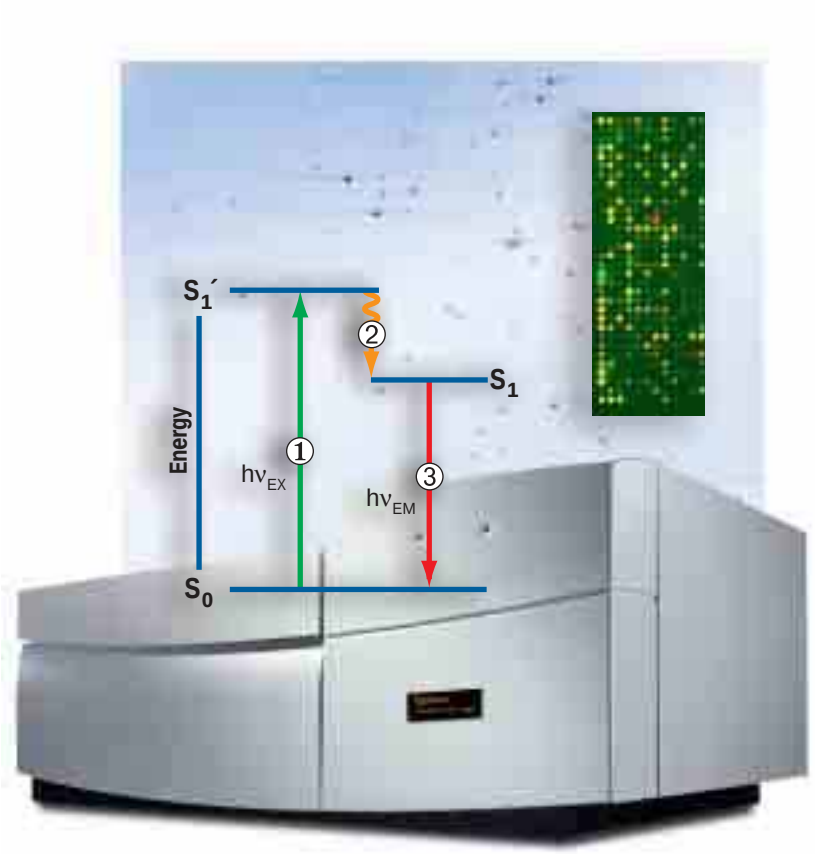
handbook

Fluorescence Imaging

principles and methods



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Amersham Biosciences

Contents

Chapter 1

INTRODUCTION TO FLUORESCENCE

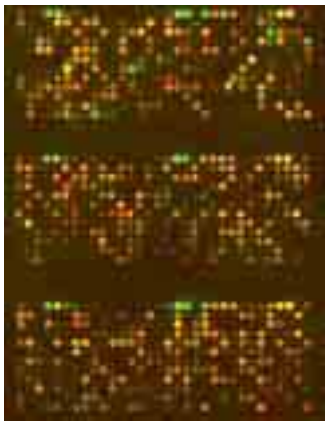


Fig 1. A microarray slide, spotted with DNA from Amersham Biosciences Lucidea™ Microarray ScoreCard™ control plate and DNA from Incyte Genomics, Inc. using Amersham Biosciences Generation III Array Spotter, was hybridized with Cy™3-labelled skeletal muscle cDNA and Cy5-labelled liver cDNA. The slide was scanned on Typhoon™ 9410 Variable Mode Imager with the 10-µm pixel size option.

1.1 Advantages of fluorescence 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 (1, 2). From genomics to proteomics, commonly used techniques such as total nucleic acid and protein quantitation, Western, Northern, and Southern blotting, PCR✧ product analysis, microarray analysis, and DNA sequencing can all benefit from the application of fluorescence detection. Fluorescence 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 generally far more sensitive than traditional colorimetric methods for detecting total DNA, RNA, and protein. Many fluorescence applications approach the sensitivity afforded by radioisotopes.

Multicolor detection

Multicolor fluorescence detection allows the detection and resolution of multiple targets using fluorescent labels that can be spectrally resolved. The ability to detect and analyze two or more labelled targets in the same sample eliminates sample-to-sample variation and is both time- and cost-effective. For example, two-color fluorescence imaging is used in differential gene profiling with microarray analysis. This is usually done by labelling cDNAs from two different samples with two different fluorochromes, such as Cy3 or Cy5 (Fig 1).

Stability

Fluorescently labelled molecules offer several distinct advantages over radiolabelled molecules with respect to stability. Fluorescent antibodies, oligonucleotide hybridization probes, and PCR primers can be stored for six months or longer, whereas 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, thus

✧ See licensing information on inside back cover.

minimizing reagent variability between assays. This is advantageous for applications such as DNA and protein sizing and quantitation, enzyme assays, immunoassays, PCR-based genetic typing assays, microarray analysis, 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, including monoclonal and polyclonal antibodies, are available in fluorochrome-labelled forms. 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). In addition, a wide variety of fluorescent labelling kits for biological molecules are commercially available. Some companies offer customized fluorescent labelling depending on the customer's specific needs.

Lower cost

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

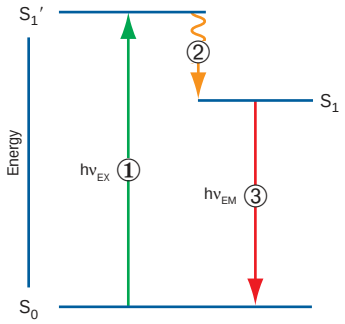


Fig 2. Jablonski diagram illustrating the processes involved in creating an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. ① Excitation; ② Vibrational relaxation; ③ Emission.

1.2 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 singlet state (S_1'). This process is distinct from chemiluminescence, in which the excited state is created by a chemical reaction.

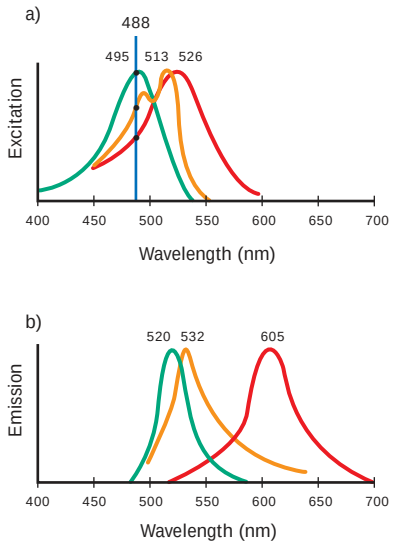


Fig 3. Excitation (a) and emission (b) spectra of fluorescein (green), DNA-bound TOTO™ (orange), and DNA-bound ethidium bromide (red). Curves are normalized to the same peak height. The wavelength at which maximum excitation (a) or maximum emission (b) occurs is shown above each curve. The position at 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 Biosciences 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 this 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 quantitate the resulting image.

1.3 Properties of fluorochromes

1.3.1 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 emitted fluorescence versus excitation wavelength, and it is identical or very similar to the absorption spectrum (Fig 3a) commonly provided by fluorochrome manufacturers.

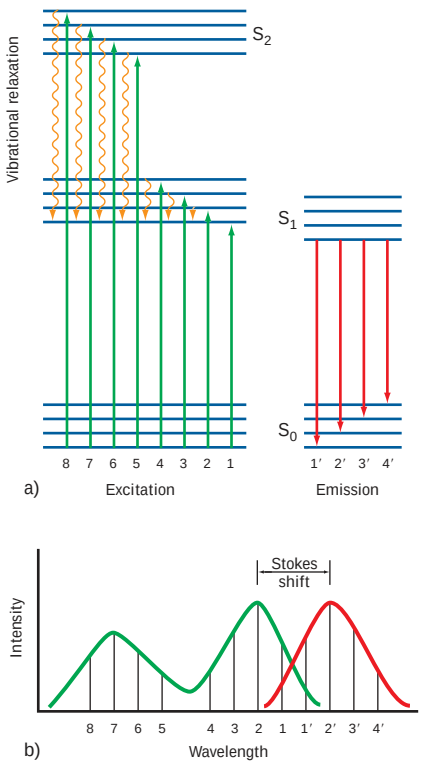


Fig 4. Diagram of the energy levels of a fluorochrome molecule, including superimposed vibrational energy levels (a) and an example of excitation and fluorescent spectra (b).

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 theory, a fluorochrome is most effectively excited by wavelengths nearest to the apex of its excitation peak. For example, the excitation of fluorescein (apex at 495 nm) at 488 nm results in high excitation efficiency, whereas exciting either DNA-bound TOTO (apex at 513 nm) or DNA-bound ethidium bromide (apex at 526 nm) at 488 nm results in less optimal excitation efficiencies compared to the maximal (Fig 3a). In practice, however, other factors including laser power and the environment of the fluorochromes can also affect the excitation efficiency.

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 equal to the energy difference between the base level of the excited state and 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

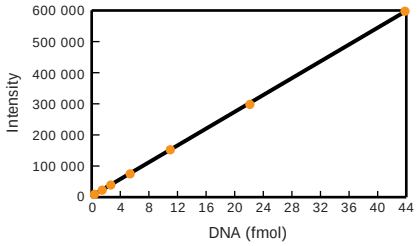


Fig 5. Fluorescence linearity. A 24-mer DNA oligonucleotide, 5' end-labelled with fluorescein (two-fold serial dilutions) was detected in a denaturing polyacrylamide gel sandwich using the Typhoon Imager with 532 nm excitation and 526 SP emission filter. The plot shows signal linearity over a range of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

excitation peak is known as the Stokes shift. This shift in wavelength (energy) represents the energy dissipated as heat during the lifetime of the excited state before the fluorescent light is emitted. Since the excitation and emission peaks are spectrally separated, the interference from excitation photons can be effectively removed from emission photons by using appropriate optics. This approach reduces background and noise, and therefore improves sensitivity of fluorescence techniques. See Appendix 2 for excitation and emission spectra of many commonly used fluorophores.

1.3.2 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).

1.3.3 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 Cy5 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. In theory, a more intense source will yield the greater fluorescence. However, in actual practice, photodestruction of the sample can occur when high intensity light is delivered over a prolonged period of time.

1.3.4 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 thereby reducing 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 excitation cycles.

1.4 Quantitation of fluorescence

As discussed previously, the energy (wavelength) of the emitted fluorescent light is a statistical function of the available energy levels in the fluorochrome, 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.

In general, when more intense light is used to illuminate a sample, more of the fluorochrome molecules are excited, and the number of photons emitted increases. 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, causing the resulting current to be 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

2.1 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.

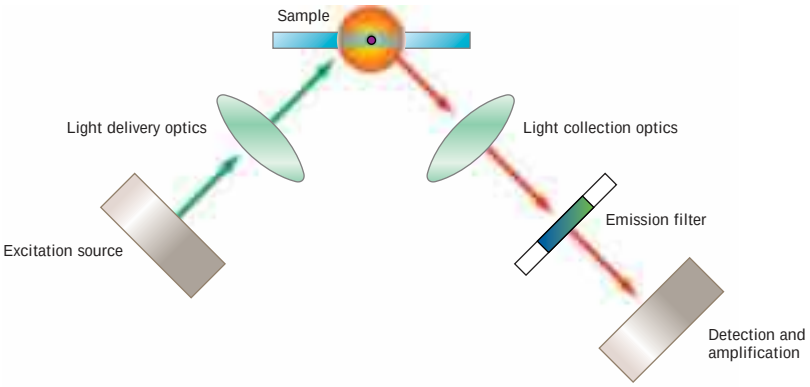


Fig 6. Components of a general fluorescence imaging system.

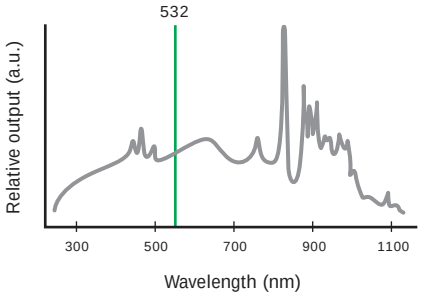


Fig 7. Spectral output of light from a xenon lamp and Nd:YAG laser. The "relative output" axis is scaled arbitrarily for the two light sources. The 532-nm line of the Nd:YAG laser is shown in green.

2.1.1 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.

2.1.2 Light collection optics

High-quality optical elements, such as lenses, mirrors, and filters, are integral components of any efficient imaging system. Optical 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.)

2.1.3 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.

2.1.4 Detection, amplification and digitization

For detection and quantitation 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.

2.2 Scanner systems

2.2.1 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 systems 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 457 nm, 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" fluorochromes. 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 "red-excited" fluorochromes such as Cy5. 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.

2.2.2 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, predominantly 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.

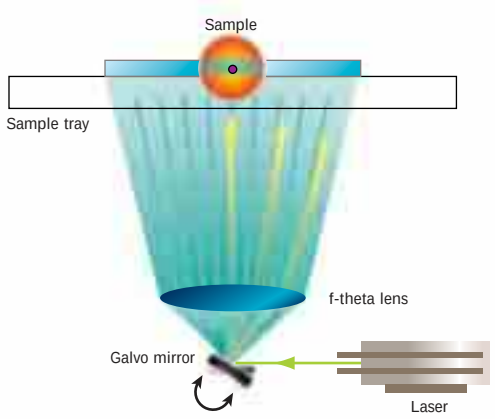


Fig 8. Galvanometer-controlled scanning mechanism. Light is emitted from the laser in a single, straight line. The galvanometer mirror moves rapidly back and forth redirecting the laser beam and illuminating the sample across its entire width (X-axis). The f-theta lens reduces the angle of the excitation 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 for accurate signal quantitation.

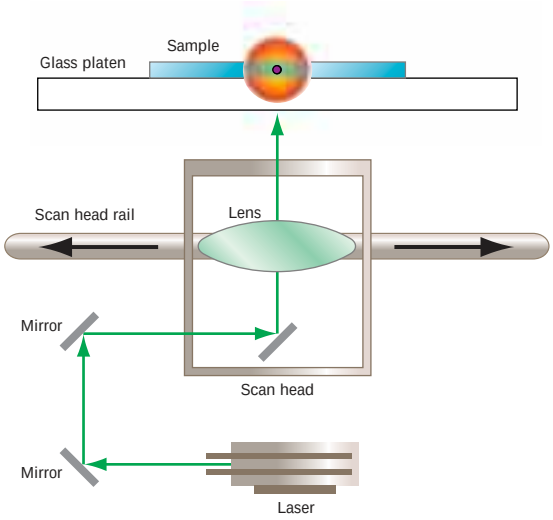


Fig 9. Moving-head scanning mechanism. The light beam from the laser is folded by a series of mirrors and ultimately reflected onto the sample. The sample is illuminated across its width as the scan head moves along the scan head rail (X-axis). The entire sample is illuminated by the scan head, laser,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

2.2.3 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 (fiber 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 artifacts. 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 artifact 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.

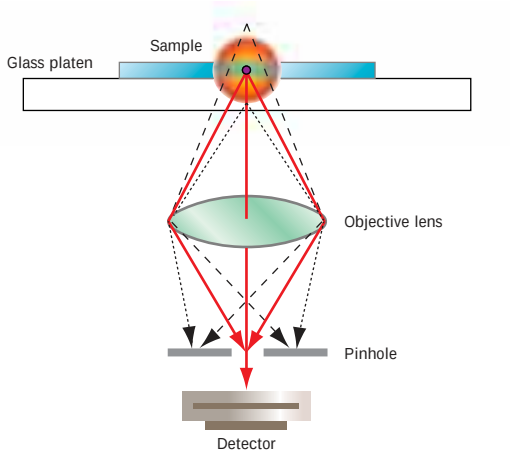


Fig 10. Illustration of confocal optics. Fluorescence from the sample is collected by an objective lens and directed toward a pinhole aperture. The pinhole allows the emitted light from a narrow focal plane (red solid lines) to pass to the detector, while blocking most of the out-of-focus light (black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

2.2.4 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.)

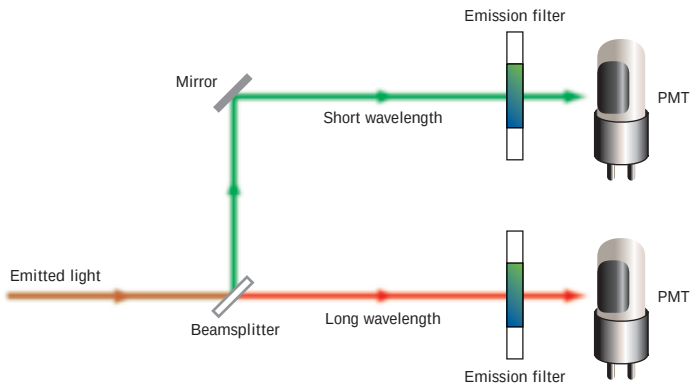


Fig 11. Use of a beamsplitter or dichroic filter with two separate PMTs. Light from a dual color sample enters the emission optics as a combination of wavelengths. A dichroic beamsplitter distinguishes light on the basis of wavelength. Wavelengths above the beamsplitter range pass through, those below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

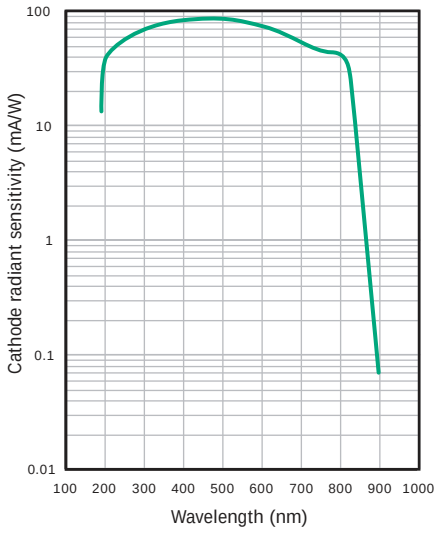


Fig 12. An example of the response of a PMT versus wavelength.

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.

2.2.5 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 of an instrument refers to its ability to distinguish between two very closely positioned objects. 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 interval (6). Thus, to resolve a 100 µm sample, the sampling interval must be at most 50 µm.

Amplitude resolution, or gray-level quantitation, 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 quantitation. A scanner with a wide dynamic range can detect and accurately quantify signals 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 quantitation. 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.

Detection limit is the minimum amount of sample that can be detected by an instrument at a known confidence level. From an economical standpoint, instruments with better detection limits are more cost-effective because they require less fluorescent sample for analysis.

2.3 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.

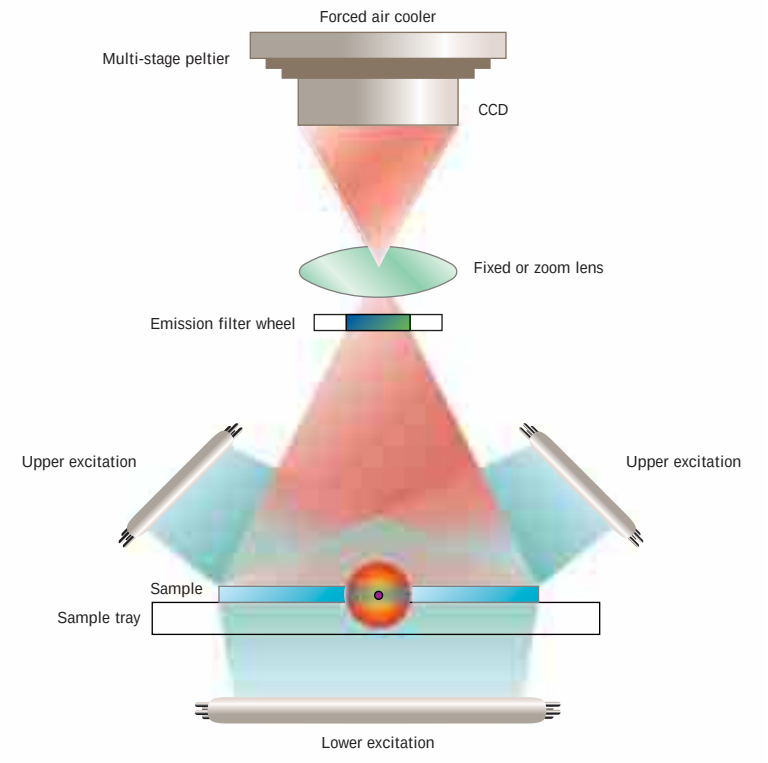


Fig 13. Components of a typical CCD camera-based imaging device. The sample can be illuminated in a variety of ways depending on the nature of the labels to be analysed. The sample is then viewed by the camera. The camera includes focusing optics to accommodate samples at different heights. Emission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

2.3.1 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.

2.3.2 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.

2.3.3 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.

2.3.4 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.

2.4 Amersham Biosciences imaging systems

Amersham Biosciences 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.amershambiosciences.com.

Table 1. Amersham Biosciences imaging systems

TYPHOON 8600, 8610, 9200, 9210, 9400, OR 9410

High performance laser scanning system

Excitation sources : 532-nm Nd:YAG and 633-nm HeNe lasers (all models) Argon Ion laser, 2 lines: 457-nm and 488-nm (9400/9410 models only)

Filters : 7 emission filters, 3 beamsplitters, and up to 13 emission filter positions (9400/9410 models)

6 emission filters, 3 beamsplitters, and up to 13 emission filter positions (9200/9210 models)

6 emission filters, 2 beamsplitters, and up to 13 emission filter positions (8600/8610 models)

Detection : 2 high-sensitivity PMTs

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

Scanning area : 35 x 43 cm

Sample types : Gel sandwiches, gels, blots, microplates, TLC plates, macroarrays, and microarrays (8610, 9210 and 9410 models)

STORM™ 830, 840 OR 860

Variable mode laser scanning system

Excitation sources : 450-nm LED and/or 635-nm laser diode

Filters : 2 built-in emission filters

Detection : High-sensitivity PMT

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

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 colorimetric detection

Scanning area : 21 x 25 cm

Sample types : Gels, blots, and TLC plates

Focus : Automated

Chapter 3

FLUOROCHROME AND FILTER SELECTION

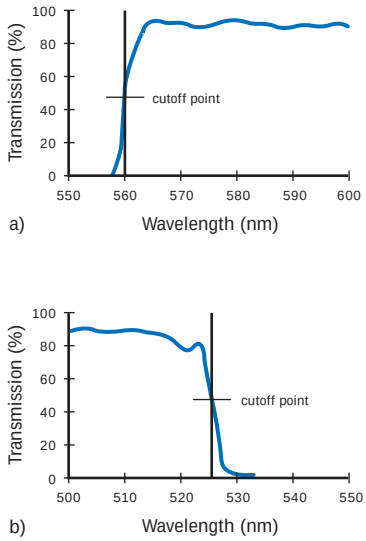


Fig 14. Transmission profiles for a 560 nm long-pass (a) and a 526 nm short-pass (b) filter. The cutoff points are noted.

3.1 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-color and multicolor imaging.

3.2 Types of emission filters

The composition of emission filters used in fluorescence scanners and cameras ranges from simple colored 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 560 LP 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 colored-glass absorption filters, whereas E, LP, and EFLP filters are coated interference filters. Colored-glass filters are less expensive and have more gradual transition slopes than coated interference filters.

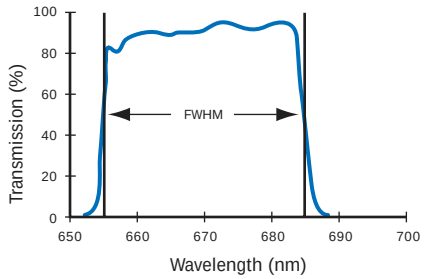


Fig 15. Transmission profile for a band-pass (670 BP 30) filter. The full-width at half maximum (FWHM) transmission of 30 nm is indicated by the arrows.

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 526 SP 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. Band-pass filters provide 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 670 BP 30 filter passes a band of light centred at 670 nm (Fig 15).
- the full-width at half-maximum transmission (FWHM). For example, a 670 BP 30 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.

3.3 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

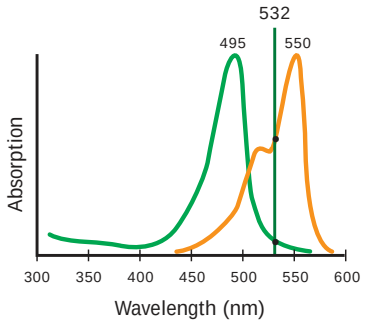


Fig 16. Excitation of fluorescein (green) and Cy3 (orange) using 532 nm laser light. The absorption spectra of Cy3 and fluorescein are overlaid with the 532 nm wavelength line of the Nd:YAG laser.

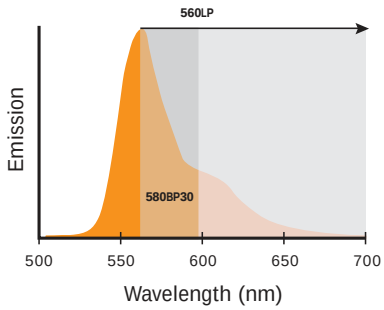


Fig 17. Emission filtering of Cy3 fluorescence using either a 580 BP 30 (dark gray area) or a 560 LP filter (light and dark gray areas).

only slight attenuation of the signal from the fluorochrome. Therefore, 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 most 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.

3.4 General guidelines for selecting fluorochromes and filters

3.4.1 Single-color 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 580 BP 30 or a 560 LP 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 Biosciences fluorescence

scanning systems.

Fig 18. Two-color 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-color 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.

3.4.2 Multicolor imaging

Multicolor 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 two-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-color Western blot experiment where two protein targets are differentially probed using antibodies conjugated with two different fluorescent tags.

The use of multicolor 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 multicolor image acquisition varies depending on the imaging system. An imager with a single detector acquires 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.

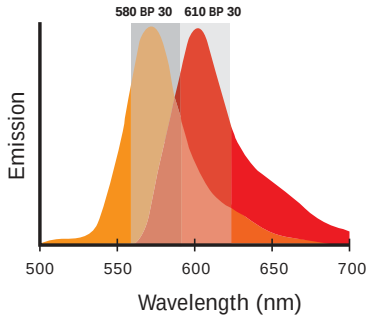


Fig 20. Emission spectra of TAMRA (orange) and ROX (red). A 580 BP 30 filter (dark gray) was used for TAMRA, and a 610 BP 30 filter (light gray) was used for ROX.

Implementation of dual detection requires a beamsplitter filter to spectrally split the mixed fluorescent signal, directing the resulting two 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 multicolor experiments

When designing multicolor 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 multicolor experiments when it provides selectable emission filters suitable for a variety of fluorochrome labels. A range of narrow band-pass filters that match the peak emission wavelengths of commonly used fluorochrome labels will address most multicolor 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

4.1 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, quantitation, 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 multicolor 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.

4.2 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 65 536 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 quantitation. 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 color) values of interest. For example, by increasing the low values, image noise or background can be visually 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. Multicolor software will also allow either side-by-side display of the individual channels or a multicolor 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 response 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.

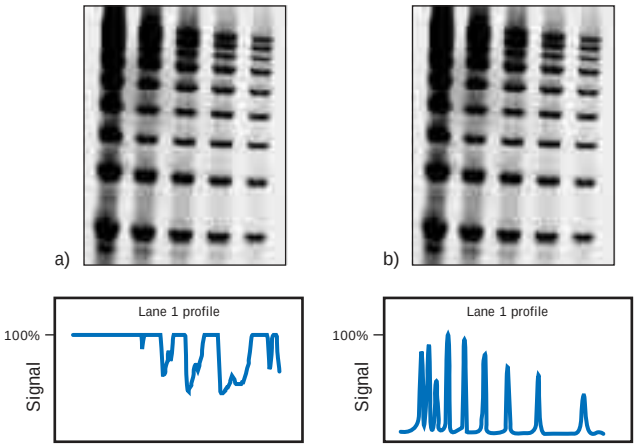


Fig 21. Effect of detector saturation on data quality. A Cy5-labelled size standard was resolved in a 10% polyacrylamide gel and imaged on Typhoon Imager using a PMT setting of 1000 V (panel a) or 500 V (panel b). The line profiles through lane 1 of each image show the response of the PMT to the fluorescent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

4.3 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).

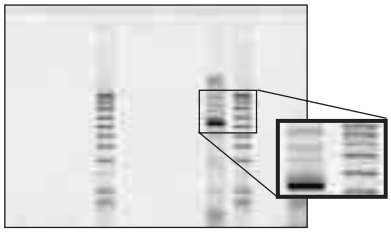


Fig 22. Magnification or zooming to view details of an image.

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.

4.4 Quantitation

4.4.1 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 quantitation. Although they all calculate integrated fluorescent signal, they do so in different ways as outlined below:

Lane profile – Area –	**Object quantitation – Volume –**	**Lane quantitation – 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

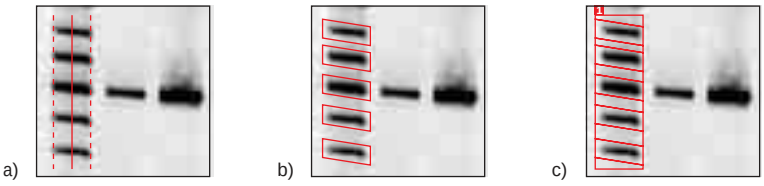
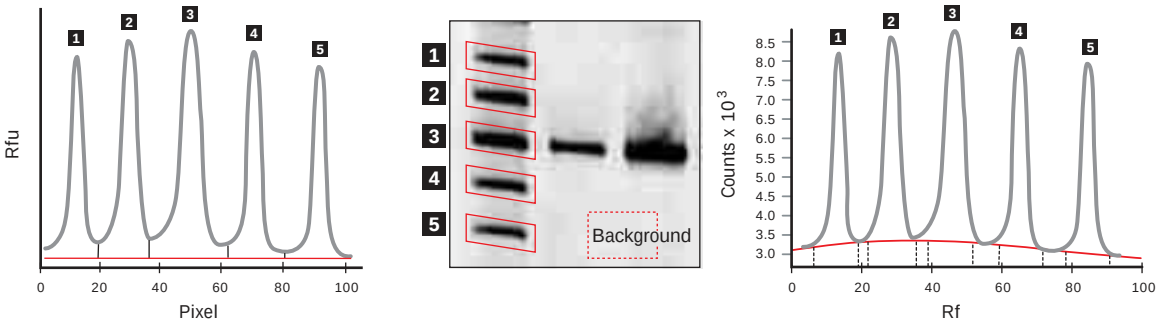


Fig 23. Three methods for signal quantitation. Line profile and integration of area under the curve (panel a); integration of signal from manually created closed objects (panel b); software-assisted detection and quantitation of lane and bands (panel c).

The lane profile quantitation 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 quantitation methods, analysis targets (i.e. bands, spots, slots) are enclosed using objects such as boxes, rectangles, polygons, or ellipses. Both manual (Fig 24b) and automated (Fig 24c) tools for lane and band identification are available. Quantitation 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 quantitation (see section 4.4). All the image pixels bounded by each object are used for quantitation. 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).



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

a)

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

b)

Band	Volume	Band %
1	750235.49	16.03
2	1018636.94	21.76
3	1326294.64	28.33
4	852357.26	18.21
5	733996.99	15.68

c)

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.

4.4.2 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).

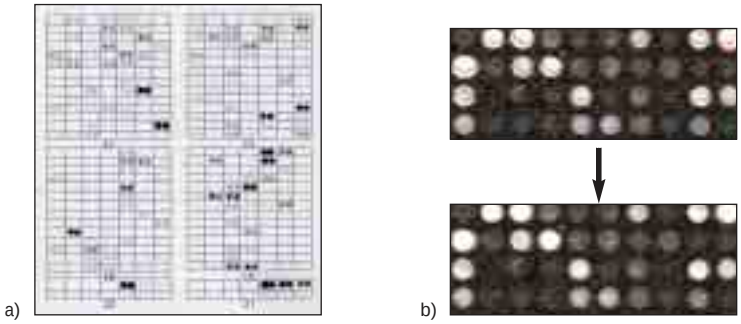


Fig 25. Approaches to software analysis of arrays. In panel a, simple arrays (low-density dot blots, microplates) are analysed using a grid or series of rectangles to surround each array element. In panel b, dedicated array software packages employ spot-finding and/or flexible array templates that find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

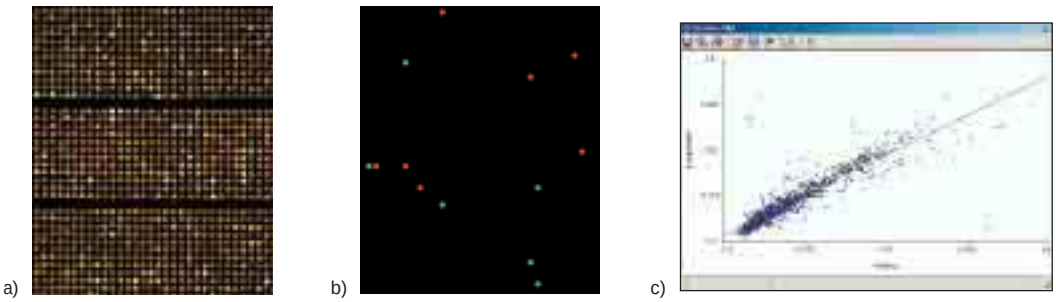
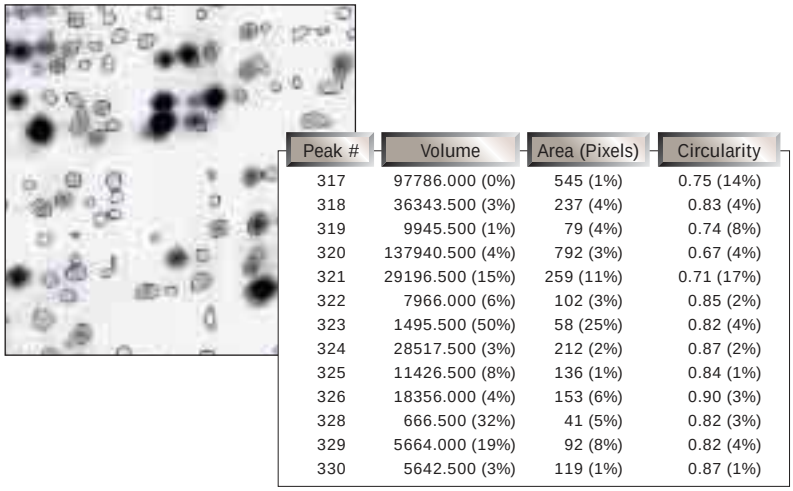


Fig 26. Display and analysis of array experiments. In panel a, images from a two-channel gene expression array using Cy3 (green) and Cy5 (red) labels are overlaid or merged. Levels of gene over- or under-expression are indicated by the relative strength of the green and red colors, respectively. Software 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.

4.4.3 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	97786.000 (0%)	545 (1%)	0.75 (14%)
318	36343.500 (3%)	237 (4%)	0.83 (4%)
319	9945.500 (1%)	79 (4%)	0.74 (8%)
320	137940.500 (4%)	792 (3%)	0.67 (4%)
321	29196.500 (15%)	259 (11%)	0.71 (17%)
322	7966.000 (6%)	102 (3%)	0.85 (2%)
323	1495.500 (50%)	58 (25%)	0.82 (4%)
324	28517.500 (3%)	212 (2%)	0.87 (2%)
325	11426.500 (8%)	136 (1%)	0.84 (1%)
326	18356.000 (4%)	153 (6%)	0.90 (3%)
328	666.500 (32%)	41 (5%)	0.82 (3%)
329	5664.000 (19%)	92 (8%)	0.82 (4%)
330	5642.500 (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.

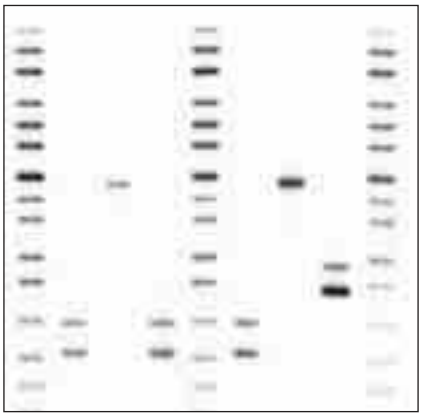
4.5 Background correction

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 fluorescence detection chemistry used, the sample matrix (i.e. gel, membrane, microplate), and the integrity or quality of the sample itself. Because fluorescence 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).

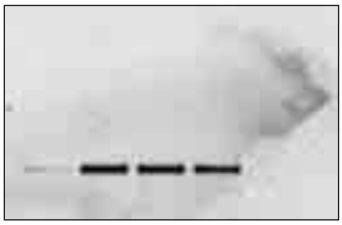
The nature of the background signal should be assessed before proceeding with image analysis (Fig 28). Background commonly appears as:

- 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

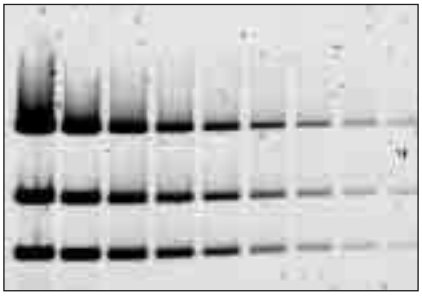
Fig 28. Examples of different types of fluorescent image background.



a) Uniform



b) Non-uniform



c) Noise spikes

d) Lane-specific

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 neighborhood of a band, spot, or slot target to be quantitated. Depending on the quantitation 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).

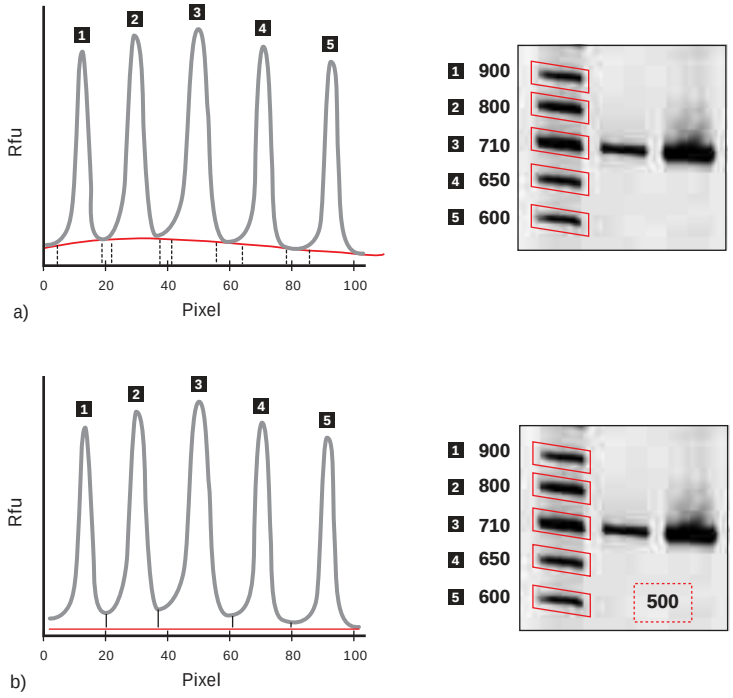


Fig 29. Comparison of local and global background correction methods applied to the same image. The local method (panel a) uses different background values at each band in the gel lane, with background based on the average signal from the boundary of each band. In panel b, a single global background 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 quantitation 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 quantitation. 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 quantitation. In the same way, the boundaries used to define analysis targets—bands, spots, or slots—will also impact the results of quantitation. 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.

4.6 Image processing tools

Software utilities for image processing functions improve the accuracy of quantitation for both single-color and multicolor images.

Resolution of fluorescent signal overlap in multicolor images

Overlap between the emission spectra of fluorochromes is a common and almost unavoidable aspect of multicolor 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 quantitation 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.

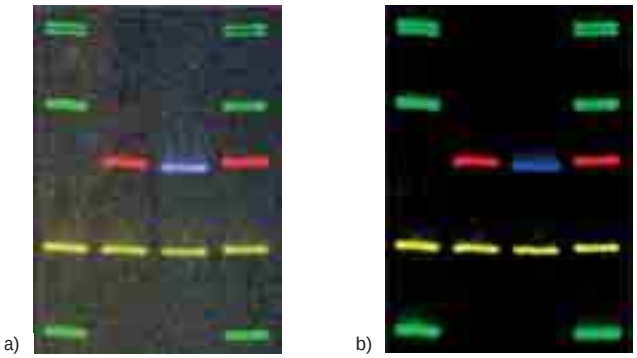


Fig 30. Multicolor image processing using a fluorochrome separation routine. Spectral contamination in this four-color image, particularly evident in the blue and yellow channels (a), is reduced to give a better representation of the signal from each of the four fluorochrome labels (b).

Image filtering

Image artifacts caused by dust or bubbles complicate fluorescence analysis of gels and membranes. Removing these artifacts 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.

4.7 Amersham Biosciences image analysis software

Image analysis is an integral part of today's life science applications. Amersham Biosciences 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 Biosciences 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

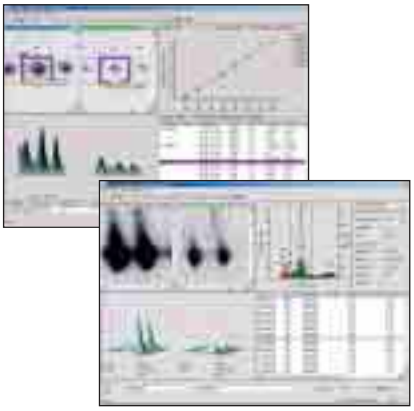
ImageQuant tools

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

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

DeCyder™ Differential Analysis Software

Powerful tool for automatic analysis of 2-D protein gels containing multi-color samples labelled with CyDye™ DIGE Fluors



DeCyder Differential Analysis Software

- Automatic analysis
- Requires no manual image editing
- No spot matching within gels
- Designed to use an internal standard

DeCyder software consists of four modules:

Differential In-Gel Analysis (DIA)

- Automatic co-detection of image pairs
- Automated detection, background subtraction, quantitation, normalisation and first level (In-Gel) matching
- Minimal user intervention

Batch processor

- Automatic DIA detection and BVA matching of several hundred images without user intervention

Biological Variation Analysis (BVA)

- Automatic gel-to-gel matching
- Automatic statistical analysis and trend visualisation
- Minimal gel-to-gel variation with use of internal standard

XML toolbox

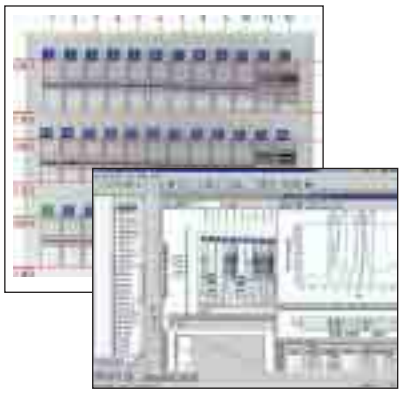
- Data extraction of relevant information
- Data conversion from XML to HTML and text files
- Automatic report generation
- Open structure for custom script generation

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

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

- Robust algorithm for spot finding and quantitation, quality metrics, and use of pre-made protocols ensure accuracy and reproducibility
- Choices for background correction, normalization, and ratio calculation between arrays provides flexibility
- Wizard guides simplify operation
- Automatic batch-processing increases throughput and saves time
- Data easily exported to Microsoft Excel or database programs for flexibility in reporting

Chapter 5

FLUORESCENCE APPLICATIONS USING AMERSHAM BIOSCIENCES IMAGING SYSTEMS

This chapter gives an overview on fluorescence imaging applications and provides the basic information necessary for maximizing the fluorescence imaging capabilities of your system. Fluorescence methods in common molecular biology methods, such as nucleic acids gel electrophoresis, one-dimensional and two-dimensional protein gel electrophoresis, blotting, solution analysis of nucleic acids and proteins, as well as DNA microarray is discussed. Typical protocols for each application area are included, together with materials, suggestions, and tips for successful implementation of fluorescence detection. Available fluorescent stains, chemifluorescent substrates, and covalent fluorochrome labels are described along with Amersham Biosciences instrument compatibility and recommendations for imaging setup and analysis.

5.1 Introduction to fluorescence imaging applications

5.1.1 Fluorescent stains

Fluorescence staining is a widely used detection method for proteins and nucleic acids. A number of stains, such as the SYPRO™ stains for protein detection and Vistra Green™ for nucleic acid detection, have a convenient property: their fluorescence quantum yields are significantly increased when non-covalently bound to biological molecules (2). Moreover, the intensity of fluorescence staining generally demonstrates a correlation with the amount of the target biological molecules, allowing the quantitation of these molecules. Many fluorescent stains offer great sensitivity and detection limits, as well as a much wider linear dynamic range than the traditional non-fluorescent staining methods such as Coomassie™ Blue staining. Because of the non-covalent binding nature of the stains, no complex chemical reactions are involved in the staining protocols. Many fluorescent stains allow a simple one-step staining procedure without a destaining step.

Fluorescent protein stains are frequently used to quantitate the amounts of proteins, visualize the separation of the protein complex mixtures on gels, verify the purity of the protein samples, or investigate the composition of protein subunits. Fluorescent nucleic acid stains are often used to quantitate the amounts of nucleic acids, visualize the results of DNA/RNA preparations, restriction digests, and PCR reactions. The uses

of various fluorescent gel and solution stains for nucleic acids and proteins are described in more detail in sections 5.2 and 5.9 respectively.

5.1.2 Covalent fluorescence labelling of nucleic acids and proteins

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 and fluorochrome-conjugated antibodies. These tagged molecules are widely used in a variety of applications, including DNA typing, differential display, RT-PCR, and Western blotting. Fluorescence energy transfer between the matching fluorochromes is also utilized in some life science applications.

Fluorochrome labels are available in reactive forms that are 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 multicolor experiments. Multicolor fluorescence imaging allows multiple fluorescent labels to be detected in the same experiment, which improves both throughput and accuracy of the analyses. See Appendix 3 for an extended list of multipurpose labels.

Nucleic acid labelling

The fluorescence modification 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—at the 5'-end, at the 3'-end, or at any internal base position. Oligonucleotides can also be designed to exhibit fluorescence resonance energy transfer (ET) properties (9). 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. Commercial 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* 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 (10, 11).

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. It is extremely important to make sure that the fluorescence labelling does not alter the function of the biomolecules.

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. A wide variety of fluorochrome-conjugated anti-species antibodies are commercially available.

5.1.3 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 (12). 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 (13).

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 sorting (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 improve upon these limitations (14). 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 and applications of green fluorescent protein and its variants are discussed in section 5.13.

Phycobiliproteins

Phycobiliproteins are stable, highly soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae. 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 center. 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 $M^{-1}cm^{-1}$ (2). 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. The spectral properties and applications of phycobiliproteins are described in section 5.13.

5.1.4 Chemifluorescence applications

Chemifluorescence has found wide applications in immunoassay and immunoblotting applications. Reagents such as primary or secondary antibodies are conjugated to enzymes. Upon the addition of a suitable fluorogenic substrate, a catalytic reaction between enzyme and substrate results in the formation of a fluorescent product at the site of the reaction as well as a significant amplification of the signal. Details of using chemifluorescence in Southern, Northern, and Western blotting applications are described in sections 5.4 and 5.12.

5.2 Detection of nucleic acids in gels

5.2.1 Nucleic acid gel stains

Fluorescence 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 detection limits 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 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 safety 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. See reference 15 for more details about this application.

Table 3. Nucleic acid gel stains

Stain	Excitation max (nm)	Emission max (nm)	Fluorescence application
Ethidium bromide	526	605	Classic general purpose nucleic acid stain
SYBR Gold	495	537	Ultrasensitive gel stain for dsDNA and oligonucleotides
SYBR Green I	497	520	Ultrasensitive gel stain for dsDNA and oligonucleotides
SYBR Green II	497	520	Ultrasensitive gel stain for RNA and ssDNA
Vistra Green	490	520	Ultrasensitive gel stain for ss or dsDNA or RNA

5.2.2 Instrument compatibility

Fluorescence imaging systems from Amersham Biosciences combine powerful excitation sources with efficient optics for sensitive fluorescence imaging of common DNA gel stains, including ethidium bromide, Vistra Green, SYBR Green, and SYBR Gold (15,16). Setup for the various instruments is given in Table 4.

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

Stain	Typhoon				FluorImager		Storm	VDS-CL	
	9400/9410		8600/8610/9200/9210						
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
Ethidium bromide	532	610 BP 30	532	610 BP 30	514	610 RG	NA	Transmission	UV high
SYBR Gold	488	520 BP 40	532	526 SP	488	530 DF 30	Blue	Transmission	UV low
SYBR Green I	488	520 BP 40	532	526 SP	488	530 DF 30	Blue	Transmission	UV low
SYBR Green II	488	520 BP 40	532	526 SP	488	530 DF 30	Blue	Transmission	UV low
Vistra Green	488	520 BP 40	532	526 SP	488	530 DF 30	Blue	Transmission	UV low

NA = not applicable

5.2.3 Typical protocol

Amersham Biosciences products available for this application

Product used	Code number
Hoefer™ EPS 301 Power Supply	18-1130-01
Hoefer HE 99X Max Submarine Unit	80-6061-57
Hoefer miniVE Vertical Electrophoresis System	80-6418-77
PlusOne™ Ethidium Bromide Solution, 10 mg/ml	17-1328-01
Vistra Green Nucleic Acid Gel Stain, (makes 5 l)	RPN5786
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog

❶

Sample preparation

1.1 Mix the DNA samples with loading buffer.

Note: Bromophenol blue, xylene cyanol, and other electrophoresis tracking dyes autofluoresce, which can potentially mask the fluorescence of bands of interest on 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.

❷

Gel electrophoresis

2.1 Load the prepared samples into the wells.

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

❸

Gel staining

3.1 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).

3.2 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 aluminum foil to prevent photobleaching of the stains.

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

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

❹

Imaging

4.1 **Wet gels:** Place the wet gel directly onto the platen (Typhoon and Storm) or onto the glass tray (FluorImager) in a small amount of water. Avoid trapping air bubbles between the gel and the glass.

Gels attached to one of the electrophoresis plates: Place the glass plate directly onto the platen (Typhoon) or in the extended universal holder tray (FluorImager). 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.

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

❺

Analysis

See Chapter 4 for information concerning image analysis.

5.2.4 Expected results

Typical results for the fluorescence detection of nucleic acids in agarose gels are given in Table 5. Figure 31 shows the detection of DNA in a polyacrylamide gel stained with Vistra Green and imaged using Typhoon.

Fig 31. Two-fold serial dilutions of the DNA mass ladder on 10% polyacrylamide gels. The gel was stained with Vistra Green and imaged using Typhoon 488-nm laser line. Starting quantities were 10 ng of 2000 bp, 6 ng of 1000 bp, 4 ng of 800 bp, 2 ng of 400 bp, 1 ng of 200 bp, and 0.5 ng of 100 bp.

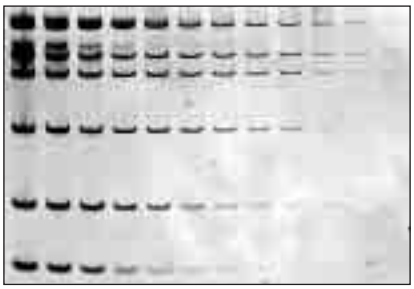


Table 5. Fluorescent gel detection of double-stranded DNA*

Stain	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (pg)	LDR (fold)	LOD (pg)	LDR (fold)	LOD (pg)	LDR (fold)	LOD (pg)	LDR (fold)
Ethidium bromide, cast with gel	50/ND	500/ND	50/ND	500/ND	NA	NA	ND	ND
Ethidium bromide, post-stain	50/6	500/1000	200/25	50/500	NA	NA	100/ND	300/ND
SYBR Gold	10/3	500/1000	10/6	500/1000	500/25	100/500	ND/20	ND/100
SYBR Green I	10/3	500/1000	10/6	500/1000	500/25	100/500	ND/20	ND/100
Vistra Green	10/3	500/1000	10/6	500/1000	500/25	100/500	ND/20	ND/100

ND = not determined; NA = not applicable.

* A dilution series of a DNA ladder was loaded onto a 1% agarose gel (3 mm) or a 10% polyacrylamide gel (1 mm). Results from the agarose/polyacrylamide gels are expressed as limit of detection (LOD) and linear detection range (LDR). Using appropriate emission filters, the 532-nm laser line on Typhoon Imager provides similar results as the 488-nm laser line.

5.3 Quantitation of nucleic acids in solution

5.3.1 Stains for quantitation 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, RNA, and contaminants from the nucleic acid preparations. Nucleic acids are more accurately quantified in solution using fluorescent stains that bind with very high specificity and sensitivity (Table 6). When bound to their target molecules (DNA or RNA), the fluorescence of these stains is greatly enhanced.

Whereas the sensitivity of non-fluorescent 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 to 1400 fold, depending on which imaging instrument is used (see Table 8).

The fluorescence 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 fluorescent enhancement specifically by binding to RNA.

Table 6. Nucleic acid solution stains

Stain	Excitation max (nm)	Emission max (nm)	Fluorescence application
PicoGreen	502	523	Quantitation of dsDNA
OliGreen	500	520	Quantitation of ssDNA and oligonucleotides
RiboGreen	500	525	Quantitation of RNA

5.3.2 Instrument compatibility

Table 7. Instrument settings for use with nucleic acid solution stains*

Stain	Typhoon				FluorImager		Storm
	9400/9410		8600/8610/9200/9210				
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode
PicoGreen	488/532†	520 BP 40/526 SP†	532	526 SP	488	530 DF 30	Blue
OliGreen	488/532	520 BP 40/526 SP	532	526 SP	488	530 DF 30	Blue
RiboGreen	488/532	520 BP 40/526 SP	532	526 SP	488	530 DF 30	Blue

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

† Note that for Typhoon 9400/9410, more than one setting could produce comparable imaging results. The most optimal setting should be experimentally determined based on individual application needs.

5.3.3 Typical protocol

Amersham Biosciences products available for this application

Product used	Code number
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog

Other materials required	Vendor
PicoGreen nucleic acid stain	Molecular Probes, Inc
Clear (polystyrene) 96-well microplate*	Corning Costar Corp

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

❶

Sample preparation

1.1 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

2.1 Prepare sufficient working solution of the PicoGreen reagent by diluting the stock 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.

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

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

2.4 Incubate for 2–5 min at room temperature.

❸

Imaging

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

3.2 Acquire the image according to the recommended instrument setup (Table 7) for the stain 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

4.1 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 quantitate integrated signal from the microplate wells.

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

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

4.4 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.

4.5 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).

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

5.3.4 Expected results

The limits of detection and linear detection ranges for quantitation of DNA in solution are given in Table 8. Similar detection limits and linear range were found for 488-nm/520 BP40 and 532-nm/526 SP settings on Typhoon Imager. Figure 32 is an image from a microplate-based PicoGreen assay detected using Typhoon Imager.

Fig 32. Detection of DNA in solution using PicoGreen and the 488-nm laser line of Typhoon Imager with PolySorp 96-well plates with removable strips. Lambda DNA was used at concentrations of 10 000, 3500, 429, 150, 52.5,18.4, 6.4, 2.25 ng/ml.

Table 8. Fluorescence-based quantitation of DNA in solution

Stain	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

ND = not determined

* First number from assay performed using Costar flat-bottomed plate/second number from assay using PolySorp 96-well plates with removable strips. A dilution series of lamda phage DNA prepared in 1× TE was used for the analysis. Results are expressed as limit of detection (LOD) and linear detection range (LDR).

5.4 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 fractionated 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.

5.4.1 Fluorogenic substrates for Southern and Northern detection

Fluorescent Southern and Northern detection chemistries employ enzyme-amplified detection schemes using alkaline phosphatase (AP) enzyme (17). 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 9.

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

Table 9. Fluorogenic substrates for Southern and Northern blots

Stain	Excitation max (nm)	Emission max (nm)	Fluorescence application
ECF	440	560	Alkaline phosphatase substrate
DDAO Phosphate	646	660	Alkaline phosphatase substrate

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 33. With some kits, the probe can be directly labelled with a thermostable enzyme (e.g., AlkPhos Direct™ Systems). Since this bypasses the hapten detection step, the signal development process is much faster.

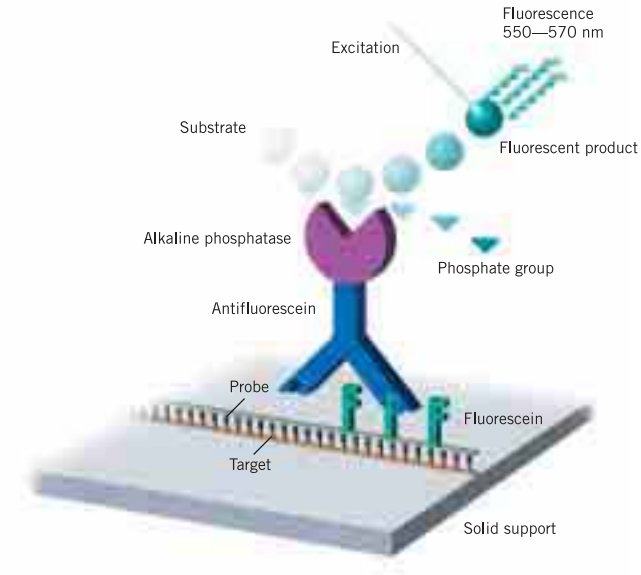


Fig 33. Schematic showing the indirect detection of a fluorescein-labelled DNA probe in a Southern blot. The ECF Signal Amplification Module boosts sensitivity by coupling alkaline phosphatase to the fluorescein-labelled DNA probe. Alkaline phosphatase catalyses the formation of stable fluorophores that remain near the probe and emit light when detected using fluorescence imaging systems.

5.4.2 Instrument compatibility

Table 10. Compatibility of selected fluorogenic substrates for Southern/Northern blot detection with fluorescence imaging systems*

Stain	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
ECF	532	526 SP	488	570 DF 30	Blue	Reflection	UV high
DDAO Phosphate	633	670 BP 30	NA	NA	Red	NA	NA

NA = not applicable

* For membrane applications, substantial background fluorescence primarily due to non-specific antibody binding may occur. Shifting the excitation and emission wavelengths slightly away from the fluorochromes' spectral maxima wavelengths sometimes greatly reduces the noise and improves the overall signal-to-noise. For the most sensitive results, we recommend that the optimal setup be experimentally determined for each of the applications.

5.4.3 Typical protocol

Fluorescent Southern or Northern blot detection using ECF Random-Prime Labelling Kit (or DDAO phosphate substrate)

Amersham Biosciences products available for this application

Products used	Code number
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog
ECF Random-Prime Labelling and Detection System	RPN5752
PlusOne Ethidium Bromide Solution, 10 mg/ml	17-1328-01
Hoefer EPS 301 Power Supply	18-1130-01
Hoefer HE 99X Max Submarine Unit	80-6061-57
Hybond™-N+ Membranes	see catalog
Vistra Green Nucleic Acid Gel Stain	RPN5786

Other materials required	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 fluorescence 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

2.1 Separate the DNA samples in a neutral agarose gel, then depurinate, denature, and neutralize the gel according to standard procedures (18).

2.2 Transfer the samples to a Hybond-N+ Nylon Transfer Membrane.

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

Northern blots

2.4 Separate denatured RNA (prepared in a glyoxal buffer) in an agarose gel prepared in 1× MOPS buffer (18).

2.5 Transfer the samples to a Hybond-N+ Nylon Transfer Membrane.

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

❸

Application of substrate

ECF substrate

3.1 Prepare the ECF substrate as directed.

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

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

3.4 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

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

3.6 Dilute the DDAO phosphate stock 1:1000 in 10 mM Tris-Cl (pH 9.5), 1 mM $MgCl^2$.

3.7 Add 50–100 µl of substrate per cm^2 of membrane, covering it evenly, and incubate for 4 h.

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Imaging

4.1 Place the covered, developed blot onto 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.

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

4.3 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. It is important to note that in Table 10 the excitation wavelength and

emission filter selection for optimal sensitivity do not necessarily match the excitation and emission maxima of the fluorochrome of interest. For membrane applications, substantial background fluorescence primarily due to non-specific antibody binding may occur. Shifting the excitation and emission wavelengths slightly away from the fluorochromes' spectral maxima wavelengths sometimes greatly reduces the noise and improves the overall signal-to-noise. For the most sensitive results, we recommend that the optimal setup should be experimentally determined for imaging each of the fluorescent blot applications.

❺

Analysis

See Chapter 4 for information concerning image analysis.

5.4.4 Expected results

Typical results from a fluorescent Southern blot of a single-copy human gene acquired using the Typhoon Imager are shown in Figure 34. Results that can be expected for other systems and substrates are given in Table 11.

Fig 34. 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 Imager. Amount of DNA per lane ranged from 10.4 µg to 0.32 µg, prepared in two-fold serial dilutions.

Table 11. Fluorescence-based DNA quantitation in genomic Southern blots*

Stain	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (pg)	LDR (fold)	LOD (pg)	LDR (fold)	LOD (pg)	LDR (fold)	LOD (pg)	LDR (fold)
ECF	0.5	25	0.25	50	0.25	50	0.25	ND
DDAO Phosphate	0.25	50	NA	NA	0.25	50	ND	ND

NA = not applicable, ND = not determined

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

5.5 Microarray applications

5.5.1 Fluorescent microarray applications

DNA microarray technology is a powerful tool to investigate global changes in gene expression of cells and tissues (19). Differential expression profiling is often used to compare the gene expression patterns of two different samples, such as diseased versus normal, or drug-treated versus control. This is usually done by labelling the cDNAs from two individual samples with two different fluorescent dyes, such as Cy3 and Cy5, followed by hybridizing the labelled cDNAs onto the microarray slides. After hybridization, the slide can be imaged by fluorescence detection. The normalized ratio of the fluorescence intensities of the two dyes from each spot is then calculated and used to determine the relative expression of the genes from the two samples for each spot. See reference 20 for details about this application.

5.5.2 Instrument compatibility

Table 12. Compatibility of Cy3, Cy5, and other fluorochromes used in fluorescent microarray with Typhoon 8610/9210/9410

Fluorochromes	Excitation (nm)	Emission filter
Cy3 (550, 570 nm)	532	580 BP 30
Cy5 (649, 670 nm)	633	670 BP 30

5.5.3 Typical protocol

An example of a microarray application and the preparation protocol are described here. Figure 35 shows the overall workflow for preparation, data acquisition, and data analysis for this application. Typhoon Imager will also image microarray slides prepared with alternative protocols and reagents. Please refer to the manufacturer's instructions for experimental details.

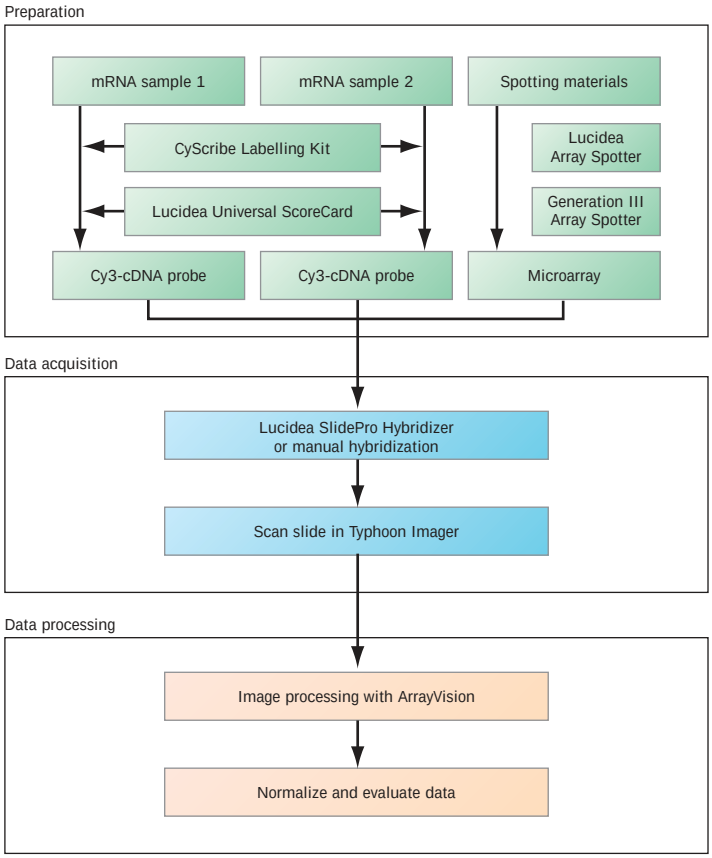


Fig 35. The overall workflow of a microarray application. All information and data can be initiated, monitored, and analyzed using Scierra™ Microarray Laboratory Workflow System.

Amersham Biosciences products available for this application

Products used	Code number
Typhoon 8610	inquire
Typhoon 9210	see catalog
Typhoon 9410	see catalog
Microarray Slide Holder	63-0039-99
ArrayVision	ARV-100
CyScribe™ First-Strand cDNA Labelling Kit with GFX™ Purification Kit	RPN6200X
Cy3-dCTP	PA53021
Cy5-dCTP	PA55021
HEPES	US16926
Humid Hybridization Cabinet for microarrays	RPK0176
Hybridization Oven/Shaker	RPN2511
Lucidea Microarray ScoreCard Kit v1.1	RPK1161
Lucidea Universal ScoreCard	63-0042-85
Lucidea Automated Spotfinder	63-0038-18
RNase-Free water	US70783
Sodium dodecyl sulphate (SDS)	US75819
SSC–20×	US19629
Microarray Hybridization Solution	RPK0325

Other materials used	Vendor
CMT-GAPS microarray slide	Corning
Human liver and skeletal muscle mRNA	Clontech
SpeedVac™ System	Savant

For this application, the microarray slides used were robotically spotted with a Generation III Array Spotter from Amersham Biosciences. Lucidea Microarray ScoreCard reagents and software were used for data validation and normalization. This kit is only compatible with human microarray slides spotted with a Generation III array spotter. A set of 4224 human genes and Lucidea Microarray ScoreCard control reagents were spotted in duplicate onto a Corning CMT-GAPS microarray slide.

For non-human and human microarray slides prepared by other types of array spotters, Lucidea Universal ScoreCard reagents are recommended as universal references for validating and normalizing microarray data. This product only contains reagents and is independent of experimental platform and data analysis software. Lucidea Universal ScoreCard Reagents display no cross-hybridization over a wide range of biological species.

For more details about reagents, instrumentation, slides, and software products that are part of Lucidea Microarray Platform, refer to the *Microarray Handbook* from Amersham Biosciences.

❶

cDNA labelling

For this application, CyScribe First-Strand cDNA Labelling Kit with Cyscribe GFX Purification Kit was used for cDNA labelling.

1.1 Add Lucidea Microarray ScoreCard spike mix to the human skeletal muscle and liver mRNA samples.

1.2 Use the CyScribe Kit to label the cDNAs according to the kit instructions. Cy3- and Cy5-dCTPs are used to label skeletal muscle and liver cDNAs, respectively.

1.3 Purify the Cy3- and Cy5-labelled cDNAs using the CyScribe GFX Purification Kit following the manufacturer's protocol.

❷

Hybridization and post-hybridization washes

Hybridization and post-hybridization washes were performed according to the protocol in reference 20.

❸

Imaging the microarray slide

For complete instructions on how to image microarray slides on Typhoon, refer to the Microarray Slide Holder Kit instructions and Typhoon Instrument Guide.

3.1 In the **Fluorescence Setup** window, select the appropriate laser/emission filter settings. Select **Normal** for sensitivity. Set an appropriate PMT voltage setting (the recommended range is between 450-800 V).

Note: For quantitative results, select sensitivity mode to perform single-channel scans.

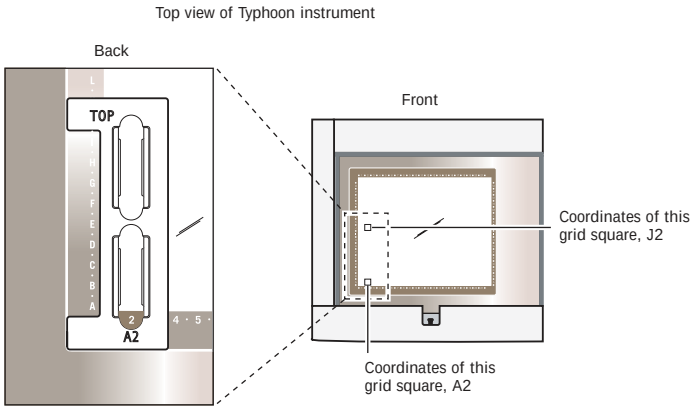


Fig 36. Positioning the microarray slide holder on the glass platen.

❹

Image processing

The following procedures are recommended for image processing:

4.1 Use ImageQuant Tools Utility 2.2 (IQ Tools) to open and process the Typhoon microarray images. After the images are opened, make sure the image orientation is appropriate for analysis using either the bar code or control spots on the slide as landmarks. If necessary, change the image orientation using the rotating or flipping tools in IQ Tools.

Note: Further data analysis after image processing may require that the bar codes be removed (cropped out) from the images.

4.1.1 If the image contains two slides, use the cropping tool to crop out each slide and use **Save Region of Interest As** to create separate image files for them.

4.1.2 If Lucidea Microarray ScoreCard is used for data validation and normalization, prepare the image files by cropping the left and right half of the image and use **Save Region of Interest As** to create individual half-image files. It is recommended to name the files as:

SlideID_left.ds
SlideID_right.ds

4.2 Use ArrayVision v5.1 or higher for image quantitation.

For more details, refer to the *ImageQuant Utilities* and *ArrayVision User Manuals*.

❺

Data validation and normalization

For the application described in this note, Lucidea Microarray ScoreCard was used for data validation and normalization. For more details, refer to the *Lucidea Microarray ScoreCard User Manua*l.

5.5.4 Expected Results

Labelled cDNA from human skeletal muscle and liver were hybridized onto a slide spotted with 4224 human genes in duplicate plus the Lucidea Microarray ScoreCard controls in 24 replicate. After washing, the hybridized slide was scanned on Typhoon 9410. The spots were about 200 µm in diameter (Figure 37).

To validate the microarray experiment, it is important that the variations in the measurements are evaluated so that accurate comparisons can be made within the experiment and across multiple experiments. This is a common requirement with any quantitative microarray application. The Lucidea Microarray ScoreCard controls include negative, dynamic range, and ratio controls for Cy3 and Cy5. Negative controls are used to evaluate the degree of nonspecific hybridization and provide the detection threshold values. Dynamic range controls are mainly used to estimate detection limits and linear range. Ratio controls provide a mechanism for verifying the accuracy of calculated gene expression ratios. Table 13 illustrates the concentrations and relative abundance in the labelled sample of the dynamic range control and ratio control elements.

To evaluate the detection limits and dynamic range of this application, six dynamic range control samples were used. The sixth dynamic range control (6DR) has the least relative abundance of 0.0033% (33 pg per µg of sample mRNA). Even at this low relative abundance level, the signal-to-noise ratios for Cy3 and Cy5 detection for 6DR were determined to be 5 and 13 respectively, demonstrating Typhoon's high sensitivity for both Cy3 and Cy5 spot detection. Typhoon's limit of detection (LOD) was determined to be 0.002% in relative abundance level (20 pg per µg of sample mRNA) for imaging Cy3 and 0.0008% (8 pg per µg of sample mRNA) for Cy5. The LOD was determined by converting the fluorescence intensity level of the detection limit to the abundance level at which the background-corrected signal-to-noise ratio is 3.

The signals were determined to be linear at least to 1% (10 000 pg) in relative abundance for both Cy3 and Cy5 detection. For higher abundance genes (such as 3% or 33 000 pg), the DNA on the microarray spot was hybridized to saturation, causing the fluorescence intensity to plateau. Typhoon has a wide linear dynamic range of five orders of magnitude (from count 1 to 100 000). Typically, the range of microarray gene expression levels is limited to 2.5 to 3.5 orders of magnitude.

Fig 37. A section of a microarray slide imaged on Typhoon Imager. Human tissue cDNA from skeletal muscle (labelled with Cy3, shown in green) and cDNA from liver (labelled with Cy5, shown in red) were mixed and hybridized onto a spotted slide with 4224 human genes printed in duplicate. Shown here are two (left and right) duplicate blocks out of a total of 24 blocks of spots on the whole slide. Row 1 of each block contains the set of Lucidea Microarray ScoreCard controls (32 spots). Rows 2 through 12 contain a duplicate set of 352 human genes.

Table 13. The relative concentration and abundance of Lucidea Microarray ScoreCard dynamic range and ratio controls. RC and DR stand for ratio control and dynamic range control, respectively.

Control sample	Cy3:Cy5 ratio	Conc. in mix (pg/5 µl mix) Cy3	Cy5	Relative abundance
1DR	1:1	33 000	33 000	3.3%
2DR	1:1	10 000	10 000	1%
3DR	1:1	1000	1000	0.10%
4DR	1:1	330	330	0.033%
5DR	1:1	100	100	0.01%
6DR	1:1	33	33	0.0033%
1RC	1:3	1000	3000	NA
2RC	3:1	3000	1000	NA
3RC	1:10	1000	10 000	NA
4RC	10:1	10 000	1000	NA

NA = not applicable

To determine the relative expression of the genes from two samples, the ratio of the fluorescence intensities of Cy3 and Cy5 needs to be evaluated and normalized. The normalization process of Lucidea Microarray ScoreCard software is designed to correct for the difference in Cy3 and Cy5 fluorescence intensities that are caused by factors other than differential gene expression, such as variations in Cy3- and Cy5-dye incorporation during labelling and Cy3 and Cy5 imaging sensitivity. Table 14 shows the result of the ratio analysis on the ratio and dynamic range controls before and after normalization. In general, the normalization procedure brings the Cy3/Cy5 ratio much closer to the expected value than the observed (unnormalized) value.

The 10-µm pixel option on Typhoon Imager allows high-resolution scanning suitable for microarray applications. Typhoon offers high sensitivity for successful detection of genes at very low abundance levels. The wide range of excitation sources and emission filters are suitable for multicolor microarray imaging. Typhoon also provides flexibility for imaging a variety of fluorescent labels in addition to Cy3 and Cy5. Typhoon microarray images can be analyzed with software such as ArrayVision.

Table 14. Lucidea Microarray ScoreCard ratio analysis. RC and DR stand for ratio control and dynamic range control, respectively.

	Ratio analysis (Cy3/Cy5)		
Control (Cy3/Cy5)	Expected	Observed	Normalized
1RC (1:3)	0.33	0.73	0.31
2RC (3:1)	3	7.8	3.2
3RC (1:10)	0.1	0.28	0.12
4RC (10:1)	10	21	8.6
1DR (1:1)	1	2.2	0.97
2DR (1:1)	1	2.2	0.96
3DR (1:1)	1	2.5	1.0
4DR (1:1)	1	2.6	1.1
5DR (1:1)	1	2.6	0.95
6DR (1:1)	1	3.0	1.1

5.6 Differential display

5.6.1 Diffferential display

Differential display is a PCR-based technique for studying broadscale gene expression (21). 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 analyzed. 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 quantitation due to the proportional relationship between signal and quantity of message (22). Additionally, fluorescently labelled PCR primers are stable for relatively long periods. Fluorescent 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. Multicolor imaging permits the detection and resolution of multiple targets using fluorescent labels that can be spectrally resolved. For example, fluorescent differential display (FDD) can be used to compare the gene expression patterns of two different samples such as diseased versus normal, or drug-treated versus control, by labelling the cDNAs from two individual samples with two different fluorochromes. The ability to detect multiple labels in the same experiment allows convenient identification and quantitation of the differentially expressed mRNAs.

5.6.2 Instrument compatibility

Refer to Appendix 3 for suggested instrument settings for imaging various fluorochromes.

5.6.3 Sample protocol

Amersham Biosciences products available for this application

Products used	Code number
Hoefer SQ3 Sequencer	80-6301-16
Low-fluorescence Glass Plate Set	see catalog
Hoefer EPS 3501 Power Supply	18-1130-04
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 830/860	see catalog
FluorImager 595	see catalog
Ettan DALT Gel Alignment Guide	80-6496-10

Other materials used

Flurochrome-labelled oligonucleotide

Primers for differential display amplification

Total RNA

❶

Preparation of sample

1.1 Follow the recommended protocol for PCR amplification from total RNA (18).

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

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

❷

Gel electrophoresis

2.1 Before casting the gel, treat one glass plate with silane (18).

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

2.3 Load the samples onto the gel, and run at 35 W from 1.5 to 2 h.

❸

Scanning the differential display gel

Typhoon

Option A: With Gel Alignment Guide:

3.1 Use Ettan™ DALT Gel Alignment Guides to place the gel glass sandwich carefully onto the glass platen. Refer to the instruction sheet of the Gel Alignment Guides for more details.

Option B: Without Gel Alignment Guide:

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

3.2 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.

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

Storm 830/860

3.1 Remove the glass plate, which has been treated with silane. Cover the gel with plastic wrap; avoid trapping air bubbles or creating 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.)

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

FluorImager

3.1 Position the gel sandwich in the universal tray.

3.2 Select the instrument settings appropriate for the fluorochrome label used (see Appendix 3).

❹

Recovering the gene fragments

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

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

5.6.4 Expected results

A two-color differential display analysis using fluorescein and rhodamine labels and imaged using Typhoon Imager is shown in Figure 38.

Fig 38. Two-color FDD analysis of normal (N) and diseased (D) kidney gene expression using either fluorescein (green)- or rhodamine (red)-labelled anchored primer H-T_{11}A in combination with the arbitrary primer H-AP1 and resolved on a 6% denaturing polyacrylamide gel. To better reveal differentially regulated genes, labelled normal and diseased PCR samples were mixed equally and loaded onto the same gel (N/D). Non-differentially expressed genes appear in yellow. The bands shown inside the box were excised for further sequencing analysis. The image was processed using FluorSep software.

5.7 In-lane PCR product analysis

5.7.1 Introduction

Fluorescently labelled DNA fragments can be generated by PCR using modified oligonucleotide primers or deoxynucleotide triphosphates (dNTPs) (10, 11). A wide selection of fluorochrome tags are 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.

Fluorescence detection offers the advantage of sensitivity, a wide linear dynamic range for quantitation, and the option for using multiple tags in analysis. Additionally, the ability to detect multiple fluorescent labels in the same experiment is both time- and cost-effective and improves accuracy. CyDye fluorochromes are bright, photostable molecules that are highly water-soluble and insensitive to pH changes. The labels are available in a range of intense colors with narrow emission bands, making them ideal for multicolor detection. Two of these, Cy3 and Cy5, are popular labels for two-channel fluorescent experiments, such as gene expression arrays. Fluorescent imaging instrumentation with 532-nm and 633-nm laser excitation sources are ideally suited for CyDye imaging.

5.7.2 Instrument compatability

Refer to Appendix 3 for suggested instrument settings for imaging various fluorochromes.

5.7.3 Sample protocol

Amersham Biosciences products available for this application

Products used	**Code number**
Hoefer miniVE Electrophoresis System	80-6418-58
Hoefer SE 400 Sturdier Vertical Unit	80-6154-86
Low-fluorescence Glass Plate Set	see catalog
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	US77212-500 µl
dNTP Set, 100 mM Solutions	27-2035-01
Taq DNA Polymerase (cloned)	T0303Y
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 830/860	see catalog
FluorImager 595	see catalog

❶

Preliminary preparations and general handling instructions

1.1 Prepare the reactions in one of the following ways:

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

Stock	Volume	Final
10× PCR Buffer*	5 µl	1×
25 mM MgCl2	3 µl	1.5 mM
10 mM dATP, dGTP, dTTP, dCTP	1 µl	200 µM
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 ddH2O	to a final reaction volume of 50 µl	

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

B. PCR with CyDye labelled dCTP

Stock	Volume	Final
10× PCR buffer*	5 µl	1×
25 mM MgCl2	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 ddH2O	to a final reaction volume of 50 µl	

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

❷

PCR

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

2.2 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

3.1 Prepare a 10% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer (18).

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

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

❹

Imaging

Typhoon

Option A: With Gel Alignment Guide:

4.1 Use Ettan DALT Gel Alignment Guides to place the gel glass sandwich carefully onto the glass platen. Refer to the instruction sheet for more details.

Option B: Without Gel Alignment Guide:

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

4.2 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.

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

FluorImager

4.1 Position the gel sandwich in the universal tray.

4.2 Select the instrument settings appropriate for the fluorochrome label used (see Appendix 3).

❺

Analysis

5.1 Display and analyze the gel image(s) using ImageQuant software, FluorSep software, or Fragmant Analysis software, as appropriate (refer to user manual for details).

5.7.4 Expected results

In-lane size analysis of CyDye labelled PCR products imaged using Typhoon Imager is illustrated in Figure 39.

Limit of detection (LOD) for CyDye labelled oligonucleotides in a denaturing polyacrylamide gel are shown is Table 15. Values are obtained from signal-to-noise measurements of a two-fold dilution series of end-labelled oligonucleotides analyzed in 0.4 mm thick denaturing polyacrylamide gels (12%). Gels were prepared using low fluorescence glass electrophoresis plates to enable direct imaging.

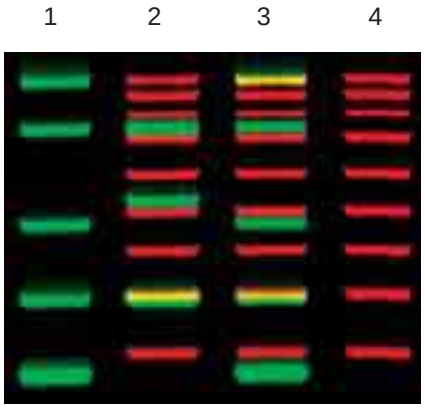


Fig 39. Cy3- and Cy5-labelled DNA fragments. Cy3-labelled fragments are in green, and Cy5-labelled fragments are in red.

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).

Table 15. Detection limits for CyDye labelled oligonucleotides

CyDye	Typhoon LOD (fmol)	FluorImager LOD (fmol)	Storm LOD (fmol)	VDS-CL LOD (fmol)
Cy2	0.6/7.5*	7.5	30	ND
Cy3	0.2	4	NA	ND
Cy3.5	0.2	ND	NA	ND
Cy5	0.2	ND	1	ND

NA = not applicable, ND = not detected

* Limit of detection (LOD) for Typhoon 9400 and 9410 series/ LOD for the Typhoon 8600, 8610, 9200, and 9210 series.

5.8 Bandshift assay

5.8.1 Introduction

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 (23). 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, that contains 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 for Fluorescence can be used for their rapid preparation. The availability of sensitive fluorescence imaging systems makes it practical to perform bandshift assays without radioactivity (24, 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 fluorescence 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.

5.8.2 Instrument compatibility

Refer to Appendix 3 for suggested instrument settings for various fluorochromes.

5.8.3 Sample protocol

Amersham Biosciences products available for this application

Products used	Code number
Hoefer SE 400 Sturdier Vertical Unit	80-6154-86
Low-fluorescence Electrophoresis Glass Plate Set	see catalog
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
PCR Nucleotide Mix	US77212
dNTP Set, 100 mM Solutions	27-2035-01
Taq DNA Polymerase (cloned)	T0303Y
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 830/860	see catalog
FluorImager 595	see catalog
Ettan DALT Gel Alignment Guide	80-6496-10

❶

Preparation of labelled DNA

1.1 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 steps 2.1–2.4 below for all duplexes to be tested.

2.1 Prepare the following mix:

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

*10× annealing buffer: 200 mM Tris-HCl, pH 7.6, 50 mM MgCl2, 1 mM DTT, 0.1 mM EDTA

2.2 Heat the reactions for 10 min at 70 °C.

2.3 Incubate for 30 min at room temperature.

2.4 Place the reactions on ice until ready to use.

❸

Preparation of binding reaction

Note: Repeat steps 3.1–3.2 for all protein-DNA combinations to be tested, including a negative control containing no protein.

3.1 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

3.2 Incubate the reactions on ice for 30 min.

❹

Gel electrophoresis

4.1 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 step 4.2).

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

4.3 Fill the reservoirs with TAE buffer containing 1 mM MgCl2 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

Option A: With Gel Alignment Guide:

5.1 Use Ettan DALT Gel Alignment Guides to place the gel glass sandwich carefully onto the glass platen. Refer to the instruction sheet for more details.

Option B: Without Gel Alignment Guide:

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

5.2 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.

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

FluorImager

5.1 Position the gel sandwich in the universal tray.

5.2 Select the instrument settings appropriate for the fluorochrome label used.

❻

Analysis

6.1 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.

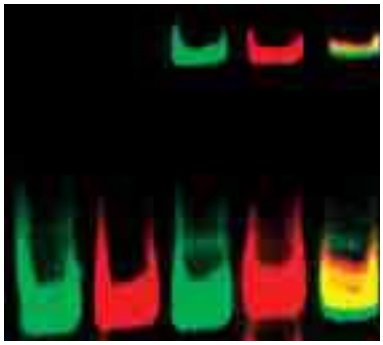


Fig 40. Multi-label gel shift experiment. First two lanes contain 0.4 pmol of two different 180-bp DNA fragments labelled with either HEX (green) or TAMRA (red). The third and fourth lanes contain the same two labelled DNA fragments after incubation with Mnt protein. The fifth lane contains mixtures of the bound labelled fragments to demonstrate multiplexing in the same gel lane (yellow color indicates overlay between green and red signal). A 532-nm excitation was used with 555 BP 30 and 580 BP 30 emission filters.

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

Alternative protocol

Eliminate Step 1 (labelling), and proceed through 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.

5.8.4 Expected results

The results of a multicolor bandshift analysis using two different DNA targets (labelled with HEX™ and TAMRA) and bacterial Mnt protein are shown in Figure 40. The gel was imaged using Typhoon Imager.

5.9 Detection of proteins in gels

5.9.1 Protein gel stains

Fluorescent protein gel staining, when combined with an appropriate imager, is a sensitive and quantitative approach for protein analysis. Fluorescent gel stains also offer advantages such as ease of use, sample stability, and safety (2). SYPRO Orange, Red, Ruby, and Tangerine

protein gel stains are one-step fluorescent stains optimal for rapid and efficient staining of one-dimensional (1-D) protein gels. These stains provide sensitivity that is equivalent to the silver staining method for 1-D gels (27) and enable protein detection that is not affected by the presence of nucleic acids and lipopolysaccharides. SYPRO Ruby stain is also suitable for two-dimensional (2-D) protein gel staining (27, 28). SYPRO Ruby offers a much wider linear dynamic range than the traditional silver staining method, and it is also compatible with mass spectrometry and Edman sequencing. See more details for the protein stain applications in reference 28 and 29.

Table 16. Fluorescent protein gel stains

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

5.9.2 Instrument compatibility

Table 17. Instrument settings for use with fluorescent protein stains

Stain	Typhoon				FluorImager		Storm	VDS-CL	
	9400/9410		8600/8610/9200/9210						
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation	Emission
SYPRO Orange	488	555 BP 20	532	555 BP 20	488	570 DF 30	Blue	Transmission	UV high
SYPRO Red	532	610 BP 30	532	610 BP 30	514	610 RG	Red	Transmission	UV high
SYPRO Ruby	457† 488/532	610 BP 30	532	610 BP 30	488	610 RG	Blue	Transmission	UV high
SYPRO Ruby IEF	457† 488/532	610 BP 30	532	610 BP 30	488	610 RG	Blue	Transmission	UV high
SYPRO Tangerine	488	610 BP 30	532	610 BP 30	488	610 RG	Blue	Transmission	UV high

† Note that for Typhoon 9400/9410, more than one setting could produce comparable imaging results. The most optimal setting could be experimentally determined by users for the individual application needs.

5.9.3 Typical protocol

A. 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 quantitation 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. Using appropriate gel systems, proteins can also be studied under non-denaturing or native conditions which preserve the higher order structure and even the biological function of some proteins.

Amersham Biosciences products available for this application

Products used	**Code number**
Hoefer EPS 301 Power Supply	18-1130-01
Hoefer miniVE Vertical Electrophoresis System	0-6418-77
Bovine serum albumin (BSA) protein standard, Dnase-Free	27-8915-01
SYPRO Orange Protein Gel Stain	RPN5801
SYPRO Red Protein Gel Stain	RPN5803
SYPRO Tangerine Protein Gel Stain	RPN5805
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog

Other materials required	**Vendor**
Protein samples prepared in appropriate loading buffer	
SYPRO Ruby protein gel stain	Molecular Probes, Inc

❶

Sample preparation

1.1 Refer to *Protein Electrophoresis* from Amersham Biosciences (30).

❷

Gel electrophoresis

2.1 Load the prepared samples onto the gel. For denaturing conditions, use a gel and/or running buffer that contains 1% SDS. Refer to *Protein Electrophoresis* from Amersham Biosciences for further details (30).

❸

Staining the gel

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

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

For SYPRO Ruby, use the stain stock directly with no dilution.

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

3.2 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 aluminum foil to prevent photobleaching of the stains.

3.3 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

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

4.2 In the **Scanner Control Setup** window, choose the appropriate laser and emission filter combinations (Table 17). For Typhoon imaging, choose "platen" for the focal depth setting. Acquire the image according to the recommended instrument setup.

❺

Analysis

See Chapter 4 for information concerning image analysis.

B. SYPRO Ruby and two-dimensional gels

Two-dimensional gel electrophoresis is used to analyze complex mixtures of proteins through the combined resolving power of two electrophoretic methods (31). 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. Direct protein labelling method using fluorochromes such as Cy2, Cy3, and Cy5 are also suitable for 2-D protein gel detection (Section 5.10).

Amersham Biosciences products available for this application

Products used	Code number
IPG Buffer pH 4-7	17-6000-86
Immobiline™ DryStrip pH 4-7, 18 cm	17-1233-01
Ettan DALT II Large Vertical System	see catalog
Multiphor™ II IEF System	18-1018-06
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog

Other materials required

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

❶

Sample preparation

The following is a specific procedure for analysis of *E. coli* proteins. For additional information, see reference 32.

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

1.2 Precipitate the sonicate overnight at -40 °C with 80 ml acetone, 10 ml 100% w/v TCA, 1 ml 2-mercaptoethanol.

1.3 Collect the precipitate by centrifugation at 20 000 rpm for 20 min. Wash the pellet with the same volume of 80% acetone, 1% 2-mercaptoethanol and leave in the freezer for a few hours.

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

1.5 Clarify the extract by centrifugation at 20 000 rpm for 30 min.

❷

Gel electrophoresis.

2.1 Prepare Immobiline Drystrips, 18 cm, pH 4-7 and IPGphor™ System.

2.2 Dilute protein extract (100 µg of total protein in 350 ml) 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).

2.3 Load proteins using rehydration loading for 12 h at 20 °C and 500 V for 500 Vhr, 1000 V for 1000 Vhr, and 8000 V for 60 000 Vhr.

2.4 Equilibrate the strip sequentially for 15 min each with SDS equilibration solution containing 1% (w/v) DTT and 2.5% iodoacetamide, respectively.

2.5 Load the equilibrated strip onto a 1-mm, 12.5% Laemmli gel cast for the Ettan DALT*twelve* System (32).

2.6 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

Staining the gel

3.1 Fix the gel in 10% ethanol, 10% acetic acid for 30–60 min.

3.2 Stain the gel directly with the working stain solution (between 5- and 10-fold greater than the gel volume) for at least 12 h.

3.3 Rinse the gels with deionized water. Destain for 30 min in 10% (v/v) methanol and 7% (v/v) glacial acetic acid.

❹

Imaging

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

4.2 In the **Scanner Control Setup** window, choose the appropriate laser and emission filter combinations (Table 17). For Typhoon imaging, choose "platen" for the focal depth setting. Acquire the image according to the recommended instrument setup.

❺

Analysis

Analyze the image using ImageMaster 2-D software.

5.9.4 Expected results

The expected limits of detection (LOD) and linear detection ranges (LDR) for protein quantitation in one-dimensional gels are given in Table 18. A Typhoon image from a one-dimensional SDS-PAGE gel stained with SYPRO Ruby is shown in Figure 41. An image of a two-dimensional protein gel stained with SYPRO Ruby is shown in Figure 42. Using Typhoon Imager, similar sensitivity and linear range were found for all three laser lines that excite SYPRO Ruby—457 nm, 488 nm, and 532 nm.

Table 18. Fluorescence gel detection using SYPRO stains*

Stain	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)
SYPRO Orange	2	1000	3	700	6	350	5	200
SYPRO Red	2	1000	2	1000	3	700	ND	ND
SYPRO Tangerine	2	1000	4	500	4	500	ND	ND
SYPRO Ruby	1	2000	2	1000	2	700	1	2000

ND = not determined

* 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 the Hoefer miniVE System. Results are expressed as limit of detection (LOD) and linear detection range (LDR).

Fig 41. SYPRO Ruby detection of BSA imaged by Typhoon: 457-nm (top image), 488-nm (middle image), and 532-nm (bottom image) laser lines with the 610 BP 30 emission filter. The amount of BSA per lane ranges from 512 ng to 1 ng, in 2-fold serial dilutions. From left to right, lanes 1 to 10 contain: 512 ng, 256 ng, 128 ng, 64 ng, 32 ng, 16 ng, 8 ng, 4 ng, 2 ng, and 1 ng of BSA.

Fig 42. SYPRO Ruby staining of a 2-D gel. A total protein extract of *E. coli* (100 µg) focused on an 18 cm Immobiline DryStrip (pH 4–7) was run out in the second dimension using a 12%, 1-mm Laemmli gel using the Ettan DALT*twelve* System. The gel was stained overnight with ~5 volumes of SYPRO Ruby and imaged using the Typhoon 532-nm laser line.

5.10 Ettan DIGE (2-D Fluorescence Difference Gel Electrophoresis)

5.10.1 Introduction to Ettan DIGE technology

Ettan DIGE, 2-D Fluorescence Difference Gel Electrophoresis, is a new technology for protein expression studies on two-dimensional gels (33). Ettan DIGE System allows the multiplexing of samples within the same two-dimensional protein gel. With Ettan DIGE System, samples are pre-labelled with CyDye DIGE Fluors that are charge- and mass-matched to each other and mixed prior to electrophoretic separation. This results in co-migration of proteins originating from separate samples. Proteins that are expressed differently in different samples will show different levels of abundance. For example, healthy and tumor cells from the same donor can be labelled with different fluorochromes and then combined and run in a single gel. The advantage of this method over conventional methods is that the samples are exposed to the same chemical environments and electrophoretic conditions. Co-migration is guaranteed for identical proteins from the separate samples, therefore reducing gel-to-gel variation. This approach also greatly improves the throughput of experiment and data analysis. The Ettan DIGE fluorescence labelling method and associated DeCyder Differential Analysis Software have been developed and optimized for this technology. Sensitive fluorescence detection is accomplished using Typhoon Imagers. This technology is able to routinely detect <10% differences in protein expression between samples.

The recommended protocol suggests an internal standard be run on all gels within an experiment which is then analyzed using DeCyder Software. The use of spectrally resolvable CyDye DIGE Fluors that are matched for both mass and charge enables up to 3 differently labelled protein samples to be separated on the same 1st and 2nd dimension gel. This provides the opportunity to include an in-gel standard for every protein in the experiment, on each gel. The internal standard is created by pooling an aliquot of all biological samples in the experiment and labelling it with one of the CyDye DIGE Fluors (usually Cy2). The internal standard is then run on every single gel along with each individual sample. This means that every protein from all samples will be represented in the internal standard. The internal standard is present within all gels.

Linking every sample in-gel to a common internal standard gives a number of advantages:

- accurate quantitation and accurate spot statistics between gels
- increased confidence in matching between gels
- flexibility of statistical analysis depending on the relationship between samples
- separation of experimental variation from sample/biological variation.

5.10.2 Instrument compatibility

Table 19. Instrument settings for using Typhoon for Ettan DIGE imaging

Fluorochrome	Typhoon			
	9400/9410		8600/8610/9200/9210	
	Excitation (nm)	Emission Filter	Excitation (nm)	Emission Filter
Cy2	488	520 BP 40	Not optimal	Not optimal
Cy3	532	580 BP 30	532	580 BP 30
Cy5	633	670 BP 30	633	670 BP 30
SYPRO Ruby	457/488/532†	610 BP 30	532	610 BP 30

† Note that for Typhoon 9400/9410, more than one setting could produce comparable imaging results. The most optimal setting could be experimentally determined by users for the individual application needs.

5.10.3 Typical protocol

Amersham Biosciences products available for this application

Products used	Code number
Immobiline DryStrip, full range including 24 cm strips	see catalog
CyDye DIGE Fluor Cy2 minimal dye	25-1900-27
CyDye DIGE Fluor Cy3 minimal dye	25-1900-28
CyDye DIGE Fluor Cy5 minimal dye	25-1900-30
IPG Buffer, full range	see catalog
IPGphor IEF System	80-6414-02
Ettan DALT*six* and Ettan DALT*twelve* Large Format Vertical System	see catalog
Ettan DALT Low-Fluorescence Glass Plates	80-6475-58
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Ettan DALT Gel Alignment Guide	80-6496-10
SE600 Gel Alignment Guide	80-6496-29
Gel Orientation Guide	80-6496-67
DeCyder Differential Analysis Software	see catalog

Other materials required

Protein samples prepared in appropriate lysis buffer

❶

Experimental design

It is recommended that all Ettan DIGE experiments are analyzed using DeCyder Differential Analysis Software, as outlined in the *Ettan DIGE User Manual*. The recommended experimental design for use with DeCyder is as follows:

- The standard sample should, if possible, be a pool combining all samples and controls.
- The standard sample should be labelled with Cy2, the experimental samples and controls should be labelled with Cy3 and/or Cy5.
- All labelled materials should be run with at least three replicates to enable statistical analysis

For full details refer to the *Ettan DIGE User Manual*.

❷

Sample preparation

Note: The CyDye DIGE Fluors minimal dyes label primary amines. Exogenous sources of amines, such as DTT or ampholytes, must not be included in the buffer prior to labelling. For additional information, refer to *Ettan DIGE User Manual*. Samples should be prepared in the following buffer:

- 7M Urea
- 2M Thiourea
- 10–40mM Tris pH 8.5
- 4% CHAPS

2.1 Sonicate the samples on ice (20 s bursts, with 60 s rest between bursts) until no particulate matter remains, or until no more of the particulate matter is being dissolved. Once completed, centrifuge the samples to remove any insoluble material.

2.2 Check the pH of the sample **on ice**. The sample pH must be 8.0–9.0. Adjust the pH using dilute NaOH if required.

2.3 Store the sample in suitable aliquots at -70 °C or lower.

❸

Labelling (CyDye DIGE Fluors only)

This labelling scheme assumes that samples will be separated on 24-cm IPG strips and Ettan DALT*six* or Ettan DALT*twelve* format gels.

3.1 Dilute the stock dyes by 1.5-fold in DMF so 1 µl contains 400 pmoles of dyes.

3.2 Label 50 µg of each sample with 400 pmoles of dye, using standard protocols. This reaction may be done in bulk for multiple samples.

3.3 Carry out the labelling reaction by incubating the sample on ice for 30 min. Add 1 µl of 10 mM lysine, followed by a further 10 min incubation on ice.

3.4 Combine the individually labelled samples.

❹

Gel electrophoresis

4.1 First-dimension electrophoresis:
The separation is normally performed on the IPGphor, using cup loading with the universal IPG strip holders. The focussing protocols used depend on the IEF strips selected, see the *Ettan DIGE User Manual* for further details.

4.2 Pre-equilibrate the IEF strips containing the focussed minimal CyDye labelled proteins immediately prior to performing second-dimension electrophoresis using DTT in equilibration buffer for 10 min followed by iodoacetamide in equilibration buffer for 10 min.

4.3 Second-dimension electrophoresis:
The second-dimension stage typically uses 12.5% isocratic or 10–16% gradient gels prepared in Ettan DALT*twelve* or Ettan DALT*six* format using low-fluorescence (LF) glass plates. Gels are run with Laemmli buffer, with running buffer containing 0.2% SDS. Gels are run at a constant power (Watts) until completion.

❺

Imaging

5.1 Rinse the gel glass sandwich with distilled water and wipe it dry with lint-free towels. Use the Gel Alignment Guide to position the gel sandwich onto the Typhoon glass platen carefully.

5.2. For Typhoon imaging, choose “+3 mm” for the focal plane setting. Acquire the image according to the recommended instrument setup. It is recommended that the “DIGE File Naming Format” is used to capture the scan file to optimize scan throughput in DeCyder Software.

❻

Analysis

6.1 Crop the images initially using ImageQuant Tools Software to remove surplus image information.

6.2 Analyze the images using DeCyder Software.

For more details, refer to the *DeCyder* and *ImageQuant Tools Software User Manuals*. For full details of correct cropping procedure, refer to the *Ettan DIGE User Manual*.

❼

Gel staining

The primary image of the gel will be obtained from the CyDye labelled proteins. If further work is required, such as picking of protein spots, then additional gels may be run with higher protein loading and stained using SYPRO Ruby. In this case, for the gel to adhere, the glass plates will have been pre-treated with bind silane.

7.1 Incubate the gel in 30% ethanol, 7.5% acetic acid for 30–60 min, and then incubate overnight at room temperature with gentle agitation in 5–10 volumes (250–500 ml per Ettan DALT gel) of SYPRO Ruby staining solution.

7.2 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.

Fig 43. A three-color Typhoon image of a 2-D gel of *E. coli* samples pre-labelled with CyDye DIGE Fluors. The *E. coli* control sample was pre-labelled with Cy3 minimal dye (red), and benzoic acid-treated *E. coli* sample was pre-labelled with Cy5 minimal dye (blue). A standard sample was made by pooling the control and treated sample, and then pre-labelling with Cy2 minimal dye (green).

5.10.4 Expected results

Figure 43 shows a 2-D protein gel of a *E. coli* sample pre-labelled with CyDye DIGE Fluors.

In order to study the quantitation ability of Ettan DIGE protein gels imaged by Typhoon Imager, a dilution series of known amounts of transferrin labelled with Cy2, Cy3, and Cy5, using the standard DIGE labelling protocol, was loaded on a 12% 1-mm Laemmli gel. This approach eliminates errors from possible protein loss during the first-dimension and second-dimension electrophoresis procedures, as well as gel-to-gel variations. Table 20 gives the least amount of transferrin that should be used for reliable detection by Typhoon as well as the linear detection range (LDR) for this application. It is important to note that these parameters are not detection limits (LOD). LOD refers to the minimal amount of fluorochrome-labelled bio-molecules that can be detected at a signal-to-noise ratio of at least 3. With the minimal CyDye Fluors used in section 5.10.3, only 1–2% of lysine residues available are actually labelled. So the amount of labelled protein detected is lower than the total amount of protein loaded.

Table 20. The least amount of transferrin used for the labelling reaction in order to be detected by Typhoon

CyDye	Typhoon	
	Transferrin (pg)	LDR (fold)
Cy2	75	100 000
Cy3	25	100 000
Cy5	25	100 000

5.11 Quantitation of proteins in solution

5.11.1 Stains for quantitation 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 colorimetric assays. Both methods, however, have limitations. For example, the sensitivity of the absorbance method is limited since detection depends on the number of aromatic amino acid residues that are present. Colorimetric 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 (18). High protein-to-protein signal variability is also common with colorimetric detection.

Proteins can be more accurately detected in solution using fluorescent dyes (Table 21). 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, NanoOrange™ binds specifically to the detergent coating on proteins and to hydrophobic regions of proteins, and it is not affected by the presence of contaminating nucleic acids and reducing agents.

Protein detection is much more sensitive using fluorescence. Whereas the sensitivity of non-fluorescent 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 0.5 µg/ml. The linear detection range of this assay is typically 10- to 20-fold.

Table 21. Stains for proteins in solution

Stain	Excitation max (nm)	Emission max (nm)	Fluorescence application
NanoOrange	470	570	Total protein quantitation
CBQCA	465	550	Protein quantitation (reacts with primary amines)

5.11.2 Instrument compatibility

Table 22. Instrument settings for use with protein solution stains*

Stain	Typhoon				FluorImager		Storm
	9400/9410		8600/8610/9200/9210				
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode
NanoOrange	488	580 BP 30	532	580 BP 30	488	570 DF 30	Blue
CBQCA	488	580 BP 30	532	580 BP 30	488	570 DF 30	Blue

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

5.11.3 Typical protocol

Amersham Biosciences products available for this application

Products used	Code number
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog

Other materials required	Vendor
NanoOrange protein solution stain	Molecular Probes, Inc.
Clear (polystyrene) 96-well micoplates*	Corning Costar Corp
Protein samples	

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

❶

Working stain preparation

1.1 Prepare sufficient working solution of the NanoOrange reagent by diluting the stock 1:500 using the 1× diluent provided.

Note: 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

2.1 Using the NanoOrange working solution from Step 1.1, 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. NanoOrange is minimally affected by the presence of salts, urea, detergents, DNA, and amino acids (see manufacturer's literature).

2.2 Pipette the samples into the microplate wells.

Note: The manufacturer's instructions recommend heating the samples at 90–96 °C for 10 min and cooling to room temperature before pipetting the samples. We find that heating the samples does not have a major effect on the assay's performances on Typhoon, Storm, and FluorImagers.

❸

Imaging

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

3.2 Acquire the image according to the recommended instrument setup 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

4.1 Display the image using ImageQuant software. 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 quantitate integrated signal from the microplate wells.

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

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

4.4 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.

4.5 Generate a standard curve from the protein standards used.

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

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

5.11.4 Expected results

The expected limits of detection and linear ranges for protein quantitation in solution are given in Table 23. Similar detection limits and linear range were found for all three laser lines (457 nm, 488 nm, 532 nm) on Typhoon Imager, although 488 nm gives the best sensitivity. Quantitation of a BSA solution using NanoOrange and Typhoon Imager is shown in Figure 44.

Fig 44. Detection of protein in solution using NanoOrange and the 488-nm laser line of Typhoon Imager with PolySorp 96-well plates with removable strips. BSA was used at concentrations of 10, 6, 3, 1, 0.6, 0.3, 0.1, 0.06 µg/ml.

Table 23. Fluorescence-based quantitation of protein in solution using NanoOrange*

Stain	Typhoon		FluorImager		Storm	
	LOD (µg/ml)	LDR (fold)	LOD (µg/ml)	LDR (fold)	LOD (µg/ml)	LDR (fold)
NanoOrange	1/0.1†	10/100	0.3	30	1.0	10

* BSA quantitation was 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 using PolySorp 96-well plates with removble strips.

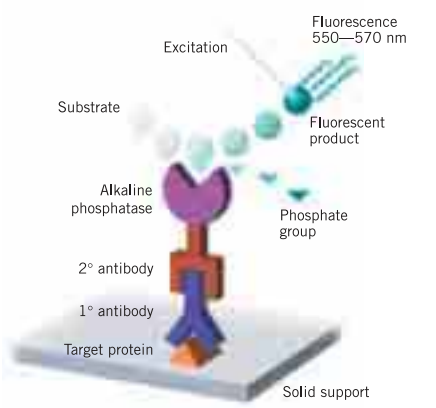


Fig 45. Schematic of the ECF Western Blotting Kit. Proteins are detected by chemifluorescence using alkaline phosphatase-labelled anti-species secondary antibody. Signal is developed with ECF substrate.

Fig 46. 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.

5.12 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 fluorescence detection chemistries, are now widely accepted. Convenient one-step fluorescence imaging does not require lengthy film exposures or development processes and generally offers better signal stability and a wider linear dynamic range than chemiluminescence methods (34) allowing more accurate quantitation.

Most of the fluorescent Western detection mechanism employs either a direct or an enzyme-amplified (chemifluorescence) method (34). The direct fluorescence method uses antibodies labelled with fluorescent dyes, such as Cy3 or Cy5. The chemifluorescence method uses enzyme-conjugated antibodies that react with a fluorogenic substrate to yield a highly fluorescent product. Chemifluorescence detection of Western blots combines the advantages of signal stability and wider linear range from fluorescence and signal amplification from chemiluminescence (Fig 45). It is potentially a more sensitive method than direct fluorescence due to signal amplification. Direct fluorescence is a simpler and more rapid method for Western blot detection. Recent developments in dye chemistry and instrumentation such as Typhoon laser-based scanning systems have significantly improved the sensitivity of Western detection by direct fluorescence. In addition, direct fluorescence imaging is particularly convenient for simultaneous detection of more than one protein target. See details for fluorescent Western application in reference 34 (Fig 46).

5.12.1 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 45). Chemifluorescence can be used in place of any standard chemiluminescence method using AP- and HRP-conjugated antibodies with minimal protocol modification. Fluorogenic substrates that are available for use with these enzymes are listed in Table 24. Note that ECL Plus™ substrate can be used for both chemifluorescence and chemiluminescence detection. Storm imaging of Western blots developed with ECL Plus results in detection limits that match those obtained with 5 min of film-based chemiluminescence detection and offers advantages of two-fold wider linear dynamic range (35). Here we focus on the chemifluorescence property of ECL Plus substrate, which is also compatible with fluorescence imaging using the Typhoon 9400 series of variable mode imagers.

Direct fluorescence detection

Direct fluorescence detection of Westerns (Fig 46) 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. 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 colorimetric stains, such as Ponceau S, Amido black, or Coomassie Brilliant Blue. Properties of commonly used fluorescent blot stains are summarized in Table 25.

Table 24. Properties of substrates for fluorescence-based Western blots

Substrate	Excitation max (nm)	Emission max (nm)	Fluorescence application
ECL Plus*	430	503	Horseradish peroxidase (HRP)
ECF	440	560	Alkaline phosphatase (AP)
DDAO Phosphate	646	660	Alkaline phosphatase (AP)

* ECL Plus substrate can be used for both chemifluorescence and chemiluminescence detection.

Table 25. Properties of fluorescent blot stains

Substrate	Excitation max (nm)	Emission max (nm)	Fluorescence application
SYPRO Ruby Blot	450	618	Blot stain (PVDF or nitrocellulose)
SYPRO Rose Plus	~350	610	Blot stain (PVDF or nitrocellulose)

5.12.2 Instrument compatibility

Table 26. Compatibility of selected instrumentation with chemifluorescent substrates (a), fluorescent labels (b), and selected fluorescent stains (c) used in fluorescent Western blots*

a) Chemifluorescent substrates

Substrate	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation (nm)	Emission filter
ECL Plus	457	520 BP 40	488	530 DF 30	Blue	NA	NA
ECF	532	526 SP	488	570 DF 30	Blue	Reflection	UV high
DDAO Phosphate	633	670 BP 30	NA	NA	Red	NA	NA

b) Fluoresent labels

Fluorochrome	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation (nm)	Emission filter
Fluorescein	532	526 SP	488	530 DF 30	Blue	Reflection	UV low
Cy3	532	580 BP 30	514	570 DF 30	NA	NA	NA
Cy5	633	670 BP 30	NA	NA	Red	NA	NA
Alexa 532	532	555 BP 20	514	570 DF 30	NA	NA	NA
Alexa 633	633	670 BP 30	NA	NA	Red	NA	NA
PBXL-3	633	670 BP 30	NA	NA	Red	NA	NA

c) Fluoresent stains

Stain	Typhoon		FluorImager		Storm	VDS-CL	
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode	Excitation (nm)	Emission filter
SYPRO Ruby Blot	457†/488†/532	610 BP 30	488	610 RG	Blue	Reflection	UV high
SYPRO Rose Plus	NA	NA	NA	NA	NA	Reflection	UV high

NA = not applicable

* For membrane applications, substantial background fluorescence primarily due to non-specific antibody binding may occur. Shifting the excitation and emission wavelengths slightly away from the fluorochromes' spectral maxima wavelengths sometimes greatly reduces the noise and improves the overall signal-to-noise. For the most sensitive results, we recommend that the optimal setup be experimentally determined for each of the applications.

† Only available on Typhoon 9400 series. Note that for Typhoon 9400/9410, more than one setting could produce comparable imaging results. The most optimal setting could be experimentally determined by users for the individual application needs.

5.12.3 Typical protocols

This section gives an overview of typical fluorescent Western protocols. See reference 34 for complete protocols.

A. Western blotting using a fluorogenic substrate

Amersham Biosciences products available for this application

Products used	Code number
Hoefer miniVE Vertical Electrophoresis System	80-6418-77
Hoefer EPS 301 Power Supply	18-1130-01
Hybond-P PVDF Membranes (20 × 20 cm)	RPN2020F
ECL Plus Western Blotting Detection Reagents	RPN2132
ECF Western Blotting Kit	RPN5780
Fluorescein-linked anti-mouse Ig	N1031
Cy3-linked anti-rabbit IgG	PA43004
Cy5-linked anti-mouse IgG	PA45002
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog

Other materials required	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 (36). 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 during the blot preparation process.
- Successful fluorescence 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 large enough 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).
- Depending on the application, air-drying a fluorescent Western blot at the end of the experiment may improve the signal-to-noise ratio of the acquired image. The suitability of drying the blot should be determined with each application.

❷

Preparation of blot

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

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

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

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

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

❸

Application of substrate

3.1 Add 50-100 ml of substrate per cm^2 of membrane (except for ECF, add 5 ml of substrate per cm^2). Incubate for 5 min or less with ECF, 5 min for ECL Plus, and 5 min for DDAO phosphate substrates.

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

3.2 After developing, seal the blot in a low-fluorescence bag or page protector or air-dry the blot (if appropriate).

❹

Imaging

4.1 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 fluorescent imager.

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

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

4.3 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

5. Analysis

See Chapter 4 for information concerning image analysis.

B. Western blotting using a fluorochrome-conjugated antibody

Amersham Biosciences products available for this application

Products used	Code number
Hoefer miniVE Vertical Electrophoresis System	80-6418-77
Hoefer EPS 301 Power Supply	18-1130-01
Hybond-P PVDF Membranes (20 × 20 cm)	RPN2020F
ECL Plus Western Blotting Detection Reagents	RPN2132
ECF Western Blotting Kit	RPN5780
Fluorescein-linked anti-mouse Ig	N1031
Cy3-linked anti-rabbit IgG	PA43004
Cy5-linked anti-mouse IgG	PA45002
Typhoon 8600/8610	inquire
Typhoon 9200/9210	see catalog
Typhoon 9400/9410	see catalog
Storm 840/860	see catalog
FluorImager 595	see catalog
ImageMaster VDS-CL	see catalog

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 full recommended time interval per wash should be adhered to strictly.
- PVDF membranes should be kept wet at all times during the preparation process.
- Successful fluorescence 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).

- Depending on the application, air-drying a fluorescent Western blot at the end of the experiment may improve the signal-to-noise ratio of the acquired image. The suitability of drying the blot should be determined with each application.

❷

Preparation of blot

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

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

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

Note: If the primary antibody is fluorochrome-labelled, go directly to step 5.

2.4 Incubate the blot with fluorochrome-linked secondary antibody for 1 h, then wash the membrane thoroughly.

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

❸

Imaging

3.1 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 fluorescence imager.

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

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

3.3 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.

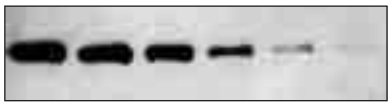


Fig 47. Typhoon image of chemifluorescent Western blot of serial two-fold dilutions of tubulin using ECL Plus substrate. Lanes from left to right contain 16, 8, 4, 2, 1, and 0.5 ng of β-tubulin.

5.12.4 Expected results

Typical results for chemifluorescent Western detection (a) and direct fluorescent Western detection (b) are given in Table 27. A Western blot developed with ECL Plus substrate and imaged using Typhoon is shown in Figure 47.

Table 27. Expected results for fluorescent Western detection of tubulin protein

a) Chemifluorescence

Substrate	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)
ECL Plus	0.5*	30*	5	3	1	15	ND	ND
ECF	2 to 4	10 to 20	2 to 4	10 to 20	2 to 4	10 to 20	2 to 4	10 to 20
DDAO Phosphate	1 to 2	10 to 20	1 to 2	10 to 20	1 to 2	10 to 20	NA	NA

NA = not applicable, ND = not determined

* ECL Plus Chemifluorescence is only available on Typhoon 9400/9410

b) Direct fluorescence

Fluorochrome	Typhoon		FluorImager		Storm		VDS-CL	
	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)	LOD (ng)	LDR (fold)
Fluorescein	1	130	1	130	4	30	ND	ND
Cy3	8	30	8	30	NA	NA	NA	NA
Cy5	8	30	NA	NA	8	30	NA	NA
Alexa 532	1	130	1	130	NA	NA	NA	NA
Alexa 633	1	130	NA	NA	1	130	NA	NA
PBXL-3	2	65	NA	NA	4	30	NA	NA

NA = not applicable, ND = not determined

* 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.

5.13 Using naturally occurring fluorescent proteins

5.13.1 Green fluorescent protein and its variants

The spectral properties of green fluorescent protein and its variants are given in Table 28.

Table 28. Spectral properties of GFP and its variants

Protein	Excitation max (nm)	Emission max (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum yield	Approximate relative brightness
EBFP	380	440	31 000	0.18	1x
ECFP	434	477	26 000	0.4	–
GFP (wt)	395, 470	508	–	–	1x
GFP-S65T	488	511	–	–	4-6x
EGFP	489	508	55 000	0.6	35x
EYFP	514	527	84 000	0.61	35x
DsRed	558	583	22 500	0.23	6x

5.13.2 Instrument compatibility with GFP and its variants

Table 29. Instrument settings for imaging 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	457	526 SP	NA	NA	Blue	NA	NA
GFP (wt)	532	526 SP	488	530 DF 30	Blue	R/T	UV low
GFP-S65T	532	526 SP	488	530 DF 30	Blue	R/T	UV low
EGFP	532	526 SP	488	530 DF 30	Blue	NA	NA
EYFP	488/532*	520 BP 20*/ 526 SP	514	530 DF 30	NA	NA	NA
DsRed	532	580 BP 30	514	570 DF 30	NA	NA	NA

NA = not applicable, R/T = reflection (opaque samples)

* Note that for Typhoon 9400/9410, more than one setting could produce comparable imaging results. The most optimal setting could be experimentally determined by users for the individual application needs.

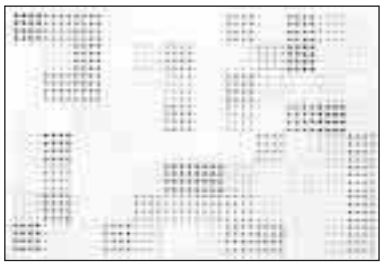


Fig 48. Varying expression levels from GFP-reporter constructs in yeast colonies. Colonies were spotted on agar plates and incubated at 37 °C. The agar plate was placed in a microplate tray and scanned using FluorImager at a resolution of 100 µm.

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

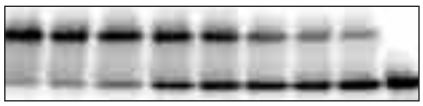


Fig 49. GFP gel shift assay showing quantitation of the interaction between S-protein and S15~GFP-S65T~His_6 using FluorImager. A constant amount of S15~GFP-S65T~His_6 (1 mM) was incubated with varying amounts (0-0.95 mM, left to right) of S-protein for 20 min at 20 °C. Samples were resolved by electrophoresis on a native 6% polyacrylamide gel.

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

5.13.3 Examples of applications using GFP

Monitoring gene expression in yeast

In this application, FluorImager SI was used to analyze transient gene expression in trans-formed 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 48).

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 (37). In this study, varying amounts of S-protein were incubated with purified S15 peptide~GFP-S65T~His6, and the complexes were then separated from free components in a native polyacrylamide gel (Fig 49). The image of the gel retardation assay was acquired using the 488-nm excitation source of a FluorImager SI system.

In another study using the 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 (38).

5.13.4 Expected results for GFP detection

Native gel electrophoresis is required to preserve the structure, and therefore the intrinsic fluorescence, of green fluorescent protein (GFP). The detection limits for purified wild-type wt-GFP for Typhoon, Storm, and FluorImager systems are shown in Table 30.

Table 30. Detection limits of GFP and variants

Protein	Typhoon 9400/9410 LOD (ng)	Typhoon 8600/8610/9200/9210 LOD (ng)	FluorImager LOD (ng)	Storm LOD (ng)
GFP (wt)	0.03	0.5	0.5	4

5.13.5 Phycobiliproteins

Table 31 shows the optical properties of some common phycobiliproteins

Table 31. Properties of phycobiliproteins

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

5.13.6 Instrument compatibility with phycobiliproteins

The broad excitation spectra, particularly of the RPE conjugates, allow phycobiliproteins to be efficiently excited using different types of imaging instrumentation with different excitation sources (Table 32). Allophycocyanin conjugates are ideal for use with helium-neon (He–Ne) laser excitation (633 nm).

Table 32. Compatibility of Amersham Biosciences instruments with phycobiliproteins

	Typhoon		FluorImager 595		Storm
	Excitation (nm)	Emission filter	Excitation (nm)	Emission filter	Fluorescence mode
B-phycoerythrin	532 nm	580 BP 30	514 nm	570 BP 30	Blue
R-phycoerythrin	532 nm	580 BP 30	514 nm	570 BP 30	Blue
Allophycocyanin	633 nm	670 BP 30	NA	NA	Red

NA = not applicable

Chapter 6

PRACTICAL RECOMMENDATIONS

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 fluorescent imaging experiment—from sample preparation to instrument operation and data analysis.

6.1 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 contaminants in buffer solutions and gels can cause minor spikes in the background, thus affecting image quality and quantitation. Filter solutions to remove dust particles, and then 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 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 of 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 fluorescent properties before they are used in experiments. The following materials are recommended for use in fluorescence imaging:

Table 33. Recommended materials

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

Avoid generating air bubbles when casting gels.

Air bubbles affect light scatter and can cause artifacts that interfere with quantitation. 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 uneven backgrounds that will affect quantitation. If a plastic gel tray is used, be sure to remove the gel from the tray prior to scanning.

Place wet 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 wet 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 Kimwipes™. 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. Clear, flat-bottom wells provide the largest imaging area with uniform surface characteristics. Microplates with clear bottoms and black or opaque walls can also be used.

6.2 Sample placement

The placement of the sample onto the imager is important to prevent the introduction of fluorescent artifacts, 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 quantitation. 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. If the platen is accidentally contaminated by fluorescent material, clean the surface with freshly made 10% hydrogen perioxide solution followed by a few rinses will distilled water.

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 quantitation. 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 can cause image artifacts. To avoid this, wear powder-free gloves and rinse gloves with

distilled water before handling samples and preparing reagents. Change gloves often.

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

A bag/sheet protector is used to cover wet 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 artifacts and interference patterns. The spacers can also protect the platen from scratching.

Use Gel Alignment Guide when scanning sandwich gels on Typhoon.

A Gel Alignment Guide (available only with Typhoon, code number 80-6496-10) can be used to scan multiple sandwich gels on Typhoon. Make sure the glass plate used for the bottom of the sandwich gel is 3-mm thick and has a low-fluorescence background. The grippers and the spacers on the guide hold the sandwich gel 0.2 mm above the glass platen which prevents optical interference.

Use the microarray slide holder when scanning microarray slides on Typhoon.

A microarray slide holder (available only with Typhoon, code number 63-0039-99) should be used to scan glass microarray slides on Typhoon. The slide holder prevents slide movement and positions the slide at the proper height above the glass platen so that Typhoon Imager can accurately collect data. Make sure to wear powder-free gloves to prevent the transfer of oils from your hands onto the glass platen or the microarray slide.

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 quantitation.

6.3 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 comprised of 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 (centered 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 so that 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 materials 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 quantitation, the sample signal should fall within the linear range of the system (count 1 to 100 000). 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. Refer to Appendix 1 and 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 fluorescence detection when using Typhoon.

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.

6.4 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 quantitation on the image, you must rescan the fluorescent sample using a lower PMT voltage setting.

Once the image is 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 coordinate (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 amount of light absorbed by a fluorochrome versus the absorption wavelength.

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

amplitude resolution — or **gray-level quantitation** 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	the release of light from a fluorochrome when an electron in the molecule falls from an excited state to a lower energy state.
emission filter	an optical filter used to enhance the collection of fluorescent signal from a fluorochrome.
emission spectrum	a plot of the relative intensity of emitted light as a function of emitted light wavelength.
energy of a photon of light	$E = hc/\lambda$, where h is Planck's constant, c is the speed of light, and λ is the wavelength of the light.
epi-illumination	illumination that impinges on a sample from the viewing direction.
excitation	the absorption of light energy by a fluorochrome, during which an electron in the fluorochrome molecule is boosted to a higher energy level.
excitation spectrum	a plot of the total fluorescence emitted as a function of incident-light wavelength.

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 color.

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, or 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 color in a digital image. The physical size of a pixel depends on the resolution of the image.

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 how well a true signal can be resolved from the noise. It is calculated by taking the signal strength divided by the standard deviation associated with measured background counts.

spatial resolution the ability to distinguish between two very closely positioned objects. Spatial resolution is related, but not equivalent, to the pixel size.

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

Should the excitation wavelength exactly match the peak of the fluorochrome's excitation spectrum in order to be excited?

No. However, the excitation efficiency is the highest when the excitation wavelength correlates closely with a fluorochrome's excitation peak. The excitation spectra of most fluorochromes are rather broad. Some fluorochromes also have long "tails" in their excitation spectra or have additional excitation peaks. Therefore it is not mandatory that the excitation wavelength match exactly with a fluorochrome's major excitation peaks in order to efficiently excite it. Note that a fluorochrome's excitation peak may shift with the changes in the binding environment or solvent. A more detailed explanation can be found in Chapter 1 and Chapter 3 of this handbook.

How is it possible to use Typhoon's 532-nm laser line and a 526 short-pass emission filter to detect fluorescein and similar "blue" dyes?

Excitation of a fluorochrome at the peak of its excitation spectrum is most efficient. However, a fluorochrome molecule can also be excited by other wavelengths of the excitation spectrum. A fluorescein molecule is successfully excited by Typhoon's powerful green laser line at 532 nm, even though the excitation peak for fluorescein is at 495 nm. However, better results for imaging fluorescein molecules are often achieved using the 488-nm laser line.

The wavelength of the emission collection is independent of the wavelength used for excitation. The center wavelength of the emission filter does not need to be at a longer wavelength than the excitation. Fluorescein is characterized by a fluorescence emission spectrum with a peak wavelength at 520 nm. Efficient collection of fluorescein emissions below 532 nm can be accomplished using a short-pass filter (526 SP), high-quality confocal optics, and a sensitive photomultiplier tube detector.

Can I scan microarrays or high-density arrays?

Fluorescently labelled microarrays (i.e on a microscope slide) typically require the 10 micron pixel option which is available on the Typhoon 9410, 9210, and 8610 models. Radiolabelled high-density membrane arrays (i.e macroarrays) can be exposed to phosphor screens which can then be imaged using Storage Phosphor mode (available on Typhoon, Storm, PhosphorImager or PSI) using the 50 micron pixel option.

Do I need to use low-fluorescence sample support materials?

Gels, membranes, glass plates, and microplates all autofluoresce to some extent. For optimal detection limits and dynamic range, low-fluorescence materials should be used. New materials should always be tested before they are used in experiments. See Table 33 for support materials recommended for fluorescence imaging.

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 with 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. If the platen is accidentally contaminated by fluorescent material, cleaning the surface with freshly made 10% hydrogen peroxide solution followed by a few rinses with distilled water.

What is spectral cross-talk and how do I avoid it?

Spectral cross-talk (cross-contamination) refers to the presence of fluorescence signal from more than one fluorochrome in a single optical channel. For best quantitative results with multicolor applications, cross-talk should be avoided or eliminated. The use of high-quality narrow band-pass emission filters and careful selection of fluorochromes with sufficient spectral separation can reduce or eliminate the cross-talk. If the cross-talk comes from the simultaneous multi-channel scans, sequential individual-channel scans should be used. In addition, FluorSep utility software (part of ImageQuant Solutions) successfully eliminates cross-talk for most multicolor applications.

I heard that the lasers in these scanners are very powerful. Is photobleaching a concern?

Powerful lasers do not necessarily cause photobleaching. Photobleaching depends on the photostability of the fluorochromes, which is affected by many environmental and experimental factors, such as solvent types, pH, temperature, etc. The amount of photobleaching is affected by the excitation intensity, efficiency, and dwell time at the sample during a scan. Short illumination dwell time helps to reduce the amount of photobleaching. Typhoon, Storm, and FluorImager all give ultra-short dwell times at the sample during the scan. With most commonly used fluorochromes, minimal to no photobleaching is observed even after multiple scans. CCD camera systems can cause significant photobleaching after only one scan.

What PMT voltage should I use for my scans? Will the increase of PMT voltage improve the signal-to-noise ratio?

The recommended PMT voltage is 450–800 V, which is the linear operating range of PMT. Using higher PMT settings within the linear range will increase the signal levels but not signal-to-noise ratios because the background and noise will go up proportionally with the signal. For quantitative results, always check to ensure that all pixels have counts between 1 and 100 000 before proceeding with data analysis.

Signal-to-noise ratio is the ultimate measure of sensitivity and detection limits for fluorescence applications. The “Normal” sensitivity setting on Typhoon, Storm, and FluorImager offer sufficient sensitivity levels for most applications. It is possible to increase signal-to-noise ratios by selecting a higher sensitivity setting that averages data for each pixel over multiple scans.

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

The "Press Sample” choice should be selected when appropriate. Remove any excess liquid from below the gel so that it does not move on the glass tray (FluorImager) or glass platen (Storm, Typhoon). Place a clean glass plate on top of a membrane or a blot that has been sealed between plastic sheets or page protectors.

How can I get the best fluorescence imaging results with my applications?

Amersham Biosciences offer many practical tips for various stages of fluorescence imaging applications – from sample preparation to instrument operation and data analysis. Please refer to a full description of practical recommendations in Chapter 6 of this manual.

What is a 16-bit GEL file and its advantages?

Amasham Biosciences' 16-bit GEL file is an extension to the industry standard TIFF, Tagged Image File Format. Amersham Biosciences uses proprietary TIFF tags to support quantitative data with a dynamic range up to 5 orders—values ranging from 0.00 to 100 000.00. A 16-bit file has only 2^{16} (65 536) levels, therefore it can not accommodate the 100 000 dynamic range from the Typhoon, Storm, and FluorImager systems. The Amersham Biosciences' 16-bit GEL file data are stored in square root scale rather than a linear scale to preserve and provide more resolution at the low-end without sacrificing the accuracy of the high-end data. Additionally the data in the GEL file is represented in floating point format not integer format. As a result, the data for the low signals are more accurate compared to the linear scale format. More accurate representation of the low-end values makes the background look more realistic and consequently smoother.

Can I view and analyze GEL images using software from suppliers other than Amersham Biosciences?

Many of the graphics or image analysis packages that can read and display 16-bit TIFF formatted files can only view GEL images for qualitative purposes. Only the software packages that support the Amersham Biosciences' GEL file format can be used for quantitative data analysis (see the list of the software products listed in Chapter 4). To avoid erroneous results, caution must be taken when converting a GEL file to a linear TIFF, as the quantitative advantages of the GEL file format are lost after the conversion.

Is my image suitable for quantitation?

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 centered on the tray. Center 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, or the signal acquisition time may have been too short and should be increased. The sample could be too thick or may not lay flat on the surface of the tray. To flatten a dry sample, place a glass plate over the sample. Remove any bubbles from below a wet gel.

Can sensitivity be improved by extending the exposure time?

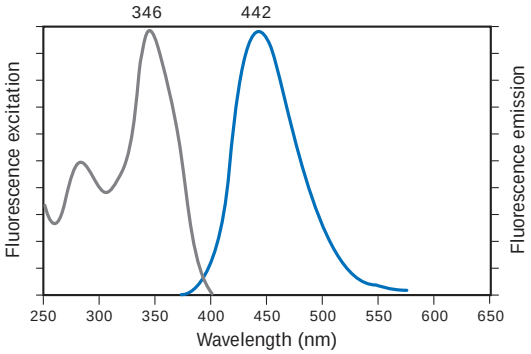
The signal from a sample is integrated over time. The sensitivity improves with exposure time, but only up to a point. The instrument noise dramatically affects the linearity of the CCD at low-light intensity and long exposure. The VDS-CL has a cooled CCD that significantly reduces the noise. However,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

Appendix 2

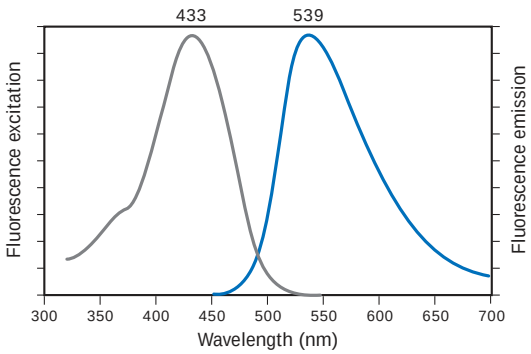
SPECTRAL CHARACTERISTICS OF COMMONLY USED FLUOROPHORES, FLUORESCENT STAINS, AND PROTEINS

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

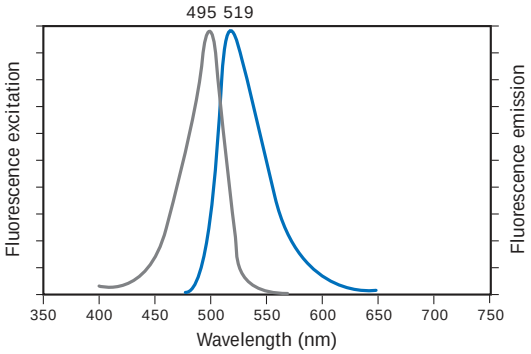
Alexa Fluor 350



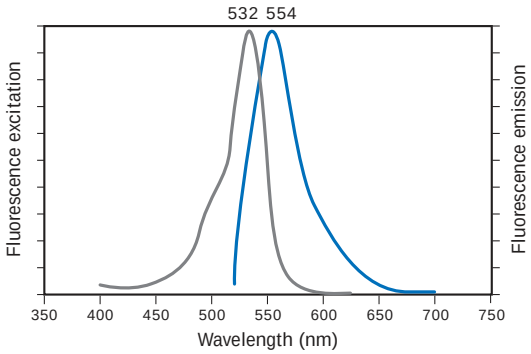
Alexa Fluor 430



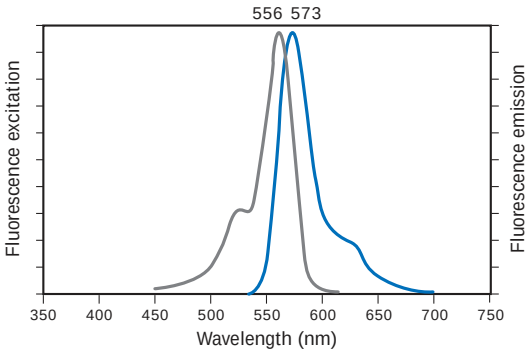
Alexa Fluor 488



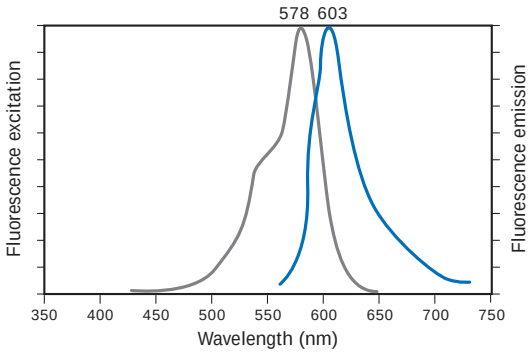
Alexa Fluor 532



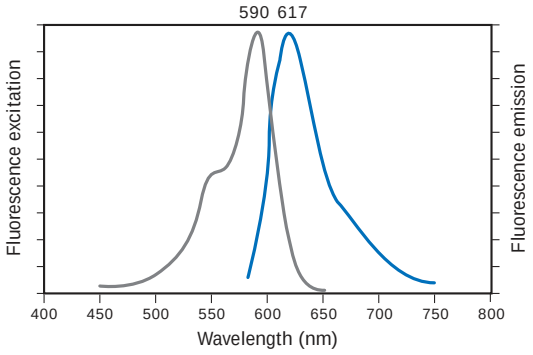
Alexa Fluor 546



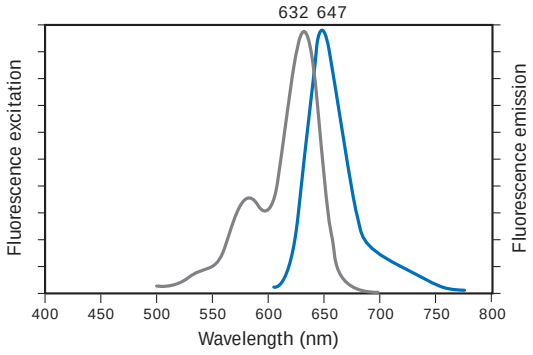
Alexa Fluor 568



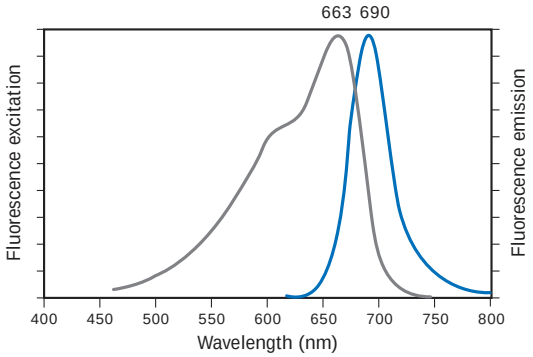
Alexa Fluor 594



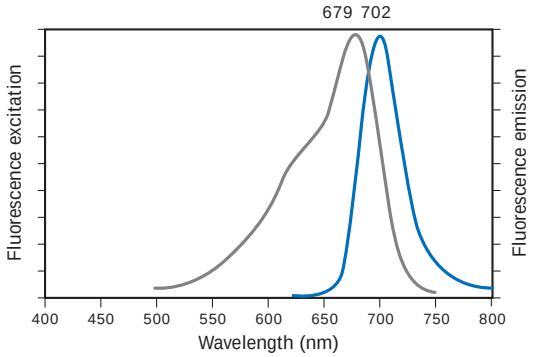
Alexa Fluor 633



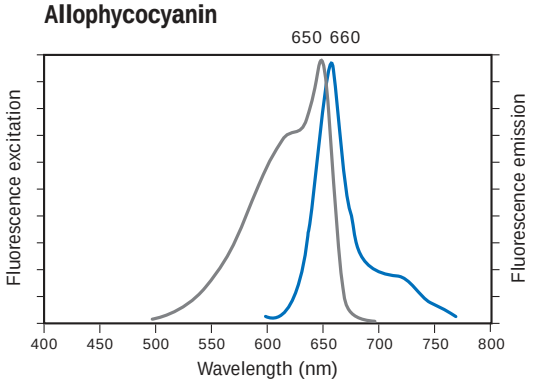
Alexa Fluor 660



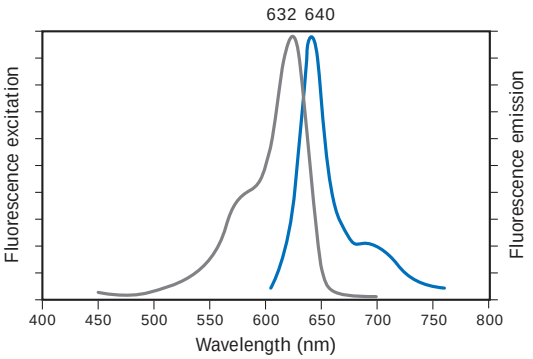
Alexa Fluor 680



Allophycocyanin



BODIPY™ 630/650



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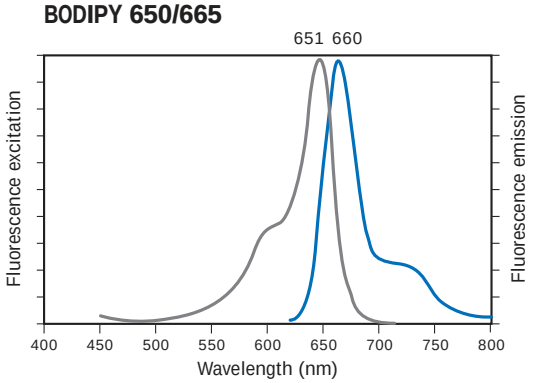
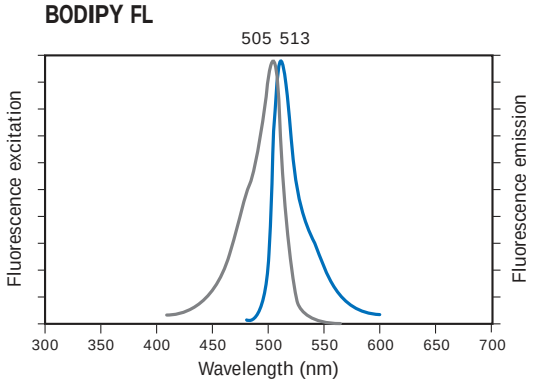
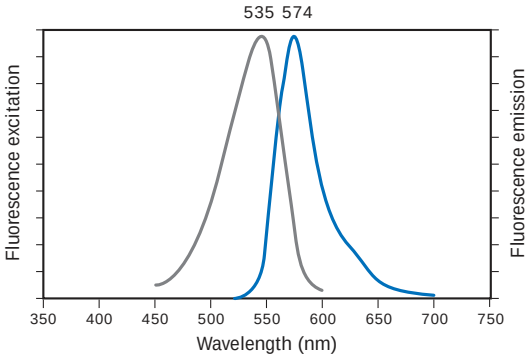


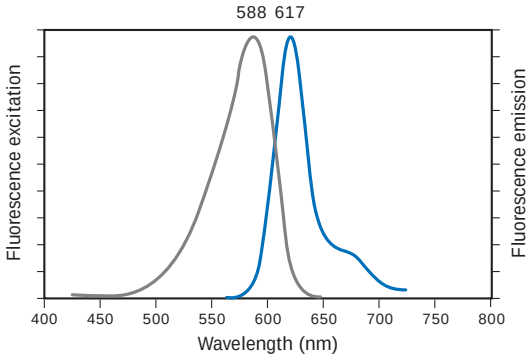
BODIPY FL



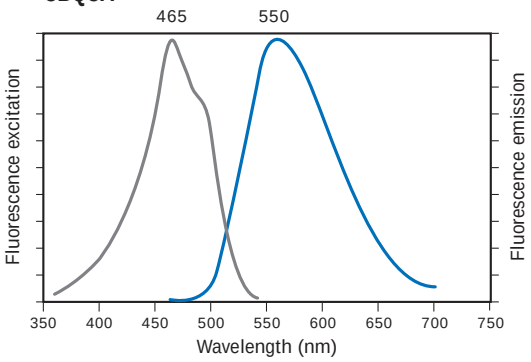
BODIPY TMR-X



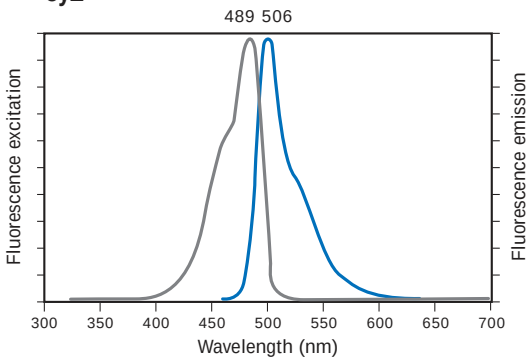
BODIPY TR-X



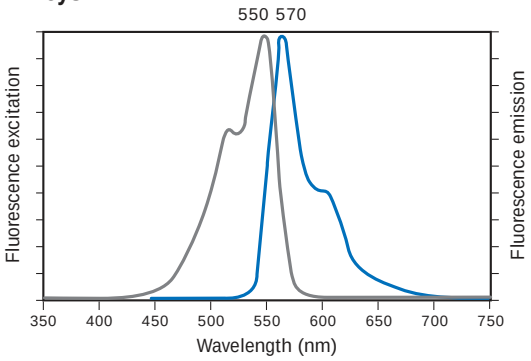
CBQCA



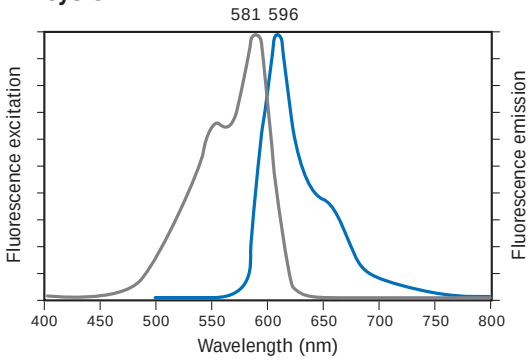
Cy2



Cy3



Cy3.5



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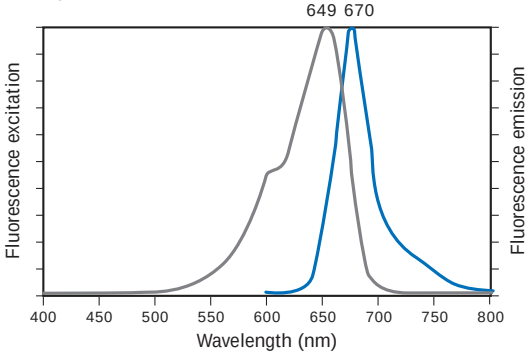

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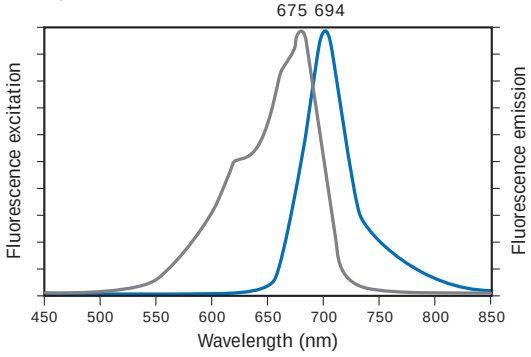

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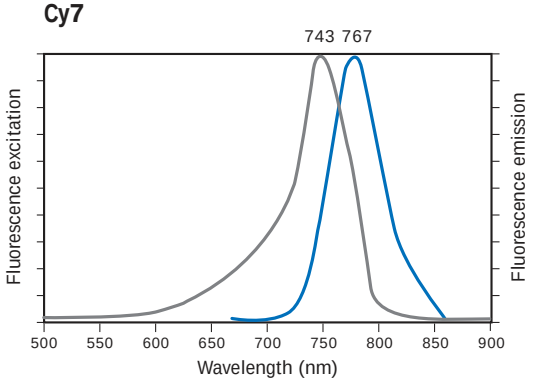
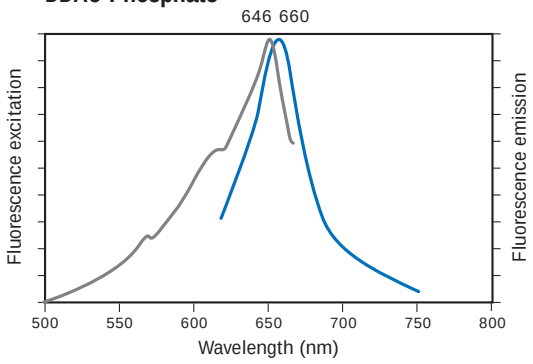
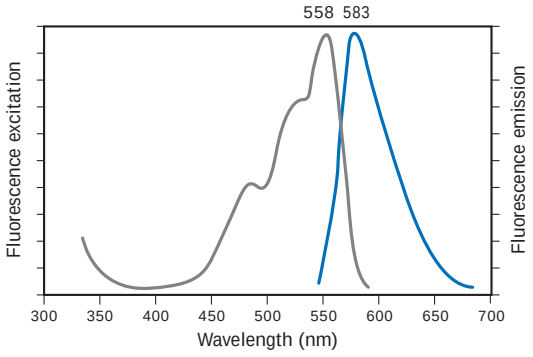


DDAO Phosphate*



DsRed



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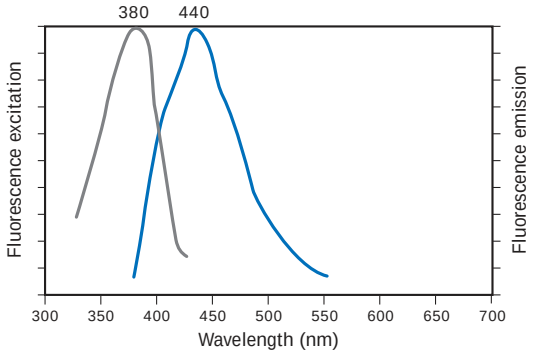

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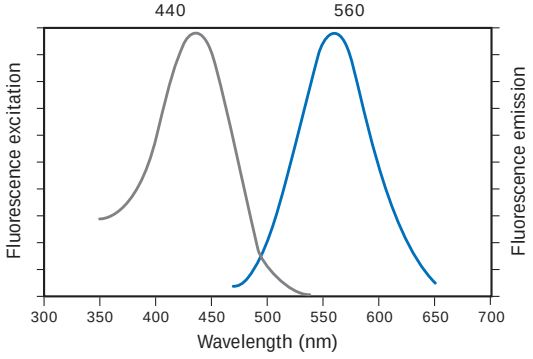
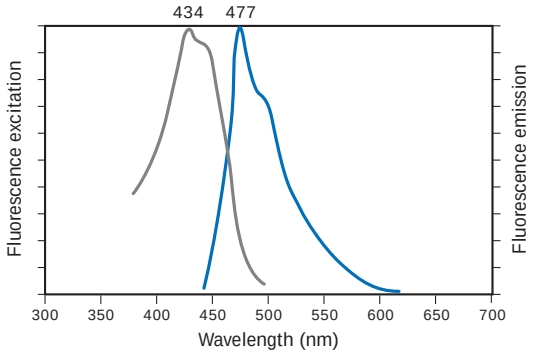


ECFP



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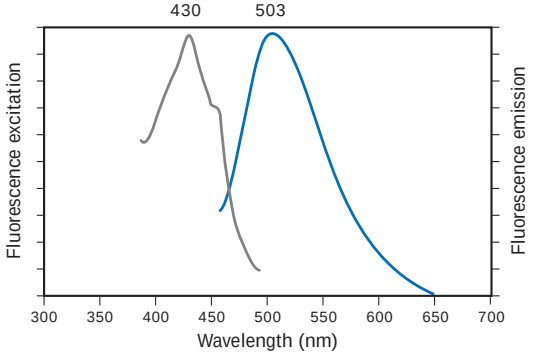
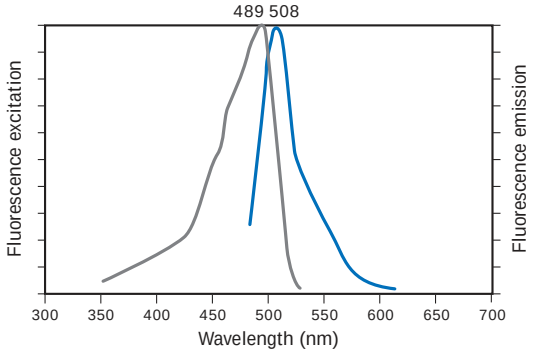
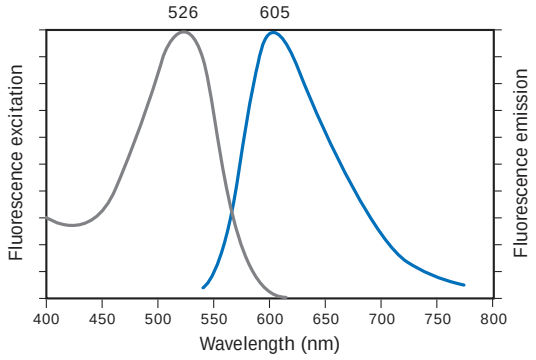


EGFP



Ethidium bromide†



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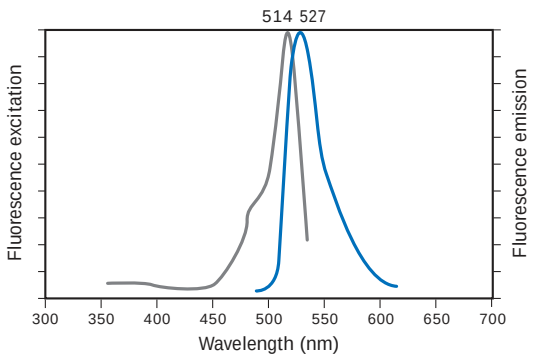
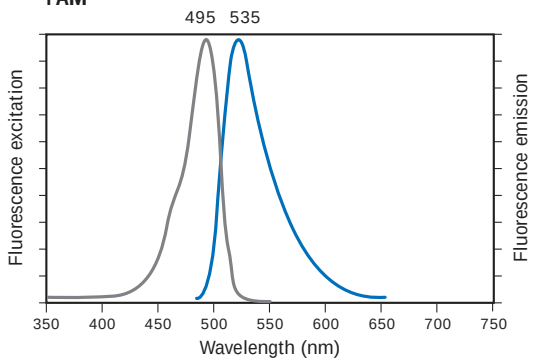


FAM™



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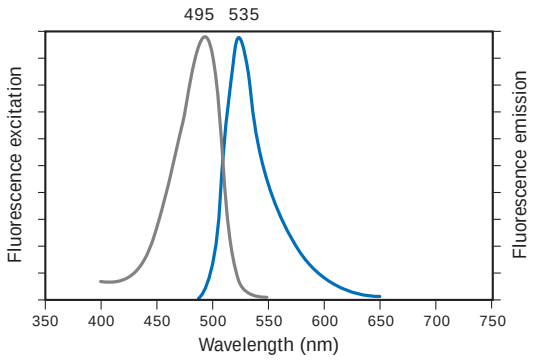
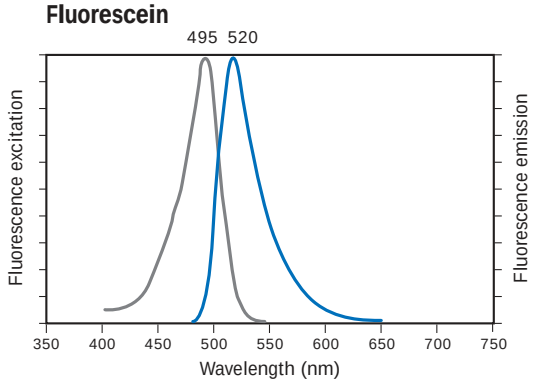


Fluorescein



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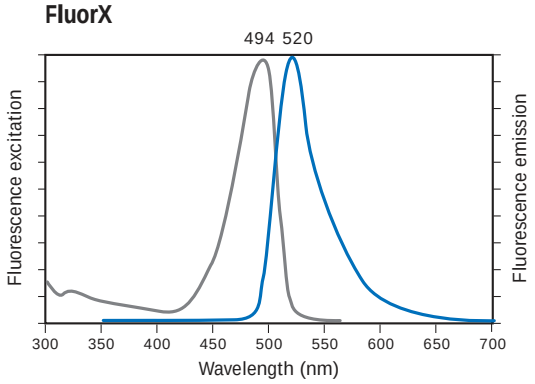
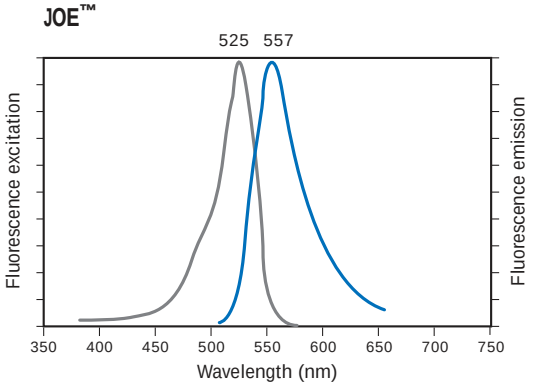
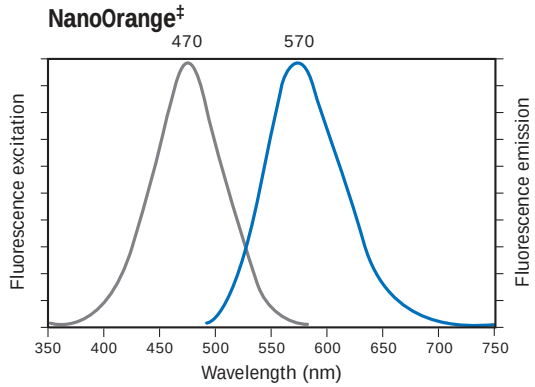


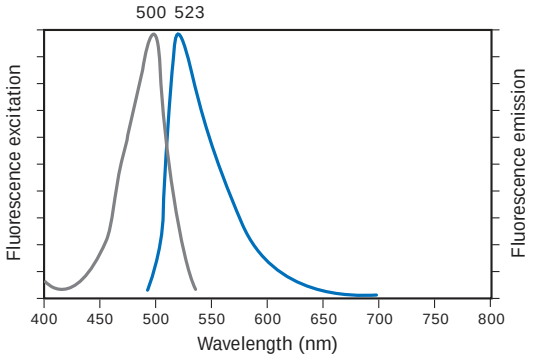
JOE™



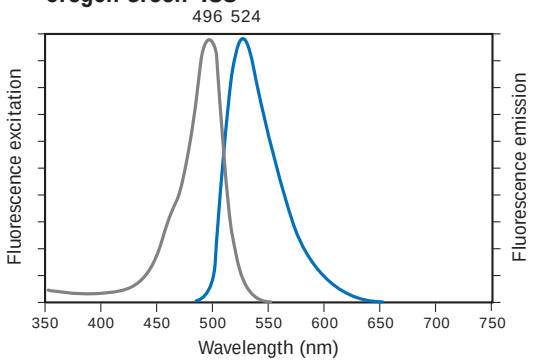
NanoOrange‡



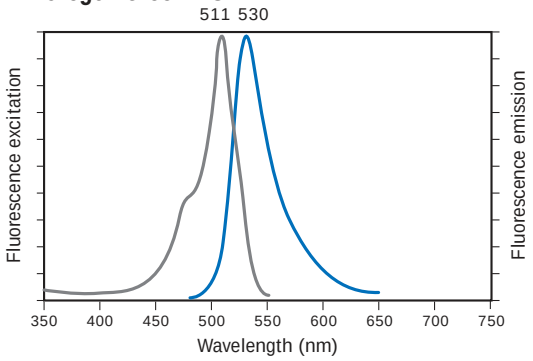
OliGreen†



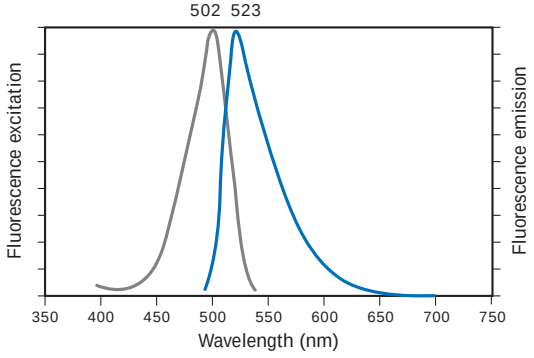
Oregon Green 488



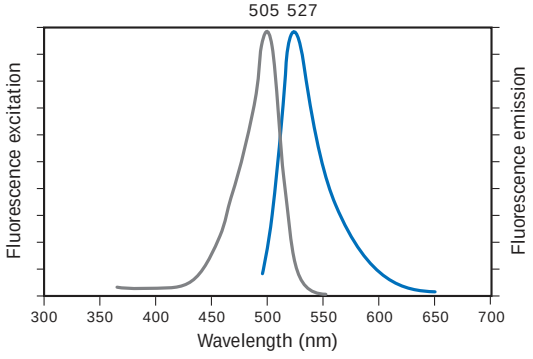
Oregon Green™ 514



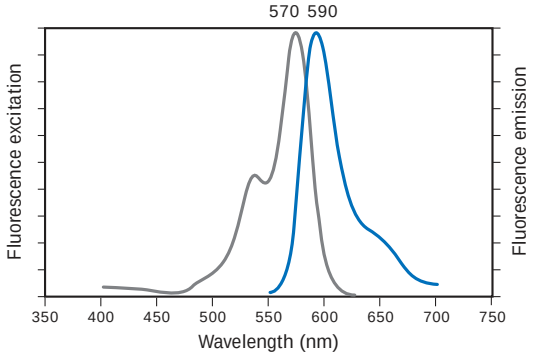
PicoGreen†



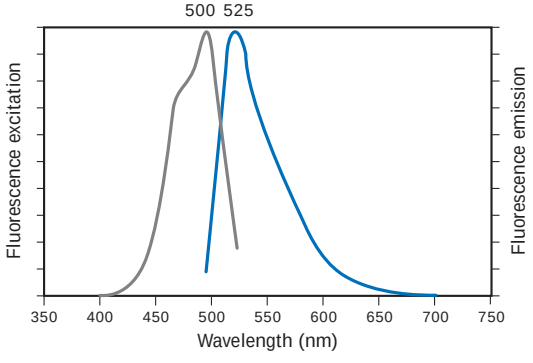
Rhodamine Green™



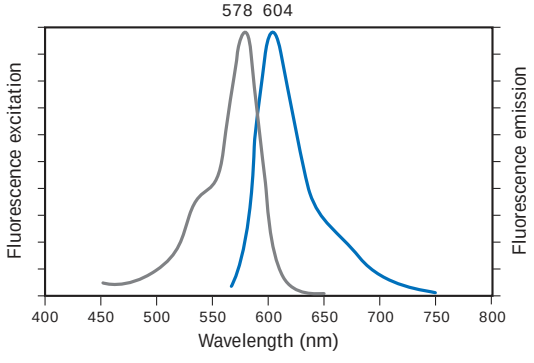
Rhodamine Red™-X



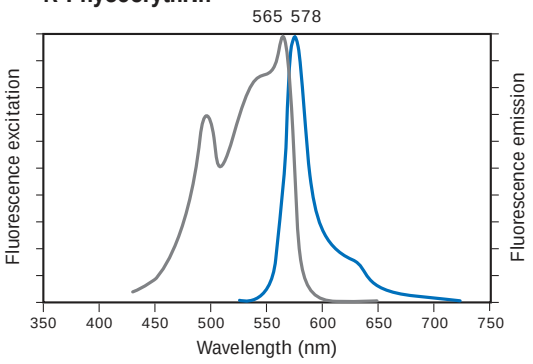
RiboGreen†



ROX



R-Phycoerythrin



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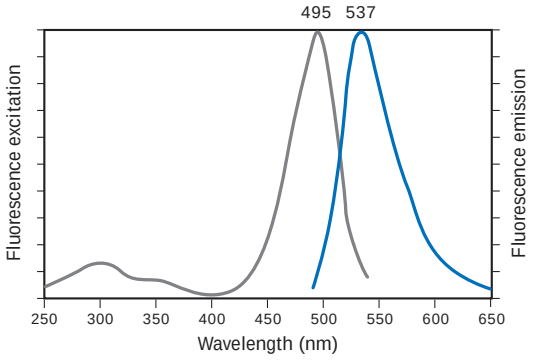

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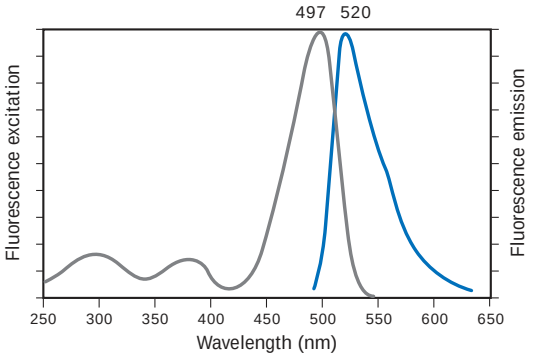

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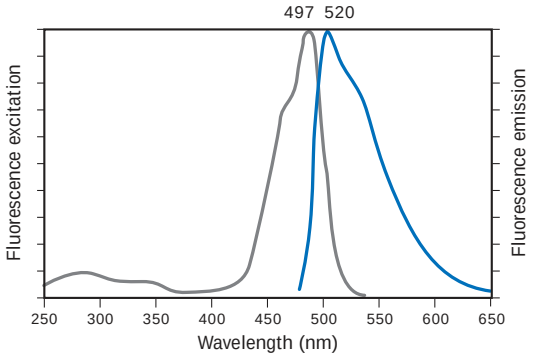
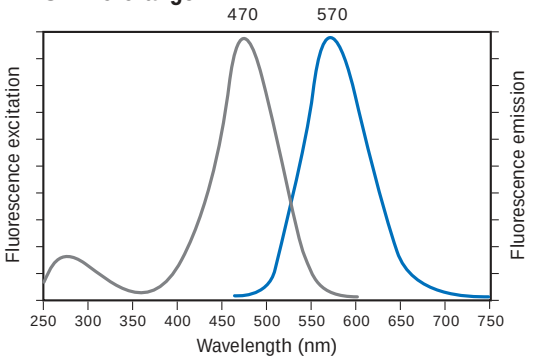


SYPRO Orange



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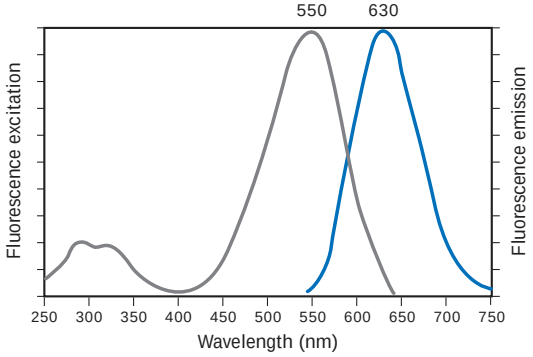
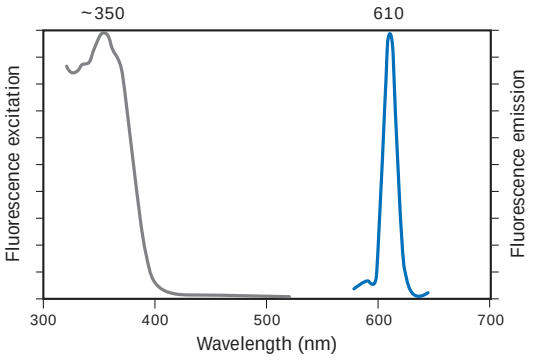
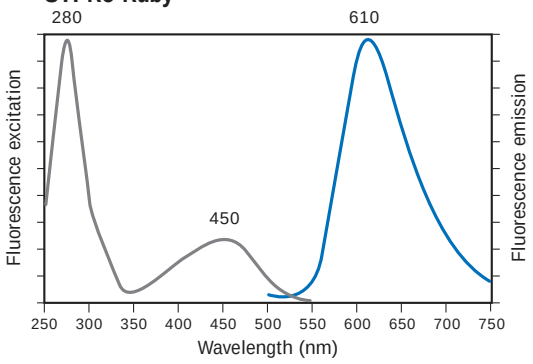


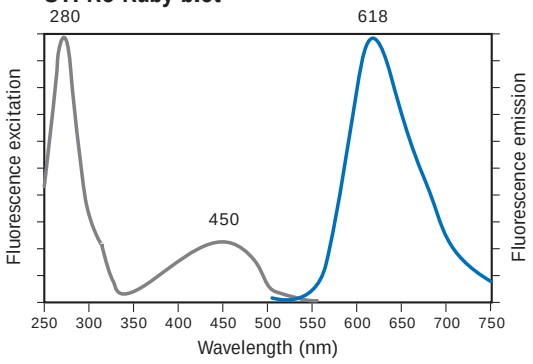
SYPRO Rose Plus



SYPRO Ruby



SYPRO Ruby blot



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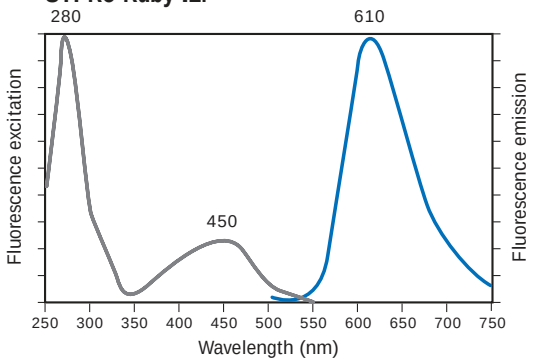
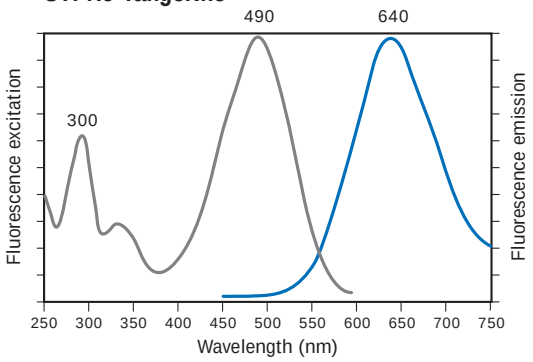
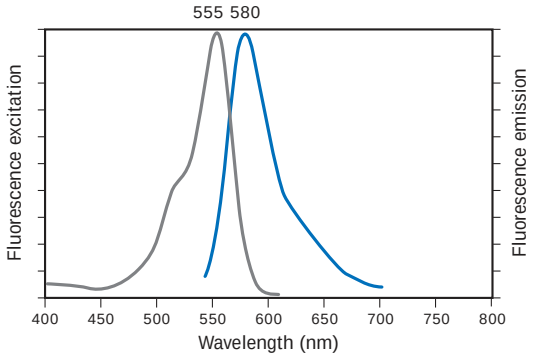


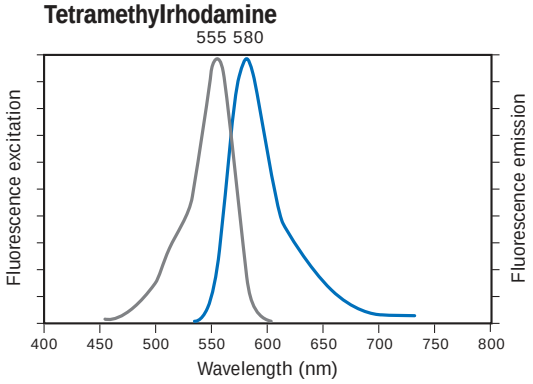
SYPRO Tangerine



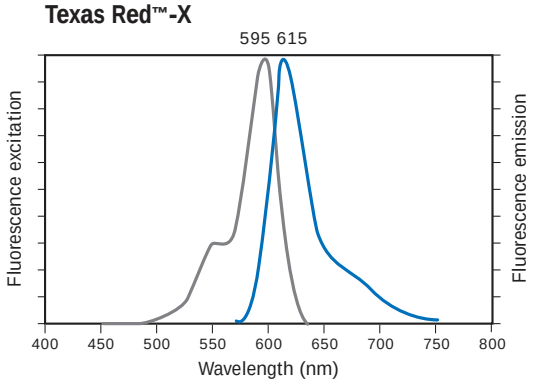
TAMRA



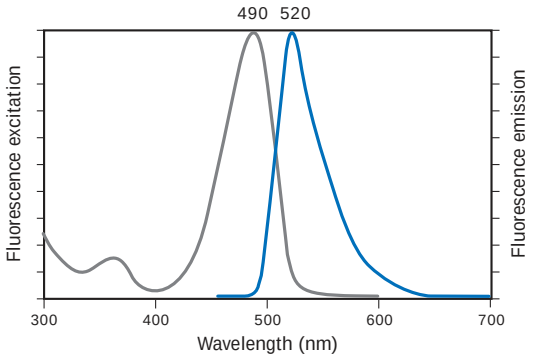
TetramethylRhodamine



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 Biosciences. 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, FLUORESCENT STAINS, AND PROTEINS

Fluorophore	Excitation max (nm)	Emission max (nm)	Typhoon 9400/9410 Excitation (nm)	Typhoon 9400/9410 Emission filter	Typhoon 9200/9210/8600/8610 Excitation (nm)	Typhoon 9200/9210/8600/8610 Emission filter
Nucleic acid gel stains						
Ethidium bromide	526	605	532	610 BP 30	532	610 BP 30
SYBR Gold	495	537	488	520 BP 40	532	526 SP
SYBR Green I	497	520	488	520 BP 40	532	526 SP
SYBR Green II	497	520	488	520 BP 40	532	526 SP
Vistra Green	490	520	488	520 BP 40	532	526 SP
Nucelic acids solution stains						
OliGreen	500	520	488/532*	520 BP 40/526 SP	532	526 SP
PicoGreen	502	523	488/532	520 BP 40/526 SP	532	526 SP
RiboGreen	500	525	488/532	520 BP 40/526 SP	532	526 SP
Substrates for Northern and Southern detection						
ODDAO Phosphate	646	660	633	670 BP 30	633	670 BP 30
ECF	440	560	532	526 SP	532	526 SP
Protein gel stains						
SYPRO Orange	300,470	570	488/532	555 BP 20	532	555 BP 20
SYPRO Red	300,550	630	532	610 BP 30	532	610 BP 30
SYPRO Ruby	280,450	610	457/488/532	610 BP 30	532	610 BP 30
SYPRO Ruby IEF	280,450	610	457/488/532	610 BP 30	532	610 BP 30
SYPRO Tangerine	300,490	640	488/532	610 BP 30	532	610 BP 30
Protein solution stains						
CBQCA	465	550	488/532	555 BP 20	532	555 BP 20
Nano Orange	470	570	457/488/532	580 BP 30	532	580 BP 30
Substrates, labels, and stains for Western blotting						
DDDAO Phosphate	646	660	633	670 BP 30	633	670 BP 30
ECF	440	560	532	526 SP	532	526 SP

Fluorophore	Excitation max (nm)	Emission max (nm)	FluorImager Excitation (nm)	FluorImager Emission filter	Storm Fluorescence mode	VDS-CL Excitation	VDS-CL Emission
Nucleic acid gel stains							
Ethidium bromide	526	605	514	610 RG	NA	Transmission	UV High
SYBR Gold	495	537	488	530 DF 30	Blue	Transmission	UV Low
SYBR Green I	497	520	488	530 DF 30	Blue	Transmission	UV Low
SYBR Green II	497	520	488	530 DF 30	Blue	Transmission	UV Low
Vistra Green	490	520	488	530 DF 30	Blue	Transmission	UV Low
Nucelic acids solution stains							
OliGreen	500	520	488	530 DF 30	Blue	NA	NA
PicoGreen	502	523	488	530 DF 30	Blue	NA	NA
RiboGreen	500	525	488	530 DF 30	Blue	NA	NA
Substrates for Northern and Southern detection							
DDAO Phosphate	646	660	NA	NA	Red	NA	NA
ECF	440	560	488	570 DF 30	Blue	Reflection	UV High
Protein gel stains							
SYPRO Orange	300,470	570	488	570 DF 30	Blue	Transmission	UV High
SYPRO Red	300,550	630	514	610 RG	Red	Transmission	UV High
SYPRO Ruby	280,450	610	488	610 RG	Blue	Transmission	UV High
SYPRO Ruby IEF	280,450	610	488	610 RG	Blue	Transmission	UV High
SYPRO Tangerine	300,490	640	488	610 RG	Blue	Transmission	UV High
Protein solution stains							
CBQCA	465	550	488	570 DF 30	Blue	NA	NA
Nano Orange	470	570	488	570 DF 30	Blue	NA	NA
Substrates, labels, and stains for Western blotting							
DDAO Phosphate	646	660	NA	NA	Red	NA	NA
ECF	440	560	488	570 DF 30	Blue	Reflection	UV High

Fluorophore	Excitation max (nm)	Emission max (nm)	Typhoon 9400/9410 Excitation (nm)	Typhoon 9400/9410 Emission filter	Typhoon 9200/9210/8600/8610 Excitation (nm)	Typhoon 9200/9210/8600/8610 Emission filter
Substrates, labels, and stains for Western blotting (continued)						
ECL Plus	430	503	457	520 BP 40	CL	CL
Fluorescein	495	520	532	526 SP	532	526 SP
Cy3	550	570	532	580 BP 30	532	580 BP 30
Cy5	649	670	633	670 BP 30	633	670 BP 30
Alexa Fluor 532	532	554	532	555 BP 20	532	555 BP 20
Alexa Fluor 633	632	647	633	670 BP 30	633	670 BP 30
PBXL-3	615	666	633	670 BP 30	633	670BP 30
SYPRO Rose Plus	~350	610	NA	NA	NA	NA
SYPRO Ruby blot	450	618	457/488/532	610 BP 30	532	610 BP 30
Multipurpose labels						
Alexa Fluor 350	346	442	NA	NA	NA	NA
Alexa Fluor 430	433	530	NA	NA	NA	NA
Alexa Fluor 488	495	520	488	520 BP 40	532	526 SP
Alexa Fluor 532	532	554	532	555 BP 20	532	555 BP 20
Alexa Fluor 546	556	573	532	580 BP 30	532	580 BP 30
Alexa Fluor 568	578	603	532	610 BP 30	532	610 BP 30
Alexa Fluor 594	590	617	532	610 BP 30	532	610 BP 30
Alexa Fluor 633	632	647	633	670 BP30	633	670 BP 30
Alexa Fluor 660	663	690	633	670 BP 30	633	670 BP 30
Alexa Fluor 680	679	702	633	670 BP 30	633	670 BP 30
BODIPY 630/650	630	650	633	670 BP 30	633	670 BP 30
BODIPY 650/665	650	665	633	670 BP 30	633	670 BP 30
BODIPY FL	505	513	488	520 BP 40	532	526 SP
BODIPY TMR-X	535	574	532	580 BP 30	532	580 BP 30
BODIPY TR-X	588	617	532	610 BP 30	532	610 BP 30
Cy2	489	506	488	520 BP 40	532	526 SP
Cy3	550	570	532	580 BP 30	532	580 BP 30
Cy3.5	581	596	532	610 BP 30	532	610 BP 30
Cy5	649	670	633	670 BP 30	633	670 BP 30
Cy5.5	675	694	633	670 BP 30	633	670 BP 30
Cy7	743	767	NA	NA	NA	NA

Fluorophore	Excitation max (nm)	Emission max (nm)	FluorImager Excitation (nm)	FluorImager Emission filter	Storm Fluorescence mode	VDS-CL Excitation	VDS-CL Emission
Substrates, labels, and stains for Western blotting (continued)							
ECL Plus	430	503	488	530 DF 30	Blue	CL	CL
Fluorescein	495	520	488	530 DF 30	Blue	Reflection	UV Low
Cy3	550	570	514	570 DF 30	NA	NA	NA
Cy5	649	670	NA	NA	Red	NA	NA
Alexa Fluor 532	532	554	514	570 DF 30	NA	R/T	UV High
Alexa Fluor 633	632	647	NA	NA	Red	NA	NA
PBXL-3	615	666	NA	NA	Red	NA	NA
SYPRO Rose Plus	~350	610	NA	NA	NA	Reflection	UV High
SYPRO Ruby blot	450	618	488	610 RG	Blue	Reflection	UV High
Multipurpose labels							
Alexa Fluor 350	346	442	NA	NA	NA	R/T	UV Low
Alexa Fluor 430	433	530	NA	NA	Blue	R/T	UV Low
Alexa Fluor 488	495	520	488	530 DF 30	Blue	R/T	UV Low
Alexa Fluor 532	532	554	514	570 DF 30	NA	R/T	UV High
Alexa Fluor 546	556	573	514	570 DF 30	NA	NA	NA
Alexa Fluor 568	578	603	514	610 RG	NA	NA	NA
Alexa Fluor 594	590	617	NA	NA	NA	NA	NA
Alexa Fluor 633	632	647	NA	NA	Red	NA	NA
Alexa Fluor 660	663	690	NA	NA	Red	NA	NA
Alexa Fluor 680	679	702	NA	NA	Red	NA	NA
BODIPY 630/650	630	650	NA	NA	Red	NA	NA
BODIPY 650/665	650	665	NA	NA	NA	NA	NA
BODIPY FL	505	513	488	530 DF 30	Blue	R/T	UV Low
BODIPY TMR-X	535	574	514	570 DF 30	NA	R/T	UV High
BODIPY TR-X	588	617	NA	NA	NA	NA	NA
Cy2	489	506	488	530 DF 30	Blue	R/T	UV Low
Cy3	550	570	514	570 DF 30	NA	NA	NA
Cy3.5	581	596	514	610 RG	NA	NA	NA
Cy5	649	670	NA	NA	Red	NA	NA
Cy5.5	675	694	NA	NA	Red	NA	NA
Cy7	743	767	NA	NA	NA	NA	NA

Fluorophore	Excitation max (nm)	Emission max (nm)	Typhoon 9400/9410 Excitation (nm)	Typhoon 9400/9410 Emission filter	Typhoon 9200/9210/8600/8610 Excitation (nm)	Typhoon 9200/9210/8600/8610 Emission filter
Multipurpose labels (continued)						
FAM	495	535	488	520 BP 40	532	526 SP
FITC	495	535	488	520 BP 40	532	526 SP
FluorX	494	520	488	520 BP 40	532	526 SP
HEX	529	560	532	555 BP 20	532	555 BP 20
JOE	525	557	532	555 BP 20	532	555 BP 20
Oregon Green 488	496	524	488	520 BP 40	532	526 SP
Oregon Green 514	511	530	532	555 BP 20	532	555 BP 20
Rhodamine Green	505	527	488/532	520 BP 40/526 SP	532	526 SP
Rhodamine Red-X	570	590	532	580 BP 30	532	580 BP30
ROX	578	604	532	610 BP 30	532	610 BP30
TAMRA	555	580	532	580 BP 30	532	580 BP30
TET™	519	545	532	555 BP 20	532	555 BP20
Tetramethylrhodamine	555	580	532	580 BP 30	532	580 BP30
Texas Red-X	595	615	532	610 BP 30	532	610 BP30
Fluorescent proteins						
Allophycocyanin	650	660	633	670 BP 30	633	670 BP 30
B-phycoerythrin	546	575	532	580 BP 30	532	580 BP 30
R-phycoerythrin	565	578	532	580 BP 30	532	580 BP 30
GFP (wt)	395,470	508	488	520 BP 40	532	526 SP
GFP-S65T	488	511	488	520 BP 40	532	526 SP
EGFP	489	508	488	520 BP 40	532	526 SP
EYFP	514	527	488/532	520 BP 20/526 SP	532	555 BP 20
DsRed	558	583	532	580 BP 30	532	580 BP 30

CL = Chemiluminescence only. Not applicable for fluorescence

UV High = 580 BP 30 filter; UV Low = 520 BP 30 filter

NA = Not applicable

R/T = Reflection for membranes/ transmission for gels

*Note that for Typhoon 9410/9400, more than one settings could produce comparable imaging results. The most optimal setting could be experimentally determined by users for the individual application needs.

Fluorophore	Excitation max (nm)	Emission max (nm)	FluorImager Excitation (nm)	FluorImager Emission filter	Storm Fluorescence mode	VDS-CL Excitation	VDS-CL Emission
Multipurpose labels (continued)							
FAM	495	535	488	530 DF 30	Blue	R/T	UV Low
FITC	495	535	488	530 DF 30	Blue	R/T	UV Low
FluorX	494	520	488	530 DF 30	Blue	R/T	UV Low
HEX	529	560	514	570 DF 30	NA	R/T	UV High
JOE	525	557	514	570 DF 30	NA	R/T	UV High
Oregon Green 488	496	524	488	530 DF 30	Blue	R/T	UV Low
Oregon Green 514	511	530	514	530 DF 30	NA	R/T	UV Low
Rhodamine Green	505	527	488	530 DF30	Blue	R/T	UV Low
Rhodamine Red-X	570	590	514	570 DF 30	NA	NA	NA
ROX	578	604	514	610 RG	NA	NA	NA
TAMRA	555	580	514	570 DF 30	NA	NA	NA
TET	519	545	514	530 DF 30	NA	R/T	UV High
Tetramethylrhodamine	555	580	514	570 DF 30	NA	NA	NA
Texas Red-X	595	615	NA	NA	NA	NA	NA0
Fluorescent proteins							
Allophycocyanin	650	660	NA	NA	Red	NA	NA
B-phycoerythrin	546	575	514	570 DF 30	Blue	NA	NA
R-phycoerythrin	565	578	514	570 DF 30	Blue	NA	NA
GFP (wt)	395,470	508	488	530 DF 30	Blue	Reflection	UV Low
GFP-S65T	488	511	488	530 DF 30	Blue	Reflection	UV Low
EGFP	489	508	488	530 DF 30	Blue	Reflection	UV Low
EYFP	514	527	514	530 DF 30	NA	Reflection	UV Low
DsRed	558	583	514	570 DF 30	NA	NA	NA

CL = Chemiluminescence only. Not applicable for fluorescence

UV High = 580 BP 30 filter; UV Low = 520 BP 30 filter

NA = Not applicable

R/T = Reflection for membranes/ transmission for gels

*Note that for Typhoon 9410/9400, more than one settings could produce comparable imaging results. The most optimal setting could be experimentally determined by users for the individual application needs.

Appendix 4

INSTRUMENT PERFORMANCE WITH COMMON FLUOROPHORES AND FLUORESCENT PROTEINS

Fluorophore	Typhoon 9410/9400	Typhoon 9210/9200/8600	FluorImager	Storm	VDS-CL
Nucleic acid gel stains (ds DNA) in agarose gel	Limit of detection (pg/band)				
Ethidium Bromide-cast w/ gel	50	50	50	NA	ND
Ethidium Bromide-poststain	50	50	200	NA	100
Vistra Green	10	10	10	500	ND
SYBR Gold	10	10	10	500	ND
SYBR Green I	10	10	10	500	ND
Nucleic acid gel stains (ds DNA) in polyacrylamide gel	Limit of detection (pg/band)				
Ethidium Bromide-cast w/ gel	ND	ND	ND	NA	ND
Ethidium Bromide-poststain	6	6	25	NA	ND
Vistra Green	3	3	6	25	20
SYBR Gold	3	3	6	25	20
SYBR Green I	3	3	6	25	20
Nucleic acids solution stains	Limit of detection (ng/ml)				
PicoGreen	10/2.5*	10/2.5*	5	50	ND
RiboGreen	ND	ND	1	10	ND
Substrates for Northern and Southern detection	Limit of detection (pg target)				
ECF	0.5	0.5	0.25	0.25	0.25
DDAO Phosphate	0.25	0.25	NA	0.25	ND
Protein gel stains	Limit of detection (ng/band)				
SYPRO Orange	2	2	3	6	5
SYPRO Red	2	2	2	3	ND
SYPRO Tangerine	2	2	4	4	ND
SYPRO Ruby	1	1	2	2	1
Protein solution stains	Limit of detection (µg/ml)				
Nano Orange	1/0.1*	1/0.1*	0.3	1	ND

Fluorophore	Typhoon 9410/9400	Typhoon 9210/9200/8600	FluorImager	Storm	VDS-CL
Substrates and labels for Western blotting	Limit of detection (ng target)				
ECF	2-4	2-4	2-4	2-4	2-4
DDAO Phosphate	1-2	1-2	1-2	1-2	NA
ECL Plus	0.5	NA	5	1	ND
Fluorescein	1	1	1	4	ND
Cy3	8	8	8	NA	NA
Cy5	8	8	NA	8	NA
Alexa 532	1	1	1	NA	NA
Alexa 633	1	1	NA	1	NA
PBXL-3	2	2	NA	4	NA
Multipurpose labels	Limit of detection (fmol band DNA in polyacrylamide gel)				
Alexa Fluor 430	ND	ND	200	100	ND
Cy2	0.6	7.5	7.5	30	ND
Cy3	0.2	0.2	4	NA	ND
Cy3.5	0.2	0.2	ND	NA	ND
Cy5	0.2	0.2	ND	1	ND
FAM	0.1	0.4	0.4	50	ND
Fluorescein	0.1	0.4	0.4	50	ND
HEX	0.2	0.2	2	NA	ND
ROX	0.2	0.2	12	NA	ND
TAMRA	0.2	0.2	4	NA	ND
TET	ND	ND	1	NA	ND
Fluorescent proteins	Limit of detection (ng protein band in SDS-PAGE)				
GFP (wt)	0.03	0.5	0.5	4	ND

NA = not applicable, ND = not determined

* First number from assay performed using PolySorp 96-well plates with removable strips.

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