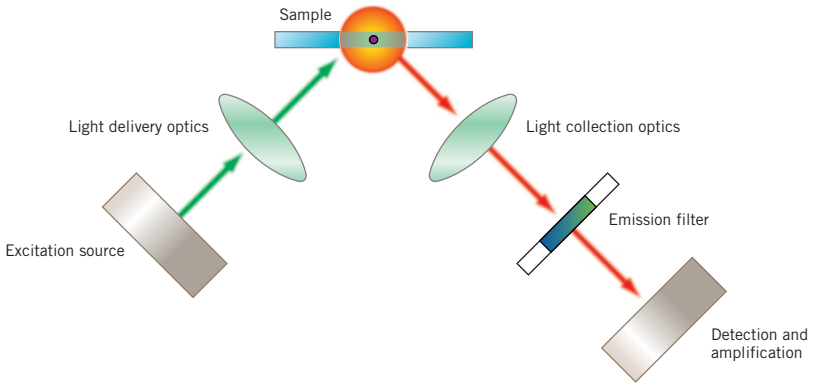

Fig 6. Components of a general fluorescence imaging system.

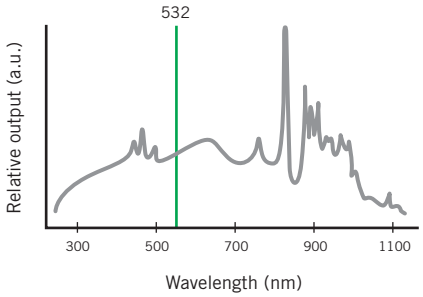

Fig 7. Spectral output of light from a xenon lamp and Nd:YAG laser. The "relative output" axis is scaled arbitrarily for the two light sources. The 532-nm line of the Nd:YAG laser is shown in green.

Excitation sources and light delivery optics

Light energy is essential to fluorescence. Light sources fall into two broad categories—wide-area, broad-wavelength sources, such as UV and xenon arc lamps, and line sources with discrete wavelengths, such as lasers (Fig 7). Broad-wavelength excitation sources are used in fluorescence spectrometers and camera imaging systems. Although the spectral output of a lamp is broad, it can be tuned to a narrow band of excitation light with the use of gratings or filters. In contrast, lasers deliver a narrow beam of collimated light that is predominantly monochromatic.

In most camera systems, excitation light is delivered to the sample by direct illumination of the imaging field, with the excitation source positioned either above, below, or to the side of the sample. Laser-based imaging systems, on the other hand, use more sophisticated optical paths, comprising mirrors and lenses, to direct the excitation beam to the sample. Some filtering of the laser light may also be required before the excitation beam is directed to the sample.

Light collection optics

High-quality optical elements, such as lenses, mirrors, and filters, are integral components of any efficient imaging system. Optical filters, generally referred to as interference filters, are typically made from laminates of multiple glass elements. Filters can be coated to selectively absorb or reflect different wavelengths of light, thus creating the best combination of wavelength selection, linearity, and transmission properties. (Refer to Chapter 3 for additional information concerning optical filters.)

Filtration of the emitted light

Although emitted fluorescent light radiates from a fluorochrome in all directions, it is typically collected from only a relatively small cone angle on one side of the sample. For this reason, light collection optics must be as efficient as possible. Any laser light that is reflected or scattered by the sample must be rejected from the collection pathway by a series of optical filters. Emitted light can also be filtered to select only the range or band of wavelengths that is of interest to the user. Systems that employ more than one detector require additional beamsplitter filters to separate and direct the emitted light along separate paths to the individual detectors.

Detection, amplification and digitization

For detection and quantification of emitted light, either a photomultiplier tube (PMT) or a charge-coupled device (CCD) can be used. In both cases, photon energy from emitted fluorescent light is converted into electrical energy, thereby producing a measurable signal that is proportional to the number of photons detected.

After the emitted light is detected and amplified, the analogue signal from a PMT or CCD detector is converted to a digital signal. The process of digitization turns a measured continuous analogue signal into discrete numbers by introducing intensity levels. The number of intensity levels is based on the digital resolution of the instrument, which is usually given as a number of bits, or exponents of 2. 8-bit, 12-bit, and 16-bit digital files correspond to the number of intensity levels allocated within that image file (256, 4096 and 65 536, respectively). Digital resolution defines the ability to resolve two signals with similar intensities.

Since only a limited number of intensity levels are available, it is unavoidable that this conversion process introduces a certain amount of error. To allow ample discrimination between similar signals and to keep the error as low as possible, the distribution of the available intensity levels should correspond well to the linear dynamic range of a detector.

There are two methods of distributing intensity levels. A linear (even) distribution has the same spacing for all the intensity levels, allowing measurement across the dynamic range with the same absolute accuracy. However, relative digitization error increases as signals become smaller. A non-linear distribution (e.g. logarithmic or square root functions) divides the lower end of the signal range into more levels while combining the high end signals into fewer intensity levels. Thus, the absolute accuracy decreases with higher signals, but the relative digitization error remains more constant across the dynamic range.

Scanner systems

Excitation sources

Most fluorescence scanner devices used in life science research employ laser light for excitation. A laser source produces a narrow beam of highly monochromatic, coherent, and collimated light. The combination of focused energy and narrow beam-width contributes to the excellent sensitivity and resolution possible with a laser scanner. The active medium of a laser—the material that is made to emit light—is commonly a solid state (glass, crystal), liquid, or gas (4). Gas lasers and solid-state lasers both provide a wide range of specific wavelength choices for different imaging needs. Other light sources used in imaging scanners include light emitting diodes (LEDs), which are more compact and less expensive than lasers, but produce a wide-band, low-power output.

Lasers

Argon ion lasers produce a variety of wavelengths including 488 nm and 514 nm that are useful for excitation of many common fluorochromes. The 488 nm line is especially well-suited for fluorescein and other related "blue-excited" dyes. Argon ion lasers are relatively large gas lasers and require external cooling.

Helium neon or HeNe lasers, which generate a single wavelength of light (e.g. 633 nm), are popular in many laser scanners, including densitometers, storage phosphor devices, and fluorescence systems. In fluorescence detection, the helium neon laser can be used to excite the Cy5 fluorochrome. These lasers are smaller than argon ion lasers and do not require independent cooling.

Neodymium: Yttrium Aluminium Garnet (Nd:YAG) solid-state lasers, when frequency-doubled, generate a strong line at 532 nm that is not readily available from other laser sources. This excitation source is useful for imaging a wide range of different fluorochromes that excite efficiently at wavelengths between 490 nm and 600 nm. Cooling is required to stabilize the output.

Diode lasers (or semiconductor diode lasers) are compact lasers. Because of their small size and light weight, these light sources can be integrated directly into the scanning mechanism of a fluorescence imager. Diode lasers are inexpensive and are generally limited to wavelengths above 635 nm.

Light Emitting Diodes (LEDs)

As a laser alternative, the LED produces an output with a much wider bandwidth (≥ 60 nm) and a wide range of power from low to moderate output. Because LED light emissions are doughnut shaped, and not collimated, the source must be mounted very close to the sample using lenses to tightly focus the light. LEDs are considerably smaller, lighter, and less expensive than lasers. They are available in the visible wavelength range above 430 nm.

Excitation light delivery

Because light from a laser is well-collimated and of sufficient power, delivery of excitation light to the sample is relatively straightforward, with only negligible losses incurred during the process. For lasers that produce multiple wavelengths of light, the desired line(s) can be selected by using filters that exclude unwanted wavelengths, while allowing the selected line to pass at a very high transmission percentage. Excitation filters are also necessary with single-line lasers, as their output is not 100% pure.

Optical lenses are used to align the laser beam, and mirrors can be used to redirect the beam within the instrument. One of the main considerations in delivering light using a laser scanning system is that the light source is a point, while the sample typically occupies a relatively large two-dimensional space. Effective sample coverage can be achieved by rapidly moving the excitation beam across the sample in two dimensions.

There are two ways to move and spread the point source across the sample, which are discussed below.

Galvanometer-based systems

Galvanometer-based systems use a small, rapidly oscillating mirror to deflect the laser beam, effectively creating a line source (Fig 8). By using relatively simple optics, the beam can be deflected very quickly, resulting in a short scan time. Compared to confocal systems, galvonometer-based scanners are useful for imaging thick samples due to the ability to collect more fluorescent signal in the vertical dimension. However, since the excitation beam does not illuminate the sample from the same angle in every position, a parallax effect can result. The term parallax here refers to the shift in apparent position of targets, predominately at the outer boundaries of the scan area. Additionally, the arc of excitation light created by the galvanometer mirror produces some variations in the effective excitation energy reaching the sample at different points across the arc. These effects can be minimized with an f-theta lens (as illustrated in Fig 8), but when the angle of incident excitation light varies over the imaging field, some spatial distortion can still occur in the resulting image.

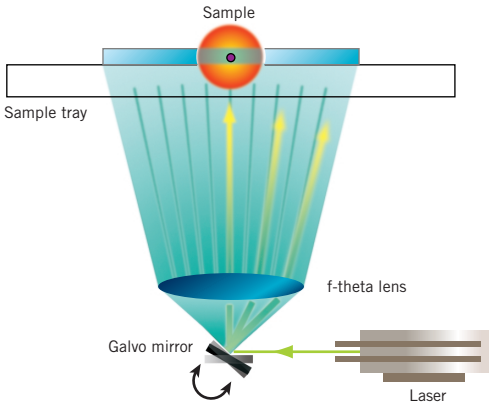

Fig 8. Galvanometer-controlled scanning mechanism. Light is emitted from the laser in a single, straight line. The galvanometer mirror moves rapidly back and forth redirecting the laser beam and illuminating the sample across its entire width (X-axis). The f-theta lens reduces the angle of the excitation beam delivered to the sample. The entire sample is illuminated either by the galvanometer mechanism moving along the length of the sample (Y-axis) or the sample moving relative to the scanning mechanism.

Moving-head scanners

Moving-head scanners use an optical mechanism that is equidistant from the sample. This means that the angle and path length of the excitation beam is identical at any point on the sample (Fig 9). This eliminates variations in power density and spatial distortion common with galvanometer-based systems. Although scan times are longer with a moving-head design, the benefits of uniformity in both light delivery and collection of fluorescence are indispensible to accurate signal quantification.

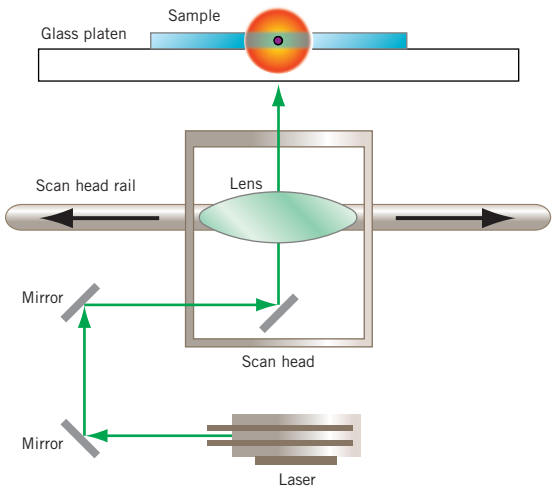

Fig 9. Moving-head scanning mechanism. The light beam from the laser is folded by a series of mirrors and ultimately reflected onto the sample. The sample is illuminated across its width as the scan head moves along the scan head rail (X-axis). The entire sample is illuminated by the scan head, laser, and mirrors tracking along the length of the sample (Y-axis).

Light collection

The light-collection optics in a scanner system must be designed to efficiently collect as much of the emitted fluorescent light as possible. Laser light that is reflected or scattered by the sample is generally rejected from the collection pathway by a laser-blocking filter designed to exclude the light produced by the laser source, while passing all other emitted light.

Light collection schemes vary depending on the nature of the excitation system. With galvanometer systems, the emitted fluorescence must be gathered in a wide line across the sample. This is usually achieved with a linear lens (fibre bundle or light bar), positioned beneath the sample, that tracks with the excitation line, collecting fluorescence independently at each pixel. Although this system is effective, it can produce image artefacts. At the edges of the scan area where the angle of the excitation beam, relative to the sample, is farthest from perpendicular, some spatial distortion may occur. Where very high signal levels are present, stimulation of fluorescence from sample areas that are adjacent to the pixel under investigation can result in an inaccurate signal measurement from that pixel, an artefact known as flaring or blooming.

With moving-head systems, emitted light is collected directly below the point of sample excitation. Again, it is important to collect as much of the emitted light as possible to maintain high sensitivity. This can be achieved by using large collection lenses, or lenses with large numerical apertures (NA). Since the NA is directly related to the full angle of the cone of light rays that a lens can collect, the higher the NA, the greater the signal resolution and brightness (5). Moving-head designs can also include confocal optical elements that detect light from only a narrow vertical plane in the sample. This improves sensitivity by focusing and collecting emission light from the point of interest while reducing the background signal and noise from out-of-focus regions in the sample (Fig 10). Additionally, the parallel motion of moving head designs removes other artefacts associated with galvanometer-based systems, such as spatial distortion and the flaring or blooming associated with high activity samples.

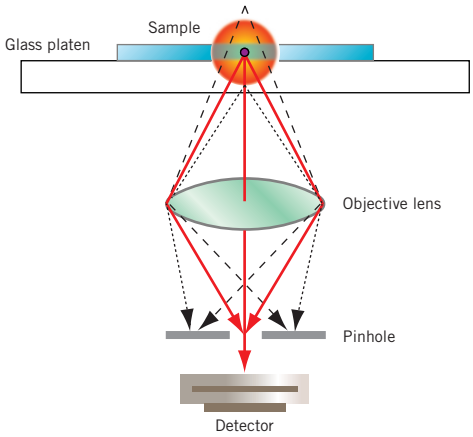

Fig 10. Illustration of confocal optics. Fluorescence from the sample is collected by an objective lens and directed toward a pinhole aperture. The pinhole allows the emitted light from a narrow focal plane (red solid lines) to pass to the detector, while blocking most of the out-of-focus light (black dashed lines).

Signal detection and amplification

The first stage in fluorescent signal detection is selection of only the desired emission wavelengths from the label or dye. In single-channel or single-label experiments, emission filters are designed to allow only a well-defined spectrum of emitted light to reach the detector. Any remaining stray excitation or scattered light is rejected. Because the intensity of the laser light is many orders of magnitude greater than the emitted light, even a small fraction of laser light reaching the detector will significantly increase background. Filtration is also used to reduce background fluorescence or inherent autofluorescence originating from either the sample itself or the sample matrix (i.e. gel, membrane, or microplate).

In multichannel or multi-label experiments using instrumentation with dual detectors, additional filtering is required upstream of the previously described emission filter. During the initial stage of collection in these experiments, fluorescence from two different labels within the same sample is collected simultaneously as a mixed signal. A dichroic beamsplitter must be included to spectrally resolve (or split) the contribution from each label and then direct the light to appropriate emission filters (Fig 11). At a specified wavelength, the beamsplitter partitions the incident fluorescent light beam into two beams, passing one and reflecting the other. The reflected light creates a second channel that is filtered independently and detected by a separate detector. In this way, the fluorescent signal from each label is determined accurately in both spatial and quantitative terms. (See Chapter 3 for additional information on multichannel experiments.)

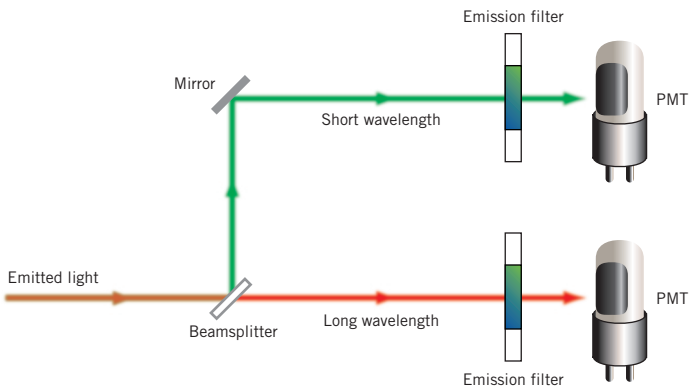

Fig 11. Use of a beamsplitter or dichroic filter with two separate PMTs. Light from a dual colour sample enters the emission optics as a combination of wavelengths. A dichroic beamsplitter distinguishes light on the basis of wavelength. Wavelengths above the beamsplitter range pass through, those below are reflected. In this way two channels are created. These two channels can then be filtered and detected independently.

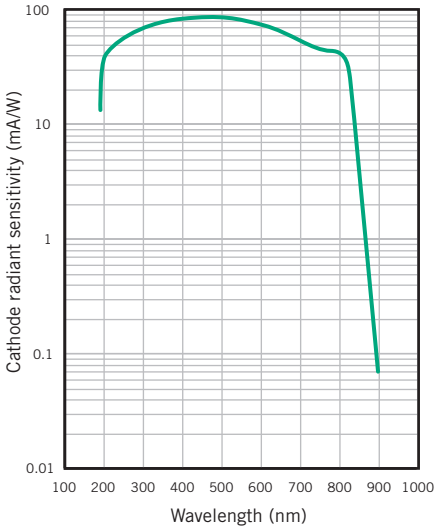

Fig 12. An example of the response of a PMT versus wavelength.

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After the fluorescent emission has been filtered and only the desired wavelengths remain, the light is detected and quantified. Because the intensity of light at this stage is very small, a PMT must be used to detect it. In the PMT, photons of light hit a photocathode and are converted into electrons which are then accelerated in a voltage gradient and multiplied between 10^6 to 10^7 times. This produces a measurable electrical signal that is proportional to the number of photons detected. The response of a PMT is typically useful over a wavelength range of 300–800 nm (Fig 12). High-performance PMTs extend this range to 200–900 nm.

System performance

The performance of a laser scanner system is described in terms of system resolution, linearity, uniformity, and sensitivity.

Resolution can be defined in terms of both spatial and amplitude resolution. Spatial resolution refers to the number of data points sampled per unit length or area. It is a function of the diameter of the light beam when it reaches the sample and the distance between adjacent measurements. Spatial resolution is dependent on, but not equivalent to, the pixel size of the image. Spatial resolution improves as pixel size reduces. Systems with higher spatial resolution can not only detect smaller objects, but can also discriminate more accurately between closely spaced targets. However, an image with a 100 µm pixel size will not have a spatial resolution of 100 µm. The pixel size refers to the collection sampling interval of the image. According to a fundamental sampling principle, the Nyquist Criterion, the smallest resolvable object in an image is no better than twice the sampling interva (6). Thus, to resolve a 100 µm sample, the sampling interval must be at most 50 µm.

Amplitude resolution, or gray-level quantification, describes the minimum difference that is distinguishable between levels of light intensity (or fluorescence) detected from the sample (7). For example, an imaging system with 16-bit digitization can resolve and accurately quantify 65 536 different values of light intensity from a fluorescent sample.

Linearity of a laser scanner is the signal range over which the instrument yields a linear response to fluorochrome concentration and is therefore useful for accurate quantification. A scanner with a wide dynamic range can detect and accurately quantify signal from both very low- and very high-intensity targets in the same scan. The linear dynamic range of most laser scanner instruments is between 10^4 and 10^5.

Uniformity across the entire scan area is critical for reliable quantification. A given fluorescent signal should yield the same measurement at any position within the imaging field. Moving-head scanners, in particular, deliver flat-field illumination and uniform collection of fluorescent emissions across the entire scan area.

Sensitivity, or detection threshold, is a measure of the lowest fluorescent signal that can be detected by the instrument. Increased sensitivity aids the detection of low abundance targets. From an economical standpoint, instruments with high sensitivities for different fluoresecent labels are very cost-effective because they enable analyses that require less label and consume less sample.

CCD camera-based systems

CCD-(charge-coupled device) based cameras are composed of an illumination system and a lens assembly that focuses the image onto the light-sensitive CCD array (Fig 13). CCD camera-based systems are area imagers that integrate fluorescent signal from a continuously illuminated sample field. Most of these systems are designed to capture a single view of the imaging area, using lens assemblies with either a fixed or selectable focal distance.

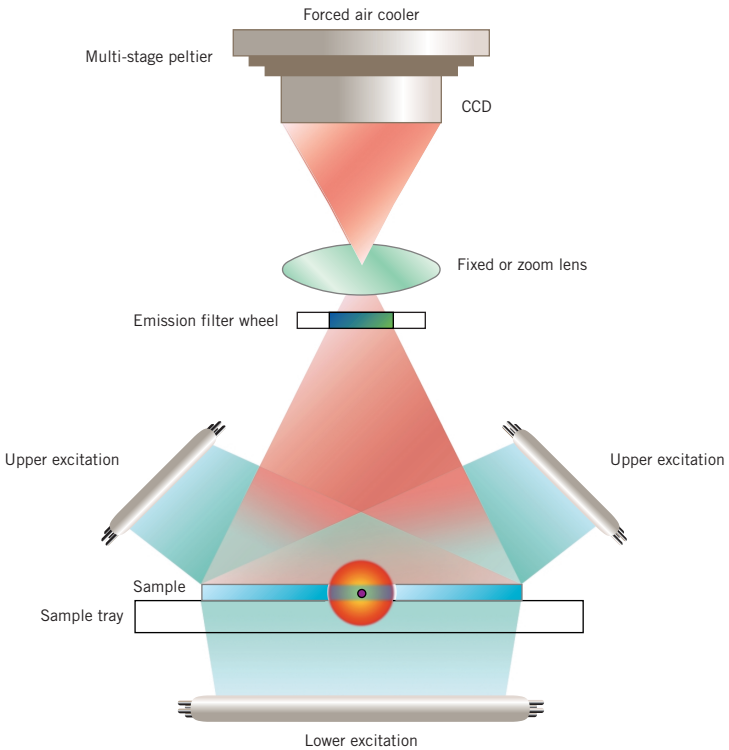


Fig 13. Components of a typical CCD camera-based imaging device. The sample can be illuminated in a variety of ways depending on the nature of the labels to be analysed. The sample is then viewed by the camera. The camera includes focusing optics to accommodate samples at different heights. Emission filters can be inserted in the light path to select specific wavelengths and eliminate background.

Excitation sources and light delivery

Illumination or excitation in CCD camera systems is provided by ultraviolet (UV) or white light gas discharge tubes, broad-spectrum xenon arc lamps, or high-power, narrow bandwidth diodes. Light is delivered to the sample either from below (trans-illumination) or from above (epi-illumination). Even with the broadband light sources used in CCD camera systems, wavelength selection is possible through the use of appropriate filters.

Light collection

Lenses are used to collect fluorescent emission from the illuminated imaging field. A lens system typically has a zoom capacity, so that different sample sizes can be captured in a single view. Some falloff in light intensity detected at the corners and edges of the field can be expected in large-field photographic imaging with a lens because light at the corners of the imaging field is farther from the centre of the lens than light on the axis (8). Such aberrations in field uniformity associated with CCD systems can be improved using software flat-field corrections.

Signal detection and amplification

An image that is focused on a two-dimensional CCD array produces a pattern of charge that is proportional to the total integrated energy flux incident on each pixel. The CCD array can be programmed to collect photonic charge over a designated period of time. The total charge collected at a given pixel is equal to the product of the photonic charge generation rate and the exposure time. Thermal cooling of the CCD can improve detection sensitivity by reducing the level of electronic noise.

System performance

The performance of any CCD camera system is dependent on the system resolution, sensitivity, linearity, and dynamic range.

Resolution

The resolution of a captured image is linked to the geometry of the CCD, with the size of each pixel varying from 6–30 µm. Currently, CCDs with formats from 512 × 512–4096 × 4096 elements are available. Image resolution is reduced when charges from adjacent pixels are combined or "binned" during image acquisition. However, it is possible to collect multiple images by moving the lens assembly and CCD detector relative to the sample, and then using software to "stitch" the images together to form a complete view of the sample. In this way, each segment of the image or "tile" can utilize the full resolution of the CCD.

Sensitivity and linearity

CCD arrays are sensitive to light, temperature, and high-energy radiation. Dark current from thermal energy, cosmic rays, and the preamplifier causes system noise that can have a profound effect on instrument performance. Cooling of the CCD significantly reduces noise levels and improves both sensitivity and linearity of the system. For example, active thermal cooling to -50 °C improves the linear response of a CCD three- to five-fold. Combining charges from adjacent pixels during acquisition can also enhance sensitivity, although image resolution may suffer.

Dynamic range

The dynamic range of a CCD is defined as the ratio of the full saturation charge to the noise level. CCD cameras typically have a dynamic range of up to 10^5. An imaging system with a 15 × 15 µm pixel has a 225 μm^2 area and a saturation level of about 180 000. If the system noise level is 10, then the dynamic range is the ratio of 180 000:10 or 18 000:1, thus demonstrating how system noise can limit the dynamic range.

Amersham Pharmacia Biotech imaging systems

Amersham Pharmacia Biotech offers a variety of imaging instrumentation, including laser scanning and CCD-based systems. A brief description of each instrument is given in Table 1. For more information, please visit www.apbiotech.com.

Table 1. Amersham Pharmacia Biotech imaging systems

TYPHOON 8600

High performance laser scanning system

Excitation sources : 532-nm Nd:YAG and 633-nm HeNe lasers

Filters : 6 emission filters and 2 beamsplitters (up to 13 emission filter positions)

Detection : 2 high-sensitivity PMTs

Imaging modes : 4-colour automated fluorescence detection, direct chemiluminescence, storage phosphor

Scanning area : 35 x 43 cm

Sample types : Gel sandwiches, agarose and polyacrylamide gels, blots, microplates, TLC plates, and macroarrays

STORM™ 830, 840 OR 860

Variable mode laser scanning system

Excitation sources : 450-nm LED and/or 633-nm laser diode

Filters : 2 built-in emission filters

Detection : High-sensitivity PMT

Imaging modes : Blue- and/or red-excited fluorescence, storage phosphor, and chemifluorescence

Scanning area : 35 x 43 cm

Sample types : Gels, blots, microplates, TLC plates, and macroarrays

Table 1. ***(continued)***

FLUORIMAGER™ 595

Dedicated fluorescence laser scanning system

Excitation sources : 488-nm and 514-nm laser lines of argon ion laser

Filters : 4 selectable emission filters

Detection : High-sensitivity PMT

Imaging modes : Blue- and green-excited fluorescence

Scanning area : 20 x 24 cm

Sample types : Gels, blots, microplates, and TLC plates

IMAGEMASTER™ VDS-CL

Automated CCD camera-based system

Excitation sources : UV, white light

Filters : 2 emission filters (up to 6 emission filter positions)

Detection : Cooled CCD

Imaging modes : Chemiluminescence, fluorescence, and colourimetric detection

Scanning area : 21 x 25 cm

Sample types : Gels, blots, and TLC plates

Focus : Automated

Chapter 3

FLUOROCHROME AND FILTER SELECTION

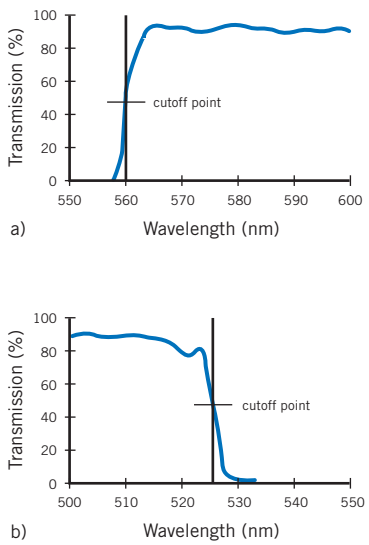


Fig 14. Transmission profiles for a 560 nm long-pass (a) and a 526 nm short-pass (b) filter. The cutoff points are noted.

Introduction

To generate fluorescence, excitation light delivered to the sample must be within the absorption spectrum of the fluorochrome. Generally, the closer the excitation wavelength is to the peak absorption wavelength of the fluorochrome, the greater the excitation efficiency. Appropriate filters are usually built into scanner instruments for laser line selection and elimination of unwanted background light. Fixed or interchangeable optical filters that are suitable for the emission profile of the fluorochromes are then used to refine the emitted fluorescence, such that only the desired wavelengths are passed to the detector. Matching a fluorochrome label with a suitable excitation source and emission filter is the key to optimal detection efficiency. In this chapter, details about the classes and use of emission filters are presented, along with general guidelines for selecting fluorochromes and emission filters for both single-colour and multicolour imaging.

Types of emission filters

The composition of emission filters used in fluorescence scanners and cameras ranges from simple coloured glass to glass laminates coated with thin interference films. Coated interference filters generally deliver excellent performance through their selective reflection and transmission effects. Three types of optical emission filters are in common use.

Long-pass (LP) filters pass light that is longer than a specified wavelength and reject all shorter wavelengths. A good quality long-pass filter is characterized by a steep transition between rejected and transmitted wavelengths (Fig 14a). Long-pass filters are named for the wavelength at the midpoint of the transition between the rejected and transmitted light (cutoff point). For example, the cutoff point in the transmission spectrum of a 560LP filter is 560 nm, where 50% of the maximum transmittance is rejected.

The name of a long-pass filter may also include other designations, such as OG (orange glass), RG (red glass), E (emission), LP (long-pass), or EFLP (edge filter long-pass). OG and RG are coloured-glass absorption filters, whereas E, LP, and EFLP filters are coated interference filters. Coloured-glass filters are less expensive and have more gradual transition slopes than coated interference filters.

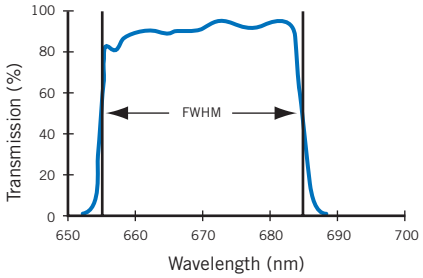


Fig 15. Transmission profile for a band-pass (670BP30) filter. The full-width at half maximum (FWHM) transmission of 30 nm is indicated by the arrows.

Short-pass (SP) filters reject wavelengths that are longer than a specified value and pass shorter wavelengths. Like long-pass filters, short-pass filters are named according to their cutoff point. For example, a 526SP filter rejects 50% of the maximum transmittance at 526 nm (Fig 14b).

Band-pass (BP) filters allow a band of selected wavelengths to pass through, while rejecting all shorter and longer wavelengths. A band-pass filter provides very sharp cutoffs with very little transmission of the rejected wavelengths. High-performance band-pass filters are also referred to as Discriminating Filters (DF). The name of a band-pass filter is typically made up of two parts:

- the wavelength of the band centre. For example, the 670BP30 filter passes a band of light centred at 670 nm (Fig 15).
- the full-width at half-maximum transmission (FWHM). For example, a 670BP30 filter passes light over a wavelength range of 30 nm (655 nm–685 nm) with an efficiency equal to or greater than half the maximum transmittance of the filter.

Band-pass filters with an FWHM of 20–30 nm are optimal for most fluorescence applications, including multi-label experiments. Filters with FWHMs greater than 30 nm allow collection of light at more wavelengths and give a higher total signal; however, they are less able to discriminate between closely spaced, overlapping emission spectra in multichannel experiments. Filters with FWHMs narrower than 20 nm transmit less signal and are most useful with fluorochromes with very narrow emission spectra.

Using emission filters to improve sensitivity and linearity range

When selectable emission filters are available in an imaging system, filter choice will influence the sensitivity and dynamic range of an assay. In general, if image background signal is high, adding an interchangeable filter may improve the sensitivity and dynamic range of the assay. The background signal from some matrices (gels and membranes) has a broad, relatively flat spectrum. In such cases, a band-pass filter can remove the portion of the background signal comprising wavelengths that are longer or shorter than the fluorochrome emissions. By selecting a filter that transmits a band at or near the emission peak of the fluorochrome of interest, the background signal is typically reduced with only slight attenuation of the signal from the fluorochrome. Therefore,

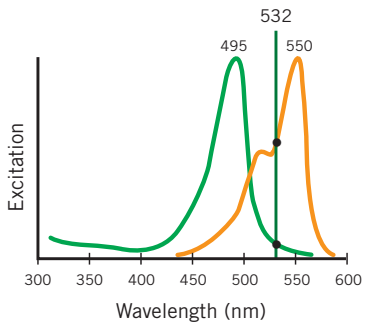


Fig 16. Excitation of fluorescein (green) and Cy3 (orange) using 532 nm laser light. The absorption spectra of Cy3 and fluorescein are overlaid with the 532 nm wavelength line of the Nd:YAG laser.

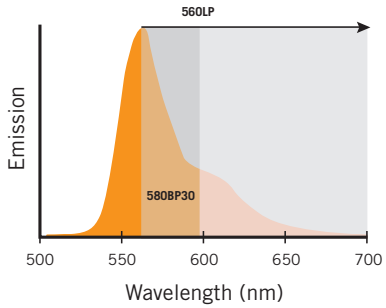


Fig 17. Emission filtering of Cy3 fluorescence using either a 580BP30 (dark gray area) or a 560LP filter (light and dark gray areas).

the use of an appropriate band-pass filter should improve the overall signal-to-noise ratio (S/N). To determine if a filter is needed, scans should be performed with and without the filter while other conditions remain constant. The resulting S/N values should then be compared to determine the more efficient configuration.

Interchangeable filters can also be used in fluorescence scanners to attenuate the sample signal itself so that it falls within the linear range of the system. Although scanning the sample at a reduced PMT voltage can attenuate the signal, the response of the PMT may not be linear if the voltage is set below the instrument manufacturer's recommendation. If further attenuation is necessary to prevent saturation of the PMT, the addition of an appropriate emission filter can decrease the signal reaching the detector.

General guidelines for selecting fluorochromes and filters

Single-colour imaging

Excitation efficiency is usually highest when the fluorochrome's absorption maximum correlates closely with the excitation wavelength of the imaging system. However, the absorption profiles of most fluorochromes are rather broad, and some fluorochromes have a second (or additional) absorption peak or a long "tail" in their spectra. It is not mandatory that the fluorochrome's major absorption peak exactly match the available excitation wavelength for efficient excitation. For example, the absorption maxima of the fluorescein and Cy3 fluorochromes are 490 nm and 552 nm respectively (Fig 16). Excitation of either dye using the 532 nm wavelength line of the Nd:YAG laser may seem to be inefficient, since the laser produces light that is 40 nm above the absorption peak of fluorescein and 20 nm below that of Cy3. In practice, however, delivery of a high level of excitation energy at 532 nm does efficiently excite both fluorochromes. (See Appendix 1 for a discussion of fluorescein excitation using 532 nm laser line.)

For emission, selecting a filter that transmits a band at or near the emission peak of the fluorochrome generally improves the sensitivity and linear range of the measurement. Figure 17 shows collection of Cy3 fluorescence using either a 580BP30 or a 560LP emission filter.

Please refer to Appendixes 2 and 3 for a list of fluorochromes and their excitation and emission maxima and spectra, as well as the appropriate instrument set-up with Amersham Pharmacia Biotech fluorescence scanning systems.

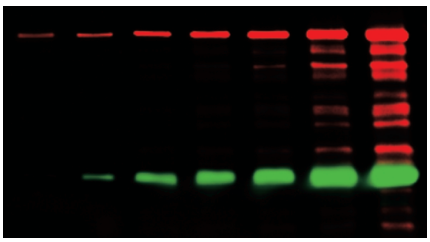


Fig 18. Two-colour fluorescent Western blot. β-galactosidase was detected using a Cy5-labelled secondary antibody (red), and tubulin was detected using an enzyme-amplified chemistry with the fluorogenic ECF™ substrate (green). Storm 860 was used for image acquisition.

Fig 19. Three-colour gel image of a DNA in-lane sizing experiment. The fluorochromes used were TAMRA™ (yellow), ROX (red), and fluorescein (green). The ROX and TAMRA bands are labelled DNA size ladders. The fluorescein fragments are PCR products of unknown size. Typhoon 8600 was used for image acquisition.

Multicolour imaging

Multicolour imaging allows detection and resolution of multiple targets using fluorescent labels with different spectral properties. The ability to multiplex or detect multiple labels in the same experiment is both time- and cost-effective and improves accuracy for some assays. Analyses using a single label can require a set of experiments or many repetitions of the same experiment to generate one set of data. For example, single-label analysis of gene expression from two different tissues requires two separate hybridizations to different gene arrays or consecutive hybridizations to the same array with stripping and reprobing. With a dual-label approach, however, the DNA probes from the two tissue types are labelled with different fluorochromes and used simultaneously with the same gene array. In this way, experimental error is reduced because only one array is used, and hybridization conditions for the two probes are identical. Additionally, by using a 2-channel scan, expression data is rapidly collected from both tissues, thus streamlining analysis. Other applications are equally amenable to dual-label analysis. For example, Figure 18 shows a two-colour Western blot experiment where two protein targets are differentially probed using antibodies conjugated with two different fluorescent tags.

The use of multicolour imaging can greatly improve the accuracy for applications such as DNA fragment sizing. This technique is usually performed by loading a DNA size ladder and an unknown DNA sample in adjacent lanes of a gel. Because variations in lane-to-lane migration rate can occur during electrophoresis, errors in size estimation may result. By labelling the standard and the unknown fragments with two fluorochromes whose spectra can be differentiated, co-resolution of the unknown and the size ladder can be achieved in the same lane (Fig 19).

The process for multicolour image acquisition varies depending on the imaging system. An imager with a single detector takes consecutive images using different emission filters and, in some cases, different excitation light. When two detectors are available, the combined or mixed fluorescence from two different labels is collected at the same time and then resolved by filtering before the signal reaches the detectors. Implementation of dual detection requires a beamsplitter filter to spectrally split the mixed fluorescent signal, directing the resulting two

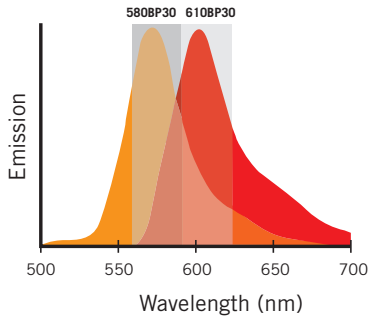


Fig 20. Emission spectra of TAMRA (orange) and ROX (red). A 580BP30 filter (dark gray) was used for TAMRA, and a 610BP30 filter (light gray) was used for ROX.

emission beams to separate emission filters (optimal for each fluorochrome), and finally to the detectors. A beamsplitter, or dichroic reflector, is specified to function as either a short-pass or long-pass filter relative to the desired transition wavelength. For example, a beamsplitter that reflects light shorter than the transition wavelength and passes longer wavelengths is effectively acting as a long-pass filter (Fig 11).

Fluorochrome selection in multicolour experiments

When designing multicolour experiments, two key elements must be considered—the fluorochromes used and the emission filters available.

As with any fluorescence experiment, the excitation wavelength of the scanner must fall within the absorption spectrum of the fluorochromes used. Additionally, the emission spectra of different fluorochromes selected for an experiment should be relatively well resolved from each other. However, some spectral overlap between emission profiles is almost unavoidable. To minimize cross-contamination, fluorochromes with well-separated emission peaks should be chosen along with emission filters that allow reasonable spectral discrimination between the fluorochrome emission profiles. Figure 20 shows the emission overlap between two common fluorochromes and the use of band-pass filters to discriminate the spectra. For best results, fluorochromes with emission peaks at least 30 nm apart should be chosen.

A fluorescence scanner is most useful for multicolour experiments when it provides selectable emission filters suitable for a variety of labels. A range of narrow band-pass filters that match the peak emission wavelengths of commonly used fluorochrome labels will address most multicolour imaging needs.

Software

To reduce the wavelength cross-contamination typically found in multichannel fluorescence images, software processing can be used. This involves applying a cross-talk algorithm to the individual channels to yield a revised image set that more ideally represents the light emitted from the different labels in the sample.

Chapter 4 gives more details about fluorochrome separation software and image analysis software in general.

Chapter 4

IMAGE ANALYSIS

Introduction

Image acquisition using a fluorescence imaging device creates one or more data files for each sample analysed. The size of these files will vary depending on sample size and the digital resolution used for acquisition. Software is used to display the image, adjust the contrast, annotate, and print the image. Image analysis tools allow fragment sizing, quantification, matching, pattern analysis, and generation of analysis reports. Some software packages also provide access to libraries or a database for sample matching and querying. Image utility functions address correction of spectral overlap in multicolour images, image filtering, rotation, pixel inversion, and image cropping. The purpose of this chapter is to provide an overview of features common to image analysis software packages and to illustrate how the software is applied to different image analysis needs.

Image display

One of the basic functions of an image analysis software package is to enable viewing, adjustment, and assessment of the acquired image. Currently, image files usually have at least a 12-bit or 16-bit data structure, which means as many as 65 356 gray levels are possible. Computer displays, printers, and humans are only capable of distinguishing approximately 256 gray levels. It is necessary for the software to adjust the gray scale so that the objects of interest in the image can be seen.

Software features allow the user to fine-tune the display range without affecting the original image data or the results of quantification. Contrast and brightness settings of the display can be adjusted to optimize the image view. The ability to change both the high- and low-display value settings is important for viewing the range of gray (or colour) values of interest. For example, by increasing the low values, image noise or background can be reduced. Reducing the high-value setting of the display increases image contrast, such that weak signals can be visualized. These adjustments are made separately to each channel in a multichannel image. Multicolour software will also allow either side-by-side display of the individual channels or a multicolour overlay of all channels together.

Image analysis software can be used to ascertain if the image contains areas that are non-quantifiable due to light saturation of the detector. When saturation occurs, the results of image analysis are likely to be in error (Fig 21). If an image is composed of pixels with saturated values, imaging should be repeated at a reduced detector sensitivity setting. Other image acquisition settings, such as the scan area, pixel size (resolution), and choice of laser or emission filter, can also be adjusted to improve the resolution, discrimination, or strength of the desired signal.

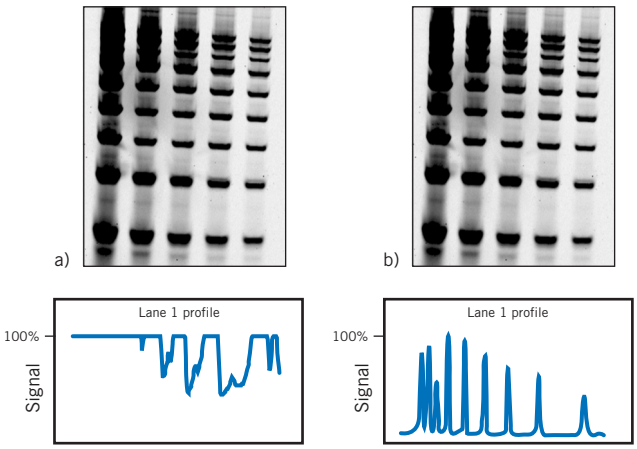


Fig 21. Effect of detector saturation on data quality. A Cy5-labelled size standard was resolved in a 10% polyacrylamide gel and imaged on Typhoon 8600. Image acquired using a PMT setting of 1000 V (panel a) or 500 V (panel b). The line profiles through lane 1 of each image show the response of the PMT to the fluorescent signal collected.

Image documentation

Investigators commonly annotate images with text, numbers, and other labels before archiving their files to disc or printing a copy for documentation. Most imaging software packages offer solutions to simple documentation, annotation, and output of image files. Enlargement, zooming, or magnification is often used to view, in detail, a subsection of a larger image (Fig 22). A scaling function that fits the image to the size of the current program window is useful when the actual (100%) size of an image is larger than the viewing area of the monitor. For some applications, image analysis software must be able to accommodate actual sample size or 1:1 printing. For example, excision and recovery of DNA fragments from fluorescent differential display analysis gels require a precise overlay of a printed copy of the fluorescent image with the original gel. In other cases, it may be desirable to subdivide large image files into separate, smaller image files or to reduce the overall file size before archiving. Other common software utilities for image manipulation include rotation, as well as filtering which reduces undesirable extraneous fluorescent signal caused by sample contamination (e.g. dust or lint).

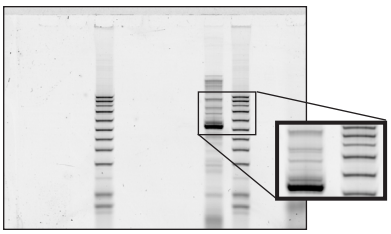


Fig 22. Magnification or zooming to view details of an image.

Documentation of image files is facilitated by the use of "region-of-interest" tools that allow images to be copied directly to a clipboard and pasted into another type of file, such as a word processing or spreadsheet document. Images can thus be readily combined with the contents of a relevant analysis sheet or experiment report. An image copy/paste function is useful in the preparation of presentations, as well as the production of publication-quality figures and illustrations for papers or journal articles.

Quantification

One-dimensional gel/blot analysis

One-dimensional (1-D) gel/blot analysis is performed by signal integration of either the lane as a whole or of the individual elements within a lane (i.e. bands) as separate items (Fig 23). Three approaches are commonly used for quantification. Although they all calculate integrated fluorescent signal, they do so in different ways as outlined below:

Lane profile – Area –	Object quantification – Volume –	Lane quantification – Volume –
■ Wide line across sample track (gel lane)	■ Bands identified manually by user	■ Wide line across sample track (gel lane)
■ Peaks identified	■ Bands bounded by separate objects	■ Bands identified as separate objects
■ Signal integrated across line	■ Total signal inside each band object used	■ Total signal inside each band object used
■ Area under the curve calculated	■ Volume = total integrated signal	■ Volume = total integrated signal
■ Benefits: objectivity, speed	■ Benefits: flexibility, accuracy, user-created objects	■ Benefits: objectivity, speed, accuracy

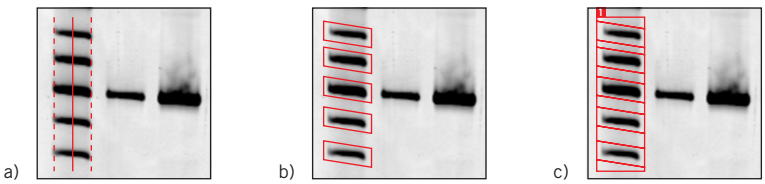
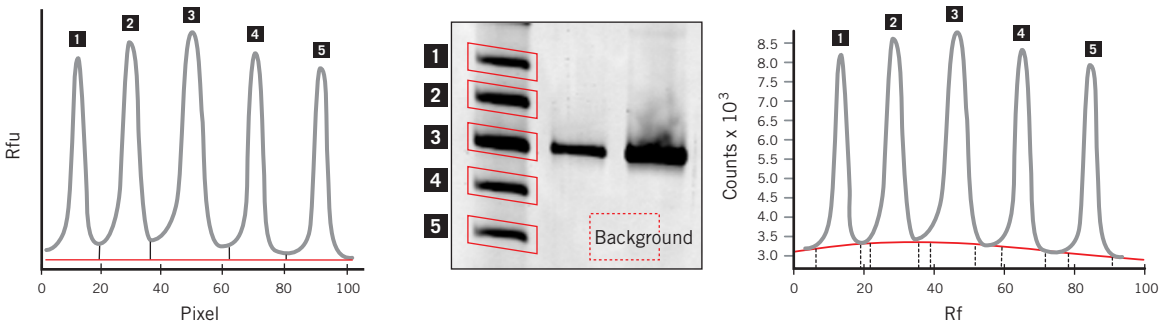


Fig 23. Three methods for signal quantification. Line profile and integration of area under the curve (panel a); integration of signal from manually created closed objects (panel b); software-assisted detection and quantification of lane and bands (panel c).

The lane profile quantification method uses a wide line spanning the width of a gel lane to generate a profile from the average signal at each row of pixels perpendicular to the line (Fig 24a). The accuracy of this approach is greatest when the wide line includes most of the target signal across the width of the lane. Each peak is identified, the area under each peak or curve is integrated, and the resulting peak area is then reported.

In the object and lane quantification methods, analysis targets (i.e. bands, spots, slots) are enclosed using objects such as bands, boxes, rectangles, polygons, or ellipses. Both manual (Fig 24b) and automated (Fig 24c) tools for lane and band identification are available. Quantification in this manner is inherently more flexible than a lane profile method since the user has more control in defining the area to be analysed and in choosing a method for background correction prior to quantification (see section 4.4). All the image pixels bounded by each object are used for quantification. While the absolute data differs between the methods, the trends or relative differences between the measurements from each method are similar (Fig 24a, b, c).



a)

Peak #	Area	Percent
1	35611.55	16.159
2	48933.02	22.204
3	64490.90	29.264
4	39506.01	17.927
5	31835.27	14.446

b)

Name	Volume	Percent
Rectangle 1	914863.7	16.81
Rectangle 2	1212416	22.27
Rectangle 3	1485657	27.29
Rectangle 4	1000343	18.38
Rectangle 5	830632.5	15.26

c)

Band	Volume	Band %
1	750.235,49	16,03
2	1.018.636,94	21,76
3	1.326.294,64	28,33
4	852.357,26	18,21
5	733.996,99	15,68

Fig 24. Comparison of results from "area" versus "volume" analysis methods. In panel a, area refers to integration of signal from each peak identified in a trace through the gel lane, with background taken as the lowest value in the wide-line profile. Volume analysis (panel b) produces a value of integrated signal from within a box surrounding each separate band in the gel lane. A background value, selected from a different region of the gel, has been applied to all calculations. Volume analysis from automated lane and band finding (panel c), with a specific background is calculated around each individual band using the lowest value.

Array and microplate analysis

Arrays range from simple dot blots with a few spots to high-density gene expression arrays with thousands of closely spaced elements. Arrays are typically configured in regular and predictable patterns of rows and columns. Simple arrays and microplates can be analysed manually using grid tools or a series of ellipse objects to identify each element of the array (Fig 25a). Automated, high-throughput analysis of high-density arrays requires sophisticated software packages, complete with algorithms for automated spot-finding (Fig 25b), data normalization, comparisons between different arrays, and database input of analysis results. Array software frequently employs a quality metrics system to assist in the identification of poorly arrayed, contaminated, or improperly detected spots. Tools for elemental display and graphical analysis provide easy visualization and interpretation of results (Fig 26).

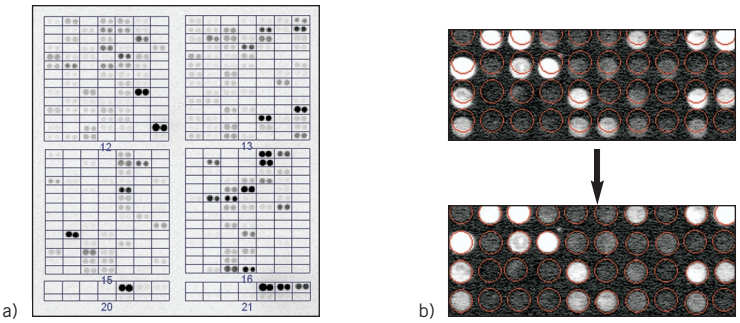


Fig 25. Approaches to software analysis of arrays. In panel a, simple arrays (low-density dot blots, microplates) are analysed using a grid or series of rectangles to surround each array element. In panel b, dedicated array software packages employ spot-finding and/or flexible array templates that find best-fits to enclosing spot elements.

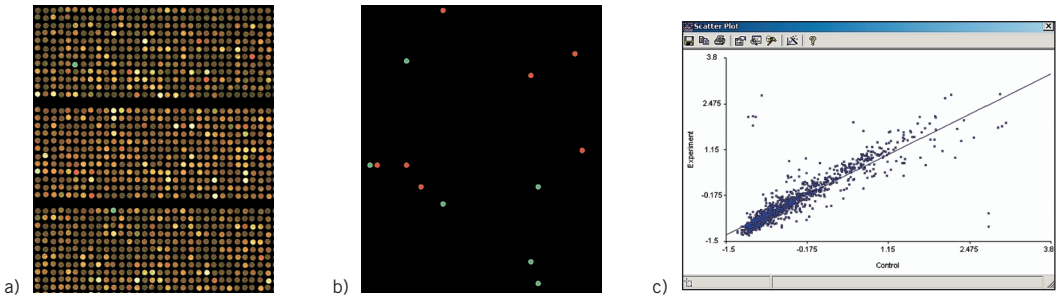
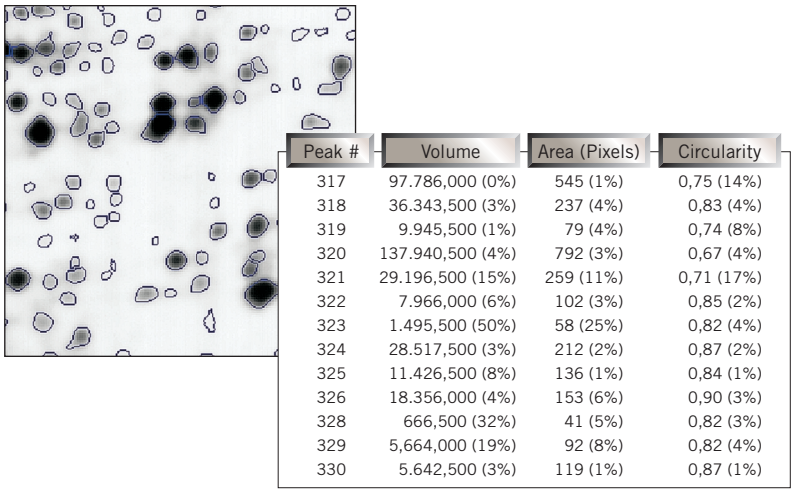


Fig 26. Display and analysis of array experiments. In panel a, images from a two-channel gene expression array using Cy3 (green) and Cy5 (red) labels are overlaid or merged. Levels of gene over- or under-expression are indicated by the relative strength of the green and red colours, respectively. Software displays yellow when signal from both fluorochromes is equal. In panel b, only the array elements exhibiting expression above (green) or below (red) a defined threshold are shown. In panel c, a scatter plot presents the normalized signal ratios of each array element.

Two-dimensional protein gel analysis

Software packages for two-dimensional (2-D) protein gel analysis feature specialized algorithms for spot-finding and analysis routines for gel-to-gel comparisons (Fig 27). Other important tools in these software packages include data normalization; background correction; gel matching and grouping; and database input of analysis results.



Peak #	Volume	Area (Pixels)	Circularity
317	97.786,000 (0%)	545 (1%)	0,75 (14%)
318	36.343,500 (3%)	237 (4%)	0,83 (4%)
319	9.945,500 (1%)	79 (4%)	0,74 (8%)
320	137.940,500 (4%)	792 (3%)	0,67 (4%)
321	29.196,500 (15%)	259 (11%)	0,71 (17%)
322	7.966,000 (6%)	102 (3%)	0,85 (2%)
323	1.495,500 (50%)	58 (25%)	0,82 (4%)
324	28.517,500 (3%)	212 (2%)	0,87 (2%)
325	11.426,500 (8%)	136 (1%)	0,84 (1%)
326	18.356,000 (4%)	153 (6%)	0,90 (3%)
328	666,500 (32%)	41 (5%)	0,82 (3%)
329	5,664,000 (19%)	92 (8%)	0,82 (4%)
330	5.642,500 (3%)	119 (1%)	0,87 (1%)

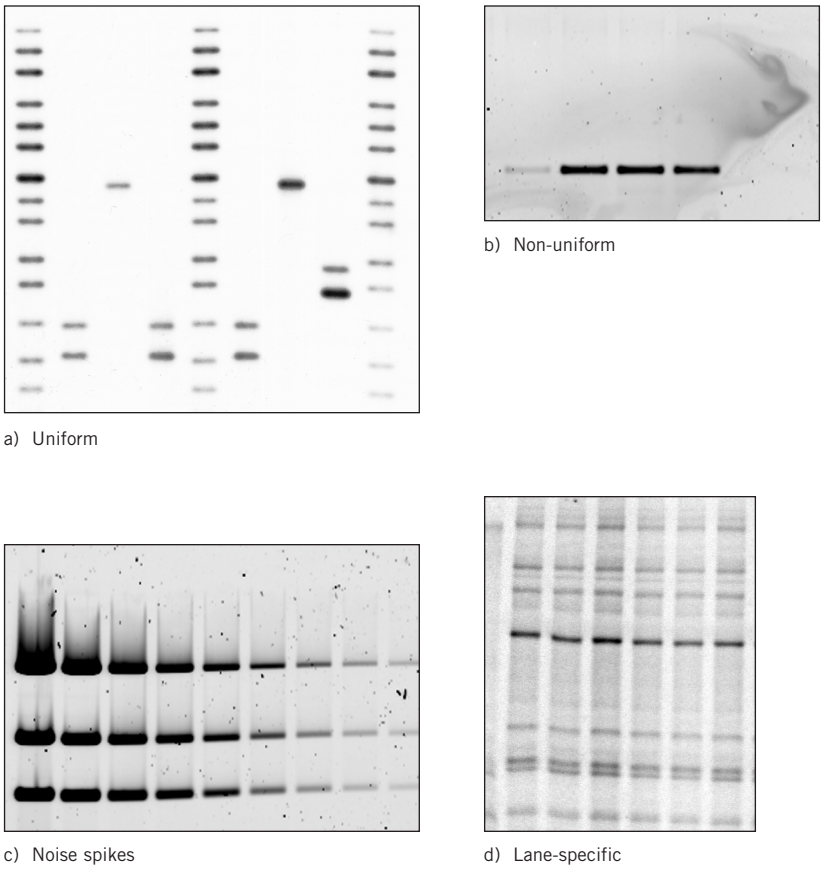
Fig 27. 2-D gel analysis software. Spot borders are identified using spot-finding algorithms. Background must be removed using a global or a local background correction method.

Background correction

Most image analysis software offers multiple choices for applying background correction to fluorescence measurements. The nature of image background can vary significantly depending on a number of factors, such as the fluorescent detection chemistry used, the sample matrix (i.e. gel, membrane, microplate), and the integrity or quality of the sample itself. Because fluorescent detection is extremely sensitive, high background levels in the scanned image can be a common problem, especially in the early stages of protocol development. Fluorescence protocols require careful attention to cleanliness and sample handling to minimize background problems (see Chapter 6 for tips).

The nature of the background signal should be assessed before proceeding with image analysis (Fig 28). Background commonly appears as:

- uniform signal across the image
- non-uniform, uneven or patchy regions
- noise spikes, or small groups of pixels with high counts
- high signal within lanes



a) Uniform

b) Non-uniform

c) Noise spikes

d) Lane-specific

Fig 27. Examples of different types of fluorescent image background.

A full range of background correction choices includes both local and global methods. Local methods account for the local environment at each region of interest—that is, in the immediate neighbourhood of a band, spot, or slot target to be quantified. Depending on the quantification method used, a local method can define background threshold by connecting the low points (or valleys) in a lane profile, or it may use the signal defined by the boundary of each closed object to determine a different background value for each object (Fig 29a).

In global methods, a single global background value is applied equally to a group of analysis targets in the same image. These correction methods include using a straight baseline below a lane profile (i.e. determined from the minimum signal in the profile) or choosing one or more representative site(s) in the image to generate a background value that is applied to multiple objects (Fig 29b).

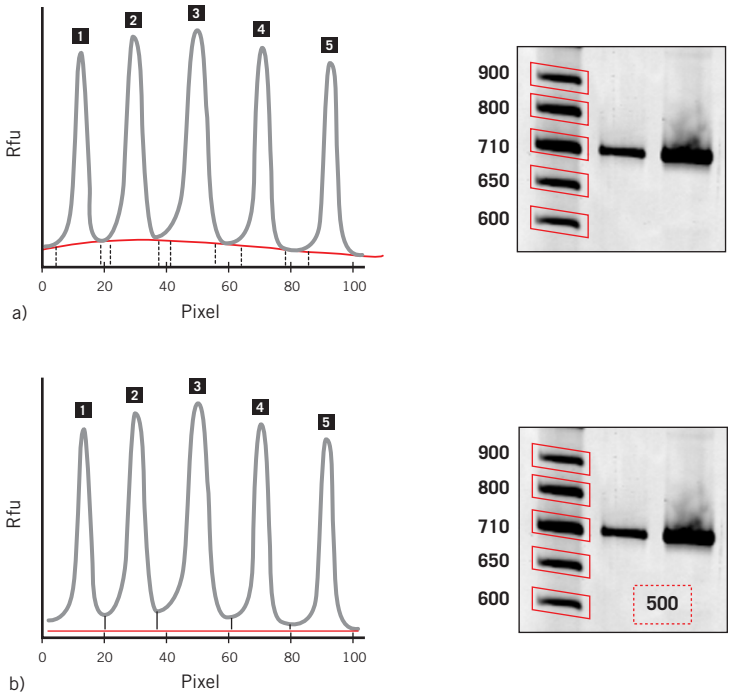


Fig 29. Comparison of local and global background correction methods applied to the same image. The local method (panel a) uses different background values at each band in the gel lane, with background based on the average signal from the boundary of each band. In panel b, a single global background value of 500 counts is applied to each band in the analysis.

The type of background pattern apparent in an image will suggest the method of background correction to apply. For example, if background signal is variable across the image, then a local method of correction may be appropriate because it can account for different background counts at each site where quantification is applied. Alternatively, one global background value for the whole image may be the best choice when background signal is uniform.

It is also important to select the most appropriate method for calculating the background value(s). The choice between an average and a median value for background calculation can significantly affect the results of quantification. For example, if high signal spikes are contributing to the background noise in an area of interest, calculation of an average background will be skewed on the high side. In this case, aberrant noise from the background calculation can be disregarded by using a median value.

The region of the image selected to represent the background signal is important for accurate quantification. In the same way, the boundaries used to define analysis targets—bands, spots, or slots—will also impact the results of quantification. If boundaries are too close to a particular band, the signal from that target will be under-represented. In contrast, a boundary that is set too far away from the target can overlap with other analyses, bringing unexpected and undesired signal into the analysis.

Image processing tools

Software utilities for image processing functions improve the accuracy of quantification for both single-colour and multicolour images.

Resolution of fluorescent signal overlap in multicolour images

Overlap between the emission spectra of fluorochromes is a common and almost unavoidable aspect of multicolour imaging. Even the best band pass filters cannot completely reject the emission from one fluorochrome when its emission spectrum overlaps that of other fluorochromes (Fig 20). When emissions from one fluorochrome contaminate the light collection for other fluorochromes in the sample, a process is needed to remove or reduce this cross-contamination for accurate quantification of each separated channel. Fluorochrome separation uses a mathematical transformation of the original images to create new images that more closely represent light emitted from the different fluorochromes used in the sample (Fig 30).

Because the original image files are left unchanged, the separation process can be undone and repeated using different settings to optimize the results. To enhance the quality of the image, software filters can also be used to eliminate variation in background without affecting target signal.

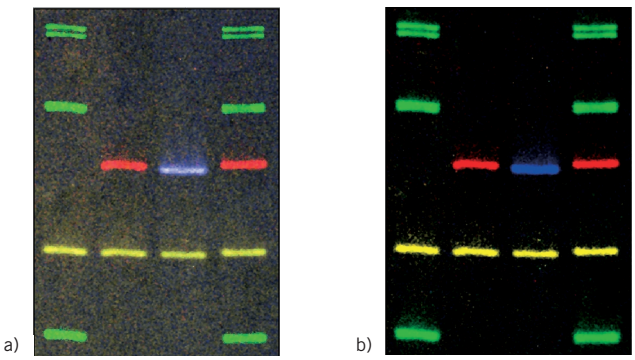


Fig 30. Multicolour image processing using a fluorochrome separation routine. Spectral contamination in this four-colour image, particularly evident in the blue and yellow channels (a), is reduced to give a better representation of the signal from each of the four fluorochrome labels (b).

Image filtering

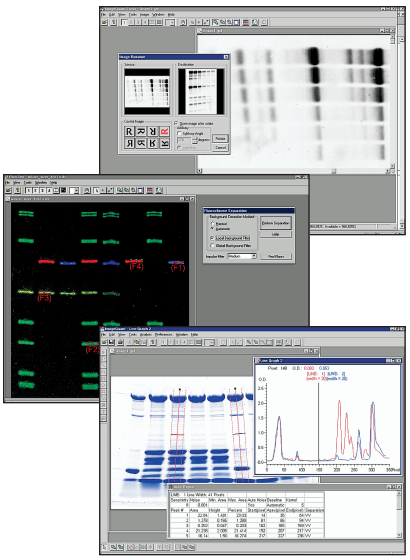
Image artefacts caused by dust or bubbles complicate fluorescence analysis of gels and membranes. Removing these artefacts can improve the quality and accuracy of image analysis by reducing background noise without affecting the integrity of the overall image.

A digital filter can reduce unusually intense or bright single pixel values, blending them more evenly into the surrounding image. For example, the single highest pixel value (noise spike) in a small group of contiguous pixels can be replaced with a lower value based on an assessment of the neighbouring pixel values. Because filtering alters the original data file, results from filtered images must be interpreted appropriately. On the other hand, intense fluorescent signal from dust and other contaminants can severely complicate analysis. For these reasons, the decision to filter, and therefore alter, an image prior to analysis must be carefully considered.

Amersham Pharmacia Biotech image analysis software

Image analysis is an integral part of today's life science applications. Amersham Pharmacia Biotech provides a comprehensive range of software products to address image analysis needs, from basic documentation and routine purity screens to the querying of entire gene expression or 2-D gel datasets (Table 2). Our image analysis software, combined with our wide range of fluorescence imaging instrumentation, deliver a complete system and a total solution to address a wide range of application needs.

Table 2. Amersham Pharmcia Biotech image analysis software



IMAGEQUANT™ SOLUTIONS

Powerful portfolio of software modules for 1-D gel and blot analysis

ImageQuant

- User-defined signal integration of regions (volume) or lane profiles and peak analysis (area)
- Support for up to four-channel images
- Text annotation of images and region-of-interest tool

Fragment analysis

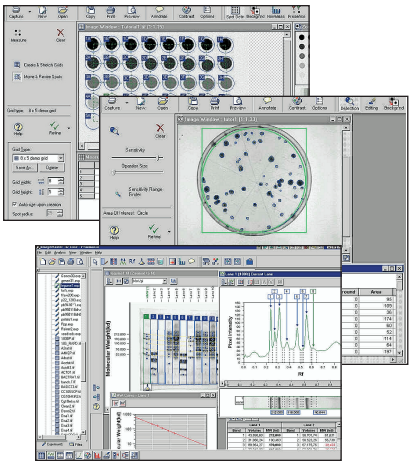
- Molecular weight, fragment size, and isoelectric point determination
- Analysis of two-channel images (with in-lane size standard)
- Assisted lane-finding and automated band-finding

FluorSep™

- Reduction of cross-contamination from multiple fluorochromes typically found in multichannel fluorescence images
- Support for two- to four-channel images

ImageQuant tools

- Image processing options for single and multichannel image files
- Signal inversion
- Noise filtration
- Image rotation

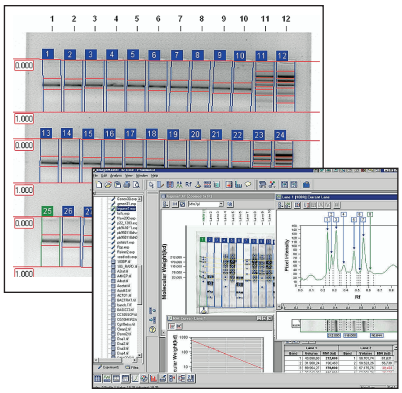


IMAGEMASTER TOTALLAB

Easy-to-use software for analysis of 1-D gels, dot and slot blots, and microplates

- Automatic lane identification and easy-to-use functions for background subtraction, band detection, and molecular weight determination
- Designed for quantitative needs in basic array analysis
- Automatic colony-counting facility
- Spot-detection algorithm
- Volume and area measurements

Table 2. ***(continued)***



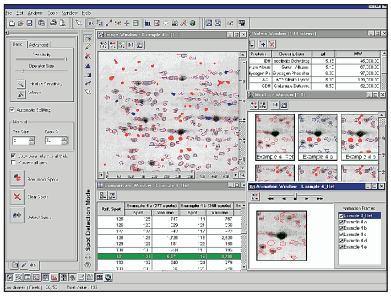
IMAGEMASTER 1D

Comprehensive software for 1-D gel image analysis

- Option of two modules: Prime (entry level) and Elite (power user level)
- Automated lane and band detection
- Account for distortion within and among gels
- Band-matching and lane-relationship studies (Elite module only)

Additional capabilities with database module

- Sample matching to user-built libraries
- A variety of clustering methods for dendrogram construction



IMAGEMASTER 2D

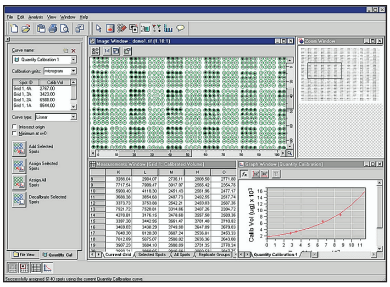
Premier tool for automated analysis of 2-D gels in proteomics

- Automated spot detection and measurement
- Batch processing of unlimited number of gels
- Grouping of multiple gel images into one experiment
- Gel averaging
- Multiple statistical tools
- Web site query
- Multiple reporting capabilities including Web page building

Additional capability with database module

- Data extraction queries
- Similar-spot queries and ratio queries to examine expression changes
- Statistical tests to help identify significant results and patterns

Table 2. ***(continued)***



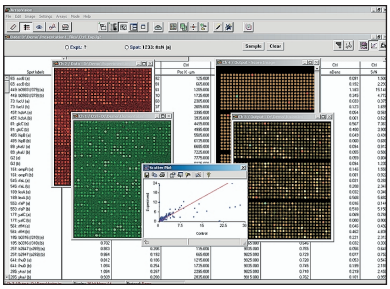
IMAGEMASTER ARRAY 2

Powerful facility for array analysis

- Automated grid production and alignment
- “Flagging” of spots
- Analysis templates
- Sample nomenclature import and automatic identification of replicate sets

Additional capabilities with database module

- Multiple array and/or ratio experiments
- Querying on experiments, arrays, and spots
- Combining a series of queries
- Identification of similar expression patterns
- Organization of data subsets into results sets



ARRAYVISION™

Premier analysis tool for array applications in medium- to high-throughput environments

- Automated template alignment and analysis
- Quality metrics and error "flagging"
- Up to three levels of template organization (spots, spot groups, and sub-arrays)
- Pre-configured and user-defined protocols
- Direct comparisons between images and Elemental Display to highlight key targets
- Batch-processing
- Wizard guides

Chapter 5

FLUORESCENCE APPLICATIONS USING AMERSHAM PHARMACIA BIOTECH IMAGING SYSTEMS

Introduction

This chapter provides the basic information necessary for maximizing the fluorescence imaging capabilities of your system. The application of fluorescence in standard molecular biology methods, such as gel electrophoresis, blotting, and solution analysis of nucleic acids and proteins, is discussed, and typical protocols for each application area are included, together with materials, suggestions, and tips for successful implementation of fluorescent detection. Available fluorescent stains, substrates, and covalent labels are described along with Amersham Pharmacia Biotech instrument compatibility and recommendations for imaging setup and analysis.

Detection of nucleic acids in gels

Nucleic acid gel stains

Fluorescent detection of nucleic acids in gels is used to visualize the results of DNA preparations, restriction digests, and PCR analyses, as well as other more specialized applications. Ethidium bromide is a popular fluorescent stain that is used for the routine detection of nucleic acids in gels. The dye binds by intercalating between the bases of nucleic acid molecules, and its fluorescence is detected by imaging the stained gel using UV or laser illumination.

More sensitive fluorescent stains, such as Vistra Green™ and SYBR™ Green, are available for nucleic acid applications requiring lower limits of detection in both agarose and polyacrylamide gel formats. These stains have a high affinity for their target nucleic acid and upon binding, their fluorescence and quantum yield are significantly enhanced. Because their background fluorescence is negligible in the absence of nucleic acids, gels

Table 3. Nucleic acid gel stains

Stain	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Application
Ethidium bromide	526	605	Red	Classic general purpose nucleic acid stain
SYBR Gold	495	537	Orange-green	Ultrasensitive gel stain for ss- or dsDNA or RNA
SYBR Green I	497	520	Green	Ultrasensitive gel stain for dsDNA and oligonucleotides
SYBR Green II	497	520	Green	Ultrasensitive gel stain for RNA and ssDNA
Vistra Green	495	520	Green	Ultrasensitive gel stain for dsDNA and oligonucleotides

Table 4. Instrument settings for use with nucleic acid gel stains.

Stain	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
Ethidium bromide	532	610BP30	514	610RG	NA*	Transmission	UV high†
SYBR Gold	532	526SP	488	530DF30	Blue	Transmission	UV low†
SYBR Green I	532	526SP	488	530DF30	Blue	Transmission	UV low
SYBR Green II	532	526SP	488	530DF30	Blue	Transmission	UV low
Vistra Green	532	526SP	488	530DF30	Blue	Transmission	UV low

* NA = Not applicable.

† UV high = 580BP30 filter; UV low = 520BP30 filter.

stained with these dyes require no destaining before imaging. Post-stain processing of nucleic acids, such as restriction digests and blot transfers, is possible as these stains do not interfere with these techniques. These stains also pose less of a health risk than ethidium bromide because they are less mutagenic. Table 3 lists different nucleic acid gel stains and the nucleic acids with which each is compatible.

Instrument compatibility

Fluorescence imaging systems from Amersham Pharmacia Biotech combine powerful excitation sources with efficient optics for sensitive fluorescence imaging of the common DNA gel stains, including ethidium bromide, Vistra Green, SYBR Green, and SYBR Gold (9, 10). Setup for the various instruments is given in Table 4.

Typical protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Hoefer™ EPS 301 Power Supply	18-1130-01
■ Nucleic acid gel stains	
Ethidium bromide solution, 10 mg/ml	17-1328-01
Vistra Green nucleic acid gel stain	RPN5786
■ Electrophoresis units	
Ready-To-Run™ Separation Unit	80-6460-95
Hoefer HE 99X Max Submarine Unit	80-6061-57
Hoefer miniVE Vertical Electrophoresis System	80-6418-77
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue
ImageMaster VDS-CL	see catalogue

❶

Sample preparation

Prepare agarose gels that are no thicker than 3 mm, if possible.

Mix the DNA samples with loading buffer.

Note: When imaging small size nucleic acids or proteins, avoid using bromophenol blue, xylene cyanol, and other electrophoresis tracking dyes because these dyes fluoresce and might mask the fluorescence of bands of interest on the gel. To avoid this problem, use a non-migrating dye, such as dextran blue, in the sample loading buffer. If it is necessary to monitor migration during electrophoresis, reduce the concentration of tracking dye to a minimum or load the tracking dye into a separate lane of the gel.

❷

Gel electrophoresis

Load the prepared samples into the wells.

Perform electrophoresis at 5 V/cm using the EPS 301 power supply.

❸

Gel staining

For Vistra Green or the SYBR stains, dilute the stains 1:10 000 in 1× TE (pH 7.5). For ethidium bromide, use a concentration of 0.25 µg/ml in 1× TE (pH 7.5).

Stain the gel in a polypropylene container for 30 min with gentle agitation (longer staining times may be needed for gels with high agarose content). Cover the staining container with aluminium foil to prevent photobleaching of the stains.

Gels attached to one of the electrophoresis plates: For Vistra and SYBR stains, pour enough staining solution on the gel to cover, and use a large pipette to distribute liquid.

If ethidium bromide was used, destain the gel for 30 min in water.

❹

Imaging

Wet gels: Place the wet gel directly onto the platen (Typhoon and Storm), glass tray (FluorImager), or platform (VDS-CL) of the imager in a small (just enough to create a film) amount of water. Avoid trapping air bubbles between the gel and the glass. For Typhoon imaging, choose “platen” for the focal depth setting. For thick agarose gels, it may be necessary to use the +3 mm focal depth setting. Acquire the image according to the recommended instrument set-up.

Gels attached to one of the electrophoresis plates: Place the glass plate directly onto the platen (Typhoon), in the extended universal holder tray (FluorImager), or platform (VDS-CL) of the imager. For optimal image quality on Typhoon, place Kapton™ tape (supplied with the Typhoon accessory kit) over each spacer on the outside of the long plate. Place water between the glass plate and Typhoon platen to minimize the appearance of interference patterns. Choose +3 mm for the focal height setting. For Storm, place the gel directly in contact with the platen.

In the Scanner Control Setup window, choose the appropriate laser and emission filter combinations (Table 4).

❺

Analysis

See Chapter 4 for information concerning image analysis.

Expected results

Typical results for the fluorescent detection of nucleic acids in agarose gels are given in Tables 5 and 6. Figure 31 shows the detection of DNA in an agarose gel stained with Vistra Green and imaged using Typhoon 8600.

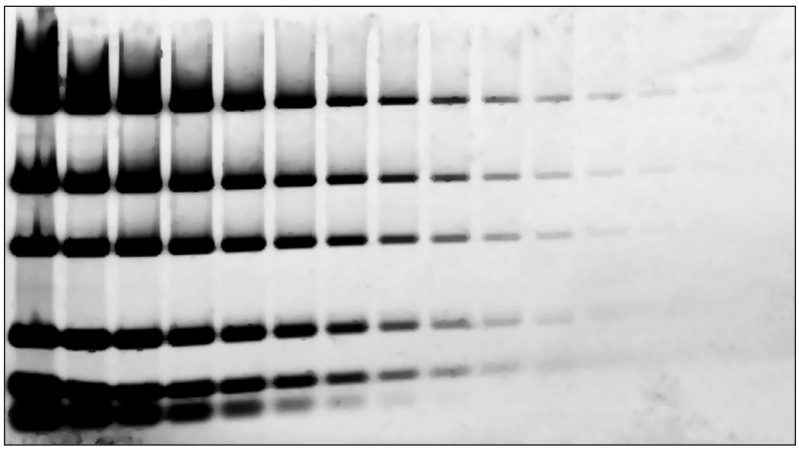


Fig 31. Detection of DNA in an agarose gel using Vistra Green and Typhoon 8600. Following electrophoresis, the gel was stained for 30 min. Amount of DNA ladder loaded per lane ranged from 120 000 pg–4 pg in two-fold serial dilutions.

Table 5. Fluorescent gel detection of double-stranded DNA*

Stain	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)
Ethidium bromide	100/ND†	500/ND	200/100	500/1000	NA†	NA	100/ND	300/ND
SYBR Gold	25/10	500/1000	40/10	500/1000	500/40	100/500	ND/20	ND/100
SYBR Green I	25/10	500/1000	40/10	500/1000	500/40	100/500	ND/20	ND/100
Vistra Green	25/10	500/1000	40/10	500/1000	500/40	100/500	ND/20	ND/100

* A dilution series of a DNA ladder was loaded onto a 1% agarose gel (3 mm) or a 10% polyacrylamide gel (1 mm). Results are expressed as limit of detection (LOD) and linear detection range (LDR) for agarose/polyacrylamide.

† ND = Not determined; NA = Not applicable.

Table 6. Fluorescent gel detection of single-stranded DNA and RNA*

Stain	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)
Ethidium bromide	5000/ND†	50/ND	10 000/ND	30/ND	NA†	NA	5000/ND	50/ND
SYBR Gold	ND/250	ND/200	ND/300	ND/150	ND/1000	ND	ND	ND
SYBR Green I	ND/250	ND/200	ND/300	ND/150	ND/1000	ND	ND	ND
SYBR Green II	10 000/ND	100/ND	10 000/ND	100/ND	100 000/ND	20/ND	ND	ND
Vistra Green	ND/250	ND/200	ND/300	ND/100	ND/1000	ND/50	ND	ND

* A dilution series of a DNA oligonucleotide or RNA ladder was separated on a formaldehyde-agarose gel or a denaturing polyacrylamide gel. Results are expressed as limit of detection (LOD) and linear detection range (LDR) for agarose/polyacrylamide.

† ND = Not determined; NA = Not applicable.

Detection of proteins in gels

Protein gel stains

Conventional colourimetric methods for visualizing proteins in gels include staining with Coomassie™ Brilliant Blue (CBB) or silver. CBB staining is commonly used even though it has low sensitivity and requires a long processing time and large volumes of organic solvents. Though more sensitive than CBB, staining with silver is expensive, labour-intensive, and exhibits protein-to-protein variation.

SYPRO™ protein gel stains (Table 7) are easy-to-use fluorescent stains with sensitivities equivalent to those of silver staining (11). After electrophoresis, the gel is simply stained, destained (optional), and then imaged. Because these stains bind to SDS-coated proteins in gels, they give more consistent staining between different types of proteins. In addition, their ability to detect proteins is not affected by the presence of contaminating nucleic acids or lipopolysaccharides. SYPRO protein gel stains can be used with both denaturing and native gels and do not interfere with upstream applications such as Western detection or microsequencing.

SYPRO Orange and Red stains are optimal for rapid and efficient fluorescent staining of one-dimensional protein gels. However, they require acetic acid fixation, which interferes with protein transfer to a membrane. For blotting techniques, SYPRO Tangerine is recommended because no acetic acid fixation is necessary. SYPRO Ruby protein gel stain provides sensitive fluorescent detection for both one- and two-dimensional protein gels and is compatible with subsequent mass spectrometry and Edman-based sequencing.

Table 7. Fluorescent protein gel stains

Stain	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Application
SYPRO Orange	300, 470	570	Orange	Routine SDS-PAGE
SYPRO Red	300, 550	630	Red	Routine SDS-PAGE
SYPRO Ruby	280, 450	610	Red	2-D gels, SDS-PAGE critical sensitivity
SYPRO Ruby IEF	280, 450	610	Red	Isoelectric focusing (IEF) gels
SYPRO Tangerine	300, 490	640	Red	SDS-PAGE followed by immunodetection or zymography

Instrument compatibility

Table 8. Instrument settings for use with protein gel stains

Stain	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
SYPRO Orange	532	580BP30	488	570DF30	Blue	Transmission	UV high
SYPRO Red	532	610BP30	514	610RG	Red	Transmission	UV high
SYPRO Ruby	532	610BP30	488	610RG	Blue	Transmission	UV high
SYPRO Ruby IEF	532	610BP30	488	610RG	Blue	Transmission	UV high
SYPRO Tangerine	532	610BP30	488	610RG	Blue	Transmission	UV high

Typical protocols

Protein detection in one-dimensional gels

One-dimensional gel electrophoresis is routinely used to study the size or molecular weight, amount, and purity of proteins. SDS polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by molecular weight, is an established tool for protein analysis. Its resolving power is useful for the routine sizing and quantification of proteins from both complex mixtures and purified fractions. The denaturing conditions used in SDS-PAGE cause the proteins to unfold, thus minimizing differences in their molecular shape and providing for more accurate molecular weight determination (12). Using appropriate gel systems, proteins can also be studied under non-denaturing or native conditions that preserve the higher order structure and even the biological function of some proteins. For example, native gel electrophoresis is required to preserve the structure, and therefore the intrinsic fluorescence, of green fluorescent protein (GFP).

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Hoefer EPS 301 Power Supply	18-1130-01
■ Hoefer miniVE Vertical Electrophoresis System	80-6418-77
■ Bovine serum albumin (BSA) protein standard	27-8915-01
■ Protein gel stains	
SYPRO Orange protein gel stain	RPN5801
SYPRO Red protein gel stain	RPN5803
SYPRO Tangerine protein gel stain	RPN5805
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue
ImageMaster VDS-CL	see catalogue

Other materials required

Product	Vendor
■ Protein samples prepared in appropriate loading buffer	
■ SYPRO Ruby protein gel stain	Molecular Probes, Inc.

❶

Sample preparation

For information on sample preparation, refer to Amersham Pharmacia Biotech Technical Manual *Protein Electrophoresis* (12).

❷

Gel electrophoresis

Load the prepared samples onto the gel. For denaturing conditions, use a gel and/or running buffer that contains 1% SDS. Refer to Amersham Pharmacia Biotech Technical Manual *Protein Electrophoresis* for further details (12).

❸

Staining the gel

For SYPRO Red or Orange, prepare a working stain solution by diluting the stain stock solution, as supplied, 1:5000 in a 7.5% acetic acid solution. Prepare enough stain solution to cover the gel (5–10 times the gel volume).

For SYPRO Tangerine, prepare a working stain solution by diluting the stain stock solution, as supplied, 1:5000 in 50 mM phosphate, 150 mM NaCl, pH 7.0.

For SYPRO Ruby, use the stain stock solution, as supplied, directly without dilution.

Note: For larger gels, prepare approximately 10 times the gel volume for staining, in order to avoid a loss of sensitivity.

Stain the gel in a polypropylene container with gentle agitation for 30 min (longer staining times may be needed for high percentage acrylamide gels). SYPRO stains are not compatible with glass or metal staining trays. Cover the staining container with aluminium foil to prevent photobleaching of the stains.

For SYPRO Red or Orange, destain the gel in a 7.5% acetic acid solution for 5–15 min. Longer destaining may result in a loss of sensitivity. For SYPRO Ruby, destain the gel for 30 min in deionized water.

❹

Imaging

Place the wet gel directly onto the platen (Typhoon and Storm), glass tray (FluorImager), or platform (VDS-CL) of the imager in a small amount of water. Avoid trapping air bubbles between the gel and the glass.

In the Scanner Control Setup window, choose the appropriate laser and emission filter combinations (Table 8). For Typhoon imaging, choose "platen" for the focal depth setting. Acquire the image according to the recommended instrument set-up.

❺

Analysis

See Chapter 4 for information concerning image analysis.

Protein detection in two-dimensional gels

Two-dimensional gel electrophoresis is used to analyse complex mixtures of proteins through the combined resolving power of two electrophoretic methods (13). In the first dimension, proteins are resolved according to their isoelectric points by isoelectric focusing (IEF). The isoelectric point of each protein relates to the pH at which the net charge of the molecule is zero. Following IEF of the proteins, SDS-PAGE is used as the second dimension to further resolve the proteins according to their molecular weights. The result is a complex pattern of spots corresponding to the many different protein molecules present in the original sample. Fluorescent gel stains have been developed and optimized for sensitive detection of proteins resolved in this format.

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Immobiline™ DryStrip pH 4–7, 18 cm	17-1233-01
■ IPG Buffer pH 4–7	17-6000-86
■ IPGphor™ IEF System	80-6414-02
■ Ettan™ DALT II Large Vertical System	see catalogue
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue
ImageMaster VDS-CL	see catalogue

Other materials required

Product	Vendor
■ Protein samples prepared in appropriate loading buffer	
■ SYPRO Ruby protein gel stain	Molecular Probes, Inc.

❶ Sample preparation

Note: The following is a general procedure for analysis of *E. coli* proteins. For additional information refer to Amersham Pharmacia Biotech Technical Manual *2-D Electrophoresis using Immobilized pH Gradients* (13).

Suspend 400 mg of lyophilized *E. coli* in 10 ml of 8 M urea, 4% (w/v) CHAPS, 20 mM triethanolamine-Cl (pH 8.0), 20 mM dithiothreitol (DTT), 1 mM PMSF. Sonicate the suspension for a few seconds per burst, chill on ice between bursts. Repeat until maximum clarification is observed.

Precipitate the sonicate overnight at –40 °C with 80 ml acetone, 10 ml 100% (w/v) trichloroacetic acid, 1 ml 2-mercaptoethanol.

Collect the precipitate by centrifugation at 105 000 × g for 20 min. Wash the pellet with the same volume of 80% (v/v) acetone, 1% (v/v) 2-mercaptoethanol and leave in the freezer for a few hours.

Collect the precipitate as before and discard the supernatant. Air-dry the pellet and resuspend in 10 ml of 8 M urea, 2% (w/v) CHAPS with sonication to aid solubilization.

Clarify the extract by centrifugation at 105 000 × g for 30 min.

❷ Gel electrophoresis

Prepare Immobiline Drystrip gels, 18 cm, pH 4–7 and IPGphor system according to manufacturer's instructions.

Dilute protein extract (100 µg of total protein in 350 µl) with rehydration solution (8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 2% (v/v) pH 4-7 IPG buffer, trace of bromophenol blue).

Load proteins using rehydration loading for 12 h at 20 °C. Separate samples using the following running conditions: 500 V for 500 Vhr, 1000 V for 1000 Vhr, 8000 V for 60 000 Vhr.

After the first-dimension separation is complete, equilibrate each strip, first with SDS equilibration solution containing 1 % (w/v) DTT for 15 min, then with 2.5% (w/v) iodoacetamide for 15 min.

Load the equilibrated strips onto a 1 mm, 12.5% Laemmli gel cast for the Ettan DALT II system (13).

Run the two-dimensional gel at 5 W/gel for 45 min and then at 26.67 W/gel until the bromophenol blue dye front runs off the gel.

❸

Gel staining

Incubate the gel in 40% ethanol, 10% acetic acid for 30–60 min, then incubate overnight at room temperature with gentle agitation in 5–10 volumes (250–500 ml per Ettan DALT II gel) of SYPRO Ruby staining solution.

Wash the gel with 2–4 changes of deionized water for approximately 2 h, and then with 10% methanol and 7% acetic acid for at least 15 min. The gel may be stored in the latter solution.

❹

Imaging

Place the wet gel directly onto the platen (Typhoon and Storm), glass tray (FluorImager), or platform (VDS-CL) of the imager in a small amount of water. Avoid trapping air bubbles between the gel and the glass.

In the Scanner Control Setup window, choose the appropriate laser and emission filter combinations (Table 8). For Typhoon imaging, choose "platen" for the focal depth setting. Acquire the image according to the recommended instrument set-up.

❺

Analysis

Analyse the image using ImageMaster 2-D software.

Expected results

The expected limits of detection (LOD) and linear detection ranges (LDR) for protein quantification in gels are given in Table 9. Images from a one-dimensional SDS-PAGE and a two-dimensional protein separation are shown in Figure 32 and Figure 33, respectively.

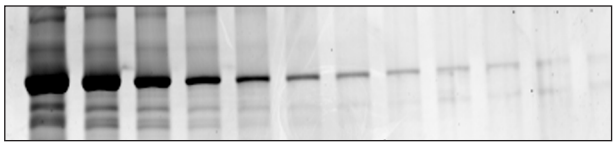


Fig 32. Proteins in an SDS-PAGE gel were stained with SYPRO Orange and imaged using Typhoon 8600. Amount of BSA per lane ranged from 1630 ng to 0.8 ng, prepared in two-fold serial dilutions.

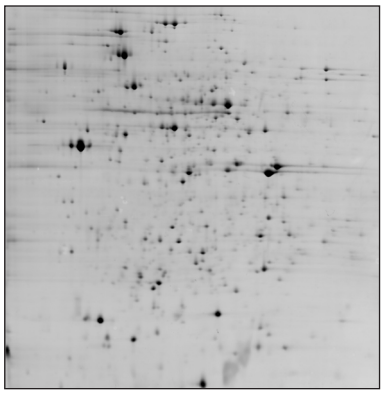


Fig 33. *E. coli* proteins in a 2-D SDS-PAGE gel were stained with SYPRO Ruby and imaged using Typhoon 8600.

Table 9. Fluorescent gel detection of protein*

Stain	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (ng/band)	LDR (~ fold)	LOD (ng/band)	LDR (~ fold)	LOD (ng/band)	LDR (~ fold)	LOD (ng/band)	LDR (~ fold)
SYPRO Orange	2	1000	3	500	6	250	5	200
SYPRO Red	2	1000	2	500	3	250	ND†	ND
SYPRO Ruby	3	500	5	ND	7	ND	3	200

* A dilution series of BSA was loaded onto a one-dimensional polyacrylamide gel (1 mm thick with 4% stacking gel and 10% resolving gel) and electrophoresed using Hoefer miniVE System. Results are expressed as limit of detection (LOD) and linear detection range (LDR).

† ND = Not determined.

Quantification of nucleic acids in solution

Dyes for quantification of nucleic acids in solution

The concentration of DNA or RNA in solution is conventionally determined by measuring the absorbance of the solution at 260 nm and 280 nm. The accuracy of this method, however, is significantly affected by the presence of free nucleotides, DNA or RNA, and contaminants from the nucleic acid preparations. Nucleic acids are more accurately quantified in solution using fluorescent dyes that bind with very high specificity and sensitivity (Table 10). When bound to their target molecules (DNA or RNA), the fluorescence of these dyes is greatly enhanced.

Whereas the sensitivity of non-fluorescence microplate-based methods is typically in the µg/ml range, fluorescence-based methods can detect nucleic acids at concentrations in the ng/ml range. In assays using the fluorescent dye, PicoGreen™, double-stranded DNA can be measured in solution at concentrations as low as 2.5 ng/ml. The linear detection range of this assay is typically 70–1400-fold, depending on which imaging instrument is used. (See Table 12.)

The fluorescent detection of nucleic acids in solution can be achieved using PicoGreen for double-stranded DNA, OliGreen™ for single-stranded DNA and oligonucleotides, and RiboGreen™ and SYBR Green II for RNA. However, it is recommended that RNA samples be treated with DNase to remove any DNA contamination, as no dye is yet available that exhibits fluorescence enhancement specifically by binding to RNA.

Table 10. Fluorescent dyes for the quantification of nucleic acids in solution

Dye	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Application
OliGreen	500	523	Green	Quantification of ssDNA and oligonucleotides
PicoGreen	502	523	Green	Quantification of dsDNA
RiboGreen	500	525	Green	Quantification of RNA

Instrument compatibility

Table 11. Instrument settings for use with nucleic acid dyes

Dye	Typhoon*		FluorImager		Storm
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode
OliGreen	532	526SP	488	530DF30	Blue
PicoGreen	532	526SP	488	530DF30	Blue
RiboGreen	532	526SP	488	530DF30	Blue

* A +3-mm focal depth setting should be used on Typhoon when imaging microplates.

Typical protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue

Other materials required

Product	Vendor
■ PicoGreen nucleic acid stain	Molecular Probes, Inc.
■ Clear (polystyrene) 96 well microplate*	Corning Costar Corp.

* Suitable clear, flat-bottomed, low-fluorescence microplates should be used. Image quality and quantification for Storm and Typhoon are improved when using Nalge-Nunc PolySorp™ 96-well plates with removable strips so that the wells sit flat directly on the platen.

❶

Sample preparation

Using TE buffer, dilute the DNA sample solution to a final volume of at least 50 µl for FluorImager and Storm or 80 µl for Typhoon.

Note: Using a higher dilution of the experimental sample ensures that any contaminants are maximally diluted. Each microplate well requires a minimum total volume of 100 µl for FluorImager and Storm or 160 µl for Typhoon.

Note: The performance of PicoGreen is minimally affected by the presence of contaminants such as salts, urea, ethanol, chloroform, detergents, proteins, and agarose. For additional information, see the manufacturer's literature.

❷

Staining the samples

Prepare sufficient working solution of the PicoGreen reagent by diluting the stock solution, as supplied, 1:200 using TE buffer.

Note: The PicoGreen working solution should be prepared in a plastic container on the day of the experiment. Glass should not be used because PicoGreen may adsorb to glass surfaces.

Pipette the PicoGreen working solution into each well of the microplate, using at least 50 µl for FluorImager and Storm or 80 µl for Typhoon.

Add an equal volume of the experimental DNA solution from step 1.1 to each well and mix thoroughly by pipetting.

Incubate 2–5 min at room temperature.

❸

Imaging

Place the microplate into the microplate tray (FluorImager) or directly onto the platen (Storm and Typhoon).

Acquire the image according to the recommended instrument setup for the fluorochrome used. Select 200 micron pixel size setting. The PMT voltage setting should be adjusted to prevent signal saturation. For Typhoon, the +3-mm focal plane setting should always be selected for imaging microplates.

❹

Analysis

Display the image using ImageQuant. If saturated pixels are present, the microplate should be rescanned at a lower PMT voltage setting. Use the Gray/Color Adjust function to adjust the image contrast. Ellipse objects can be used to quantify integrated signal from the microplate wells.

Draw an ellipse object within the inner walls of one well and copy it to the other wells.

Report the median values with background correction set to "None".

In Microsoft Excel™, subtract the median value of the negative control well from each of the other wells. This is important for good low-end linearity.

Generate a standard curve from the DNA standards used.

> **Note:** For the greatest accuracy, the DNA standards should be similar to the unknown DNA (i.e. similar size and source).

Determine the unknown DNA concentration by extrapolating from the standard curve.

Expected results

The limits of detection and linear detection ranges for quantification of DNA in solution are given in Table 12. Figure 34 is an image from a PicoGreen microplate assay detected using Typhoon 8600.

Fig 34. Detection of DNA in solution using PicoGreen and Typhoon 8600. Lambda DNA was used at concentrations of 3,500 ng/ml, 429 ng/ml, 150 ng/ml, 52.5 ng/ml, 18.4 ng/ml, 6.4 ng/ml, 2.25 ng/ml, and 0.79 ng/ml.

Table 12. Fluorescence-based quantification of DNA in solution*

Dye	Typhoon		FluorImager		Storm	
	LOD (ng/ml)	LDR (~ fold)	LOD (ng/ml)	LDR (~ fold)	LOD (ng/ml)	LDR (~ fold)
PicoGreen	10/ 2.5†	350/ 1400	5	700	50	70
RiboGreen	ND‡	ND	1	1000	10	100

* A dilution series of lambda phage DNA prepared in 1× TE was used for the analysis. Results are expressed as limit of detection (LOD) and linear detection range (LDR).

† First number from assay performed using Costar flat-bottomed plate/Second number from assay performed using Nunc Separable Strips.

‡ ND = Not determined.

Quantification of proteins in solution

Dyes for quantification of proteins in solution

Protein concentration in solution can be determined directly by measuring the absorbance of the solution at 280 nm, or indirectly by using colourimetric assays. Both methods, however, have some limitations. For example, the sensitivity of the absorbance method is limited because detection depends on the number of aromatic amino acid residues present. Colourimetric methods, such as the Bradford and Lowry assays, do not work well in the presence of contaminants and must be read within a very limited period of time (14). High protein-to-protein signal variability is also common with colourimetric detection.

Proteins can be more accurately detected in solution using fluorescent dyes (Table 13). As free molecules, the dyes are not very fluorescent, but when they bind to proteins, they exhibit enhanced fluorescence. Because they are typically quite specific for their target molecules, these fluorochromes work well even in the presence of various contaminants. For example, the dye NanoOrange™ binds specifically to the detergent coating on proteins and to hydrophobic regions of proteins and is not affected by the presence of contaminating nucleic acids or reducing agents.

Protein detection is much more sensitive using fluorescence. Whereas the sensitivity of non-fluorescence microplate-based detection methods is typically in the µg/ml range, fluorescence-based detection is generally in the ng/ml range. For example, with NanoOrange, protein can be measured in solution at concentrations as low as 300 ng/ml. The linear detection range of this assay is typically 10–30-fold.

Table 13. Fluorescent dyes for the quantification of proteins in solution

Dye	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Application
CBQCA	465	550	Orange	Protein quantification based on the number of primary amines
NanoOrange	470	570	Orange	Total protein quantification

Instrument compatibility

Table 14. Instrument settings for use with protein solution dyes

Dye	Typhoon*		FluorImager		Storm
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode
CBQCA	532	580BP30	488	570DF30	Blue
NanoOrange	532	580BP30	488	570DF30	Blue

* A +3-mm focal depth setting should be used on Typhoon when imaging microplates.

Typical protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue

Other materials required

Product	Vendor
NanoOrange protein quantification kit	Molecular Probes, Inc.
Clear (polystyrene) 96-well microplate*	Corning Costar Corp.
Protein standards	

* Suitable clear, flat-bottomed, low-fluorescence microplates should be used. The recommended microplate is manufactured by Corning Costar. Image quality and quantification for Storm and Typhoon are improved when using Nalge-Nunc 96-well plates with removable strips so that the wells sit flat directly on the platen.

❶

Working stain preparation

Prepare sufficient working solution of the NanoOrange reagent by diluting the stock solution 1:500 using the 1× diluent prepared according to manufacturer's instructions.

Note: the NanoOrange working solution should be protected from light to prevent photodegradation and should be used within a few hours of its preparation.

❷

Sample staining

Using the NanoOrange working solution from the previous step, dilute the protein sample solution in microcentrifuge tubes to a final volume of at least 100 µl for FluorImager and Storm or 160 µl for Typhoon.

Note: Using a higher dilution of the experimental sample ensures that any contaminants are maximally diluted.

Note: NanoOrange is minimally affected by the presence of salts, urea, detergents, DNA, and amino acids (see manufacturer's literature).

Heat the sample at 90–96 °C for 10 min. Cool to room temperature.

Pipette the samples into the microplate wells.

❸

Imaging

Place the microplate in the microplate tray (FluorImager) or directly onto the platen (Storm and Typhoon).

Acquire the image according to the recommended instrument set-up for the fluorochrome used. The choice of pixel size will depend on the individual experiment. The PMT voltage setting should be adjusted to prevent signal saturation. For Typhoon, the +3-mm focal plane setting should always be selected for imaging microplates.

❹

Analysis

Display the image using ImageQuant. If saturated pixels are present, the microplate should be rescanned at a lower PMT voltage setting. Use the Gray/Color Adjust function to adjust image contrast. Ellipse objects can be used to quantify integrated signal from the microplate wells.

Draw an ellipse object within the inner walls of one well and copy it to the other wells.

Report the median values with background correction set to "None".

In Microsoft Excel, subtract the median value of the negative control well from each of the other wells. This is important for good low-end linearity.

Generate a standard curve from the protein standards used.

Note: For the greatest accuracy, the protein standards should be similar to the unknown protein (i.e. similar size and source).

Determine the unknown protein concentration by extrapolating from the standard curve.

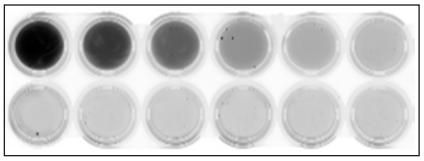


Fig 35. Detection of protein in solution using NanoOrange and Typhoon 8600 with Nunc microplate strips. BSA was used at concentrations of (starting at the top left well) 10, 6, 3, 1, 0.6, 0.3, 0.1, 0.06, 0.03, and 0.01 g/ml. The last two wells in the bottom row contained negative controls, which was NanoOrange working solution only.

Expected results

The expected limits of detection and linear ranges for protein quantification in solution are given in Table 15. Quantification of a BSA solution using NanoOrange and Typhoon 8600 is shown in Figure 35.

Table 15. Fluorescence-based quantification of protein in solution using NanoOrange*

Dye	Typhoon		FluorImager		Storm	
	LOD (µg/ml)	LDR (~ fold)	LOD (µg/ml)	LDR (~ fold)	LOD (µg/ml)	LDR (~ fold)
NanoOrange	1/0.3†	10/30	0.5	20	1	10

* BSA diluted in 1× TE was used used for analysis. Results are expressed as limit of detection (LOD) and linear detection range (LDR).

† First number from assay performed using Costar flat-bottomed plate/Second number from assay performed using Nunc Separable Strips.

Southern and Northern blotting

The transfer of DNA from an electrophoresis gel to a membrane is termed a Southern transfer or blot. In this technique, a complex mixture—usually genomic DNA—is probed to detect individual target DNA molecules. Similarly, in a Northern blot, RNA—either mRNA or total cellular RNA—is transferred from a gel to a membrane and probed for the presence of specific mRNA transcripts. Both methods permit the sensitive measurement of nucleic acid size and quantity. In traditional Southern and Northern procedures, probes are labelled with radioactive isotopes (e.g. ^{32}P) for detection. With radioactivity, however, safety issues must be considered. In contrast, non-radioactive detection methods, such as fluorescence, provide a safe alternative and deliver comparable sensitivity. Additionally, unlike radioactively labelled probes, fluorescent probes are stable for long periods.

Fluorogenic substrates for Southern and Northern detection

Fluorescent Southern and Northern detection chemistries employ enzyme-amplified detection schemes using alkaline phosphatase (AP) enzyme (15). Enzymatic turnover of a fluorogenic substrate gives the highest sensitivity because each enzyme molecule produces multiple fluorescent products. ECF reagent and DDAO phosphate are fluorogenic substrates commonly used with the AP enzyme and suitable for Southern and Northern detection. Their spectral characteristics are shown in Table 16.

Table 16. Fluorogenic substrates for Southern and Northern blots

Substrate	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Enzyme
DDAO phosphate	646	660	Red	Alkaline phosphatase
ECF	440	560	Green	Alkaline phosphatase

The direct detection of a fluorescently labelled nucleic acid probe on a membrane usually does not provide adequate sensitivity for Southern or Northern analysis. Consequently, most non-radioactive Southern and Northern detection schemes use a hapten to label the nucleic acid probe. The hapten (i.e. fluorescein, digoxigenin, or biotin) provides a target recognized by an antibody or other binding molecule that is conjugated to an enzyme. Signal amplification results from the conversion of multiple substrate molecules to fluorescent products by each enzyme. An indirect detection scheme in which fluorescein is used as a hapten is illustrated in Figure 36. With some kits, the probe can be directly labelled with a thermostable enzyme (e.g. AlkPhos Direct™ systems). Because this system bypasses the hapten detection step, the signal development process is much faster.

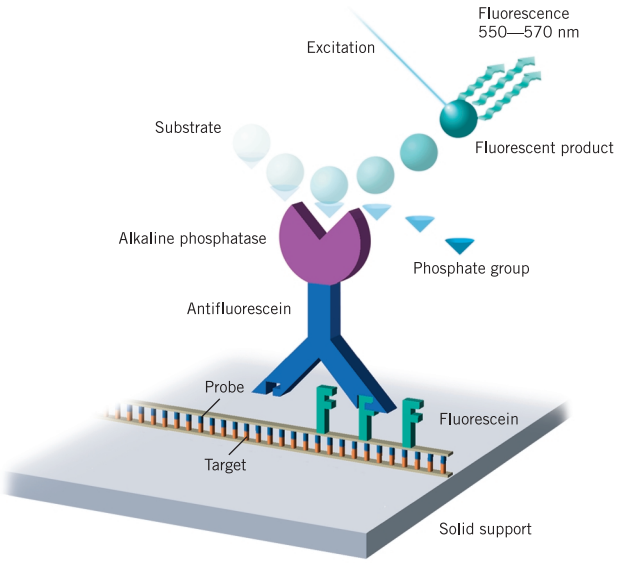


Fig 36. Schematic showing the indirect detection of a fluorescein-labelled DNA probe in a Southern blot. The ECF Signal Amplification Module boosts sensitivity by coupling alkaline phosphatase to the fluorescein-labelled DNA probe. Alkaline phosphatase catalyses the formation of stable fluorophores that remain near the probe and emit light when detected using fluorescence imaging systems.

Instrument compatibility

Table 17. Compatibility of selected fluorogenic substrates with fluorescence imaging systems

Substrate	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
DDAO phosphate	633	670BP30	NA*	NA	Red	NA	NA
ECF	532	526SP	488	570DF30	Blue	Reflection	UV high

* NA = Not applicable

Typical protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Hoefer HE 99X Max Submarine Unit	80-6061-57
■ Hoefer EPS 301 Power Supply	18-1130-01
■ Nucleic acid gel stains	
Ethidium bromide solution, 10 mg/ml	17-1328-01
Vistra Green nucleic acid gel stain	RPN5786
■ Hybond™-N+ membranes	see catalogue
■ ECF Random-Prime Labelling and Detection System	RPN5752
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue
ImageMaster VDS-CL	see catalogue

Other materials required

Product	Vendor
DDAO phosphate	Molecular Probes, Inc.

❶

Preliminary preparations and general handling instructions

- Prepare the probe according to the instructions or directions provided with the labelling kit.
- Successful fluorescent detection protocols require that background be carefully controlled. Special attention to cleanliness is required with alkaline phosphatase-based detection.
- Block the membrane thoroughly by incubating in blocking buffer with agitation on an orbital shaker. Use at least the minimum suggested volume of buffer for washing steps.
- Always wear powder-free gloves when handling membranes, solutions, and dishes used for washing.
- Adjust the hybridization or stringency wash temperature, or add more washes if necessary. For other factors that may affect the quality of detection, refer to the troubleshooting guide included with the labelling and detection kit.

❷

Preparation of blot

Southern blots

Separate the DNA samples in a neutral agarose gel, then depurinate, denature, and neutralize the gel according to standard procedures (14).

Transfer the samples to a Hybond-N+ nylon transfer membrane.

Process the Southern blot through hybridization, stringency washes, and detection of the fluorescein hapten.

Northern blots

Separate denatured RNA (prepared in a glyoxal buffer) in an agarose gel prepared in 1× MOPS buffer (14).

Transfer the samples to a Hybond-N+ nylon transfer membrane.

Process the Northern blot through hybridization, stringency washes, and detection of the fluorescein hapten.

❸

Application of substrate

ECF substrate

Prepare ECF substrate as directed.

After the final washing step, position the wet blot (sample-side up) in an open low-fluorescence bag or page protector.

Add the prepared substrate to the blot so that it is coated completely and evenly.

Cover the blot with the top sheet of the bag or page protector, squeeze out excess substrate, and incubate for up to 24 h. Make sure the blot is kept wet during the development process.

Note: Signal development can be monitored by periodic imaging.

DDAO phosphate substrate

To prepare the stock solution, dissolve the DDAO phosphate in water at a concentration of 1.25 mg/ml.

Dilute the DDAO phosphate stock 1:1000 in 10 mM Tris-Cl (pH 9.5), 1 mM $MgCl_2$.

Add 5 ml of substrate per cm^2 of membrane, covering it evenly, and incubate for 4 h.

❹

Imaging

Place the covered developed blot face down onto the glass platen (Storm, Typhoon) or glass tray (FluorImager), or face up on the platform of VDS-CL.

Note: Water can be used between the plastic bag and the platform to minimize the occurrence of interference patterns in the image.

Use a glass plate to hold the blot flat during imaging (optional).

Acquire the image according to the recommended instrument setup. The choice of pixel size and PMT voltage settings will depend on the individual experiment. Reduce the PMT voltage setting or signal integration time (for VDS-CL) to prevent signal saturation.

❺

Analysis

See Chapter 4 for information concerning image analysis.

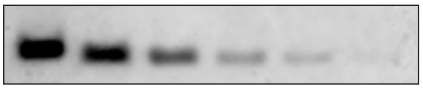


Fig 37. Southern blot of *Eco*R I digested human genomic DNA. β-actin cDNA was labelled and detected with ECF Random-Prime Labelling and Detection System and imaged on Typhoon 8600. Amount of DNA per lane ranged from 10.4 µg to 0.32 µg, prepared in two-fold serial dilutions.

Expected results

Typical results from a fluorescent Southern blot of a single-copy human gene acquired using the Typhoon 8600 scanner are shown in Figure 37. Results that can be expected for other systems and substrates are given in Table 18.

Table 18. Fluorescence-based quantification of DNA in genomic Southern blots*

Substrate	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)	LOD (pg/band)	LDR (~ fold)
DDAO phosphate	0.25	50	NA	NA	0.25	50	NA	NA
ECF	0.5	25	0.25	50	0.25	50	0.25	ND

* Results are expressed as limit of detection (LOD) and linear detection range (LDR). LOD values are given as amount of target detected.

† NA = Not applicable.

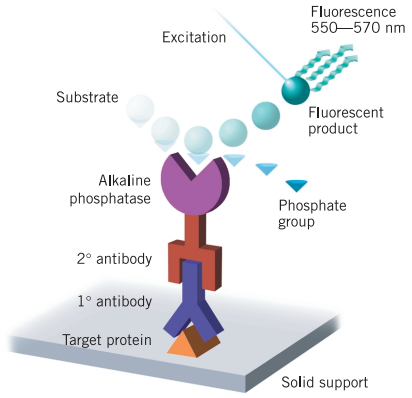


Fig 38. Schematic of the ECF Western Blotting Kit. Proteins are detected by chemifluorescence using alkaline phosphatase-labelled anti-species secondary antibody. Signal is developed with the ECF substrate.

Western blotting

Immunodetection of proteins that have been electrophoretically separated and then immobilized on a membrane is traditionally accomplished using isotope-labelled antibodies (e.g. ^{125}I). However, non-radioactive alternatives, including chemiluminescent and fluorescent detection chemistries, are now widely accepted and much preferred as the result of their safety, sensitivity, and convenience.

Fluorescent Western detection employs either a direct or enzyme-amplified format. The greatest sensitivity is achieved using fluorogenic substrates in an enzyme-amplified format with horseradish peroxidase or alkaline phosphatase (16, 17). However, direct fluorescent detection, using labels such as fluorescein, Cy3, and Cy5 conjugated to antibodies, is simpler and provides more accurate quantification (16). Fluorescent stains optimized for protein blot detection can be used for the rapid and sensitive assessment of Western transfer efficiency.

Western detection strategies

Enzyme-amplified detection (chemifluorescence)

The most common Western detection chemistries employ enzyme-amplified detection schemes using either horseradish peroxidase (HRP) or alkaline phosphatase (AP) (Fig 38). Fluorogenic substrates that are available for use with these enzymes are listed in Table 19. While the sensitivity of chemifluorescence-based Westerns is comparable to that of chemiluminescence, quantification is improved when compared with film detection (Fig 39).

Table 19. Fluorogenic substrates for Western blots

Substrate	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Enzyme
DDAO phosphate	646	660	Red	Alkaline phosphatase
ECF	440	560	Green	Alkaline phosphatase
ECL Plus™	430	503	Blue	Horseradish peroxidase

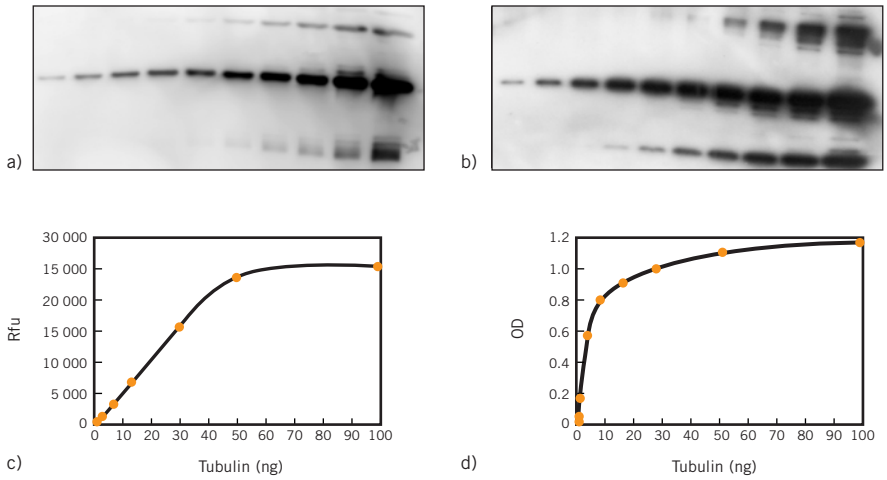


Fig 39. Limits of detection for chemifluorescence (a) and chemiluminescence (b).

A dilution series of purified tubulin was prepared in duplicate, separated by SDS-PAGE, and transferred to two PVDF membranes. Blots were incubated with mouse-anti-tubulin monoclonal primary antibody, followed by incubation with secondary antibody. For chemifluorescent detection, one blot (a) was incubated with goat-anti-mouse-IgG-alkaline phosphatase, followed by ECF substrate. The blot was imaged using FluorImager with a PMT setting of 500 V. For chemiluminescent detection, the other blot (b) was incubated with sheep-anti-mouse-IgG-horseradish peroxidase and then developed using ECL Western Blotting Kit. The blot was exposed to Hyperfilm ECL for 5 min. The developed film was scanned using Personal Densitometer™ SI. For both blots the dilution series, from left to right, was 1.6, 3.12, 6.25, 12.5, 25, 50, 100, 200, 300, and 400 ng of tubulin.
The signals for 1.6–100 ng were quantified using ImageQuant Software. The average signals in relative fluorescence units (rfu) for chemifluorescence (c) and optical density (OD) for chemiluminescence (d) were plotted against the amount of tubulin loaded.

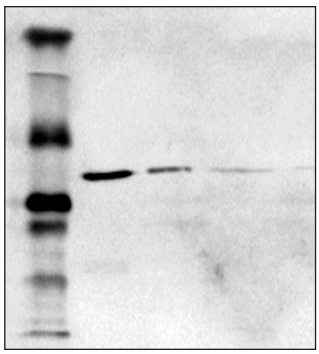


Fig 40. Example of a direct fluorescent Western blot developed using secondary antibody conjugated to Cy5. A purified recombinant protein was resolved at 120 ng, 60 ng, 30 ng, and 15 ng. The blot was imaged using Storm 860. The far left lane contains Full-Range Rainbow™ Molecular Weight Markers.

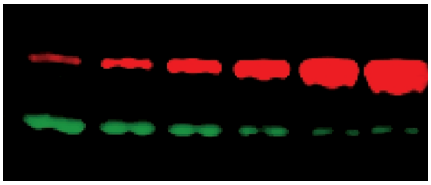


Fig 41. A dual-target Western blot showing detection of actin and tubulin. Proteins were serially diluted two-fold and resolved by gel electrophoresis. Tubulin (red) was detected using anti-β-tubulin monoclonal antibody and Cy5 linked anti-mouse IgG.

Amounts of tubulin from left to right were 31 ng, 62 ng, 125 ng, 250 ng, 500 ng and 1000 ng. Actin (green) was detected with rabbit anti-actin antibody and Cy3 linked anti-rabbit IgG. Amounts of actin from left to right were 640 ng, 320 ng, 160 ng, 80 ng, 40 ng, and 20 ng.

Direct fluorescent detection

Direct fluorescent detection of Westerns (Fig 40) is an alternative to enzyme-amplified fluorescence. Because the secondary antibody is conjugated directly with a fluorochrome, there is no need for substrate development steps. Though simpler than the enzyme method, direct fluorescent detection is less sensitive because there is no signal amplification. However, it is easier to quantify, and by using combinations of fluorochromes and/or fluorogenic substrates, it is possible to detect more than one target on the same Western blot (Fig 41). The development of direct fluorescent detection schemes is also facilitated by the wide availability of secondary (anti-species) antibodies conjugated to a variety of different fluorochromes, such as fluorescein and the CyDye™ and Alexa Fluor™ series (see Appendix 3 for a list of multipurpose labels).

Total protein stains for Western blots

Blot stains facilitate the direct comparison of total and target protein from the same blot, thus eliminating uncertainty associated with the transfer efficiency. Fluorescent blot stains are more sensitive than common colourimetric stains, such as Ponceau S, amido black, or Coomassie Brilliant Blue. Properties of commonly used fluorescent blot stains are summarized in Table 20.

Table 20. Properties of fluorescent blot stains

Stains	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Application
SYPRO Rose Plus	~ 350	610	Red	Blot stain (PVDF or nitrocellulose)
SYPRO Ruby blot	280, 450	618	Red	Blot stain (PVDF or nitrocellulose)

Instrument compatibility

Table 21. Instrument settings for fluorescent detection of Western blots

Substrates

Substrate	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
DDAO phosphate	633	670BP30	NA*	NA	Red	NA	NA
ECF	532	526SP	488	570DF30	Blue	Reflection	UV high
ECL Plus	CL†	CL	488	530DF30	Blue	CL	CL

Labels

Substrate	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Flourescence mode	Excitation	Emission
Fluorescein	532	526SP	488	530DF30	Blue	Reflection	UV low
Cy3	532	580BP30	514	570BP30	NA	NA	NA
Cy5	633	670BP30	NA	NA	Red	NA	NA

Stains

Substrate	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Flourescence mode	Excitation	Emission
SYPRO Rose Plus	NA	NA	NA	NA	NA	Reflection	UV high
SYPRO Ruby blot	532	610BP30	488	610RG	Blue	Reflection	UV high

* NA = Not applicable.

† CL = Chemiluminescence only. Not applicable for fluorescence.

Typical protocols

Western blotting using a fluorogenic substrate

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Hoefer miniVE Vertical Electrophoresis System	80-6418-77
■ Hoefer EPS 301 Power Supply	18-1130-01
■ Hybond-P PVDF membranes	RPN2020F
■ ECL Plus Western Blotting Detection System	RPN2132
■ ECF Western Blotting Kit	RPN5780
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue
ImageMaster VDS-CL	see catalogue

Other materials required

Product	Vendor
DDAO phosphate	Molecular Probes, Inc.

❶

Preliminary preparations and general handling instructions

- For superior results with low-fluorescence background, the optimal antibody dilution for detection of the target protein must be determined (18). Although blocking and washing are important, they are only temporary measures until the optimal antibody dilutions are determined. For a primary antibody or antiserum of unknown activity, use a dot or slot blot to quickly determine optimal antibody dilutions.
- An excess of buffer should be used for washing steps following blocking and antibody incubations. The blot should be agitated during washing, and the recommended time interval per wash should be adhered to strictly.
- PVDF membranes should be kept wet at all times.
- Successful fluorescent detection protocols require careful control of background by thoroughly blocking and washing the blot. A minimum of 2.5 ml of wash solution should be used for every cm^2 of membrane. The blot should be incubated in a dish that is sufficiently large for the blot to circulate freely with orbital shaking. Alkaline phosphatase-based chemistries require particular attention to cleanliness—transfer pads and all dishes and containers that come into contact with the blot should be cleaned using a combination of boiling water and ethanol (when appropriate).

❷

Preparation of blot

Transfer the separated proteins from the gel to the PVDF membrane.

Block the membrane for at least 1 h at room temperature.

Incubate the blot with primary antibody against the target protein for 1 h, then wash the membrane thoroughly.

Incubate the blot with enzyme-conjugated secondary antibody for 1 h, then wash the membrane thoroughly.

After the final washing step, position the blot in an open low-fluorescence bag or page protector.

❸

Application of substrate

Add 50-100 µl of substrate per cm^2 of membrane. Incubate for 5 min.

Note: The blot can be air-dried to slow or stop signal development.

After developing, seal the blot in a low-fluorescence bag or page protector.

❹

Imaging

Place the sealed, developed wet blot (or dry blot) sample-side down on the glass platen (Storm, Typhoon), glass tray (FluorImager), or platform (VDS-CL) of the imager.

Note: Water can be used between the plastic bag and the platform to minimize the occurrence of interference patterns in the image.

Use a glass plate to hold the blot flat during imaging.

Acquire the image according to the recommended instrument setup. The choice of pixel size and PMT voltage settings will depend on the individual experiment. Adjust the PMT voltage setting to prevent signal saturation.

❺

Analysis

See Chapter 4 for information concerning image analysis.

Western blotting using a fluorochrome-conjugated antibody

Amersham Pharmacia Biotech products available for this application

Product	Product number
Hoefer miniVE Vertical Electrophoresis System	80-6418-77
Hoefer EPS 301 Power Supply	18-1130-01
Hybond-P PVDF membranes	RPN2020F
CyDye FluoroLink™ Antibody Labelling Kits	see catalogue
Fluor-Linked secondary antibodies	see catalogue
Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue
ImageMaster VDS-CL	see catalogue

❶ Preliminary preparations and general handling instructions

- An excess of buffer should be used for washing steps following blocking and antibody incubations. The blot should be agitated during washing, and the recommended time interval per wash should be adhered to strictly.
- PVDF membranes should be kept wet at all times.
- Successful fluorescent detection protocols require careful control of background by thoroughly blocking and washing the blot. A minimum of 2.5 ml of wash solution should be used for every cm^2 of membrane. The blot should be incubated in a dish that is sufficiently large for the blot to circulate freely with orbital shaking.
- Depending on the fluorochrome, air-drying a direct fluorescent Western blot may be possible and may even improve the signal-to-noise ratio of the acquired image. The suitability of drying the blot should be determined with each fluorochrome.

❷

Preparation

Transfer the separated proteins from the gel to the PVDF membrane.

Block the membrane for at least 1 h at room temperature.

Incubate the blot for 1 h with primary antibody against the target protein. Wash the membrane.

> **Note:** If the primary antibody is fluorochrome-labelled, skip the next step.

Incubate the blot with fluorochrome-linked secondary antibody for 1 h and wash.

After the final washing step, either seal the blot in a low-fluorescence bag or page protector or air dry (if appropriate).

❸

Imaging

Place the sealed wet blot (or dry blot) sample-side down on the glass platen (Storm, Typhoon), glass tray (FluorImager), or platform (VDS-CL) of the imager.

> **Note:** Water can be used between the plastic bag and the platform to minimize the occurrence of interference patterns in the image.

Use a glass plate to hold the blot flat during imaging.

Acquire the image according to the recommended instrument setup. The choice of pixel size and PMT voltage settings will depend on the individual experiment. Adjust the PMT voltage setting to prevent signal saturation.

❹

Analysis

See Chapter 4 for information concerning image analysis.

Expected results

Typical results for fluorescent Western detection are given in Table 22. A Western blot developed with ECL Plus substrate and imaged using Storm is shown in Figure 42.

Table 22. Expected results for fluorescent Western detection of tubulin*

Substrate/Label	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (ng/band)	LDR (~ fold)	LOD (ng/band)	LDR (~ fold)	LOD (ng/band)	LDR (~ fold)	LOD (ng/band)	LDR (~ fold)
DDAO phosphate	4	10	NA†	NA	4	10	NA	NA
ECF	8	10	4	10	4	10	4	10
ECL Plus	CL‡	CL	5	30	1–2	30	CL	CL
Fluorescein	15–30	20	15–30	20	NA	NA	ND†	ND
Cy3	30	20	30	20	NA	NA	ND	ND
Cy5	15–30	20	NA	NA	15–30	20	NA	NA

* Results are expressed as limit of detection (LOD) and linear detection range (LDR). LOD values are given as ng of tubulin protein. Detection limits, or sensitivities, for Western blots depend on multiple experimental factors, including the type and concentrations of protein target and antibodies used. Each new Western detection protocol should be optimized for concentrations of both primary and secondary antibodies.

† NA = Not applicable; ND = Not determined.

‡ CL - Chemiluminescence only. Not applicable for fluorescence.

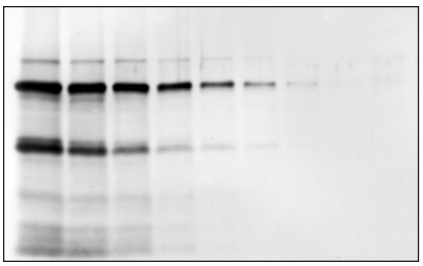


Fig 42. Detection of tubulin using ECL Plus fluorescent signal. The blot was imaged using Storm 860. Beta-tubulin was detected in a serial two-fold dilution of rat brain homogenate that was purified by SDS-PAGE and blotted to Hybond-P PVDF membrane.

Using covalent labels for nucleic acid and protein analysis

Because of their characteristic spectral properties, fluorochromes that are covalently attached to nucleic acids, proteins, and antibodies permit the identification and measurement of specific target molecules, even against the background of a complex mixture. Unlike general gel stains, covalent labels can be used to specifically tag a molecule or class of molecules, as with the generation of fluorescently labelled PCR primers, fluorochrome-conjugated antibodies, and recombinant proteins fused with a naturally fluorescent protein marker. These tagged molecules are widely used in a variety of applications, including PCR-based DNA assays (e.g. DNA typing, differential display, and RT-PCR), Western blotting, and cellular localization of fluorescent fusion proteins.

Fluorochrome labels are available in a reactive form that is suitable for attachment to the primary amines and thiol groups of biomolecules. Although both groups occur naturally in protein molecules, as for example at lysine and cysteine side chains, nucleic acids must be chemically modified to produce a site that will bind with a reactive dye. Additionally, one or more fluorochromes can be incorporated during synthesis of DNA oligonucleotides, and nucleic acids can be labelled internally by the enzymatic incorporation of fluorochrome-linked nucleotides. For example, fluorescein-linked UTP can be added to RNA during *in vitro* transcription reactions, or Cy3-labelled dCTP can be incorporated into newly synthesized DNA fragments during PCR. The choices for covalently attaching fluorophores to nucleic acids and proteins present numerous options for matching labels with the capabilities of a fluorescence imaging instrument. The broad selection of available fluorophores also facilitates the design of multi-label or multicolour experiments (Table 23). See Appendix 3 for an extended list of multipurpose labels.

Table 23. Properties of common fluorescent labels

Fluorophore	Colour of fluorescence	Excitation max (nm)	Emission max (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum yield for protein conjugates	Formula weight (g/mol)	
						Mono-reactive	Bis-reactive
FluorX	Green	494	520	68 000	0.30	586.60	-
Cy2	Green	489	506	~ 150 000	> 0.12	713.78	896.95
Cy3	Orange	550	570	150 000	> 0.15	765.95	949.11
Cy3.5	Scarlet	581	596	150 000	> 0.15	1102.37	1285.54
Cy5	Far-Red	649	670	250 000	> 0.28	791.99	975.15
Cy5.5	Near IR	675	694	250 000	> 0.28	1128.41	1311.58
Cy7	Near IR	743	767	~ 250 000	~ 0.28	818.02	1001.19

Nucleic acid labelling

The fluorescent labelling of DNA and RNA molecules can be achieved in a number of ways. The automated chemistry of oligonucleotide synthesis permits the covalent attachment of fluorophores at virtually any position in the single-stranded DNA. Oligonucleotides can also be designed to exhibit fluorescence resonance energy transfer (ET) properties (19). In this case, the oligonucleotide is modified to contain a pair of fluorochromes (donor and acceptor) spaced at a defined distance from each other in the DNA molecule.

Alternatively, oligonucleotides can be synthesized with an amino linker that can subsequently be labelled by reaction with an amine-reactive form of the fluorochrome. Kits are also available for modifying the 5'-ends of pre-existing oligonucleotides to generate reactive forms.

If DNA polymerization reactions are carried out in the presence of fluorochrome-linked deoxynucleotide triphosphates (dNTPs), using enzymes such as the Klenow fragment or *Taq* DNA polymerase, then DNA with multiple internal fluorescent labels can be generated. End-labelled DNA fragments can also be produced by PCR amplification using end-labelled primers (20, 21).

Protein labelling

Most proteins, peptides, and antibodies can be directly labelled with fluorochromes via their available amine or thiol groups. While virtually all proteins and antibodies have primary amine groups in their lysine side chains and at their N-termini, thiol groups are available only in cysteine side chains. A wide variety of anti-species antibodies are commercially available already conjugated to different fluorochromes.

Under ideal conditions, a fluorescent conjugate retains the key function of the unlabelled biomolecule, such as selective binding to a protein or nucleic acid target, or modulation of a particular enzyme activity. While conjugation of fluorochromes to biomolecules is usually a relatively straightforward reaction, preparation of the optimal conjugate may require considerable experimental manipulation. Although conjugates can be prepared with very high degrees of substitution or labelling, they frequently precipitate or bind non-specifically. Therefore, to preserve function or binding specificity, it is usually necessary to use labelling conditions that result in a submaximal fluorescence yield. After the labelling reaction, it is important to remove as much unconjugated dye as possible because the presence of free reactive dye can complicate subsequent experiments.

Several forms of reactive fluorochromes are commonly used. Isothiocyanates, such as fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC), are amine-reactive and widely used for preparing fluorescent antibody conjugates. Succinimidyl esters are excellent reagents for amine modification and form extremely stable amide bonds. The succinimidyl esters will also react with thiol groups. Some fluorochrome derivatives of sulfonyl chlorides are also highly reactive with amines, and react more mildly with thiol groups.

Instrument compatibility

Table 24. Instrument settings for use with common fluorescent labels

Fluorophore	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
FluorX	532	526SP	450	530DF30	Blue	Transmission	UV low
Cy2	532	526SP	488	530DF30	Blue	Transmission	UV low
Cy3	532	580BP30	514	570DF30	NA*	NA	NA
Cy3.5	532	610BP30	514	610RG	NA	NA	NA
Cy5	633	670BP30	NA	NA	Red	NA	NA
Cy5.5	633	670BP30	NA	NA	Red	NA	NA

* NA = Not applicable.

Applications and protocols

Differential display analysis

Differential display is a PCR-based technique for studying broad-scale gene expression (22). It enables direct side-by-side comparisons of complex expression patterns from multiple samples in a one-dimensional gel format. Using reverse transcription, the technique resolves the 3'-termini of messenger RNA (mRNA) molecules. This step is followed by PCR amplification using additional upstream arbitrary primers. PCR products are then separated on high-resolution denaturing polyacrylamide gels, from which bands of interest can be isolated and further analysed. By using multiple primer combinations, the differential display method can potentially screen all the expressed genes (up to 15 000 different mRNAs) in a mammalian cell. More importantly, the desired PCR product bands can be recovered from the gel and used as probes to isolate cDNA and genomic DNA for further molecular characterizations.

Fluorescent differential display offers fast results and easy quantification due to the proportional relationship between signal and quantity of message (23). Additionally, fluorescently labelled PCR primers are stable for relatively long periods. Fluorescence digital imaging of differential display gels provides a wide linear dynamic range and high sensitivity. With its high resolution and magnification capabilities, tightly spaced bands can be resolved and accurately excised, and gel data can immediately be archived in a digitized format for future analysis.

Protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Hoefer SQ3 Sequencer	80-6301-16
■ Low-fluorescence glass plate set	see catalogue
■ Hoefer EPS 3501 Power Supply	18-1130-04
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 840/860	see catalogue
FluorImager 595	see catalogue

Other materials required

Product
■ Fluorochrome-labelled oligonucleotide primers for differential display amplification
■ Total RNA

❶

Preparation of sample

Follow the recommended protocol for PCR amplification from total RNA (14).

Prepare the amplified products for electrophoresis using denaturing formamide sample buffer with 5 mg/ml of Dextran Blue 2000.

Heat the samples at 85 °C for 5 min, and then place the tubes directly on ice.

❷

Gel electrophoresis

Before casting the gel, treat one glass plate with silane (14).

Prerun a 6% denaturing polyacrylamide gel at 35 W for 45 min using 0.6× TBE as the electrophoresis running buffer.

Load the samples onto the gel and run at 35 W for 1.5–2 h.

❸

Scanning the differential display gel

Typhoon 8600

Affix two Kapton tape strips over each spacer on the outside of the long glass plate.

Place water between the glass plate and the glass platen to minimize the appearance of interference patterns. Avoid trapping air bubbles between the glass plate and the platen.

Select the appropriate settings for laser excitation and emission filter (see Table 24 or Appendix 3). Select a focal plane of +3 mm.

Storm 830/860

Be sure to use Cy5-labelled primers.

Remove the glass plate that has been treated with silane. Cover the gel with plastic wrap, being careful not to trap air bubbles or create wrinkles. Place the gel face down on the glass platen.

(Other options: transfer the gel to Whatman™ 3MM filter paper and dry it. Use Bind Silane to fix the gel to one glass electrophoresis plate and dry the gel directly on the glass plate.)

Select the appropriate instrument settings for the fluorochrome label used (see Table 24 or Appendix 3).

FluorImager 595

Position the gel sandwich in the universal tray.

Select the instrument settings appropriate for the fluorochrome label used (see Table 24 or Appendix 3).

❹

Recovering the gene fragments

Using image analysis software, print a 1:1 representation of the gel image on a transparency sheet.

Use the transparency sheet to locate the region of the gel containing the fragments and excise the fragments.

Expected results

A differential display analysis using a Cy5 label and imaged using Typhoon 8600 is shown in Figure 43.

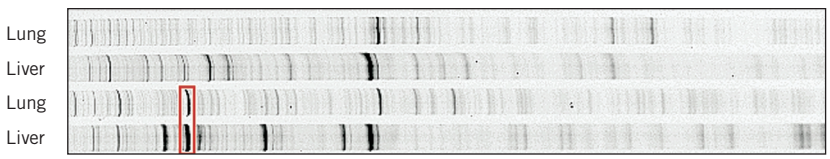


Fig 43. Differential display analysis. cDNA from rat liver and lung tissue was labelled with Cy5 and electrophoresed on a 6% denaturing polyacrylamide gel. The red box surrounds one species of cDNA that is differentially expressed in both tissue types. The image was acquired using Typhoon 8600.

In-lane PCR product analysis

Fluorescently labelled DNA fragments can be generated by PCR using modified oligonucleotide primers or deoxynucleotide triphosphates (dNTPs) (20, 21). A wide selection of fluorochrome tags is available for oligonucleotide end-labelling. For PCR products that are generated using an end-labelled PCR primer, an equimolar relationship exists between the label and the DNA molecule. In contrast, PCR products that are produced using fluorescently modified dNTPs are internally labelled at multiple sites per molecule and consequently deliver the greatest sensitivity.

Fluorescent detection offers the advantages of sensitivity, a wide linear dynamic range for quantification, and the option for using multiple tags in analysis. The CyDye series of fluorochromes are bright, photostable molecules that are highly water-soluble and insensitive to pH changes. The labels are available in a range of intense colours with narrow emission bands, making them ideal for multicolour detection. Two of these, Cy3 and Cy5, are popular labels for two-channel fluorescence experiments, such as gene expression arrays. Fluorescence imaging instrumentation with 532 nm and 633 nm laser excitation sources are ideally suited for CyDye imaging.

Protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
Hoefer SE 400 Sturdier Vertical Unit	80-6154-86
Low-fluorescence glass plate set	80-6442-14
Hoefer EPS 301 Power Supply	18-1130-01
Cy3 mono-Reactive Dye Pack	PA23001
Cy5 mono-Reactive Dye Pack	PA25001
Cy3-dCTP	PA53021
Cy5-dCTP	PA55021
PCR Nucleotide Mix	US77212
dNTP Set, 100 mM solutions	27-2035-01
Taq DNA Polymerase (cloned)	T0303Y
Imaging systems	
Typhoon 8600	see catalogue
FluorImager 595	see catalogue
Storm 830/860	see catalogue

❶

Preparation of sample

Prepare the reactions in one of the following ways:

A. PCR with CyDye-5' end-labelled oligonucleotide primer

Stock	Volume	Final
PCR Buffer*	5 µl	1 ×
25 mM $MgCl_2$	3 µl	1.5 mM
10 mM dATP, dGTP, dTTP, dCTP	1 µl	200 µM each
Forward primer (CyDye labelled)		0.5 µM
Reverse primer		0.5 µM
DNA template		70 ng
Taq DNA polymerase (5 units/µl)	0.2 µl	1 unit
Sterile ddH_2O to a final reaction volume of	50 µl	

* PCR Buffer: 500 mM KCl, 100 mM Tris-Cl (pH 9.0)

B. PCR with CyDye labelled dCTP

Stock	Volume	Final
PCR Buffer*	5 µl	
25 mM $MgCl_2$	5 µl	2.5 mM
2 mM dGTP, dATP, dTTP	1.25 µl	50 µM each
1 mM dCTP (CyDye-dCTP:dCTP, 1:10)	2.5 µl	50 µM dCTP
Forward primer		0.5 µM
Reverse primer		0.5 µM
DNA template		70 ng
Taq DNA polymerase (5 units/µl)	0.2 µl	1 unit
Sterile ddH_2O to a final reaction volume of	50 µl	

* PCR buffer: 500 mM KCl, 100 mM Tris-Cl (pH 9.0)

❷

PCR

Place the samples into the thermal cycler and heat for 1 min at 95 °C to denature them.

Run the following program for 30 cycles: 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 30 s. Complete the program by incubating the samples for 2 min at 72 °C.

❸

Gel electrophoresis

Prepare a 10% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer (14).

Mix 1–5 µl of the amplified product with TE buffer and 6× sample buffer for a final volume of 6 µl.

Load the samples onto the gel and run for 1.5 h at 100 V.

❹

Imaging

Typhoon 8600

Affix two Kapton tape strips over each spacer on the outside of the long glass plate.

Place water between the glass plate and the Typhoon glass platen to minimize the appearance of interference patterns. Avoid trapping air bubbles between the glass plate and the Typhoon platen.

Select the appropriate settings for laser excitation and emission filter (see Table 24 or Appendix 3). Select a focal plane of +3 mm.

Storm 830/860

Be sure to use Cy5-labelled primers.

Remove the glass plate that was treated with silane. Cover the gel with plastic wrap being careful not to trap air bubbles or create wrinkles. Place the gel face down on the glass platen.

(Other options: transfer the gel to Whatman 3MM filter paper and dry it. Use Bind Silane to fix the gel to one glass electrophoresis plate and dry the gel directly on the glass plate.)

Select the appropriate instrument settings for the fluorochrome label used (see Table 24 or Appendix 3).

FluorImager 595

Position the gel sandwich in the universal tray.

Select the instrument settings appropriate for the fluorochrome label used (see Table 22 or Appendix 3).

❺

Analysis

Display and analyse the gel image(s) using ImageQuant software,

FluorSep software, or Fragment Analysis software, as appropriate (refer to user documentation for details).

Expected results

In-lane size analysis of CyDye labelled PCR products imaged using Typhoon 8600 is illustrated in Figure 44.

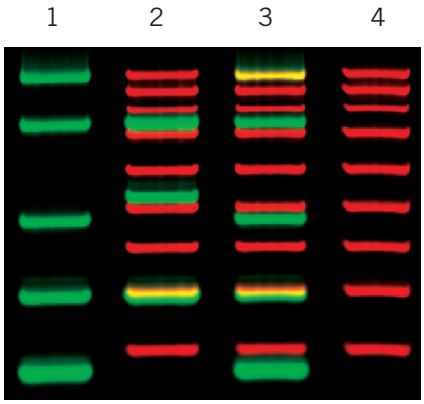


Fig 44. Cy3- and Cy5-labelled DNA fragments. Cy3-labelled fragments are in green, and Cy5-labelled fragments are in red.

Lane 1, Cy3-labelled fragments (500 bp, 365 bp, 230 bp, 150 bp, 88 bp); lane 2, Cy5 size ladder (500 bp, 450 bp, 400 bp, 350 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp) with Cy3-labelled fragments (365 bp, 268 bp, 150 bp); lane 3, Cy3-labelled fragments (same as in lane 1) with Cy5 size ladder; lane 4, Cy5 size ladder. The presence of both Cy3 and Cy5 signal in the same region of the gel is displayed as yellow by the ImageQuant software (lanes 2 and 3).

Bandshift assay

The gel mobility shift assay (also called the bandshift assay, gel shift assay, or gel retardation assay) is a useful tool for identifying protein–DNA interactions that can mediate gene expression, DNA repair, or DNA packaging (24). It can also be used to determine the affinity, abundance, binding constants, and binding specificity of DNA-binding proteins. The assay is performed by incubating a labelled DNA fragment, containing the test binding sequence, with an extract containing one or more binding protein(s). The mixture is then separated on a non-denaturing polyacrylamide gel. DNA fragments that are bound by protein migrate more slowly than free fragments and appear as bands that are shifted relative to the bands from the unbound duplexes.

Traditionally, the DNA fragments or oligonucleotides are end-labelled with ^{32}P. However, fluorescent end-labelled oligonucleotides are now commonly used, and kits such as the 5'-Oligolabelling Kit can be used for their rapid preparation. The availability of sensitive fluorescence imaging systems makes it practical to perform bandshift assays without radioactivity (25, 26). Gels containing bandshift products can also be stained after electrophoresis with Vistra Green or other sensitive DNA-intercalating dyes. In either case, with fluorescent labelling, gels can be scanned shortly after electrophoresis, with no film exposure step needed. Fluorescence imaging thus provides a rapid, convenient, safe, sensitive, and quantitative alternative to radioactivity for performing this important procedure.

Protocol

Amersham Pharmacia Biotech products available for this application

Product	Product number
■ Hoefer SE 400 Sturdier Vertical Unit	80-6154-86
■ Low-fluorescence glass plate set	80-6442-14
■ Hoefer EPS 301 Power Supply	18-1130-01
■ 5'-Oligolabelling Kit for Fluorescence	RPN5755
■ Cy3 mono-Reactive Dye Pack	PA23001
■ Cy5 mono-Reactive Dye Pack	PA25001
■ Imaging systems	
Typhoon 8600	see catalogue
Storm 830/860	see catalogue
FluorImager 595	see catalogue

❶

Preparation of labelled DNA

Label the oligonucleotides at their 5'-ends with fluorescein according to the instructions provided with the 5'-Oligolabelling Kit, or using another label, such as a CyDye, and a comparable approach.

❷

Preparation of DNA for annealing

Note: Repeat the steps below for all duplexes to be tested.

Prepare the following mix:

Labelled oligonucleotide #1	14 pmol
Labelled oligonucleotide #2	14 pmol
10× annealing buffer*	5 µl
H_2O to a total volume of	50 µl

* 10x annealing buffer: 200 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA

Heat the reactions for 10 min at 70 °C.

Incubate for 30 min at room temperature.

Place the reactions on ice until ready to use.

❸

Preparation of binding reaction

Note: Repeat the steps below for all protein-DNA combinations to be tested, including a negative control containing no protein.

Prepare the following mix:

Fluorescent oligonucleotide duplex	1.4 pmol
Binding protein	1.0 pmol (or as required)
H_2O to a total volume of 10 µl	

Incubate the reactions on ice for 30 min.

❹

Gel electrophoresis

To 3 µl of the protein–DNA mixture from each binding reaction, add 1 µl of a 50% (w/v) sucrose solution and mix gently.

Note: Do not mix tracking dye with the sample. Place tracking dye in a separate lane, if needed (see next step).

Load 2 µl of the protein–DNA/sucrose mixture onto a 6% non-denaturing polyacrylamide gel. Load tracking dye in a separate lane.

Fill the reservoirs with TAE buffer containing 1 mM $MgCl_2$ and run the gel at 10 V/cm at 4 °C until the tracking dye has migrated approximately halfway down the gel.

❺

Imaging

Typhoon 8600

Affix two Kapton tape strips over each spacer on the outside of the long glass plate.

Place water between the glass plate and the Typhoon glass platen to minimize the appearance of interference patterns. Avoid trapping air bubbles between the glass plate and the Typhoon platen.

Select the appropriate settings for laser excitation and emission filter (see Appendix 3 for appropriate settings). Select a focal plane of +3 mm.

Storm 830/860

Be sure to use Cy5-labelled primers.

Remove the glass plate that has been treated with silane. Cover the gel with plastic wrap being careful not to trap air bubbles or create wrinkles. Place the gel face down on the glass platen.

(Other options: transfer the gel to Whatman 3MM filter paper and dry it. Use Bind Silane to fix the gel to one glass electrophoresis plate and dry the gel directly on the glass plate.)

Select the appropriate instrument settings for the fluorochrome label used (see Table 24 or Appendix 3).

FluorImager 595

Position the gel sandwich in the universal tray.

Select the instrument settings appropriate for the fluorochrome label used.

❻

Analysis

Using image analysis software, determine the signal from each shifted band and divide by the total signal in the lane to calculate the percent of the signal in the shifted bands.

Alternative protocol

Eliminate Step 1 (labelling), and proceed with Steps 2–4. After electrophoresis, separate the gel sandwich and stain the gel for 20 min with a 1:10 000 dilution of Vistra Green in TAE buffer. Rinse the gel and wipe the excess liquid off the bottom of the glass plate. Image the gel using Typhoon or FluorImager system.

Expected results

The results of a multicolour bandshift analysis using two different DNA targets (labelled with HEX and TAMRA) and bacterial Mnt protein are shown in Figure 45. The gel was imaged using Typhoon 8600.

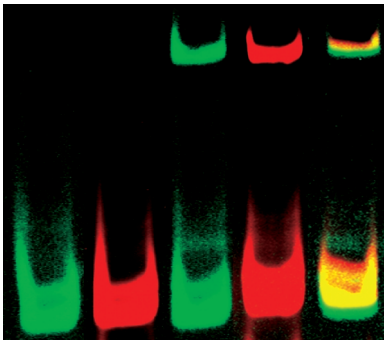


Fig 45. Multi-label gel shift experiment. First two lanes contain 0.4 pmol of two different 180-bp DNA fragments labelled with either HEX (green) or TAMRA (red). The third and fourth lanes contain the same two labelled DNA fragments after incubation with Mnt protein. The fifth lane contains mixtures of the bound labelled fragments to demonstrate multiplexing in the same gel lane (yellow colour indicates overlay between green and red signal). A 532-nm excitation was used with 555BP30 and 580BP30 emission filters.

Samples courtesy of Chris Man, Washington University School of Medicine, Department of Genetics, St. Louis, MO, USA.

Using naturally occurring fluorescent proteins

Green fluorescent protein and its variants

Green fluorescent protein (GFP) is widely used as a reporter molecule for the study of protein localization, protein binding events, and gene expression (27). Using recombinant DNA technology, the coding sequence for GFP can be spliced with that of other proteins to create fluorescent fusion proteins. GFP fusion proteins can then be used *in vivo* to localize proteins of interest to specific cell types and subcellular sites and *in vitro* to study protein–protein interactions. In gene-expression studies, when GFP expression is placed under the control of a specific promoter or DNA regulatory sequence, GFPs serve as reporters of transcriptional activity. GFP is uniquely suited as a reporter molecule in these applications because it can be expressed in many different cell types and organisms with no need for additional substrates or cofactors. Fluorescence from GFP is direct, stable, and readily observed using common modes of fluorescence detection (28).

Wild-type GFPs are not optimal for some reporter-gene applications. For example, when excited by the 488-nm argon-ion laser blue light commonly used in fluorescence microscopy and fluorescence-activated cell sorter (FACS), the fluorescence intensity from wild-type GFPs is relatively low. In addition, a significant lag in the development of fluorescence after protein synthesis can occur and complex photoisomerization of the GFP chromophore may result in the loss of fluorescence. Furthermore, wild-type GFPs are expressed at low levels in many higher eukaryotes. Numerous GFP variants have therefore been engineered to overcome these limitations (29). For example, several GFP variants are available with a significantly larger extinction coefficient for excitation at 488 nm and a modified gene sequence with codon usage that is preferentially found in highly expressed eukaryotic proteins. The spectral properties of green fluorescent protein and its variants are given in Table 25.

Table 25. Spectral properties of GFP and its variants

Protein	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum yield	Approximate relative brightness
EBFP	380	440	Blue	31 000	0.18	1×
ECFP	434	477	Blue	26 000	0.40	-
GFP (wt)	395, 470	508	Green	-	-	1×
GFP-S65T	488	511	Green	-	-	4–6×
EGFP	489	508	Green	55 000	0.60	35×
EYFP	514	527	Yellow-green	84 000	0.61	35×
DsRed	558	583	Red	22 500	0.23	6×

Instrument compatibility

Table 26. Instrument settings for use with GFP and its variants

Protein	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
EBFP	NA*	NA	NA	NA	NA	NA	NA
ECFP	NA	NA	NA	NA	Blue	NA	NA
GFP (wt)	532	526SP	488	530DF30	Blue	R/T†	UV low
GFP-S65T	532	526SP	488	530DF30	Blue	R/T	UV low
EGFP	532	526SP	488	530DF30	Blue	NA	NA
EYFP	532	555BP20	488	530DF30	NA	NA	NA
DsRed	532	580BP30	514	570BP30	NA	NA	NA

* NA = Not applicable.

† R/T = Reflection (opaque samples); Transmission (clear samples).

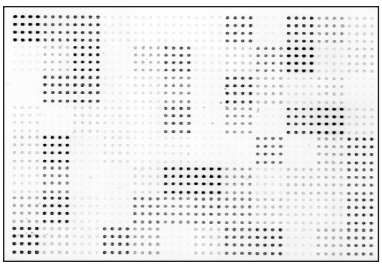


Fig 46. Varying expression levels from GFP-reporter constructs in yeast colonies. Colonies were spotted on agar plates and incubated at 37 °C. The agar plate was placed in a microplate tray and scanned using FluorImager at a resolution of 100 µm.

Image kindly provided by Drs. John Phillips and Matt Ashby, Acacia Biosciences, Richmond, CA.

Examples of applications using GFP

Monitoring gene expression in yeast

In this application, FluorImager was used to analyse transient gene expression in transformed yeast cells expressing GFP as a reporter. GFP-transformed colonies were spotted and grown on agar plates. Expression of GFP was observed by scanning the agar plate using 488-nm excitation (Fig 46).

Study of protein–protein interactions

When used as a probe in a fusion protein, GFP functions as an independent domain without altering the properties of the protein of interest. As such, GFP and its variants are effective tools for *in vivo* and *in vitro* functional analyses of protein–protein interactions. For example, GFP has been used to demonstrate the interaction between the S-peptide and S-protein fragments of ribonuclease A (30). In this study, varying amounts of S-protein were incubated with purified S15 peptide~GFP-S65T~His_6, and the complexes were then separated from free components in a native polyacrylamide gel (Fig 47). The image of the gel retardation assay was acquired using the 488-nm excitation source of FluorImager SI system.

In another study using Storm system for imaging, fusion proteins created between calmodulin (CaM) or calmodulin-like protein (CLM) and the GFP-S65T variant were used in a "gel overlay" assay to rapidly screen for interacting proteins (31).

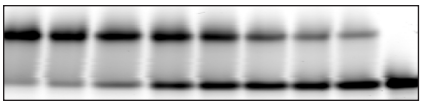


Fig 47. GFP gel shift assay showing quantification of the interaction between S-protein and S15~GFP-S65T~His_6 using FluorImager. A constant amount of S15~GFP-S65T~His_6 (1 mM) was incubated with varying amounts (0-0.95 mM, left to right) of S-protein for 20 min at 20 °C. Samples were resolved by electrophoresis on a native 6% polyacrylamide gel.

Image kindly provided by Sang-Hyun Park and Ronald Raines, University of Wisconsin, Madison, WI.

Expected results

The detection limits for purified wild-type GFP, EGFP, and GFP-S65T (Table 27) were determined by gel electrophoresis using Typhoon, Storm, and FluorImager systems. The greatest sensitivity for detection of GFP-S65T and EGFP was achieved using FluorImager system (488-nm excitation). The same two GFPs were also detected at the lowest concentrations when imaged using Storm in the blue-fluorescence mode, followed by the wild-type protein. Wild-type GFP was less compatible than the other variants when imaged using FluorImager system. The linear range of detection for each GFP was between 1.5 and 3 orders of magnitude.

Table 27. Detection limits of GFP and variants

Protein	Limit of detection (ng) Typhoon	FluorImager	Storm
GFP (wt)	13	2	13
GFP-S65T	ND*	0.3	8
EGFP	ND	0.3	8

* ND = Not determined.

Phycobiliproteins

Phycobiliproteins are stable, highly soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae (Table 28). These proteins contain covalently linked tetrapyrrole groups that play a biological role in collecting light and, through fluorescence resonance energy transfer, conveying it to a special pair of chlorophyll molecules located in the photosynthetic reaction centre. Because of their role in light collection, phycobiliproteins possess exceptional spectral properties—quantum yields up to 0.98 and molar extinction coefficients of up to 2.4×10^6 $cm^{-1}M^{-1}$. Phycobiliproteins have been covalently conjugated to antibodies and other proteins to generate probes that are readily detectable and which may be useful for Western blotting applications.

Table 28. Properties of phycobiliproteins

Protein	Excitation max (nm)	Emission max (nm)	Fluorescence emission colour	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum yield
Allophycocyanin	650	660	Red	700 000	0.68
B-phycoerythrin	546	575	Orange-red	2 410 000	0.98
R-phycoerythrin	565	578	Orange-red	1 960 000	0.82

Instrument compatibility

The broad excitation spectra, particularly of the R-phycoerythrin conjugates, allow phycobiliproteins to be efficiently excited using different types of imaging instrumentation with different excitation sources (Table 29). Allophycocyanin conjugates are ideal for use with helium-neon (HeNe) laser excitation (633 nm).

Table 29. Instrument settings for use with phycobiliproteins

Protein	Typhoon		FluorImager		Storm
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode
Allophycocyanin	633	670BP30	NA*	NA	Red
B-phycoerythrin	532	580BP30	514	570BP30	Blue
R-phycoerythrin	532	580BP30	514	570BP30	Blue

* NA = Not applicable.

Chapter 6

PRACTICAL RECOMMENDATIONS

Introduction

There are a number of ways to improve the results of fluorescence imaging. This chapter will describe useful recommendations for the various stages of a fluorescence imaging experiment—from sample preparation to instrument operation and data analysis.

Sample preparation

Careful sample preparation can minimize sample background fluorescence and non-uniformity, resulting in improved image quality and detection sensitivity.

Avoid using fluorescent indicator dyes.

Bromophenol blue, xylene cyanol, and other electrophoresis tracking dyes can fluoresce, potentially masking the fluorescence of the bands of interest in the gel. To avoid this problem, use a non-migrating sample loading buffer, such as dextran blue. If it is necessary to monitor migration during electrophoresis, reduce the concentration of tracking dye to a minimum or load the tracking dye into a separate lane of the gel.

Avoid excessive exposure of fluorochromes to direct light.

To prevent photobleaching, fluorochromes and fluorescently labelled samples should be protected from light. Wrap aluminium foil around individual storage tubes, plates, or racks to reduce sample exposure to light during handling and storage.

Use chemicals of highest purity.

To minimize autofluorescence from contaminants, use sequencing grade acrylamide or urea. Use powder-free gloves to eliminate fluorescent talcum particles.

Purify stock buffer solutions if necessary.

Dust in buffer solutions and gels can cause minor spikes in the background, thus affecting image quality and quantification. Filter solutions to remove dust and store the solutions in clean, rinsed containers. Spectroscopic-grade solvents should be used in the preparation of buffers because of their low autofluorescence. When appropriate, autoclave or filter-sterilize solutions and buffer stocks to eliminate the possibility of microbial contamination. Use filter-filled pipette tips.

Use appropriate staining containers for post staining.

For post-staining procedures, utilize containers that will not interfere with your stain. It is known that SYPRO and SYBR stains are adsorbed by glass surfaces, while propylene containers are better suited for these stains. Refer to the dye manufacturer's product information for details on handling specific dyes.

Use sample support materials with low-fluorescence properties.

Gels, membranes, glass plates, and microplates all autofluoresce to some extent. Using materials with low-fluorescence properties improves the limit of detection and linear range. New materials should always be tested to determine their fluorescence properties before they are used in experiments. See Table 30 for materials recommended for use in fluorescence imaging.

Table 30. Recommended materials

Material	Type	Vendor
Membranes	■ Hybond N+ membrane (nucleic acids) ■ Hybond-P membrane (proteins)	■ Amersham Pharmacia Biotech ■ Amersham Pharmacia Biotech
Membrane protection	■ Detection bags*	■ Amersham Pharmacia Biotech
Glass electrophoresis plates	■ Low-fluorescence glass plates	■ Amersham Pharmacia Biotech
Microplates	■ Clear, flat-bottom polystyrene microplates ■ Polysorp 96-well plates, with removable strips	■ Corning Costar ■ Nalge-Nunc

* Detection bags are a component of the ECF kits.

Avoid generating air bubbles when casting gels.

Air bubbles affect light scatter and can cause artefacts that interfere with quantification. Background fluorescence contributed by the gel matrix increases with gel thickness. Therefore, use the thinnest gel practical for your experiment. When preparing agarose gels, make sure the agarose is completely dissolved and well mixed before casting the gel. Uneven agarose concentration will cause non-uniform backgrounds that will affect quantification. If a plastic gel tray is used, be sure to remove the gel from the tray prior to scanning.

Place membranes between low-fluorescence transparent plastic bags.

To prevent contamination of the sample and glass tray or imaging platform, use low-fluorescence hybridization bags to sandwich the membrane.

Use low-fluorescence glass plates of optimal thickness.

When imaging sandwich gels, the position of the sample should be within the focal depth of the imaging instrument. Make sure that the thickness of the glass electrophoresis plates is optimal for the imaging system by consulting the instrument user's guide. Clean the glass with distilled water and a clean, lint-free cloth or Kimwipe™ tissue. If visible spots remain, clean the glass first with 75% ethanol and then with distilled water. Household glass cleaners should not be used because they contain ingredients that fluoresce.

Use flat-bottom microplates.

For microplates, the shape of the well is critical for proper excitation and collection of fluorescent light. Flat-bottom wells provide the largest imaging area with uniform surface characteristics. Microplates with clear bottoms and clear, black, or opaque walls should be used. Image quality and quantification are improved when using Nalge-Nunc PolySorp 96-well plates with removable strips.

Sample placement

The placement of the sample onto the imager is important to prevent the introduction of fluorescent artefacts, such as air bubbles, dust, or interference patterns.

Clean the glass platen/glass tray before and after imaging.

Dust, dried buffer and/or fluorescent stains, and skin oils from fingerprints increase background fluorescence which, in turn, can interfere with image quality and quantification. Clean the glass with distilled water and a clean, lint-free cloth or Kimwipe. If visible spots remain, clean the glass first with 75% ethanol and then with distilled water. Household glass cleaners should not be used for cleaning because they contain ingredients that fluoresce. Volatile organic solvents, such as acetone, and the excessive use of ethanol should be avoided since they can damage the glass surface.

Protect glass from scratches.

Scratches in the glass platen or tray will scatter laser light and collect dirt/solutions that will interfere with data collection and quantification. Gently place samples on the glass to prevent scratches. When handling glass electrophoresis plates on a glass tray or platen, take care not to scratch the platen.

Handle the sample with powder-free gloves.

Dust and powder fluoresce and scatter light, which cause image artefacts. To avoid this, wear powder-free gloves and rinse gloves with distilled water before handling samples and preparing reagents. Change gloves often to prevent contamination of samples and reagents.

Use a low-fluorescence bag/sheet protector for placing membranes on the glass platen.

A bag/sheet protector is used to cover membranes to avoid contaminating the glass platen or tray and to prevent contamination of the membrane. Lay one edge of the membrane down inside an open bag/sheet protector, then slowly lower the entire membrane while working any bubbles out to the edges of the membrane. Close the bag/sheet protector. A low-fluorescence glass plate can be placed on top of the sample to keep it flat.

Use thin (0.2–0.4 mm) spacers when scanning through another glass plate on a glass platen.

Sequencing gel spacers, Kapton tape (supplied with Typhoon), or a thin layer of water can be inserted between the glass plate and the glass platen to minimize optical refraction artefacts and interference patterns, and to protect the platen from scratching.

Place one-sided, opaque samples (such as membranes or thin-layer chromatographs) face down.

If the sample is physically uneven on one side (such as an agarose gel), place the smooth side down on the glass surface. For opaque samples, such as membranes, place the side with the nucleic acid or protein face down. The sample should be positioned to create a smooth and even surface. Avoid trapping air bubbles as they can appear on the scanned image and interfere with quantification.

Instrument operation

The detection and measurement of the emitted fluorescent signal can be enhanced in a number of ways:

Add optical filters to reduce background fluorescence from the sample matrix.

When the background signal from the sample matrix (e.g. some gels, TLC plates, and membranes) has a broad, flat spectrum, a band-pass optical filter can be used to remove background signal comprising wavelengths longer or shorter than the fluorochrome emissions. This type of filter rejects wavelengths that are shorter and longer than the selected band, while allowing wavelengths in the selected range (centred around the fluorescent emissions of the sample) to pass through to the collection pathway.

Increase the dwell time or accumulate multiple scans for mathematical processing.

Detection of weak fluorescent signals can be improved by increasing the dwell time because the instrument can excite and collect more emitted fluorescent light from the sample. Multiple scans of the sample can also be accumulated and subjected to mathematical processing (e.g. averaging, summing, or other accumulation methods). This increases fluorescence sensitivity by reducing the amount of background fluorescence. Averaged results, for example, represent the average of the constant signal and a reduction of random background effects (averaged noise).

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 the 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 can be increased to 10^4 or greater.

Add optical filters to eliminate excitation light in laser-based scanners.

Stray laser light that is reflected or scattered by the sample can be rejected from the collection pathway by adding an optical filter that rejects the laser light, while allowing fluorescent emission light to pass through.

Change the PMT (photomultiplier tube) voltage to improve signal collection in laser-based scanners.

For accurate quantification, the sample signal should fall within the linear range of the system. For intensely fluorescent samples that saturate the system, decrease the PMT voltage to bring high-intensity signals into the linear range of the scanner. For weak samples, increase the PMT voltage to increase the signal. Otherwise, you may lose sensitivity and accuracy of quantification at the lower end of the signal range. Refer to the instrument user's manual for additional information.

Change the lens aperture to improve signal collection for CCD cameras.

For intense signals that saturate the system, reduce or close the lens aperture to reduce the amount of light entering the camera. For weak signals, open the lens aperture to collect more light.

Adjust the focal plane to optimize fluorescent detection when using Typhoon scanner.

Different matrices (e.g. thick agarose gels, sandwich gels, and microplates) can change the spatial location, and thus the focal plane of the fluorescently labelled target. To achieve optimal results, adjust the focal point of the optics.

Maintain the instrument under proper environmental conditions.

Keep the instrument in a clean, relatively dust-free environment and away from direct sunlight, heat, and air-conditioning ducts. Maintain the instrument's proper temperature and humidity requirements. To avoid electrical noise, connect the instrument to a dedicated, properly grounded AC circuit. An uninterruptible power supply is recommended to prevent malfunction and loss of data caused by unexpected power failures, power surges, or AC line fluctuations. Refer to the instrument user's manual for additional information.

Data evaluation

The digital image acquired from a fluorescent sample should be evaluated for pixel saturation before proceeding to analysis. It is also important to apply an appropriate background correction method to the quantification process.

Check the image for signal saturation.

If the instrument's control software displays a preview image of the sample, monitor the preview and check for saturated data. In Scanner Control software, saturated data appear as red areas in the image. If key areas of the image are saturated and you want to perform quantification on the image, you must rescan the fluorescent sample using a lower PMT voltage setting.

Once the image is captured or acquired, it can be displayed using image analysis software. Adjust the image contrast settings and assess pixel values by using a pixel measurement tool. Alternatively, data from a line profile across the image will display signal intensity versus pixel co-ordinate (or distance). Use these tools to determine if any signal has saturated the detector at the high end of the intensity scale.

Use background correction and analysis tools that are appropriate for the image.

(For discussion and suggestions, see Chapter 4.)

Glossary

TERMS DEFINED

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

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

algorithm — a mathematical or computational procedure for solving a recurrent problem.

amplitude resolution — or **gray-level quantification**
describes the minimum difference that is distinguishable between levels of light intensity (fluorescence) detected from a sample.

aperture — an optical opening that admits light.

autofluorescence — an inherent or intrinsic property of a material to fluoresce.

background — undesired signal often resulting from autofluorescence or light-scatter from a matrix or sample support.

band-pass filter — an optical filter that transmits a band of light between two specified wavelength cutoffs. The filter rejects light with wavelengths shorter than the first cutoff and longer than the second cutoff.

beamsplitter — a dichroic optical filter used to separate the fluorescent signal of two distinct fluorochromes from a mixed-emission beam.

brightness — the level of fluorescence intensity of a fluorochrome. Brightness depends on the extinction coefficient and the quantum efficiency.

CCD — (**charge-coupled device**)
a two-dimensional photosensitive array that produces a pattern of charge that is proportional to the total integrated energy flux incident on each array element (pixel).

chemifluorescence — the chemical and/or enzymatic production of fluorescence.

chemiluminescence — the emission of light from a molecule as a result of a chemical reaction.

coherent — a property of light where all the waves are at the same frequency and phase. Only light that is monochromatic can be completely coherent.

collimated light	light that is radiated in only one direction.
cone angle	the full angle between the extreme off-axis rays in a converging or diverging beam of light.
confocal imaging	the detection of fluorescent light only from those points on a sample that are within the desired focal plane. Confocality is controlled by an aperture (pinhole) placed in front of the detector that greatly reduces the passage of out-of-focus information, both above and below the desired focal plane.
cutoff point	the wavelength of light at which transmission through an optical filter is 50% of the maximum transmission.
dataset	the files and folder that make up a multichannel image.
dichroic filter	a coated glass filter used to split light by reflecting one wavelength range and transmitting another range.
diode laser	a semiconductor device that produces coherent radiation in the visible or infrared transmission spectrum when current passes through it.
dwell time	the amount of time the excitation light illuminates a spot (pixel) in a sample.
dynamic range	the range over which a detected signal can be quantified.
emission	

extinction coefficient (ε)
the amount of light absorbed. The molar extinction coefficient is the optical density of a one-molar solution of a 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. Emission continues only as long as the stimulus producing it continues and persists with a half-life of less than ~ 10^{-8} second.

fluorochrome or **fluorophore**
a fluorescent dye.

FWHM (**full-width at half-maximum transmission**)
defines the width of the pass-band of a band-pass filter. It is referenced to the points on the cutoff edge where the transmission is one-half of the maximum transmission.

galvanometer a device used to determine the presence, direction, and strength of electric current in a conductor.

gel sandwich a vertical gel (typically polyacrylamide) cast between two supporting glass electrophoresis plates.

glass platen a horizontal glass stage or platform used to support samples (i.e. gels, membranes, microplates) for imaging; typically used in imagers with moving-head mechanisms.

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.

Kapton tape thin adhesive tape that is used to raise a gel sandwich a defined distance above a glass platen.

laser an acronym for light amplified stimulated emission of radiation. A laser produces highly monochromatic, coherent, and collimated light.

LED (**light-emitting diode**)
a semiconductor device that emits visible light when an electric current passes through it.

linearity the signal range over which a laser scanner yields a linear response to fluorochrome concentration.

limit of detection the smallest amount of a sample that can be reliably detected.

long-pass filter — an optical filter that transmits light of wavelengths longer than a specified cutoff. The filter rejects light with wavelengths that are shorter than the cutoff.

monochromatic — light of a single frequency, single wavelength, or single colour.

multichannel image — a set of images that can be viewed as a composite when overlaid or viewed as individual images. Each separate image of the set represents a single channel.

noise — the statistical uncertainty inherent in a measurement, such as the standard deviation associated with measured background counts.

numerical aperature — (**NA**)
a number that expresses the ability of a lens to resolve fine detail in an object being observed. The NA is related to the angular aperture of the lens and the index of refraction of the medium found between the lens and the specimen.

optical filter — a glass designed to specifically attenuate, reflect, and transmit only selected wavelengths of light.

parallax — a shift in the apparent position of an object that occurs when it is viewed from different vantage points.

photobleaching — or **photodestruction**
the irreversible destruction of an excited fluorophore upon exposure to an intense light source, resulting in loss of the emission-light intensity (brightness).

PMT — (**photomultiplier tube**)
a photoelectric device 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 states that the energy of an oscillating system can have only discrete (quantized) values.

pixel — the basic unit of programmable gray or colour in a digital image. The physical size of a pixel depends on the resolution of the image.

quantification — a process in which the signal intensity of a sample is calculated.

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

rfu (**relative fluorescence units**)
the arbitrary units in which fluorescence intensity is reported by the fluorescence imaging systems.

resolution see amplitude resolution or spatial resolution.

saturation the reception of excess light by a photosensitive detector, resulting in loss of signal discrimination.

sensitivity threshold or **detection threshold**
a measure of the lowest signal that can be accurately detected by an instrument.

short-pass filter an optical filter that transmits light of wavelengths that are shorter than a specified cutoff value while rejecting light of wavelengths that are longer than the cutoff.

spectral cross-contamination the presence of fluorescent signal from more than one fluorochrome in a single optical channel; spectral contamination in a single optical channel that cannot be separated by optical filtering.

signal-to-noise ratio (**S/N**)
a measure of the brightness of a desired fluorescent signal relative to the brightness of the background.

spatial resolution the number of data points sampled per unit length or area. Spatial resolution is a function of the distance between adjacent measurements.

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).

trans-illumination delivery of light through a sample with detection of the resulting signal from the opposite side.

transmission the passage of light through a filter element.

uniformity describes the evenness of illumination or collection of light from an imaging area.

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

Appendix 1

FREQUENTLY ASKED QUESTIONS

Typhoon, Storm, and FluorImager Systems

How do I clean the glass sample tray, glass platen, or sample lid?

To clean the platen and sample lid (Storm, Typhoon) or glass tray (FluorImager), dampen a lint-free cloth with distilled water and wipe the surfaces. Alternatively, you can use a lint-free cloth dampened with 75% ethanol to wipe the surfaces, and then wipe the surface again using distilled water.

Because laboratory alcohol formulations may contain residue that is highly fluorescent, make sure that surfaces cleaned with alcohol are always wiped with distilled water afterwards.

How is it possible to use 532-nm laser light to excite fluorescein and similar dyes?

Fluorochrome molecules have different rotational and vibrational energies associated with them; these are represented in the excitation spectrum or the probability that the fluorochrome will be excited by a particular wavelength of light. (Refer to Chapter 1.) Excitation of a fluorochrome at the peak of its excitation spectrum is most efficient, since the majority of fluorochrome molecules are able to absorb this energy. However, a small population of fluorochrome molecules can also be excited at other regions of the excitation spectrum. The emission profile for a fluorochrome is always independent of the wavelength used for excitation.

A small population of fluorescein molecules accepts 532 nm excitation energy, even though the excitation maximum for the fluorochrome is 488 nm. Fluorescein, in turn, is characterized by a fluorescence emission spectrum with a peak wavelength of 520 nm. Efficient collection of fluorescein emissions on the short side of the spectrum (below 532 nm) is accomplished using a short-pass filter (526SP), high quality confocal optics, and a highly sensitive PMT detector.

Why does my image contain a double or ghost image?

The sample may have moved after its initial placement on the glass plate or platen of the instrument. If fluorescent material from the sample has contaminated the glass, carefully remove the sample and clean the glass. Place the sample on the glass plate or platen again and do not readjust the placement.

How do I keep the sample from moving during the scan?

Remove any excess liquid from below the gel so that it does not move on the glass tray or glass platen.

Place a clean electrophoresis glass plate on top of a membrane or a blot that has been sealed between plastic sheets or page protectors.

Why is the sensitivity of my image unexpectedly poor?

The choices of excitation and emission filters may not have been optimal. Make sure that the correct excitation light and the proper emission filters have been selected for the dyes used in the sample. Check that the combination of excitation light and emission filter are compatible. (Refer to Appendix 3 and Chapter 3 for more details.)

Why do I have high image background or inaccurate readings?

The instrument may not have been warmed up before the sample was scanned. Allow at least a 30-minute warm-up time.

The instrument may be damaged and is no longer light-tight. If this has occurred, do not continue to use the instrument. Contact Technical Support to arrange for repair.

Dust, fingerprints, or other dirt may have contaminated the screen, sample, or glass platen. Clean the glass platen. If necessary, for fluorescence scans, filter the liquid samples, reagents, and components used to prepare the gels.

The sample support may be highly autofluorescent. Use a low-fluorescence material.

If using FluorImager, the filter door may have been open during the scan. Close the filter door and repeat the scan. Make sure there are no obstructions that prevent the door from closing completely.

The wrong light source or emission filters may have been used for the fluorescent sample.

Why are there streaks or other artefacts in my image?

The instrument may not have been warmed up before the sample was scanned. Allow at least a 30-minute warm-up time.

Diagonal streaks may indicate a light leak during scanning. Check for damaged panels on the instrument. Contact Technical Support.

The glass sample tray or glass platen may be scratched. If possible, scan the sample on another area of the glass. Contact Technical Support to order replacement glass and arrange for a service call.

Fingerprints appear in the scan. Clean the glass sample tray or glass platen. If the fingerprints are on the gel, rinse the gel briefly in 0.1% Tween™ or SDS. Rinse the gel again in distilled water and then rescan it. If the fingerprints persist, you may need to prepare a new gel and handle it more carefully.

Dust specks appear in the scan. Rinse wet gels in filtered distilled water to remove surface dust prior to scanning. Filter liquid reagents that are used in gel and buffer preparation. Make sure to dissolve agarose completely before pouring the gel. Clean the glass sample tray or glass platen of the instrument with a damp, lint-free cloth.

The tracking dye is fluorescing. Place the tracking dye in a single well, or dilute the tracking dye with sample buffer.

The sample may have stained unevenly. Make sure you mix staining solutions thoroughly, use a large excess of staining solution, and rock or shake the gels during staining, if possible.

How do I reduce the appearance of diffraction patterns in the image from a glass plate placed on a platen?

Diffraction patterns are caused by the interface between two different pieces of glass. To reduce their appearance, use two Kapton strips (supplied in the Typhoon accessory kit) positioned over the spacers on the outside edges of the 3-mm thick plate to raise the sandwich gel slightly above the glass platen Fill the gap between the platen and the bottom of the 3-mm electrophoresis glass plate with distilled water.

If water is used, be sure to avoid trapping air bubbles between the sandwich gel and the glass platen. Rest one side of the sandwich gel on the glass platen and slowly lower it. When you can no longer lower the sandwich gel using your fingers, insert the Wonder Wedge tool (supplied in the Typhoon accessory kit) between the glass platen and the 3-mm electrophoresis glass plate, then slowly remove the wedge. After scanning, use the Wonder Wedge to help remove the sandwich gel.

Is my image suitable for quantification?

Display the scanned image in ImageQuant and use the Gray/Color Adjust, Pixel Locator, or Create Graph features to assess the signal values across the image. If saturated values are present in the image, consider rescanning the sample using a lower PMT voltage setting.

VDS-CL System

Why can't I focus on my image?

The sample may not be centred on the tray. Centre the sample on the tray in the middle of the imaging area.

The autofocus algorithm requires a sharp edge as a reference for focusing. If the lens is zoomed in and the edge of the sample is not visible, autofocus will not function properly. To avoid this situation, place a piece of white paper (e.g. a business card) adjacent to the area of interest on the sample.

The object or sample may be too thick. Make sure the object or sample is no thicker than 3 mm for an iris below 1.8. For thicker samples, use a higher iris value and increase acquisition time accordingly.

Why does my image appear dirty, fuzzy, or uneven?

The sample tray or the optical surfaces may need cleaning.

The signal acquisition time may have been too short and should be increased.

The sample is too thick or is not flat on the surface of the tray. Place a glass plate over dry samples to flatten them. Remove any bubbles from below a wet gel.

Can sensitivity be improved by extending the exposure time?

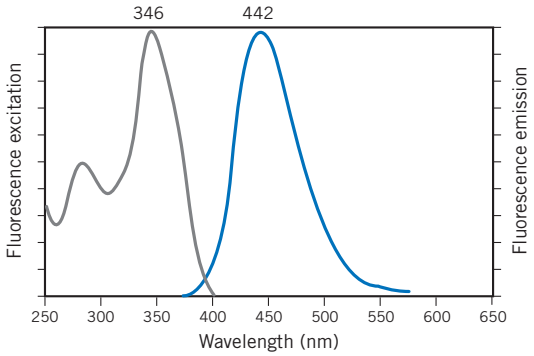
The signal from a sample is integrated over time. The sensitivity improves with exposure time, but only up to a point. The instrument noise dramatically affects the linearity of the CCD at low light intensity and long exposure. The VDS-CL has a cooled CCD that significantly reduces the noise, however exposures longer than 30 minutes do not improve the sensitivity.

Appendix 2

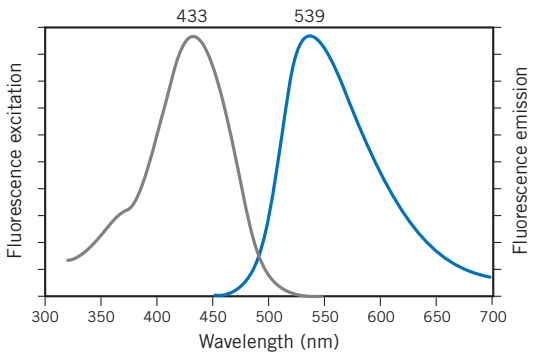
SPECTRAL CHARACTERISTICS OF COMMONLY USED FLUOROPHORES AND FLUORESCENT PROTEINS

Note: Gray line = excitation; blue line = emission.

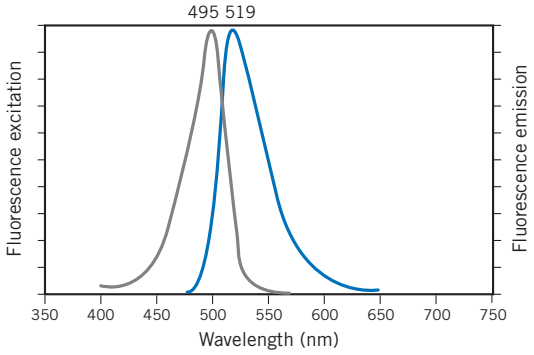
Alexa Fluor 350



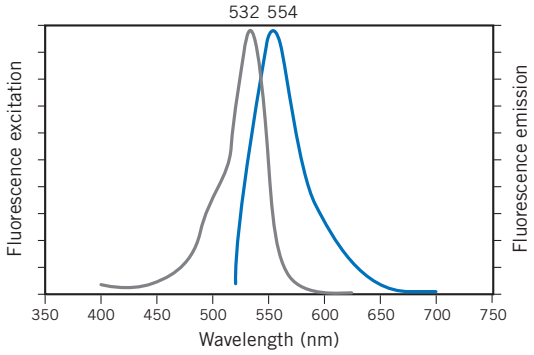
Alexa Fluor 430



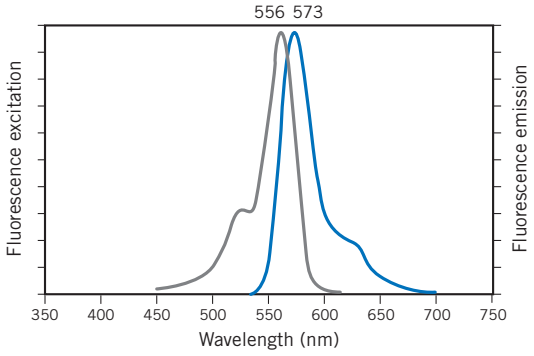
Alexa Fluor 488



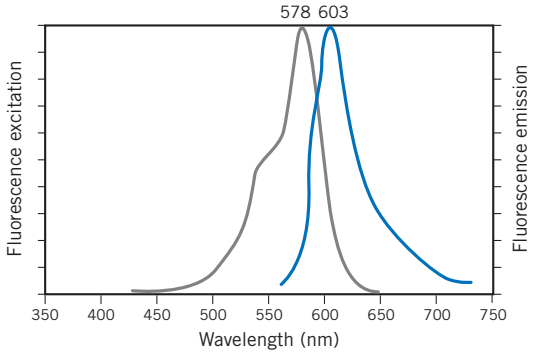
Alexa Fluor 532



Alexa Fluor 546



Alexa Fluor 568



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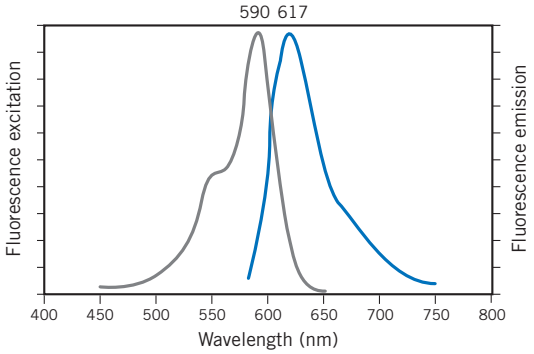
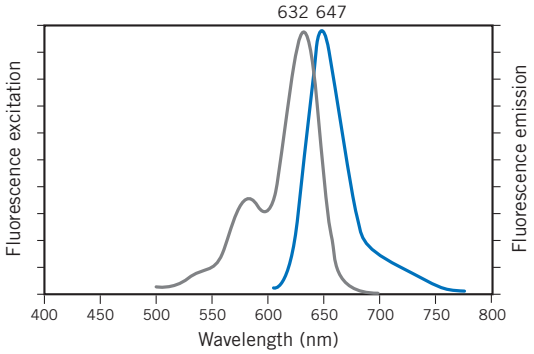
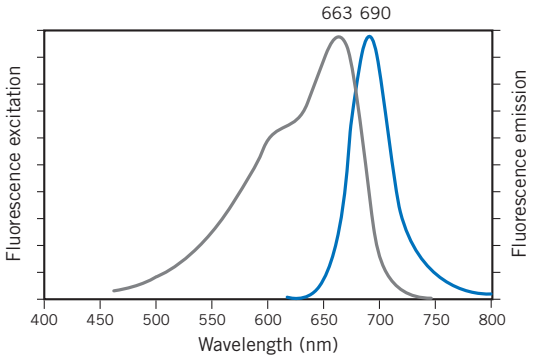


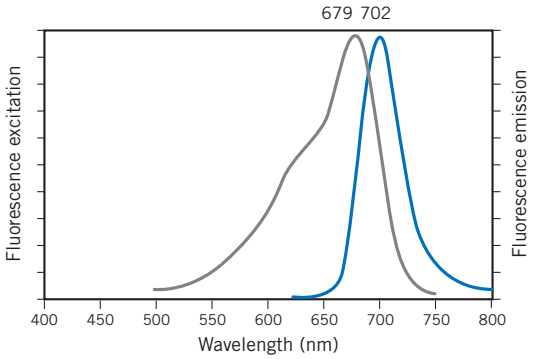
Alexa Fluor 633



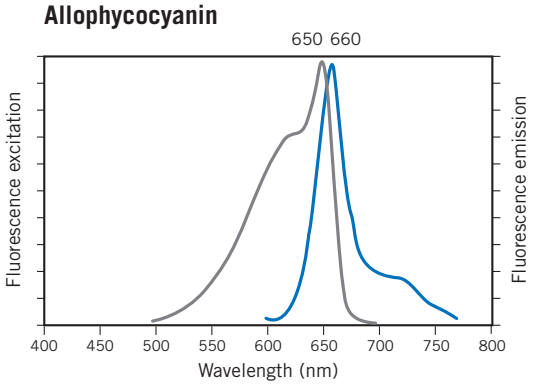
Alexa Fluor 660



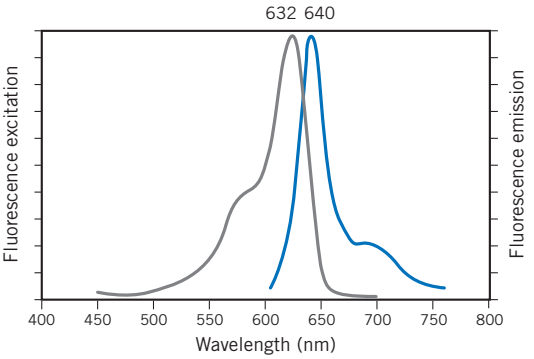
Alexa Fluor 680



Allophycocyanin



BODIPY™ 630/650



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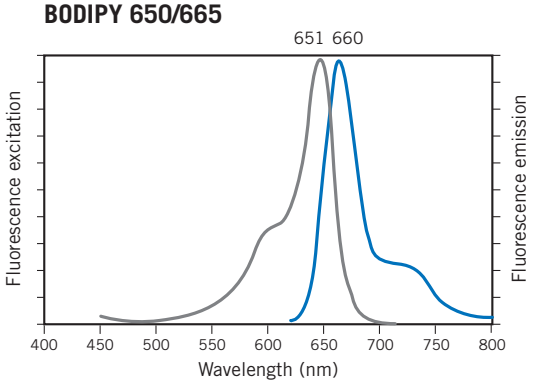
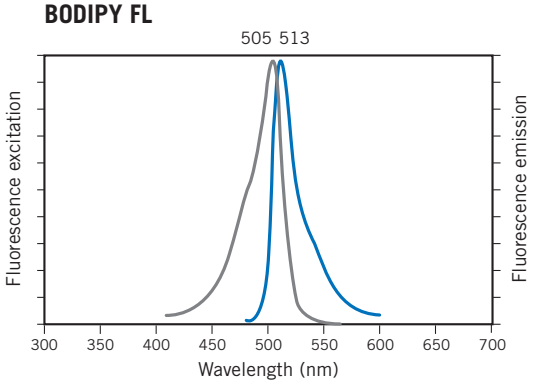
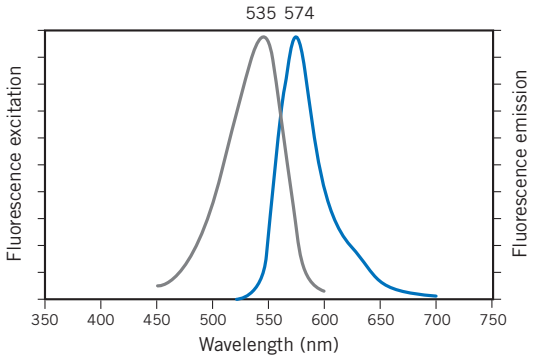


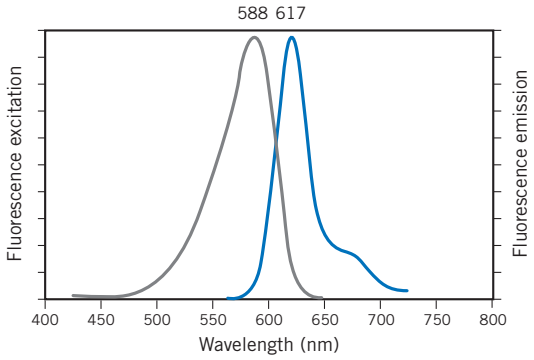
BODIPY FL



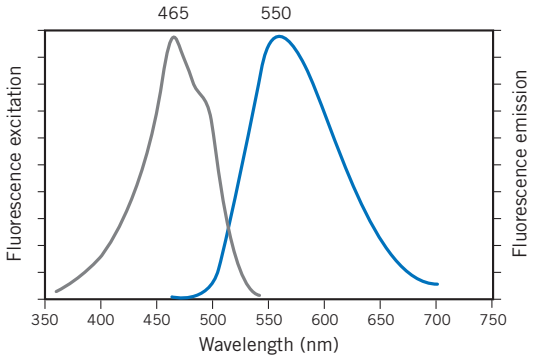
BODIPY TMR-X



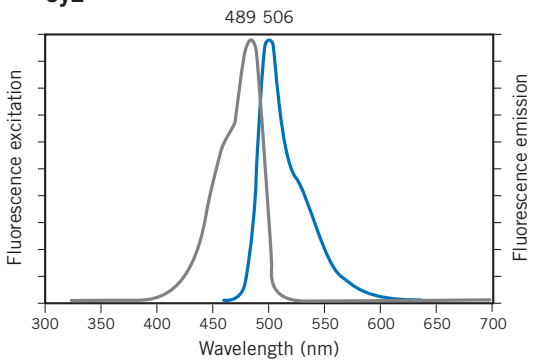
BODIPY TR-X



CBQCA



Cy2



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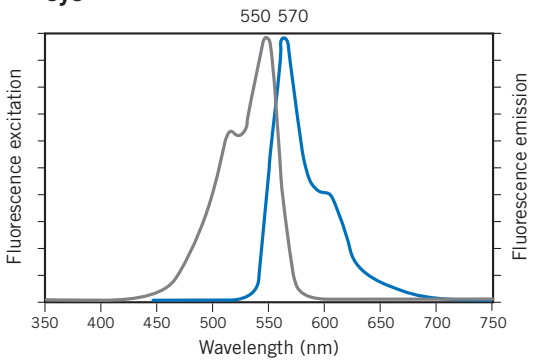
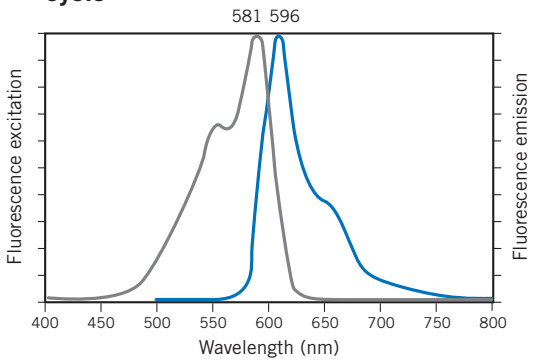
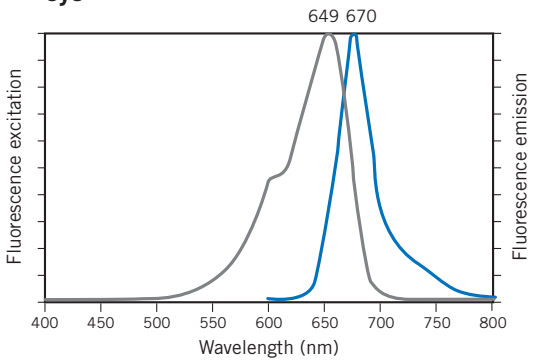


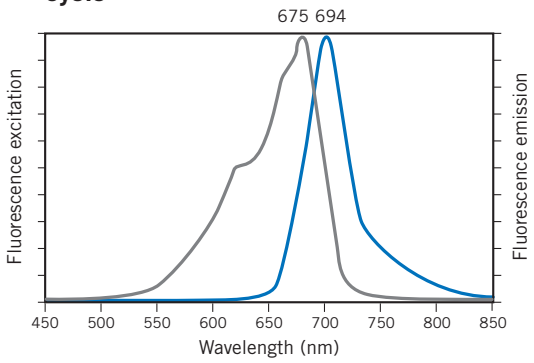
Cy3.5



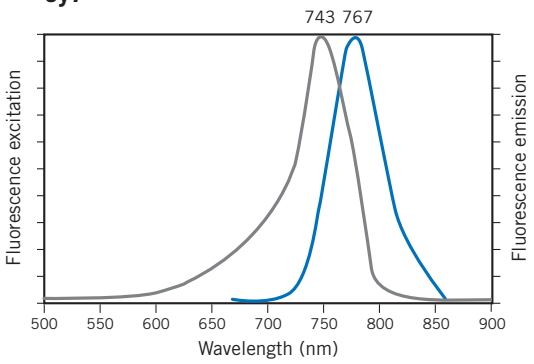
Cy5



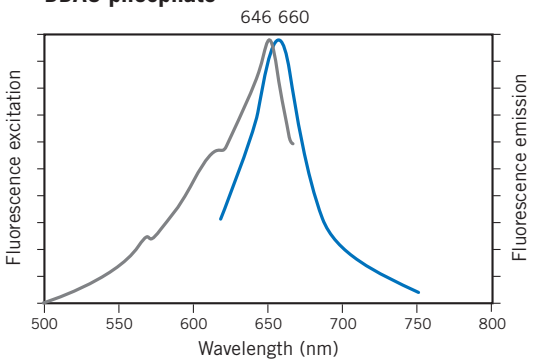
Cy5.5



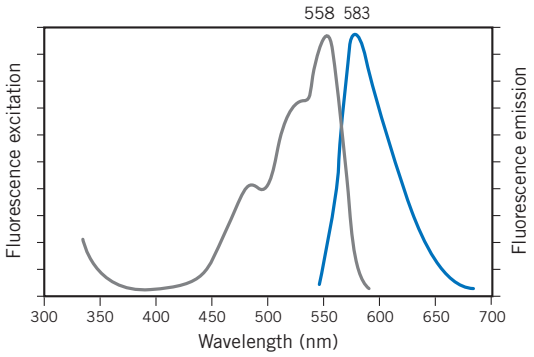
Cy7



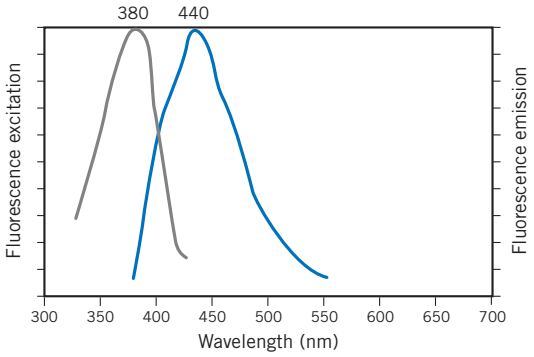
DDAO phosphate*



DsRed



EBFP



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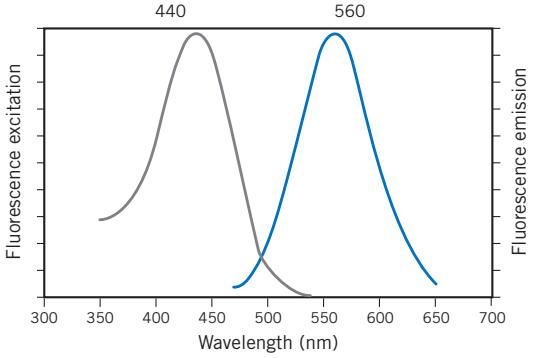
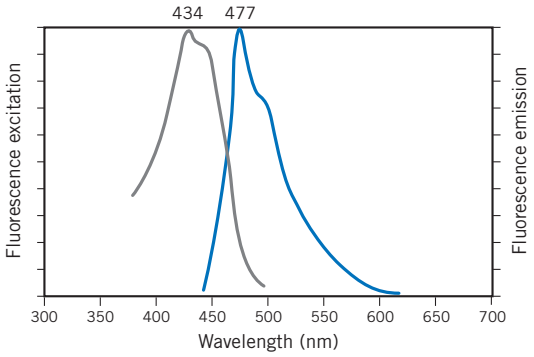
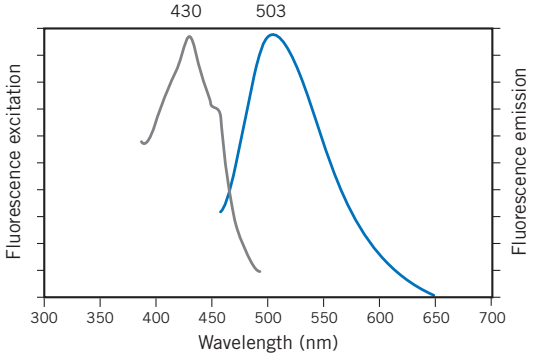


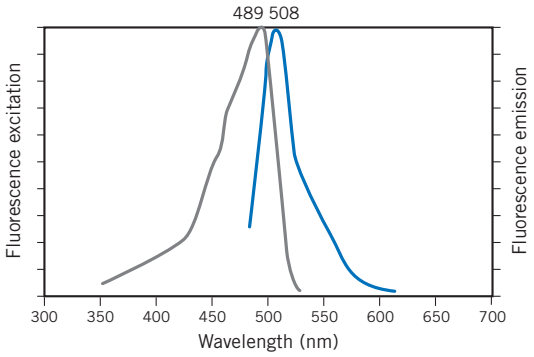
ECFP



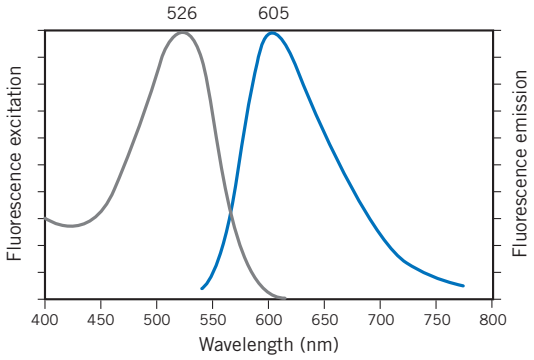
ECL Plus*



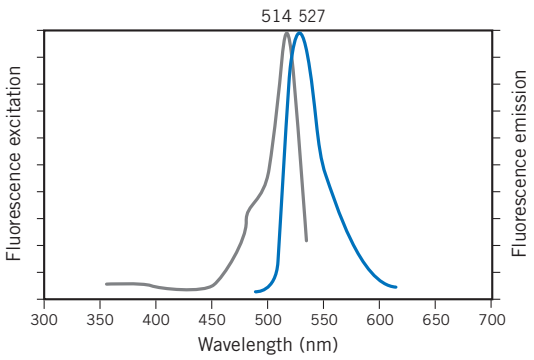
EGFP



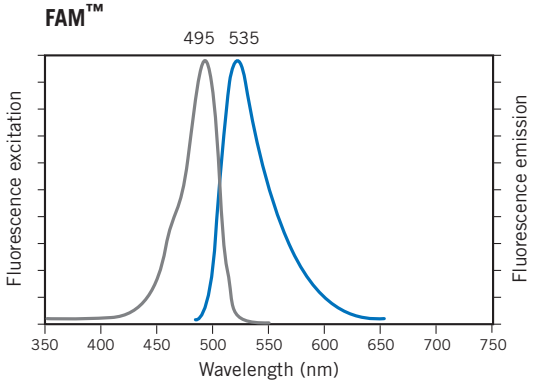
Ethidium bromide†



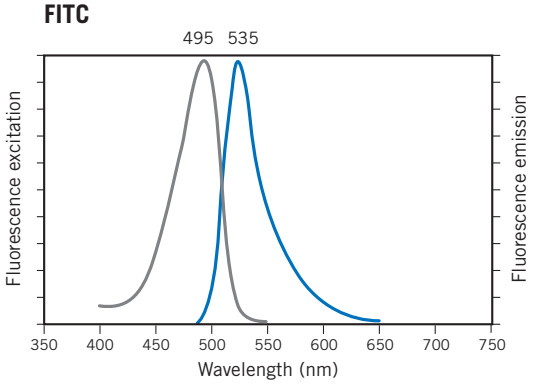
EYFP



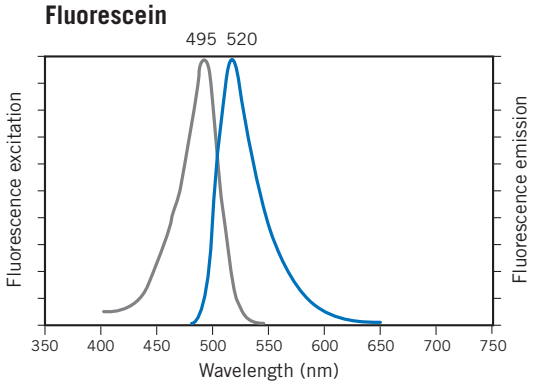
FAM™



FITC



Fluorescein



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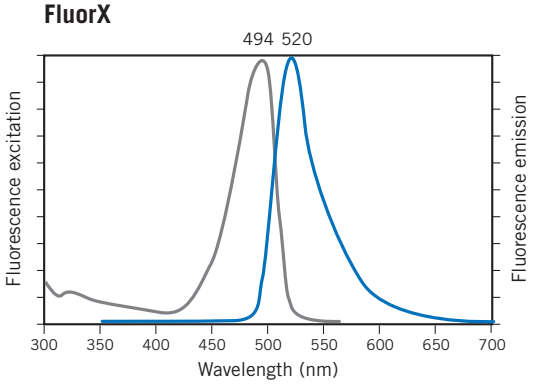
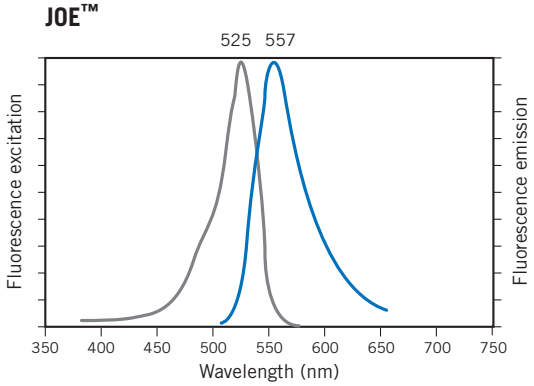
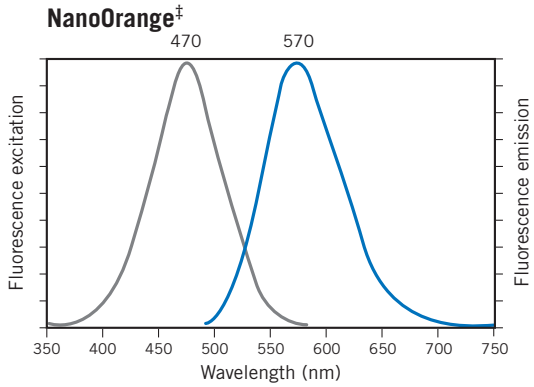


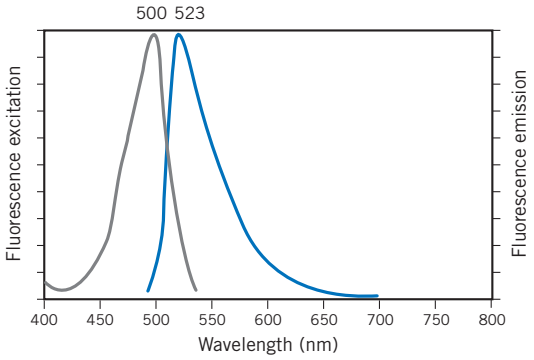
JOE™



NanoOrange‡



OliGreen†



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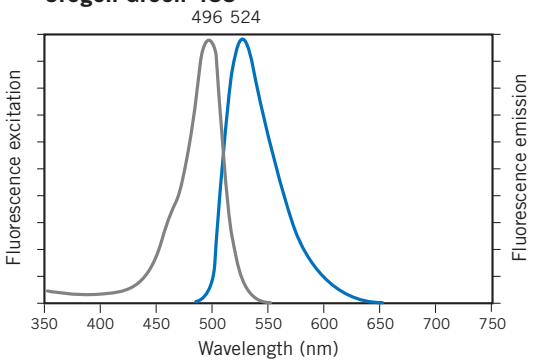

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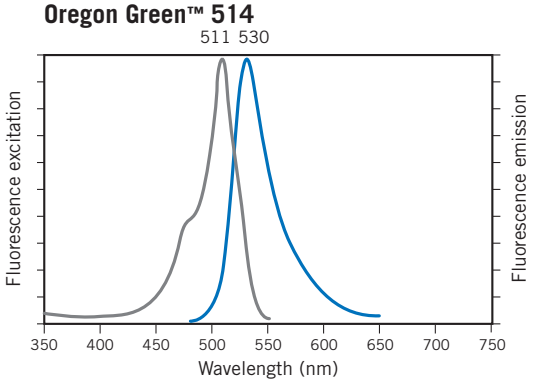

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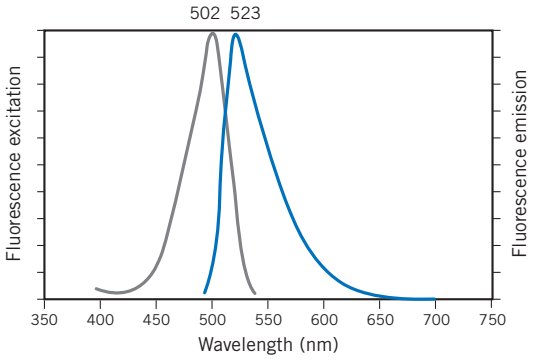

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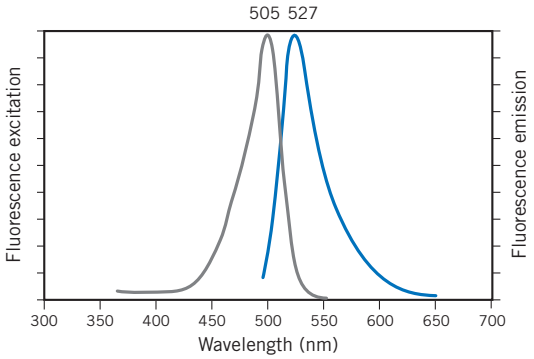

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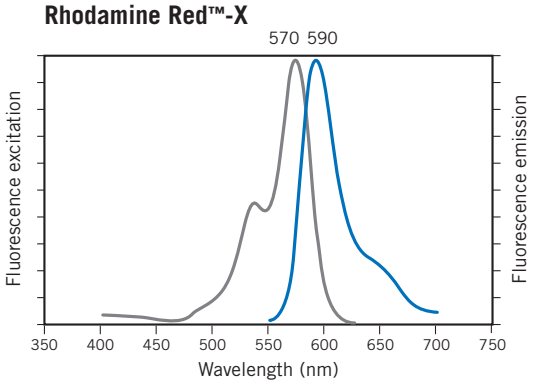

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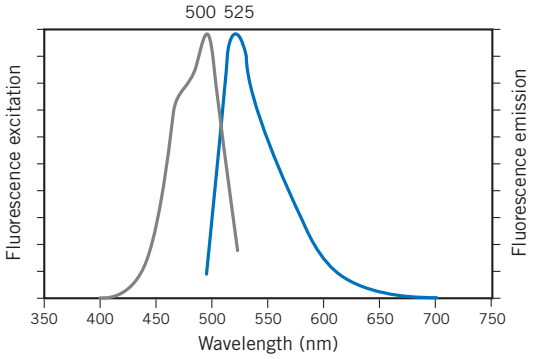

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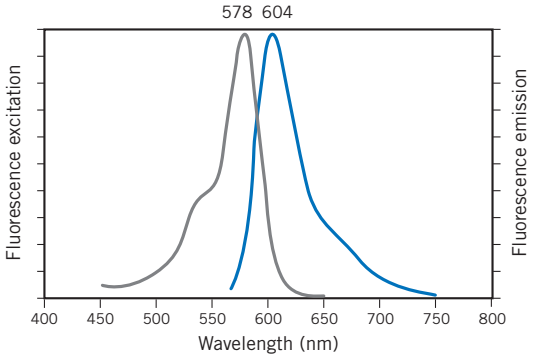
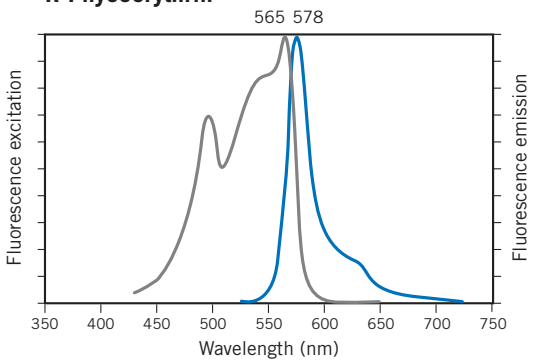


R-Phycoerythrin



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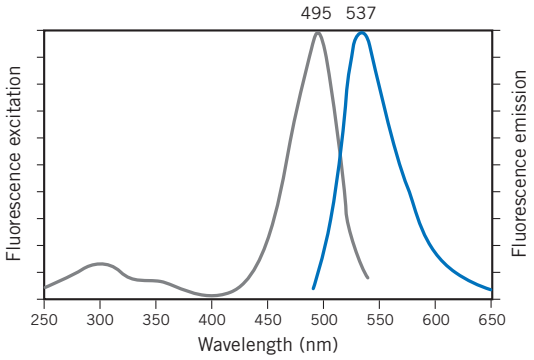

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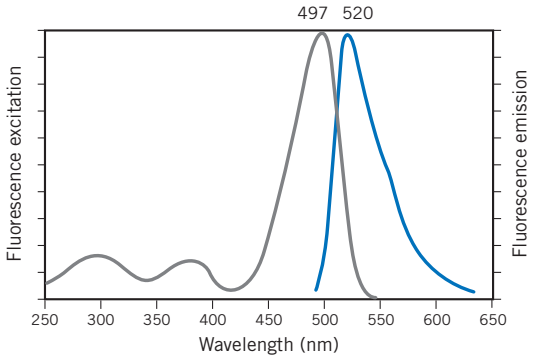

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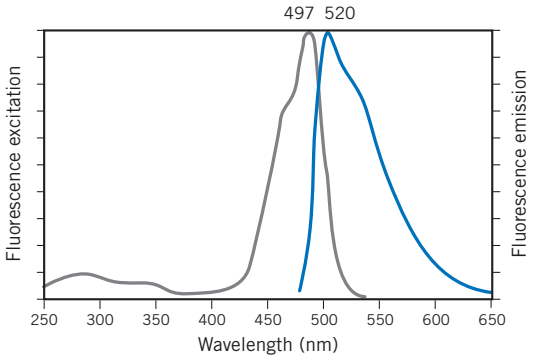

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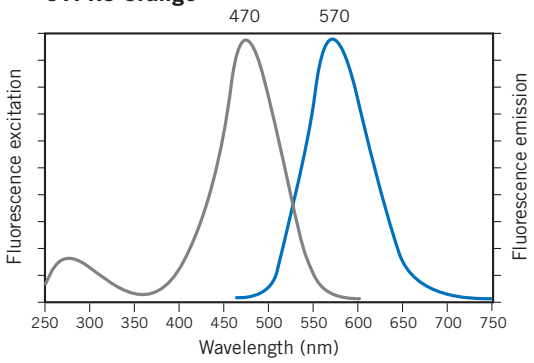

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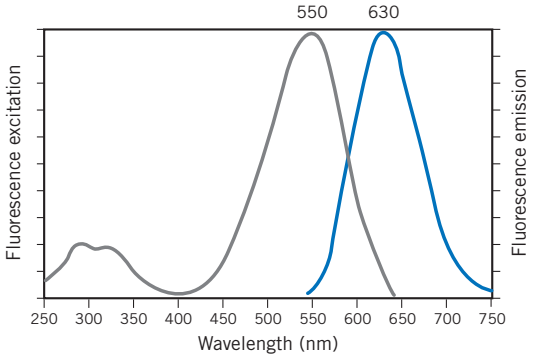

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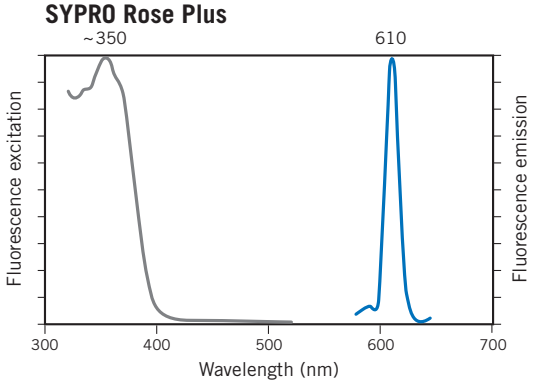

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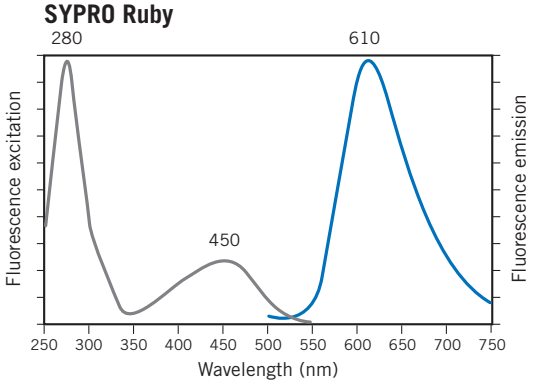
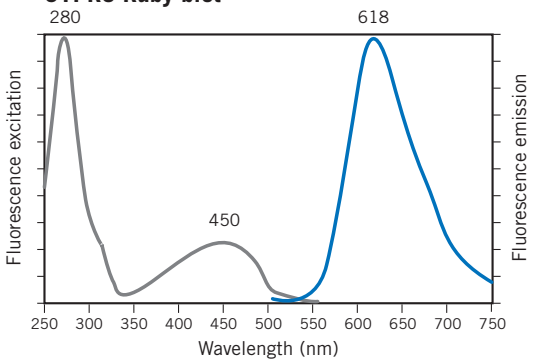
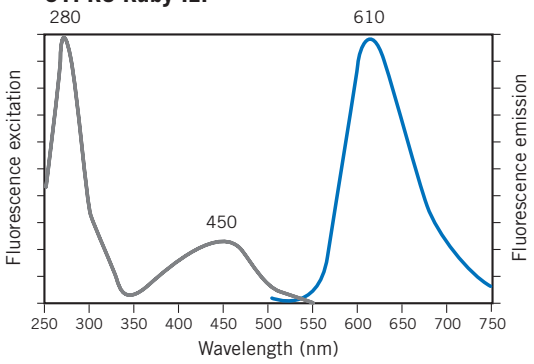


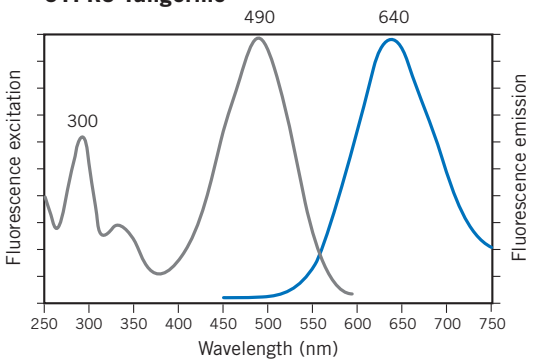
SYPRO Ruby blot



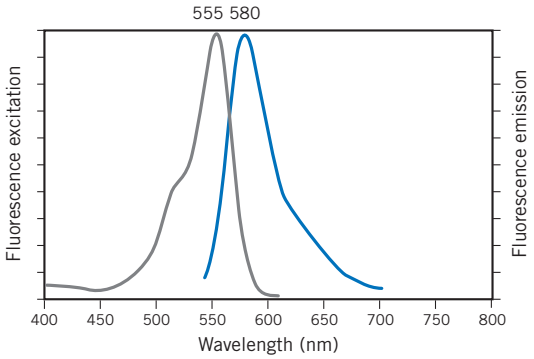
SYPRO Ruby IEF



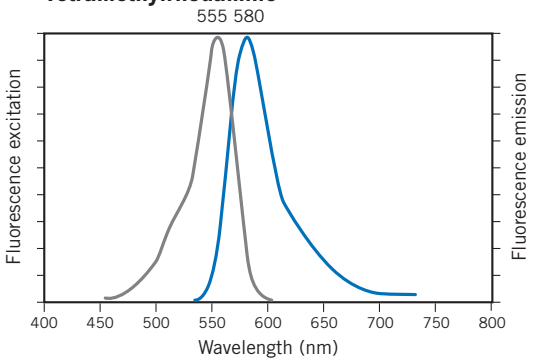
SYPRO Tangerine



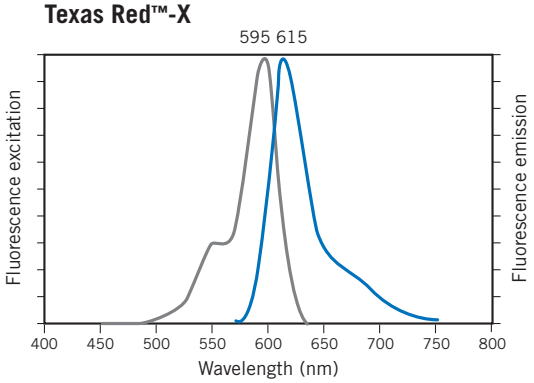
TAMRA



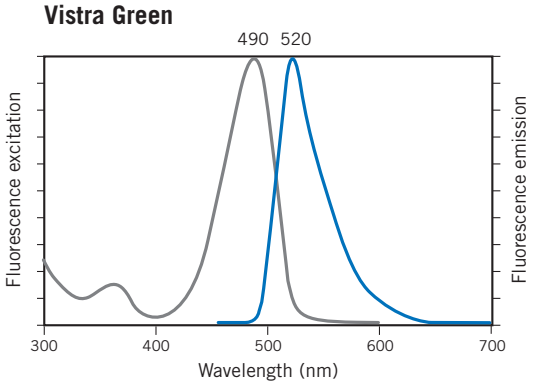
Tetramethylrhodamine



Texas Red™-X



Vistra Green



* Spectra were obtained for the product of the enzymatic reaction on PVDF membrane.

† Spectra were obtained in the presence of nucleic acids.

‡ Spectra were obtained in the presence of protein.

Spectra of Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, DDAO phosphate, ECF, ECL Plus, and FluorX were obtained at Amersham Pharmacia Biotech. DsRed, EBFP, ECFP, EGFP and EYFP spectra are courtesy of Clontech. All other spectra are courtesy of Molecular Probes, Inc.

Appendix 3

INSTRUMENT COMPATIBILITY AND SETUP WITH COMMON FLUOROPHORES AND FLUORESCENT PROTEINS

Fluorophore	Excitation max (nm)	Emission max (nm)	Typhoon		FluorImager		Storm	VDS-CL	
			Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
Nucleic acid gel stains									
Ethidium bromide	526	605	532	610BP30	514	610RG	NA*	Transmission	UV high‡
SYBR Gold	495	537	532	526SP	488	530DF30	Blue	Transmission	UV low‡
SYBR Green I	497	520	532	526SP	488	530DF30	Blue	Transmission	UV low
SYBR Green II	497	520	532	526SP	488	530DF30	Blue	Transmission	UV low
Vistra Green	490	520	532	526SP	488	530DF30	Blue	Transmission	UV low
Protein gel stains									
SYPRO Orange	300, 470	570	532	580BP30	488	570DF30	Blue	Transmission	UV high
SYPRO Red	300, 550	630	532	610BP30	514	610RG	Red	Transmission	UV high
SYPRO Ruby	280, 450	610	532	610BP30	488	610RG	Blue	Transmission	UV high
SYPRO Ruby IEF	280, 450	610	532	610BP30	488	610RG	Blue	Transmission	UV high
SYPRO Tangerine	300, 490	640	532	610BP30	488	610RG	Blue	Transmission	UV high
Nucleic acids solution stains									
OliGreen	500	523	532	526SP	488	530DF30	Blue	NA	NA
PicoGreen	502	523	532	526SP	488	530DF30	Blue	NA	NA
RiboGreen	500	525	532	526SP	488	530DF30	Blue	NA	NA
Protein solution stains									
CBQCA	465	550	532	555BP20	488	570DF30	Blue	NA	NA
NanoOrange	470	570	532	580BP30	488	570DF30	Blue	NA	NA
Substrates for Northern and Southern detection									
DDAO phosphate	646	660	633	670BP30	NA	NA	Red	NA	NA
ECF	440	560	532	526SP	488	570DF30	Blue	Reflection	UV high

Fluorophore	Excitation max (nm)	Emission max (nm)	Typhoon		FluorImager		Storm	VDS-CL	
			Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
Substrates and stains for Western blotting									
DDAO phosphate	646	660	633	670BP30	NA	NA	Red	NA	NA
ECF	440	560	532	526SP	488	570DF30	Blue	Reflection	UV high
ECL Plus	430	503	CL[†]	CL	488	530DF 30	Blue	CL	CL
SYPRO Rose Plus	~ 350	610	NA	NA	NA	NA	NA	Reflection	UV high
SYPRO Ruby blot	280, 450	618	532	610BP30	488	610RG	Blue	Reflection	UV high
Multipurpose labels									
Alexa Fluor 350	346	442	NA	NA	NA	NA	NA	R/T[§]	UV low
Alexa Fluor 430	433	539	NA	NA	NA	NA	Blue	R/T	UV low
Alexa Fluor 488	495	520	532	526SP	488	530DF30	Blue	R/T	UV low
Alexa Fluor 532	532	554	532	555BP20	514	570DF30	NA	R/T	UV high
Alexa Fluor 546	556	573	532	580BP30	514	570DF30	NA	NA	NA
Alexa Fluor 568	578	603	532	610BP30	514	610RG	NA	NA	NA
Alexa Fluor 594	590	617	532	610BP30	NA	NA	NA	NA	NA
Alexa Fluor 633	632	647	633	670BP30	NA	NA	Red	NA	NA
Alexa Fluor 660	663	690	633	670BP30	NA	NA	Red	NA	NA
Alexa Fluor 680	679	702	633	670BP30	NA	NA	Red	NA	NA
BODIPY 630/650	632	640	633	670BP30	NA	NA	Red	NA	NA
BODIPY 650/665	651	660	633	670BP30	NA	NA	NA	NA	NA
BODIPY FL	505	513	532	526SP	488	530DF30	Blue	R/T	UV low
BODIPY TMR-X	535	574	532	580BP30	514	570DF30	NA	R/T	UV high
BODIPY TR-X	588	617	532	610BP30	NA	NA	NA	NA	NA
Cy2	489	506	532	526SP	488	530DF30	Blue	R/T	UV low
Cy3	550	570	532	580BP30	514	570DF30	NA	NA	NA
Cy3.5	581	596	532	610BP30	514	610RG	NA	NA	NA
Cy5	649	670	633	670BP30	NA	NA	Red	NA	NA
Cy5.5	675	694	633	670BP30	NA	NA	Red	NA	NA
Cy7	743	767	NA	NA	NA	NA	NA	NA	NA

Fluorophore	Excitation max (nm)	Emission max (nm)	Typhoon		FluorImager		Storm	VDS-CL	
			Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
Multipurpose labels *(continued)*									
FAM	495	535	532	526SP	488	530DF30	Blue	R/T	UV low
FITC	495	535	532	526SP	488	530DF30	Blue	R/T	UV low
Fluorescein	495	520	532	526SP	488	530DF30	Blue	R/T	UV low
FluorX	494	520	532	526SP	488	530DF30	Blue	R/T	UV low
HEX™	529	560	532	555BP20	514	570DF30	NA	R/T	UV high
JOE	525	557	532	555BP20	514	570DF30	NA	R/T	UV high
Oregon Green 488	496	524	532	526SP	488	530DF30	Blue	R/T	UV low
Oregon Green 514	511	530	532	555BP20	488	530DF30	NA	R/T	UV low
Rhodamine Green	505	527	532	526SP	488	530DF30	Blue	R/T	UV low
Rhodamine Red-X	570	590	532	580BP30	514	570DF30	NA	NA	NA
ROX	578	604	532	610BP30	514	610RG	NA	NA	NA
TAMRA	555	580	532	580BP30	514	570DF30	NA	NA	NA
TET™	519	545	532	555BP20	514	530DF30	NA	R/T	UV high
Tetramethylrhodamine	555	580	532	580BP30	514	570DF30	NA	NA	NA
Texas Red-X	595	615	532	610BP30	NA	NA	NA	NA	NA
Fluorescent proteins									
Allophycocyanin	650	660	633	670BP30	NA	NA	Red	NA	NA
B-phycoerythrin	546	575	532	580BP30	514	570BP30	Blue	NA	NA
R-phycoerythrin	565	578	532	580BP30	514	570BP30	Blue	NA	NA
GFP (wt)	395, 470	508	532	526SP	488	530DF30	Blue	Reflection	UV low
GFP-S65T	488	511	532	526SP	488	530DF30	Blue	Reflection	UV low
EGFP	489	508	532	526SP	488	530DF30	Blue	Reflection	UV low
EYFP	514	527	532	555BP20	488	530DF30	NA	Reflection	UV low
DsRed	558	583	532	580BP30	514	570BP30	NA	NA	NA

* NA = Not applicable.

† CL = Chemiluminescence only. Not applicable for fluorescence.

‡ UV high = 580BP30 filter; UV low = 520BP30 filter.

§ R/T = Reflection (membranes); Transmission (gels).

Appendix 4

INSTRUMENT PERFORMANCE WITH COMMON FLUOROPHORES AND FLUORESCENT PROTEINS

Fluorophore	Typhoon	FluorImager	Storm	VDS-CL
Nucleic acid gel stains (ds DNA)	Limit of detection (pg/band) in agarose/polyacrylamide gel			
Ethidium Bromide	100/ND*	200/100	NA*	100/ND
SYBR Gold	25/10	40/10	500/40	ND/20
SYBR Green I	25/10	40/10	500/40	ND/20
Vistra Green	25/10	40/10	500/40	ND/20
Nucleic acid gel stains (ss DNA)	Limit of detection (pg/band) in agarose/polyacrylamide gel			
Ethidium Bromide	5000/ND	10 000/ND	NA	5000/ND
SYBR Gold	ND/250	ND/300	ND/1000	ND
SYBR Green I	ND/250	ND/300	ND/1000	ND
SYBR Green II (RNA)	10 000/ND	10 000/ND	100 000/ND	ND
Vistra Green	ND/250	ND/300	ND/1000	ND
Protein gel stains	Limit of detection (ng/band)			
SYPRO Orange	2	3	6	5
SYPRO Red	2	2	3	ND
SYPRO Ruby	3	5	7	3
Nucelic acids solution stains	Limit of detection (ng/ml)			
PicoGreen	10/2.5†	5	50	ND
RiboGreen	ND	1	10	ND
Protein solution stains	Limit of detection (µg/ml)			
NanoOrange	1/0.3†	0.5	1	ND

Fluorophore	Typhoon	FluorImager	Storm	VDS-CL
Substrates for Northern and Southern detection		Limit of detection (pg target)		
DDAO phosphate	0.25	NA	0.25	NA
ECF	0.5	0.25	0.25	0.25
Substrates for Western blotting‡		Limit of detection (ng target)		
DDAO phosphate	4	NA	4	NA
ECF	8	4	4	4
ECL Plus	CL§	5	1–2	CL
Multipurpose labels	Limit of detection (fmol DNA/band) in polyacrylamide gel			
Alexa Fluor 430	NA	200	100	ND
Cy2	7.5	7.5	30	ND
Cy3	0.2	4	NA	ND
Cy3.5	0.2	ND	NA	ND
Cy5	0.2	ND	1	ND
FAM	0.4	0.4	50	ND
Fluorescein	0.4	0.4	50	ND
HEX	0.2	2	NA	ND
ROX	0.2	12	NA	ND
TAMRA	0.2	4	NA	ND
TET	ND	1	NA	ND
Fluorescent proteins	Limit of detection (ng protein/band) in SDS-polyacrylamide gel			
GFP (wt)	13	2	13	ND
GFP-S65T	ND	0.3	8	ND
EGFP	ND	0.3	8	ND

* ND = Not determined; NA = Not applicable.

† First number from assay performed using Costar flat-bottomed plate/Second number from assay performed using Nunc Separable Strips.

‡ Detection limits, or sensitivities, for Western blots depend on multiple experimental factors, including the types and concentrations of protein target and antibodies used. Each new detection protocol should be optimised for concentrations of both primary and secondary antibodies.

§ CL = Chemiluminescence only. Not applicable for fluorescence.

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