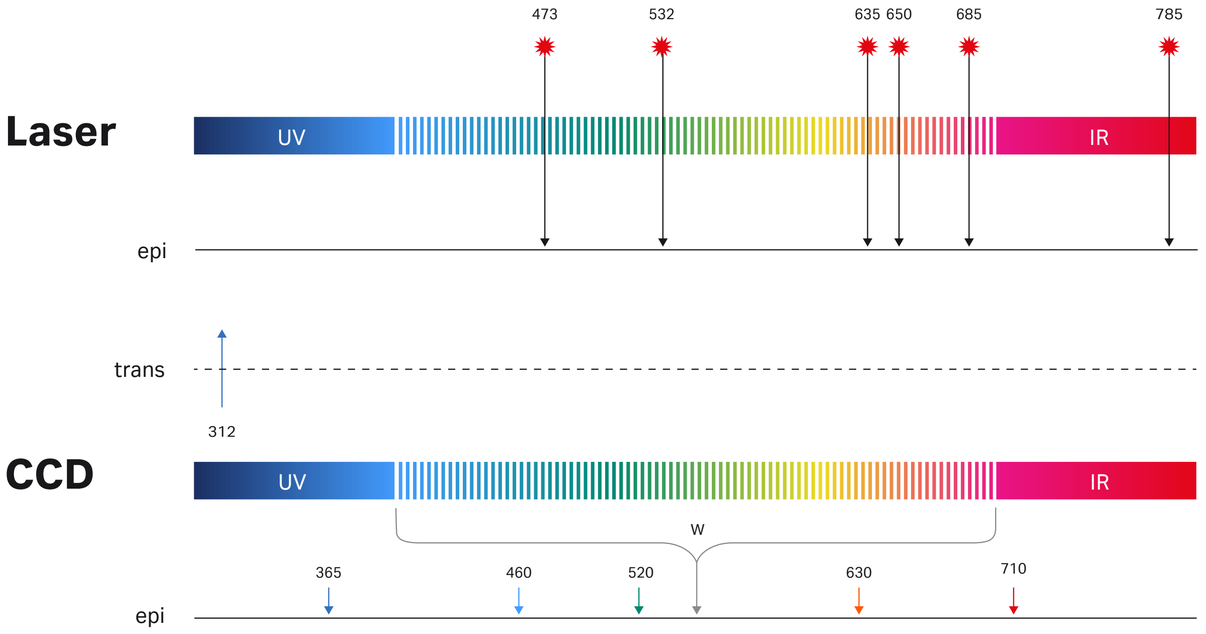
Imaging



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Contents

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Preface

Long before 1997 when the term “proteomics” saw the light of day scientists were taking images of proteins and other biomolecules. However, the rapid expansion of imaging technologies in the last decade has provided new tools and methods that are more sensitive, affordable and safer to use. The objective of “Imaging – principles and methods” is to provide readers with a theoretical overview and practical guide to protein imaging and quantitation.

The first three chapters give the reader a thorough background in how proteins are detected, the variety of imaging instruments and how image capture and filters work. Chapter four shows how data can be refined and analyzed after image acquisition using Cytiva’s software packages.

Chapter five gives a summary and protocols for some of the most widely used imaging applications, such as Western blotting and naturally fluorescent proteins. We hope the practical approach in this chapter will provide readers with the necessary knowledge to maximize their imaging capabilities. To help avoid typical imaging pitfalls, chapter six provides a range of practical advice based on the experience of Cytiva’s own scientists.

Symbols

This symbol indicates general advice on how to improve procedures or recommends measures to take in specific situations

This symbol indicates where special care should be taken

Chemicals, buffers and equipment

Experimental protocol

01

Imaging and detection

1.1 Introduction

Some of the most outstanding advances in genomics, proteomics, protein research, and drug discovery during the 20th century were based on images and the information they revealed. Since the advent of electrophoresis-based blotting in the mid-1970s, there has been a rapid evolution of the tools and the methods used to produce and interpret these images.

Capturing images has a long and varied history that spans many scientific disciplines. From Isaac Newton's discovery that light was composed of different colors it took over 150 years before the first image was captured by Joseph Niepce in 1825. The invention of film by George Eastman in the early 20th century led to its widespread use. His invention of film rolls as well as Kodak™ cameras set the standard for image capture until the mid 1960's and the advent of Polaroid™ instant color photography. The development of the charge-coupled device (CCD) chip in 1969 by Willard S. Boyle and George E. Smith heralded the beginning of a technological line of development that eventually led to the replacement of film by digital imaging. However, older photochemical methods continue to serve users in specialist applications.

The detection of biological molecules on gels, blots and arrays can be performed using a wide variety of labeling and staining techniques in combination with the appropriate imaging device. Therefore, it is important to first consider the quality and quantity of data required from your experiments. Do you want to know whether a protein is present or absent in a sample or simultaneously detect multiple proteins down to picogram quantities? The answers to these types of questions will decide the type of workflow, imaging detection, method and system you use.

Historically, colorimetric stains such as Coomassie™ Blue and silver stain held positions as the 'gold-standard' techniques in protein imaging. Coomassie Blue stain has its origins in an acid wool dye developed in the late 19th century, and is named after the town of Kumasi, in Ghana. Coomassie Brilliant Blue was first used to visualize proteins in 1963 by Fazekas de St. Groth and colleagues (1), while silver staining was first used in the 14th century to color glass. The first biological applications, however, emerged early in the 20th century when Camillo Golgi used silver staining to study the nervous system (2). In 1973, silver staining was introduced by Kerenyi and Gallyas as a sensitive procedure to detect trace amounts of proteins in gels (3).

The last decade has seen a shift from colorimetric stains to the use of fluorescent labels and stains. However, colorimetric staining is still widely used to verify and evaluate the efficiency of transfer of proteins from gels to membranes. Generally, colorimetric staining methods have a narrower dynamic range and more variability in the specificity with which the stain binds to different proteins. The use of fluorescent stains and labels, in combination with advanced imaging devices, allows for a much broader dynamic range and more sensitive detection when compared to traditional colorimetric staining methods. Fluorescent stains and labels such as Deep Purple Total Protein Stain and CyDye™ Fluors from Cytiva have become essential tools for the analysis of proteins. The potential of fluorescent technologies in multiplexing applications (the simultaneous use of two or more labels directed against different targets in the same sample) makes it the technology of choice in several situations. Fluorescent labeling of specific proteins has allowed scientists to localize subcellular structures or to identify multiple protein targets on a single blot, even if they have identical molecular weights.

Green fluorescent protein (GFP), first isolated from the jellyfish *Aequorea victoria*, emits bright green light when exposed to blue light. Importantly, GFP is not toxic when illuminated in living cells and has become an important tool for *in vivo* analysis. Through cloning and genetic modification, a wide variety of GFP derivatives are in use today. It is widely used as a reporter for gene expression of fusion proteins and in the study of gene regulation in living organisms.

In 1975, the British biologist, Edwin Southern, pioneered the use of isotope-labeled nucleotides for detection of specific sequences in DNA (4). This technique to transfer DNA fragments from an agarose gel to a membrane, known as Southern blotting, opened the way for other blotting techniques based on electrophoresis, such as Northern blotting for RNA in 1977 and Western blotting (also known as immunoblotting) for the transfer of proteins.

The name “Western blot” was given to the technique by Burnette in 1981 (5) although the method was introduced in 1979 by Towbin and colleagues (6). To this day, Western blotting remains one of the most powerful methods for the separation and quantitation of proteins, although radioisotopes have lost their position as the principle technology for labeling and detection to safer, cheaper, and more effective antibody labeling and light-emitting technologies.

Depending on experimental needs, a large number of options are available for luminescent labeling and detection of biomolecules. Chemiluminescent or enhanced chemiluminescent (ECL) systems now deliver high sensitivity for solution or blot-based assays. Amersham™ ECL™ was the world’s first commercially available non-radioactive method for detecting proteins, and nucleic acids enabling detection of minute quantities of a specific protein, based on a signal proportional to the amount of protein present over a wide range of concentrations.

Bioluminescence is an aspect of chemiluminescence in which living organisms, such as fireflies, create their own light when an enzyme acts on a chemical substrate. This application has grown considerably over the past decade as the traditional use of reporter genes has enabled the production of proteins with a bioluminescent tag. While a chemiluminescent system must be assayed *in vitro*, bioluminescent systems allow biological mechanisms to be visualized and assayed *in vivo*.

1.2 Detection principles

The need to detect low abundance proteins has increased the requirements for sensitivity and linear dynamic range when using techniques such as gel- and/or blot-based quantitative analysis. A wide linear dynamic range is particularly important for applications where weak and strong signals are compared in the same experimental series.

Components of imagers such as the excitation source (for fluorescent imaging), lens, emission filter, detector and the basic technical principles used (e.g., confocal optics, moving scanner head or galvanometer technology) all impact on linear dynamic range and image quality. Digital resolution (signal intensity levels distinguished by the imager) and spatial resolution (related to photosite configuration as well as the number of lines per millimeter that can be resolved in an image) are also important.

CCD camera-based systems are composed of an illumination source and optics that focus the image onto a CCD chip. They are area imagers that integrate chemiluminescent signals or fluorescent signals 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 variable focal length. Some flatbed scanners, such as ImageScanner III from Cytiva, are also CCD camera-based and capture the illuminated section during a scan cycle.

In addition, the most appropriate choice of label or stain will also enhance performance in terms of both sensitivity and dynamic range. Fluorescent labels and stains can be more sensitive and have a broader dynamic range than colorimetric stains. These are important considerations when large variations in signal intensity are expected.

Fluorescence based detection is replacing chemiluminescence based detection due to improved sensitivity, the development of new fluorescent labels and the reduced cost of fluorescent detection systems. Fluorescent detection also opens the possibility of multiplexing, where samples containing differentially labeled proteins can be simultaneously detected on a single gel or blot.

The type of imager to use depends heavily upon the detection system or systems you choose and the quality and quantity of data required from your experiments. Densiometers, such as ImageScanner III from Cytiva, provide high resolution densiometry for X-ray films exposed to radioisotopes or light and gels stained with reagents such as Coomassie Blue or silver. With CCD camera-based systems, such as the ImageQuant™ LAS series from Cytiva it is possible to cover a full range of tasks, from sample documentation to fluorescence and chemiluminescence. For maximum versatility in imaging, high performance variable mode imagers are available, such as those in the Typhoon™ FLA series from Cytiva. These imagers make it possible to handle phosphorimaging, multiplex fluorescence, and chemifluorescence with high throughput, whilst maintaining quantitative precision and low detection limits.

1.3 Fluorescence detection

The light phenomenon of fluorescence occurs when molecules called fluorophores absorb light. In their ground state, fluorophores do not emit light, but when subjected to light (excitation) their energy levels are raised to a brief but unstable excited state. As fluorophores return to their ground state, they emit light at a lower energy, (longer wavelength) than the excitation light (Fig 1.1).

Fluorescence is a regenerative process; fluorophores can repeatedly undergo excitation, which means that a fluorophore can produce a signal several times, making this method potentially very sensitive and stable.

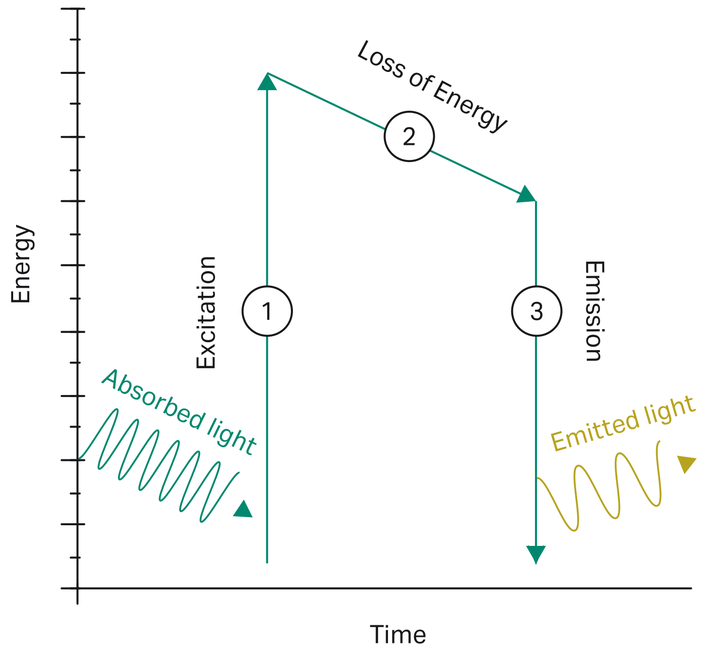

Fig 1.1. Fluorescence can be summarized as a three step process: (1) Excitation of a fluorophore at ground state, by absorption of light, (2) a short period in an excited but unstable state that usually relaxes towards the lowest vibrational energy level within the excited state before, (3) returning to ground state with the emission of light at a lower energy and longer wavelength than the absorbed light.

Proteins can be pre-labeled with fluorescent dyes that are spectrally resolvable to allow for multiplexing, with the resulting ability to accurately quantitate changes in expression. The ability to multiplex can also be used in Western blotting where different proteins can be targeted by antibodies conjugated to different fluorescent dyes (Fig 1.2).

A vast range of fluorochromes (fluorophores conjugated to another molecule), fluorescent stains and proteins are available from many vendors (see Appendix 2). From genomics to proteomics, commonly used techniques such as total protein and nucleic acid quantitation, Western, Northern, and Southern blotting, PCR product analysis, microarray analysis, and DNA sequencing can all benefit from the application of fluorescence detection.

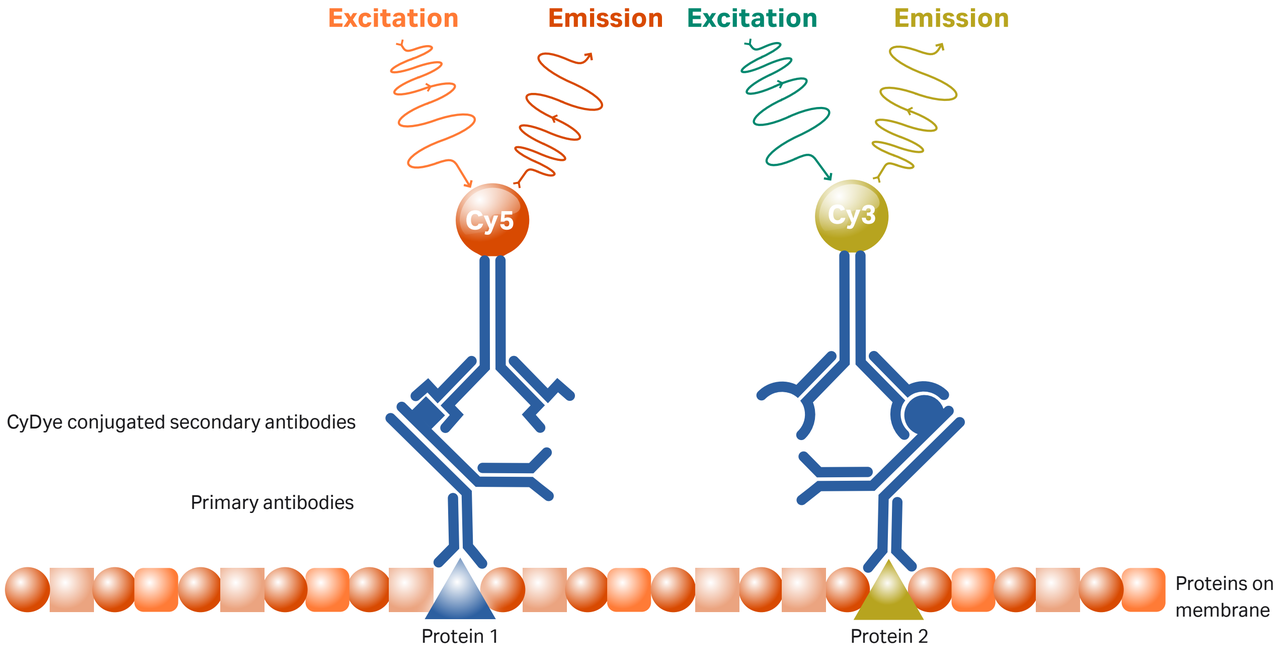

Fig 1.2. Fluorescence-based, multiplexed protein detection. Here, primary antibodies against two different proteins are recognized by species-specific secondary antibodies conjugated to the fluorescent dyes, Cy5 and Cy3. After exposure to light at specific wavelengths in separate channels, the signals emitted as the fluorophores return to their ground states can be measured using a multichannel fluorescence imager.

1.3.1 Excitation and emission spectra

1.3.1.1 Excitation spectrum

The relative probability that a fluorophore will be excited by a given wavelength of incident light is shown in its excitation and emission spectra (Fig 1.3).

The photon energy at the apex of the excitation peak equals the energy difference between the ground state and the excited state of the fluorophore.

The distribution of the excitation spectrum reflects the fact that the fluorophore 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 fluorophore is most effectively excited by wavelengths nearest to the apex of its excitation peak. For example, the excitation of Cy™3 with light close to the maximum absorption wavelength of that fluorophore (550 nm) will result in high excitation of Cy3, whereas Cy2 and most of Cy5 will remain in their ground states. However, remember that other factors including laser power and the environment the fluorophores are in can also affect the excitation efficiency.

1.3.1.2 Emission spectrum

The emission spectrum is generated by exciting the fluorophore 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 excited state and the ground state.

The emission spectrum is always shifted toward a longer wavelength (lower energy) than that of the excitation spectrum (Fig 1.3). The difference in wavelength between the apex of the emission peak and the apex of the excitation peak 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 is essential for measuring fluorescence since the intensity of excitation light is much higher than the emitted light. Optimization of filter optics reduces background and noise, and therefore improves sensitivity of fluorescence techniques.

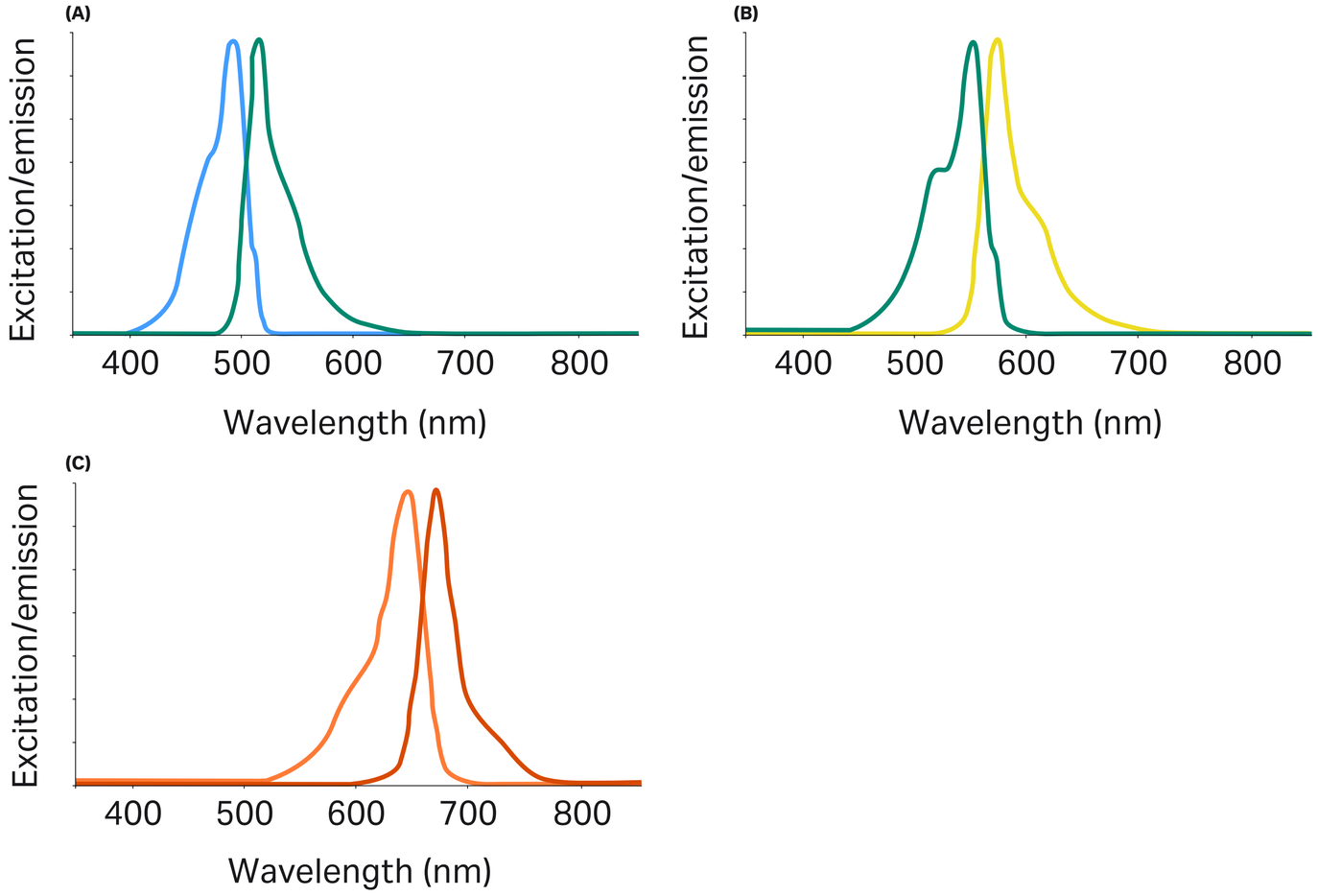

Fig 1.3. Excitation and emission spectra for CyDye DIGE Fluor dyes (A) Cy2, (B) Cy3, and (C) Cy5.

1.3.2 Signal linearity

The intensity of emitted fluorescent light is a linear function of the amount of fluorophore present when the wavelength and intensity of the illuminating light are constant, for example, when using a controlled laser light source in a scanner. Linearity of response is maintained over several orders of magnitude of concentrations of target molecules (Fig 1.4).

1.3.3 Signal intensity

Fluorophores differ greatly in the signal intensity of their emitted signals, a property impacting on sensitivity. Signal intensity depends on two properties of the fluorophore:

- Ability to absorb light (extinction coefficient, ε)
- Efficiency with which absorbed light is converted to emitted light (quantum yield, φ)

The brightness (signal intensity) of a fluorophore is given by the equation, Brightness = εφ. ε is the amount of light that a fluorophore absorbs at a particular wavelength and the molar extinction coefficient is defined as the optical density of a 1 M solution of the fluorophore measured through a 1 cm light path. For fluorophores that are useful molecular labels, the molar extinction coefficient at peak absorption is in the tens of thousands.

For example, fluorescein (ε ≈ 70 000, φ ≈ 0.9) and Cy5 (ε ≈ 200 000, φ ≈ 0.3) are fluorophores, with quite similar brightness (ε × φ), however their quantum yields and extinction coefficients are quite different. It is thus important to consider both ε and φ when evaluating new fluorophores.

Fluorescence signal intensity is also directly related to the intensity of excitation light. A more intense source will yield the greater fluorescence. Excess intensity however, can cause photodegradation of the sample resulting in loss of linearity.

1.3.4 Susceptibility to environmental effects

The quantum yield and excitation and emission spectra of a fluorophore 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 non-covalent 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 fluorophore when excited. Since the excited state is generally much more chemically reactive than the ground state, a small fraction of the excited fluorophores molecules can participate in chemical reactions that alter the molecular structure of the fluorophore thereby reducing fluorescence. The rate of these reactions depends on the sensitivity of the particular fluorophore to bleaching, the chemical environment, the excitation light intensity, the dwell time of the excitation beam or time of exposure, and the number of excitation cycles.

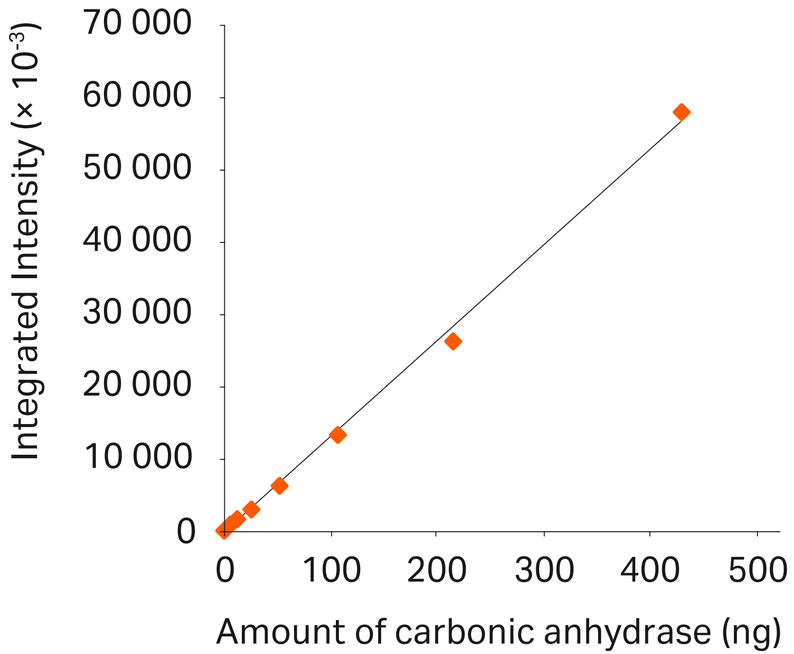

Fig 1.4. A two fold dilution series of carbonic anhydrase was labeled with CyDye DIGE Fluor, Cy3 minimal dye and subjected to 1-D electrophoresis. The gel was imaged with Typhoon FLA 9500. The Limit of Detection (LOD) was 0.2 ng carbonic anhydrase and the linear dynamic range was 3.4 orders of magnitude.

1.4 Chemiluminescence detection

Chemiluminescence occurs when a chemical reagent containing stored energy releases light. The reagent is normally stable and does not emit light, but can be converted into a light emitting product, for example after interaction with a specific enzyme. In most contemporary ECL systems, the enzyme horseradish peroxidase (HRP) conjugated to a secondary antibody is the catalyst that fulfills this function (Fig 1.5).

The light produced is proportional to the amount of labeled compound in the sample and can be detected on X-ray films as well as by using CCD camera-based imagers. ECL is more versatile than general colorimetric methods as these antibody-based systems are usually designed to target specific biomolecules. In addition, the technique is fast and sensitive; signals are generated in seconds and relatively small quantities of antigens and antibodies are normally consumed. As light is generated without an external excitation source, there is no risk of photodamage to samples.

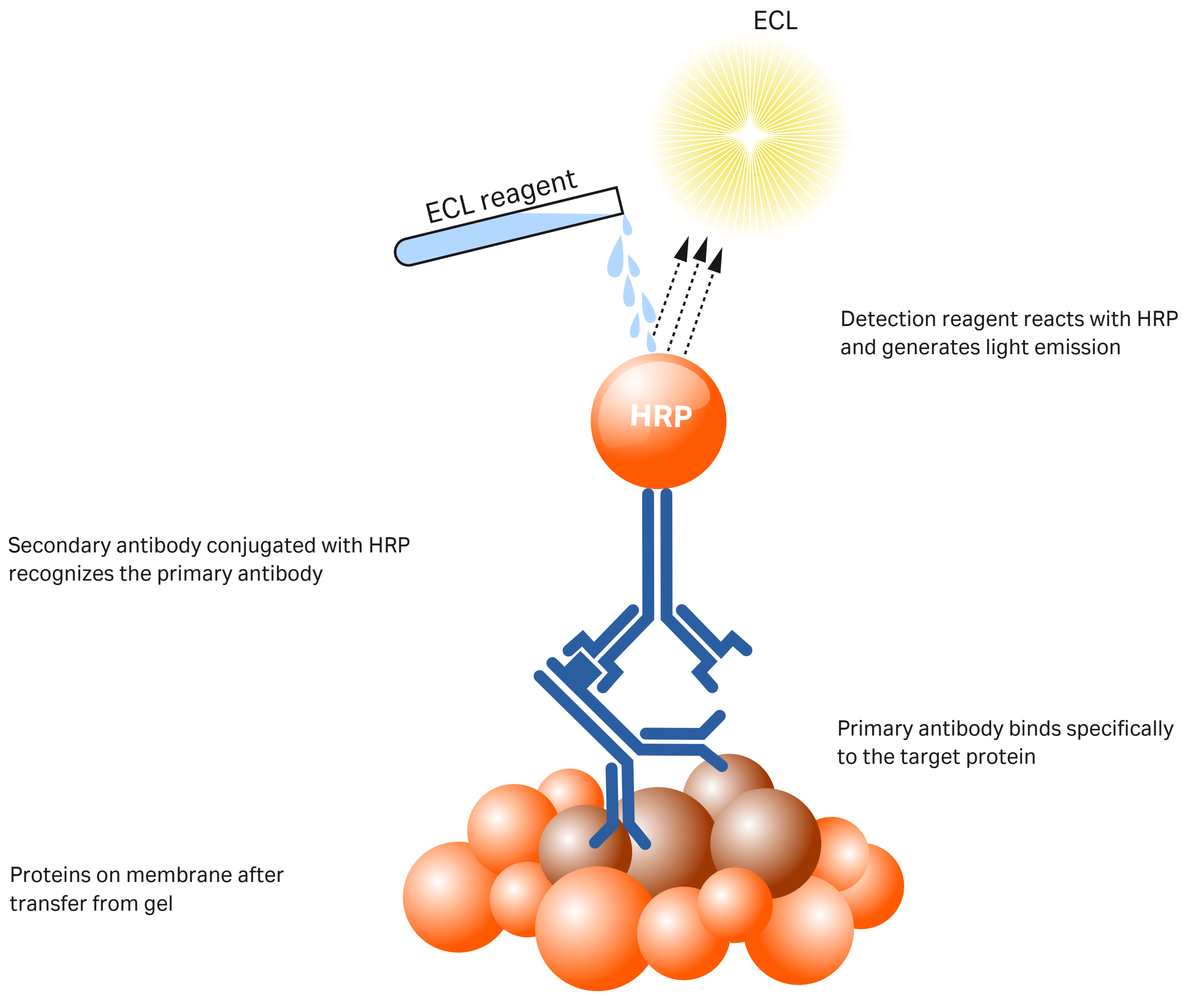

Fig 1.5. Chemiluminescence-based protein detection. The HRP conjugated secondary antibody will recognize the primary antibody directed against the protein of interest. This enzyme catalyzes the conversion of the ECL substrate into a sensitized reagent, which on further oxidation produces an excited state that emits light (428 nm) when it decays.

1.4.1 Sensitivity and precision

The most recent ECL reagents from Cytiva give rise to high sensitivity and linearity of signal response over a wide range of protein levels. The results shown in Figure 1.6 were obtained after 75 s exposure using Amersham ECL Prime, and enable detection and precise quantitation of both high and low abundant proteins on the same blot after a single exposure.

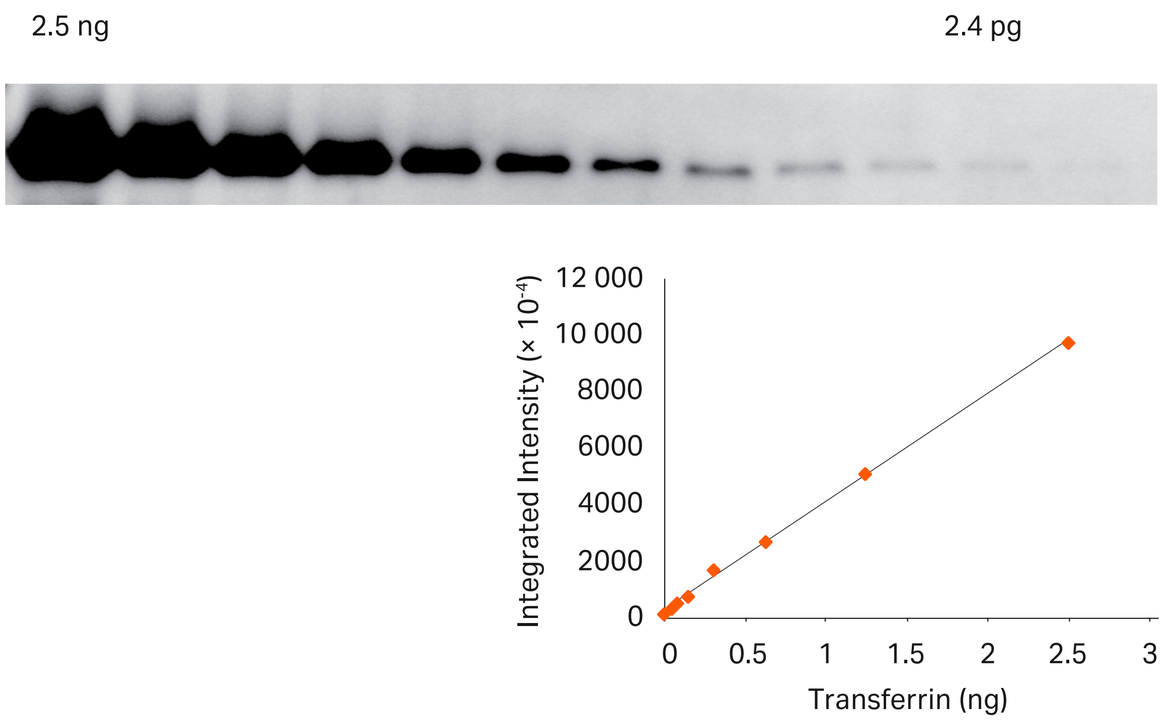

Fig 1.6. Western blotting detection of transferrin in a 2-fold dilution series using Amersham ECL Prime. The LOD was 2.4 pg transferrin and the linear dynamic range was 3.0 orders of magnitude. The blot was imaged using a CCD-based ImageQuant LAS 4000 mini system.

1.4.2 Signal stability

The light signal emitted by an ECL reagent reaches its maximum intensity shortly after activation and then diminishes with time. The rate of signal quenching varies among the available reagents but usually lies in the range of a few minutes to 2 h. The chemiluminescent signal produced by Amersham ECL Prime is highly stable, with measureable signals remaining up to 3 h after starting the reaction between HRP and the luminescent reagent, even for the lowest amounts of protein tested. This enables multiple exposures and a convenient time window between the end of the experiment and image capture (Fig 1.7).

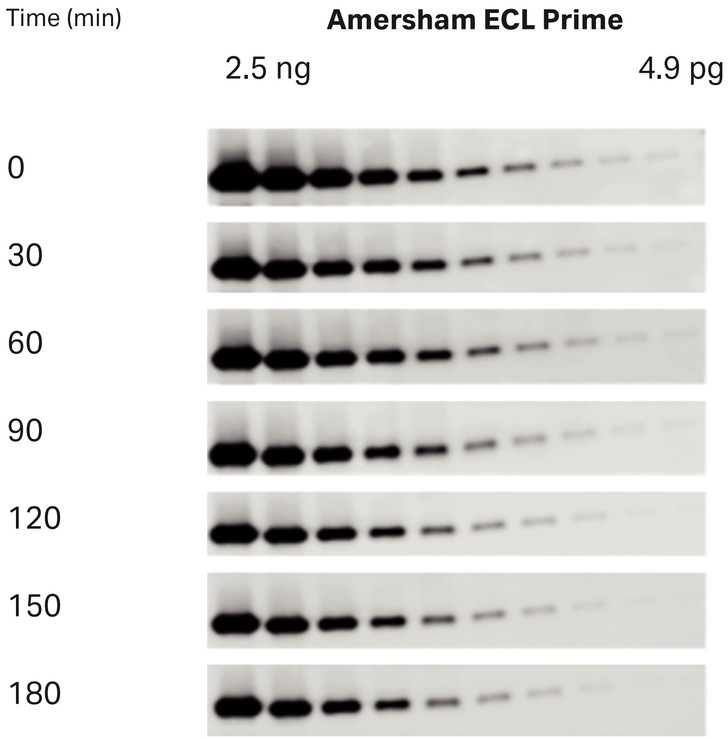

Fig 1.7. Signal intensities 5 minutes after Amersham ECL Prime reagent addition and at time intervals up to 3 h after reagent addition. Image exposure was set at 3 min for all the samples.

1.5 Other detection systems

Chemifluorescence refers to the enzymatic production of fluorescence. As with chemiluminescence, a substrate is needed. A catalytic reaction between enzyme and substrate results in the formation of a fluorescent product at the site of the reaction as well as an amplification of the signal. The newly generated fluorescent product can be induced to emit light based on the same excitation/emission principle described for fluorescence. The signal can be detected by using a suitably equipped CCD camera-based imager or laser scanner.

Colorimetric methods using dyes such as Coomassie Blue and silver staining are still widely used for visualization and detection of proteins. Silver is about 10- to 100-fold more sensitive than Coomassie Blue and can be used to detect as little as sub-nanogram quantities of protein. Silver staining is therefore the method of choice where sensitivity is more important than accurate quantitation, due to a relatively narrow dynamic range of detection.

Staining with Coomassie Blue is simpler than silver staining and is reasonably sensitive. Images of lanes of blue bands are universally recognized among researchers as resolved proteins on polyacrylamide gels. Coomassie Blue reliably detects protein in the 5 to 500 ng range, but staining and destaining usually takes at least 2 h and demands large quantities of noxious solvents. In addition, Coomassie Blue binds proteins with variable affinity; glycoproteins, for example, which make up more than half the total complement of proteins, stain poorly with Coomassie Blue.

Although alternatives to **radioisotope-based detection systems** have emerged, there are still some areas in which radioisotopes continue to offer some advantages. Radioactive systems in combination with phosphorimaging screens are still used in Southern and Northern blotting because of their sensitivity and speed. Phosphorimaging screens are sensitive to any source of ionizing radiation, including commonly used isotopes such as ^{32}P, ^{33}P, ^{35}S, ^{14}C, ^{3}H and ^{125}I. They generate signals across a linear dynamic range of up to five orders of magnitude, allowing precise quantitation of weak and strong signals in one exposure.

1.6 References

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02

Imaging systems

A variety of imaging systems are designed for different techniques and types of samples. Fluorescence imaging systems capture signals emitted by fluorochromes after excitation by light of specific wavelengths. Filtration of light is an important part of the system.

The amount and intensity of light emitted during chemiluminescence is a function of the rate of the reaction between an enzyme (usually horseradish peroxidase (HRP)) and a potentially luminescent substrate. Image capture of chemiluminescence requires neither external excitation nor filters. Systems designed only for the quantitation of signals emitted via chemiluminescence are thus intrinsically simpler.

The technique used to detect radioisotope-labeled compounds using storage phosphor screens, is often referred to as phosphorimaging. Ionizing radiation such as X-rays, β-emissions, and γ-emissions from the sample is captured, but remains latent, in the storage phosphor screen. Upon light induced stimulation of the storage phosphor screen, light is emitted in proportion to the amount of radioactivity in the sample. The technique uses the same principle as fluorescence imaging, where signals are emitted from the screen after excitation with red light, however the emission in this case is at a lower wavelength than the excitation source.

This chapter focuses primarily on the principles of fluorescence imaging systems. The other techniques are also described, and can be understood by adapting the principles of fluorescence imaging to them. Cytiva supplies imaging systems for fluorescence, chemiluminescence and phosphorimaging, (see *section 2.5*).

2.1 Introduction

Fluorescence imaging systems consist of some, but not necessarily all, of the following components:

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

The components and design of a typical fluorescence detection system are illustrated in Figure 2.1. The following paragraphs provide additional details concerning the elements that comprise the system.

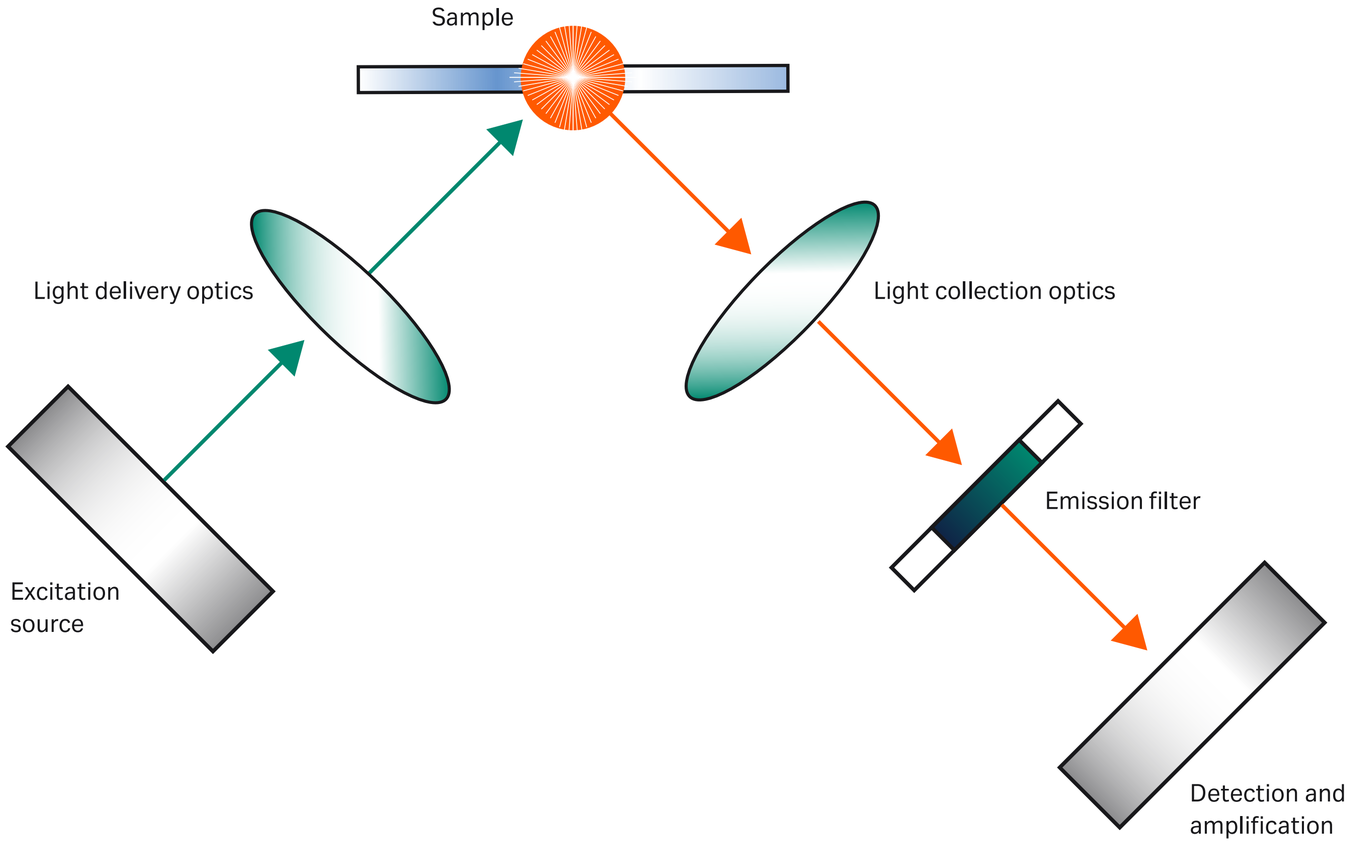


Fig 2.1. Components of a fluorescence imaging system.

2.1.1 Excitation sources and light delivery optics

Light energy is essential for the generation of fluorescence. Light sources fall into two broad categories — wide-area, broad-wavelength sources, such as LED's, UV and xenon arc lamps, and line sources with discrete wavelengths, such as lasers (Fig 2.2).

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 monochromatic light.

In most camera systems, excitation light is delivered to the sample by direct illumination of the imaging field, with the excitation source positioned above, below, or to the side of the sample. Laser-based imaging systems have more sophisticated optical paths that use 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 laser-based imaging system. Filters can be coated to selectively absorb or reflect light to give the best combination of wavelength selection, linearity, and transmission properties (see Chapter 3 for more information about optical filters).

2.1.3 Filtration of emitted light

Fluorophores emit light that radiates in all directions. However, the light that is collected requires highly efficient collection optics since it comes from a relatively small cone angle to one side of the sample. Any laser light reflected or scattered by the sample must be blocked from the collection pathway by optical filters. Emitted light can also be filtered to select only the range or band of wavelengths of interest to the user. Systems that employ more than one detector at a time require additional beamsplitter filters to separate and direct the emitted light along separate paths to the individual detectors.

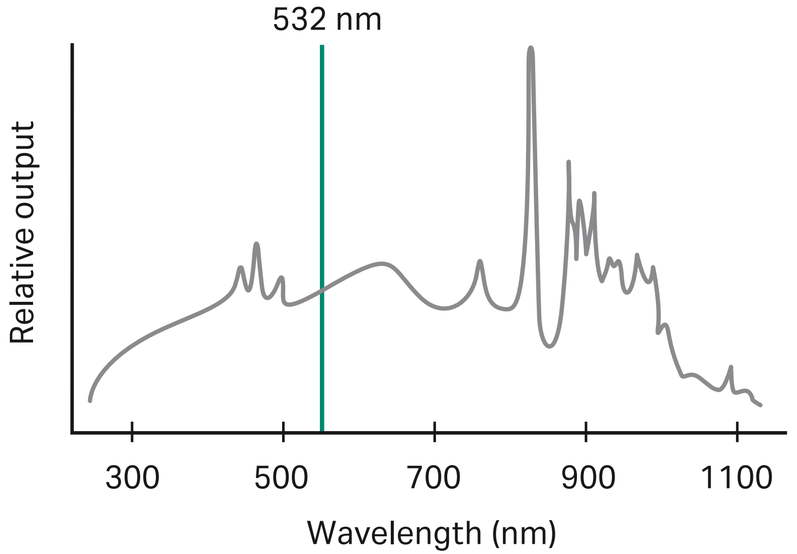


Fig 2.2. Spectral output of light from a xenon lamp (gray) and a 532 nm SHG laser (green line). The “relative output” axis is scaled arbitrarily for the two light sources.

2.1.4 Detection, amplification and digitization

Emitted light can be detected and quantitated using either a photodetector, such as a photomultiplier tube (PMT), or a detector array such as a charge-coupled device (CCD). In both cases, photon energy from emitted fluorescent light is converted into electrical energy, producing a signal proportional to the number of photons detected. A PMT is an electro-optic device that converts light energy into electrical current and amplifies the current, whilst a CCD is a silicon-based integrated circuit consisting of a dense matrix of photodiodes that operate by converting light energy in the form of photons into an electric charge.

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 indicated by the number of bits. Bit values correspond to the total possible number of gray scale values based on the exponent of 2. Therefore, 8-, 12- and 16-bit digital files have 256, 4096 and 65 536, allocated levels of gray respectively. Digital resolution is defined as the ability to resolve two signals with similar, but slightly different intensities (for example, resolving 10 000 from 10 050 on a 65 536 scale).

As the number of intensity levels is limited, the conversion process introduces a degree of error. To discriminate between similar signals and to minimize this error, the distribution of the available intensity levels should closely correspond to the linear dynamic range of the detector for the best digital resolution.

There are two methods of distributing intensity levels. A linear distribution has the same spacing for all the intensity levels, allowing measurement across the dynamic range with the same absolute precision. However, relative digitization error increases as signals become lower. A non-linear distribution (e.g. logarithmic or square root functions) imparts more intensity levels to the lower end of the signal range and fewer intensity levels to the higher end. Absolute precision, therefore, decreases with higher signals, but the relative digitization error remains more constant across the whole dynamic range.

2.2 Understanding the digital image

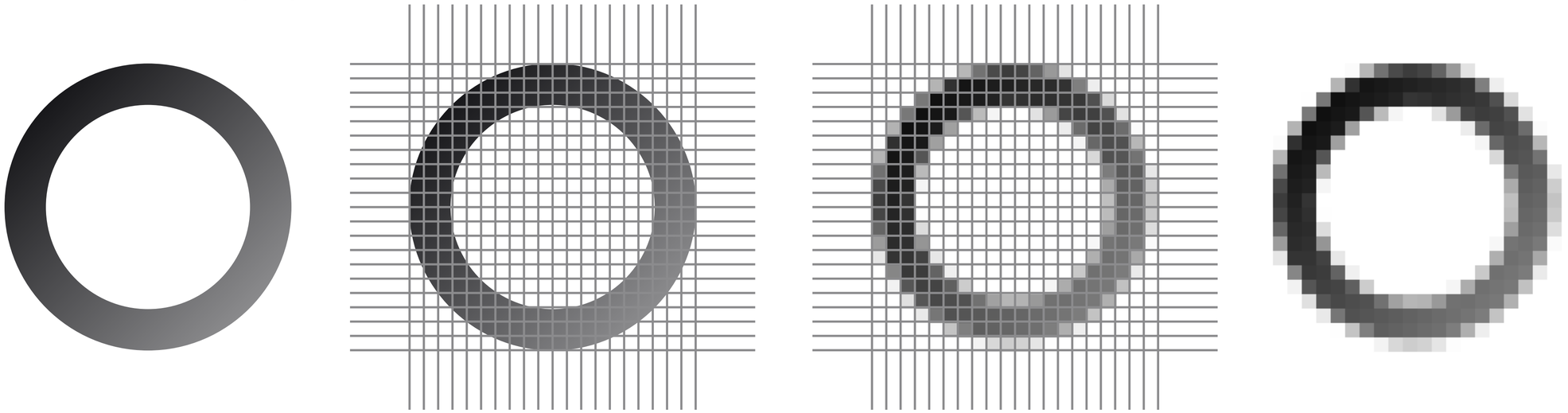
This section deals with the acquisition and definition of the digital image. It explains some of the more common terms used in association with digital images and explains Cytiva's .GEL file format.

2.2.1 Image acquisition

Image acquisition is a critical step. Image capture requires that all the raw data hare stored precisely and without significant data compression to maintain accuracy. Acquisition can be achieved with a variety of scanners or digital imagers that are usually based on a PMT or a CCD (see section 2.1.4).

2.2.2 The digital image

The most practical way of storing an image as digital data is to divide the image into a grid of very small regions or "pixels". In the computer this digital grid or "bitmap" represents the image. Each pixel is identified by its position in the grid, as referenced by its row (x) and column (y) number. Each pixel has a different color or gray scale value and together they form a representation of the image (Fig 2.3).



Original image

The image is partitioned into an array of squares

Each square is used to form a pixel

The scanning device collects a single value representing the entire square, which it then transmits to the computer

The computer reconstructs the entire image using the pixel (x, y) values

Fig 2.3. The digitization procedure.

2.2.3 Formatting graphic files

After the image is acquired, the data are converted to a particular file format such as Tagged Image File Format (TIFF or .TIF) or Graphics Interchange Format (GIF) for storage. Some formats are software-specific, whilst others are universally used for transfer and presentation in web pages.

Image files can include information, which is stored in an image header or tag. The tag is used to display the dimensions of the image in pixels and can also identify the name, source and/or owner of the image.

Scientific images should be acquired and archived using an information preserving or non-destructive format. Some file formats actually distort some of the pixel information when the file is saved, while others do not. Loss of data may be unacceptable for use in quantitative analysis and therefore Cytiva's image analysis software only supports specific compression formats.

2.2.3.1 Tagged Image File Format images

Tagged Image File Format (TIFF), is an image format widely used for file transfer between platforms and software applications. TIFF files consists of a number of tags that describe properties such as; gray levels, color table, byte format, and compression size. The image data follows the initial tags, which can be interrupted by more descriptive tags.

Although the TIFF file is an industry standard, it has many variants. The 16-bit TIFF format has 2^{16} = 65 536 levels of signal resolution and is the most commonly used file format for images (Fig 2.4).

2.2.3.2 .GEL image file format

Images scanned from Cytiva imagers can also be stored as .GEL files, extensions of the 16-bit TIFF format that only contain gray scale information at 16 bit per pixel. These images have very wide dynamic ranges; with a possible 10^5 or 1 to 100 000 levels of signal resolution. The .GEL format uses a square root algorithm to compress the possible 100 000 levels of an image into the 65 536 levels. Square root compression provides higher signal resolution for low light emission. The benefit of the .GEL format is that it accurately represents small differences in data, whereas a TIFF format may assign the same number to two values, particularly in the fainter part of the image.

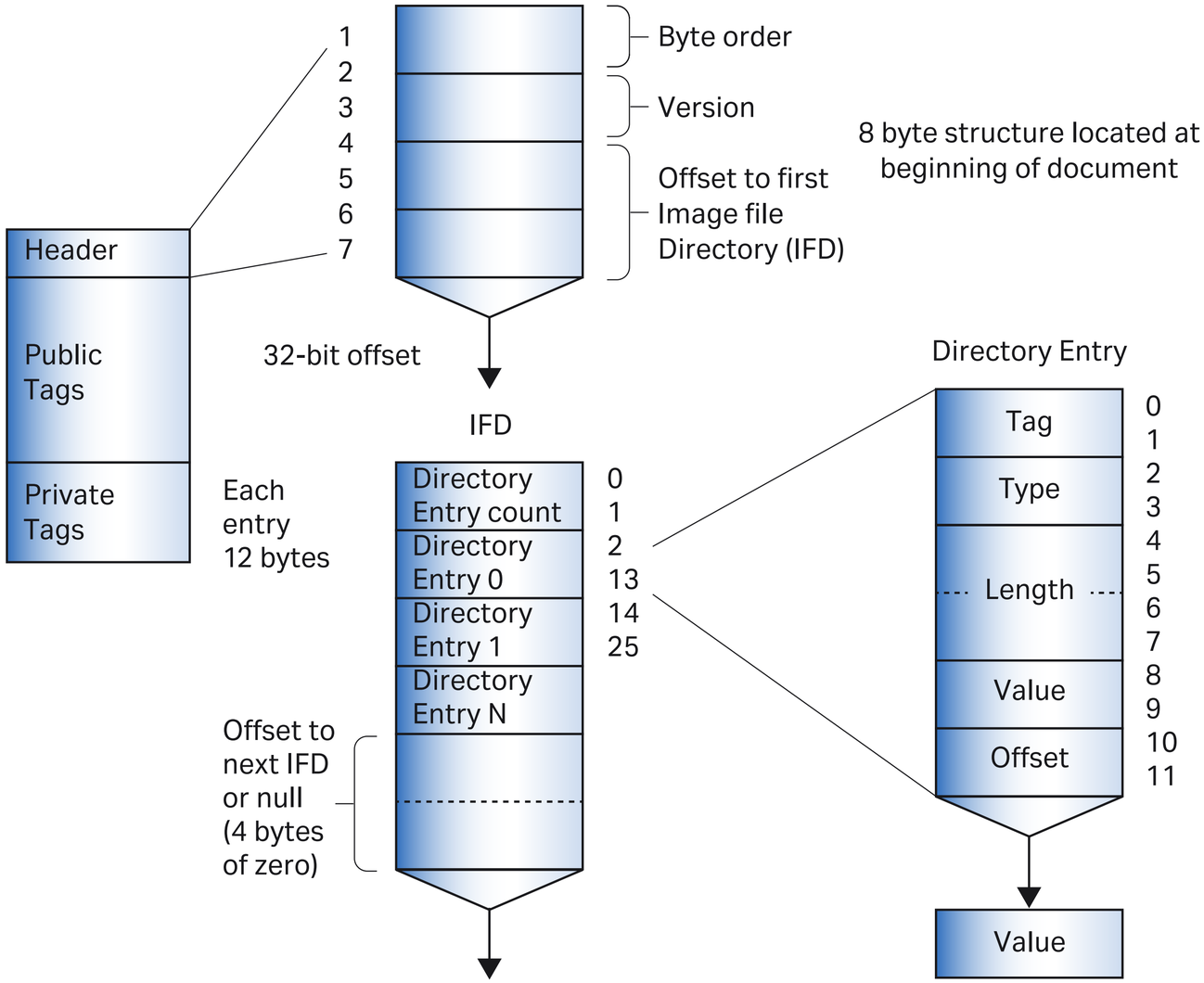


Fig 2.4. Schematic diagram of a TIFF file.

2.2.3.3 Pixel intensity value

Each pixel in a stored image has a pixel intensity value that describes its brightness or amount of emitted light. This value is the average for the whole area covered by the pixel. The "address" of a pixel is defined by its coordinates in the two dimensional image.

2.2.3.4 Bit depth

Bit depth refers to the amount of information allocated to each pixel in a graphic image. Pixels have different bit depths, which determine how many shades of gray (grayscale levels) are available to the image. Most image file formats store grayscale information in the header section of the file. An 8-bit image has 2^8 or 256 grayscale levels, while a 16-bit image has 2^{16} or 65 536 grayscale levels.

Due to variations in software and conversion algorithms, it is best to start with as much bit depth as possible. Larger bit depths increase the dynamic range available for image collection from gels with protein or nucleic acid bands that are going to be quantitated by pixel intensity. For black and white (binary) images, pixels need only two possible combinations of information (i.e., black and white) and hence the bit depth is 1 (Table 2.1).

Table 2.1. Bit depths and their corresponding number of different combinations available

Depth	Example	Combinations
1-bit	0	2
2-bit	10	4
4-bit	0110	16
8-bit	01010011	256
12-bit	011010011101	4096
16-bit	1011000100101101	65 536

2.2.3.5 Pixel frequency histogram

The frequency histogram refers to the frequency representation of different shades of gray or color in the image. A frequency histogram displays the number of pixels representing each grayscale level or color value. Frequency histograms most commonly represent grayscale images and have many uses: the histogram may reveal an under-or overexposed image (too many pixels with values close to 0, or too many with values close to 65 535 or 100 000 respectively), and the histogram can be adjusted to change how the image is displayed on a monitor. This is particularly useful when an image has more grayscale levels than a monitor can display or that the eye can see. The histogram helps to set the thresholds for displaying the levels of interest (Fig 2.5).

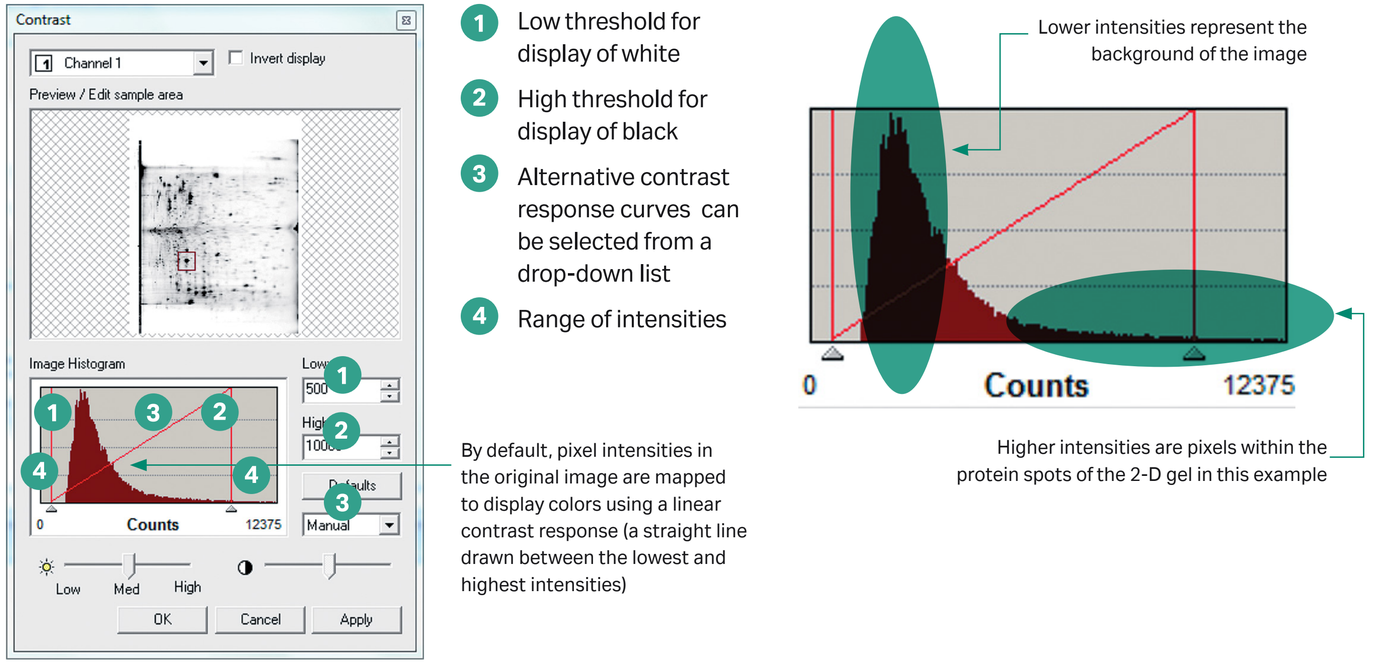


Fig 2.5. Pixel frequency histogram displays a barchart of the pixel intensity distribution within the threshold set.

2.2.4 Image dimensions

2.2.4.1 Resolution

Image resolution is defined as the number of pixels displayed per unit length of an image. Pixel resolution is the fineness of the divisions into which the imager partitions the image. Therefore, a high resolution imager has extremely small divisions per unit length. Resolution determines the area occupied by images in conjunction with the pixel dimension and is a measurement of clarity, or detail. It can also refer either to an image file or a display monitor.

The relationship between number of pixels and area is commonly expressed by pixels per inch (ppi); the more pixels per inch the better the resolution. Output (print or display) resolution is typically expressed in dots per inch (dpi). The resolution of a scanned image is also expressed in dpi where the number of dpi is equal to the number of ppi i.e., an image scanned in at 300 dpi will give you an image resolution of 300 ppi. Alternatively, resolution can be expressed by stating the size of each pixel in μm (microns). For example, 300 ppi equals 1 inch (or 0.0254 m) divided by 300. A single pixel thus represents 85 μm.

Image-file resolution and output resolution combine to influence the apparent clarity of a digital image when it is viewed. The display monitor also influences apparent image quality. Image resolution needs to be matched to the purpose of the scan. A resolution of 100 dpi is recommended for web images and 300 dpi is often sufficient for printing. Table 2.2 shows the size of an uncompressed 1" × 1" image in different bit sizes and resolutions.

Table 2.2. The size of uncompressed 1" × 1" image files at different resolutions

Resolution (dpi)	1-bit black and white (kb)	8-bit grayscale (kb)	16-bit grayscale (kb)	24-bit color (kb)
400 × 400	20	156	312	469
300 × 300	11	88	176	264
200 × 200	5	39	78	117
100 × 100	1	10	20	29

2.2.4.2 Dynamic range

Dynamic range is a measure of the ability of an imaging system to quantitatively detect very dim and very bright features within a single image, in a single reading and is related to bit depth. It is a measurement of the number of bits used to represent each pixel in an image and hence determines the number of colors or grayscale levels that can be represented in a digital image.

The dynamic range of a system is a function of the analogue-to-digital converter, the purity of the illuminating light, filters, and system noise. It is measured on a logarithmic scale. The dynamic range is actually the difference between the darkest and brightest intensity that the scanner can quantitate. The bigger the difference, the larger the dynamic range, and the better the image quality. Variations in dynamic range of a system impact the quality of the digitized image more than simple resolution. High end imaging systems are more sensitive to a wide range of intensities and can record minor differences between two almost identical intensities.

2.2.5 Image quality

Image quality is the cumulative result of the scanning resolution, the dynamic range of the scanned image and the scanning device or technique used. Image quality is often expressed in terms of resolution, but other factors also affect the quality of an image file. Images are often stored at much higher quality than they are displayed on a monitor because most printing devices are capable of a much higher resolution than screen displays.

A key trade-off in defining an appropriate level of image quality is the balancing of file size and resulting storage requirements with quality needs. Since pixel dimensions and color depth of a graphic image are directly proportional to the file size of the image (Table 1.2), the higher the quality of an image, the more storage space it will occupy. High quality images also require more system resources e.g., higher bandwidth, networks, increased memory requirements and increased time and cost of the scanning process. Analysis of images can also be affected by their quality. Generally, very large files tend to slow the analysis process and can occasionally prevent the analysis software from opening. Effective image compression provides a key to maintaining quality while using less storage space and system resources.

2.2.5.1 Background and noise

Background is defined as undesired signal often resulting from autofluorescence or light scatter from a matrix or sample support. It can be minimized by the selection of an appropriate matrix or sample support e.g., low fluorescence glass. Noise is defined as the statistical uncertainty inherent in a measurement, such as the standard deviation associated with measured background counts in fluorescent gels. Noise can be caused by excitation light fluctuation, electronic component performance, or contaminants with fluorescent properties. Noise can be reduced by using high quality reagents and following recommended procedures. The sensitivity of the imaging system can be adjusted by changing exposure times for CCD camera-based systems or voltage settings for PMT-based systems. The specific signal can be optimized to give the highest signal-to-noise ratio thus ensuring that the maximum amount of information is obtained from the image.

2.3 Scanner systems

2.3.1 Excitation sources

Many fluorescence scanner devices used in life science research use laser light for excitation. 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 made to emit light — is commonly solid state (glass, crystal), or gas. Gas lasers and solid-state lasers both provide a wide range of specific wavelength choices for different imaging needs. LEDs have increasingly been used as an alternative to lasers as a light source — they produce wider bands of light and have low power outputs while at the same time are more compact and less expensive than lasers.

2.3.1.1 Lasers

Argon ion lasers produce light at a variety of wavelengths including 457 nm, 488 nm, and 514 nm that are useful for excitation of many common fluorophores. The 488 nm line can be used for Cy2 and other related "blue-excited" fluorophores. Argon ion lasers are relatively large gas lasers and require external cooling.

Neodymium solid-state lasers, that are also known as SHG (Second harmonic generation) lasers, have an yttrium aluminium garnet (Nd:YAG) crystal that produces powerful green light at 532 nm. This excitation source is useful for imaging a wide range of different fluorochromes that excite efficiently at wavelengths between 490 and 600 nm (e.g., Cy3). Cooling is required to stabilize the output.

Helium neon lasers generate a single wavelength of light (e.g., 633 nm) and are popular in many laser scanners, storage phosphor devices, and fluorescence systems. In fluorescence detection, the helium neon laser can be used to excite "red-excited" fluorochromes such as Cy5. These lasers are smaller than argon ion lasers and do not require independent cooling.

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.

2.3.1.2 Light-emitting diode (LED)

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. LED light emissions are not collimated, the source must be mounted relatively close to the sample or lenses are needed to focus the light. LEDs are considerably smaller, lighter, and less expensive than lasers and cover a wide range of wavelengths.

2.3.2 Light excitation 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. At times, excitation filters can even be used with single-line lasers, as their output is not 100% pure.

Optical lenses align the laser beam, and mirrors can be used to redirect the beam within the instrument. A major consideration when delivering light with laser scanning systems is that samples typically occupy large areas whereas light is delivered from sources with small areas. Effective sample coverage can be achieved by rapidly moving the excitation beam across the sample in two dimensions. The two most used ways to move and spread the point source across samples are discussed below.

2.3.2.1 Galvanometer-based systems

Galvanometer-based systems use a small, rapidly spinning or oscillating mirror to deflect the laser beam, effectively creating a line source (Fig 2.6).

Using relatively simple optics, the beam can be deflected very quickly, resulting in a short scan time. Compared to confocal systems, galvanometer-based scanners are useful for imaging thick samples due to the ability to collect more fluorescent signal in the vertical plane. 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 2.6), but when the angle of incident excitation light varies over the imaging field, some spatial distortion can still occur in the resulting image.

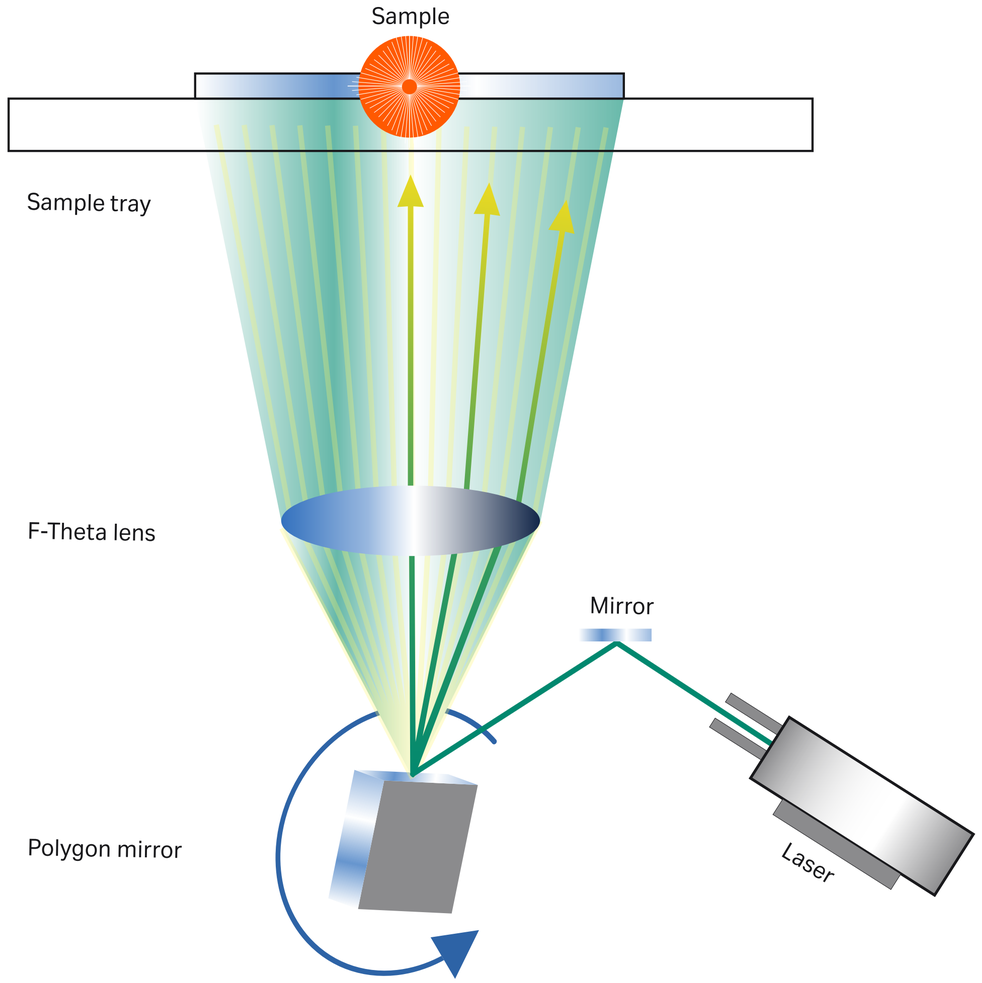


Fig 2.6. Galvanometer-controlled scanning mechanism. Light is emitted from the laser in a single, straight line. The galvanometer mirror rotates or moves rapidly back and forth redirecting the laser beam and illuminating the sample across its entire width. The F-Theta lens reduces the angle of the excitation beam delivered to the sample. The entire sample is illuminated either by the galvanometer mechanism moving along the length of the sample or the sample moving relative to the scanning mechanism.

2.3.2.2 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 2.7).

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.

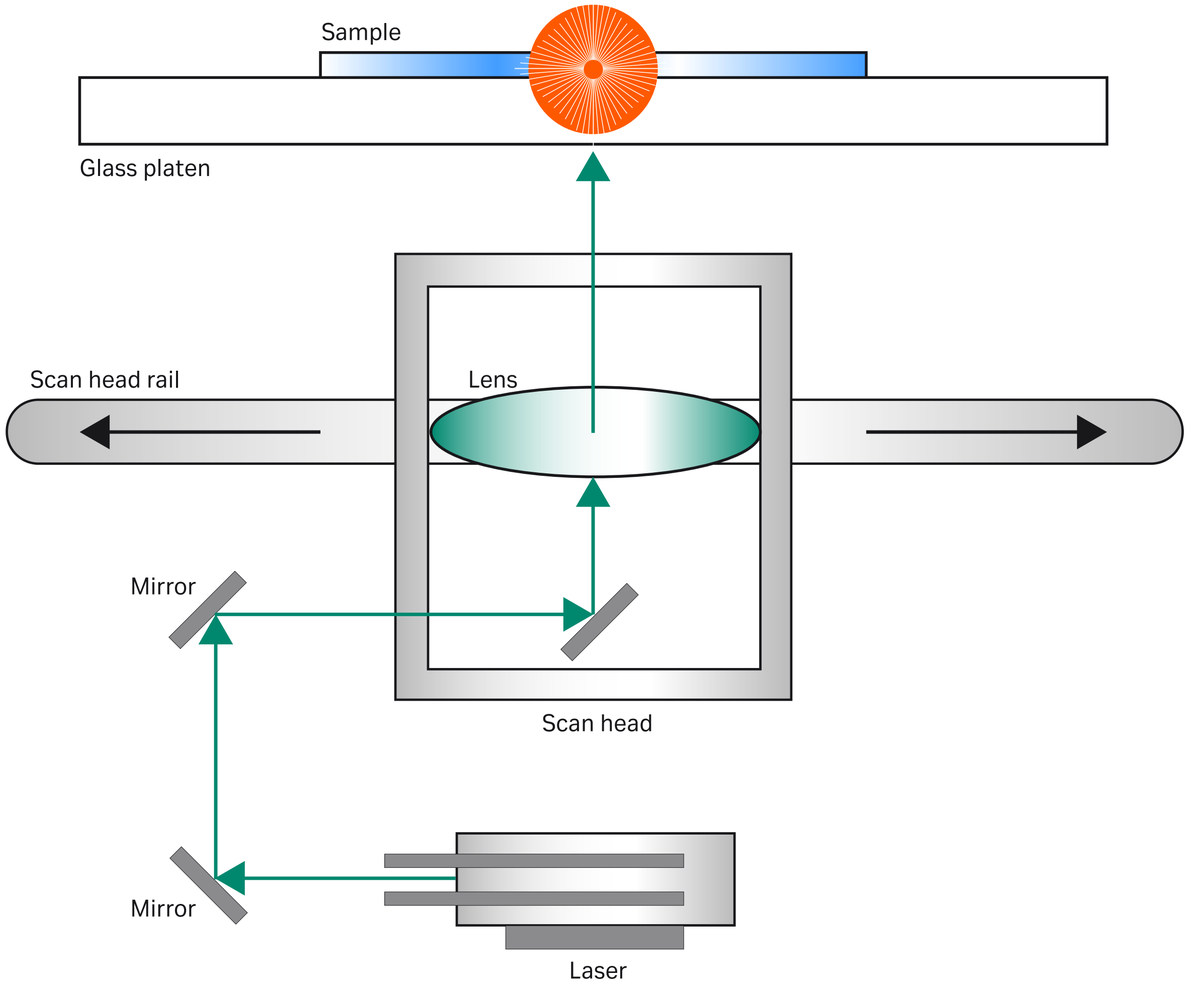


Fig 2.7. Moving head scanning mechanism. The light from the laser beam passes a series of mirrors before hitting the sample. The sample is illuminated across its width as the scan head moves along the scan head rail. The entire sample is illuminated by the scan head, laser, and mirrors tracking along the length of the sample.

2.3.3 Light collection

The light-collection optics in a scanner system are 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, light collecting guide or light bar), positioned beneath the sample, that tracks with the excitation line, collecting fluorescence independently at each pixel. Although this system is fast and effective, there are some drawbacks. 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 for maximum 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. 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 2.8).

Additionally, the parallel motion of moving head designs removes other artifacts associated with galvanometer-based systems, such as flaring or blooming associated with high signal samples.

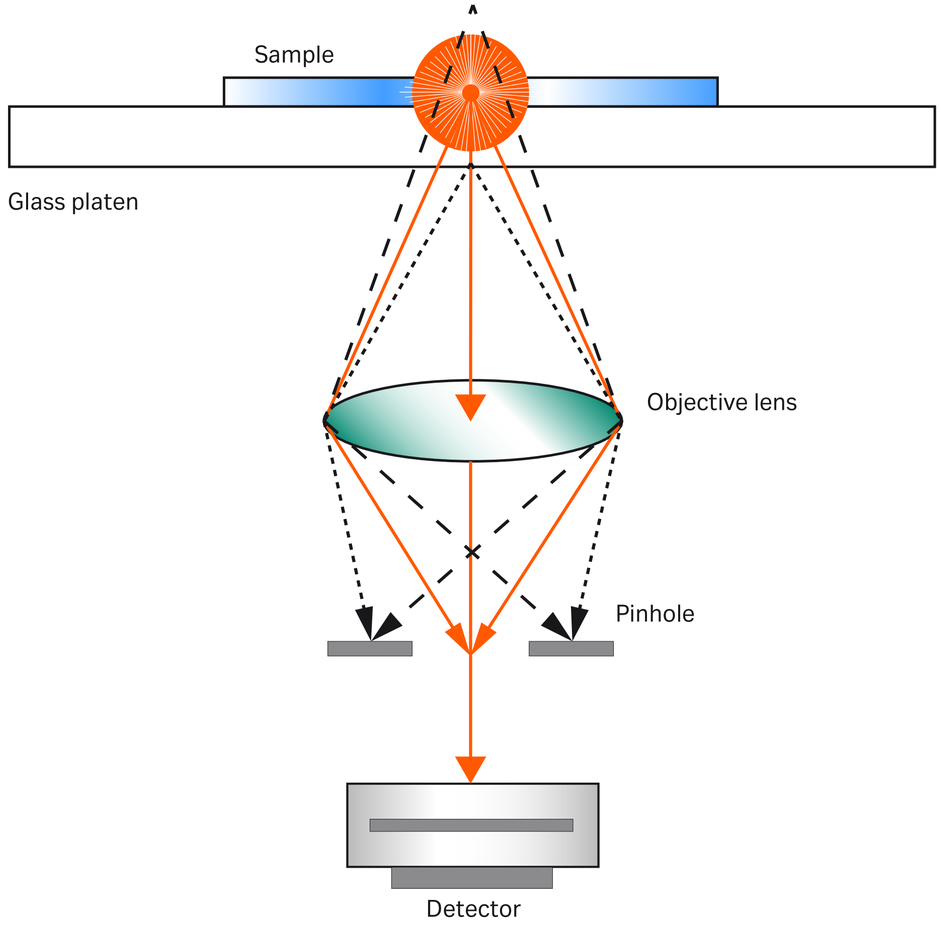


Fig 2.8. Illustration of confocal optics. Fluorescence from the sample is collected by an objective lens and directed toward a pinhole aperture. The pinhole allows the emitted light from a narrow focal plane (orange solid lines) to pass to the detector, while blocking most of the out-of-focus light (black dashed lines).

2.3.4 Signal detection and amplification

The first step 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 incident light is many orders of magnitude greater than the emitted light, even a small fraction of incident 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 (such as a gel, membrane, or microplate).

In multichannel or multilabel experiments, the preferred technique is to scan the different channels in a consecutive manner. However, some combinations of fluorochromes can be simultaneously detected in two channels in scanners with dual detectors. This technique can save time but it demands an experienced user. Multichannel imaging instruments with dual detectors requires additional filtering upstream of the 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 is included to spectrally resolve (or split) the contribution from each label and then direct the light to appropriate emission filters (Fig 2.9).

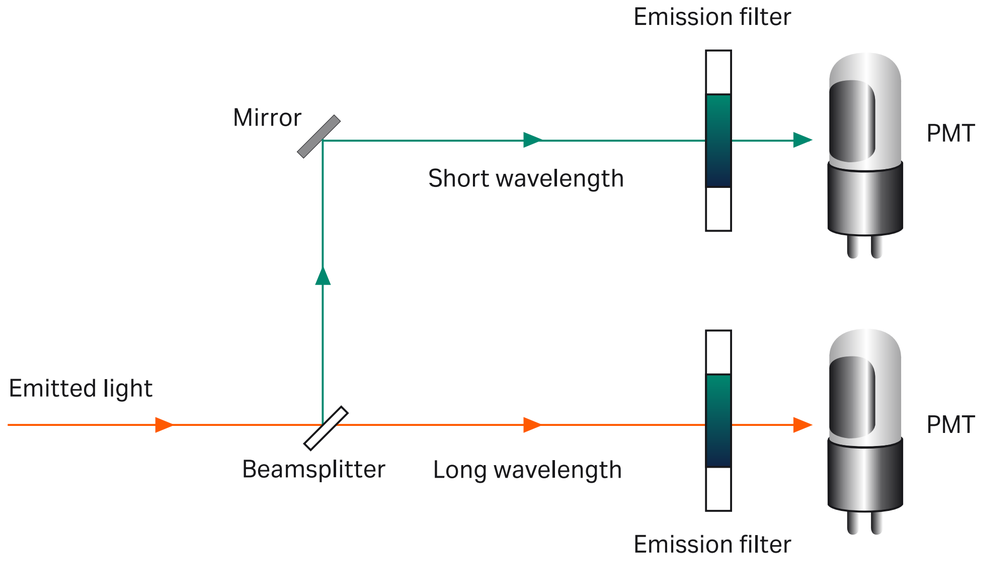


Fig 2.9. Use of a beamsplitter or dichroic filter with two separate PMTs. Light from a dual color sample enters the emission optics as a combination of wavelengths. A dichroic beamsplitter distinguishes light on the basis of wavelength. Wavelengths above the beamsplitter range pass through, those below are reflected. In this way two channels are created, which can then be filtered and enable independent detection.

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 more information about multichannel experiments).

After the fluorescent emission has been filtered, the remaining light is detected and quantitated with a PMT. In the PMT, photons of light hit a photocathode and are converted into electrons which are then accelerated over a voltage gradient and amplified between six and seven orders of magnitude. 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 to 800 nm (Fig 2.10).

In contrast to PMT-based systems, CCD-based systems require no amplification since the samples have long exposure times (see section 2.4).

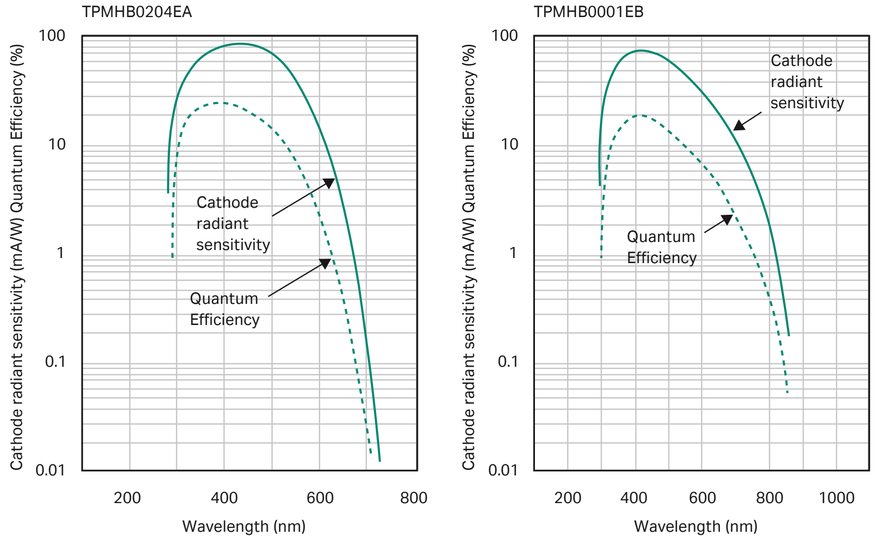


Fig 2.10. Examples of Bi-alkali (BiA) and Multi-alkali (MA) PMT's response curves of efficiency versus wavelength. Copyright© 1994, Hamamatsu Photonics K.K. Used with permission.

2.3.5 System performance

The quality of performance of a laser scanner system depends on system resolution, linearity, uniformity, pixel accuracy, sensitivity, and additionally, in the case of multichannel detection, the alignment between the different channels.

2.3.5.1 Resolution

Resolution can be defined in terms of 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 on the sample, the distance between adjacent measurements and the pixel size of the image. 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. If information is collected every 100 µm, each piece of information represents a square with 100 µm sides. According to a fundamental sampling principle, the Nyquist Criterion, the smallest resolvable object in an image is no better than twice the sampling interval. Thus, to resolve a 100 µm sample, the sampling interval must be less than 50 µm. 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.

Amplitude resolution, or grayscale quantitation, describes the minimum distinguishable difference between levels of light intensity (or fluorescence) detected from the sample.

2.3.5.2 Linear dynamic range

The linear dynamic range of a laser scanner is the signal range over which the instrument yields a linear response to fluorophore concentration and is therefore useful for accurate quantitation. A scanner with a wide dynamic range can detect and accurately quantitate signals from both very low intensity and very high intensity targets in the same scan. The linear dynamic range of most laser scanner instruments is between four and five orders of magnitude.

2.3.5.3 Uniformity

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.

2.3.5.4 Pixel accuracy

Pixel accuracy defines how well the imager can reconstruct the scanned object, which is important for gel spot picking applications. Optical distortion and resolution are among the factors that impact on pixel accuracy.

2.3.5.5 Sensitivity

Sensitivity is measured by estimating the limit of detection (LOD), which is the minimum amount of sample that can be detected by an instrument. The increasing demand for lower detection limits is driving the development of more sensitive imagers. Several of the elements described in this chapter affect the detection limit. However, the properties of the stains and labels chosen, together with the design of experiments and practical skills of the user, also play a major role.

2.4 CCD camera-based systems

CCD cameras are composed of an illumination system and a lens assembly that focuses the image onto a light-sensitive CCD array (Fig 2.11).

CCD camera-based systems are area imagers that integrate chemiluminescent signals, visible light or fluorescent signals from an 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 variable focal distance.

2.4.1 Excitation sources and light delivery

Illumination or excitation in CCD camera-based systems is typically from high power, narrow bandwidth LEDs. Other sources include UV or white light gas discharge tubes, broad-spectrum xenon arc lamps. Light is delivered to the sample either from below (trans-illumination) or from above (epi-illumination). When broadband light sources are used in CCD camera-based systems, filters can be used for wavelength selection.

2.4.2 Light collection

Lenses are used to collect light from the imaging field. Different sample sizes are normally captured in a single view by adjusting the height of the sample tray or by using a zoom lens system. Some reduction 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 center of the lens than light on the axis (5). Such aberrations in field uniformity associated with CCD camera-based systems can be improved using software flat-field corrections.

2.4.3 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 improves detection sensitivity by reducing the level of electronic noise.

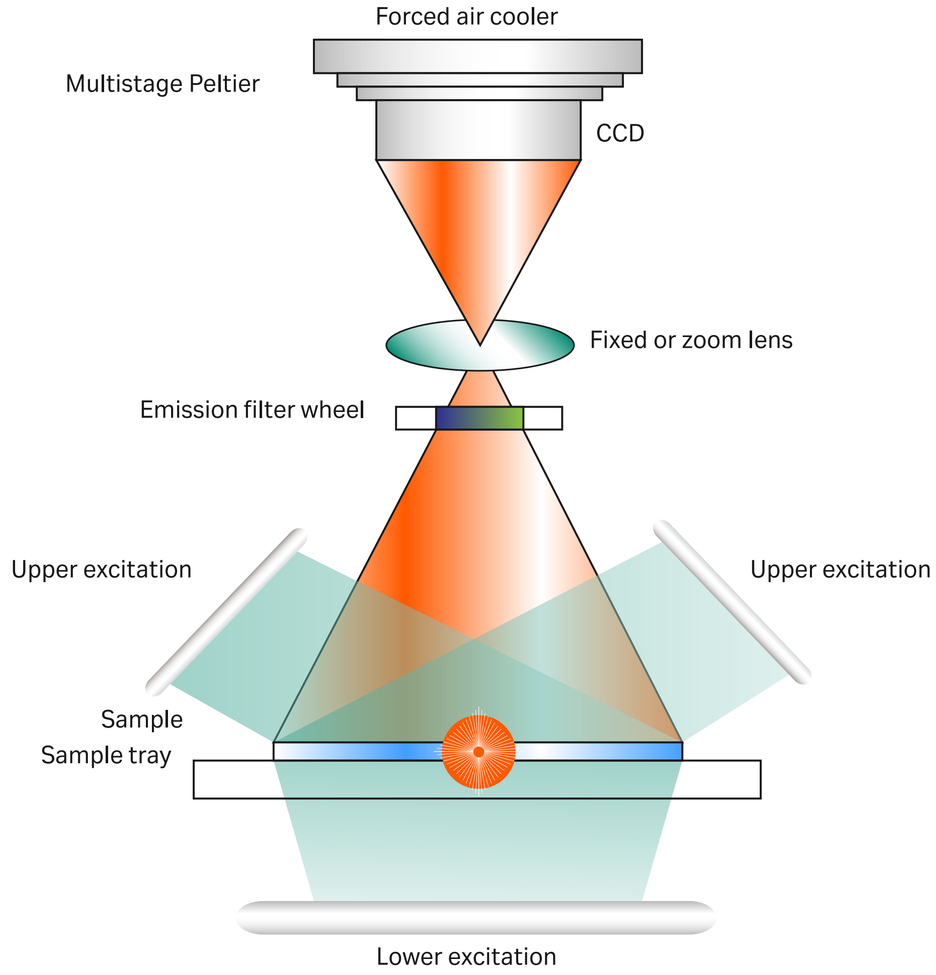


Fig 2.11. Components of a typical CCD camera-based imaging device. The sample can be illuminated in a variety of ways depending on the nature of the labels to be analyzed. The sample is then viewed by the camera. The camera includes focusing optics to accommodate samples at different heights. Emission filters can be inserted in the light path to select specific wavelengths and significantly reduce background.

2.4.4 System performance

The performance of any CCD camera-based system depends on the system resolution, uniformity, sensitivity, linearity, and dynamic range.

2.4.4.1 Resolution

The resolution of a captured image is linked to the geometry of the CCD, with the size of each pixel varying from 6 to 30 µm. Currently, CCDs with formats from 512 × 512 to 4096 × 4096 elements are common, but the technique is constantly developing. Image resolution is reduced and sensitivity increased when charges from adjacent pixels are combined or "binned" during image acquisition.

The quality and design of the CCD chip also affects image resolution. For example, ImageQuant LAS imagers from Cytiva have a denser octagonal matrix of photosites (Fig 2.12) that has a larger relative area of pixels than conventional CCD patterns.

2.4.4.2 Uniformity

Uniformity across the entire image area is critical for reliable quantitation. A given signal should yield the same measurement at any position within the imaging field. Optical distortion requires flat-frame calibration to ensure uniform collection of signals across the entire image area.

2.4.4.3 Sensitivity and linearity

CCD arrays are sensitive to light, temperature, and high energy radiation. System noise caused by dark current from thermal energy, cosmic rays, and the preamplifier 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. Combining charges from adjacent pixels during acquisition, or 'binning', can also enhance sensitivity, although image resolution is compromised.

2.4.4.4 Dynamic range

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

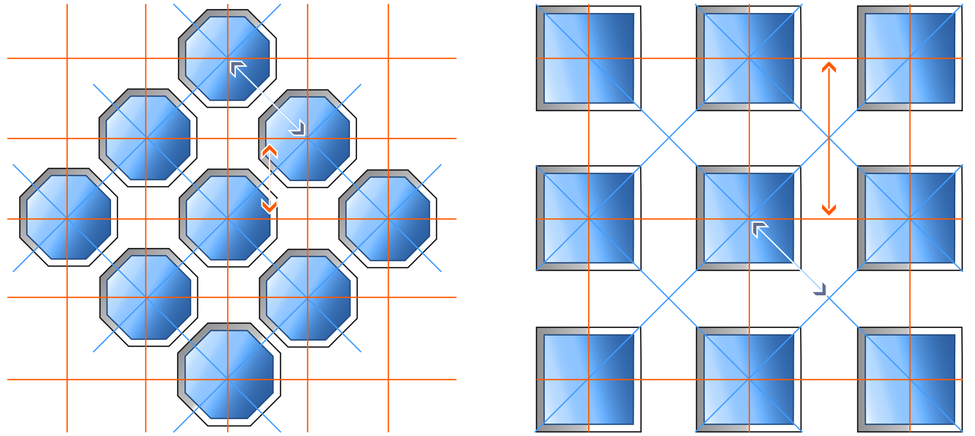


Fig 2.12. An interwoven octagonal matrix of photosites gives a denser matrix for better resolution than a standard square pattern.

2.5 Imaging systems from Cytiva

Cytiva offers a broad range of products for quantitative biomolecular imaging. The imagers fit seamlessly into the application driven workflows, such as Western blotting, 1-D or 2-D gel electrophoresis and 2-D DIGE (Difference Gel Electrophoresis). These imagers cover several types of detection modes — from densitometry, through phosphorimaging to fluorescence and chemiluminescence.

2.5.1 Laser scanning systems

2.5.1.1 Typhoon variable mode imagers

Typhoon imagers are used to support storage phosphor, enzyme-catalyzed chemifluorescence and fluorescence imaging applications. Typhoon imagers deliver outstanding linearity, quantitative accuracy, and extremely low LOD. Automated multicolor scanning permits the detection of multiple samples in the same experiment.

Typhoon FLA 9500 (Fig 2.13) is a robust and versatile laser scanner that is ideally suited to multi-user environments. Biomolecular imaging applications include sensitive and quantitative measurements of fluorescent Western blots, multiplexed fluorescence, and radioisotopic labels by storage phosphor as well as digitization of colorimetric stains.

Table 2.3. Typhoon FLA 9500 specifications

	Typhoon FLA 9500
Excitation sources	473 nm (blue LD laser), 532 nm (green SHG laser), 635 nm (red LD laser), 685 nm (optional red LD laser), and 785 nm (optional near IR LD laser)
Filters	IP (B390), LPB (510LP), LPG (575LP), LPR (665LP), LPR-Ch2 (665LP), BPB1 (530DF20), and BPG1 (570DF20)
	Optional filters: BPFR700 (R715), BPFR800 (R810), DBR1 (530DF20/665LP), and DGR1 (570DF20/665LP)
Scanning area	40 × 46 cm
Pixel sizes	10, 25, 50, 100, 200 µm and prescan 1000 µm
Dynamic range	5 orders of magnitude
Bit depth	16-bit
Imaging modes	Fluorescence, phosphorimaging, digitization
Sample types	Gel sandwiches, gels, blots, microplates, TLC plates, macroarrays, and storage phosphor screens

Fig 2.13. Typhoon FLA 9500.

Typhoon FLA 7000 (Fig 2.14) is a fast and versatile laser scanner for biomolecular imaging applications, including sensitive, quantitative phosphorimaging, fluorescent Western blots (by single and dual detection with some well separated fluors), colorimetric stains, such as Coomassie Blue and silver. It is a non-confocal variable mode laser scanner based on the galvanometer technique with a light collecting guide, with four pre-installed lasers, offering high speed and resolution for precise quantitation.

Table 2.4. Typhoon FLA 7000 specifications

	Typhoon FLA 7000	Typhoon FLA 7000 IP
Excitation sources	473 nm (blue LD laser), 532 nm (green SHG laser), 635 nm (red LD laser), and 650 nm (red LD laser)	650 nm (Red LD laser)
Filters	Y520, O580, R670, and IP (B390)	IP (B390)
Scanning area	**Phosphorimaging** 20 × 40 cm **Fluorescence and digitization** 24 × 40 cm	**Phosphorimaging** 20 × 40 cm
Pixel sizes	25, 50, 100, and 200 µm	25, 50, 100, and 200 µm
Dynamic range	5 orders of magnitude	5 orders of magnitude
Bit depth	16-bit	16-bit
Imaging modes	Fluorescence, phosphorimaging, and digitization	Phosphorimaging
Sample types	Gel sandwiches, gels, blots, microplates, TLC plates, macroarrays, and storage phosphor screens	Storage phosphor screens

Fig 2.14. Typhoon FLA 7000.

2.5.2 CCD camera-based imaging systems

2.5.2.1 ImageQuant LAS imagers

ImageQuant imagers use advanced Fujifilm™ CCD chips and optics to provide high sensitivity for chemiluminescence, chemifluorescence and fluorescent probes for protein and nucleic acid detection and quantitation, as well as fluorescent and colorimetric post stains.

ImageQuant LAS 4000 (Fig 2.15) is a sensitive CCD system for quantitative imaging of gels and blots, by chemiluminescence and fluorescence. A flexible combination of light sources and filters can be incorporated into the imager according to your needs.

ImageQuant LAS 4010 is additionally equipped for epifluorescent RGB applications, as well as white light transillumination.

ImageQuant LAS 4000 can be upgraded at any time to allow for UV-, IR- and RGB-based applications and also white light illumination for documenting gels stained with Coomassie Blue and silver. ImageQuant LAS 4010 features preinstalled components to enable trans-UV and epi-RGB fluorescence detection.

Table 2.5. ImageQuant LAS 4000/4010 specifications

	ImageQuant LAS 4000	ImageQuant LAS 4010
Excitation sources	UV transillumination Epi white light	UV transillumination Epi R, G, B, and white light White trans illumination
Filters	Automated, up to 4 filters 605DF40 filter	Automated, up to 4 filters 605DF40, Y515-Di, 575DF20, R670 filter
Image area	21 × 14 cm (25 × 25 cm with SIGMA wide view lens)	21 × 14 cm (25 × 25 cm with SIGMA wide view lens)
Image resolution	Maximum 3072 × 2048, 6.3 Mpixels	Maximum 3072 × 2048, 6.3 Mpixels
Dynamic range	4.8 orders of magnitude	4.8 orders of magnitude
Bit depth	16-bit	16-bit
Imaging modes	Chemiluminescence, fluorescence (optional), and digitization	Chemiluminescence, fluorescence, and digitization
Sample types	Gels, blots, and TLC plates	Gels, blots, and TLC plates

Fig 2.15. ImageQuant LAS 4000.

ImageQuant LAS 4000 mini (Fig 2.16) is a CCD system for sensitive, quantitative imaging of gels and blots by chemiluminescence and white light epi-illumination gel documentation. The system can be upgraded at any time to UV transillumination and epi-UV or also to perform epifluorescence with blue excitation.

Table 2.6. ImageQuant LAS 4000 mini specifications

	ImageQuant LAS 4000 mini
Excitation sources	Epi white light Optional trans-UV - or epi-UV and epi blue illumination
Filters	Manual, single filter holder
Image area	18 × 12 cm
Image resolution	Maximum 3072 × 2048, 6.3 Mpixels
Dynamic range	4.8 orders of magnitude
Bit depth	16-bit
Imaging modes	Chemiluminescence, fluorescence (optional), digitization (optional)
Sample types	Gels, blots and TLC plates

Fig 2.16. ImageQuant LAS 4000 mini.

ImageQuant LAS 500 (Fig 2.17) is a cooled CCD imager for detection of chemiluminescent Western blots, imaging of fluorescent protein and DNA gel stains and white light imaging of colorimetric stains and markers.

Table 2.7. ImageQuant LAS 500 specifications

	ImageQuant LAS 500
Excitation sources	White epi illumination Combined UV and blue epi illumination
Filters	Manual, single filter O560
Image area	105 × 105 mm
Image resolution	Maximum 3358 × 2536, 8.5 Mpixels
Dynamic range	4.8 orders of magnitude
Bit depth	16-bit
Imaging modes	Chemiluminescence, fluorescence (UV, blue), colorimetric
Sample types	Gels and blots

Fig 2.17. ImageQuant LAS 500.

2.5.3 Flatbed image scanners

2.5.3.1 ImageScanner III

ImageScanner III is well suited to densitometric applications, especially gel electrophoresis of all sizes (Fig 2.18). It offers high resolution with a wide optical density range to scan gels, blots, membranes, and slides. ImageScanner III is highly sensitive and can capture faint signals without the need for a diffusion plate. It can scan in either reflection or transmission mode for optimal data quality.

Table 2.8. ImageScanner III specifications

	ImageScanner III
Sample type	Densitometric applications, particularly gel electrophoresis with 1-D and 2-D gels stained with Coomassie or silver
Excitation source	Xenon gas fluorescent
Scan area	310 × 437 mm
Scanning resolution	up to 600 dpi
Optical density range	0.0 up to 3.6 OD
Scan speed	20 × 20 cm in 25 s at 300 dpi
Scanning color selection	Green, red, blue, or white
Bit depth	Grayscale: 16 bits/pixel

Fig 2.18. ImageScanner III.

03

Fluorochrome and filter selection

3.1 Introduction

The key to optimal detection efficiency is the successful matching of a fluorochrome label with a suitable excitation source and emission filter. To generate fluorescence, the excitation light delivered to a sample must be within the absorption spectrum of the fluorochrome. The highest excitation efficiencies occur when the excitation and peak absorption wavelengths of a fluorochrome coincide. Fixed or interchangeable optical filters that match fluorochrome emission profiles are then used to further refine the emitted fluorescence, so that excitation light is excluded and only the desired fluorescent emission wavelengths pass to the detector. This chapter gives details about the classes and use of emission filters and general guidelines for the selection of 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 have excellent performance through their selective reflection and transmission properties. Colored glass filters are less expensive and have more gradual transition slopes than coated interference filters.

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 3.1a). 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 665 LP filter is 665 nm, where 50% of the maximum transmittance is rejected.

The name of a long-pass filter may also include other designations, such as O or OG (orange glass), R or RG (red glass), E (emission), or EFLP (edge filter long-pass). O, OG, R , and RG are colored-glass absorption filters, whereas E, LP, and EFLP filters are coated interference filters.

Short Pass (SP) filters reject wavelengths that are longer than a specified value and suit shorter wavelengths. 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 3.1b).

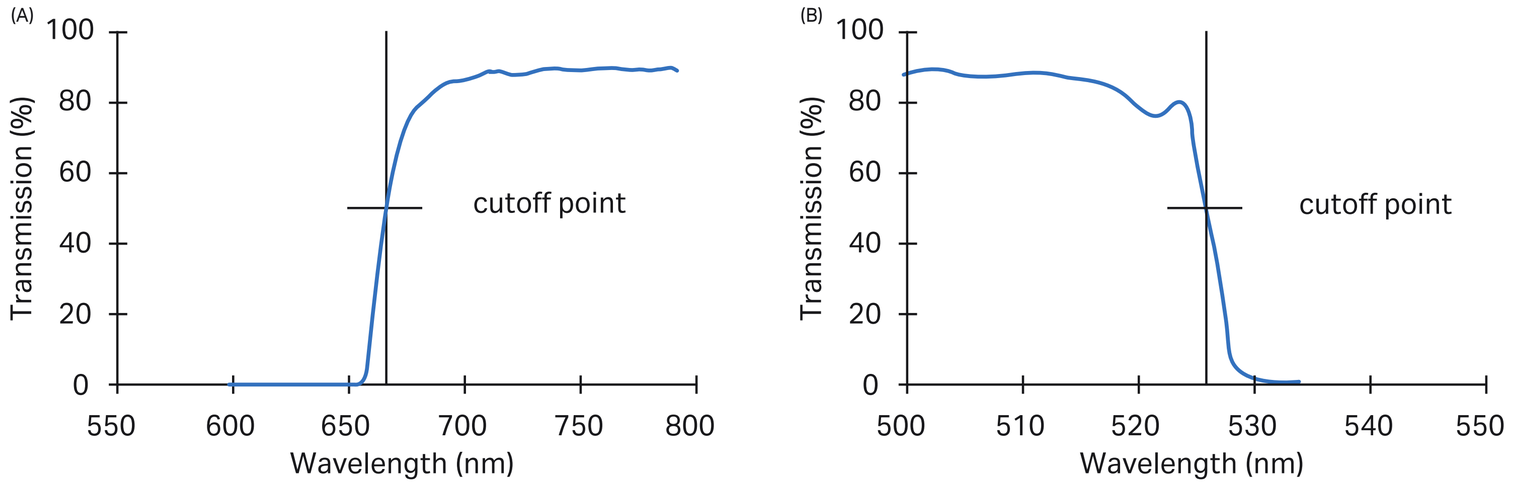


Fig 3.1. Transmission profiles for a 665 nm long-pass (a) and a 526 nm short-pass (b) filter. The cutoff points are noted.

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 center. For example, the 570 BP 20 filter passes a band of light centered at 570 nm (Fig 3.2).
- the full-width at half-maximum transmission (FWHM). For example, a 570 BP 20 filter passes light over a wavelength range of 20 nm (560 nm - 580 nm) with efficiency equal to or greater than 50% of 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 FWHM greater than 30 nm allow collection of light at more wavelengths and give a higher total signal; however, they give less discrimination 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

The choice of filters influences 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 portions of the background signal that are comprised of wavelengths that are longer or shorter than the fluorochrome emissions. By selecting a filter that transmits a band of light at or near the emission peak of interest, the background signal is typically reduced with 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 (S/N ratio). To decide if a filter is needed, scans should be performed with and without the filter while keeping other conditions constant. The resulting S/N values should then be compared to determine the most efficient configuration.

Selectable 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 lower than the instrument manufacturer's recommendation. If further attenuation is necessary to prevent saturation of the PMT, the signal reaching the detector can be reduced by adding an appropriate emission filter such as a neutral density (ND) filter.

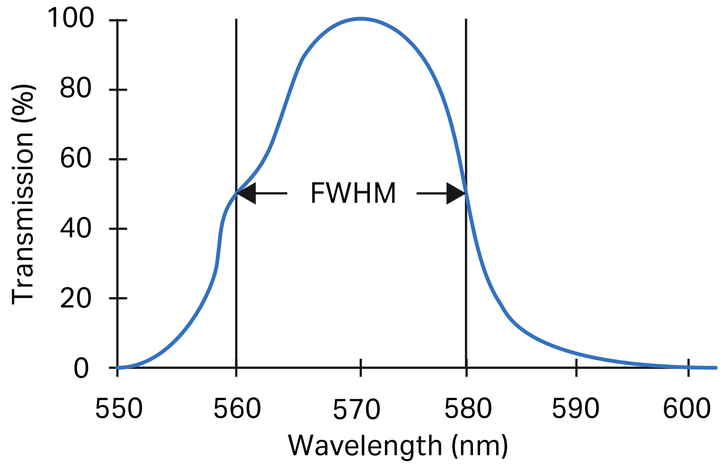


Fig 3.2. Transmission profile for a band pass (570 BP 20) filter. The full-width at half maximum (FWHM) transmission of 20 nm is indicated by the arrows.

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, many fluorochromes have broad absorption profiles, and some have an additional absorption peak or a long "tail" Therefore, efficient excitation does not require that the available excitation wavelength exactly matches the fluorochrome's major absorption peak. It is also important to consider other fluorochrome properties, such as brightness or signal intensity (see section 1.3.3). For example, the absorption maxima of the fluorescein and Cy3 fluorochromes are 495 nm and 550 nm, respectively (Fig 3.3). Excitation of either dye using the 532 nm wavelength line of the green 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, supplying a high level of excitation energy at 532 nm efficiently excites both fluorochromes.

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 3.4. shows collection of Cy3 fluorescence using either a 570 BP 20 or a 575 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 for fluorescence scanning systems from Cytiva.

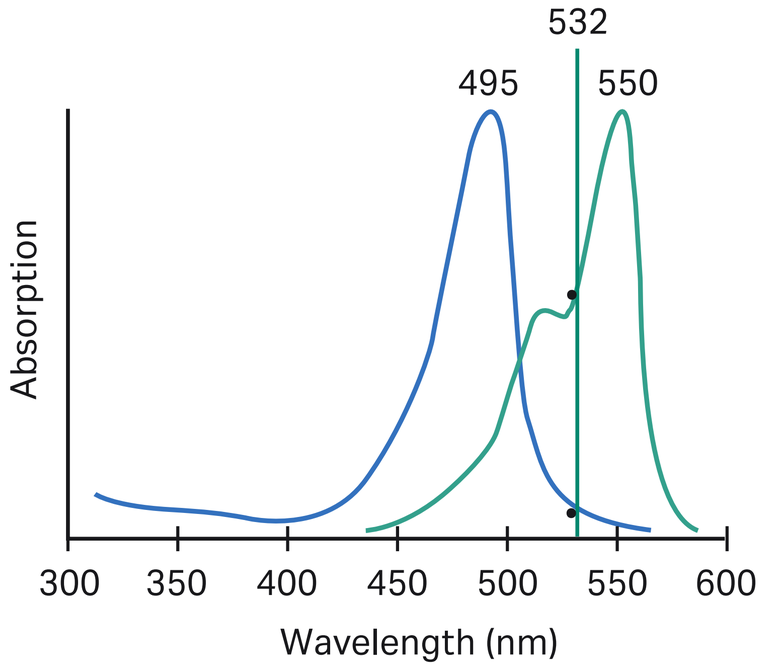


Fig 3.3. Excitation of fluorescein (blue) and Cy3 (green) using 532 nm laser light. The absorption spectra of Cy3 and fluorescein are overlaid with the 532 nm wavelength line of the green laser.

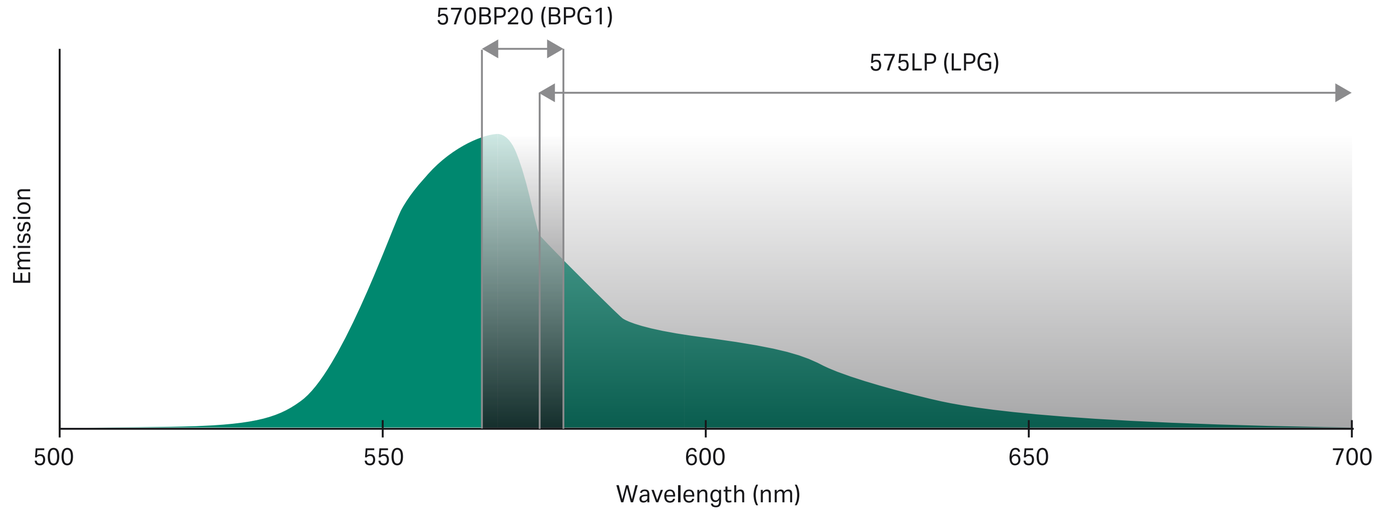


Fig 3.4. Emission filtering of Cy3 fluorescence with either a 570 BP 20 (dark gray area) or a 575 LP filter (light and dark gray areas). The emission spectra of Cy3 shown in green.

3.4.2 Multicolor imaging

Multicolor imaging enables 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, Figure 3.5 shows a two-color Western blot experiment where two protein targets are differentially probed using two antibodies, each conjugated with different fluorescent dyes.

By using antibodies conjugated with fluorescent molecules for Western blotting it is also possible to simultaneously detect two proteins of the same molecular weight or post-translational modified proteins on the same membrane without stripping and reprobing. This is particularly useful in studies of low-abundant proteins, such as phosphorylated proteins. The technique increases the dynamic range and sensitivity to give more reliable quantitation and reduced variability of activated low-abundant targets (Fig 3.6).

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 sources. If two detectors are available, the mixed fluorescence emitted from the two labels can be collected and resolved by filters before simultaneous detection of the signals by their respective detectors. Cross-talk between signals during simultaneous detection can be effectively eliminated by selecting fluorochromes with completely separate emission spectra.

Simultaneous 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, functions 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 2.9).

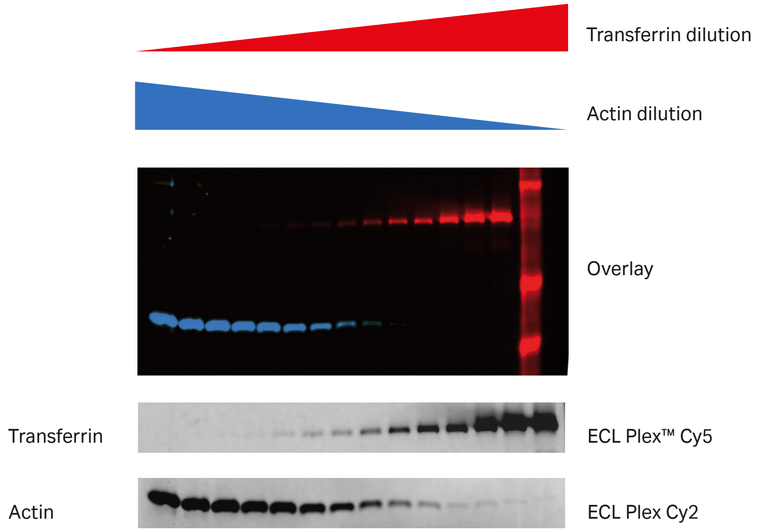


Fig 3.5. Two-color fluorescent Western blot. Transferrin was detected using a Cy5-conjugated secondary antibody (red), and actin was detected using a Cy2-conjugated secondary antibody (blue). Typhoon FLA 7000 was used for image acquisition.

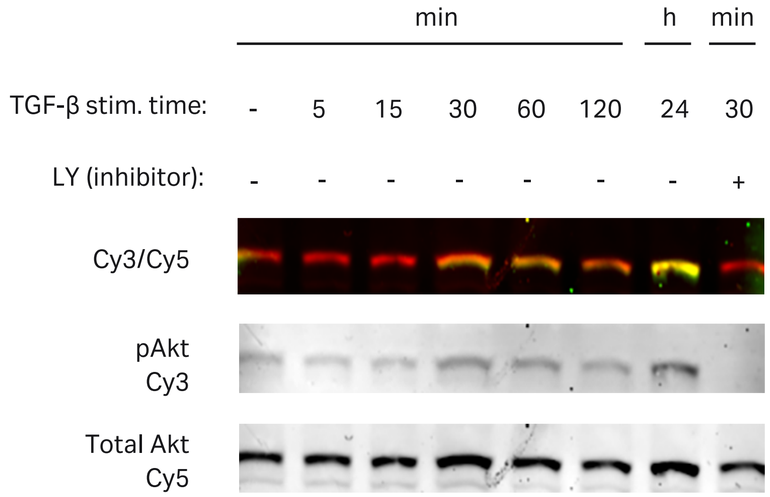


Fig 3.6. The simultaneous detection of two targets with the same molecular weight using Amersham ECL Plex. The results show detection of the low-abundant phosphoprotein, pAkt (Cy3) and the total amount of Akt (Cy5) in separate channels. In this example, human prostate cancer cells were stimulated with TGF-β for different lengths of time. Phosphorylation of Akt above base level was seen after 30 min. Treatment with kinase inhibitor in the lane furthest to the right totally blocked the phosphorylation of Akt. The images of Cy3 and Cy5 are shown in black and white to make the weak pAkt signals visible. Data courtesy of Marene Landström, Ludwig Institute for Cancer Research, Uppsala, Sweden.

3.4.3 Fluorochrome and filter selection in multicolor experiments

The two key elements to consider when designing multicolor experiments are the choice of fluorochromes and the emission filters available. To generate a fluorescent signal, the excitation light directed at the sample must be within the absorption spectrum of the fluorochrome.

The emission spectra of the fluorochromes used in multicolor experiments should ideally be well segregated to avoid spectral overlap. However, slight spectral overlap can sometimes be expected. For more detailed information about the excitation and emission spectra of commonly used fluorochromes and stains, see Appendix 2.

To minimize cross-talk in multicolor detection, fluorochromes should be chosen that have well-separated emission peaks (Figs 3.5 and 3.6). Good results can be achieved by choosing fluorochromes with emission peaks that are at least 50 nm apart. Moreover, emission filters should be used to further discriminate between the light emitted from the different fluorochromes.

Figure 3.7 shows multicolor detection of two well-segregated fluorochromes, Cy2 and Cy5. Since the excitation spectra of Cy2 and Cy5 do not overlap, it is possible to detect discrete Cy2 and Cy5 emission spectra by two consecutive scans using long-pass filters. Discrete detection using simultaneous multicolor scanning would require an imager that can be equipped with a narrow band pass filter optimized for Cy2 emission.

Figure 3.8 illustrates the detection of Cy3 and Cy5. These two fluorochromes have slightly overlapping spectra. To achieve discrete detection of Cy3 and Cy5, consecutive scanning and a narrow band pass filter optimized for Cy3 are recommended. If simultaneous detection is used there is a risk of cross-talk between the Cy3 and Cy5 detection channels.

In conclusion, to achieve optimal results in multicolor experiments, it is necessary to have an understanding of fluorochrome excitation- and emission spectra, the principles of filter selection, and which detection methods are available in the imaging system you use.

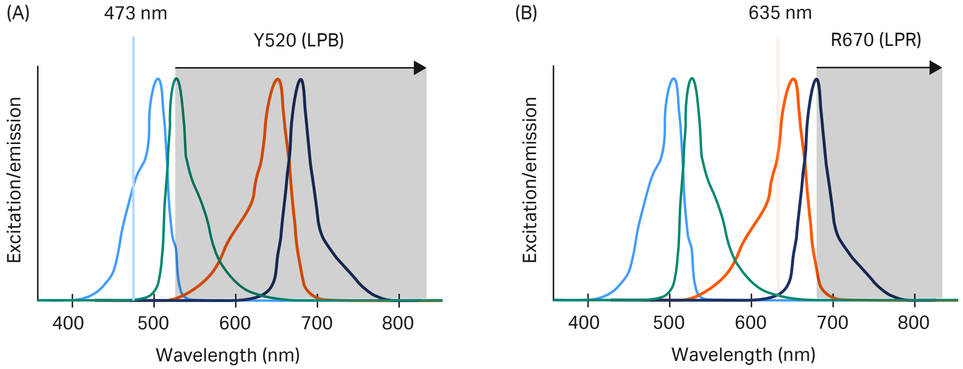


Fig 3.7. (A) Consecutive detection of Cy2 and Cy5 with Typhoon FLA 7000. Excitation of Cy2 at 473 nm (blue line) creates Cy2 emission (green curve) that passes through the LPB filter (grey area). Since the two fluorochromes are spectrally segregated, Cy5 is not excited (orange curve). **(B)** Excitation of Cy5 with a 635 nm laser (orange line) creates Cy5 emission (dark blue curve) that passes through the LPR filter (grey area). No Cy2 emission occurs since the excitation spectrum of Cy2 (blue curve) is far from the excitation wavelength of the 635 nm laser and any potential emission under 670 nm is blocked by the LPR filter.

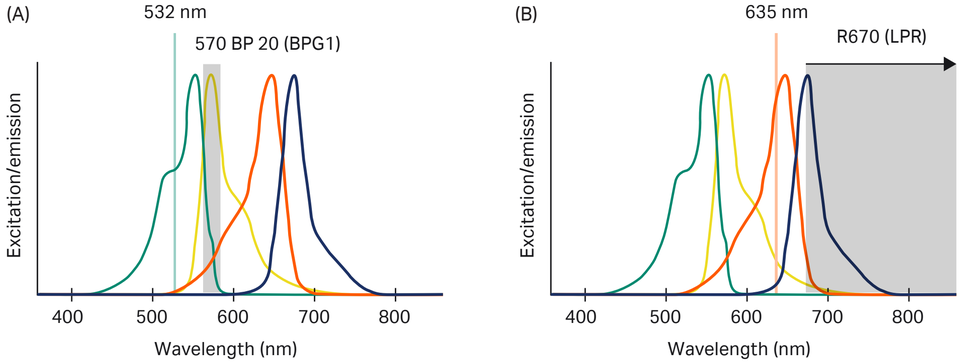


Fig 3.8. (A) Consecutive detection of Cy3 and Cy5 with Typhoon FLA 9500. The green 532 nm laser (green line) effectively excites Cy3 (green curve) and Cy3 emission (yellow curve) passes the narrow BP filter (grey area). A small quantity of Cy5 is excited (orange curve) but any signal from Cy5 (dark blue curve) is blocked by the BP filter. **(B)** Cy5 is excited with a 635 nm laser (orange line) and the emission (dark blue curve) passes the LPR filter (grey area). No Cy3 is detected since its excitation spectrum (green curve) does not overlap with the 635 nm laser line. Should any emission of Cy3 (yellow curve) occur, then emission signal is blocked by the LPR filter.

04

Image analysis

4.1 Introduction

Imaging systems create one or more data files for each sample during image acquisition and the size of the files varies depending on sample size and the digital resolution used. The analysis software is then used to display the image, adjust the contrast, annotate, and print the image. Moreover, the software can include analytical tools for fragment sizing, quantitation, matching, pattern analysis, and generation of analysis reports. Some software packages also provide access to libraries or to a database for target matching and querying. Image utility functions manage 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.

One of the basic functions of an image analysis software package is to assist the viewing, adjustment, and assessment of images. The software should allow users to fine-tune the display range without affecting original data or results.

The contrast and brightness of displays can be adjusted to optimize the image view. High- and low-display gray (or color) values can be adjusted to assist viewing the range of interest (See section 2.2.4). 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, so that weak signals can be visualized. High contrast settings; can however, pixelate images whereas increased high-value settings tend to give a smoother appearance. These adjustments are made separately to each channel in multichannel images. The analysis software also allows display of the individual channels or a multicolor overlay of all channels together.

4.2 Analysis software

Cytiva imaging instruments and software offer total solutions to many challenging applications. Cytiva offers three software packages, ImageQuant TL, DeCyder 2D and, ImageMaster 2D Platinum. These software packages can be used for a variety of tasks from basic documentation and purity screening to complex queries of an entire gene expression and analyses of 2-D gel datasets.

General graphics software packages ignore or even remove calibration information and can also corrupt the image file for quantitative analysis. Therefore, they should not be used to flip, rotate, crop or invert images. Instead, use the analysis software of choice.

4.2.1 ImageQuant TL

ImageQuant TL (IQTL) is a suite of image analysis tools for a broad range of requirements. It offers a high degree of automation yet allows editing and override at any stage of analysis. Image data can be extracted easily and quickly, reducing analysis time to a minimum while giving consistently reliable results. IQTL supports multiplexing for advanced fluorescence applications.

The IQTL software consists of the following modules:

- 1-D gel analysis for analysis of 1-D electrophoresis gels and blotting results
- Colony Counting for analysis of colonies on agar culture
- Array analysis for analysis of arrays such as microtiter plates as well as dot and slot blots
- Analysis Toolbox for area and profile analysis of acquired images

Each module is started from the ImageQuant TL Control Centre. Multiple modules may be run simultaneously, but the modules are independent of each other.

IQTL can analyze .TIF, .GEL, .DS and .IMG type of files.

4.2.2 DeCyder

DeCyder 2D Software is an automated software suite for detection, quantitation, matching and, analysis of 2-D DIGE gels. The 2-D electrophoresis gels are used for separating complex protein mixtures labeled with up to three CyDye DIGE Fluor dyes, for more information see section 5.2.4.1. The software also includes univariate and multivariate analysis of large and combined data sets and can link external protein databases to the data set.

4.2.3 ImageMaster 2D Platinum

ImageMaster 2D Platinum is a flexible solution for comprehensive visualization, exploration and analysis of 2-D gel data. The software is available for both conventional 2-D electrophoresis and for 2-D DIGE gels. It is fully functional, enabling import of DIGE gels directly into the workspace. It is also possible to co-detect DIGE gels using the same algorithm as DeCyder and match, report, plot histograms and perform statistical analyses on DIGE gels.

4.3 Image acquisition optimization

Analysis software can identify images that contain inaccurate, non-quantifiable areas caused by light saturation of the detector. If an image contains saturated values, then it should be acquired with a shorter exposure time (CCD cameras) or lower PMT voltage (laser scanners), so that pixels in the areas of interest are unsaturated. Adjustments to other settings, such as the scan area, pixel size, and choice of laser or emission filter, can also improve the resolution, discrimination, or strength of signals. ImageQuant LAS 500 has an instrument control screen that displays intensities directly and allows adjustment of exposure time without the need for analysis software.

There are several different ways of using IQTL and the Analysis toolbox module to evaluate acquired images. The two different approaches presented here, the box approach and the line approach, can be used separately or in combination.

Box approach

After image acquisition, open IQTL and select Analysis Toolbox. Open the image file with extension .TIF, .GEL, .DS or .IMG (if multiple images) and select one or several areas suitable for the optimization. This could be the strongest band or specific protein spot that represents the highest signal to be included in the analysis.

- In Analysis menu, select shape definition and use the Rectangle shape tool and draw a box around the area (Fig 4.1)
- Check the maximum intensity of the box in the table in Area window (If Max intensity isn't displayed, make sure the checkbox of Max intensity is checked under the Tables tab located in Options.)
- If the Max intensity is 100 000 (valid for .GEL files from scanners) or 65 536 pixels are saturated within the area and optimization is needed. If the intensity is very low (judged differently depending on the application needs, but a rule of thumb would be below 20 000), a wider dynamic range can be achieved by longer exposure or higher amplification.

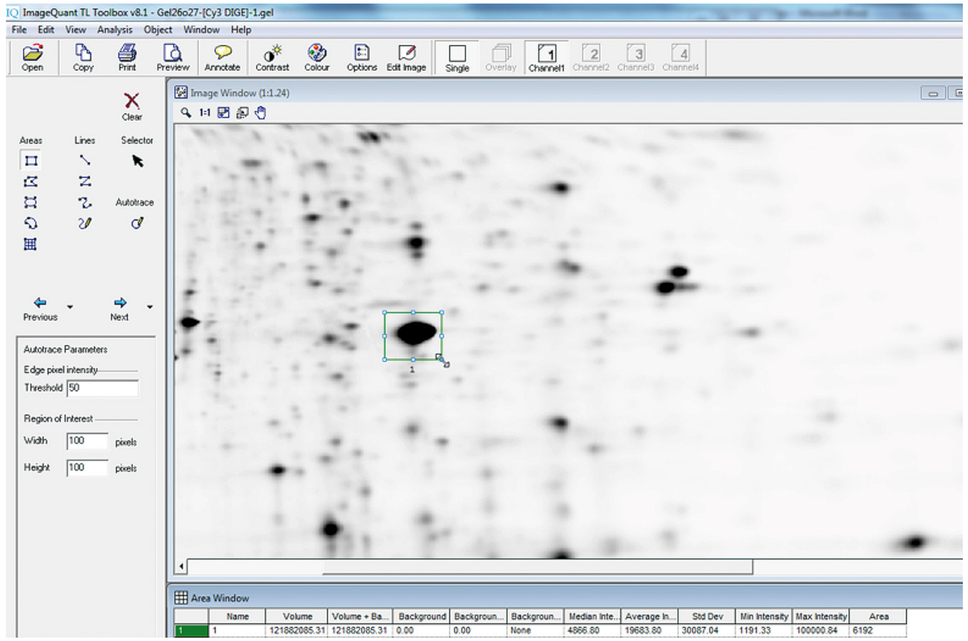


Fig 4.1. Box approach for evaluating intensity levels of acquired image. The Max intensity in the table (red field) shows 100 000 (indicating saturated pixels within the area).

Line approach

After image acquisition, open IQTL and select Analysis Toolbox. Open the image file with extension .TIF, .GEL, .DS or .IMG (if multiple images) and select one or several areas suitable for the optimization. This could be the strongest band or specific protein spot that represents the highest signal to be included in the analysis.

- In Analysis menu, select shape definition and use the Line tool to draw a line through the area
- Check the scale of intensities to the left in Line window to see the level of intensity for the area. Also check the shape of the line
- If the area where the line is drawn is saturated, the peak of the line is truncated. The scale of intensities gives indications of how optimized the image is. If top values are very low (judged differently depending on the application needs, but a rule of thumb would be below 20 000), a wider dynamic range can be achieved by longer exposure or higher amplification (Fig 4.2)

4.4 Multi-channel images

Multi-channel images can be created by repeated acquisition from one target, typically using different settings to allow for separate analyses of multiple fluorochromes such as Cy2, Cy3 and Cy5. In addition, a white light image can be combined with a chemiluminescent image to show colorimetric molecular size markers together with the protein of interest in a Western blot.

A multichannel image is created in the analysis software from imager source files. The source files must be the same size and type (photometric interpretation value) and also be aligned since channels cannot be moved relative to each other.

There are different methods to display multichannel images that depend on which analysis software is used. DeCyder 2D and ImageMaster 2D Platinum use the DIGE file naming convention (see section 5.2.4.8) to group source files for multichannel display, whereas IQTL uses a special text file with the extension .DS. The .DS file is either created by the imager when the source files are acquired or can be created manually in IQTL. When opening the .DS file in IQTL, the text file points out the source files (all source files have to be located in the same folder) and opens them together in a multi-channel image.

The images can be displayed as overlay images where each source image can be represented by a color or individually as single images. See sections 4.6 and 4.10 for details about analysis of multichannel images.

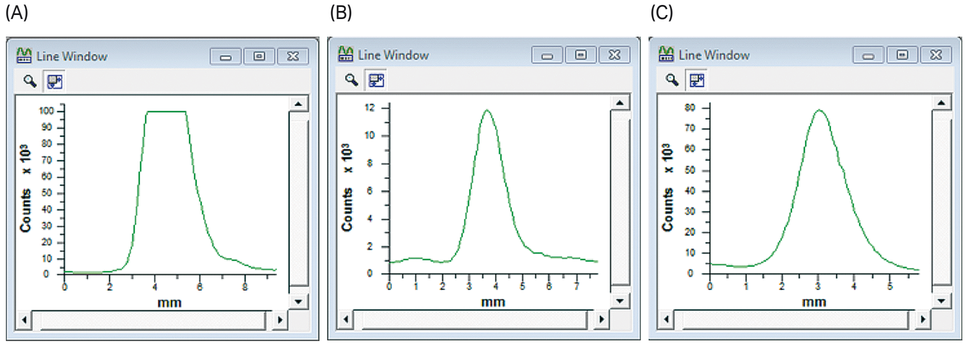


Fig 4.2. Three examples of line analysis on the most intense area of interest for image acquisition optimization. (A) Saturated spot, the peak is truncated. (B) Intensity could be optimized by increasing exposure or amplification. (C) Optimal conditions regarding intensities.

4.5 Background correction

The nature of image background can vary significantly depending on factors, such as the detection chemistry used, the sample matrix (e.g. gel, membrane, microtiter plate), and the integrity or quality of the sample. Therefore, analysis software offer several ways to apply background correction to intensity measurements.

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.

The nature of the background signal should be assessed before proceeding with image data analysis (Fig 4.3). The type of background pattern in an image is a good indicator of which background correction to apply. For example, if background signal is variable across the image, then a local method of correction may be appropriate. Alternatively, one global background value for the whole image may be the best choice when background signal is uniform. 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

Background correction can be applied either locally or globally. Local methods account for the local environment in the immediate vincinity 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. Alternatively, signals from boundaries surrounding objects can be used to determine different background values for each object.

Global methods use one background value applied equally to a group of analysis targets in one 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.

Choosing the most appropriate method to calculate the background value(s) is also important. The difference between a statistical mean and a median value for background calculation can significantly affect the results of quantitation. If for example, high signal spikes contribute to the background noise, calculation of mean average background will be skewed on the high side. Therefore, calculating a median value is recommended since it disregards aberrant noise from the background calculation.

The region of an 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.

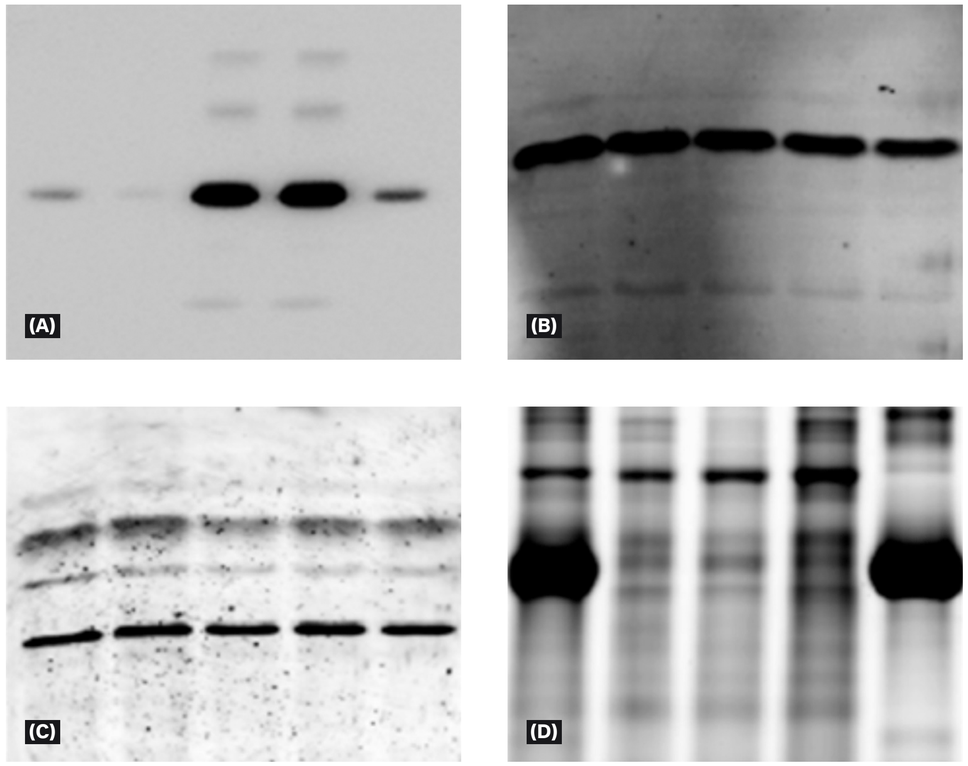


Fig 4.3. Examples of different types of image background: (A) Uniform. (B) Non-uniform. (C) Noise spikes. (D) Lane-specific.

4.6 1-D analysis

The 1D gel analysis module in IQTL analyzes one-dimensional electrophoresis gel, blot and thin-layer chromatography (TLC) images, where a lane in the image represents each sample. The analysis assesses the amounts of separated components from the integrated intensity of bands as well as component properties (e.g., fragment size, molecular weight or isoelectric point) from the position of bands in relation to calibration standards. Volume quantitation (integrated intensity) of the bands is calculated from the area under the average lane profile and the width of the lane. The method uses a 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. The accuracy of this approach is greatest when the line includes most of the target signal across the width of the lane. The automated workflow is recommended since it prevents bias from user interaction, but when the nature of the sample requires flexibility, manual actions can be applied.

Analysis can be performed on single images and multichannel images. Most analytical procedures apply to all channels in a multichannel image, even if they are performed in single channel view mode. Some procedures can only be performed in single channel view mode.

- Contrast changes can be made in either display mode, but are applied only to one channel at a time. In overlay view mode, select the channel to work with in the Contrast dialog box.
- False color changes can only be applied in channel overlay view mode, and apply only to the channel displayed and to the lane image in the analysis window. The display colors can be changed for each channel individually.
- Lane creation and editing can be performed in either display mode and applies to all channels regardless of whether they are visible or not
- Band detection can only be performed in single channel display mode and applies only to the selected channel
- Quantity calibration can only be performed in single channel view mode and applies independently to each channel. Note that the quantity calibration curve is only displayed in single channel view mode. However, the calibration results are shown in the Measurements window in both display modes.
- Normalization can only be applied in single channel view mode, and applies independently to each channel

There are many different options for exporting and displaying the results of the analysis. Exports can be made for each window independently either as data points for data collection or processing in external programs such as Microsoft Excel® or images for publication or presentation purposes. Options in the Reports menu allow analysis reports to be created as PDF-files for printing and archiving.

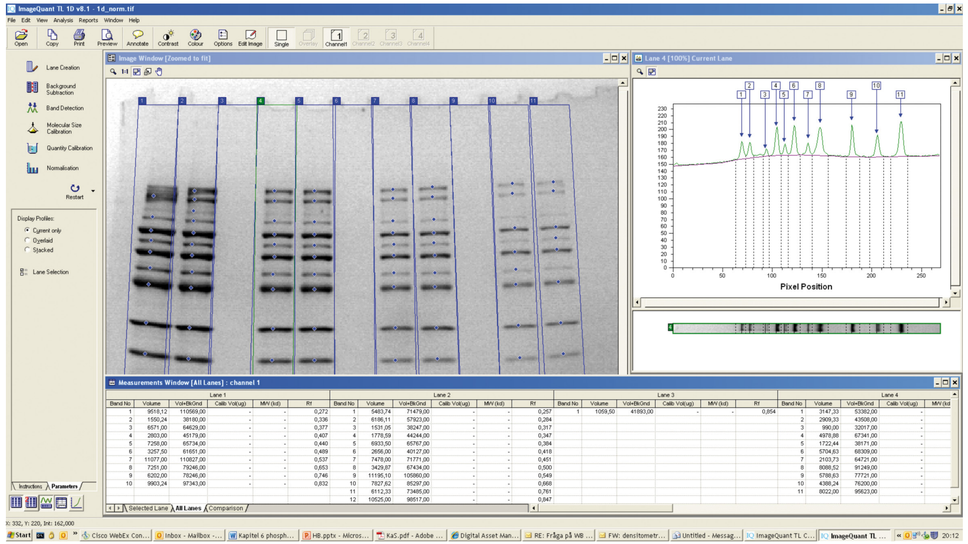


Fig 4.4. Illustration of an analysis of a SDS-PAGE gel using the automated workflow in the 1D gel analysis module in IQTL.

4.6.1 Analysis of a channel and a marker

A typical sample for this workflow would be a chemiluminescent Western blot with a colorimetric marker. The analysis is based on two images: an image of the colorimetric marker and an image of the chemiluminescent sample. The images are either linked from image acquisition by a .DS file, or come as two separate images that can be linked with the function "Create multiplex image..." in the IQTL 1D gel analysis module for display and analysis (Fig 4.5).

The IQTL 1D gel analysis module workflow of an image would preferably be automated and would include the following steps:

- Lane detection
- Background subtraction
- Band detection
- Molecular size calibration
- Quantity calibration or normalization
- Result display

☞ Molecular size calibration can be made either for one channel applying to all channels, or calibration is applied separately and independently to each channel. For the purpose of the example in this section, the calibration should be performed in one channel and applied to both.

☞ When calibrating (both molecular size and quantity), the selection of curve type used for the curve fitting is important for your result. Choose the type of curve that applies to the conditions in the experiment and check the calibration curve in IQTL before deciding which gives the best fit to the data.

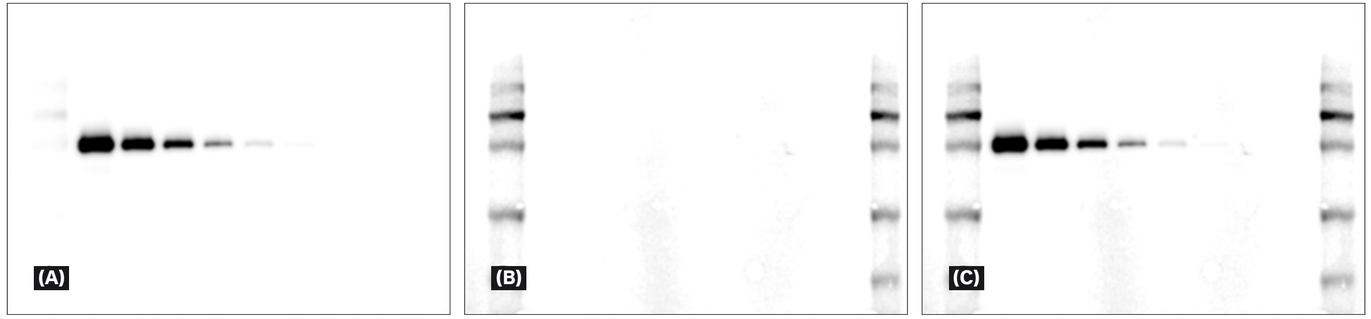


Fig 4.5. The three images of one chemiluminescent membrane showing (A) the chemiluminescent image, (B) the white light image of the colorimetric marker and (C) the overlay image in IQTL for display and analysis.

4.6.2 Multiplex analysis

When two or more different labels are used, it is preferable that each channel is analyzed separately and the resulting quantities are used in relation to each other. A good example of this approach is when quantitating a protein sample by comparing it to a housekeeping protein.

The typical sample used in this workflow could be a membrane with ECL Plex Western blot detection system. The analysis includes two images, one image showing the Cy3 conjugated secondary antibody for detection of one specific protein and another image showing the Cy5 conjugated secondary antibody for detection of the housekeeping protein. The images are either linked from the acquisition by a .DS file, or provided as two separate images to be linked with the function "Create multiplex image..." in the IQTL 1D gel analysis module (the .DS file is created in IQTL), for display and analysis.

The IQTL 1D gel analysis module workflow of the images would preferably be automated and would include the same steps as for analysis of one channel and a colorimetric marker:

☞ Molecular size calibration can be made either for one channel applying to all channels, or calibration is applied separately and independently to each channel. For the purpose of the example in this section, the calibration should be applied separately and independently to each channel.

☞ Quantity calibration or normalization cannot be performed across different channels and applies independently to each channel. There is nothing linking the intensities of the different channels together and hence, in sense of quantities, they have to be analyzed separately. However the experimental design links the quantities together between the channels, the results can then be used for inter-channel quantitation.

4.7 The toolbox approach

The Analysis Toolbox module in IQTL can be used for a number of applications. It is flexible and uses different shapes and objects for quantitative analysis.

Object quantitation analyzes target bands, spots and slots, by enclosing them with objects such as boxes, rectangles, polygons, or ellipses to include all the image pixels within the object. A number of background subtraction methods can be applied as well. This method of quantitation is more flexible than the lane profile method since the user defines the area to be analyzed. However, the method also increases the risk for user-induced variation, which may affect reproducibility and objectivity.

The IQTL Analysis Toolbox can be used for image acquisition optimization (see section 4.3) and many other calculations, such as the evaluation of limit of detection (LOD), that are not normally part of specific workflows.

4.7.1 Determine signal to noise

The signal to noise ratio (S/N) indicates how well an object can be distinguished from background noise and distortions of the image. A signal to noise value can be used in an evaluation at a set threshold value to decide whether an object is detectable or not (the value differs depending on the application, but in many cases the S/N threshold is set to 3). Figure 4.6 shows the parameters that are included in the definition of signal to noise.

One way to calculate signal to noise in Analysis Toolbox is to select a rectangular area within the area of interest and extract the S_{tot} for the signal component of S/N ratio. To avoid contribution from spiked artifacts, use the median value as long as the area of interest is larger than the analysis area rectangle. Thereafter, copy the rectangle and place these around the signal area to measure the background and noise. The selected areas give data to calculate average background and its standard deviation represent the noise for the S/N ratio (Fig 4.7).

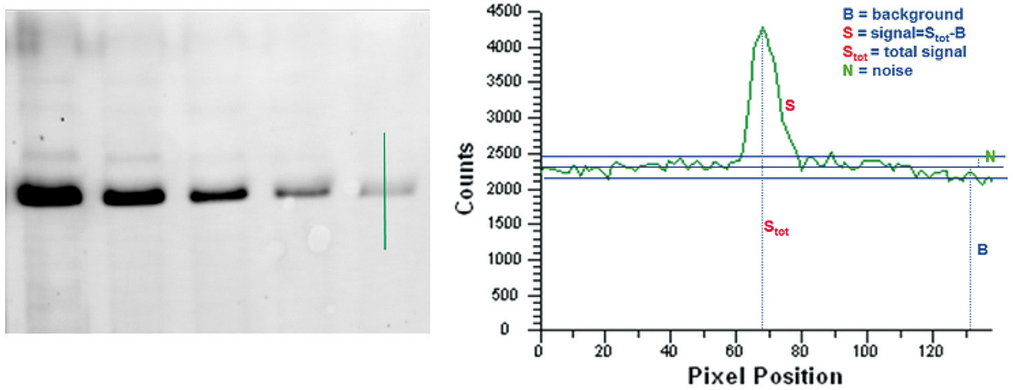
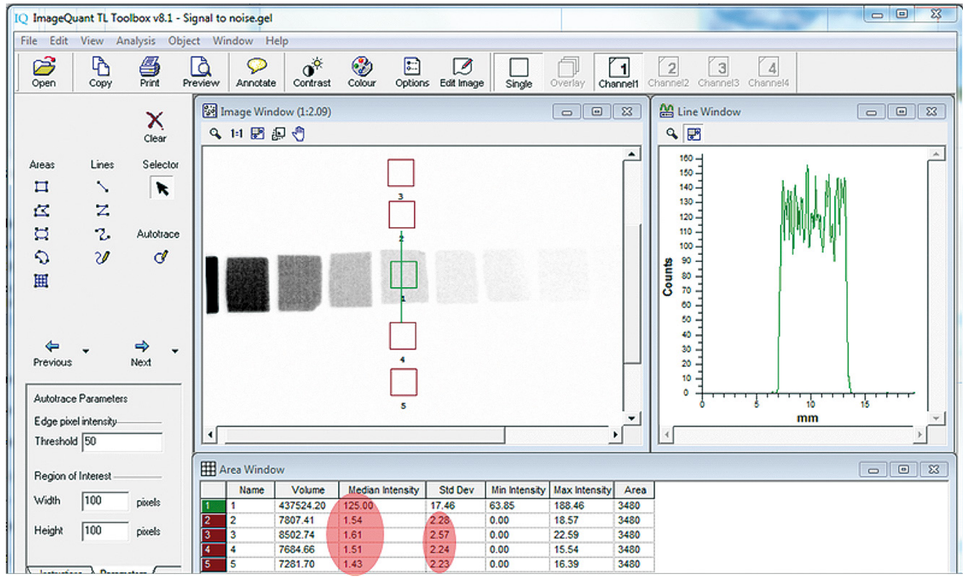


Fig 4.6. The lane profile (green line) of the protein band of the left image is shown to the right. The intensity is shown as a function of the pixel position and parameters defining S/N such as background, signal, total signal and noise.



Sample	S_b	N	$S_{corr} = S_{tot} - S_b$	$S/N=S_{corr}/N$
1	1.52	2.33	123.48	53.0

Box	Median Intensity	Standard deviation
1	125.00	17.46
2	1.54	2.28
3	1.61	2.57
4	1.51	2.24
5	1.43	2.23

Fig 4.7. Signal to noise (S/N) determination from a ^{14}C standard exposed to a phosphorimaging plate. S_{tot} is given by the median intensity of rectangle 1, S_b is given by the calculated average of the median intensity from rectangle 2 to 5, N is given by the calculated average of the Std Dev of rectangle 2 to 5 and S is given from $S_{cor}r=S_{tot} - S_b$.

4.8 Colony counting

The colony counting module provides functions for analysis of spots and is designed primarily for use with images of microbial colonies on for example, agar plates. Spot analysis provides data on number, size (area and volume), average intensity and circularity of spots (Fig 4.8). The module can also be used for very basic analysis of traditional 2-D gel images.

Global and local background subtraction methods are available. Image rectangle, the global background subtraction method, uses the average intensity level within a specified rectangle on the image. The image rectangle will produce a single value for background. This is removed from each pixel in a spot. If the background value is higher than the pixel value then the value zero is used. The total background can therefore vary between spots.

The local method, Mode non-Spot, uses the lowest pixel intensity in a rectangular border enclosing the colony, with a width specified as the Margin Value. Any colonies in the border area are ignored. However, 16-bit images can exhibit high variation in pixel intensities in the boundaries that surround colonies. In this case, pixels are assigned into groups of intensities, and the average intensity in the lowest group is used to determine the background.

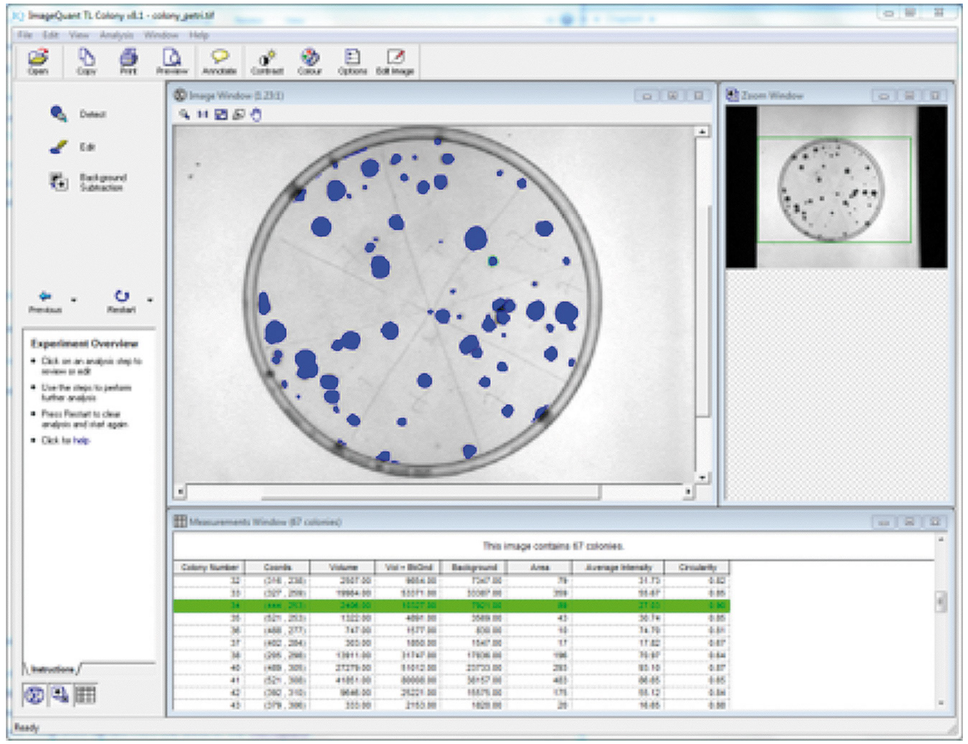


Fig 4.8. Detection and analysis of colonies on an agar plate.

4.9 Array 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.

IQTL has an array analysis module for analysis of images consisting of rectangular arrays, such as microtiter plates, dot blot and slot blot images. Array analysis assists in the identification of poorly arrayed, contaminated, or improperly detected spots. Spots or slots in selected images can be analyzed for volume (total pixel intensity) and can be flagged as present or absent on the basis of user-defined thresholds (Fig 4.9).

Background subtraction can be carried out with local methods such as spot surface minimum and spot edge average or with global methods such as negative control and image rectangle. The Normalization protocol allows the use of known values, or the spots can be expressed as a percentage or a proportion of one or more selected spots. If the actual volumes are not known, the spot volume can be set to 100% and the software calculates the unknown spots in relation to that spot. Normalization is carried out with a single click and has a choice of 10 quantity units.

For more information about functions in the IQTL software, please refer to the ImageQuant TL User Manual (1).

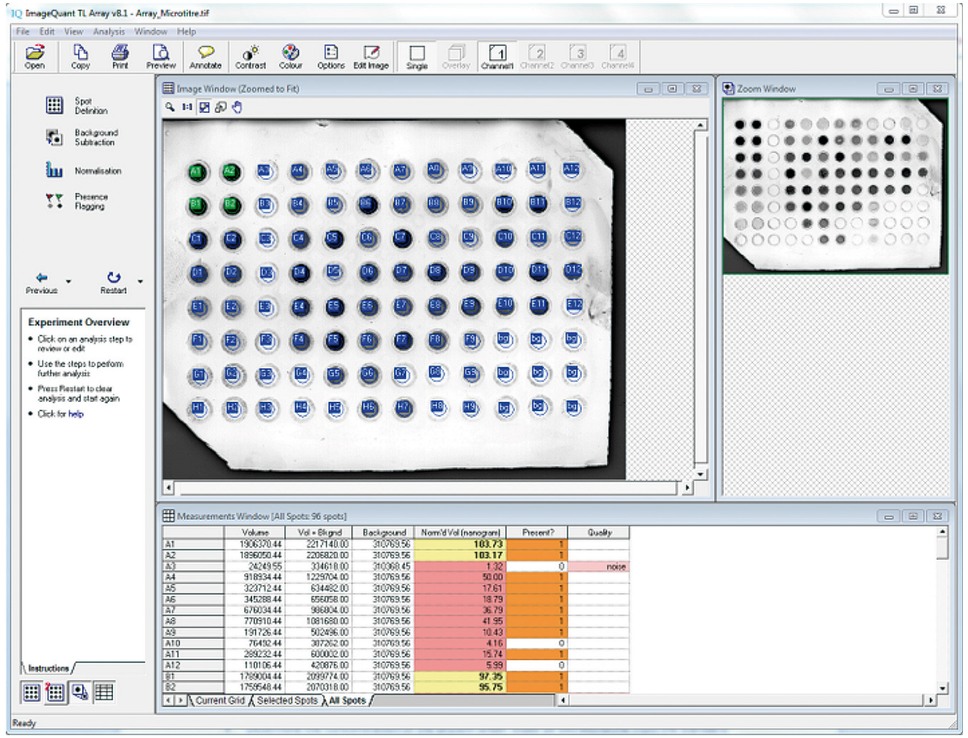


Fig 4.9. Analysis of a 96-well plate.

4.10 2-D analysis

4.10.1 Introduction

Software packages for 2-D protein gel analysis feature specialized algorithms for spot-finding and analysis routines for gel-to-gel comparisons. Other important tools in these software packages include data normalization, background correction, gel to gel matching, statistical tools and database input of analysis results.

4.10.2 DeCyder 2-D DIGE Analysis

DeCyder 2D Software was specifically developed to exploit all the advantages of 2-D DIGE. The software supports simple control-treated experiments as well as complex multi-condition experiments to enable investigation of factors such as dose and time in a single analysis. The relationships between samples can be accurately quantitated and statistically analyzed by using an internal standard on every gel (see section 5.3). This approach is very accurate, and allows experimental conclusions to be made with high confidence.

A novel co-detection algorithm uses the identical spot patterns that are generated when multiple samples are resolved on the same gel. The algorithm generates identical spot detection patterns on all images derived from the same gel. Hence all spots on the same gel are effectively matched with the identical spot boundaries. Spot quantitation is performed automatically after normalizing spot volumes against the internal standard. This results in a highly accurate and robust protein quantitation.

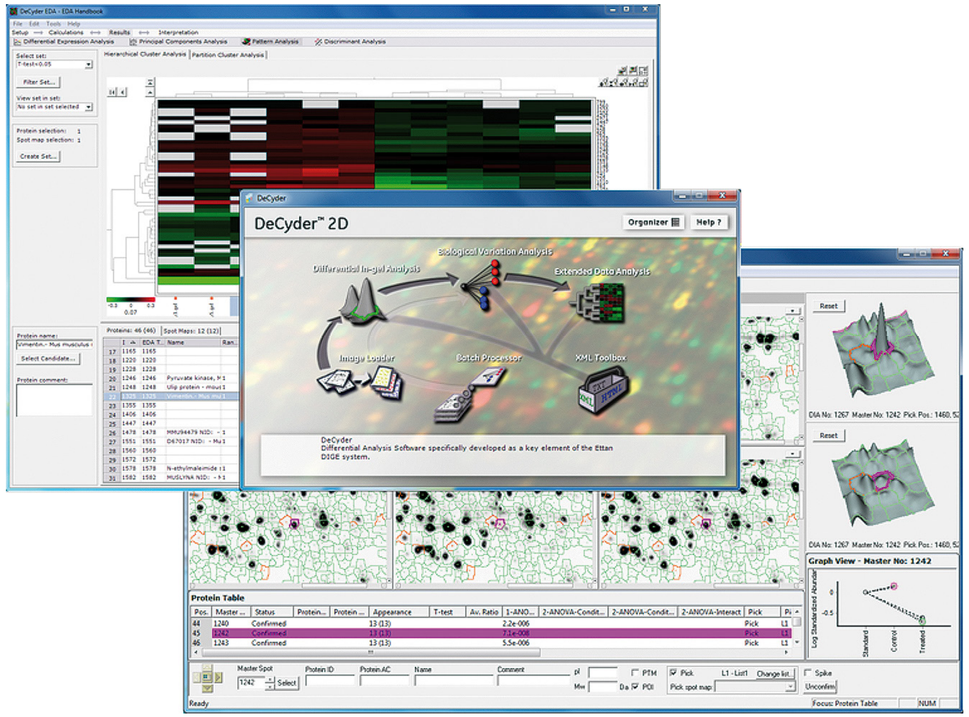


Fig 4.10. DeCyder 2D Software

DeCyder 2D Software can be used from preparing images after scanning to statistical analysis and data interpretation. The DeCyder 2D analysis workflow is shown in Figure 4.11.

A more automated workflow is recommended to minimize bias of user interaction. Here are some useful hints of how to get the best results in a normal 2D DIGE experiment. For more information, please refer to the DeCyder 2D Software User Manual (2) or the DeCyder Extended Data Analysis User Manual (3).

Cropping of images:

- Remove all areas with unnecessary information since it can interfere with spot finding
- Ensure that all relevant spots remain inside the image
- It is more important to ensure consistent patterns than equal sizes

☞ Pick gels should not be cropped as described above. The pick gel must still contain the internal reference markers. To facilitate proper matching define an “area of interest” in the DIA module instead that is similar to the crop pattern used.

Spot detection:

- Version 6 spot detection algorithm is the default setting and is recommended for normal gel quality, version 5 has a higher tendancy to split spots that might give better results when many spots appear merged
- Select 10 000 as estimated number of spots (overestimation) to compensate for the detection of non-protein objects on the image, e.g., dust particles which are subsequently excluded from the analysis
- Exclude spots with volumes less than 30 000 with an exclusion filter

DeCyder 2D analysis workflow

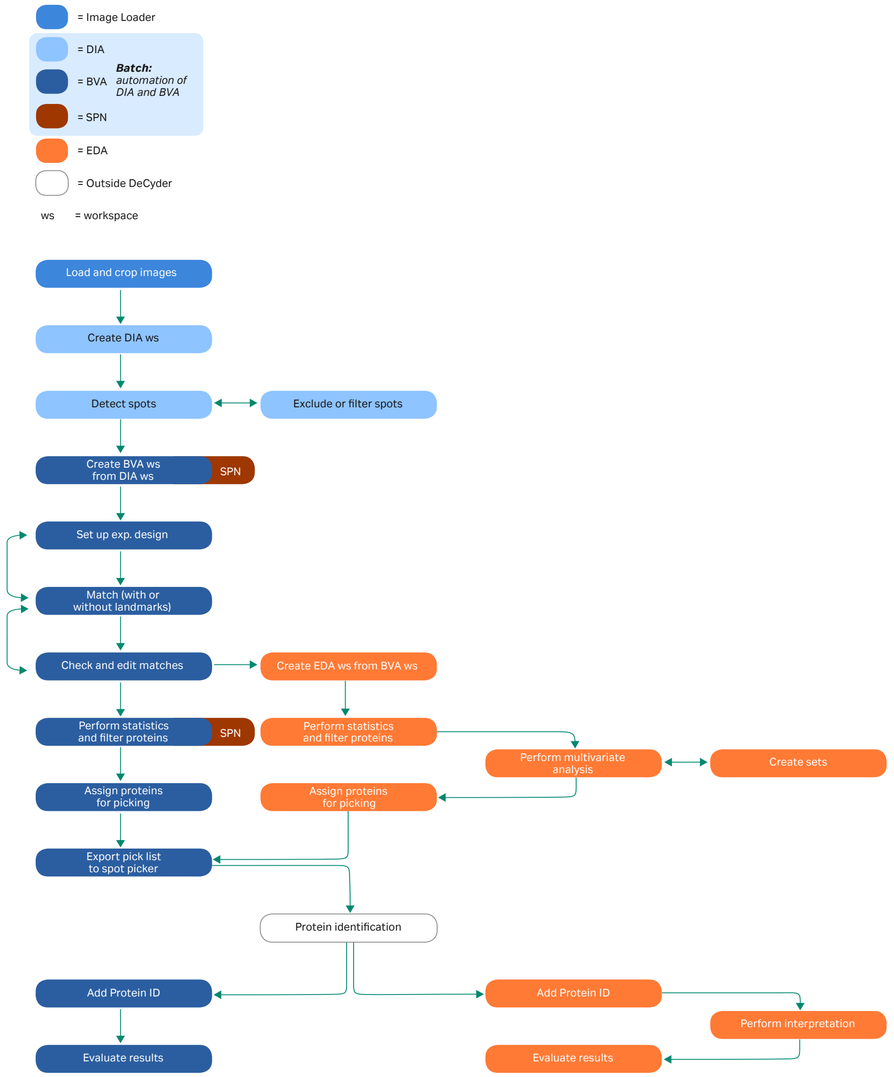


Fig 4.11. DeCyder 2D Differential Analysis Software v7.2 analysis workflow.

- Image Loader — importing images into an Oracle database
- Administration Tools — database administration, maintenance, and user access control
- Batch Processor — automated detection, quantitation, matching, and comparison of multiple 2-D DIGE gels
- Differential In-gel Analysis (DIA) — co-detection, background subtraction, normalization, and quantitation of spots in images from a single 2-D DIGE gel
- Biological Variation Analysis (BVA) — matching (with warping aid) of multiple 2D-DIGE gels for comparison and statistical analysis of protein abundance changes
- Extended Data Analysis (EDA) — advanced statistical analysis for characterization and classification of biological samples based on protein expression data.
- Spike Protein Normalization (SPN) — normalizes data to spike proteins and enables analysis of protein abundance with non-normal distribution
- XML Toolbox — export of spot data from DIA or BVA modules for further downstream analysis

Matching:

- Matching with a few landmarks normally gives good results. If possible the spots selected as landmarks should be isolated, well-shaped (circular) and of medium abundance
- Warping can help evaluate the matching quality and the overlay function in BVA can help find areas (if a gel with lower quality was used) where matching needs correction

Protein statistics:

- Define to calculate average ratio and Student's t-test for two experimental groups. If more than two experimental groups are analyzed, additionally select one-way ANOVA.
- Use two-way ANOVA for analysis of two conditions in an experimental design comprised of two independent factors (e.g., time-dose study). This analysis allows the study of internal and mutual effects of the two factors.
- Use Protein filter to assign protein of interest and/or pick status

☞ Do not apply the False Discovery Rate (FDR) correction unless the data set is statistically significant. We recommend using a suitable protein filter instead.

The Batch Processor is for automated processing of images and links the DeCyder 2D Software DIA and BVA modules to perform all stages of the 2-D DIGE analysis process. After set-up, the Batch Processor processes gels one after another without user intervention. The normal workflow contains 4 main steps:

1. Set up the DIA batch list
    a. Select images to add.
    b. Enter settings
2. Set up the BVA batch list
    a. Define spot map attributes (analysis, master, pick gel)
    b. Define protein statistics settings (average ratio, Student's t-test, one-way ANOVA)
    c. Define protein filter values
3. Save the Batch
4. Run the Batch

☞ Review the BVA workspace prior to generating the final pick list

☞ The choice of the master gel is important since it assists the matching process. Chose a representative gel that is neither the best nor the worst, without too few or too many spots.

Extended data analysis (EDA):

- Create workspace then select and import one or several BVA workspaces

☞ No other values than standardized log abundance will be imported

- Check experimental design and change if necessary
- Generate a "base set" consisting of proteins that show a minimum frequency of appearance (e.g., in 70% of the images, filter 1). Too many "missing values" (protein spots) can cause the calculation to abort. Exclude images that do not belong to an experimental group (filter 2).
- Perform PCA (principal component analysis) to identify outliers and EDA (differential expression analysis) (Student's t-test and ANOVA). With this a tool is created for the separation of differentially expressed from the non-differentially expressed proteins.

4.10.3 ImageMaster 2D Platinum

ImageMaster 2D Platinum offers comprehensive visualization, exploration and analysis of 2-D gel data. It is available in two versions, one for analysis of conventional 2-D gels and the other for use with DIGE gels and conventional 2-D gels. Some useful hints on how to get the most out of the analysis are presented below. For more information, please refer to the ImageMaster 2D Platinum User Manual (4).

4.10.3.1 Traditional 2-D Analysis

ImageMaster 2D Platinum offers analysis of traditional 2-D electrophoresis datasets but uses a less automated workflow for spot detection when compared to 2-D DIGE datasets.

The ImageMaster spot detection algorithm is optimized to give relevant biological results with minimum user interaction. It is possible to preview spot detection and fine-tune parameters before automatically locating spots in images. There are three spot detection parameters to consider and adjust for traditional 2-D analysis.

- Smooth: low value = oversplit
  high value = undersplit
- Saliency: low values = lower probability
  high values = higher probability
- Min Area: removes spikes

Quantitation of spots on a 2-D gel is automated by defining spot boundaries as 75% of peak maximum and calculating the volume that lies above the boundary level. Background is automatically corrected by the local method, subtracting the lowest value in the vicinity of the spot and normalization is calculated with the relative volume.

4.10.3.2 2-D DIGE Analysis in ImageMaster 2D Platinum

ImageMaster 2D Platinum for DIGE analysis enables the addition and import of DIGE gels directly in to the workspace. The software can perform matching and reporting, as well as statistical analyses and plotting histograms. The manual approach to analysis is flexible and provides control of the dataset. ImageMaster 2D Platinum uses the same co-detection algorithm as DeCyder software, however, it does not share the same functionality and can not manage complex experimental designs.

Since the software has many tools and options, we recommend creating a customized toolbar when you are familiar with the software. A customized toolbar is a useful aid when following an established workflow.

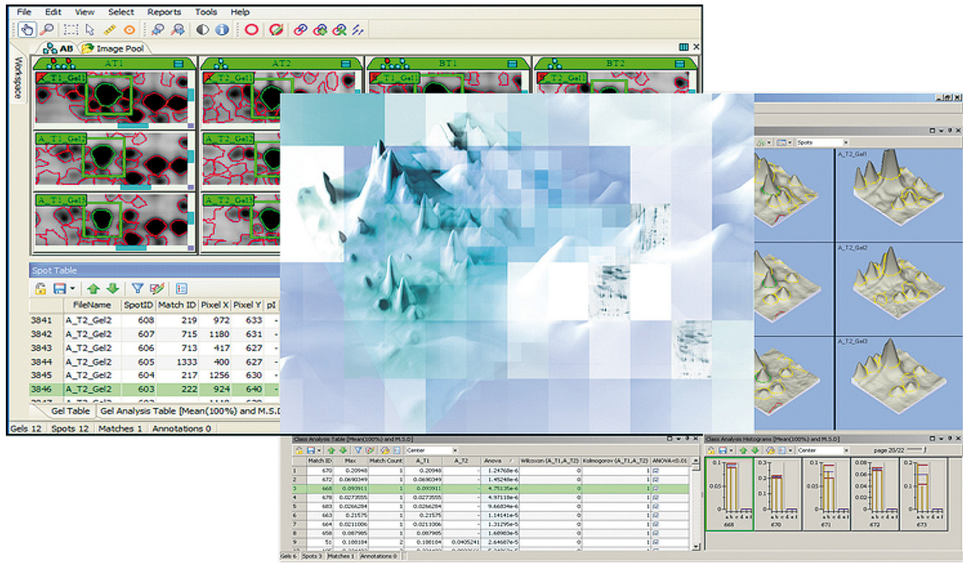


Fig 4.12. ImageMaster 2D Platinum Software.

Adding images:

- Select appropriate staining or labeling method. Pick gels within the DIGE experiment should be Cy-labelled

Gel quality evaluation:

- Use viewing tools like contrast, overview, profile, 3-D to assess image quality. Poor quality images can cause insufficient spot detection and/or matching

Matching:

- Place one or several landmarks to support automatic matching. Keep landmarks at a minimum. Place the landmark on well-defined smaller spots that are clearly identified on all gels in the dataset

Data Analysis:

- Option 1. Use the ratio between means and gaps to find the most interesting potential marker proteins
- Option 2. Use statistical analyses such as ANOVA, Wilcoxon, or Kolmogorov. They are automatically calculated within classes (experimental groups) and presented in the Class Analysis Table

Statistical tests:

- ANOVA and normal distribution
- Mann-Whitney (non-parametric). This test should usually be used for non-normal distributions; it tests the differences in means for non-normal distributions
- Kolmogorov-Smirnov (non-parametric). This test will test for differences in distribution along with differences in mean. This should be used in addition to Mann-Whitney, if there is a reasonable assumption that the distribution is different

There are different tables providing the result of the analysis. To find the appropriate information check the appropriate table that summarizes the data needed:

- Spot Table: gives summarized information about enabled spots
- Gel Analysis Table: Gel Analysis Table and Histograms provide valuable tools for checking spot detection, verifying matches, analyzing expression changes within a class
- Class Analysis Table: provides information about expression changes between classes (experimental groups)

4.11 References

1. *ImageQuant TL User Manual*, Cytiva, 29-0006-10 Edition AA (2011)
2. *DeCyder 2D Software*, Version 7.2 User Manual, Cytiva, 28-9414-47 Edition AB (2010).
3. *DeCyder Extended Data Analysis User Manual*, Cytiva 28-9414-44 Edition AA (2008)
4. *ImageMaster Platinum 2D User Manual*, Cytiva, 28-9381-02 Edition AA (2008)

05

Protein imaging applications

This chapter provides a summary of some of the most widely used imaging applications and provides the necessary knowledge for maximizing your imaging capabilities. The applications discussed in this chapter cover 1-D and 2-D gel electrophoresis of proteins and their associated transfer (Western blotting) and analysis. Phosphorimaging and naturally occurring fluorescent protein applications are also discussed. Typical protocols for each application area are included, together with tips and recommendations for successful implementation of detection and imaging. Available fluorescent stains, chemifluorescent substrates, and covalent fluorochrome labels are described with Cytiva's instrument compatibility for imaging setup and analysis.

5.1 Detection of proteins in gels

5.1.1 Introduction

Gel electrophoresis under denaturing conditions, can resolve complex protein mixtures into numerous bands on a gel. After staining, the position of a protein gives an approximation of its size and the band intensity is an estimate of the amount present in the sample. This dual ability is useful in various applications from: estimating purity, level of gene expression, and as a precursor to immunoblotting (see section 5.3, Western blotting), to preparation for protein sequencing or mass spectrometry (MS), and generating antibodies.

Highly sensitive detection methods have been developed to monitor and analyze electrophoretic separations. One-dimensional (1-D) SDS polyacrylamide gel electrophoresis (SDS-PAGE) is routinely used as a relatively rapid analytical tool. PAGE is based on the principle that proteins migrate through gel pores when placed in an electrical field. As proteins have different electrical charges that affect their mobility, the denaturing agent sodium dodecyl sulfate (SDS) is usually added to the samples and buffers. The SDS disrupts hydrogen bonds, blocks hydrophobic interactions, and unfolds protein molecules, which reduces differences in molecular form by eliminating the tertiary and secondary structures. SDS also breaks up aggregates and non-covalently bound multimers. For almost all proteins, SDS binds at a ratio of approximately 1.4 g SDS per gram of protein. SDS gives proteins a net negative charge that is proportional to their size and ensures that they will migrate toward a positively charged anode.

SDS-PAGE is run as the second gel in 2-D (two-dimensional) electrophoresis, see section 5.2 in Ettan™ DIGE and 2-D Electrophoresis Principals and Methods 80-6429-60 AD 06/2010 (1).

- By using appropriate gel systems, proteins can also be studied under non-denaturing or native conditions that preserve the higher order structure and even the biological function of some proteins
- Estimating the size or molecular weight of a protein is straightforward with SDS gels. The procedure uses a standard set of proteins with known molecular weights in parallel with the sample protein. The gel concentration should be chosen so that the positions of the molecular weight standards on the calibration curve are at approximately the molecular weight of your target proteins.

Polyacrylamide gel composition is indicated by two different percentages: the percentage of acrylamide (%T), which is usually between 5 to 20%, and the percentage of cross-linker used (%C), which is usually 2 to 3% for SDS-PAGE electrophoresis of proteins. The higher the %T, the smaller the pore sizes, and the more difficult it is for proteins to migrate through the gel. Table 5.1 shows the percentage of acrylamide required for the molecular weight of target proteins.

Table 5.1. Recommended acrylamide content in SDS-containing polyacrylamide gels for linear separation of target proteins within defined size ranges.

Target size range	%T in separation gel
36 to 205 kDa	5.0%
24 to 205 kDa	7.5%
14 to 205 kDa	10.0%
14 to 66 kDa	12.5%
14 to 45 kDa	15.0%

- Although gels are easy to store, it is more convenient to store a photograph, printout, or a digital image of a gel. Numerous methods exist for capturing images for subsequent analysis or storage.
- Image analysis software allows easy and rapid analysis of separated proteins, including automatic calculation of amount and molecular weight (see Chapter 4). For quantitative analysis, it is always advisable to have known standards as controls for staining efficiency and recovery yields.

Molecular weight markers are usually run in separate lanes on the gel together with the samples (Fig 5.1). The markers will help to determine the size of proteins in sample bands by eye or, more accurately with analytical software such as ImageQuant TL. In addition, with a prestained marker it is easy to monitor protein electrophoresis progression and transfer efficiency of proteins blotted to a membrane.

Fig 5.1. (A) Rainbow™ Molecular Weight Markers are available in three size ranges for use with SDS-PAGE. (B) Amersham ECL Plex Fluorescent Rainbow Markers imaged on a Typhoon scanner. From left to right: Full color Cy3 and Cy5, Cy3 channel, Cy5 channel and Visible spectrum.

5.1.2 Protein gel detection

After completing the electrophoresis, most proteins are not directly visible and must be stained to determine their location and amount. This is typically referred to as post-staining.

Colorimetric stains

Colorimetric stains, such as Coomassie Blue or silver staining, are widely used. Once the gel is stained, it can be imaged or dried on a transparent backing or filter paper as a record of the position and intensity of each band.

Fluorescent stains

Fluorescent protein gel staining using reagents such as Deep Purple Total Protein Stain, when combined with an appropriate imager, is a sensitive and quantitative approach for protein analysis that offer advantages such as ease of use, sample stability, and safety. These stains provide sensitivity approaching that of silver staining but with a much wider linear dynamic range. Most fluorescent stains are also compatible with mass spectroscopy and Edman sequencing.

Prelabeling of proteins

Prelabeling is the practice of labeling samples before electrophoresis. Previously this was performed with radioisotopes and detection by autoradiography or phosphorimaging (see section 5.6, Phosphorimaging). The development of sensitive optimized fluorescent stains and labels have replaced the use of radioisotopes in most applications. Direct protein labeling methods using fluorochromes such as Cy2, Cy3, and Cy5 are suitable for the detection of proteins in 1-D and 2-D gels (see section 5.2), and enable multiplexing when used in combination with an appropriate imager.

Table 5.2 lists performance characteristics of some of the more popular protein detection reagents in contemporary use.

Table 5.2. The sensitivity and dynamic range of some commonly used protein stains. Note that quantities in the table depend on the protein source and detection system used.

Staining	Detection limit	Dynamic range		Number of steps	Total time
		Orders of magnitude	Range		
Coomassie Blue	10 ng	1.5 to 2.0	8 to 1000 ng	3	3 h to over night
Silver	0.5 to 2 ng	0.6 to 0.9	1 to 8 ng	8 to 15	5 to 8 h
Modified* Silver (compatible with MS analysis)	10 ng	0.6 to 0.9	10 to 80 ng	8 to 15	5 to 8 h
Deep Purple Total Protein Stain	0.2 to 1 ng	3.0 to 3.3	0.25 to 500 ng	4	3 h to over night
CyDye (prelabeling)	0.02-0.2 ng**	3.6 to 4.2	0.02 to 250 ng	1	45 min

*By omitting glutardialdehyde from the sensitizer and formaldehyde from the silver solution, this method is compatible with MS analysis. This procedure, however, leads to a less sensitive stain.
** This detection limit is reached in a highly diluted sample prelabelled at a much higher concentration.

Instrument compatibility

Table 5.3 shows the recommended filter settings for imagers from Cytiva when used for the analysis of gels containing proteins detected with Coomassie Blue or silver.

Table 5.3. Recommended filter settings on imagers for the analysis of Coomassie Blue or silver-stained proteins in gels.

			Stain	
			Coomassie Blue	**Silver**
Densitometric flatbed scanner	ImageScanner III	Mode	Transparent	Transparent
		Filter	Blank, red or green	Blank, blue or green
Laser scanners	Typhoon FLA 9500 (Digitization mode with digitization plate)	Excitation (nm)	532	473
		Emission filter (nm)	LPG (575LP)	LPB (510LP)
	Typhoon FLA 7000 (Digitization mode with digitization plate)	Excitation (nm)	532	473
		Emission filter (nm)	O580	Y520
Charged-coupled device (CCD) systems	ImageQuant LAS 4010	Light source	White light table	White light table
		Emission filter (nm)	None (Through)	None (Through)
	ImageQuant LAS 4000*	Light source	NA	NA
		Emission filter (nm)	NA	NA
	ImageQuant LAS 4000 mini (with upgrade configuration)**	Light source	UV table + pink plate	UV table + pink plate
		Emission filter (nm)	None (Through)	None (Through)
	ImageQuant LAS 500	Colorimetric	Colorimetric	Colorimetric

* ImageQuant LAS 4000 can be upgraded with a white light table for gel documentation.
** Use UV transillumination in combination with the pink calibration plate.

Table 5.4 shows the recommended filter settings for imagers from Cytiva when used for the analysis of gels containing proteins labeled with fluorophores.

Table 5.4. Recommended filter settings on imagers for the analysis of fluorophore labeled proteins in gels.

			Fluorophore			
			Cy2	**Cy3**	**Cy5**	**Deep Purple**
Laser scanners	Typhoon FLA 9500	Excitation (nm)	473	532	635	532
		Emission filter	BPB1	BPG1	LPR (665)	LPG (575)
	Typhoon FLA 7000	Excitation (nm)	473	532	635	532
		Emission filter	Y520	O580	R670	O580
Charged-coupled device (CCD) systems	ImageQuant LAS 4010	Light source	Blue epi-illuminator (460)	Green epi-illuminator (520)	Red epi-illuminator (630)	Green epi-illuminator (520)
		Emission filter	Y515Di	575DF20	R670BP	605DF40
	ImageQuant LAS 4000*	Light source	NA	NA	NA	NA
		Emission filter	NA	NA	NA	NA
	ImageQuant LAS 4000 mini (with upgrade configuration)**	Light source	Blue epi-illuminator**	NA	NA	NA
		Emission filter	Y515Di	NA	NA	NA
	ImageQuant LAS 500	Mode	Fluorescence (Nonoptimal)	Fluorescence	NA	Fluorescence

* ImageQuant LAS 4000 can be upgraded with RGB/IR and UV epi-illumination to the same performance as ImageQuant LAS 4010.
** Upgrade.

Table 5.5 shows the gel dimensions suited to specific imagers from Cytiva.

Table 5.5. Maximal imaging area for different sized gels.

		Maximal imaging area
Densitometric flatbed scanner	ImageScanner III	31 × 43.7 cm
Laser scanners	Typhoon FLA 9500 (Digitization mode)	25 × 20 or 40 × 46 cm
	Typhoon FLA 7000 (Digitization mode)	20 × 40 cm
Charged-coupled device (CCD) systems	ImageQuant LAS 4010/4000	14 × 21 cm
	ImageQuant LAS 4000 mini (with upgrade configuration)	12 × 18 cm
	ImageQuant LAS 500	10.5 × 10.5 cm

5.1.3 Protocol for the analysis of proteins in a 1-D gel

Sample preparation

The procedure for sample preparation depends on the application and sample type. 1-D SDS PAGE is often used as a rapid analytical method for estimation of protein purity or level of expression in cell lysates. Usually, a small aliquot of the sample is mixed with sample loading buffer and analyzed directly. For dilute samples in high salt or other disturbing agents, a concentration or buffer exchange step might be needed. The amount of protein to load on the gel will depend on the stain used, the gel type, and the electrophoresis system.

☞ Rule of thumb: The total protein load for a complex sample, such as, cell lysates should be 50 to 100 ug of protein per lane for Coomassie Blue staining and 5 to 100 ng of protein per lane for silver or Deep Purple staining. The protein load for highly purified proteins should be 0.3 to 1 µg of protein per lane for Coomassie Blue staining and 2.5 to 5 ng of protein per lane for silver or Deep Purple staining.

☞ Load one or two lanes with a molecular weight standard (0.2 to 1 µg per lane if the gel is to be stained with Coomassie Blue and 10 to 50 ng per lane for silver or Deep Purple staining).

Typical sample loading buffers (SLB) for SDS PAGE

Non-reducing SDS-treatment:

2× SDS loading buffer (store aliquots at -20°C)
0.5 M Tris pH 6.8 to 8.0
10% SDS (w/v)
20% glycerol (v/v)
0.02% (w/v) Bromophenol Blue (BPB)

☞ Non-reducing SDS-treatment unfolds the protein but leaves inter-and intra- disulfide bridges intact.

Reducing SDS-treatment:

2× SDS reducing loading buffer (store aliquots at -20°C)
0.5 M Tris pH 6.8 to 8.0
10% SDS (w/v)
20% glycerol (v/v)
0.02% BPB (w/v)
1% DTT* (w/v) (or 5% β-mercaptoethanol (v/v))

* Add fresh

☞ Reducing agents such as DTT or β-mercaptoethanol reduce inter- and intra- disuphide bridges in proteins to disrupt their tertiary and quaternary structure.

Reducing SDS treatment followed by alkylation:

2× reducing loading buffer (store aliquots at -20°C)
400 mM Tris pH 8.0
2 to 4% SDS (w/v)
0.004% BPB (w/v)
0.3% DTT (w/v)

* Add fresh before use of SLB

Alkylation solution 15% (w/v) iodoacetamide (IAA), freshly prepared

☞ If there are concerns about re-oxidation of the free sulfhydryl groups then alkylation with IAA prevents this problem and results in very sharp bands after electrophoresis. Proteins with high amounts of cysteine may show a minor increase in molecular weight.

Gel electrophoresis and gel staining

1. Add sample and appropriate loading buffer to a tube so that loading buffer reaches a working concentration.
2. Vortex briefly and heat for 2 to 5 min at 90 to 100°C.
3. Place samples on ice until ready for loading.
4. Centrifuge briefly and load the sample onto an SDS-polyacrylamide gel.

☞ For alkylation treatment, add 1:10 sample volume of IAA solution and load the sample onto an SDS-polyacrylamide gel.

5. Run the gel.
6. Stain the gel with Coomassie Blue, silver or Deep Purple Total Protein Stain according to the instructions.

Image acquisition

Select an imaging system that matches the staining technique and the gel size. Use the recommended settings and, if applicable, adjust for band intensities to avoid saturation. For more information on performance, see Application note, 28-9794-23 *A comparative evaluation of quantitative imaging of DNA and protein using stains and labels*. Resolution and file format are important properties of images that can affect analysis in software and the suitability of images for publication.

Image analysis

Crude analysis of 1-D gels can be made by eye. An advanced software package is needed for accurate analysis of the quantity and molecular weight of the samples. ImageQuant TL 1-D Analysis software offers a high level of automation for the analysis of 1-D gels (see section 4.2.1 ImageQuant TL) to ensure reproducible evaluations.

5.1.4 Typical results

1-D gels are routinely used as a relatively rapid and sensitive analytical tool. Figure 5.2 a two-fold dilution series of HeLa cell lysate, post stained with Deep Purple Total Protein Stain and Coomassie Blue.

Sample: Two-fold dilution series of HeLa cell lysate from 10 µg to 625 ng
Detection: Coomassie Blue (A), Deep Purple (B)
Imaging: ImageQuant LAS 500
Imaging method: Colorimetric (A), Fluorescence (B)
Analysis: ImageQuant TL v8.1

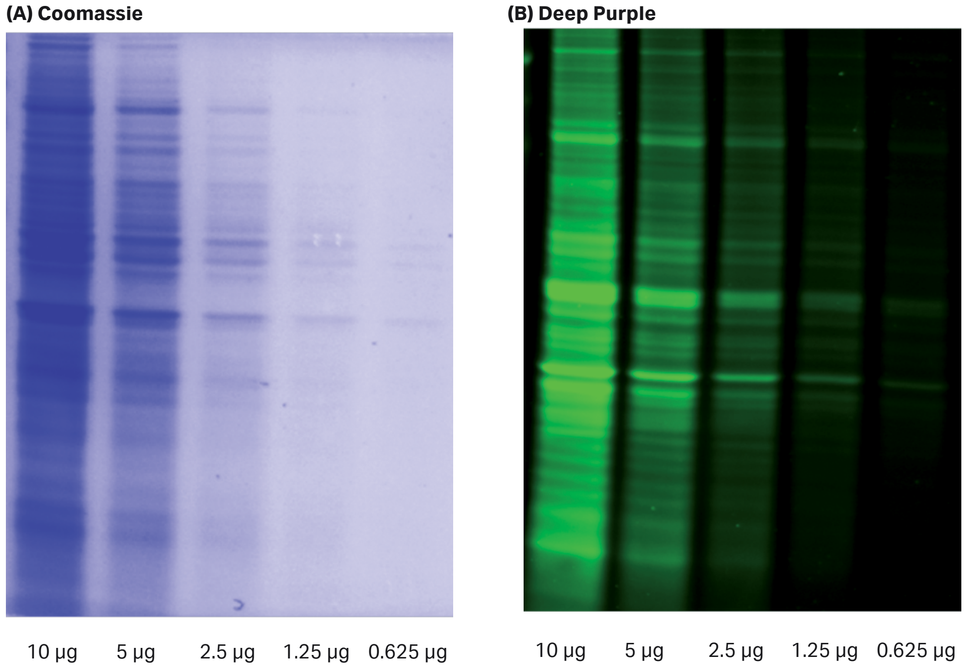


Fig 5.2. Protein stains after SDS-PAGE. (A) colorimetric method with Coomassie Blue. (B) fluorescent method with Deep Purple Total Protein Stain.

5.2 2-D Difference Gel Electrophoresis (2-D DIGE)

5.2.1 Introduction

2-D DIGE is extensively used for the characterization of complex protein mixtures in proteomics applications. It is a powerful tool for separating proteins by charge and size and for analyzing protein differences in the second dimension SDS PAGE gel images (2-D map). By combining the technologies of fluorescence, sample multiplexing and image analysis, the DIGE technology offers significant benefits over traditional 2-D electrophoresis.

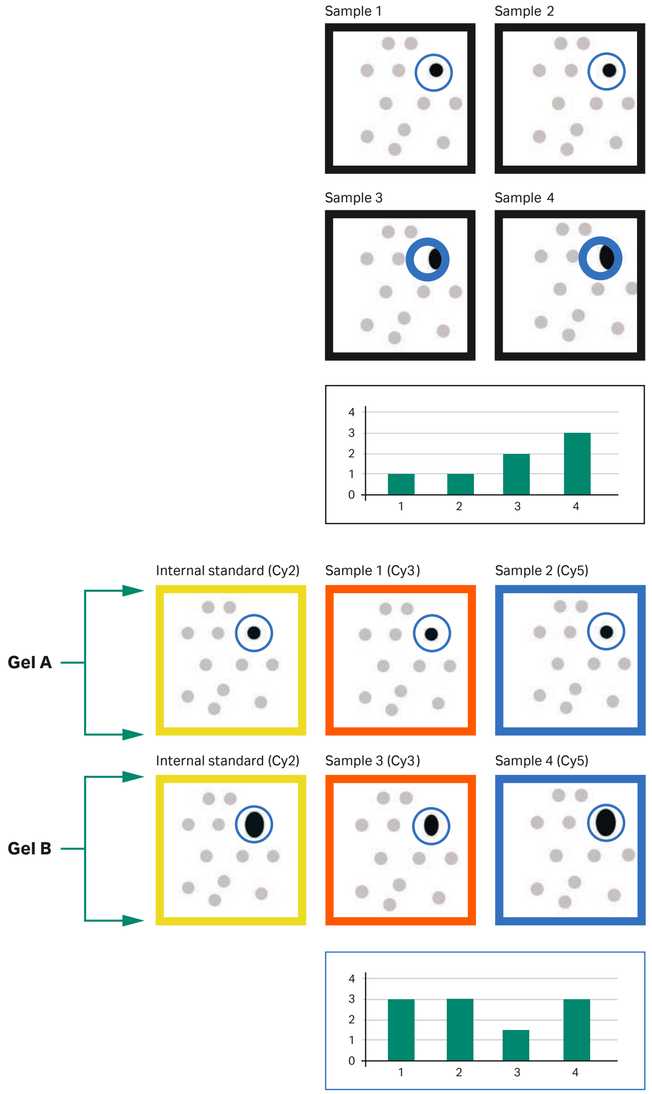
The fundamental difference between 2-D DIGE and traditional 2-D electrophoresis is the use of one internal standard for all samples in an experiment. This eliminates virtually all gel-to-gel variation and allows the multiplexing capability of 2-D DIGE to be used for all samples including those run on different gels (Fig 5.3). Moreover, as each protein spot has its own internal standard the accuracy and reproducibility of data is significantly increased. This means that the DIGE System is capable of detecting and measuring differences as small as 10% between samples (above system variation) with greater than 95% statistical confidence.

2-D DIGE works with protein samples that are labeled with CyDye DIGE Fluor dyes and co-separated on the same 2-D electrophoresis gel. CyDye DIGE fluors are available as minimal and saturation labeling dyes. Minimal dyes are intended for applications where sufficient amounts of sample are available, whereas saturation dyes are for use in applications where only small amtounts of sample are available.

CyDye DIGE Fluors are:

- Size and charge-matched; similarly labeled proteins from different samples will co-migrate to the same position, regardless of the dye used
- Spectrally resolvable; the distinct signal from each fluor contributes to accuracy. They are highly sensitive, bright, and photostable, with a minimal loss of signal during labeling, separation, and scanning
- pH and pI insensitive; pI of proteins are not changed after labeling and signals do not change over the wide pH range used during first dimension IEF or during migration in SDS PAGE gels

Quantitation of results using DeCyder 2D Differential Analysis Software can be performed on studies ranging from a simple control versus treated experiment to multivariable experiments (e.g., dose and time) in a single analysis.



Traditional 2-D electrophoresis

Four different samples run on four different gels

The abundance of this particular protein spot appears to be increasing in samples 3 and 4. Is this increase due to system variation or induced biological change?

Experimental conclusion

Without running a significant number of replicates to average the results, the conclusion would be an increase in abundance in samples 3 and 4.

2-D DIGE using a pooled internal standard

Four different samples, plus one internal standard, on two different gels.

Experimental conclusion

The same internal standard is run on both gels. The increase in abundance of protein in gel B, as shown by the increase in the internal standard, is due to gel-to-gel variation. When the internal standard is normalized between gels A and B, the conclusion is that the abundance of protein in sample 3 has actually decreased.

Fig 5.3. A comparison of traditional 2-D electrophoresis and 2-D DIGE.

5.2.2 Instrument compatibility

Table 5.6. Instrument settings for 2-D DIGE imaging with Typhoon.

	Typhoon FLA 9500	
Fluorochrome	**Excitation (nm)**	**Emission filter**
Cy2	473	BPB1
Cy3	532	BPG1
Cy5	635	LPR
Deep Purple	532	LPG

5.2.3 Typical 2-D DIGE workflow

5.2.3.1 Sample preparation

Proteins are extracted from cells or tissues of interest

5.2.3.2 Sample labeling with CyDye DIGE fluors

CyDyes are size and charge-matched, spectrally resolvable dyes that enable simultaneous separation and analysis of samples on a single multiplexed gel.

5.2.3.3 Addition of DTT and ampholytes

Following sample labeling, DTT and ampholytes are added to each sample (Fig 5.4).

5.2.3.4 2-D electrophoresis

Up to three samples (one of which is the internal pooled standard) can be simultaneously separated on a single 2-D gel.

5.2.3.5 Image acquisition

The gel is scanned using the highly sensitive Typhoon FLA 9500, optimized for 2-D DIGE. This ensures quantitation of protein expression levels and identification of spots with high precision.

5.2.3.6 Image analysis

DeCyder 2-D Differential Analysis Software locates spots and analyzes multiplexed samples. The software allows complex analysis of multiple gels to provide comparative and accurate measurement of differential protein expression.

5.2.3.7 Spot Processing for MS analysis

When protein spots of interest have been identified they are matched to a preparative picking gel and automatically excised by a picking robot. The sample plugs are then transferred to a digestion station for digestion with trypsin. The resulting peptide fragments are analyzed by mass spectrometry before searching databases to identify the proteins.

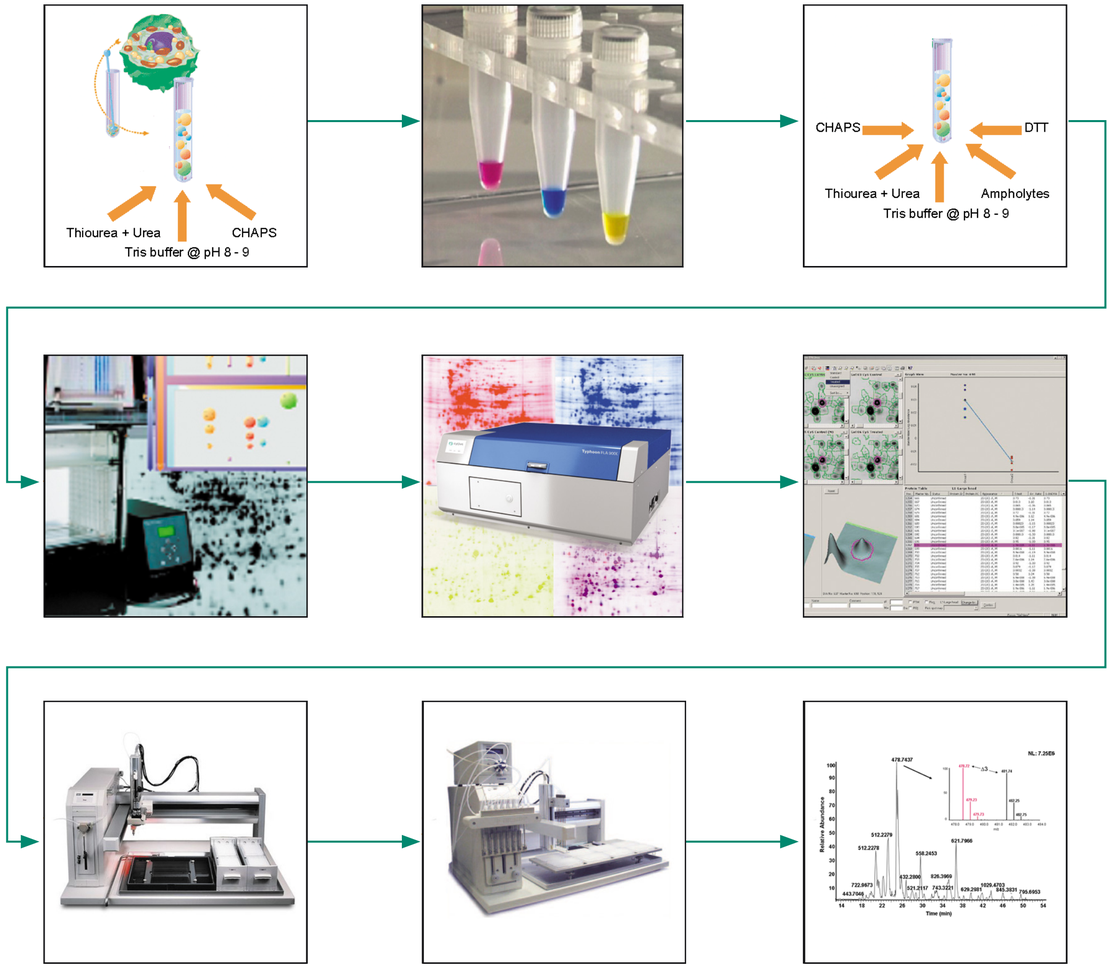


Fig 5.4. A typical 2-D DIGE workflow.

5.2.4 Experimental design

Multiplexing with the 2-D DIGE System virtually eliminates gel-to-gel variation by using an internal standard. The system exhibits very low experimental variation when compared to conventional 2-D electrophoresis and the use of biological replicates rather than technical replicates are recommended when designing an experiment.

The following example illustrates an experimental design of four experimental groups with four biological replicates in each group. A similar experiment using conventional 2-D electrophoresis with one sample per gel would require at least 16 gels. To gain any meaningful statistical data using this approach would require a minimum of three technical replicates of each sample, making 48 gels in total.

For further information relating to experimental design and statistical tools in the analysis software, refer to the Ettan DIGE user manual (2) and DeCyder 2D Software, Version 7.2 User Manual (3).

5.2.4.1 Experimental design using CyDye DIGE Fluor minimal dyes

With CyDye DIGE Fluor minimal dye, three protein samples labeled with different CyDye DIGE Fluors can be run on the same 2-D gel simultaneously. The pooled internal standard is preferably labeled with the Cy2 DIGE Fluor minimal dye and is run together with individual samples labeled with Cy3 and Cy5 DIGE Fluor minimal dyes (Fig 5.5).

For co-separation on a 24 cm IPG strip and precast DIGE Gel it is recommended that 50 µg of sample is labeled with each CyDye DIGE Fluor minimal dye.

An example of an experimental design to enable derivation of statistical data on differences between a control and three treatment regimens A, B and C labeled with CyDye DIGE Fluor minimal dyes Cy2, Cy3, and Cy5 is in Table 5.6. Four biological replicates are included in each experimental group, with every sample having a common internal standard. An internal (pooled) standard is prepared by mixing equal amounts of each sample. The samples have been arranged between gels to ensure an even distribution of biological replicates labeled with CyDye DIGE Fluor Cy3 minimal dye and those labeled with CyDye DIGE Fluor Cy5 minimal dye. This setup avoids repeatedly linking the same two treatment types (experimental group) on multiple gels.

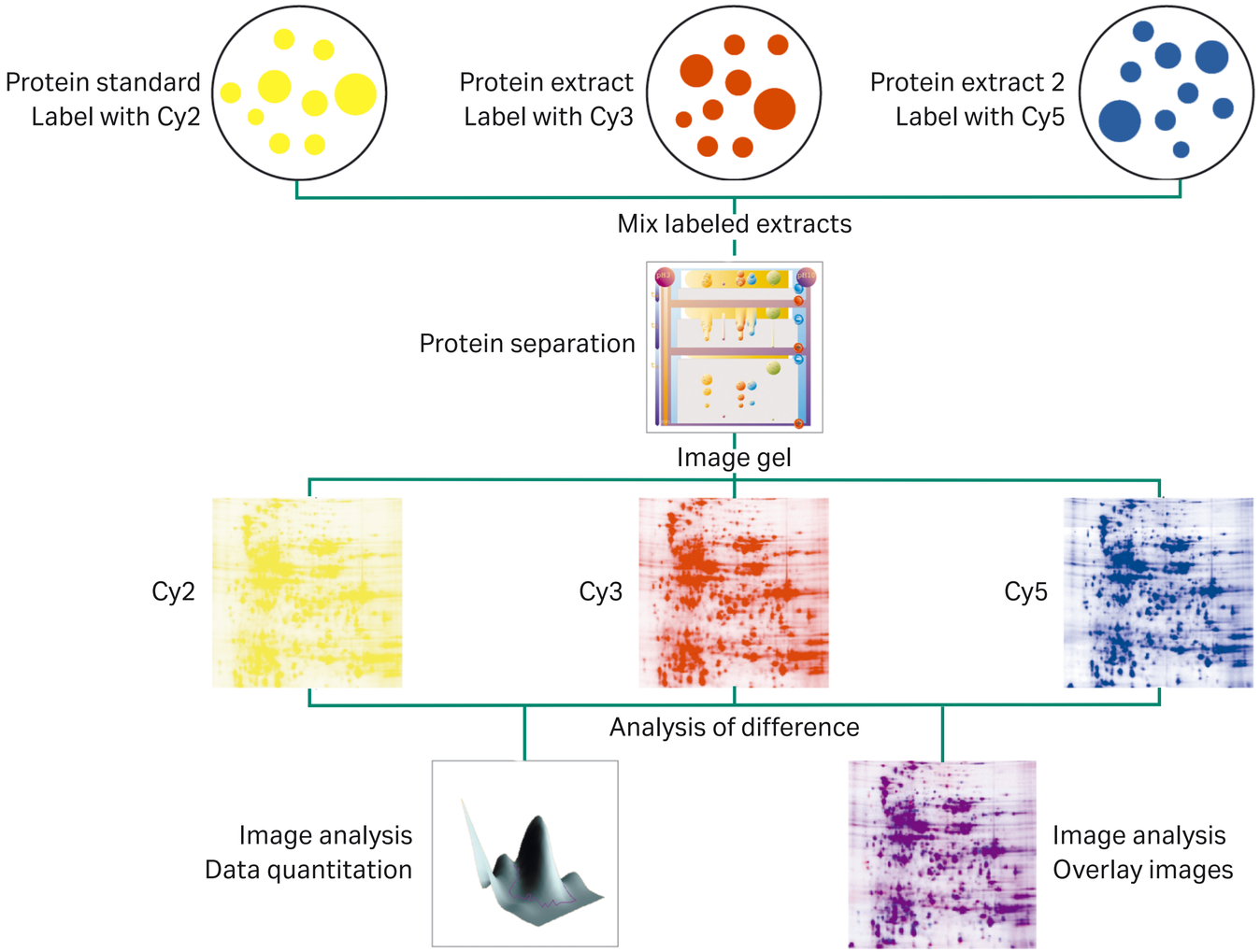


Fig 5.5. Multiplexing using the CyDye DIGE Fluor minimal dye option with the 2-D DIGE system.

Table 5.6. Recommended experimental design for the comparative analysis of three treatment regimens using CyDye DIGE Fluor minimal dyes; Cy2, Cy3 and Cy5.

Gel number	Cy2	Cy3	Cy5
1	Pooled standard	Control 1	Sample C3
2	Pooled standard	Sample A1	Control 3
3	Pooled standard	Sample B1	Sample A3
4	Pooled standard	Sample C1	Sample B3
5	Pooled standard	Control 2	Sample B4
6	Pooled standard	Sample A2	Sample C4
7	Pooled standard	Sample B2	Control 4
8	Pooled standard	Sample C2	Sample A4

5.2.4.2 Experimental design using CyDye DIGE Fluor saturation dyes from the Scarce Sample Labeling Kit

With the saturation dyes from the CyDye DIGE Fluor Labeling Kit for Scarce Samples, two protein samples labeled with different CyDye DIGE fluors can be run on the same 2-D gel simultaneously. The pooled standard is preferably labeled with the Cy3 CyDye DIGE Fluor saturation dye and is run together with an individual sample labeled with the Cy5 CyDye DIGE Fluor saturation dye. The saturation dyes have been developed specifically for use with protein samples, which under normal circumstances may be more challenging due to limited sample quantities (Fig 5.6).

For co-separation on a 24 cm IPG strip and precast DIGE Gel, it is recommended that 5 µg of sample is labeled with each CyDye DIGE Fluor saturation dye.

An example is outlined here of an experimental design to enable derivation of statistical data on any differences between control and three treatment regimens A, B and C labeled with CyDye DIGE Fluor saturation dyes Cy3 or Cy5 (Table 5.7). Four biological replicates are included in each experimental group, each sample having its own internal standard. Adding equal amounts of each sample together creates a pooled standard.

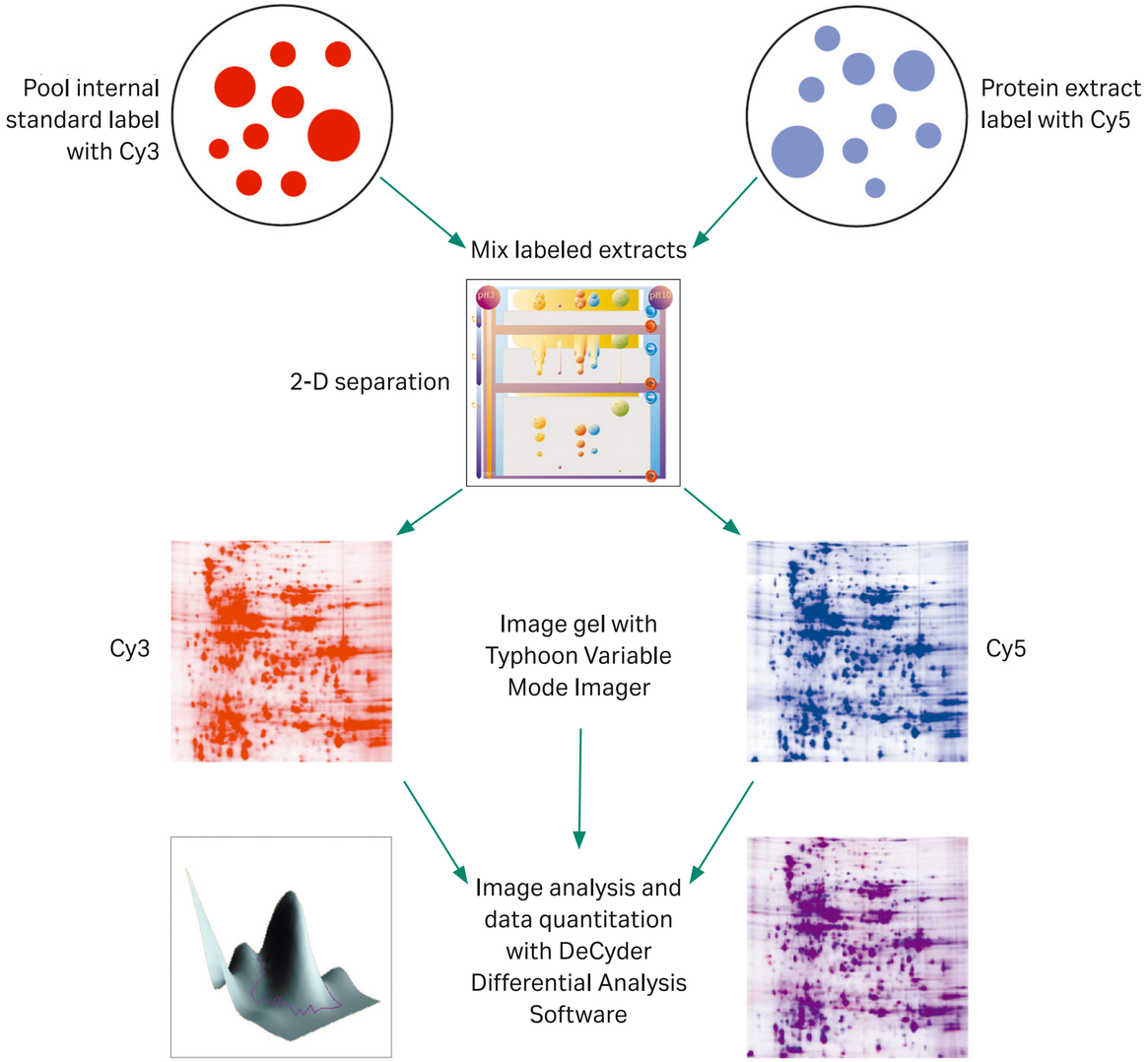


Fig 5.6. Outline of the 2-D DIGE system workflow for saturation labeling.

Table 5.7. Recommended experimental design for the comparative analysis of three treatment regimens using CyDye DIGE Fluor saturation dyes. Cy2, Cy3 and Cy5.

Gel number	Cy3	Cy5
1	Pooled standard	Control 1
2	Pooled standard	Sample A1
3	Pooled standard	Sample B1
4	Pooled standard	Sample C1
5	Pooled standard	Control 2
6	Pooled standard	Sample A2
7	Pooled standard	Sample B2
8	Pooled standard	Sample C2
9	Pooled standard	Control 3
10	Pooled standard	Sample A3
11	Pooled standard	Sample B3
12	Pooled standard	Sample C3
13	Pooled standard	Control 4
14	Pooled standard	Sample A4
15	Pooled standard	Sample B4
16	Pooled standard	Sample C4

5.2.4.3 Protein sample preparation

2-D electrophoresis is typically used to study a proteome from a cell culture or tissue. In whole cell lysates, proteins are present over a wide range of concentrations and may mask less abundant proteins of interest. Effective proteome analysis requires removal of abundant proteins and enrichment of low-abundance proteins to detectable levels. This allows improved resolution of individual fractions; less crowded 2-D maps, simpler analysis and interpretation, and a better opportunity to discover proteins of interest. If only a subset of the proteins in a tissue or cell type is of interest, different strategies of fractionation can be employed during sample preparation. The decision to employ these steps depends on the nature of the sample and the experimental goal. For more information refer to *Protein Sample Preparation Handbook* from Cytiva (4).

- Cultured cells or tissue samples are typically used in 2-D DIGE experiments. To prevent proteolysis and other types of protein degradation it is important to quickly disrupt the cells and solubilize as many proteins as possible. For the best results with the least complications when working with native proteins we strongly recommend using denaturing conditions.
- Isoelectric focusing (IEF) as the first dimension separation in 2-D electrophoresis, gives the highest resolution and the cleanest results when performed under denaturing conditions. Solubilization treatments before IEF must not affect protein pI, nor should samples be left in a highly conductive solutions such as those containing salts.
- Cell disruption should ideally be carried out directly in a strongly denaturing solution compatible with the CyDye labeling step. Solid particles and lipids must be removed by centrifugation to prevent blockage of pores in the electrophoresis gel.

Combinations of urea, thiourea and detergent are in general used for sample preparation for 2-D electrophoresis. Urea solubilizes and unfolds most proteins, exposing all ionizable groups to solution. Thiourea with urea further improves solubility, particularly of membrane proteins. The 2-D Protein Extraction Buffers I to VI available from Cytiva (Table 5.8.) offer combinations of denaturing reagents and detergent suitable for a diverse range of proteins. 2-D Protein Extraction Buffers are modifications of well-studied protein solubilization and IPG-strip rehydration buffers designed to produce better spot resolution for 2-D gel analysis.

Table 5.8. Composition of 2-D Protein Extraction Buffers available from Cytiva.

Buffer	Composition
2-D Protein Extraction Buffer-I[1]	Urea (< 10 M) and NP-40 (< 10%)
2-D Protein Extraction Buffer-II	Urea (< 10 M) and CHAPS (< 10%)
2-D Protein Extraction Buffer-III[2]	Urea (< 8 M), thiourea (< 5 M), CHAPS (< 5%), and ASB-16 (< 5%)
2-D Protein Extraction Buffer-IV[2]	Urea (< 8 M), thiourea (< 5 M), CHAPS (< 5%), and SB 3-10 (< 5%)
2-D Protein Extraction Buffer-V	Urea (< 8 M), thiourea (< 5 M), and CHAPS (< 10%)
2-D Protein Extraction Buffer-VI	Urea (< 8 M), thiourea (< 5 M), CHAPS (< 5%), and NDSB 201 (< 4%)
2-D Protein Extraction Buffer Trial Kit	2-D Extraction Buffer- I, -II, -III, -IV, -V and VI

[1] 2-D Protein Extraction Buffer-I can slightly reduce the labeling efficiency when CyDye DIGE Fluor minimal dye is used.
[2] 2-D Protein Extraction Buffer-III and -IV are not suitable when CyDye DIGE Fluor Labeling Kit for Scarce Samples is used. Labeling efficiency is significantly reduced.

5.2.4.4 Cell lysis

It is important to wash the cells or tissue before cell disruption to remove culture medium and cells, such as blood cells. The wash buffer and procedure will depend on the sample type.

1. Pellet the washed cells in a microcentrifuge at 12 000 × g for 4 min at 4°C.
2. Remove and discard the supernatant and ensure the wash buffer has been completely removed.
3. Suspend the cells in 2-D Extraction Buffer of choice complemented with 30 mM Tris (pH 8. 5 for CyDye minimal labeling and pH 8.0 for saturation labeling) and leave on ice for 10 min.

Additional cell disruption may be used depending on sample properties. Sonication with a micro-tip, or homogenization with Sample Grinding kit or freeze/thaw treatments are commonly used methods.

Sonication generates heat and therefore must be performed on wet ice to control the temperature.

To avoid modification of proteins, never heat a sample after adding urea. Temperatures must not exceed 37°C, as urea will hydrolyze to isocyanate, which modifies proteins by carbamylation, causing “charge train” artifacts in the 2-D map.

4. Centrifuge the cell lysate for 10 min at 12 000 × g in a microcentrifuge at 4°C.
5. Transfer supernatant to a marked tube for CyDye labeling. Discard the pellet.
6. Cell lysates can be stored in aliquots at -70°C before labeling and analysis.

Additional sample preparation steps may improve the quality of the results, but at the possible expense of selective protein loss. Repeated freezing and thawing of samples may also cause protein losses.

5.2.4.5 Determination of protein concentration

The concentration of protein in all lysates should be determined using a suitable protein assay. The assay must be compatible with detergents and thiourea if these are present in the cell lysate. 2-D Quant Kit is suitable.

If the total protein concentration is less than 1 mg/mL after protein quantitation for CyDye minimal labeling, suspend cells in a smaller volume of lysis buffer to bring the concentration to at least this level in subsequent experiments.

If the protein concentration is less than 0.55 mg/mL in analytical samples or 1.2 mg/mL in preparative samples after quantitation for CyDye saturation labeling, then suspend cells in a correspondingly smaller volume of cell lysis buffer to bring the concentration to at least these levels in subsequent experiments.

When the protein concentration of the lysate is low. Use 2-D Clean-Up Kit or Vivaspin™ Concentrators to concentrate the sample. This procedure removes contaminating substances and small endogenous molecules, improves labeling efficiency, enhances spot resolution and increases the number of detectable spots.

The 2-D Clean-Up Kit lowers pH significantly and it may be necessary to adjust the pH.

5.2.4.6 Protein CyDye labeling

CyDye DIGE Fluors are available as minimal and saturation dyes, and their use depends on the type of sample to be labeled. Minimal dyes are often the first choice since three samples can be multiplexed on one gel. Saturation (or scarce sample) dyes enable a full 2-D analysis of samples that are normally difficult to analyze due to limited sample quantities of sample.

Minimal dyes

CyDye DIGE Fluors Cy2, Cy3 and Cy5 minimal dyes have an NHS ester reactive group, that covalently bind to lysine residues of proteins by an amide linkage (Fig 5.7). Since the quantity of dye added to the sample is limited, this method is referred to as 'minimal' labeling. Minimal dyes label approximately 2% to 5% of the total available lysine. Lysine in proteins carries a single positive charge at neutral or acidic pH. CyDye DIGE Fluors also carry an intrinsic positive charge which, when coupled to lysine, substitutes the charge on lysine to ensure that the pI of the protein is not altered. CyDye DIGE Fluors add approximately 500 Da to the protein's mass in a uniform manner, and give images that are comparable to silver stained gels but with many added benefits.

Saturation dyes

CyDye DIGE Fluor Cy3 and Cy5 saturation dyes have a maleimide reactive group that forms a covalent bond with the thiol group of cysteine residues on proteins by a thioether linkage (Fig 5.8). The purpose of "saturation" labeling is to increase the limit of detection by marking all available cysteines on each protein with a high dye-to-protein labeling ratio.

- It is very important when handling cell lysates to pay attention to maintaining the optimal pH. A pH below the optimal level makes labeling ineffective and a higher than optimal pH leads to lack of specificity.
- Check that the pH of the cell lysate is still optimal just before preparing for the labeling reaction. Add a drop of the cell lysate to a narrow range pH test strips pH 7.5-9.5. If pH has fallen then adjust the pH of the lysate.
- The optimal pH for minimal labeling is 8.5. For saturation labeling, the optimal pH is 8.0.

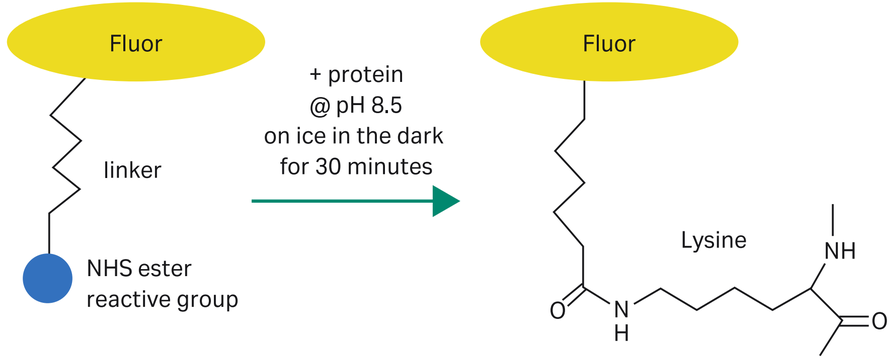


Fig 5.7. A schematic diagram of the labeling reaction. CyDye DIGE Fluor containing NHS-ester active group covalently binds to lysine residue of protein by an amide linkage.

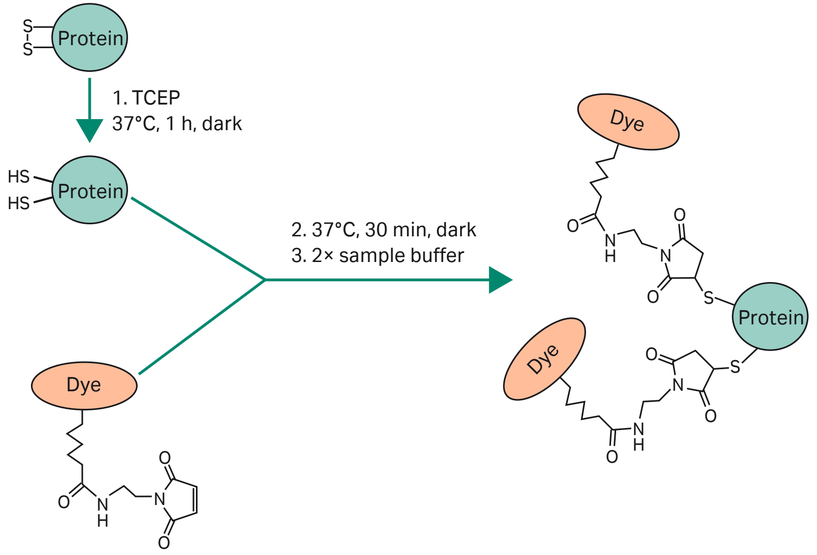


Fig 5.8. Schematic of a labeling reaction between CyDye DIGE Fluor saturation dye and the cysteine residues of a protein.

Minimal labeling

The labeling protocol for minimal dyes is outlined in Figure 5.9.

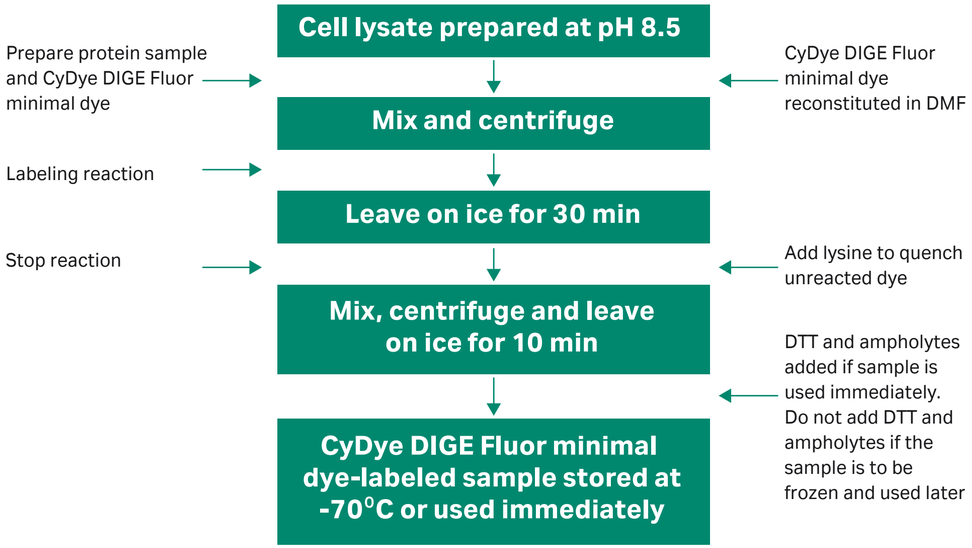


Fig 5.9. CyDye DIGE Fluor minimal dye-labeled protein sample is prepared in 40 min.

Preparing the labeling solution

1. Take a small volume of dimethylformamide (DMF) from its original container and dispense into a fresh microfuge tube.
2. Remove the CyDye from the freezer and leave to warm for 5 min at room temperature.
3. After 5 min, add the specified volume of DMF to each new vial of CyDye (see specification sheet supplied with the dye). This gives a CyDye stock solution of 1 mM. For the 2 nmol pack size, this gives a CyDye working solution of 0.4 mM and should be used immediately.
4. Close cap and vortex vigorously for 30 s.
5. Centrifuge the microfuge tube for 30 seconds at 12 000 × g in a microcentrifuge.
6. Prepare enough 0.4 mM working solution for the samples to be labeled.
7. The dye can now be used.

- Concentrated stocks (1 mM) of CyDye DIGE Fluor dyes (minimal or saturated) are stable for 2 months at -15°C to -30°C or until the expiry date on the tube, whichever is sooner.
- The working concentration of the dyes is usually 0.4 mM and they are stable for 1 week at -15°C to -30°C when stored in the dark.
- The amount of CyDye used in the labeling reaction must be determined empirically for different types of protein. Between 100 and 1000 pmol per 50 µg of protein can be used. Typically, 50 µg of protein is labeled with 400 pmol of CyDye.
- When labeling more than 50 µg of protein, use the same fluor: protein ratio for all samples in the same experiment. For example, the internal standard pool can be labeled in bulk.

Labeling the samples

The recommended concentration of the protein lysate is between 5 and 10 mg/mL total protein. Samples containing from 1 to 20 mg/mL have been successfully labeled using the protocol below.

Prepare a pooled internal standard by mixing equal amounts of each experimental protein sample. Ensure that there is enough pooled standard sample for each gel in the experiment.

1. Add a volume of protein lysate, pH 8.5, equivalent to 50 µg total protein to a microfuge tube. Bulk labeling reactions of the internal standard pool can also be performed using more protein and dye.
2. Add 1 µL of diluted CyDye working solution to the microfuge tube containing the protein sample i.e., 400 pmol of dye for the labeling reaction.
3. Mix and centrifuge briefly in a microcentrifuge. Leave on ice for 30 min in the dark.
4. Add 1 µL of 10 mM lysine to stop the reaction. Mix by pipetting and spin briefly in a microcentrifuge.
5. Leave for 15 min on ice in the dark.
6. Samples can now be prepared for IEF or stored at -70°C in the dark for at least three months before analysis.

Mixing the samples and preparing for IEF

2× sample buffer: Take 2-D Extraction Buffer V (or 7 M urea, 2 M thiourea, 4% CHAPS) add IPG buffer (the final concentration will depend on the choice of method for IEF) and DTT (final concentration 130 mM). For more information, see the *2-D Electrophoresis, Principles and Methods Handbook* from Cytiva (1).

1. After the protein samples have been labeled with CyDye, add an equal volume of 2× sample buffer and leave on ice for 10 min.
2. Pool the protein samples that shall be separated on the same first and second dimension gel (i.e. internal standard pool and two experimental samples according to the experimental design).

Saturation labeling

The labeling protocol for saturation dyes is outlined in Figure 5.10.

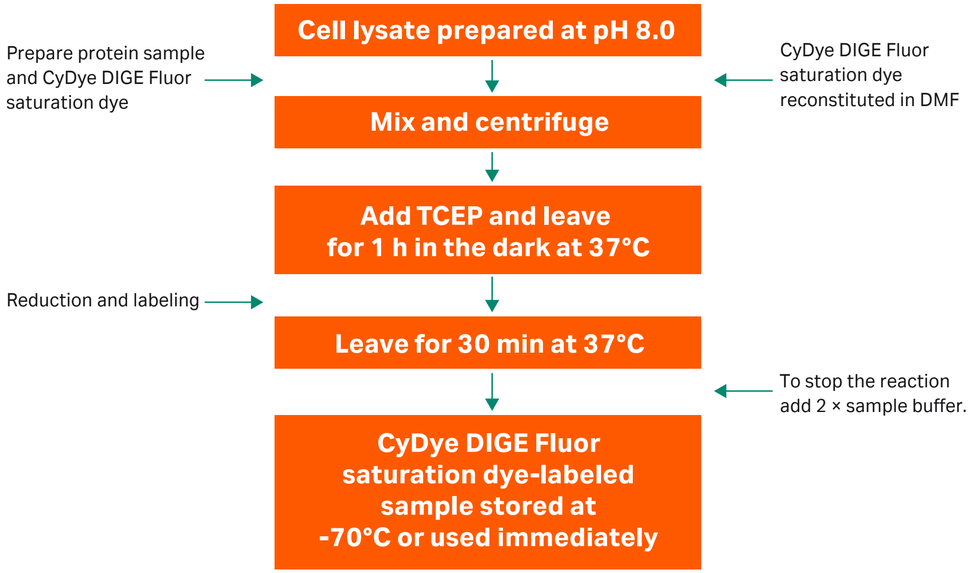


Fig 5.10. CyDye DIGE Fluor Labeling Kit for Scarce Samples labels proteins in 90 min.

Saturation dyes

- The molar ratio of TCEP (Tris (2-carboxyethyl)phosphine)) to dye should always be kept at 1: 2 to ensure efficient labeling.
- Samples with high cysteine content will require more TCEP to reduce the disulphide bonds and more dye to label the thiol groups. Typically, 5 µg of protein lysate requires 2 nmol TCEP and 4 nmol of dye for the labeling reaction (assuming an average cysteine content of 2%).
- The amount of TCEP and CyDye DIGE Fluor saturation dye used in the labeling reaction needs to be determined empirically for each protein sample type or when a nonstandard cell lysis buffer is used.
- Prepare a pooled internal standard by mixing equal amounts of each experimental protein sample together, ensuring that there is enough of the resulting pooled standard sample to include on each gel in the experiment.

1. Add a volume of protein lysate pH 8.0 equivalent to 5 µg total protein to a microfuge tube.
2. Make up the volume to 9 µL with cell lysis buffer, pH 8.0.
3. Prepare 2 mM TCEP solution by dissolving 2.8 mg TCEP in 5 mL water. TCEP solution is unstable and should be used immediately. Discard any unused material.
4. Add the required volume of 2 mM TCEP appropriate for 5 µg protein, for example 1 µL TCEP (or as determined in a labeling optimization experiment).
5. Mix vigorously by pipetting.
6. Spin down the sample in a microcentrifuge and incubate at 37°C for 1 h in the dark.

Preparing the labeling solution

- Once reconstituted at 2 mM (analytical gels) or 20 mM (preparative gels), CyDye DIGE Fluor saturation dyes are stable for up to 2 months at -15°C to -30°C or until the expiry date on the tube, whichever is sooner. Once reconstituted, dyes do not need to be diluted further.
- If labeling more than 5 µg of protein, the same fluor: protein ratio must be used for all samples on the same gel. The internal standard pool, for example, can be labeled in bulk

1. Take a small volume of DMF from its original container and dispense into a fresh microfuge tube.
2. Remove the CyDye DIGE Fluor saturation dye from the -15°C to -30°C freezer and leave unopened for 5 min, to warm to room temperature.
3. Once at ambient temperature, add the required volume of DMF to each new vial of CyDye DIGE Fluor saturation dye. For 5 µg analytical labeling reactions, reconstitute 100 nmol dye in 50 µL DMF to give a 2 mM working dye solution. For preparative labeling reactions, reconstitute 400 nmol dye in 20 µL DMF to give a 20 mM working dye solution.
4. Close the cap on the dye microfuge tube and vortex vigorously for 30 s.
5. Centrifuge for 30 s at 12 000 × g in a microcentrifuge.
6. The dye can now be used.

Labeling the samples

2 × sample buffer: To 2-D Extraction Buffer V (or 7 M urea, 2 M thiourea, 4% CHAPS) add the appropriate IPG buffer (the final concentration and pH range will depend on the choice of method for IEF) and DTT (final concentration 130 mM). For more information refer to *2-D Electrophoresis, Principles and Methods* from Cytiva (1).

1. Add the required volume of suspended 2 mM CyDye DIGE Fluor saturation dye appropriate for 5 µg protein, for example 2 µL dye (4 nmol) or as determined in a labeling optimization experiment.
2. Mix vigorously by pipetting.
3. Spin down the sample in a microcentrifuge and incubate at 37°C for 30 min, in the dark.
4. To stop the reaction, calculate the total volume of the labeling reaction and add an equal volume of fresh 2 × sample buffer.
5. Mix vigorously by pipetting.
6. Spin down the sample in a microcentrifuge.
7. Samples are ready for IEF and can be stored on ice or frozen for up to one month, at -70°C, in the dark.

Mixing the samples and preparing for IEF

1. After the protein samples have been labeled with CyDye, combine the protein samples for separation on the same first and second dimension gel i.e., the internal standard pool and one experimental sample according to the experimental design.

5.2.4.7 Protein separation

First dimension isoelectric focusing

The isoelectric point (pI) is the specific pH at which the net charge of a protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. IEF is an electrophoretic method used to separate proteins according to pI. The IPGphor™ 3 Isoelectric Focusing System and Multiphor™ II Electrophoresis System are both suitable for 2-D DIGE system applications. Detailed instructions for use of the systems are given in *2-D Electrophoresis Principles and Methods* (1).

IPG strips contain a preformed pH gradient immobilized in precast polyacrylamide gels on a plastic backing. A comprehensive range of overlapping IPG strips covering narrow, medium, and wide pH ranges are available in various strip lengths, and the strips are individually packaged and identified with a bar code and a unique numerical code for convenient sample tracking and handling.

IPG Buffers are mixtures of carrier ampholytes in a buffer solution specifically formulated for use with Immobiline™ DryStrip gels. The buffers enhance protein solubility, and improve separations without disturbing IEF or affecting the shape of the pH gradient.

DeStreak Reagent prevents unspecific oxidation by transforming thiol groups into stable disulfides. The preparation of IPG strips with DeStreak Reagent yields 2-D maps with reduced horizontal streaking between spots in the basic range above pH 7. It will also simplify the spot pattern as it reduces the number of spots caused by oxidation of proteins.

2-D DIGE analysis of protein samples is particularly sensitive to contamination. Use clean equipment when handling IPG strips and the samples.

As a first screening experiment using 2-D DIGE, a broad pH interval on an IPG strip (for example 3-11 NL, 24 cm) may be most suitable. More information on alternative pH ranges with specific recommendations on rehydration, sample loading and running condition can be found in the *Immobiline DryStrip Instructions* (28-9537-55 AA).

Materials

Immobiline DryStrip pH 3-11 NL, 24 cm
IPGbox and kit
2-D Protein Extraction Buffer-V
DeStreak Reagent
IPGphor 3 and control software
IPGphor Manifold
Immobiline DryStrip Cover Fluid
Sample cups and electrode papers

Rehydration buffer: To 2-D Extraction Buffer-V (or 7 M urea, 2 M thiourea, 4% (w/v) CHAPS) add IPG Buffet 3-11 NL (0.5 % (v/v) final concentration), DeStreak Reagent (15 mg = 12 µL/mL final concentration) and BPB 1% stock (0.002% (w/v) final concentration).

Rehydration

Place the IPGbox on a level surface during rehydration.

1. Pipett 450 µL of the rehydration buffer evenly over a slot in the disposable reswell tray for each 24 cm strip. The IPGbox can hold up to 12 strips.
2. Remove the protective cover foil from the Immobiline DryStrip gel. Use forceps to position the strip with the gel side down in the slot with rehydration buffer. Be careful not to trap bubbles under the Immobiline DryStrip.
3. Gently close the lid of the IPGbox and allow the Immobiline DryStrip gels to rehydrate at room temperature for 10 to 24 h.

IEF using the IPGphor 3 Isoelectric Focusing Unit is conducted at very high voltages (up to 10 000 V) depending on the length of the DryStrip used and very low currents (typically less than 50 µA per Immobiline DryStrip gel). During IEF, the current decreases while the voltage increases as proteins and other charged components migrate to their equilibrium positions.

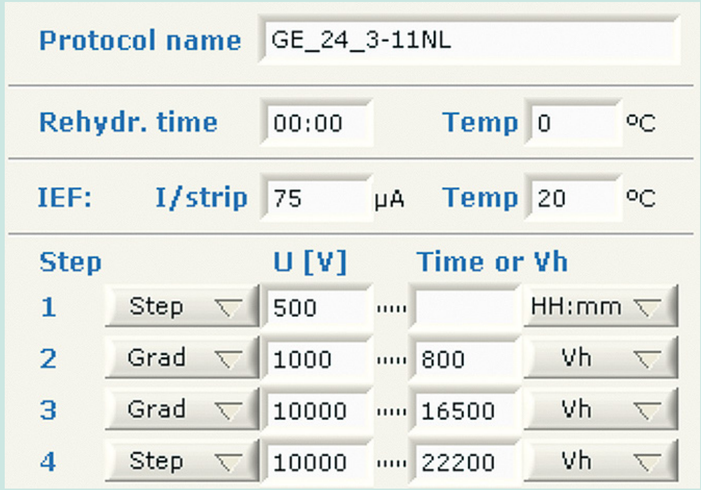
A typical IEF protocol generally follows a series of voltage steps starting at a relatively low value. Voltage is gradually increased to the final desired focusing voltage, which is maintained for several hours. A low initial voltage minimizes sample aggregation and allows the parallel separation of samples with differing ionic concentrations.

Preparing for IEF with IPGphor 3 and anodic cup loading in the manifold

1. Place the Manifold in the IPGphor 3 platform and ensure that the platform is level.
2. Measure out 108-120 mL of Immobiline DryStrip Cover Fluid and distribute evenly between the 12 manifold channels. Use 120 mL of cover fluid even when running one strip.
3. Place the strips under the cover fluid, gel side up in the tray with the anodic (+) end of the IPG strip oriented toward the anodic side of the instrument.
4. Place a strip of cups at the anodic end of the strips and fill the cups with cover fluid. Ensure that the cups are sealed.
5. Prepare two paper electrodes (precut paper wicks) for each strip. Add 150 µL of distilled water to the anode wick (+) and 150 µL of the rehydration buffer with DeStreak reagent (or DeStreak rehydration solution) to the cathode wick (-).
6. Place the wicks on the IPG strips such that one end of the wick overlaps the end of the gel on the IPG strip and then place each electrode assembly on top of the respective wicks.
7. Briefly centrifuge the CyDye labeled protein samples at top speed in a microcentrifuge prior to loading to remove insoluble material and particulate matter. Load samples into the sample cups. A maximum of 150 µL of sample may be placed in each cup. When sample is introduced into the cups, the sample will sink and come into contact with the IPG strip.

Running IEF separation in IPGphor 3

1. Close the lid of the IPGphor 3 and connect the instrument to the control software if desired.
2. Select the fast protocol for a 24 cm strip, 3-11 NL pH range and the number of strips to run. If the run time needs to be adjusted for an overnight run, select advanced protocol, and prolong the time for up to 8 h in step 1.



3. Save the protocol and start IEF. Run until the total Vh is reached and the machine stops.

If the Immobiline DryStrips are not run immediately in the second dimension gel, they can be stored at –70°C or colder in a sealed container for at least one week.

Frozen Immobiline DryStrips are very brittle and can easily be damaged. Handle with care!

Second dimension SDS PAGE

In the second-dimension electrophoresis step, proteins are separated according to their molecular weight in a polyacrylamide gel containing sodium dodecyl sulfate (SDS). A 12.5% gel is commonly used and run in a Laemmli (Tris-glycine) buffer system (5) cast in low fluorescent glass plate cassettes.

Ettan DIGE gels use PPA (piperidinopropionamide) as the counter ion rather than the standard Laemli buffer system.

The IPG strip is equilibrated, laid on top of the polyacrylamide gel, and sealed with an agarose solution. Equilibration maintains the IPG strip in a pH range appropriate for electrophoresis. SDS denatures proteins and forms negatively charged protein-SDS complexes. A tracking dye, such as bromophenol blue (BPB), allows the user to monitor the progress of electrophoresis.

Dithiothreitol (DTT) in the first equilibration step preserves the fully reduced state of denatured, non-alkylated proteins. Iodoacetamide (IAA), which is introduced in a second equilibration step, alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis. Protein reoxidation during electrophoresis can result in streaking and other artifacts. Equilibration with IAA minimizes unwanted reactions of cysteine residues, for example, when mass spectrometry is to be performed on the separated proteins. Second dimension electrophoresis is run in either of the Ettan DALT electrophoresis systems for six or twelve large format vertical gels. For detailed descriptions of Ettan DALTsix and Ettan DALTtwelve, please refer to the system user manuals.

If different strip lengths are used then use the appropriate sized second dimension gel unit. If DIGE is the detection methodology, then the use of LF glass plates is strongly recommended.

Equilibration should be performed immediately before the second-dimension run, and not before storage of the IPG gels.

For saturation DIGE dye labeled samples, omit the IAA equilibration step and repeat the equilibration with SDS equilibration solution containing DTT.

Precast DIGE gels offer high quality and performance with convenience and timesavings. DIGE Gel should be used with the DIGE Buffer Kit, which consists of concentrated running buffers and Sealing Solution for sealing IPG strips to the top of the polyacrylamide gel.

Preparing for second dimension SDS PAGE

Materials needed:

Ettan DALTsix or Ettan DALTtwelve electrophoresis system
DIGE Gel and DIGE Buffer Kit
DTT
IAA

SDS equilibration buffer: 50 mM Tris pH 8.8, 6 M Urea, 30% glycerol (v/v), 2% SDS (w/v), 0.001% BPB (w/v)

For DTT equilibration: SDS equilibration buffer, 0.5% DTT (w/v), 15 to 20 mL/strip

For IAA equilibration: SDS equilibration buffer, 4.5% IAA (w/v), 15 to 20 mL/strip

Equilibration of IPG strips

1. Place strips in individual tubes, trays or Petri dishes and add 15 to 20 mL DTT SDS equilibration solution.
2. Incubate with gentle agitation for 15 min at room temperature.
3. Pour off the DTT SDS equilibration solution. For samples labeled with CyDye DIGE fluor minimal dyes, add 15 to 20 mL of IAA SDS equilibration solution. For samples labeled with CyDye DIGE fluor saturation dyes, add 15 to 20 mL DTT SDS equilibration solution.
4. Incubate with gentle agitation for 15 min at room temperature.
5. Pour off the solution and drain thoroughly.

Loading and sealing IPG strips

1. Rinse the IPG strips briefly in cathode buffer and carefully place the strips in the glass cassette. Make sure the strips have contact with the gel and avoid trapping air bubbles between strips and gel.
2. Seal the IPG strips in place using an aliquot of hot agarose sealing solution. Carefully pipette across the length of the IPG strip, taking care not to introduce bubbles.

Run electrophoresis

1. Insert the prepared gel cassettes into the electrophoresis system. For detailed descriptions of Ettan DALTsix and Ettan DALTtwelve, refer to the system user manuals and *DIGE Gel and DIGE Buffer Kit User Manual* (28-9460-89 Edition AB).
2. Start the electrophoresis.
3. Continue the electrophoresis until the BPB front reaches the end of the gel. The front can be run off the gel if desired.
4. Stop the run and unload the gels. To minimize spot diffusion, the gels should be scanned as soon as possible.

If the gels are not scanned immediately, they should be stored in a refrigerator in a closed container and kept moist.

5.2.4.8 Image acquisition

Typhoon FLA 9500 is optimized to detect signals from CyDye DIGE Fluor dyes and is recommended for use with the DIGE system.

If spots on the 2–D gel image show saturated signals (i.e., if pixel values exceed 100 000 for .GEL image files) then quantitation may not be accurate. When optimizing scan conditions, the maximum detected pixel value within the region of interest on the gel should lie between 50 000 to 80 000 (see section 4.3). To achieve this, adjust the PMT until the maximum pixel value falls within this range.

DIGE gels should be kept in their glass cassettes throughout scanning, to prevent drying and shrinkage, and to allow rescanning if required. Allow the gels to reach room temperature before scanning.

Cysteine-rich proteins label more strongly than other proteins CyDye DIGE Fluor saturation dyes. This results in more intense spots than other proteins on the gel. By scanning at a higher PMT it is possible to saturate these spots to enhance the detection of the low abundance proteins. Data from the saturated protein spots will not be quantitative and should be disregarded during the analysis.

Avoid contamination from fluorescent material by careful handling of the instrument and gels. Wear powder-free gloves. The powder used in laboratory gloves can fluoresce and may also scatter light, affecting image quality.

Optimizing scan conditions

1. Place the dry and clean gel cassettes in the instrument. Wipe the glass with lint-free tissue. For detailed instructions, refer to the instrument User Manuals.
2. In the scanner control software, select the 2-D DIGE acquisition mode and select the appropriate settings for DIGE format gels and the CyDye dyes to be analyzed.
3. Set the PMT voltage for each scan wavelength and do a quick pre-scan at low resolution. This should be performed initially to identify a suitable voltage.
4. Open the pre-scan image (files with .DS or .GEL extensions) in ImageQuant TL software to check the area of the most intense spots to be included in the analysis. The maximum pixel value detected within the region of interest on the gel should be in the range around 50 000 to 80 000. Disregard intense, or saturated spots of no interest due to sample specific properties, such as over expressed or high abundance proteins.
5. Adjust the PMT voltage settings if needed and perform a new pre-scan of the gel.
6. Select a small scan area with the most intense spot and perform a high-resolution scan to verify that the spot intensity is within the pixel range. Repeat adjustments of the PMT voltage settings if needed.
7. Once the voltage has been optimized for one gel in an experiment, these settings can usually be used for all similar gels within the same experiment, but it is usually best to verify with a quick prescan. The maximum pixel value should be within the specified range for all gels within an experiment, to enable accurate quantitation of spot volumes.

Naming image files

For convenient image handling in DeCyder 2D software, follow the gel naming recommendations. This assures that the Image Loader automatically groups two or three images (Cy2, Cy3, Cy5) into one gel and that BVA automatically groups the internal (pooled) standard images into the standard group.

The name includes four parts: **Part 1** is a general description, normally *Gel* followed by a number or the unique ID number of the IPG strip or DIGE gel. This part will be in common to all images of the gel. **Part 2** contains a description of the sample within brackets; for example, sample ID, time and dose. If the image contains the pooled internal standard sample, **std** or **STANDARD** without brackets should be used. Use short names with the most important parts of the name first to simplify sorting and finding. **Part 3** contains information of the labeling used to create the images (Cy2, Cy3 or Cy5). By default, the Cy2 gel will be placed as image 1, the Cy3 gel as image 2, and the Cy5 gel as image 3. **Part 4** is the extension, .GEL or .TIF, added at the end of the file name.

This is an example of how three images that group into a Gel named 55720 in the Typhoon FLA 9500 may be identified:

55720 STANDARD [Cy2].gel

55720 (Time1_Dose2) [Cy3].gel

55720 (Time2_Dose2) [Cy5].gel

Cropping images

DeCyder software uses an Oracle Database. Images must first therefore be uploaded to the database before they can be analyzed. This is performed in the Image Loader module. Cropping and flipping images is easily performed in the Image Editor software that can be accessed from the Image loader module, or can be accessed as a standalone tool. Images can also be cropped and edited in ImageQuant TL software.

Crop images to remove irrelevant information and ensure that all spots of interest remain within cropped area. Note that preserving consistent patterns within the image to improve matching is more important than having equally sized images.

Image analysis

DeCyder 2D Differential Analysis Software and ImageMaster 2D Platinum DIGE Enabled software have been specifically designed for the DIGE system to accurately measure protein changes and detect small changes in protein expression. The software packages use algorithms to co-detect differentially labeled samples within the same gel.

DeCyder 2D Differential Analysis Software was developed in parallel with the 2–D DIGE methodology to exploit all the advantages of the technique. The software was developed for detection, quantitation, positional matching and differential protein expression analysis on images generated using the DIGE system. For an overview of the software and its capabilities see Chapter 4, and for detailed information refer to *DeCyder 2D Differential Analysis Software User Manual* (code 28-9435-85).

The co-detection algorithm in DeCyder 2D Differential Analysis Software takes advantage of the identical spot patterns generated when multiple samples are resolved on the same gel. The algorithm generates identical spot boundaries for spots on images derived from the same gel.

Most alternative 2–D image analysis packages allow extensive user intervention during spot detection and editing. This can lead to subjective data analysis and may result in biased conclusions. DeCyder 2D Differential Analysis Software is designed to provide automated spot detection, normalization, background subtraction, matching and spot statistics. The automated spot detection algorithms are optimized for work with multiplexed fluorescently labeled proteins and provide objective data analysis with minimal user intervention.

An experimental design based on an internal standard allows gel-to-gel matching of pooled internal standard samples, which allows confident inter-gel matching of similar images. As matching across internal standards is completed, the individual sample images co-detected with each internal standard are simultaneously matched into the dataset.

Image analysis

1. Add all gel images from the experiment to Image Loader.
2. Crop the gels in Image Editor.
3. Import the images to a project in the DeCyder database.
4. Start an automatic analysis with the Batch Processor.
5. Check spot detection and matching and edit if needed in DIA and BVA.
6. Use the statistics tools in BVA and EDA to find interesting spots that are differentially expressed, find expression profiles between experimental groups and find sub clusters within experimental groups.
7. Prepare a preparative pick gel and subsequently perform protein identification with peptide mass fingerprinting (PMF) and MS analysis on the picked spots.
8. Import the protein ID information from the search results to EDA. Use the interpretation tools in EDA to perform queries in different databases.

5.2.4.9 Spot Processing for MS analysis

When spots with interesting expression profiles are discovered a preparative workflow with a pick gel should be prepared and matched to the analytical gel set. Interesting spots are assigned for picking and polyacrylamide gel plugs containing proteins from the 2-D gel are automatically excised with the Spot Picker. For details, refer to the Ettan Spot Picker User Manual (6). The gel plug usually contains a sufficient amount of protein for protein identification by PMF and MS analysis and a protein database search.

Preparative DIGE gel

A preparative pick gel must be bound to a gel backing for processing in the Spot Picker. Two reference markers are attached to the backing of the pick gel and are also detected during image analysis in DeCyder 2D Differential Analysis Software BVA when the pick list (text files containing the Cartesian coordinates of spot pick positions) is created. The reference markers enable the Spot Picker to transform image (pixel) Cartesian coordinates for each spot.

- Precast DALT Gel 12.5 is a large format polyacrylamide gel bound to a plastic support film and is suitable for the preparation of pick gels in the 2-D DIGE workflow. The 2-D spot map on a DALT Gel 12.5 is equivalent to the spot map on a DIGE Gel of the same sample due to similar gel and buffer composition.
- Using the CyDye DIGE Fluor **saturation dyes**, a Cy3-labeled sample is used to prepare a preparative gel for spot picking. For details, refer to *CyDye DIGE Fluor Labeling Kit for Scarce Samples* (28-9531-07AD). The background fluorescence of the plastic support in the DALT Gel 12.5 will not disturb the identification of Cy3-labeled spots to pick after matching the pick gel to the analytical gels in DeCyder 2D Differential Analysis Software BVA. No post staining of the preparative gel is required.
- Using the CyDye DIGE Fluor **minimal dyes**, an unlabeled sample spiked with a small portion of Cy5-labeled sample can be used to prepare a preparative gel for spot picking. The gel must be post-stained using a fluorescent stain such as Deep Purple before scanning. CyDye labeling of proteins does not affect the mass spectrometry data used to identify proteins as only 2 to 5% of lysine residues are labeled on a single protein. CyDye labeling of lysine will only result in the loss of a single trypsin digest site per labeled protein. The migration difference between the unlabeled and labeled protein is due to the addition of a single CyDye molecule to the protein. This is more significant for low molecular weight proteins. The preparative gel is scanned in two channels; one for Cy5 and one for Deep Purple. The Cy5 image facilitates matching to the analytical gels in DeCyder 2D Differential Analysis Software BVA and the Deep Purple image is used to identify and edit positions for the spots to pick. The background fluorescence of the plastic support in the DALT Gel 12.5 will not disturb the detection of Cy5-labeled spots.
- Deep Purple is compatible with the image analysis softwares and the stain is compatible with manual or automated spot picking and MS for protein identification applications.

Spot picking

The preparative pick gel and the pick list generated in DeCyder 2D Differential Analysis Software is loaded in the Spot Picker. Gel plugs are processed.

Spot digestion

The sample plugs are then transferred to a digestion station for digestion with trypsin. The resulting peptide fragments are analyzed by mass spectrometry before searching databases to identify the proteins.

Protein identification

An important development in protein identification is peptide mass fingerprinting, where MS of peptide fragments of proteins and MS-MS sequencing of peptide fragments, combined with searches of public databases of known protein sequences allows the identification of proteins in a sample.

Labeling proteins with CyDye DIGE Fluor saturation dyes does not affect identification by MS. Labeling of cysteine residues does not reduce the efficiency or specificity of enzymatic digestion. Cysteine-labeled proteins generate equivalent levels of peptide mass fingerprint (PMF) and sequence data as unlabeled proteins.

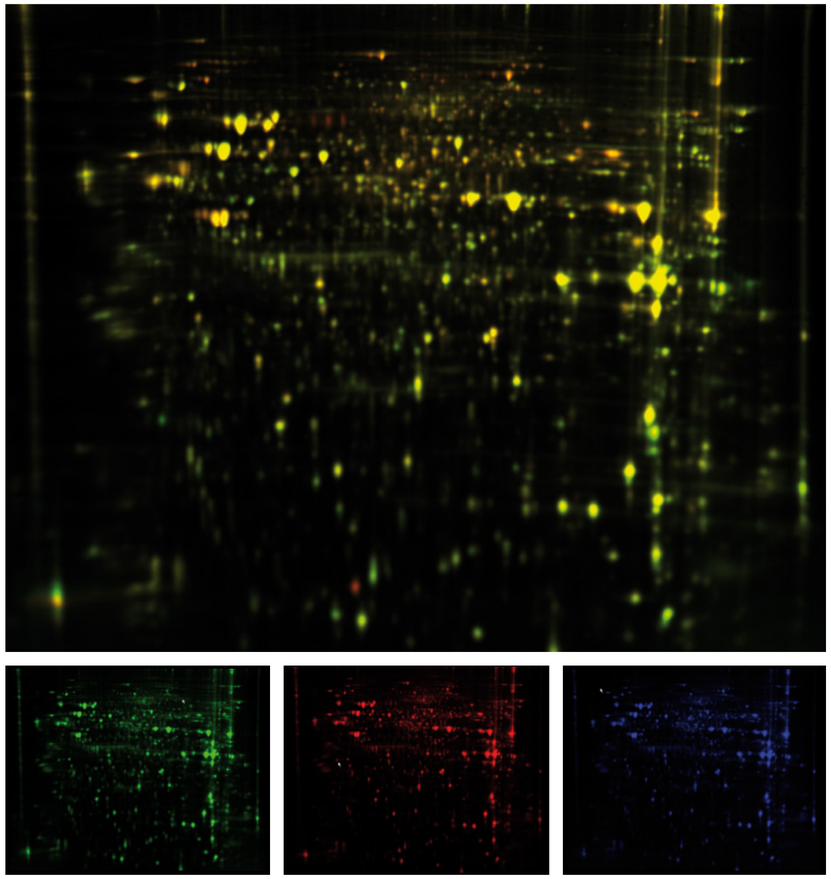
5.2.5 Typical results

Finding new cancer targets with 2-D DIGE and siRNA

Small interfering RNA (siRNA) can be used to down regulate specific genes that may result in small or large proteomal changes. This example illustrates how 2-D DIGE can be used as an effective method for the analysis of differential protein expression caused by growth factor stimulation and siRNA knock down of a protein in a signaling pathway.

The study used a human prostate cancer cell line (PC-3U) that was stimulated at different time intervals with a growth factor after gene silencing treatment with siRNA. Silencing of the target protein revealed that over 300 proteins were affected either by the presence of siRNA, the growth factor treatment, or by a combination of the factors and time.

(A)



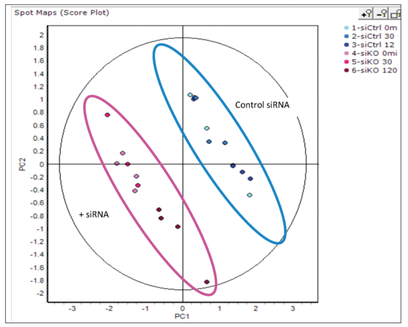
Down-regulated proteins after 120 min stimulation and siRNA treatment

Up-regulated proteins after 120 min stimulation and siRNA treatment

Non-regulated proteins after 120 min stimulation and siRNA treatment

Sample: Human prostate cancer cell line (PC-3U)
Strip: Immobiline DryStrip pH 3-11 NL
Detection: CyDye DIGE Fluor minimal dyes
Imaging: Typhoon FLA 9000
Analysis: DeCyder 2D software v 7.2

(B)



(C)

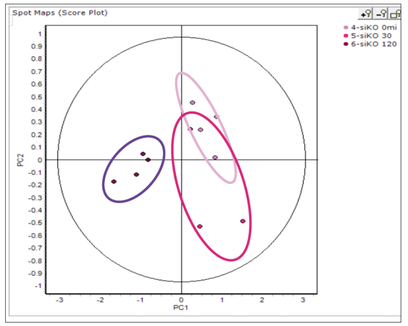


Fig 5.11. A visual example of one of the DIGE gels in the experimental set-up. The Cy3 labeled control sample and Cy5 labeled siRNA treated sample were run in the same 2-D gel together with the Cy2 pooled internal standard. The protein pattern for each individual sample can be detected separately by imaging with a laser scanner Typhoon FLA 9000. In addition Cy5, Cy3 and Cy2 images can be viewed as overlaid images (A). The control siRNA and siRNA treated PC-3U cells cluster separately in the PCA plot, indicating different protein expression patterns (B). In addition a clear cluster is found for the different time points of growth factor stimulation (C) indicating different protein expression patterns between these groups.

Characterization of host cell protein patterns using 2-D DIGE

Quality by Design (QbD) is based on a thorough product and process understanding to ensure product safety. As part of QbD, processes must be characterized by impurities such as host cell proteins (HCP). Therefore, it is important to investigate manufacturing processes at an early stage by varying upstream conditions (for example cell clone and media composition) and analyzing the effects on downstream processes (purification).

In this study, variations in HCP patterns in monoclonal antibodies (MAb) purified from Chinese hamster ovary (CHO) cells cultured in different media compositions were characterized. 2-D DIGE was used to analyze individual HCP in the starting material, and in the flowthrough and eluate fractions after purification with MabSelect SuRe™.

The results showed that 2-D DIGE facilitated the selection and optimization of growth conditions and purification methods by allowing analysis of individual HCP profiles and HCP patterns (Fig 5.12). Moreover, the study demonstrated that 2-D DIGE is a promising tool for thorough process characterization.

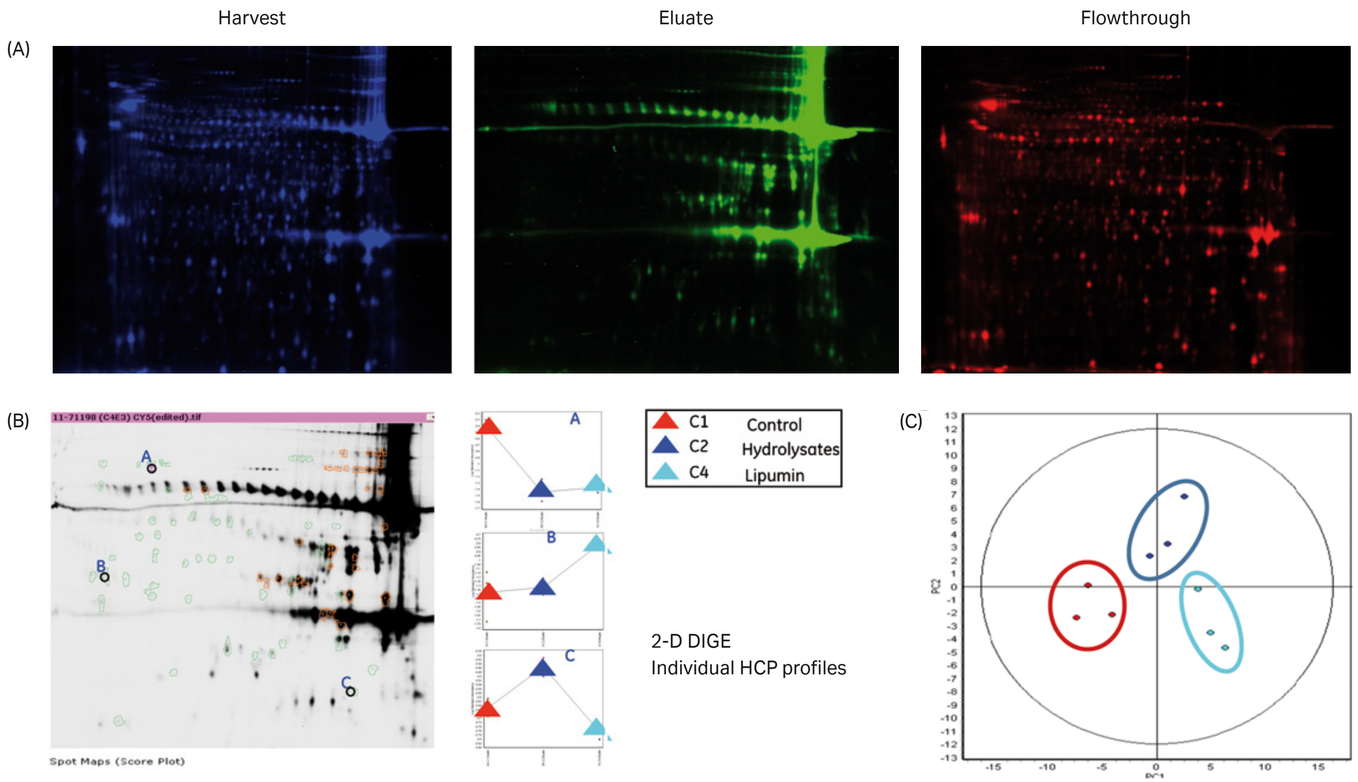


Fig 5.12. CHO cells expressing MAb were cultured with different additives. After the proteome was harvested and fractionated, 2-D DIGE analysis indicated that most of the HCP was in the flowthrough fraction. The eluate fraction mostly contained MAb and traces of HCP that could be detected and separated (A). The growth conditions were evaluated by analysis of cell culture supernatants for individual HCP profiles with 2-D DIGE (B). PCA analysis revealed that the cell culture conditions caused different HCP patterns that were detectable after purification with MabSelect SuRe™ resin (C).

5.3 Western blotting

Western blotting is a well-established and widely used technique for the detection and analysis of proteins. The method is based on the construction of an antibody:protein complex with specific antibodies that bind to proteins immobilized on a membrane. In the last decade there have been great improvements in detection methods and software that have made quantitative Western blotting safer and more sensitive and efficient.

Regardless of application or adaptations for protein characteristics, Western blotting typically follows some common basic steps (Fig 5.13) This section provides a brief summary of the most used antibody:protein detection systems as well as tips and protocols to help you improve your methods and get the best quality of data. More detailed information about Western blotting can be found in the handbook, *Western Blotting – Principles and Methods* (7).

5.3.1 Western blotting detection

A variety of detection systems, based on chemiluminescence, chemifluorescence, fluorescence, chromogenic or radioisotopic detection are available. Radioisotopic and chromogenic reagents have seen a steady decline due to safety issues with handling radioactive isotopes and poor sensitivity with chromogenic reagents. Consequently, enzyme-based chemiluminescence, chemifluorescence and direct fluorescence have now become the systems of choice, offering both high sensitivity and wide dynamic ranges (Fig 5.14).

Fig 5.13. The Western blot workflow.

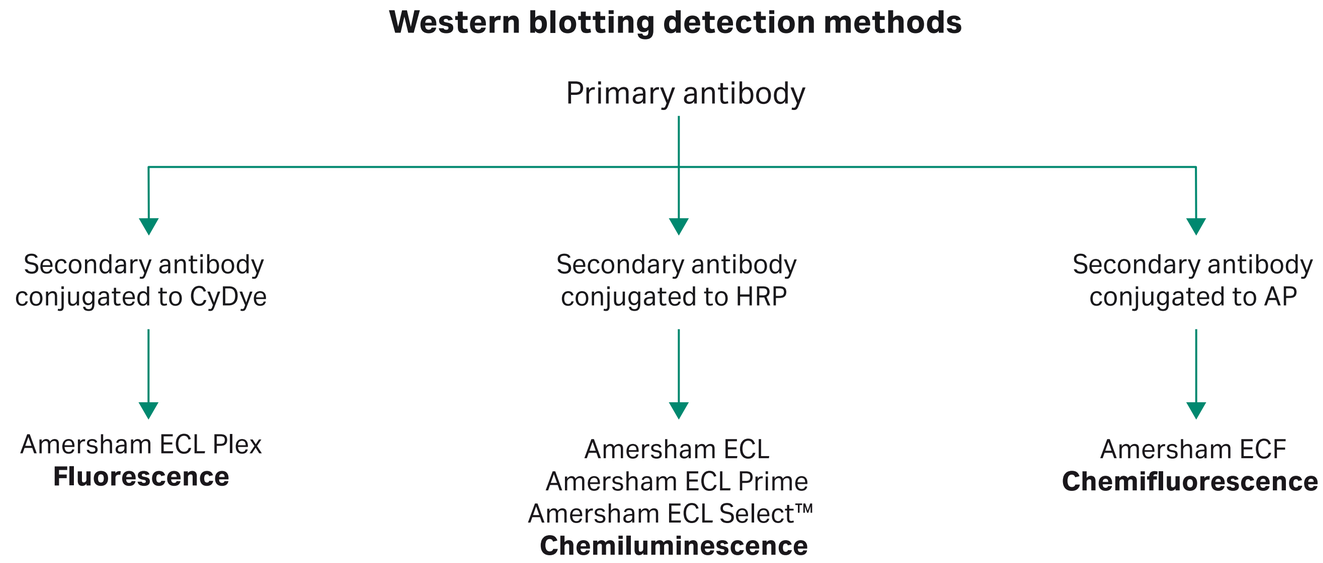


Fig 5.14. Cytiva reagents for Western blotting detection. Amersham ECL Plex consists of a series of CyDye (Cy2, Cy3, and Cy5)-conjugated secondary antibodies. The fluorescent signals from these antibodies can be directly detected using laser scanners, such as Typhoon FLA 9500. Amersham ECL, Amersham ECL Prime, and Amersham ECL Select are chemiluminescent reagents that require HRP-conjugated secondary antibodies to generate signals that can be detected with ImageQuant LAS systems. Amersham ECF is designed for chemifluorescence and requires alkaline phosphatase (AP) conjugated secondary antibodies to generate signals.

5.3.1.1 Chemiluminescence

Cytiva has continuously developed chemiluminescence detection systems that are now amongst the most widely used for Western blotting applications. Chemiluminescence detection in Western blotting is based on antibodies conjugated to the enzyme horseradish peroxidase (HRP) that catalyzes the oxidation of luminol in the presence of peroxide, and results in light emission.

Normally the reaction produces low intensity emission of light at 428 nm. However, in the presence of enhancers, such as, modified phenols and, especially p-iodophenol, the emitted light is enhanced up to 1000-fold. This simplifies detection and increases the sensitivity of the reaction; the whole process is known as enhanced chemiluminescence (ECL). The intensity of signal is a result of the number of reacting enzyme molecules and is thus proportional to the amount of antibody, which is related in turn to the amount of protein on the blot (Fig 5.15).

Several horseradish peroxidase (HRP)-based systems are available, with the best choice depending on the aims of the experiment (Table 5.9). Amersham ECL is a highly cited detection reagent that allows fast detection of high abundance proteins with little effort required for optimization. It is suitable for applications such as:

- Verification of expression of recombinant proteins
- Verification of highly expressed proteins
- Studies with tagged proteins
- Confirmatory studies

Amersham ECL Prime is recommended if high sensitivity or more accurate quantitation is important. The signal emitted by Amersham ECL Prime is very intense and highly stable, which allows repeated exposures for the detection of low levels of proteins. It is suitable for applications such as:

- Applications where high sensitivity is needed
- Changes in protein abundance
- Protein:protein interactions
- Detection of protein isoforms
- Post-translational modifications (PTM)

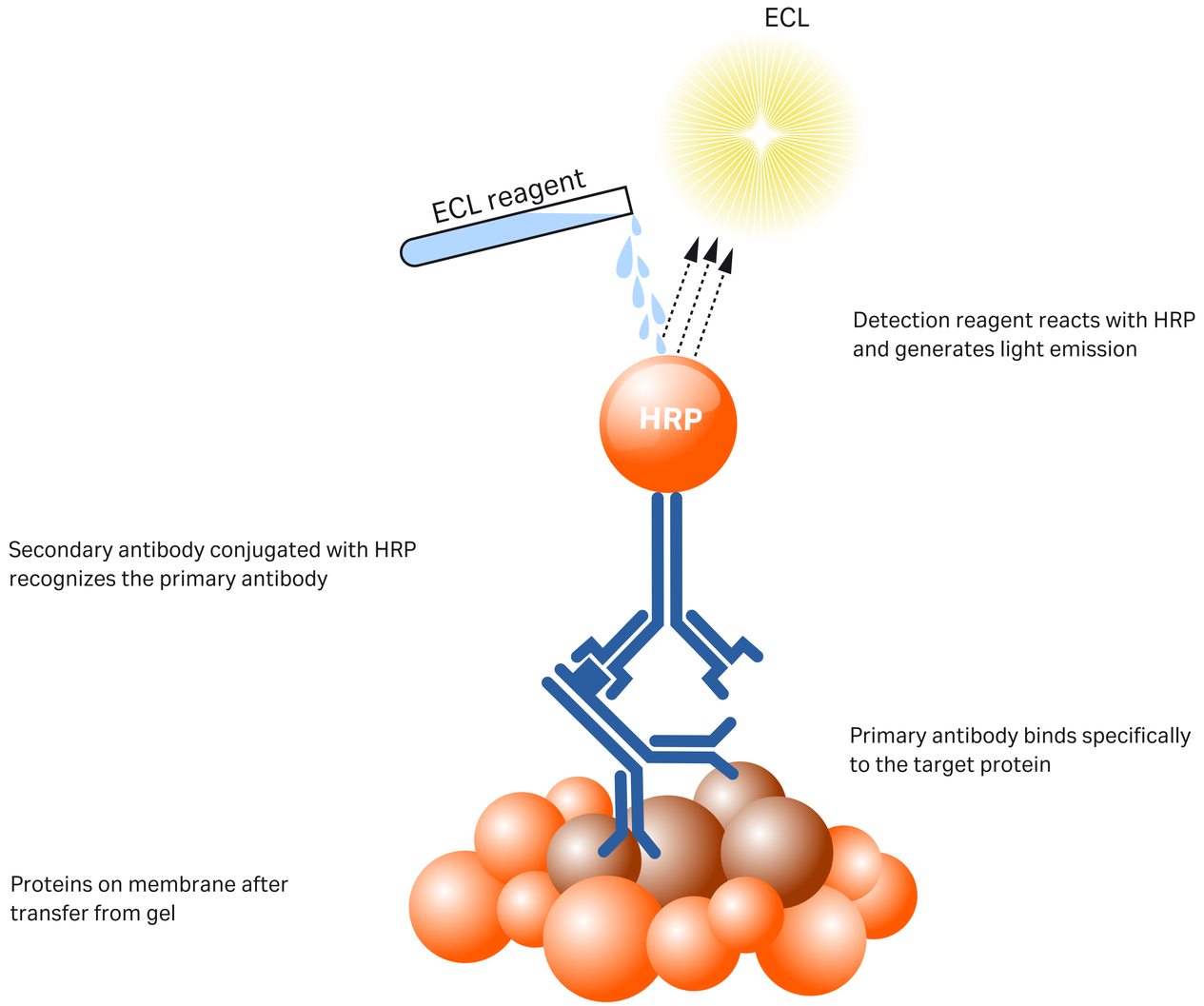


Fig 5.15. The principle of chemiluminescence detection in Western blotting.

Alternatively, Amersham ECL Select is recommended for qualitative and quantitative detection of proteins present at very low levels. High signal intensity and low background make Amersham ECL Select suitable for the most demanding Western blotting applications such as:

- Applications where very high sensitivity is needed
- Analysis of protein regulation
- When sustained performance is needed with highly dilute or valuable antibodies
- Post-translational modifications (PTM)

Table 5.9. Amersham ECL reagents are suited to different Western blotting applications depending on the required sensitivity, availability of primary antibody, and detection method.

	Amersham ECL	Amersham ECL Prime	Amersham ECL Select
Detection method	Chemiluminescence	Chemiluminescence	Chemiluminescence
Recommended membrane	Hybond™ ECL	Hybond P Hybond ECL	Hybond P Hybond ECL
Recommended primary antibody dilution	1:100-1:5000	1:1000-1:30 000	1:5000-1:30 000
Recommended secondary antibody dilution	1:10 000-1:15 000	1:50 000-1:250 000	1:100 000-1:500 000
Recommended signal detection	Hyperfilm™, ImageQuant LAS series	ImageQuant LAS series, Hyperfilm	ImageQuant LAS series, Hyperfilm
Sensitivity	Medium	High	Very high
Signal duration	Medium 1-2 hours	Very long 2-12 hours	Long 2-6 hours
Typical applications	• Detection of high to medium protein levels • Overexpressed proteins • Qualitative analysis	• Detection of medium to low protein levels • Endogenous proteins • Quantitative analysis	• Detection of medium to very low protein levels • Weakly expressed proteins • Quantitative analysis

More information on Amersham ECL reagents is provided in the handbook, *Western Blotting – Principles and Methods* (7).

Chemiluminescence hints and tips

- To increase the signal-to-noise ratio, spend time on optimizing blocking agents and antibodies. Always start with the manufacturers' recommendations.
- If the membrane is to be reused, place it in a plastic file folder to prevent drying, before imaging. Be sure to remove any air bubbles.
- Do not allow the membrane to dry out at any time during the immunodetection procedure or between rounds of immunodetection. Any residual molecules will bind permanently to the membrane if it is allowed to dry, making it impossible to strip the membrane. This is particularly important when using polyvinylidene fluoride (PVDF) membranes.

5.3.1.2 Fluorescence

Fluorescence detection is a direct method that does not require additional reagents since the secondary antibody is conjugated to a fluorophore. Different fluorophores are available, either for detection of signals at wavelengths of visible light or at near infrared wavelength. The system is highly sensitive, delivers a broad linear dynamic range, and is well adapted to quantitative Western blotting (Fig 5.16).

The fluorescence-based Amersham ECL Plex system uses discrete primary antibodies that are recognized by species-specific secondary antibodies conjugated to fluorescent Amersham CyDyes. All CyDyes have their own specific excitation and emission wavelengths in the visible light spectra and are spectrally differentiated from each other, resulting in minimal cross talk (Fig 5.17).

Fluorescent detection enables the simultaneous detection of more than one protein target or more than one protein target (multiplex detection). Figure 5.18 illustrates how two primary antibodies that are raised in different species can be used to recognize two protein targets. A third primary antibody can be directly labeled with Cy2 for simultaneous identification of three target proteins. The fluorescent emission signals are captured by a multichannel fluorescent imager such as Typhoon FLA 9500.

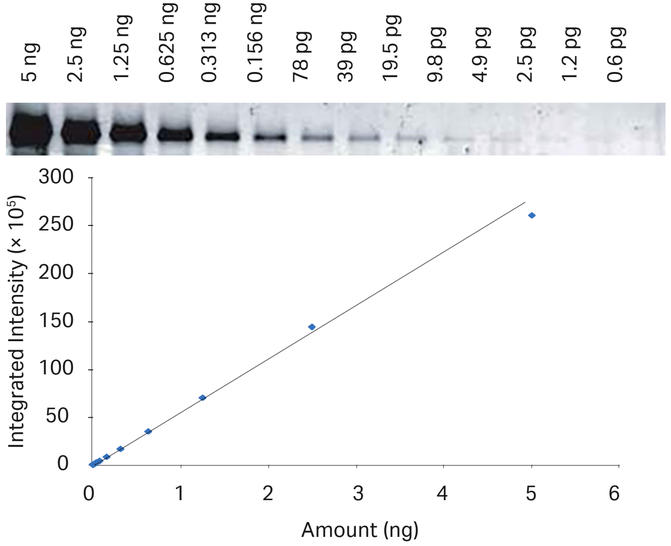


Fig 5.16. Fluorescence detection with Amersham ECL Plex gives a broad linear dynamic range, to allow detection and comparison of weak and strong bands on the same blot. In this example, a dilution series of transferrin detected with Cy3-labeled secondary antibodies. The dynamic range is 3.6 orders of magnitude and very linear with an LOD of 1.2 pg.

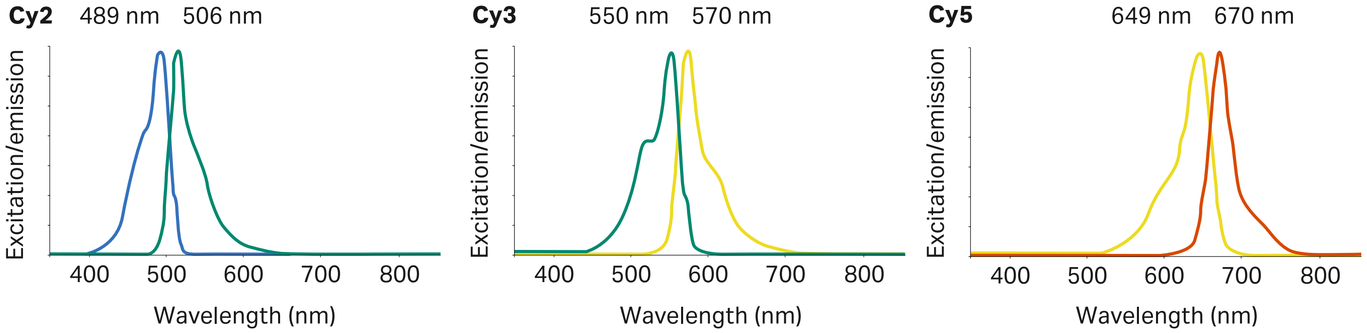


Fig 5.17. Excitation and emission spectra for fluorescent Amersham ECL Plex CyDye antibody conjugates Cy2, Cy3, and Cy5. In each example, the effect of the light wavelength generating the maximum efficiency of emission is shown.

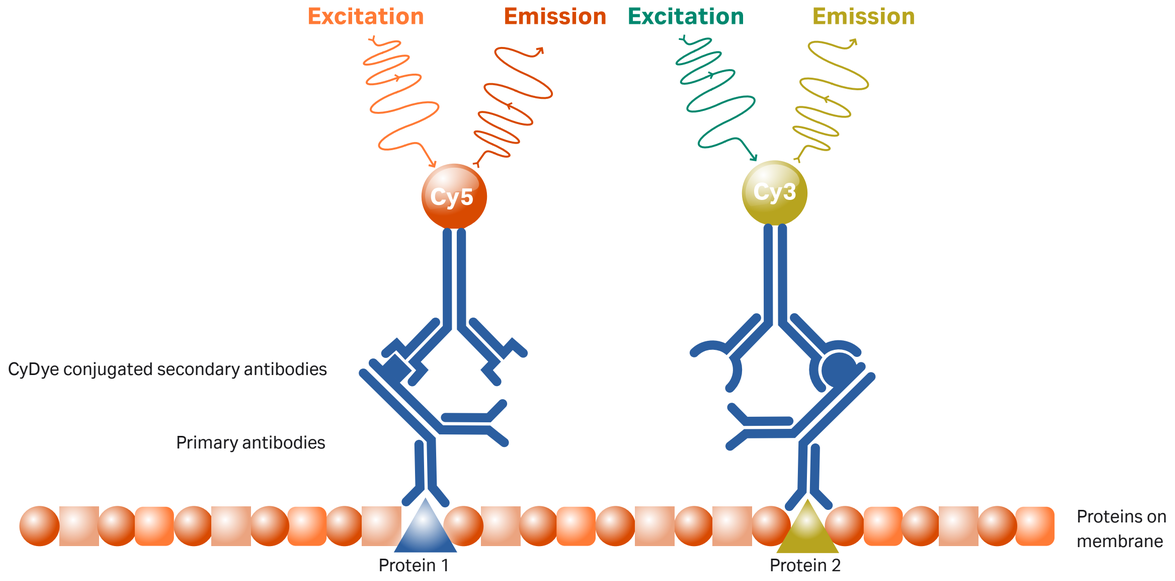


Fig 5.18. Using the direct fluorescence technique of Amersham ECL Plex, primary antibodies against two proteins are recognized by species-specific secondary antibodies conjugated to fluorescent dyes Cy3 and Cy5. Detection by direct fluorescence reduces the number of processing and imaging steps, which saves time and reduces errors in quantitation.

Amersham ECL Plex has the added benefits of high photostability compared to other fluorophores, and a wide pH tolerance (3 to 10) that makes them compatible with most Western blotting buffers. Finally, fluorecent detection systems have few handling steps and a longer signal duration, which allows the comparison of data over several experiments.

Fluorescence-based detection is recommended for:

- Multiplexing — the simultaneous detection of up to three different proteins
- Detection of different proteins of identical molecular weights
- Quantitative Western blotting applications
- Applications where high sensitivity is needed
- Changes in protein abundance
- Protein-protein interactions
- Protein localization
- Protein stability
- Post-translational modifications
- Studies of low abundant proteins
- Studies of low and high abundant proteins at the same time

High quality Western blotting results depend on general concerns regarding fluorescent detection described in this handbook. In addition, you may find the following points useful to help you solve problems related to low signal-to-noise ratios.

- Use Amersham ECL Plex Cy5-conjugated secondary antibodies for the protein at the suspected lowest concentration in your sample. The signals emitted by Cy5 are slightly more intense than those emitted by Cy3 and especially Cy2.
- To increase weak signals, use phosphate-buffered saline (PBS) supplemented with 0.1% Triton™ X-100 instead of 0.1% Tween™-20 throughout the protocol. Note that Triton X-100 is NOT compatible with the Cy3 channel and its use may lead to high background.
- If very strong signals from the markers, overwhelm signals from low abundance proteins, then use a smaller amount of ECL Plex Rainbow Markers (1.5 µL). It maybe necessary to dilute the Rainbow Marker with sample buffer to ensure full coverage of the bottom of the well. If possible, add sample loading buffer in one lane between the markers and the sample.
- Cytiva imagers are highly sensitive to fluorescent signals and enable detection of a wide linear dynamic range of protein quantities. As both sensitivity and dynamic range are also functions of characteristics of the antibody:protein pair, it is critical to optimize the concentrations of both the primary and secondary antibodies.
- Remove any residual pieces of gel from your membranes
- The presence of bromophenol blue (BPB) on the membrane can generate unwanted fluorescent signals. Remove all traces of BPB from the bottom of sodium dodecyl sulfate (SDS)-PAGE gels before transfer to a membrane.
- Ensure that your trays, forceps and containers are clean and free of Coomassie Blue stain, as this can also cause background problems
- Avoid labeling the membrane with a ballpoint pen as this can generate contaminating signals. Cut one corner of your membrane (and note which corner you have cut!).
- Do not handle the membrane with your fingers; use clean forceps
- Wear powder-free gloves when handling membranes. The powder used in laboratory gloves can fluoresce and may also scatter light, complicating the interpretation of images.
- A concentration of Tween-20 greater than 0.1% may result in a significantly increased background on PVDF membranes
- Rinse the membrane in PBS to remove Tween-20 before imaging

More information about Amersham ECL Plex is available in the handbook, *Western Blotting – Principles and Methods* (7).

5.3.1.3 Chemifluorescence

Chemifluorescence uses alkaline phosphatase (AP) conjugated antibodies that react with a fluorogenic substrate to generate a stable fluorophore (Fig 5.19). The advantage of AP is that its linear reaction time allows increased sensitivity by prolonging incubation time with substrate. ECF has a similar limit of detection as ECL, but has the advantage that the signal from the stable fluorophore can be detected on multiple occasions. ECF is recommended for:

- Verification of the expression of recombinant proteins
- Verification of over-expression of proteins in cell lysates
- Studies of tagged proteins
- Studies that are not limited by low amounts of protein

Chemifluorescence hints and tips

- A wetted blot must not dry out during the immunodetection steps or before incubation in ECF substrate. If a membrane dries, then rinse it quickly with methanol (PVDF only) followed by PBS or Tris-buffered saline.
- Adding ECF substrate to blots requires a flat, clean surface. Smoothing Saran™ Wrap on to a bench is usually sufficient. Drain the excess washing buffer and lay the blot, protein side down, into the ECF substrate solution.
- Once ECF substrate incubation is complete, scan the wet blot immediately with the protein side facing the excitation source

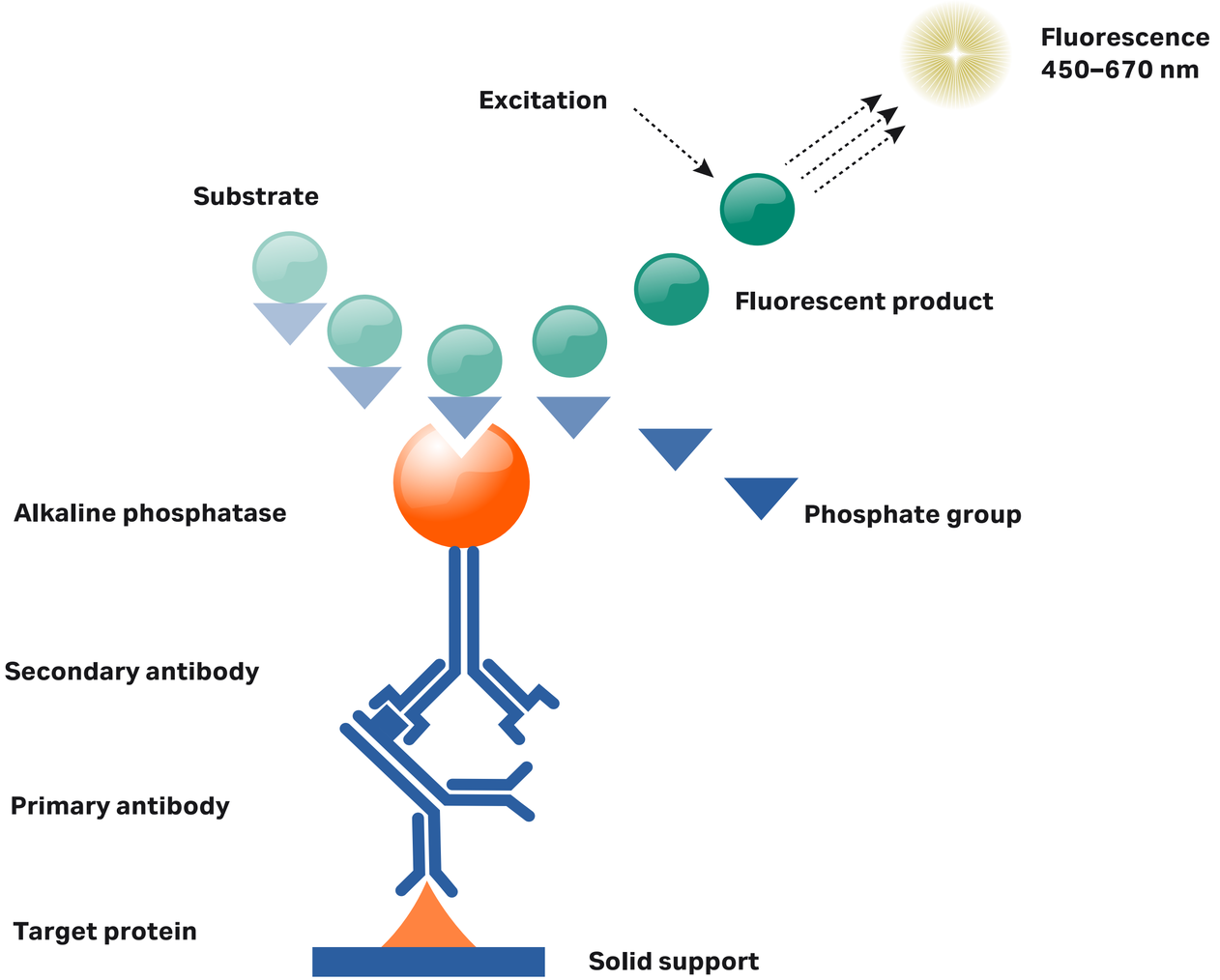


Fig 5.19. ECF Western blotting detection. Proteins are detected by chemifluorescence using an AP-labeled secondary antibody. AP cleaves a phosphate group from the ECF substrate to yield a highly fluorescent product.

5.3.2 Instrument compatibility

Cytiva provides complete solutions for the labeling and detection of proteins, delivering the highest quality of quantitative data (Table 5.10). Typhoon and ImageQuant systems are crucial components for accurate imaging. They are made to work together with Amersham ECL, Amersham ECL Prime, Amersham ECL Select (ImageQuant) and Amersham ECL Plex and phosphor screens (Typhoon FLA).

CCD-based cameras have a critical advantage over laser-based scanners for the detection of chemiluminescent emissions. In laser scanners, the light collecting scan head, moves over the sample at high speed and records data in less than a millisecond, while a CCD-chip can be exposed to chemiluminescent light from a fraction of a second to minutes before integrating the signal for the best possible data quality.

Table 5.10. Compatibility of imagers from Cytiva with Western blotting detection modes.

	Amersham ECL, ECL Prime, and ECL Select	Amersham ECL Plex	Fluorescent labels	ECF	Colorimetric stains	Radioisotopes
Typhoon FLA 9500	Not recommended	Fluorescence Full Multiplex	Fluorescence	Fluorescence	—	Phosphorimaging
Typhoon FLA 7000	Not recommended	Fluorescence Multiplex (Cy2/Cy5)	Fluorescence	Fluorescence	—	Phosphorimaging
ImageQuant LAS 4010	Chemiluminescence	Fluorescence Multiplex (Cy3/Cy5)	Fluorescence	Fluorescence	White light	For documentation of X-ray film
ImageQuant LAS 4000	Chemiluminescence	Fluorescence* Multiplex* (Cy3/Cy5)	Fluorescence*	Fluorescence*	White light*	For documentation of X-ray film
ImageQuant LAS 4000 mini	Chemiluminescence	Single detection* (Cy2)	Fluorescence* (UV or Blue)	Fluorescence* (Blue)	White light*	For documentation of X-ray film
ImageQuant LAS 500	Chemiluminescence	Single detection (Cy2)	Fluorescence (UV/Blue)	Fluorescence (UV/Blue)	Colorimetric (White light)	For documentation of X-ray film
ImageScanner III	For documentation of X-ray film	—	—	—	Colorimetric (White light)	For documentation of X-ray film

* Upgrade options

5.3.3 Typical protocols

Successful Western blotting is the result of carefully following each step of a workflow that starts with protein sample preparation, protein electrophoresis and transfer of proteins from a gel to a blot. Thereafter, the choice of methods for antibody probing depends on the detection systems that best suit your target protein(s) and imaging instrument. A detailed account of the entire workflow and a standard Western blot procedure can be found in the handbook, *Western Blotting – Principles and Methods* (7).

There are many detection systems based on chemiluminescent, fluorescent, chemifluorescent, colorimetric or radioisotopic techniques. The following probing and detection protocols are examples of the most frequently used methods: chemiluminescence with Amersham ECL, Amersham ECL Prime, and Amersham ECL Select, fluorescence with ECL Plex and chemifluorescence (ECF).

Amersham ECL, Amersham ECL Prime and Amersham ECL Select detection systems

1. Dilute the primary antibody in PBS-Tween or TBS-Tween.
2. Place the membrane (protein side up) in the primary antibody solution and incubate with agitation for 1 h at room temperature or overnight at 4°C. Always refer to manufacturers' recommendations.
3. Wash the membrane three to six times in PBS-Tween or TBS-Tween for 5 min per wash or according to manufacturers' recommendations.
4. Place the membrane in the secondary antibody diluted in PBS-Tween or TBS-Tween and incubate with agitation for 1 h at room temperature or overnight at 4°C.
5. Place the membrane in washing solution and wash four to six times for 5 min per wash.
6. Continue with detection as recommended for the selected detection reagent and imaging system.

Charge-coupled device (CCD) camera-based imaging

1. Allow the detection solutions to equilibrate to room temperature before opening the vials.
2. Mix an equal volume of detection solutions A and B, allowing sufficient total volume to cover the membranes. A volume of 0.1 mL/cm^2 of membrane is required.
3. Drain the excess washing solution from the washed membranes and place them, protein side up, on a sheet of Saran Wrap or other suitable clean surface. Pipette the mixed detection reagent onto the membrane.
4. Incubate for 1 min (Amersham ECL) or 5 min (Amersham ECL Prime, Amersham ECL Select) at room temperature.
5. Drain excess detection reagent by holding the membrane gently with forceps and touching the edge against a tissue.
6. Place the membrane, protein side up, on the CCD camera sample tray.
7. Place the sample tray in the CCD-camera and operate according to instructions. Choose automatic exposure time and capture the image.

Choose exposure times according to expected signal intensity if you are familiar with your system. Recommended is to begin with automatic exposure time and then adjust the time to find the optimal exposure. Alternatively, an increment function can be used (ImageQuant LAS 4000 series), in which the camera captures images at predetermined time points during a given time. Optimization of exposure time is further described in chapter 4.

Amersham ECL Plex detection system

1. Dilute the primary antibody of mouse or rabbit origin to optimal concentration in washing solution or blocking solution.
2. Incubate a blocked membrane (protein side up) with the diluted primary antibody for 1.5 h at room temperature, or over night at 4°C.
3. Rinse the membrane twice in washing solution, then wash the membrane twice for 5 min per wash in washing solution, with shaking, at room temperature.
4. Dilute the ECL Plex CyDye-conjugated secondary antibody, (prepared at a concentration of 1 µg/mL) to optimal concentration.
5. Incubate the washed membrane in the secondary antibody solution, protected from light, for 1 h at room temperature, with shaking.
6. Rinse the membrane three times in washing solution, followed by four washes in washing solution for 5 min per wash, with shaking, at room temperature and protected from light.
7. Rinse the membrane three times in PBS or TBS (without Tween-20).
8. Detect the secondary antibody signal by scanning the membrane using a laser scanner or CCD-camera with fluorescent capability. For best results, dry the membrane before scanning by placing it on Hybond blotting paper and incubate at 37°C to 40°C for 1 h, or at room temperature. Protect the membrane from light.

Chemifluorescence detection system using ECF substrate

1. Place the washed blot, protein side up, on an open sheet protector (made of a low fluorescence material to minimize background).
2. Apply the ECF substrate at 5 µg/cm^2 of membrane. Close the sheet protector slowly so the blot is completely covered with the substrate. Extrude any bubbles along with excess substrate using a laboratory wipe, and incubate at room temperature for 1 to 5 min.
3. The incubation time for optimal sensitivity depends on the target concentration on the blot and should be determined empirically for each new experiment.
4. Position the blot, protein side down, on the clean glass platen. If needed, blots can be air dried in the dark before imaging.
5. Detect the ECF signal by scanning the membrane using a laser scanner or CCD-camera with fluorescent capability. Protect the membrane from light.

5.3.4 Typical results

Monitoring signaling pathway activation with chemiluminescent detection of low abundance proteins

The activation of signaling pathways by drugs can be indicated by post-translational modifications (PTMs), such as protein phosphorylation. However phosphorylated proteins are often present in very low levels compared to the parent protein. For this reason, very sensitive detection is needed to measure a wide range of protein quantities. Western blotting followed by detection using chemiluminescent Amersham ECL Prime offers a practical solution when both high sensitivity and a broad linear dynamic range are important.

In this example, phosphorylation of STAT3 is evaluated in HeLa cells after treatment with IFN-α. Samples of lysates from HeLa cells (IFN-α-treated and untreated controls), were separated by gel electrophoresis and, blotted onto an Amersham Hybond-P membrane and probed with a phospho STAT3 specific antibody. Phosphorylated STAT3 (pSTAT3) was detected with Amersham ECL Prime (Fig 5.20.) and imaged using ImageQuant LAS 4000 mini. The membrane was then stripped and reprobed for actin, a housekeeping protein. By monitoring the expression levels of actin, it was possible to compensate for uneven sample loads on the gel. Levels of pSTAT3 were then normalized against corresponding actin levels.

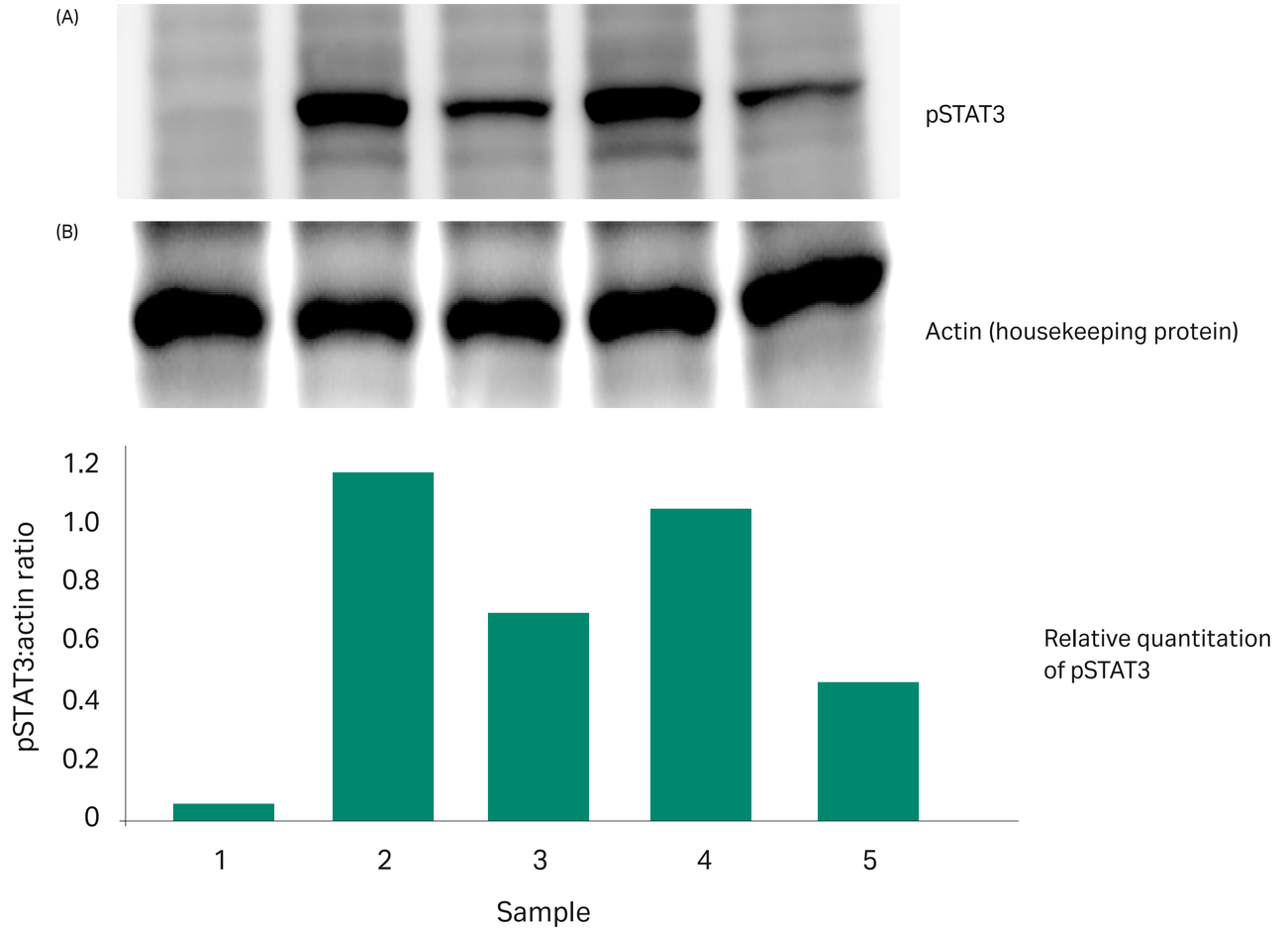


Fig 5.20. Amersham ECL Prime Western blotting detection of pSTAT3 and actin in cell lysates from untreated (1) and IFN-α treated HeLa cells (2 to 5). The result shows IFN-α-induced phosphorylation of STAT3 (A). The levels of pSTAT3 (A) are normalized to the levels of a housekeeping protein, actin (B), to correct for variation in total sample amount loaded. The biological variation of pSTAT3 levels in the samples is relatively quantitated to monitor the changes in STAT3 phosphorylation as a response to IFN-α treatment.

Multiplexed detection for normalizing against a housekeeping protein

Chemiluminescent Western blotting can require stripping and reprobing to normalize target protein levels against a housekeeping protein. However, stripping brings the risk of uneven loss of proteins. By using multiplexed detection with Amersham ECL Plex, the target protein and the housekeeping protein are probed with different CyDyes which eliminates the need to strip and reprobe the membrane.

The activation of ERK 1/2 was studied in the lysates of wildtype and knockout fibroblasts treated with fibroblast growth factor-2 (FGF-2). After the lysates were separated and blotted, the membrane was simultaneously probed with mouse anti-ERK 1/2 and rabbit anti-GAPDH, followed by Amersham ECL Plex anti-mouse Cy5 and Amersham ECL Plex anti-rabbit Cy3. Although protein quantitation analysis indicated that a similar amount of total protein was loaded in each lane, the intensity of the signals emitted due to detection of the housekeeping protein, GAPDH, showed that this was not the case (Fig 5.21). Without relating to the GAPDH levels, no significant pattern of ERK 1/2 activation related to wild type and knockout cells was seen. However, when normalized against GAPDH signals, ERK 1/2 levels increased in knockout cells after stimulation with 2 and 4 ng/mL FGF-2.

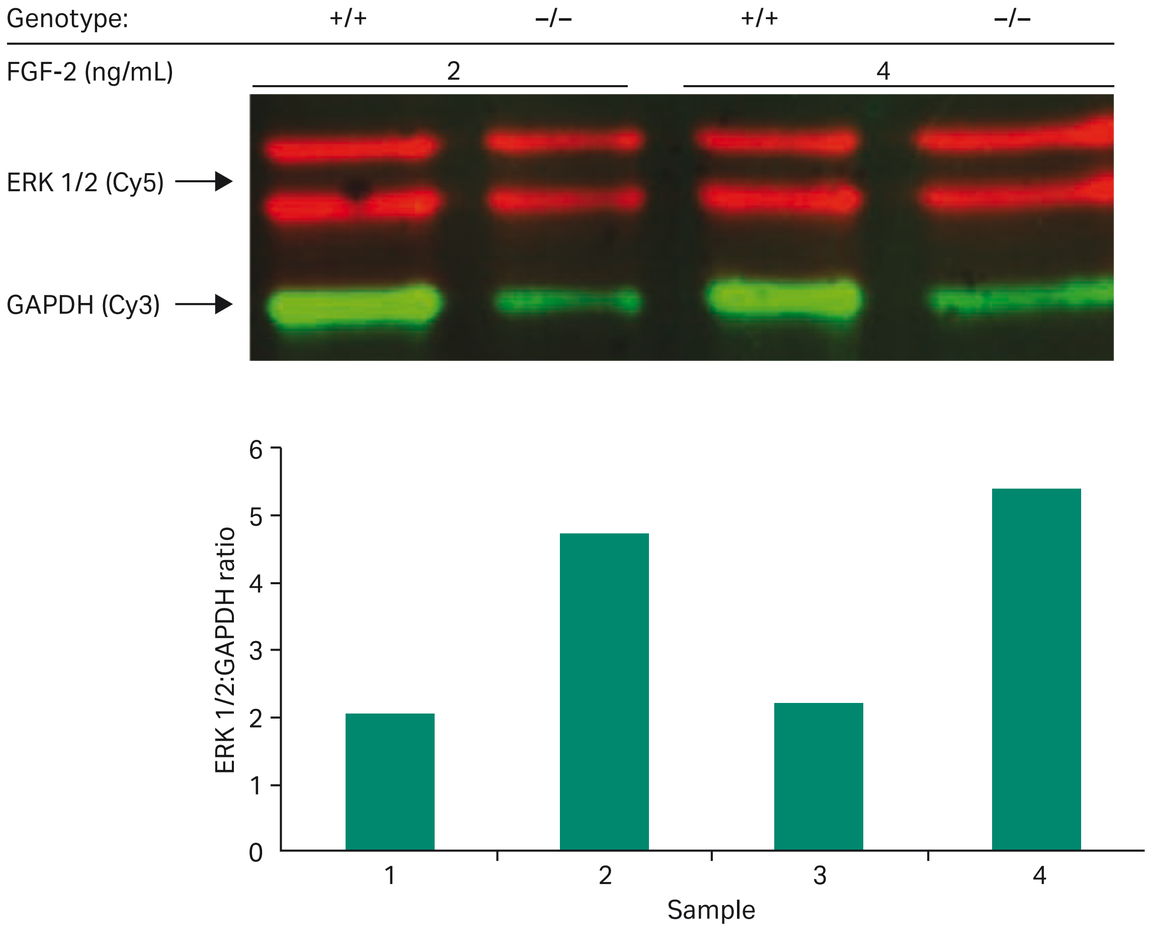


Fig 5.21. Protein expression can be quantitated in relation to a housekeeping protein (GAPDH) by detecting two proteins on the same blot. This image shows Amersham ECL Plex detection of ERK 1/2 in wild type (+/+) and knockout (-/-) mouse embryonic fibroblasts after treatment with FGF-2. ERK 1/2 (Cy5) and GAPDH (Cy3) were targeted using specific primary antibodies followed by secondary ECL Plex Cy5 and Cy3 antibodies. The diagram shows expression levels of ERK1/2 after FGF-2 stimulation following normalization to GAPDH. Data courtesy of Dr. Jin-Ping Li and Dr. Juan Jia, Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden.

5.4 Quantitation of proteins in solution

5.4.1 Dyes 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 in the protein sample. Colorimetric methods, such as the Bradford or Lowry assays, do not work well in the presence of contaminants and must be read within a very limited period of time. High protein-to-protein signal variability is also common with colorimetric detection (7).

The 2-D Quant Kit provides indirect colorimetric quantitation of proteins in solution by measuring absorbance at 480 nm. The kit is suitable for high-resolution electrophoresis techniques such as 2-D electrophoresis or SDS-PAGE or IEF. As detergents, reductants, chaotropes and carrier ampholytes are widely used in the preparation of such samples and are incompatible with other protein assays. This kit quantitatively precipitates proteins while leaving interfering substances in solution. The assay is based on the specific binding of copper ions to protein. Precipitated proteins are suspended in a copper solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration.

Proteins can be more accurately detected in solution using fluorescent dyes. In their free state the dyes are not very fluorescent, but when bound to proteins, their fluorescence is enhanced. The dyes are typically quite specific for their target molecules, and thus work well even in the presence of contaminants. Some dyes bind specifically to, for example, the detergent coating on proteins or to hydrophobic regions, and are unaffected by the presence of contaminating nucleic acids and reducing agents.

Protein detection using fluorescence is much more sensitive than non-fluorescent methods. Whereas non-fluorescent microtiter plate detection methods have sensitivity in the µg/mL range, fluorescence-based detection is generally in the ng/mL range. Appendix 3 gives a comprehensive list of fluorescent dyes and the wavelengths for maximum excitation and emission.

5.4.2 Instrument compatibility

The available choices of fluorochrome depend on the type of instrument you use. For details of instrument and fluochrome compatibility, see Appendix 3. The type of instrument used also determines the type of sample plate to use. For all fluorescence detection, sample plates must be made of low-fluorescent material. Transparent plates are essential if samples are imaged from below as with laser scanners in the Typhoon-series. CCD cameras acquire images from above the sample, and can use both epi-illumination and trans illumination. Therefore, the sample plates must suit the type of illumination used. Other aspects of sample plates to consider include: the shape of the wells, transparency, and volume.

5.4.3 Typical protocol

1. Working stain preparation

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

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

2. 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 160 µL for Typhoon.

   2.2 Pipette the samples into the wells of the microtiter plate.

   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 manufacturers' literature).

   The manufacturers' instructions recommend heating the samples at 90°C to 96°C for 10 min and cooling to room temperature before pipetting the samples. We find that heating the samples does not effect the performance of assays when using Typhoon.

3. Imaging

   Imaging with Typhoon scanners:

   3.1 Attach the microtiter plate holder to the Multi Stage according to the user manual of the instrument. Place the microtiter plate in the microtiter plate holder.

   3.2 Acquire the image with the recommended instrument setup for the fluorochrome used (see Appendix 3). The choice of pixel size will depend on the individual experiment. The PMT voltage setting should be adjusted to prevent signal saturation.

4. Analysis

   4.1 Display the image using the analysis toolbox of the ImageQuant TL software. If saturated pixels are present, a new image should be acquired with either a lower PMT voltage or a shorter exposure time. Image analysis of the microtiter plate can be made in the Array module of the ImageQuant TL software, which is described in Chapter 4.

   For the greatest accuracy, the standards should be of a similar size and source the protein under study.

5.5 Using naturally occurring fluorescent proteins

5.5.1 Green fluorescent protein and its variants

Green fluorescent protein (GFP), first isolated from the jellyfish *Aequorea victoria*, was cloned in 1994. Genetic engineering of the original green fluorescent protein from Aequorea victoria has resulted in new fluorescent probes. A wide variety of GFP derivatives are commercially available, and range in color from blue to yellow. Today, they are some of the most widely used in vivo reporter molecules used in biological research. Although native or wild type (wt) green fluorescent protein produces significant fluorescence and is extremely stable, the excitation maximum is close to the ultraviolet range. By introducing a single point mutation altering the serine at position 65 into a threonine residue the excitation maximum of green fluorescent protein is shifted to 488 nanometers. This mutation is featured in the most popular variant of green fluorescent protein, termed enhanced GFP (EGFP). Enhanced GFP can be imaged using commonly available filters designed for fluorescein and is among the brightest of the currently available fluorescent proteins. The spectral properties of green fluorescent protein and some of its variants are given in Table 5.11.

Table 5.11. 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 (% of EGFP)
EBFP	383	445	29 000	0.31	27
ECFP	439	476	32 500	0.4	39
GFP (wt)	395, 470	509	21 000	0.77	48
EGFP	489	508	56 000	0.6	100
EYFP	514	527	83 400	0.61	151
DsRed (RFP)	558	583	75 000 (tetramer)	0.79	176

5.5.2 Instrument compatibility with GFP and its variants

For detailed information of the compatibility of Cytiva instruments with GFP and its variants see Appendix 3.

5.5.3 Examples of applications using GFP

Recombinant protein vectors are commonly used to overexpress cloned proteins. The advantage of using a histidine-tagged GFP fused to a recombinant protein is to facilitate its purification and detection. In some cases, tags may improve the stability and solubility of recombinant proteins. In this example a $(histidine)_8$-tagged membrane protein, YedZ-TEV-GFP-$(His)_8$ was purified from a crude *Escherichia coli* lysate by immobilized metal ion affinity chromatography (IMAC) using a HisTrap™ FF crude column. Figure 5.22 shows the chromatogram of the purification.

Fractions were analyzed in SDS-PAGE and the gel was scanned to detect the fluorescent GFP portion of the recombinant protein and then post stained with Coomassie Blue to detect total protein (Fig 5.23). A one step purification of a crude lysate is often sufficient as demonstrated by the gel analysis.

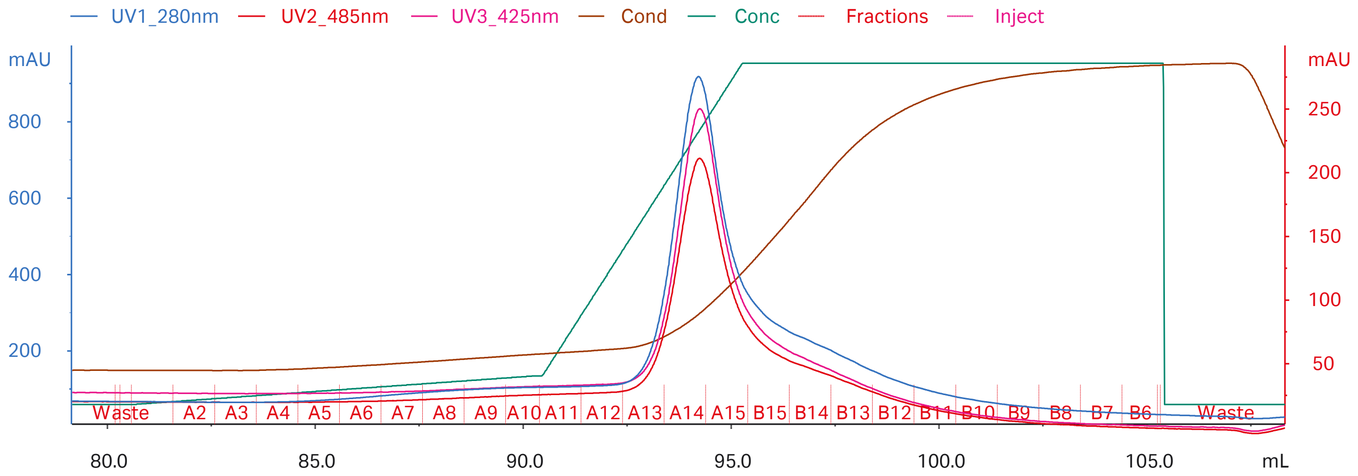


Fig 5.22. Purification of a $(histidine)_8$-tagged membrane protein, YedZ-TEV-GFP-$(His)_8$ directly from crude, solubilized *E. coli* lysate using HisTrap FF crude, 1 mL. In the chromatogram, blue =A280; cerice = A425 (to detect YedZ); red = A485 (to detect GFP); brown = conductivity; green = % elution buffer; red = collected fractions. The overexpression vector was kindly provided by Dr J.-W. deGier, Centre for Biomembrane Research, Stockholm, Sweden.

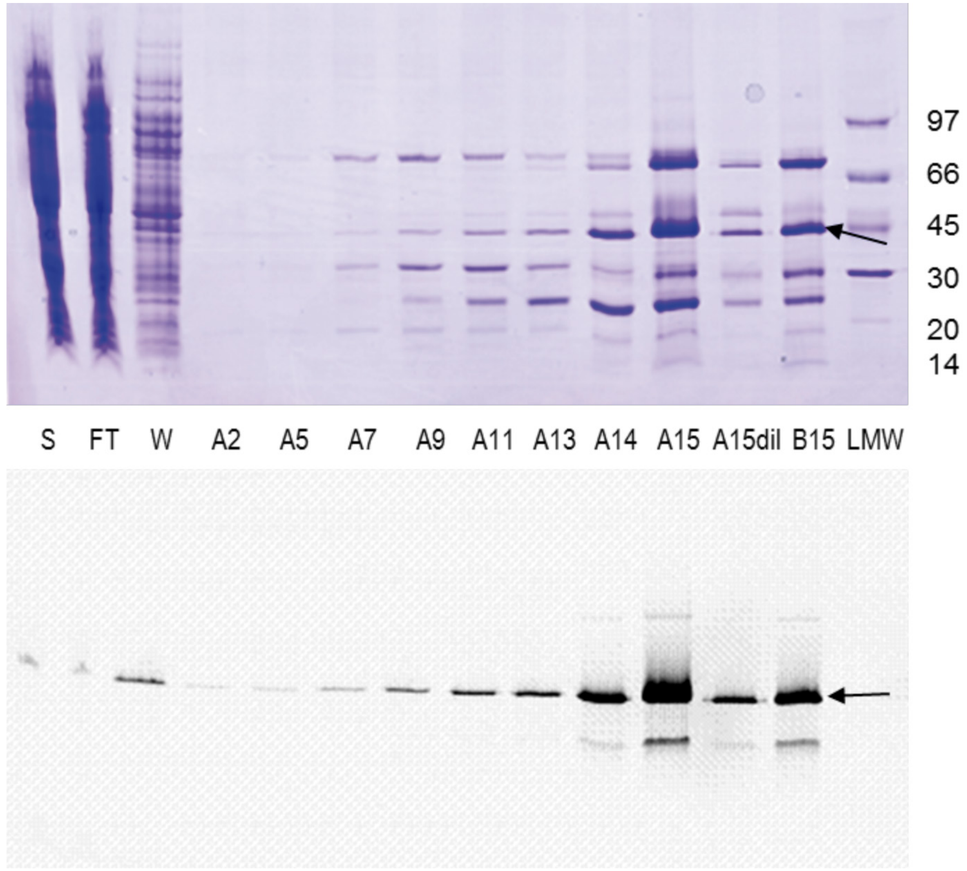


Fig 5.23. Different fractions from the purification process were analyzed on ExcelGel SDS Gradient 8-18 gel. S = start sample of solubilized *E. coli* lysate; FT = flow though, W = wash and eluted fractions from chromatogram in Figure 5.22. LMW, low molecular marker loaded on the right side, size in kDa. The gel was scanned to detect the fluorescent GFP portion of the fusion protein (bottom) and then post stained with Coomassie Blue to detect total protein (top). An arrow indicates the band corresponding to YedZ-TEV-GFP-$(His)_8$.

5.5.4 Phycobiliproteins

Phycobiliproteins are stable and 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 (FRET), conveying it to a special pair of chlorophyll molecules located in the photosynthetic reaction center. Because of their biological role, phycobiliproteins possess exceptional spectral properties. Quantum yields up to 0.98 and molar extinction coefficients of up to 2.4×10^6 have been reported (8). Phycobiliproteins have been covalently conjugated to antibodies and other proteins to generate probes that are readily detectable and which may be useful for various applications. Highly purified phycobiliproteins are commercially available and the optical properties of most common phycobiliproteins are listed in Table 5.12.

Table 5.12. Properties of phycobiliproteins

Protein	Excitation Max (nm)	Emission Max (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum Yield
B-phycoerythrin	546, 565	575	2 410 000	0.98
R-phycoerythrin	496, 546, 565	576	1 960 000	0.82
Allophycocyanin	652	658	700 000	0.68

The broad excitation spectra, particularly of the R-phycoerythrin conjugates, allow phycobiliproteins to be efficiently excited using different types of imaging instrumentation with different excitation sources.

5.5.5 Instrument compatibility with phycobiliproteins

Compatibility of Cytiva instruments with phycobiliproteins see detailed list in Appendix 3.

5.6 Phosphorimaging

Despite the alternatives to radioisotope-based detection systems, there are still some areas in which radioisotopes continue to offer some advantages. Radioactive systems in combination with phosphorimaging screens continue to be used in Southern and Northern blotting because of their sensitivity and speed. Phosphorimaging screens are 10 to 100 times more sensitive than film for most radioisotopes, and detect almost any source of ionizing radiation, including commonly used isotopes such as ^{32}P, ^{33}P, ^{35}S, ^{14}C, ^{3}H and ^{125}I. They generate signals across a linear dynamic range of up to five orders of magnitude; they also offer precise quantitation of signals in approximately one-tenth the exposure time of traditional autoradiography film.

5.6.1. Autoradiography

Radioactive labeling of biomolecules followed by autoradiography on photographic film can still be used as a detection method. Autoradiography provides good spatial resolution without destroying the sample when the film has close contact with a flat sample, such as a blotted membrane. Crystals of silver halide in photographic emulsion respond directly to β-particles and γ-rays by converting ionic silver to atomic silver, which produces a stable latent image. During film development, the silver atoms catalyze the reduction of the silver halide crystals to metallic silver to give an autoradiographic image.

The blackening of the film is directly proportional to the amount of radiation in the sample. Scanning the film with a densitometer can therefore roughly quantitate the distribution of radioactivity in a direct autoradiograph.

For most isotopes, direct autoradiography is limited due to inefficient transfer of emission energies to film. Low energy emissions from isotopes such as ^{3}H, ^{35}S and ^{14}C fail to reach the film since they are absorbed within the sample, while highly energetic β-particles from ^{32}P or γ-rays from ^{125}I, ^{131}I, ^{51}Cr or ^{75}Se (Table 5.13) pass through the film so that only a fraction of their energy is captured and recorded. These issues can be overcome by converting the emitted energy to light.

Table 5.13. Radioisotopes most commonly used to label biomolecules.

Radioisotope	Emitted radiation	Energy (max) MeV	Half life
^{3}H	β	0.0186	12.43 years
^{14}C	β	0.156	5730 years
^{35}S	β	0.167	87.4 days
^{33}P	β	0.249	25.4 days
^{32}P	β	1.709	14.3 days
^{125}I	γ	0.035	60.0 days
^{133}I	γ	0.364	8.04 days

5.6.2 Converting radiation to light — Fluorography

Conversion of radioisotopic emissions to light greatly increases the sensitivity of image acquisition. A dense scintillator emits photons of blue or ultraviolet light in response to excitation by β-particles or γ-rays, and is recorded on radiographic film or exploited in scintillation counters. Fluorography and screen scintillators are typically used for low energy emissions and to increase the capture of highly penetrative emissions.

In fluorography, a scintillator solution provides maximum contact between the isotope and the scintillator. This allows energy from weak emissions to be transferred to the scintillator molecules that then emit ultraviolet light. Provided the sample is translucent and colorless, light can travel further than β-particles, to produce an image on film. Fluorography is relatively insensitive for ^{14}C, ^{35}S or ^{33}P on very thin samples such as blotted proteins but substantially increases sensitivity for ^{3}H.

5.6.2.1 Screen scintillators

Screen scintillators or intensifying screens are plastic screens with a dense layer scintillator material, usually calcium tungstate, that are placed behind a film. Emissions that pass through the film are absorbed by the scintillator, which produces light. Screen scintillation is recommended as a method that prevents wastage of γ-rays or β-particles that can penetrate through and beyond film.

The light produced by intensifying screens superimposes a photographic image on the autoradiographic image. The screens can be used for blots containing ^{32}P or γ-emitting isotopes. Screens do not improve detection efficiency of ^{3}H, ^{14}C or ^{35}S since their β-particles do not penetrate through film. It is essential to use a "screen-type" film such as Hyperfilm-MP. For more details about films and accessories, see the *Autoradiography Product Guide* (11) and Table 5.14.

Table 5.14. Summary of films for imaging in Western blotting analysis

Film	Applications	Emulsion type	Performances
Hyperfilm ECL	ECL	Single coated	Sensitive, high contrast
Hyperfilm MP	Direct ^{32}P, ^{33}P, ^{125}I Fluorography ^{3}H, ^{14}C, ^{35}S	Double coated	Sensitive, high resolution

The increased sensitivity gained from converting radioactive emissions to light is partly offset by two disadvantages. First, resolution is decreased, as both the primary emissions and the secondary scintillations disperse. Furthermore, when radioactive emissions are converted to light the response of film is no longer linear.

Low light intensities produce disproportionately faint images that can potentially result in misinterpretation of data. This problem can partly be overcome by pre-exposing film to a hypersensitizing light flash and then cooling the film to -70°C. Hypersensitization of film by pre-flashing requires a short flash exposure of about 1 msec. A flash of this duration can be provided by photographic flash units that filter light to an appropriate intensity and wavelength.

Long duration flashes tend to increase the background without further hypersensitization.

Scintillation reagents and intensifying screens are not recommended for storage phosphor work. Do not store screens and samples at -70°C for exposure when performing storage phosphor work.

5.6.2.2 Storage phosphor screens

Storage phosphor screens or phosphorimaging plates (IP) are reusable (except for tritium screens) and can capture two-dimensional images of radiolabels. The image plate was introduced in 1983 by Fujifilm as an X-ray detector system that is analogous to the intensifying screen. The functional layer consists of an organic binder in which grains of photo-stimulable phosphor (PSP) or storage phosphor is imbedded.

The energy of the ionizing radiation is absorbed in crystals of, for example, BaFBr that is doped with traces of Eu^{2+}. The energy is released when stimulated by additional light, in a process known as photostimulated luminescence (PSL) (9). In principle, the deposited energy from an X-ray photon (or β- or γ-rays) forms metastable excited states in the crystal called "luminescence centers". The energy can be released as fluorescence (prompt release of light), phosphorescence (spontaneously fading over time) or with PSL. When an exposed storage phosphor screen is scanned with the appropriate wavelength, local photostimulation releases the trapped energy as light (PSL) with a higher energy and shorter wavelength than the stimulation light (10).

Storage phosphor screens are the best option for high sensitivity radioisotopic imaging. They offer a wide linear dynamic range (up to 5 orders of magnitude) to allow quantitation and visualization of both weak and strong signals in a single exposure. Moreover, they capture latent images produced by ionizing radiation (X-rays, β and γ emissions from isotopes such as ^{14}C, ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{33}P, and ^{35}S).

Light from storage phosphor screens is released in proportion to the amount of radioactivity after laser-induced stimulation. Laser-based systems, such as Typhoon, produce digital images that allow quantitation of subtle differences in signal intensity over a wide linear dynamic range using storage phosphor. Typically, exposure time is reduced by 50% to 90% when compared to exposures with conventional film. Storage phosphor screens are not degraded by exposure to working levels of radioactivity and they can be reused after exposure in an ImageEraser light box.

☞ Tritium screens can not be re-used once they are exposed. However unexposed parts of the screen can then be used on subsequent exposures.

The spatial resolution of an image plate is determined by the grain size (typically around 5 µm for the BAS-IP), and the structure and thickness of the storage phosphor layer. However, the resolution of the resulting image also depends on sample preparation, the radioisotope used, and the distance between the radiation source and the imaging plate. For laser scanners, the sizes of the laser spot and how the PSL is collected also affects resolution of the final image.

5.6.3 Instrument compatibility

Table 5.15 shows the compatibility of Cytiva's imaging systems for different modes of phosphorimaging.

Autoradiography film can be documented with either ImageScanner III or ImageQuant LAS-series using white trans-illumination. However, quantitation is limited due to the low linear dynamic range of film. Typhoon FLA scanners can also be used for documentation purposes by the digitization function with a fluorescent plate on top of the film. The latter method is strictly for documentation without any quantitative possibilities.

Phosphorimaging with Typhoon laser-based scanners using imaging plates provides excellent images for quantitative analysis of radioisotope labeled samples.

Table 5.15. The compatibility of Cytiva's imaging systems with different phosphorimaging modes.

		Method	
		Film documentation	**Phosphorimaging**
ImageScanner III	Mode	Transparent	NA
	Filter	Blank	NA
ImageQuant LAS 500		Colorimetric	NA
ImageQuant LAS 4000 mini (with upgrade configuration)	Light source	Trans UV + pink plate*	NA
	Emission filter	None (Through)	NA
ImageQuant LAS 4000*/4010	Light source	White light table	NA
	Emission filter	None (Through)	NA
Typhoon FLA 9500	Excitation (nm)	473/532	635
	Emission filter	LPB/LPG	IP
Typhoon FLA 7000/7000 IP	Excitation (nm)	473/532	650
	Emission filter	Y520/O580	IP

* Upgrade option

5.6.4 Phosphor screen compatibility

A variety of phosphor screens are available depending on sample type and need for resolution (Table 5.16). Multipurpose imaging plates can be used for most radioisotopes. Whereas ^{3}H-imaging requires a TR-screen (without a protective layer) and the sample has to be placed in close direct contact with the screen since the low energy β-emission is unable to penetrate through dense materials. Ideally, TR-screens should only be used once since direct contact with the sample causes lasting contamination. However, it is possible to use plates more than once as long as samples are placed on previously unexposed areas.

Make sure that ^{3}H samples are completely dried for example in an vacuum oven.

Most ^{3}H samples can lead to an irreversible contamination due to diffusion of ^{3}H into the TR-screen.

All phosphor screens from Cytiva are compatible with the Typhoon laser-based scanners and other similar scanners with red excitation and suitable filters (B390 or similar). The BAS-IP series of imaging plates have magnetic backing and fit the Phosphor stage of the Typhoon laser scanners. No glass plate lies between the imaging plate and the optics. Other imaging plates, such as mounted or unmounted GP imaging plates can be used if they are placed on the glass Fluor stage of Typhoon FLA-series. See Table 5.16 for detailed information about compatibility of Cytiva's imaging systems with different phosphor screens.

The cassettes used for exposure are mainly similar to the cassettes used for film exposure. The purpose of the cassettes is to protect the screen from light and background radiation during exposure. Phosphor screens are significantly more sensitive than X-ray film, and therefore it is important to check that no radioisotope contamination is present in the cassettes. Mounted screens are used with cassettes where the backing of the screen serves as a top cover for the cassette. Unmounted screens can be used in any cassette as long as the size of the screen fits into the cassette. The screens should be erased immediately before and after use.

Table 5.16. The compatibility of Cytiva's imaging systems with different phosphor screens.

	Typhoon FLA 9500	Typhoon FLA 7000/7000 IP
Maximal scanning area	35 × 43 cm on Phosphor Stage 40 × 46 cm on Fluor Stage	20 × 40 cm on Phosphor stage 24 × 40 cm on Fluor Stage
Phosphor Screen type		
BAS-IP MS Magnetic for Multipurpose use	BAS-IP MS 2025 BAS-IP MS 2040 BAS-IP MS 3543	BAS-IP MS 2025 BAS-IP MS 2040
BAS-IP SR Magnetic, High resolution	BAS-IP SR 0813 BAS-IP SR 12.7 × 12.7 BAS-IP SR 2025 BAS-IP SR 2040	BAS-IP SR 0813 BAS-IP SR 12.7 × 12.7 BAS-IP SR 2025 BAS-IP SR 2040
BAS-IP TR Magnetic, Single-use for ^{3}H	BAS-IP TR 2025 BAS-IP TR 2040	BAS-IP TR 2025
Unmounted GP Multipurpose use	Unmounted GP 20 × 25 cm Unmounted GP 35 × 43 cm	Unmounted GP 20 × 25 cm
Mounted GP Multipurpose use	Mounted GP 20 × 25 cm	Mounted GP 20 × 25 cm
Unmounted Tritium Single-use for ^{3}H	Unmounted Tritium 19 × 24 cm	Unmounted Tritium 19 × 24 cm
Mounted Tritium One time use for ^{3}H	Mounted Tritium 19 × 24 cm	Mounted Tritium 19 × 24 cm

5.6.5 Workflow

1. Clean the surface of the storage phosphor screen and the inside of cassette with a soft tissue.
2. Erase the storage phosphor screen completely. Image Eraser can typically erase a storage phosphor screen in 15 minutes

☞ If the screen does not clear after repeated erasing cycles, check for contamination.

3. Wrap the radioisotope sample with thin plastic film. Make sure not to wrinkle the wrapping film. Use double layers to protect the screen if the sample contains volatile solvents

☞ Tritium storage phosphor screens require samples to be placed in direct contact with the screen. Tritium storage phosphor screens are easily contaminated and can be used only once.

4. Open the top cover of the cassette and place the sample into the cassette. Make sure that the sample surface faces up.
5. Place the storage phosphor screen in the cassette, with the exposure surface of the storage phosphor screen facing the sample. Note the position of the notch on the screen to keep track of the direction of the sample and then close the cassette.

☞ The scanner may need some time to warm-up. Make sure to start up the scanner in good time before exposure.

☞ The exposure time of the storage phosphor screen is approximately one-twentieth of the time required for X-ray film. That should be considered when setting the initial exposure time. For increased image quality, keep the time between exposing the screen and reading it with the scanner to a minimum.

☞ Increased exposure time will increase the intensity of the signal.

☞ Background radiation can vary depending on your location and can affect the lower limits of detection. Therefore, proper shielding may be needed to lower background radiation levels.

✋ Do not stack cassettes during exposure.

6. Dim the ambient light to 20 lux or less before opening the cassette or moving an uncovered storage phosphor screen and protect exposed storage phosphor screens from light until the reading is finished.
7. If a Typhoon FLA scanner is used, position the phosphor stage with the storage phosphor screen face down. The PMT voltage setting may need optimization by using a short exposure time and then reading the result before scaling up the exposure time proportionally. As a general recommendation Typhoon FLA 7000 and Typhoon FLA 7000 IP can be set at 700 volts and Typhoon FLA 9500 can be set at 800 volts.

5.6.6 Typical results

Figure 5.24 shows a series of ^{14}C standards scanned in a Typhoon FLA 9500 to show detection over a wide dynamic range between 0.087 µCi/g and 1000 µCi/g.

Figure 5.25 shows autoradiographs of a rat brain using three different types of imaging plate.

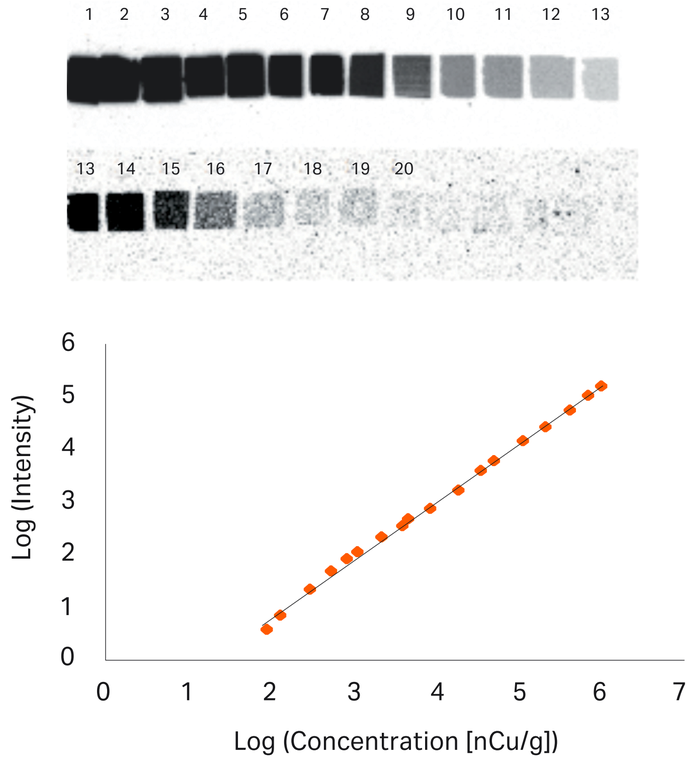


Fig 5.24. [^{14}C] Standards for autoradiography exposed for 5 hours to a BAS-IP MS imaging plate. The plate was scanned in Typhoon FLA 9500. The scanned image is divided into two images to visualize the dynamic range (overlap of sample 13 between the images). Samples 1 to18 gave a linear response over 4 orders of magnitude (from 1000 µCi/g to 0.087 µCi/g) as shown in the log-log plot. Longer exposure time gives higher intensities and allow detection of samples with lower radiation concentrations.

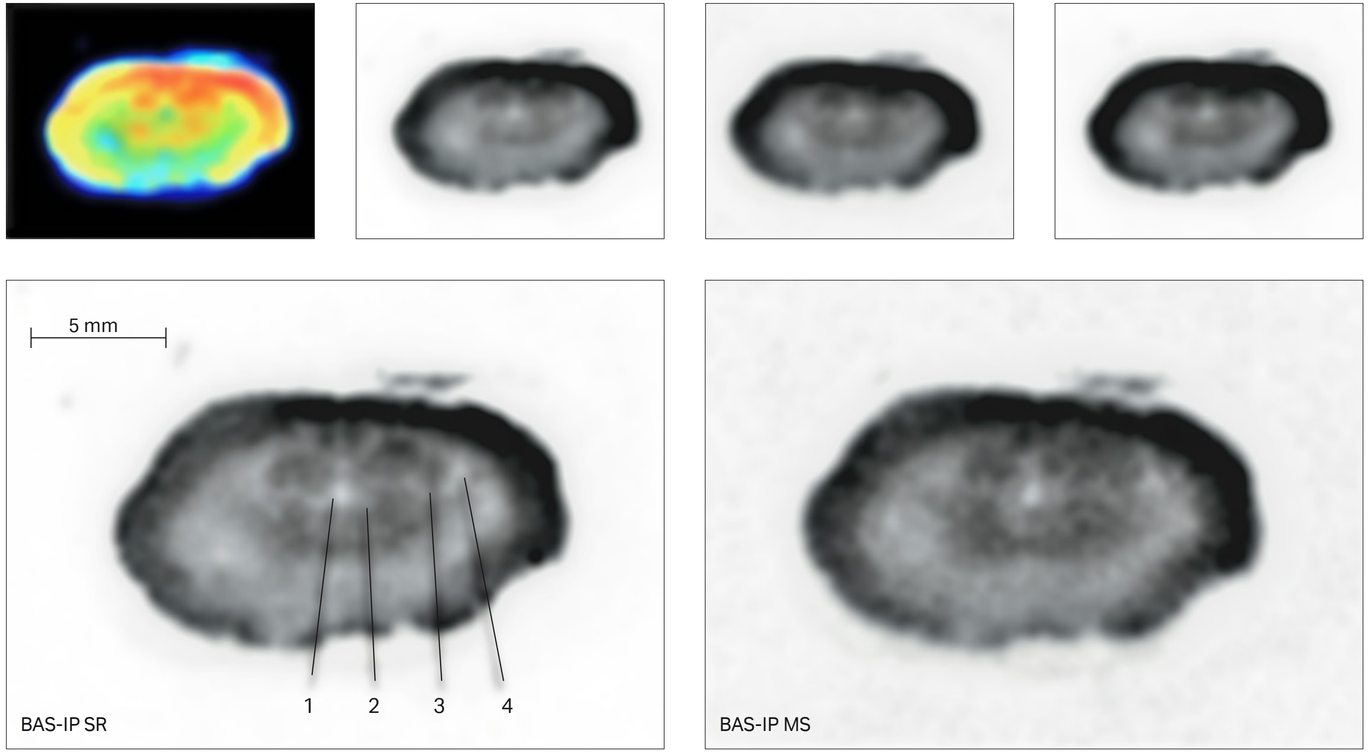


Fig 5.25. Sample: Autoradiography images of rat brain. [^{125}I] Somatostatin (0.6 nM, 0.05 MBq/mL) on cryosections from brain exposed for 24 hours to three different kind of imaging plates. All plates are scanned in Typhoon FLA 7000. The upper left corner shows a color-coded autoradiogram of ^{125}I. Small images in the grayscale in upper row show from left to right exposure to BAS-IP SR, BAS-IP MS and Mounted GP. The lower row shows the gain in resolution between BAS-IP SR and BAS-IP MS. The regions 1-4 can clearly be distinguished in the lower left image. 1) Third ventricle 2) Thalamus 3) Hippocampus 4) Lateral ventricle.
Images courtesy of Håkan Hall, Preclinical PET Platform, Dept. of Medicinal Chemistry, Uppsala University and Kristina Magnusson, Dept. of Public Health and Caring Sciences, Uppsala University.

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06
Practical recommendations

6.1 Introduction

There are many ways to improve the quality of imaging results. In this chapter you will find useful tips and recommendations for the various stages of an imaging experiment, from transfer of proteins from gel to membrane through to instrument operation and data analysis.

6.2 Sample preparation

The importance of good sample preparation cannot be stressed enough and performance of even the most sophisticated imagers is limited by sample quality. Sample quality can be affected at all experimental stages as a direct result of sample preparation. This section lists some points to consider during protein electrophoresis and transfer of proteins from gel to membrane in Western blotting applications.

To increase your chances of a successful analysis it is important to start any imaging experiment with an understanding of your sample material and have a clear picture of the quality of information you expect to gain. More detailed information on sample preparation can be found in the handbook, *Protein Sample Preparation* from Cytiva (1).

Use appropriate sample support material

As a general recommendation, when working with fluorescence applications use low-fluorescent sample support materials (e.g., gels, membranes, glass plates and microplates) since they will improve the analytical sensitivity and linear range. The shape of the wells in microplates, is critical to excitation and collection of fluorescent light. Therefore, clear, flat-bottom wells are recommended as they provide a large imaging area and uniform surface characteristics. Microplates with clear bottoms and black or opaque walls can also be used. Use clean containers that are only used for fluorescent staining of gels and do not mix different trays used for different fluorescent stains (i.e., one tray used per specific fluorescent stain). To prevent an uneven background, stain one gel per container. Remember to handle gels with care since physical damage may cause background staining.

☞ Some containers are known to absorb certain fluorescent stains. Use containers that do not interfere with your stain in post-staining procedures.

Glass is not always inert; SYPRO™ and SYBR™ stains are adsorbed by glass surfaces, while polypropylene containers are better suited for these stains. Refer to the dye manufacturer's product information for details on handling of specific dyes.

Beware of fluorescent contamination

Indicator dyes such as, bromophenol blue, xylene cyanol and other tracker dyes, have fluorescent properties. Avoid using dyes in fluorescent applications. since they can mask fluorescence bands and cause problems with high background on Western blotting membranes. If tracker dyes are present then remove the section of gel where they are present prior to transfer or before imaging.

☞ Wear powder free gloves.

Dust and powder from gloves is a possible source of background contamination. Use powder free gloves instead and rinse gloves with distilled water before handling samples and preparing reagents. Dust contaminant in buffer solutions and gels can cause of elevated backgrounds that negatively effect image quality and quantitation. To obtain optimal results, use high-grade chemicals and prepare buffers with spectroscopic grade solvents that have low autofluorescence. When appropriate, autoclave or filter-sterilize solutions and buffer stocks to eliminate the possibility of microbial contamination.

Avoid excessive exposure of fluorochromes to direct light

To prevent photobleaching, fluorochromes and fluorescently labeled 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.

Optimize imaging conditions for gels

There are several steps before gel imaging that can affect the final result. Select a gel density that provides the best resolution and separation profile for your proteins of interest (see section 5.1).

If you are casting your own gels, avoid generation of air bubbles when casting gels, since they can cause artifacts and interfere with quantitation.

When staining, ensure that the containers used are clean and are only used for fluorescent staining of gels. Make sure not to mix up different trays used for different fluorescent stains — one tray used per specific fluorescent stain. To prevent an uneven background, only stain one gel per container. It is important to handle gels with care since physical damage may cause background staining.

Optimize imaging conditions for membranes

Fluorescent Western blotting results are affected by several parameters that can be improved by small adjustments. Initially, use a low fluorescent membrane and if you use a fluorescent size marker. Leave an empty lane between the size marker and samples to prevent the marker signal interfering with sample signal. Do not mark membranes with ballpoint pens since the ink can contaminate membranes and trays and is visible in fluorescent scans. Mark membranes by cutting a corner off the membrane to ease identification during handling. This is also very useful in a multi-membrane scan to be able to discriminate them from each other during image analysis.

- When assembling a Western blot sandwich, remember to remove the stacking gel and remove all air bubbles between the different layers, as bubbles will obstruct protein transfer.
- If you have small pieces of gel on the membrane after transfer, ensure that these are removed before blocking the membrane. Pieces of gel can cause black spots on fluorescent images.
- Do not touch membranes probed with the secondary antibodies since this could leave marks on the fluorescent image. Always use forceps (slide forceps are recommended) when handling membranes.

6.3 Pre-imaging

Final preparation involves handling gels or membranes prior to imaging and care should be taken to minimize sample background and non-uniformity.

Handle glass stages and trays with care

Scratches on glass stages or trays scatter light and collect dirt or solutions that interfere with data collection and quantitation. Use clean plates and trays and avoid leaving fingerprints since they increase background in both fluorescent and colorimetric applications.

- If the surface of a glass stage is soiled or contaminated then it can be cleaned by following this three-step procedure; wash with isopropanol, then with 10% hydrogen peroxide, and finally rinse the plate a few times with distilled water.
- SYBR Gold binds strongly to glass stage and can cause contamination.
- If a tray is stained then rinse it with isopropanol or NaOH and then wash with water and dry it sufficiently before use. For difficult cases of persistent background contamination a platen can be cleaned by photobleaching with 254 nm UV light.

Handling of gels

If a sample is physically uneven on one side (such as an agarose gel), then place the smooth side down on the glass surface. Avoid trapping air bubbles as they can appear on the scanned image and interfere with quantitation. By placing a small amount of water on the scanner bed, it is possible to prevent the gel from drying and the formation of air bubbles. Moreover, water on the scanner bed simplifies the handling of gels. However be aware that too much water can also cause the sample to move.

For CCD camera imaging, using a UV trans tray, we recommend using a gel sheet slightly larger than the sample size. The gel sheet can be used repeatedly up to 20 times. After use, wash the gel sheet with mild detergent, rinse with water and dry it well.

Adjust membrane conditions to imaging and detection method

- It is important to have protein side towards the detector, regardless of the detection method you use.

Imaging results are also affected by the condition of the membrane. In chemiluminescent applications the membranes are initially wet but can dry out depending on the exposure time. However, this does not affect the results negatively. In fluorescent applications it is recommended, for PVDF, that dried membranes are used, since they produce an even background and a higher signal to noise ratio.

- When scanning dried membranes place a low fluorescent glass plate on top of the membranes to secure them on the laser scanner bed.

Nitrocellulose membranes should preferably be scanned while they are wet since they are fragile when dry. Moreover, scanning wet membranes on a wetted scanner bed reduces the risk of trapping air bubbles. Should air be trapped then it can easily be expelled by rolling a 10 mL pipette or similar across the membrane. To prevent contamination of the sample, glass tray and imaging platform, use low-fluorescence hybridization bags to sandwich the wet membrane. Lay one edge of the membrane inside an open bag/sheet protector, and then slowly lower the entire membrane while displacing any bubbles 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.

6.4 Sample placement

The careful placement of the sample on the imager is important since it prevents the introduction of artifacts, such as air bubbles, dust, or interference patterns.

Save time by placing the sample in an optimal scanning direction

The scanning direction and starting points for scanning vary between different models of scanners. If the sample is smaller than the entire scan area, you can save time by placing the sample close to the scanning start point. For example, place the sample in the lower left corner of the scanning area if the scanner head starts from that area. Place additional samples so that they line up along the scanning direction to minimize scanning time.

Use microarray slide holders when scanning glass slides on Typhoon

A microarray slide holder should be used to scan glass microarray slides on Typhoon. The slide holder fixes the slide in position so that the imager can accurately collect data. Wear powder free gloves to prevent oils from your hands from coming in contact with the glass platen or the microarray slide and make sure the sample on the slides are facing down in the slide holder.

6.5 Instrument operation

The detection and measurement of signals can be enhanced in a number of ways.

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 the instrument's control software, saturated data appear as red fields in the image. If key areas of the image are saturated and you want to perform quantitative analysis, you must repeat the image process using a lower PMT voltage setting or adjust exposure time accordingly.

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. See section 4.3 for more information about enhancing signals.

6.5.1 CCD camera operation

Focus adjustment with CCD camera

Chemiluminescent imaging applications with CCD cameras may need manual focus adjustments. This can be done by placing an object on the tray, with the same thickness as the membrane, with a clear marking to focus on. This feature can also be used to center the sample in the field of view.

Optimize the exposure time for maximal dynamic range

When using CCD-cameras it is convenient to start the image acquisition with an automatic exposure. Note the exposure time, and then evaluate the area where the most intense signal of interest is. Thereafter, adjust the exposure time so that this area has an intensity just below saturation (65 535). The relation between exposure time and intensity is almost linear for CCD-based imaging systems and the optimal exposure time for the sample can be easily calculated from the automatically exposed image.

CCD camera detection of weak signals

General recommendation for CCD Imaging is to use the auto exposure mode. However, for membranes with weak signals this function may result in very long exposure times >10 min. In such cases manual exposure is recommended, starting at 1 min and subsequently a stepwise increase to 2 min, 3 min etc. Imaging such as this can be performed using either the Incremental, Repetition or Program settings in the LAS 4000 series imaging control software.

6.5.2 Scanner operation

Select pixel size to match application demand

Adjust the selected pixel size to match your needs.

It is not always beneficial to select smaller pixel sizes, even though the quality of the image might be higher. Smaller pixel sizes can cause longer scan times, slow down analysis software and create unnecessary data.

For 2-D DIGE, a 100-µm pixel size is recommended. 1-D applications such as Western blotting rarely benefit from pixel sizes smaller than 100 µm, and 200 µm is usually sufficient for most purposes. Use the smallest possible pixel sizes for imaging of tissue sections and other high-resolution and demanding samples.

Be careful when scanning large images with small pixel size since the file may be too large for the analysis software to open.

Strategy for PMT setting optimization

The difference in pixel size between the pre-scan function and the high-resolution scan affects how well pre-scan images and high-resolution images correspond to each other in intensity. A good strategy for optimization is to start by pre-scanning of the entire sample and identify where the most intense signal for your analysis is by using the IQTL Analysis Toolbox.

Select a small area around the intense signal in the control software and scan the area with the desired pixel size. Optimize the PMT setting by evaluating the image and then adjust the PMT voltage so that intensity is in a suitable range below the saturation level. Note that the relation between intensity and PMT voltage is not linear and that optimization might have to be made stepwise. When the small area of interest is optimized, it is possible to scan the whole sample at the correct PMT voltage.

☞ With fast galvanometer-based instruments a pre scan is usually not necessary.

6.6 Data evaluation

Use automated analysis functions when possible

Use software with automated workflows to prevent user bias caused by manual intervention and arbitrary decisions.

General graphics software can corrupt image files

General graphics software can ignore or even remove calibration information and can even corrupt image files making them useless for quantitative analysis. Therefore, do not use them to flip, rotate, crop or invert images.

☞ Always use dedicated analysis software.

Open several instances in parallel for side by side comparison of images

In IQTL v8.1, several instances of the same module can be open at the same time, thus enabling different images to be compared and analyzed side by side.

Use the original 16-bit image files for analysis

To preserve the dynamic range and possibilities of quantitative analysis, remember not to alter the input data. Cytiva Analysis Software described in this handbook doesn't alter the image data. During the analysis workflow, experimental files are created where the analysis steps are saved, preserving the original files. For molecular imaging applications, 16-bit data is recommended (see Chapter 2). If the imaging device gives an 8-bit output, converting it to 16-bit will not add value to the image. It is important that the imaging device fully supports 16-bit storage of data.

☞ Use uncompressed file formats for data storage.

6.7 Phosphorimaging

A variety of phosphor screens are available depending on sample type and need for resolution.Multipurpose imaging plates can be used for most radioisotopes. However, ^{3}H-imaging requires TR-screens (without a protective layer) where the sample is placed in close direct contact with the screen to allow the detection of the low energy β-emission that is unable to penetrate through dense materials. Ideally, TR-screens should only be used once since direct contact with the sample results in lasting contamination. However, it is possible to use plates more than once as long as samples are placed on previously unexposed areas. Avoid exposing storage phosphor screens in places where the environmental radiation may be increased, for example rooms with concrete walls or in basements. Background radiation can vary depending on your location and can affect the lower limit of detection. Therefore, proper shielding may be needed to lower background radiation levels.

Pre-scanning of storage phosphor screens is not recommended due to loss of up to 80% of the signal. Best practice is to use a short exposure first and detect signal and then multiply time proportionally assuming a linear relationship to make full use of the dynamic range.

Avoid wet samples being in direct contact with a screen, since that can contaminate the screen. To prevent contamination of the screen use appropriate bags/sheet protector to sandwich the sample.

When transferring the screen from the phosphor cassette to the imager keep the plate shielded from the light as much as possible.

Traditionally the recommendation has been to store films used in phosphor work in the freezer to obtain optimal results and low background. However storage phosphor screens capture the signal via a different process so do not need to be exposed at low temperatures such that room temperature exposure is usually recommended.

However storage phosphor screens, with a different mode of action, there is no need of doing that, there is rather a danger of damaging the screens added. Sometimes a sample may need to be exposed at very low temperatures (freezer) due to a stability issue — in this instance, to avoid damage to the storage phosphor screen, it is recommended to place the cassette in a plastic bag and then after exposure the bag and cassette are removed from the freezer and slowly allowed to come back to room temperature before removal of the screen. If there is an absolute need to place the phosphor screen in a freezer, then place it in a plastic bag, and slowly allow it to warm back up to room temperature upon removal.

6.8 References

1. *Protein Sample Preparation Handbook*, Cytiva, 28-9887-41 Edition AA (2010).

07
Glossary

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.
Analysis software	Tools to enable the automated analysis, storage and archiving of images collected on imagers.
Aperture	An optical opening that admits light.
Autofluorescence	An inherent or intrinsic property of a material to fluoresce.
Autoradiography	The process of capture and analysis of an image on an x-ray film/phosphoscreen produced by the decay emissions of a radioactive substance.
β-mercaptoethanol $HOCH_2CH_2SH$	A reducing reagent used to reduce disulfide bonds in proteins, resulting in unfolded proteins.
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.
Bisacrylamide $C_7H_{10}N_2O_2$	A cross-linking agent used to polymerize acrylamide to polyacrylamide in the formation of gels.
	The process of transferring proteins from a gel to membrane, usually in an electric field. Also known as immunoblotting.
Brightness	The level of fluorescence intensity of a fluorochrome. Brightness depends on the extinction coefficient and the quantum efficiency.
Bromophenol blue $C_{19}H_{10}Br_4O_5S$	A blue colored compound that is a component in sample loading buffer. Migrates at the front in PAGE and thus serves as a marker for progress of electrophoresis.
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	Enzymatically induced generation of an active fluorophore which emits light after excitation with light of a specific wavelength.
Chemiluminescence	The emission of light by 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
Cone angle	The full angle between the extreme off-axis rays in a converging or diverging beam of light.
Conjugate	Any chemical stably attached to a carrier molecule of interest and which can then serve as a marker. The conjugate must not interfere with the function of its carrier.
Cutoff point	The wavelength of light at which transmission through an optical filter is 50% of the maximum transmission.
CyDye	Fluorophores from Cytiva that emit red (Cy5), green (Cy3) or blue (Cy2) light after excitation with light of the appropriate wavelengths.
Dataset	The files and folder that make up a multichannel image.
Denaturing gel	A polyacrylamide gel containing SDS.
Densitometry	The quantitative measurement of optical density on film. Optical density is usually given a relative value in a scale.
Detection limit (LOD)	The smallest amount of protein that can be detected using given detection reagents and systems.
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.
Dithiothreitol (DTT) $C_4H_{10}O_2S_2$	A reducing reagent used to reduce disulfide bonds in proteins, resulting in unfolded proteins.
Dwell time	The amount of time the excitation light illuminates a spot (pixel) in a sample.
Dynamic range	The range of protein quantities that can be measured using a given system. The greater the linearity of the dynamic range, the more precisely proteins can be quantitated over that range.
Electrophoresis (1-D)	The process of the separation of a mixture of proteins on a gel in an electric field according size, shape and charge.

Electrophoresis (2-D)	Separation of proteins in two dimensions, first according to isoelectric point (pI) and subsequently according to molecular weight.
Emission	The release of light from a fluorophore 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.
Enhanced chemiluminescence (ECL)	HRP-catalyzed conversion of an ECL substrate into a sensitized reagent, which on further oxidation by hydrogen peroxide, emits detectable light when it decays.
Epi-illumination	Illumination that impinges on a sample from the viewing direction.
Excitation	Absorption of light energy by a fluorophore, during which an electron in the fluorophore 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.
Filter	A component of an imager that allows light of a certain wavelength to pass while obstructing light of other wavelengths.
Fluorescence	Light of a specific wavelength emitted by a fluorophore after excitation via a light source of shorter wavelength.
Fluorochrome	Or fluorochrome, a fluorescent dye.
Fluorophore	Any compound, which, when transformed to a temporary high-energy state, emits light as it returns to its ground state.
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.
Glass platen	A horizontal glass stage or platform used to support samples (i.e., gels, membranes, microplates) for imaging.
Horseradish peroxidase (HRP)	An enzyme that catalyzes the conversion of an ECL reagent into a reactive, light emitting compound.
Housekeeping protein	Any intracellular protein that does not significantly change in expression level in response to external stimulation.
Imaging	The process of converting the signals generated by a detection system into a format that enables visualization, analysis and storage of data.
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.
Isoelectric focusing (IEF)	The separation of proteins on a pH gradient according to isoelectric point (pI).
Isoelectric point (pI)	The pH at which a protein has no net charge.
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.
Limit of detection (LOD)	The smallest amount of a sample that can be reliably detected.
Linearity	The signal range over which a laser scanner yields a linear response to fluorochrome concentration.
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.
Molecular weight markers	A mixture of proteins of known molecular weights.
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.
Multiplexing	The practice of detecting several different proteins on a single blot, in a single experiment.
Noise	The statistical uncertainty inherent in a measurement, such as the standard deviation associated with measured background counts.

Normalization	The process of adjusting for variations in the amount of total protein from lane to lane in order to reliably quantitate protein levels.
Numerical aperture (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.
Optimization	A general term used to cover the preliminary steps that should be carried out to determine optimal conditions for a specific experimental system e.g., blocking reagent, antibody concentrations, as well as incubation times/temperatures.
Parallax	A shift in the apparent position of an object that occurs when it is viewed from different vantage points.
Photomultiplier tube (PMT)	A photoelectric device that converts light into electric current and amplifies the current.
Photon of light	A quantum of light based on Planck's quantum theory of light, which states that the energy of an oscillating system can have only discrete (quantized) values.
Photosites	Are light-sensitive diodes that cover CCD chips. Each photosite measures the amount of photons that hit a particular point, and converts light to electrical charges. Brighter images are represented by higher electrical charge, and a darker images are represented by a lower electrical charge.
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.
PMT	Photomultiplier tube, a photoelectric device that converts light into electric current and amplifies the current.
Post-translational modification (PTM)	Chemical modification of a protein after translation that regulates and changes the function of the protein. Typical PTMs include phosphorylation, glycosylation and acetylation.
Primary antibody	The first antibody, specific to a target protein, used as a probe on a blotted membrane. Primary antibodies are usually unlabeled.
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.
Resolution	The quality of separation of protein bands in a gel after electrophoresis of a sample.

rfu	Relative fluorescence units — arbitrary units in which fluorescence intensity is reported by the fluorescence imaging systems.
Saturation	The reception of excess light by a photosensitive detector, resulting in loss of signal discrimination.
SDS-PAGE	Electrophoresis in a polyacrylamide gel carried out in the presence of SDS (see sodium dodecyl sulfate).
Secondary antibody	A labeled antibody directed to the constant region of a primary antibody. Increases sensitivity of the assay by multiple binding of a labeled antibody to a primary antibody.
Sensitivity threshold	The sensitivity or detection threshold is 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.
Signal duration	The time interval over which a signal can be detected.
Signal stability	The quality of consistency of signal intensity over time.
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.
Sodium dodecyl sulfate (SDS) $C_{12}H_{25}SO_4Na$	An anionic detergent used in SDS-PAGE to denature and coat proteins with a negative charge. Also known as sodium lauryl sulfate.
Spatial resolution	The ability to distinguish between two very closely positioned objects. Spatial resolution is related, but not equivalent, to the pixel size.
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.
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.
Western blotting	The process of transferring proteins from a gel to membrane, usually in an electric field. Also known as immunoblotting.

08
Appendix

Appendix 1

Frequently asked questions

Q. How do I clean glass surfaces?

A. Clean the glass surface in three steps; first with isopropanol, then 10% hydrogen peroxide solution followed by a few rinses with distilled water. Finally use a lint-free cloth to dry the glass surface.

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

A. Spectral cross-talk 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 multichannel scans, sequential individualchannel scans should be used.

Q. How can I avoid problems with high background for fluorescent signals?

A. As a general recommendation, when working with fluorescence applications use low-fluorescent sample support materials (e.g., gels, membranes, glass plates and microplates) since they will improve the analytical sensitivity and linear range. To avoid problems with high background caused by tracker dyes make sure to remove the front after finishing the electrophoresis.

Q. Which is the best way to find optimal scanning conditions for a storage phosphor plate?

A. Pre-scanning of storage phosphor plates is not recommended due to loss of up to 80% of the signal. Best practice is to use a short exposure first and detect signal and then multiply time proportionally assuming a linear relationship to make full use of the dynamic range.

Q. Can I re-use a tritium (TR) screen?

A. Ideally, TR-screens should only be used once since direct contact with the sample causes lasting contamination. However, it is possible to use plates more than once as long as samples are placed on previously unexposed areas.

Q. I have followed all recommendations regarding sample preparation, sample placement, pre-imaging and instrument operations but I still have high background. How can I improve my results?

A. If problems with backgrounds persist after following general recommendations for sample preparation and imaging, run system without the sample in place to see if artifact may be related to the imaging system.

Q. I have problems with a lot of speckling on my fluorescent image. What can I do to improve the results?

A. Check for dust in the whole workflow; use powder free gloves, clean glass stage and support material with lint free tissues, check filters for dust and clean lenses and filters with Whatman™ lens cleaning tissue (cat. No. 2105 84).

Q. How and where do I get a customized filter for a new fluorophore and how do I set it up in the camera/laser system?

A. The Image Quant CCD cameras contain standard objectives and there are several manufactures available providing filters on the market. In addition, there are also several manufactures providing filters for the laser systems. The specific information needed for ordering (objective types and filter size) and how to install filters are found in the manuals of each respective imager.

Q. Why is .GEL from ImageQuant LAS system a 65 000 gray scale image whereas an image from Typhoon FLA system is 100 000 grayscale?

A. .GEL file formats can manage both quadratic and linear data. CCD-based LAS systems generate linear signals that are sampled and stored linearly in .GEL files. Whereas, FLA systems amplify signals from the PMT logarithmically before sampling which gives better dynamic resolution.

Q. Can I view and analyze .GEL images using software from suppliers other than Cytiva?

A. Many of the graphics or image analysis packages that can read and display 16-bit .TIF formatted files can only view .GEL images for qualitative purposes. Only the software packages that support the Cytiva's .GEL file format can be used for quantitative data analysis (see products in Chapter 4). To avoid inaccurate results, caution must be taken when converting a square root encoded .GEL file to a linear .TIF, as the quantitative advantages of the .GEL file format might be lost after the conversion.

Q. Is my image suitable for quantitation?

A. Display the acquired image in ImageQuant TL 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, alternatively decrease the exposure time. For more information see section 4.3.

Q. How can I convert a .GEL file into a .TIF file?

A. IQTLv8.1 can be used for converting .GEL files into .TIF files. This is done by choosing "Save as" in the Edit image function, and then change "Save as type" from .GEL to .TIF.

Q. How can I create a multiplexed image in IQTLv8.1 for a chemiluminescent sample and a acolorimetric marker?

A. A multiplex black and white image between a chemiluminescent sample and a colorimetric marker is easily created by the "Create multiplex image..." function in the IQTL 1D gel analysis module. A prerequisite for generation of a multiplex image is that source file must be the same size and in the same folder. The different channels are acquired without changing the position of the membrane. They must also be aligned since channels cannot be moved relative to each other in the IQTL modules. Source images are typically created by repeated acquisition of the same sample at different wavelengths.

Q. When scanning 2-D gels, what is the recommended resolution to use if DeCyder software is used for data analysis?

A. The recommended resolution for 2-D gels is 100 µm if data analysis is performed using DeCyder software.

Scanner Systems

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

A. 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 fairly broad. Some fluorochromes also have long "tails" in their excitation spectra or have additional excitation peaks. Therefore, it is not essential that an excitation wavelength exactly matches a fluorochrome's major excitation peaks 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.

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

A. 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.

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

A. 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 systems have very short dwell times on the sample during scanning. With most commonly used fluorochromes, little or no photobleaching is observed even after multiple scans.

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

A. The recommended starting voltage is 450–800 V. Using higher PMT settings within the linear range will increase the signal levels but not signal-to-noise ratios since the background and noise increases proportionally with the signal. For quantitative results, always check that all pixels have counts between 1 and 100 000 before proceeding with data analysis. Signal-to-noise ratio is the critical measure of sensitivity and detection limits for fluorescence applications.

Q. Which PMT is optimal to use for my fluorochrome?

A. A bi-alkali PMT is optimal for detection of fluorochromes with emission in the blue to yellow wavelength spectra. A multi-alkali PMT can be used for detection of fluorochromes with emission from blue up to the near IR spectra, but is not as sensitive as a bi-alkali PMT. For multiplex applications with fluorochromes that emit at blue/yellow wavelengths as well as red wavelengths, optimal results are obtained by combining the two types of PMTs.

Q. Why is the chemiluminescent function of the Typhoon/FLA series not "ideal" for chemiluminescent applications?

A. Point scanners collect light point by point so there is a limited opportunity to collect the weak light signal from chemiluminescent (CL) reaction coupled with the change in CL signal over time. CCD cameras allow you to collect light for as long as you want and is therefore a far better alternative for chemiluminescent applications.

Q. Why does the Typhoon FLA7000 have both a 650nm and a 635nm red laser?

A. Typhon FLA7000 has been equipped with two red lasers to provide optimal results for fluorescent applications and phosphor screen applications, respectively. The 635nm red laser is optimal for scanning of storage phosphor screens, and the 650nm red is optimal for fluorescent applications in this particular instrument.

Q. What's the difference between L4 and L5 selection on FLA7000?

A. The dynamic range that can be detected is bigger with L5 than with L4. If the signals of the sample are in the L4 range, the density gradation is represented more finely if L4 is selected. Thus if you have signals that are close in intensity the use of L4 will better enable their differentiation

Q. Why can't band pass filters be used with the FLA7000?

A. The physics/optics of the light collection guide do not allow for this possibility.

Q. When I'm using the digitization function on FLA9500 for analysis of my sample the system displays an error message? Why is this happening and how can I solve the problem?

A. The Typhoon FLA9500 system may be unable to complete a scan when a sample is placed towards the edges of the plate resulting in a too intense signal. To avoid this make sure to scan the sample using a scan area well within the beige digitization plate.

Q. When should I use the calibration convertor with ImageScanner III?

A. The ImageQuant TL (IQTL) calibration converter makes it possible to take images in .MEL file format from ImageScanner III/LabScan 6 and convert them into .TIF files for image analysis using IQTL.

CCD based systems

Q. Why can't I focus on my image?

A. The sample may not be centered on the tray. By centering the sample, the autofocus algorithm will be better able to find a sharp edge as a reference for focusing. If the lens is closely zoomed in on the sample, the autofocus will not function properly since the edge of the sample will lie outside the field of view. To avoid this, place a piece of white paper or 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.

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

A. 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 lie unevenly on the surface of the tray. Remove any bubbles from below a wet gel.

Q. Can sensitivity be improved by extending the exposure time?

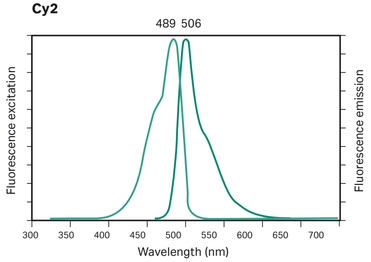
A. The signal from a sample is integrated over time. The sensitivity improves with exposure time, but only up to a point. Instrument noise can dramatically affect the linearity of a CCD at lowlight intensities and long exposure times. The Image Quant LAS systems have a cooled CCD that significantly reduces the noise.

Appendix 2

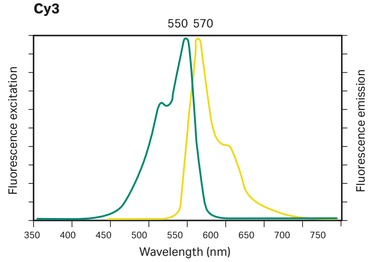
Spectral characteristics of commonly used fluorophores, fluorescent stains, and proteins available from Cytiva.

Spectra of Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, DDAP Phosphate, ECF, ECL Plus, and FluorX were obtained at Cytiva. The EGFP spectrum is reproduced courtesy of Clontech. All other spectra are reproduced courtesy of Molecular Probes, Inc.

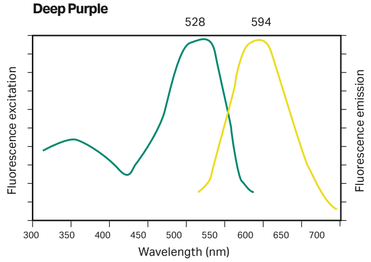
Cy2



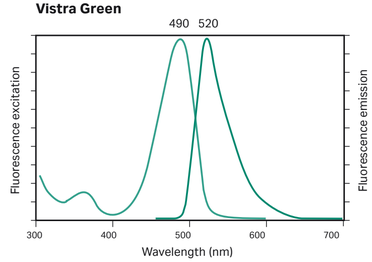
Cy3



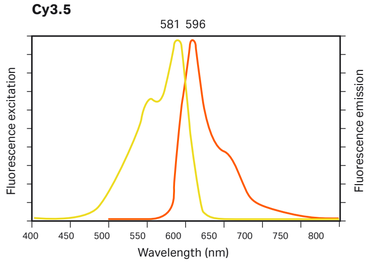
Deep Purple



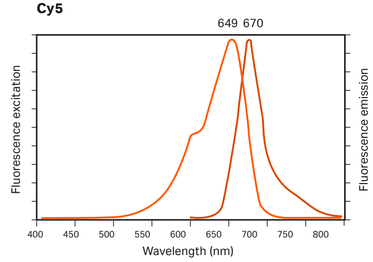
Vistra Green



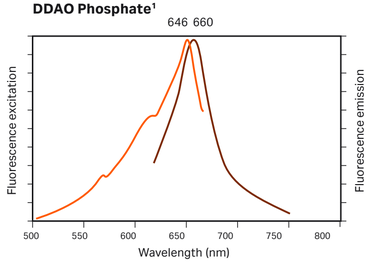
Cy3.5



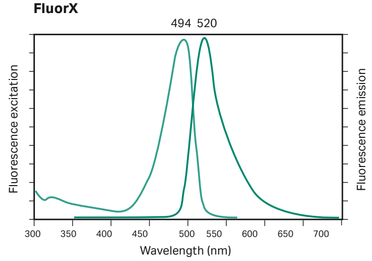
Cy5



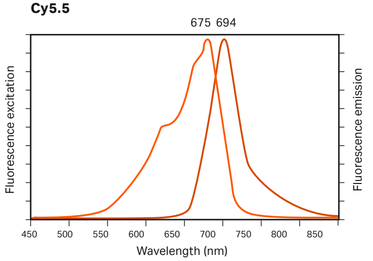
DDAO Phosphate[1]



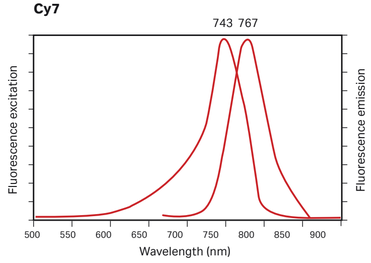
FluorX



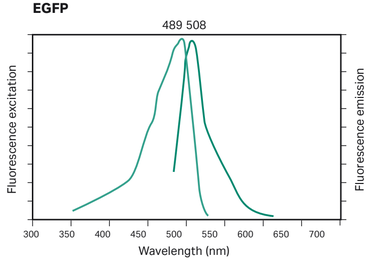
Cy5.5



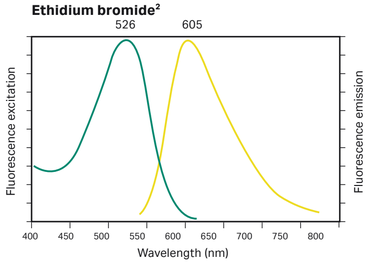
Cy7



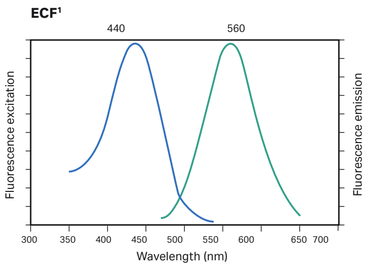
EGFP



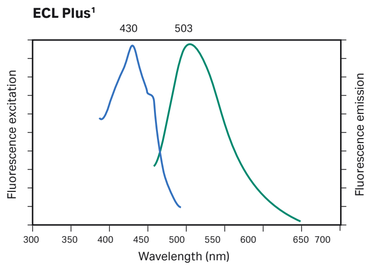
Ethidium bromide[2]



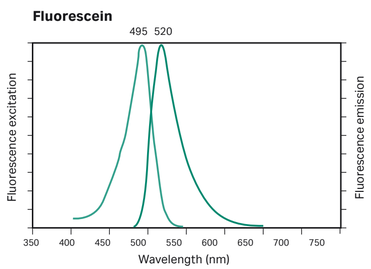
ECF[1]



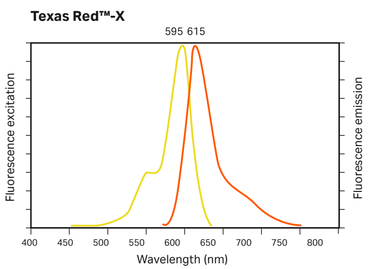
ECL Plus[1]



Fluorescein



Texas Red™-X



[1] Spectra were obtained for the product of the enzymatic reaction on a PVDF membrane.

[2] Spectra were obtained in the presence of nucleic acids.

Appendix 3

This table is based on theoretical spectral analysis and estimation out of standard configuration, with optional components where needed. There are variations in published excitation and emission spectra depending on supplier and the form of the fluorochrome and its environment. Not all of these fluorochomes have been tested on the instruments. Use the table as a support for selection of fluorochrome for your instrument. For precise evaluation, always check the excitation and emission spectra from the supplier.

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
[FxCycle] Far Red stain	640	658	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
[FxCycle] Violet stain	359	461	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41/Y515-Di	UV	L41
[Qnuclear] Deep Red stain	642	657	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
[ThiolTracker] Violet+GSH	404	526	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
1,8-ANS/BSA	270 375	477	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
2-dodecylresorufin/lipid	582	595	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
5-chloromethylfluorescein	491	517	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
5-FAM	492	518	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
5-ROX	578	604	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
5-TAMRA™/MeOH	543	567	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
5-TAMRA/pH 7.0	553	576	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
6-FAM™	495	517	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
6-JOE™	520	548	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
7-amino-4-methylcoumarin	346	442	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
7-aminoactinomycin D	548	648	532	LPR	532	R670	UV/Blue	Orange	Blue	R670	Green	R670	Green	R670
7-hydroxy-4-methylcoumarin/pH 9.0	360	448	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Acridine orange	502	526	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Alexa Fluor® 350 antibody conjugate/pH 8.0	343	441	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Alexa Fluor® 350 hydrazide/H2O	347	444	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Alexa Fluor® 405 antibody conjugate	379 401	422	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Alexa Fluor® 430 antibody conjugate	277 431	540	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV/Blue	Y515-Di	UV/Blue	Y515-Di	Blue	Y515-Di

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
Alexa Fluor® 488 antibody conjugate/pH 8.0	499	520	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Alexa Fluor® 488 dye labeled oligonucleotide/H_2O	493	519	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Alexa Fluor® 488 hydrazide/H_2O	493	517	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Alexa Fluor® 514 antibody conjugate/pH 8.0	518	543	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
Alexa Fluor® 532 antibody conjugate	534	553	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	Y515-Di	Green	575DF20	Green	575DF20
Alexa Fluor® 546 antibody conjugate	561	572	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
Alexa Fluor® 546 dye labeled oligonucleotide/H_2O	554	574	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Alexa Fluor® 555 dye labeled oligonucleotide/H_2O	552	567	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Alexa Fluor® 555 goat anti-mouse IgG antibody	553	568	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Alexa Fluor® 555 hydrazide/H_2O	551	564	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Alexa Fluor® 568 antibody conjugate	579	603	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Alexa Fluor® 568 hydrazide/H_2O	573	600	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Alexa Fluor® 594 antibody conjugate	280 590	618	532	LPG	532	O580	UV/Blue	Orange	UV	605DF40	UV/Green	605DF40	Green	605DF40
Alexa Fluor® 594 cadaverine/H_2O	588	614	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Alexa Fluor® 594 dye labeled oligonucleotide/H_2O	591	621	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Alexa Fluor® 610 antibody conjugate	612	630	635	LPR	635	R670	NA	NA	NA	NA	Red	605DF40	Red	605DF40
Alexa Fluor® 610 R-phycoerythrin streptavidin	496 567	627	473/532	LPG/LPR/BPG1	473/532	O580	UV/Blue	Orange	UV/Blue	605DF40	UV/Green	605DF40	Green	605DF40
Alexa Fluor® 633 antibody conjugate	632	647	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 635 antibody conjugate	285 633	648	635	LPR	635	R670	UV/Blue	Orange	UV	R670	UV/Red	R670	Red	R670
Alexa Fluor® 647 antibody conjugate	650	671	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 647 dye labeled oligonucleotide/H_2O	650	668	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 647 hydrazide/H_2O	649	670	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 647 R-phycoerythrin streptavidin	497 568	666	473/532/635	LPR	473/532/635	R670	UV/Blue	Orange	UV/Blue	R670	UV/Green	R670	Green	R670
Alexa Fluor® 660 antibody conjugate	663	691	635/685	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 680 allophycocyanin streptavidin	655	704	635/685	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 680 antibody conjugate	681	704	685	LPR	635	NA	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 680 R-phycoerythrin streptavidin	498 570	702	473/532/635	LPR	473/532/635	R670	UV/Blue	Orange	UV/Blue	R670	UV/Green	R670	Green	R670
Alexa Fluor® 700 antibody conjugate	696	719	685	BPFR700	NA	NA	NA	NA	NA	NA	Red	R670	Red	R670
Alexa Fluor® 750 antibody conjugate	752	776	785	BPFR700	NA	NA	NA	NA	NA	NA	IR	IR785	IR	IR785

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
Alexa Fluor® 790 antibody conjugate	782	805	785	BPFR800	NA	NA	NA	NA	NA	NA	IR	IR785	IR	IR785
Allophycocyanin	652	658	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Amplex® UltraRed peroxidation product	568	581	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
ANSA	388	470	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
BOBO -1	461	484	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
BOBO -3/DNA	570	605	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
BODIPY® 630/650	630	650	635	LPR	635	R670	UV/Blue	Orange	UV	R670	Red	R670	Red	R670
BODIPY® 630/650-X/MeOH	625	641	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
BODIPY® 650/665	650	665	635	LPR	635	R670	UV/Blue	Orange	UV	R670	Red	R670	Red	R670
BODIPY® 650/665-X/MeOH	646	664	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
BODIPY® FL antibody conjugate/pH 7.2	505	512	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
BODIPY® FL/MeOH	502	511	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
BODIPY® R6G/MeOH	528	547	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
BODIPY® TMR-X antibody conjugate/pH 7.2	542	573	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
BODIPY® TMR-X/MeOH	544	570	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
BODIPY® TR-X phallacidin/pH 7.0	590	621	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
BODIPY® TR-X/MeOH	588	621	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
B-phycoerythrin	546	575	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Calcein/pH 9.0	494	514	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Calcium Crimson /Ca^{2+}	589	609	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Calcium Green -1/Ca^{2+}	507	529	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Calcium Orange /Ca^{2+}	549	574	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
Carboxynaphthofluorescein/pH 10.0	293 599	674	532/635	LPR	532/635	R670	NA	NA	UV/Blue	R670	UV/Red	R670	Red	R670
Carboxyrhodamine 6G	525	547	532	LPG/BPG1	532	O580	NA	NA	Blue	Y515-Di	Green	575DF20	Green	575DF20
Cascade Blue® BSA/pH 7.0	380 401	419	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Cascade Yellow antibody conjugate/pH 8.0	399t	549	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
CBQCA	465	550	473	LPB/BPB1/ LPG/BPG1	473	Y520/O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
CellMask Deep Red plasma membrane stain	659	676	635/685	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
CellMask Orange plasma membrane stain	556	572	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
CellTrace BODIPY® TR methyl ester/lipid	598	625	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
CellTrace calcein red-orange	575	591	532	LPG	532	O580	UV/Blue	Orange	UV	605DF40	Green	605DF40	Green	605DF40
CellTrace calcein violet	400	452	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
CellTrace Violet stain	392	456	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
CellTracker Red CMTPX	586	614	532	LPR	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
CellTracker Violet BMQC+GSH	406	526	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
CFP	433	475	NA	NA	NA	NA	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
CMR	547	575	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Cy2	489	506	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Cy3	550	570	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Cy3.5	581	596	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Cy5	649	670	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Cy5.5	675	694	685	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Cy7	743	767	785	BPFR700	NA	NA	NA	NA	NA	NA	IR	IR785	IR	IR785
CyQUANT® GR/DNA	502	523	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
DAF-FM/NO	495	519	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Dansyl cadaverine/MeOH	334	526	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Dansyl chloride	337	492	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
DAPI/DNA	359	461	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Dapoxyl® (2-aminoethyl)sulfonamide/MeOH	280 373	579	NA	NA	NA	NA	UV/Blue	Orange	UV	575DF20	UV	575DF20	UV	575DF20
DCF 5-(and-6)-carboxy-2',7'-dichlorofluorescein/pH 9.0	504	525	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
DDAO	646	660	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Deep Purple	528	594	532	LPG	532	O580	UV/Blue	Orange	UV	605DF40	Green	605DF40	Green	605DF40
Di-8-ANEPPS/lipid	467	631	473	LPG/LPR/BPG1	473	O580/R670	UV/Blue	Orange	Blue	605DF40	Blue	605DF40	Blue	605DF40
DiA/lipid	457	586	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	575DF20	Blue	575DF20	Blue	575DF20
DiD/lipid	648	670	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
DiFMU/pH 3.0	320	451	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
DiFMU/pH 9.0	358	450	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
DiI/lipid	551	569	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
DiO/lipid	489	506	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
DiR/lipid	750	782	785	BPFR800	NA	NA	NA	NA	NA	NA	IR	IR785	IR	IR785
DsRed (RFP)	558	583	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
EBFP	383	445	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
ECF	440	560	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
ECFP	439	476	473	LPB	473	Y520	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
ECL Plus	430	503	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
EGFP	489	508	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
ELF® 97 alcohol/pH 8.0	253 292 342	536	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Eosin antibody conjugate/pH 8.0	525	546	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
ER-Tracker Blue-White DPX/lipid	372	557	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Ethidium bromide	300 526	605	532	LPG	532	O580	UV/Blue	Orange	UV	605DF40	UV/Green	605DF40	UV/Green	605DF40
EYFP	514	527	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
F2N12S/lipid	421	575	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FAM	494	518	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FITC	494	518	473	LPB/BPB2	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FITC antibody conjugate/pH 8.0	495	519	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FlAsH CCPGCC	511	530	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	605DF40	Green	605DF40	Green	605DF40
Flamingo Fluorescent Gel Stain	271.512	535	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	Y515-Di	UV/Green	575DF20	Green	575DF20
Fluo-3/Ca^{2+}	506	527	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
Fluo-4/Ca^{2+}	494	516	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Fluorescein antibody conjugate/pH 8.0	283 498	517	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV/Blue	Y515-Di	UV/Blue	Y515-Di	UV/Blue	Y515-Di
Fluorescein dextran/pH 8.0	501	524	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Fluorescein/pH 13.0	493	513	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Fluorescein/pH 9.0	490	513	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FluoSpheres® blue fluorescent microspheres/ H_2O	356	414	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
FluoSpheres® crimson fluorescent microspheres/H_2O	620	646	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
FluoSpheres® dark red fluorescent microspheres/H_2O	656	683	635/685	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
FluoSpheres® nile red fluorescent microspheres/H_2O	535	641	532	LPG/LPR	532	O580/R670	UV/Blue	Orange	Blue	605DF40	Green	605DF40	Green	605DF40

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
FluoSpheres® orange fluorescent microspheres/H_2O	540	559	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
FluoSpheres® red fluorescent microspheres/H_2O	576	607	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
FluoSpheres® yellow-green fluorescent microspheres/H_2O	503	514	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Fluram	390	475	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
FluxOR thallium indicator	493	521	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FM® 1-43/lipid	473	579	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
FM® 4-64/2% CHAPS	505	725	473	LPR	NA	NA	UV/Blue	Orange	Blue	R670	Green	R670	Green	R670
Fura Red	420 480	660	473	LPR	473	R670	UV/Blue	Orange	UV/Blue	605DF40	UV/Blue	605DF40	Blue	605DF40
Fura-2	335 363	505 512	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
GFP (native)	395 470	509	473	LPB/BPB1	473	Y520	NA	NA	UV/Blue	Y515-Di	UV/Blue	Y515-Di	Blue	Y515-Di
HCS [NuclearMask] Red stain	622	644	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
HCS CellMask Blue stain	347	444	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
HCS CellMask Deep Red stain	649	670	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
HCS CellMask Green stain	493	517	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
HCS CellMask Orange stain	551	564	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
HCS CellMask Red stain	588	614	532	LPR	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
HCS LipidTOX Deep Red neutral lipid stain/triglyceride	635	652	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
HCS LipidTOX Green neutral lipid stain/triglyceride	498	507	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
HCS LipidTOX Green phospholipidosis detection reagent	504	536	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	575DF20	Green	575DF20	Green	575DF20
HCS LipidTOX Red neutral lipid stain/triglyceride	582	616	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
HCS LipidTOX Red phospholipidosis detection reagent	585	608	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
HCS NuclearMask Deep Red stain	638	686	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Hoechst™ 33258	352	455	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Indo-1	330 346	401 475	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
InVision™ His-tag In-gel Stain	300 560	590	532	LPG	532	O580	UV/Blue	Orange	UV	605DF40	UV/Green	605DF40	Green	605DF40
JC-1/pH 8.2	498 593	595	473/532	BPB1/LPB/LPG	473/532	Y520/O580	UV/Blue	Orange	Blue	605DF40	Green	605DF40	Green	605DF40
JOJO -1	530	544	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	Y515-Di	Green	575DF20	Green	575DF20
Krypton	520	580	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Krypton Glycoprotein	654	673	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
Krypton Infrared Protein Stain	690	718	685	BPFR700	NA	NA	NA	NA	NA	NA	IR	R670	IR	R670
LDS 751/DNA	560	711	532	LPR	532	R670	UV/Blue	Orange	Blue	R670	Green	R670	Green	R670
LIVE/DEAD® Fixable Aqua Dead Cell Stain/pH 7.2	367	526	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
LIVE/DEAD® Fixable Blue Dead Cell Stain	343	441	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
LIVE/DEAD® Fixable Far Red Dead Cell Stain	650	671	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
LIVE/DEAD® Fixable Green Dead Cell Stain	498	526	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
LIVE/DEAD® Fixable Near-IR Dead Cell Stain	752	776	785	BPFR800	NA	NA	NA	NA	NA	NA	IR	IR785	IR	IR785
LIVE/DEAD® Fixable Red Dead Cell Stain	595	613	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
LIVE/DEAD® Fixable Violet Dead Cell Stain	404	455	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
LIVE/DEAD® Fixable Yellow Dead Cell Stain/pH 7.2	400	551	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
LOLO -1	568	580	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Lucifer yellow CH/H_2O	428	544	473	LPB/BPB1/BPG1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
LUCY® 506	506	520	473	LPB/BPB2	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
LUCY® 565	565	588	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
LUCY® 569	569	585	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Lumitein™	280	610	473	LPG	473	O580	UV/Blue	Orange	UV	605DF40	UV	605DF40	UV	605DF40
LysoSensor Blue/pH 5.0	374 395	401 424	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
LysoSensor Green/pH 5.0	274 447	502	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV/Blue	Y515-Di	UV/Blue	Y515-Di	UV/Blue	Y515-Di
LysoTracker® Blue/MeOH	373 394	399 422	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
LysoTracker® Green/pH 5.2	501	509	473	LPB/BPB1	473	Y520	NA	NA	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
LysoTracker® Red/pH 5.2	573	592	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
LysoTracker® Yellow HCK-123	488	565	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Magnesium Green /Mg^{2+}	507	531	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Marina Blue® antibody conjugate/pH 8.0	279 364	461	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
mBBr+GSH/pH 8.0	394	490	NA	NA	NA	NA	UV/Blue	Orange	UV	L41	UV	L41	UV	L41
MitoTracker® Deep Red FM/MeOH	641	662	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
MitoTracker® Green FM/MeOH	490	512	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
MitoTracker® Orange/MeOH	551	575	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
MitoTracker® Red/MeOH	578	598	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
mRFP/pH 7.4	555	583	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
NanoOrange®	470	570	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
NBD-X/MeOH	467	538	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
NeuroTrace® 500/525 green fluorescent Nissl stain/RNA	497	524	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Nile blue/EtOH	631	660	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Nile red/phospholipid	553	637	532	LPG/LPR	532	O580/R670	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Nile red/triglyceride	510	583	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
OFP	547	567	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	575DF20	Green	575DF20	Green	575DF20
Oregon Green® 488 antibody conjugate/pH 8.0	276 498	526	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV/Blue	Y515-Di	UV/Blue	Y515-Di	Blue	Y515-Di
Oregon Green® 488 BAPTA-1/Ca^{2+}	493	522	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Oregon Green® 488 carboxylic acid/pH 9.0	492	518	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Oregon Green® 488 DHPE	504	536	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Oregon Green® 514 antibody conjugate/pH 8.0	513	533	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
Oriole Fluorescent Gel Stain	271	604	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Pacific Blue antibody conjugate/pH 8.0	278 404	455	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Pacific Blue dye labeled oligonucleotide/H_2O	402	455	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
Pacific Orange antibody conjugate/pH 7.2	400	551	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
pHrodo, succinimidyl ester	560	587	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
POPO -1	433	457	NA	NA	NA	NA	NA	NA	Blue	L41	Blue	L41	Blue	L41
POPO -3	533	574	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
PO-PRO -1	434	457	NA	NA	NA	NA	NA	NA	Blue	L41	Blue	L41	Blue	L41
PO-PRO -3	539	571	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
Propidium iodide	305 538	617	532	LPG	532	O580	UV/Blue	Orange	UV	605DF40	UV/Green	605DF40	UV/Green	605DF40
Pro-Q® Diamond phosphoprotein gel stain	556	583	532	LPR	532	R670	UV/Blue	Orange	UV	575DF20	Green	575DF20	Green	575DF20
Pro-Q® Emerald 300 reagent	289	530	NA	NA	NA	NA	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Qdot® 525 streptavidin	300	525	473	LPB/BPB1	473	Y520	NA	NA	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Qdot® 545 streptavidin	300	543	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Qdot® 565 streptavidin	300	564	473	LPG/BPG1	473	O580	UV/Blue	Orange	UV	Y515-Di	UV	Y515-Di	UV	Y515-Di
Qdot® 585 streptavidin	300	588	473	LPG/BPG1	473	O580	UV/Blue	Orange	UV	575DF20	UV	575DF20	UV	575DF20

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
Qdot® 605 streptavidin	300	603	473	LPG	473	O580	UV/Blue	Orange	UV	605DF40	UV	605DF40	UV	605DF40
Qdot® 625 streptavidin	300	621	473	LPG	473	O580	UV/Blue	Orange	UV	605DF40	UV	605DF40	UV	605DF40
Qdot® 655 streptavidin	300	654	473	LPR	473	R670	UV/Blue	Orange	UV	R670	UV	R670	UV	R670
Qdot® 705 streptavidin	300	702	473	LPR	473	R670	NA	NA	UV	R670	UV	R670	UV	R670
Qdot® 800 streptavidin	300	792	473	BPFR800	NA	NA	NA	NA	UV	IR785	UV	IR785	UV	IR785
Quant-iT™ OliGreen® ssDNA Reagent	500	525	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Quant-iT PicoGreen® dsDNA quantitation reagent	502	523	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Quant-iT RiboGreen® RNA Reagent	500	525	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Quinine sulfate/0.5 M H2SO4	349	461	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
ReAsH CCPGCC	592	606	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Resorufin/pH 9.0	571	584	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Rhod-2/Ca^{2+}	553	577	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Rhodamine 110/pH 7	497	520	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Rhodamine 123/MeOH	507	529	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Rhodamine Green	503	528	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Rhodamine phalloidin	558	575	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Rhodamine Red -X antibody conjugate/pH 8.0	279 573	591	532	LPG/BPG1	532	O580	UV/Blue	Orange	UV	605DF40	UV/Green	605DF40	Green	605DF40
R-phycoerythrin	565	576	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	575DF20	Green	575DF20	Green	575DF20
RuBPS	462	603	473	LPG	473	O580	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Sodium Green /Na^{+}	507	532	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
Sulforhodamine 101/EtOH	578	593	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
SYBR Gold nucleic acid gel stain	260 495	537	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV	Y515-Di	UV/Blue	Y515-Di	Blue	Y515-Di
SYBR Green I nucleic acid gel stain	497	520	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYBR Green II RNA gel stain	497	520	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYBR Safe DNA gel stain	509	526	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYPRO® Orange	300 470	570	473	LPG/BPG1	473	O580	UV/Blue	Orange	UV/Blue	575DF20	UV/Blue	575DF20	Blue	575DF20
SYPRO® Orange protein gel stain/BSA	491	586	473	LPG/BPG1	473	O580	UV/Blue	Orange	Blue	605DF40	Blue	605DF40	Blue	605DF40
SYPRO® Red	300 550	630	532	LPG/LPR	532	O580/R670	UV/Blue	Orange	UV	605DF40	UV/Green	605DF40	Green	605DF40
SYPRO® Rose Plus Protein Blot Stain	350	610	NA	NA	NA	NA	UV/Blue	Orange	UV	605DF40	UV	605DF40	UV	605DF40

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
SYPRO® Ruby	280 450	610	473	LPG/LPR	473	O580	UV/Blue	Orange	UV/Blue	605DF40	UV/Blue	605DF40	UV/Blue	605DF40
SYPRO® Ruby IEF	280 450	610	473	LPG/LPR	473	O580	UV/Blue	Orange	UV/Blue	605DF40	UV/Blue	605DF40	UV/Blue	605DF40
SYPRO® Ruby protein blot stain	280 450	618	473	LPG/LPR	473	O580/R670	UV/Blue	Orange	UV/Blue	605DF40	UV/Blue	605DF40	UV/Blue	605DF40
SYPRO® Tangerine	300 490	640	473	LPG	473	O580	UV/Blue	Orange	UV/Blue	Y515-Di	UV/Blue	Y515-Di	Blue	Y515-Di
SYTO® 11	507	525	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYTO® 13	257 488	506	473	LPB/BPB1	473	Y520	UV/Blue	Orange	UV	L41	UV/Blue	L41/Y515-Di	Blue	Y515-Di
SYTO® 16	489	520	473	LPB/BPB2	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYTO® 17	619	638	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
SYTO® 45	258 452	485	NA	NA	NA	NA	NA	NA	UV	L41	UV/Blue	L41/Y515-Di	Blue	Y515-Di
SYTO® 59	622	644	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
SYTO® 60	650	681	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
SYTO® 61	618	650	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
SYTO® 62	650	681	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
SYTO® 82	540	559	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
SYTO® 9	483	500	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYTO® RNASelect green fluorescent cell stain	503	527	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYTOX® AADvanced dead cell stain	546	647	532	LPR	532	R670	UV/Blue	Orange	Blue	R670	Green	R670	Green	R670
SYTOX® Blue	257 445	470	NA	NA	NA	NA	NA	NA	UV	L41	UV/Blue	L41/Y515-Di	Blue	Y515-Di
SYTOX® Green	504	524	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
SYTOX® Orange	547	570	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
SYTOX® Red	640	658	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Tetramethylrhodamine antibody conjugate/pH 8.0	552	578	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Tetramethylrhodamine dextran/pH 7.0	554	582	532	LPG/BPG1	532	O580	NA	NA	NA	NA	Green	575DF20	Green	575DF20
Texas Red® dextran/H_2O	592	614	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Texas Red® DHPE	584	608	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Texas Red®-X antibody conjugate	595	613	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
TO-PRO®-1	515	531	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
TO-PRO®-3	642	657	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
TOTO®-1/DNA	514	531	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20

			Typhoon FLA 9500		Typhoon FLA 7000		ImageQuant LAS 500		ImageQuant LAS 4000 mini		ImageQuant LAS 4000		ImageQuant LAS 4010	
Stain/label	**Excitation maximum**	**Emission maximum**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**	**Excitation source**	**Emission filter**
TOTO®-3	642	661	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
TRITC	529	596	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
Vybrant® DyeCycle Green stain	506	534	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
Vybrant® DyeCycle Orange stain	519	563	532	LPG/BPG1	532	O580	UV/Blue	Orange	Blue	Y515-Di	Green	575DF20	Green	575DF20
Vybrant® DyeCycle Ruby stain	637	685	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
Vybrant® DyeCycle Violet stain	369	437	NA	NA	NA	NA	NA	NA	UV	L41	UV	L41	UV	L41
X-rhod-1/Ca^{2+}	580	601	532	LPG	532	O580	NA	NA	NA	NA	Green	605DF40	Green	605DF40
YO-PRO®-1	491	506	473	LPB/BPB1	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
YO-PRO®-3	613	629	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670
YOYO®-1	491	508	473	LPB/BPB2	473	Y520	UV/Blue	Orange	Blue	Y515-Di	Blue	Y515-Di	Blue	Y515-Di
YOYO®-3	612	631	635	LPR	635	R670	NA	NA	NA	NA	Red	R670	Red	R670

For Typhoon FLA 9500: 685nm Laser=Upgrade, 785nm Laser=Upgrade, BPFR700=Upgrade, BPFR800=Upgrade

For ImageQuant LAS 4000mini: UV=upgrade, Blue=Upgrade, L41=Upgrade, Y515-Di=Upgrade, 575DF20=Upgrade, R670=Upgrade, IR785=Upgrade 605DF40=Upgrade, Y515-Di=Upgrade

For ImageQuant LAS 4000: Blue=Upgrade, Green=Upgrade, Red=Upgrade, L41=Upgrade, Y515-Di=Upgrade, 575DF20=Upgrade, R670=Upgrade, IR=Upgrade, IR785=Upgrade

For ImageQuant LAS 4010: L41=Upgrade, IR=Upgrade, IR785=Upgrade

Ordering information

Product	Quantity	Code No.
Sample preparation		
Mammalian Protein Extraction Buffer	1 × for 500 mL	28-9412-79
Yeast Protein Extraction Buffer Kit	1	28-9440-45
Sample preparation kits and reagents		
2-D Protein Extraction Buffer Trial Kit	1 × for 50 mL	28-9435-22
2-D Protein Extraction Buffer-I	1 × for 50 mL	28-9435-23
2-D Protein Extraction Buffer-II	1 × for 50 mL	28-9435-24
2-D Protein Extraction Buffer-III	1 × for 50 mL	28-9435-25
2-D Protein Extraction Buffer-IV	1 × for 50 mL	28-9435-26
2-D Protein Extraction Buffer-V	1 × for 50 mL	28-9435-27
2-D Protein Extraction Buffer-VI	1 × for 50 mL	28-9435-28
TriplePrep Kit	50 preps	28-9425-44
2-D Quant Kit	500 assays	80-6483-56
2-D Clean-Up Kit	50 samples	80-6484-51
SDS-PAGE Clean-Up Kit	50 samples	80-6484-70
Amersham markers		
Low-Range Rainbow Molecular weight Markers	250 µL	RPN755E
High-Range Rainbow Molecular weight Markers	250 µL	RPN756E
Full-Range Rainbow Molecular weight Markers	250 µL	RPN800E
Full-Range Rainbow Molecular weight Markers	120 µL	RPN850E
Full-Range Rainbow Molecular weight Markers	500 µL	RPN851E
Sample labeling		
CyDye DIGE Fluor minimal dye labeling kit	2 nmol	28-9345-30
CyDye DIGE Fluor, minimal labeling kit	5 nmol	25-8010-65
CyDye DIGE Fluor Labeling Kit for Scarce Samples	1	25-8009-83
CyDye DIGE Fluor Labeling Kit for Scarce Samples and Preparative Gel Labeling	1	25-8009-84
1D gel electrophoresis, transfer and blotting equipment		
Transfer units		
TE 62 Transfer Unit	1	80-6209-58
TE 22 Mini Tank Transfer Unit	1	80-6204-26
TE 70 Semi-Dry Transfer Unit, 14 × 16 cm	1	80-6210-34
TE 70 PWR Semi-Dry Transfer Unit, 14 × 16 cm	1	11-0013-41
TE 77 Semi-Dry Transfer Unit, 21 × 26 cm	1	80-6211-86
TE 77 PWR Semi-Dry Transfer Unit, 21 × 26 cm	1	11-0013-42
Multiphor II Nova Blot Kit, Semi-Dry	1	18-1016-86
Blotting Equipment		
ECL Multiprobe	1	11-0033-95
ECL Multiprobe XL	1	11-0033-96
PR Deca-Probe Incubation Manifold	1	80-6087-98
Processor Plus	1	80-6444-04
Amersham ECL Gel Electrophoresis System		
Amersham ECL Gel Box	1	28-9906-08
Amersham ECL Gel 10%, 10wells	Pack of 10	28-9898-04
Amersham ECL Gel 12%, 10wells	Pack of 10	28-9898-05
Amersham ECL Gel 4-12%, 10wells	Pack of 10	28-9898-06
Amersham ECL Gel 8-16%, 10wells	Pack of 10	28-9898-07
Amersham ECL Gel 4-20%, 10wells	Pack of 10	28-9901-54
Amersham ECL Gel Running Buffer	10 runs	28-9902-52
Vertical electrophoresis systems		
miniVE Vertical Electrophoresis system	1	80-6418-77
miniVE Blot Module	1	80-6418-96
SE 600 Ruby Standard Dual Cooled Vertical Unit	1	80-6406-99
Power Supplies		
EPS 301 Power supply	1	18-1130-01
EPS 2A200 Power supply	1	80-6406-99
2-D gel electrophoresis equipment		
Sample separation, first dimension		
DeStreak Rehydration Solution	5 × 3 mL	17-6003-19
DeStreak Reagent	1 mL	17-6003-18
Ettan IPGphor 3 Isoelectric Focusing Unit	1	11-0033-64
Immobiline DryStrip Kit	1	18-1004-30
IPGbox 1 IPGbox + IPGbox Kit		28-9334-65
IPGbox Kit 10 Reswell Trays + IPGbox Insert		28-9334-92
IPG Buffer pH 5.5-6.7	1 mL	17-6002-06
IPG Buffer pH 4-7	1 mL	17-6000-86
IPG Buffer pH 6-11	1 mL	17-6001-78
IPG Buffer pH 7-11 NL	1 mL	17-6004-39
IPG Buffer pH 3-10 NL	1 mL	17-6000-88
IPG Buffer pH 3-10	1 mL	17-6000-87
IPG Buffer pH 3-11 NL	1 mL	17-6004-40
Ettan IPGphor Manifold, Complete	1	80-6498-38
Ettan IPGphor Manifold, Light Complete	1	11-0026-88
Multiphor II Electrophoresis System	1	18-1018-06

Product	Quantity		Code No.
Immobiline DryStrip Gels for IEF*			
pH Range		**18 cm**	**24 cm**
3.5–4.5	12/pack	–	17-6002-38
3–7 NL	12/pack	–	17-6002-43
4–7	12/pack	17-1233-01	17-6002-46
6–9	12/pack	17-6001-88	17-6002-47
6–11	12/pack	17-6001-97	–
3–10	12/pack	17-1234-01	17-6002-44
3–10 NL	12/pack	17-1235-01	17-6002-45
3–5.6 NL	12/pack	17-6003-56	17-6003-57
5.3–6.5	12/pack	17-6003-61	17-6003-62
6.2–7.5	12/pack	17-6003-66	17-6003-67
7–11 NL	12/pack	17-6003-71	17-6003-72
3–11 NL	12/pack	17-6003-76	17-6003-77
Sample separation, second dimension			
Ettan DALT*six* Electrophoresis Unit, 115V	1		80-6485-08
Ettan DALT*six* Electrophoresis Unit, 220V	1		80-6485-27
EPS 601 Power Supply	1		18-1130-02
DALT*six* Gel Caster	1		80-6485-46
DIGE Gel	3 pk		28-9374-51
DIGE Buffer Kit	2 × DALT*six* runs		28-9374-52
Low-fluorescence Glass Plates,	27 × 21 cm (including spacers)		80-6475-58
MultiTemp™ IV, Thermostatic Circulator, 115 V	1		28-9941-72
MultiTemp IV, Thermostatic Circulator, 230 V	1		28-9941-71

Product	Quantity	Code No.
Imaging systems		
Typhoon Variable Mode Imagers		
Typhoon FLA 9500	1	29-0040-80
Typhoon FLA 7000	1	28-9558-09
Typhoon FLA 7000 IP	1	28-9836-18
ImageQuant Imagers		
ImageQuant LAS 500	1	29-0050-63
ImageQuant LAS 4000	1	28-9558-10
ImageQuant LAS 4010	1	28-9558-11
ImageQuant LAS 4000 mini	1	28-9558-13
Other imaging systems		
ImageScanner III	1	28-9076-07
Analysis software		
ImageQuant TL		
ImageQuant TL single user license	1	29-0007-37
ImageQuant TL 5-user network license	1	29-0008-10
ImageQuant TL 8.1 and ImageQuant TL Sec urlTy 8.1 Software Package	1	29-0006-05
ImageMaster 2D Platinum		
ImageMaster 2D Platinum 7.0 DIGE	1	28-9380-55
ImageMaster 2D Platinum 7.0 upgrade to DIGE	1	28-9398-10
ImageMaster 2D Platinum 7.0	1	28-9380-91
ImageMaster 2D Platinum 7.0 software package	1	28-9408-30

Product	Quantity	Code No.
DeCyder 2D		
DeCyder 2D 7.2 Software package	1	28-9964-35
DeCyder 2D 7.2 concurrent network license		28-9854-18
DeCyder 2D 7.2 SPN 1 concurrent network license		28-9854-11
DeCyder 2D 7.2 SPN 1 concurrent network license Upgrade		28-9854-17
DeCyder 2D 7.2 SPN 1 concurrent network license Internal FOC		28-9854-14
DeCyder 2D 7.2 Upgrade from 7.0		28-9854-19
DeCyder 2D Oracle 11g 5 user lic, lic no 28945780		28-9957-36
Protein staining reagents		
Deep Purple Total Protein Stain	5mL	RPN6305
Deep Purple Total Protein Stain	25mL	RPN6306
Autoradiography Films		
Amersham Hyperfilm ECL (5 × 7 inches)	50 sheets	28-9068-35
Amersham Hyperfilm ECL (18 × 24 cm)	50 sheets	28-9068-36
Amersham Hyperfilm ECL (18 × 24 cm)	100 sheets	28-9068-37
Amersham Hyperfilm ECL (8 × 10 inches)	50 sheets	28-9068-38
Amersham Hyperfilm ECL (8 × 10 inches)	100 sheets	28-9068-39
Amersham Hyperfilm ECL (24 × 30 cm)	50 sheets	28-9068-40
Amersham Hyperfilm ECL (24 × 30 cm)	50 sheets	28-9068-41

* IPG strips are also available in 7, 11 and 13 cm strip lengths. For information, contact your local Cytiva representative or consult www.cytiva.com

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For local office contact information, visit cytiva.com/contact

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