About Fluorescence

A Practical Guide to Fluorescence and Optical Filters

CHROMA®

Optical Filters for Scientific, Imaging, and Detection Industries

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Introduction

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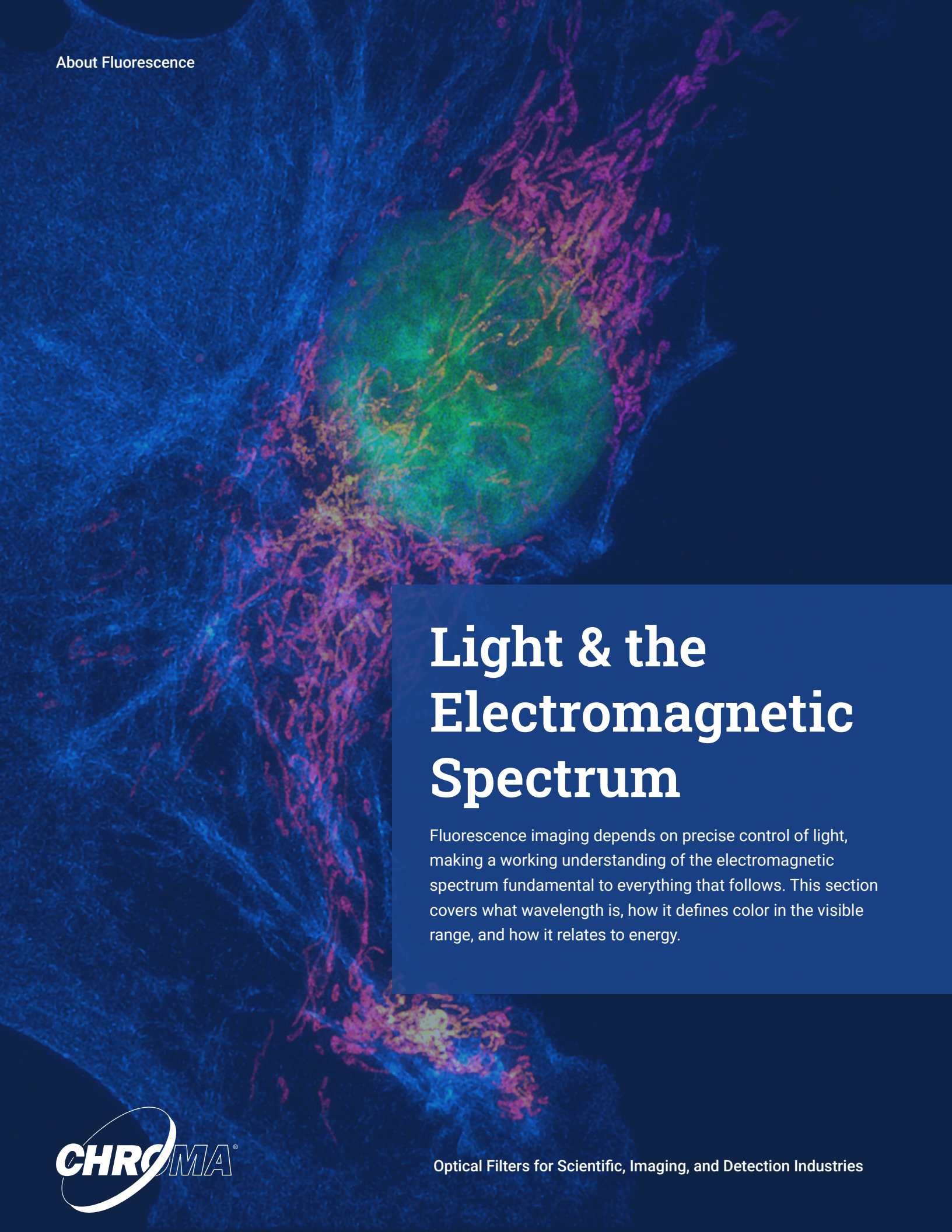
Introduction

Fluorescence has become one of the most powerful and widely used tools in modern optical imaging. From basic biological research to advanced analytical instrumentation, fluorescence-based techniques make it possible to detect, isolate, and visualize signals that would otherwise be difficult or impossible to observe. At the center of many of these systems is the controlled use of light: selecting the right wavelengths for excitation, separating weak emitted fluorescence from much stronger illumination, and preserving image quality throughout the optical path.

Because fluorescence depends on precise spectral discrimination, optical filters play a critical role in overall system performance. Excitation filters, dichroic beamsplitters, and emission filters work together to isolate fluorescence signals, reduce background, and improve contrast. The effectiveness of these components directly impacts the quality, accuracy, and sensitivity of fluorescence measurements.

This guide introduces the core ideas behind fluorescence and fluorescence filtering, beginning with the fundamentals of light and wavelength and progressing through fluorescence microscopy, filter function, performance considerations, and selected applications. Its purpose is to provide a practical foundation for understanding how fluorescence filter systems work and why they are so important in modern optical instruments.

Selected applications are included to illustrate how fluorescence filter requirements vary across different techniques and instrument configurations. Throughout, the emphasis is on building a clear, working understanding of how fluorescence systems operate and how optical filters contribute to their performance.



Light & the Electromagnetic Spectrum

Fluorescence imaging depends on precise control of light, making a working understanding of the electromagnetic spectrum fundamental to everything that follows. This section covers what wavelength is, how it defines color in the visible range, and how it relates to energy.

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The Electromagnetic Spectrum

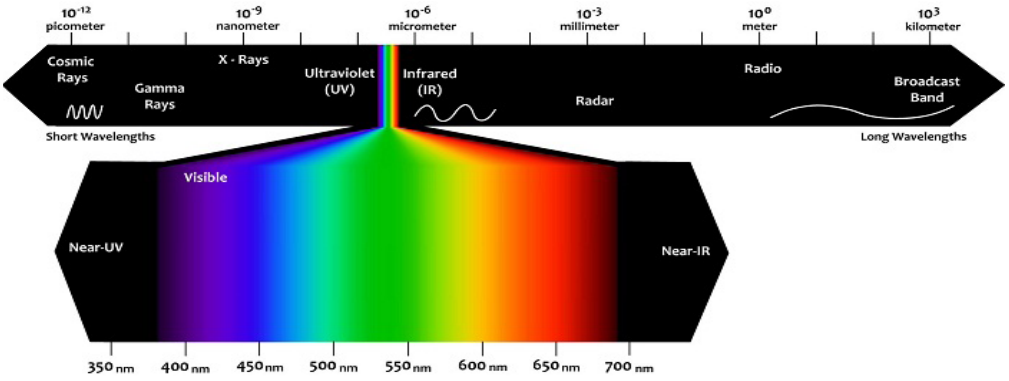


Figure 1: Visualization of the electromagnetic spectrum, quantified using wavelengths, with the visible region highlighted

Any portion of the electromagnetic spectrum is qualitatively defined by the wavelength of energy that comprises it. In other words, the wavelength of a light wave tells us almost everything we would want to know about it. In defining the visible portion of the electromagnetic spectrum, the nanometer (nm) is the most commonly used unit and the portion of the spectrum that is typically reported as perceptible by humans ranges from about 380nm to 710nm, although the exact limits vary among individuals. Sensitivity decreases near both ends of this range, particularly as wavelengths approach the ultraviolet and infrared regions.

The Visible Spectrum

The visible spectrum can be broken up into particular ranges of wavelengths, each corresponding to what we refer to as colors:

Violet & Indigo	**380 - 450nm**
Blue & Aqua	**450 - 500nm**
Green	**500 - 570nm**
Yellow & Orange	**570 - 610nm**
Red	**610 - 710nm**

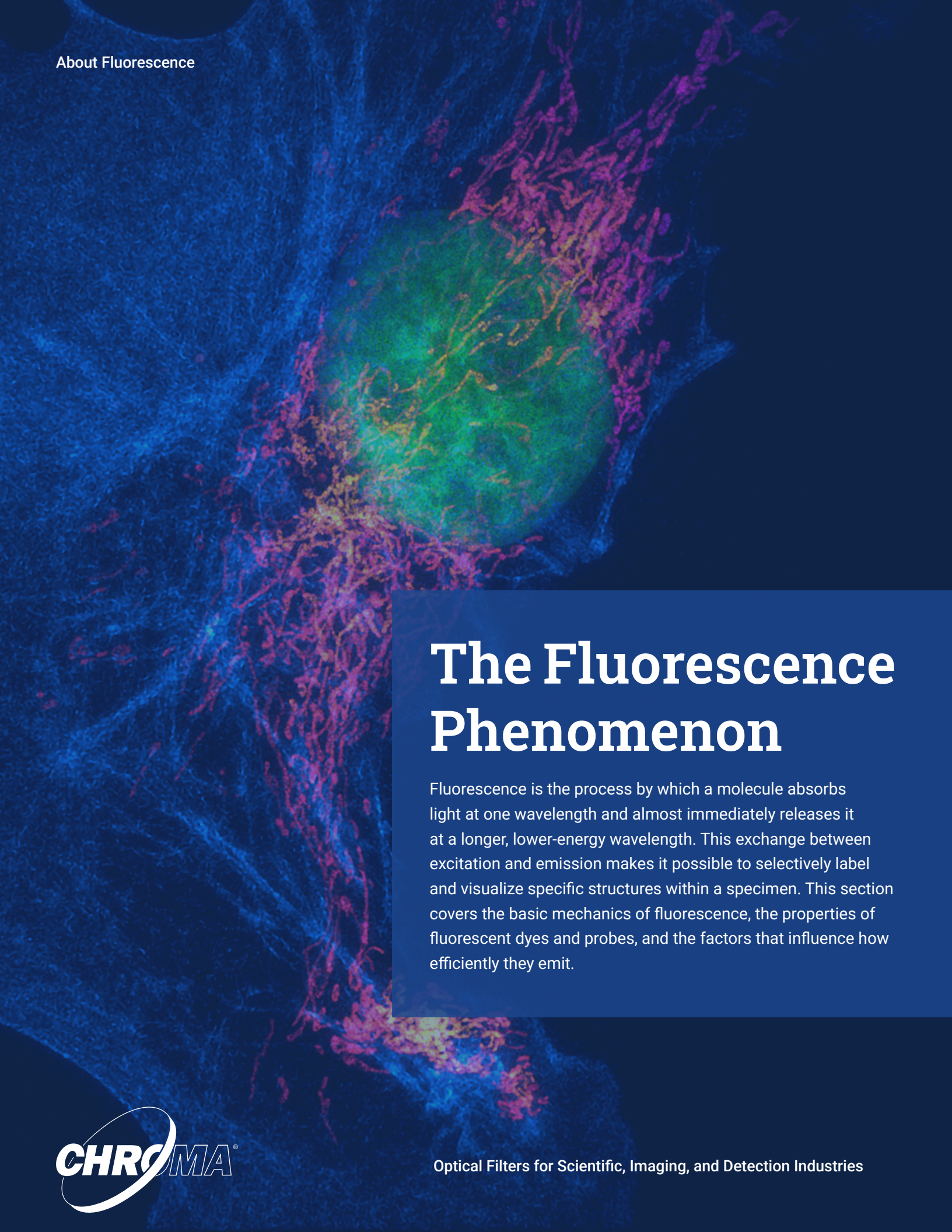
The wavelengths that directly flank the visible spectrum are also useful to the world of fluorescence. They are the short-wavelength band from 320 to 400nm (near-UV) and the long-wavelength band from 750 to approximately 2500nm (near-IR).

Wavelength, Frequency, & Energy

In optics, wavelength is often chosen over frequency to quantify light, but they are both proportional and either is accurate. Energy is directly proportional to frequency and inversely proportional to wavelength, as originally described by Max Planck with the following equation:

$$E = hc/\lambda$$

Where E is energy, h is Planck's constant, c is the speed of light, and λ is the wavelength of light. This relationship expresses the idea that light of a shorter wavelength (i.e. violet) has more energy than light of a longer wavelength (i.e. red).



The Fluorescence Phenomenon

Fluorescence is the process by which a molecule absorbs light at one wavelength and almost immediately releases it at a longer, lower-energy wavelength. This exchange between excitation and emission makes it possible to selectively label and visualize specific structures within a specimen. This section covers the basic mechanics of fluorescence, the properties of fluorescent dyes and probes, and the factors that influence how efficiently they emit.

The Fluorescence Phenomenon

Fluorescence is a molecular phenomenon in which a substance radiates light energy almost immediately upon being struck with light from another source. A portion of this incident light is absorbed by this substance, meaning that the radiated light is typically of lower energy (and thus longer wavelength) than that of the source. The process of light absorption and radiation is known as fluorescence excitation and emission.

Fluorescence is exhibited by most organic and non-organic substances and in the early days of fluorescence microscopy, microscopists primarily observed this natural, intrinsic fluorescence, called autofluorescence.

The use of fluorescence as an imaging modality has since become an invaluable tool in biological and materials science research. By labeling specific structures with fluorescent molecules (fluorochromes or fluorophores), researchers can selectively visualize components that would otherwise be difficult or impossible to detect using other microscopy techniques.

The image in figure 2, for example, was created utilizing optical filters to image the conjugation of three different fluorochromes to three different cellular targets. In this image, MitoTracker Red, Alexa Fluor 488 and DAPI are conjugated to the cell's mitochondria, actin filaments, and nucleus/nucleoli, respectively.

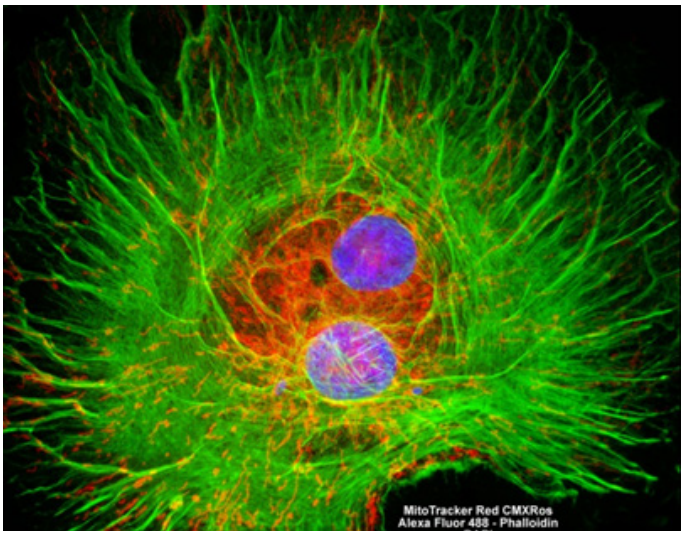


Figure 2: Three-color TIRF image of a normal African green monkey kidney fibroblast cell (CV-1). Image courtesy of Michael W. Davidson of the National High Magnetic Field Laboratory, The Florida State University

Excitation & Emission

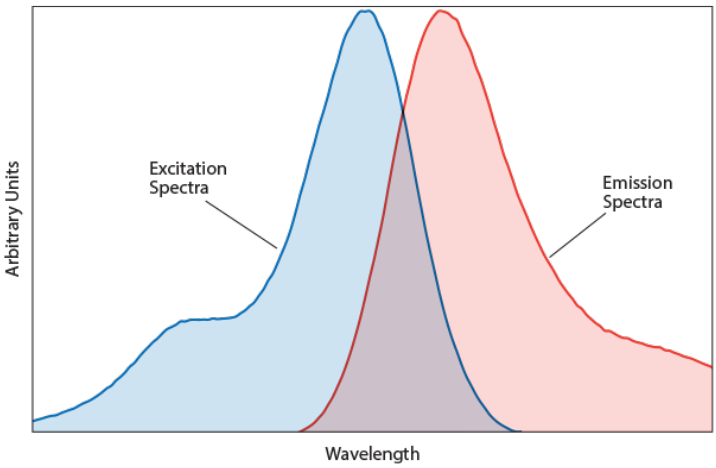


Figure 3: Generic excitation and emission spectra for a fluorescent dye.

Most commercially available fluorochromes exhibit well-defined excitation and emission spectra, as shown in Figure 3. The difference in wavelength between the peaks of these spectra is referred to as the Stokes shift, and it is a key parameter in fluorescence imaging.

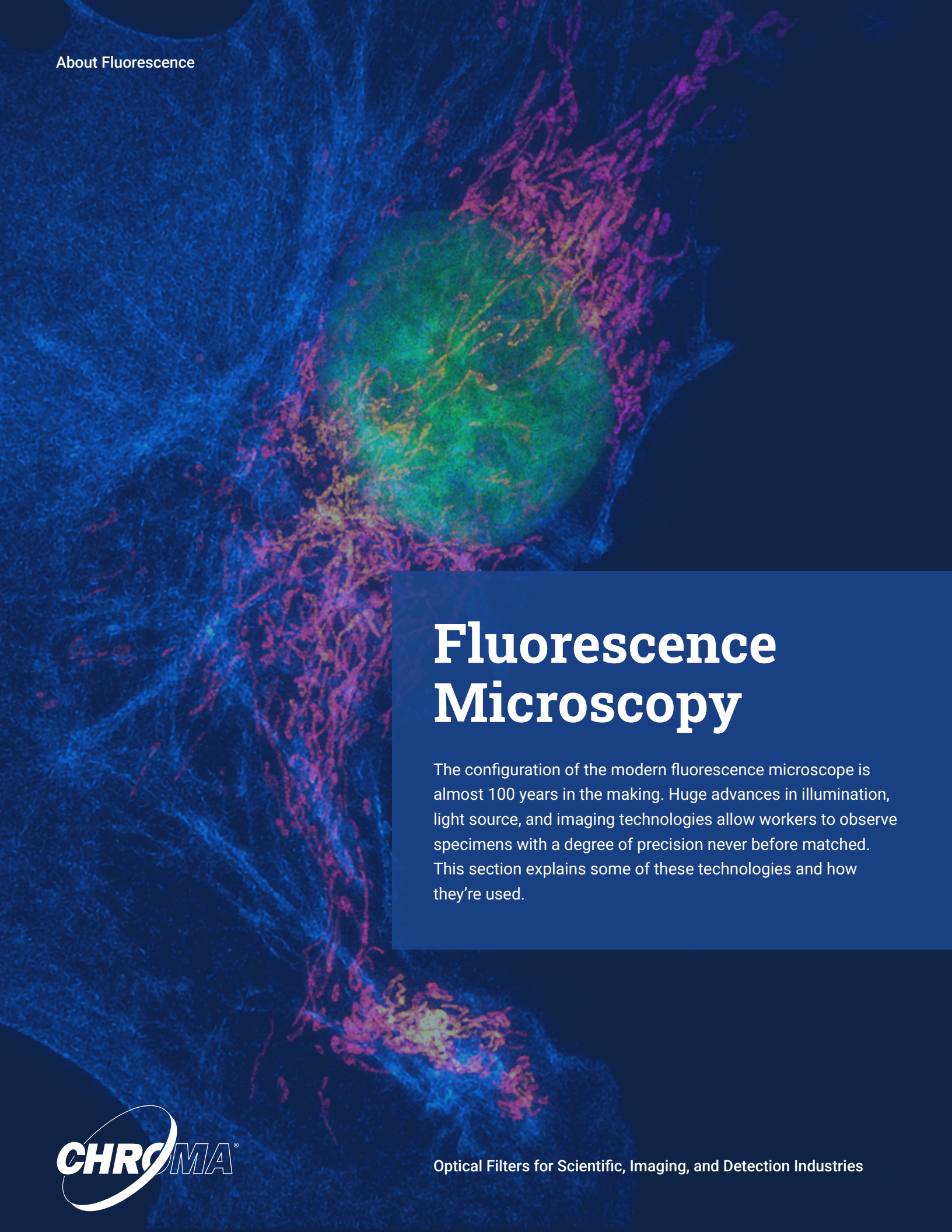
Although the overall intensity of emission varies with excitation wavelength, the spectral distribution of emitted light is largely independent of the excitation wavelength.

Fluorochrome behavior can also vary depending on changes in cellular environment such as pH level, dye concentration, and conjugation to other substances. Several dyes, such as BCECF, Fura-2, and Indo-1, are useful expressly because they have large shifts in their excitation or emission spectra with changes in concentration of ions such as H^+, Ca^{2+}, Na^+.

It is important to note that published spectra are often measured in standardized, non-cellular environments or using organic solvents that are not typically found in active biological systems. Actual cellular environments or solvent conditions may affect the accuracy of the reported spectra for a given fluorochrome, for the reasons outlined above.

Fluorescence efficiency can also decrease over time due to photochemical reactions, a process called photobleaching (or fading). Additionally, cellular quenching or simple repetitive fluorescence measurements or exposures can decrease fluorochrome efficiency over time.

Fluorescence spectra are typically measured using an instrument called a spectrofluorimeter, which consists of two spectrometers: an illuminating spectrometer and an analyzing spectrometer. The dye sample is strongly illuminated by a color of light that is found to cause some fluorescence. A spectrum of the fluorescent emission is then obtained by scanning with the analyzing spectrometer using this fixed illumination color. The analyzer is then fixed at the brightest emission color, and a spectrum of the excitation is obtained by scanning with the illuminating spectrometer and measuring the variation in emission intensity at this fixed wavelength. For the purpose of designing filters, these spectra are normalized to a scale of relative intensity.



Fluorescence Microscopy

The configuration of the modern fluorescence microscope is almost 100 years in the making. Huge advances in illumination, light source, and imaging technologies allow workers to observe specimens with a degree of precision never before matched. This section explains some of these technologies and how they're used.

Epifluorescence Microscope Overview

Today's most common fluorescence set-up is the epifluorescence microscope, named for the way it uses episcopic light to illuminate the sample. With this configuration (shown below), fluorescence filters need only filter out excitation light scattering back from the sample or reflecting off glass surfaces, as opposed to direct excitation light. The use of high quality oil-immersion objectives (made with materials that have minimal autofluorescence and using low fluorescence oil) eliminates some of these surface reflections, which can reduce the level of back-scattered light to as little as 1/100th of the incident light.

In addition, the dichroic beamsplitter plays an important role in suppressing excitation light that is scattered back from the specimen. Its primary function is to reflect excitation wavelengths into the objective and transmit fluorescence emission to the detector. It also provides partial rejection of reflected excitation light, typically by an additional factor of 10 to 500.

Even with oil-immersion objectives that minimize reflections and autofluorescence, some residual excitation light can still reach the detector. In systems that rely only on a high-quality dichroic beamsplitter, this residual background may be comparable to the fluorescence signal for bright specimens, but can easily overwhelm the signal when fluorescence is weak.

For this reason, fluorescence microscopes typically include additional excitation and emission filters. These filters further suppress stray excitation light, often by factors of 10^6 or more, while transmitting the desired fluorescence signal with high efficiency. This additional filtering is essential for maintaining good image contrast, particularly when imaging weakly fluorescing specimens.

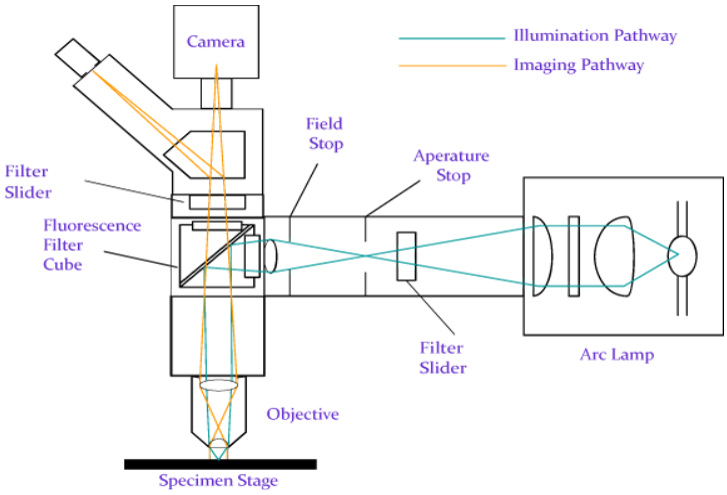


Figure 4: Schematic of a wide-field epifluorescence microscope, showing the separate optical paths for illuminating the specimen and imaging the specimen.

The optical design described above, combining efficient illumination, carefully designed objectives, and specialized filters, was made possible by a series of technological advances in microscopy and optical components. This configuration became commercialized after the 1960's, but there were other important technical advances that contributed to the progression of fluorescence microscopy, occurring both before and after this historical period. A few examples include:

1. The development of compact mercury-vapor and xenon arc lamps (1935);

2. Advances in the manufacture of colored filter glasses, which enabled the use of fluorochromes that were efficiently excited by visible light (thus allowing, for example, the use of simple tungsten filament light sources);

3. Advances in microscope objective design; and

4. The introduction of anti-reflection coatings for microscope optics (c. 1940).

More recent technological developments have enabled fluorescence microscopy to keep pace with the remarkable advances in the biological and biomedical sciences over the years. These include ultrasensitive cameras, laser illumination, confocal and multi-photon microscopy, digital image processing, new fluorochromes and fluorescent probes, and, of course, great improvements in optical filters and beamsplitters.

Light Sources

Arc Lamps (Mercury and Xenon)

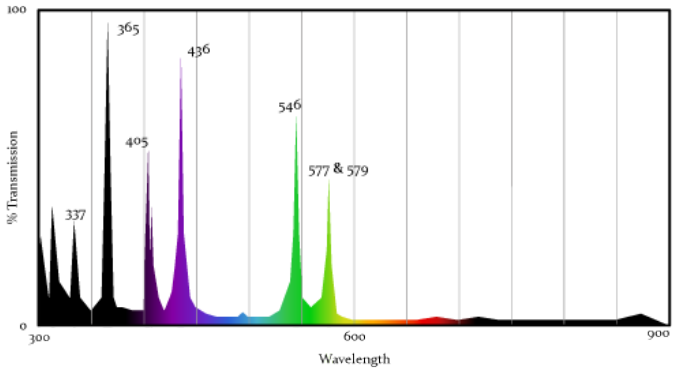


Figure 5: Spectrum of a mercury arc lamp. (Mid-UV output below 300nm is not shown.)

In the past, the most common light source for fluorescence microscopy, chosen for its high brightness (known technically as luminance or radiance) in the ultraviolet and visible spectrum, was the mercury arc lamp. The spectrum of this light source (Figure 5) outputs most of its light in a few concentrated, narrow bands called lines or spikes – with each line being approximately 10nm wide. Conveniently, most general-purpose filter sets have excitation filters that transmit one or more of these lines.

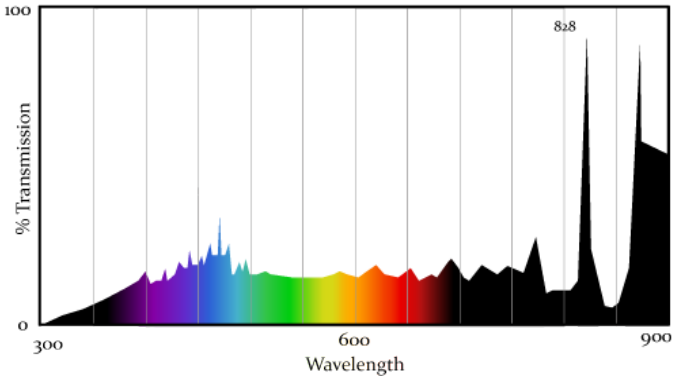


Figure 6: Spectrum of a xenon arc lamp

Another light source used in fluorescence microscopy is the xenon arc lamp (Figure 6), which exhibits a relatively continuous output spectrum in the visible range. Xenon arc lamps are preferred in systems where the spectral characteristics of dyes and/or specimens are being analyzed quantitatively, but they are not as bright as a mercury lamp of equivalent wattage. Even in the region of FITC excitation (between 450 and 500nm) where the mercury lamp is relatively weak, the xenon arc lamp is only marginally brighter. This is primarily a result of the fact that the light-producing arc of the xenon lamp is about twice the size of the arc in the equivalent mercury lamp, which reduces the amount of available light that can be focused onto the specimen using a typical microscope configuration.

LED Light Sources

In recent years, light-emitting-diode- or LED-based light sources have become the source of choice for modern microscopists. These sources often possess multiple LEDs with distinct spectral outputs. These can be combined within the source itself to form a semblance of a “white-light” source, akin to the older arc lamps, or can be kept separated to allow for rapid switching between them for sequential imaging with a multi-band filter set.

Some caution should be taken when choosing an LED-based light source to determine the spectral output of the source and filter sets should be chosen that matches that output. Also, in practically all cases, filter sets with excitation filters MUST be used to account for the relatively wide-band output of the individual LEDs.

Lasers

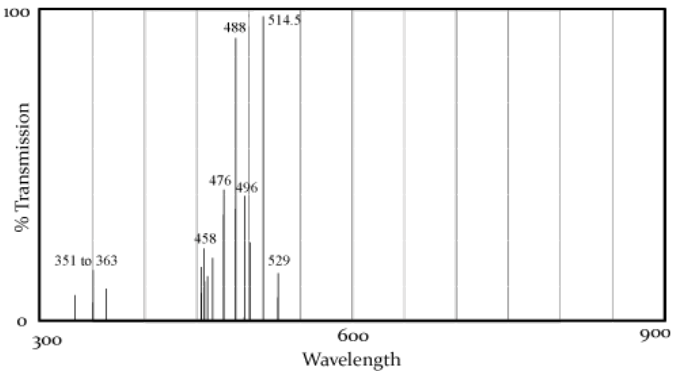


Figure 7: Typical spectrum of an argon-ion laser. (Data from Spectra-physics Lasers, Inc.)

Laser light sources have become an increasingly common choice for several forms of fluorescence microscopy. The output spectrum produced by a laser is confined to a smaller range of wavelengths due to their coherence. This means that the blocking range of excitation filters need only cover the range of output of the laser. For example, IR blocking is not required for the argon-ion laser (Figure 7).

Two general types of laser sources are available: continuous wave (CW) lasers and pulsed lasers. CW lasers emit light in a constant stream of continuous and constant power. Pulsed lasers emit light in brief bursts that are separated by a user-defined periodicity. When compared to CW lasers, pulsed lasers generally possess a higher peak power output during each pulse, but a lower power average overall. CW lasers are used in a variety of applications including laser-scanning confocal, Raman spectroscopy and TIRF microscopy, while pulsed lasers are a necessity for multi-photon microscopy.

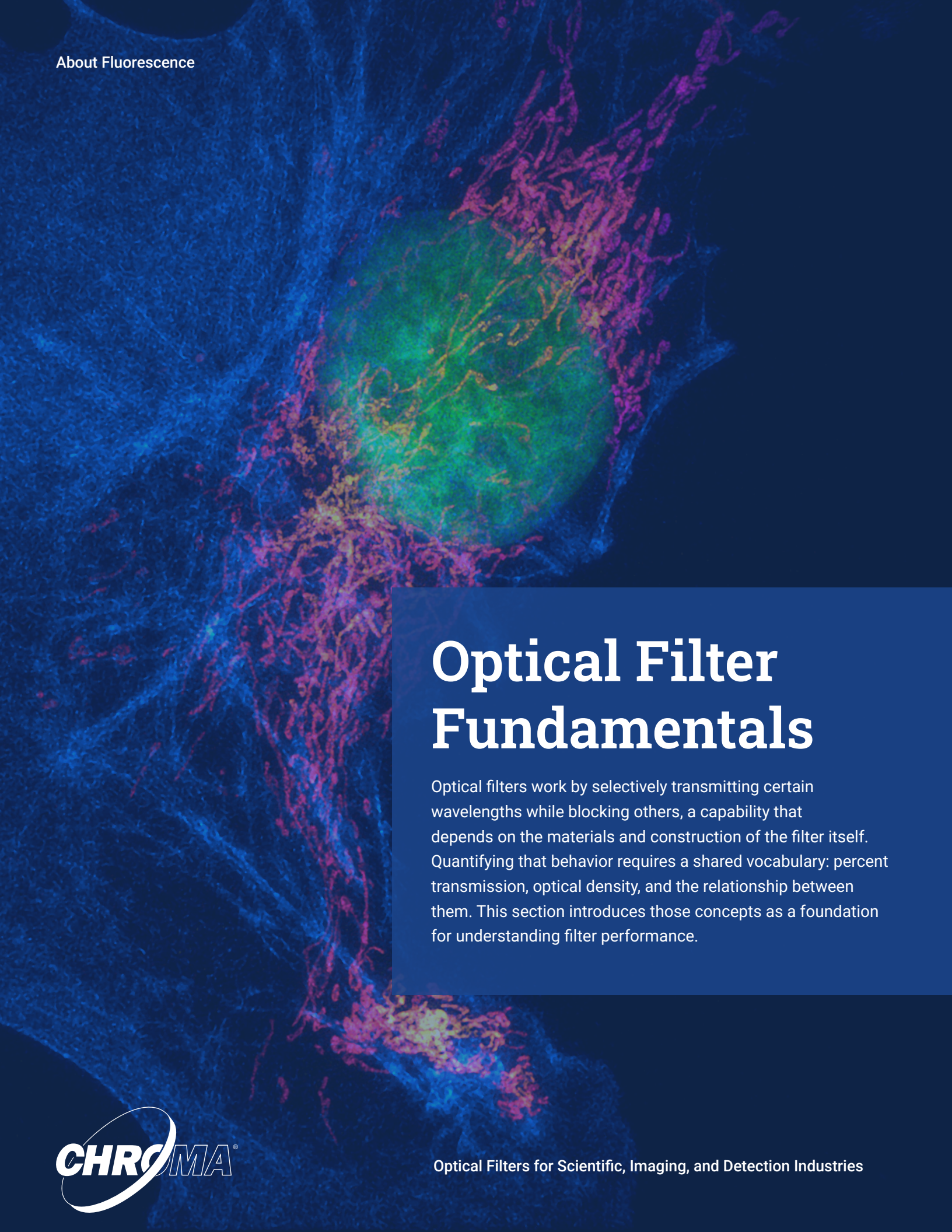
The use of lasers as the illumination source for fluorescence applications requires special consideration for the optical path. This special attention centers on the coherent nature of the light, the small beam diameter, the polarization, and the power. There are also circumstances where cone angle and scatter within the system play significantly into the design of the optics. For these reasons, laser-based fluorescence systems often require filters and beamsplitters specifically designed for laser use. In particular, the optics in the excitation and detection paths must be carefully selected to provide appropriate transmission, blocking, and wavefront performance while handling the unique characteristics of laser illumination.

Image Acquisition

In any microscopy set-up – fluorescence or brightfield - the optical detector being utilized must be carefully selected to deliver the highest quality image possible for the application. This selection is not often trivial, as the researcher must take into account a myriad of variables, most of which are mutually exclusive. Oftentimes, one variable is optimized while others are negatively impacted. Therefore, in most live cell microscopy situations, the primary task becomes balancing the health of the cells with the quality of the image; taking into consideration factors like cell viability, signal-to-noise ratio, and required resolution or speed of acquisition. The aims and goals of the particular investigation should determine the order in which these factors are prioritized.

Detector Types:

Detector choices include color CCD cameras to capture brightfield images or samples containing multiple fluorochromes, infrared sensitive cameras often utilized in DIC mode imaging, electron-multiplying CCD cameras to capture low-level fluorescence signals, high-resolution monochrome sensors for TIRF and other specific fluorescence techniques, and many more.



Optical Filter Fundamentals

Optical filters work by selectively transmitting certain wavelengths while blocking others, a capability that depends on the materials and construction of the filter itself. Quantifying that behavior requires a shared vocabulary: percent transmission, optical density, and the relationship between them. This section introduces those concepts as a foundation for understanding filter performance.

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Transmission, Reflection, and Absorption

When light strikes a discontinuity in the medium through which it is propagating, the wavelengths that pass through are said to be transmitted and wavelengths that do not pass through are said to be blocked or attenuated. When working with fluorescence, optical filters serve as the discontinuity and can be made to selectively transmit or block certain wavelengths.

Blocking (attenuation) can be accomplished by reflecting or absorbing light by the filter and the degree to which a filter transmits or attenuates light is completely dependent on its constituent materials and the way they are used in its construction.

The rainbow seen after shining light through a prism, for example, is a transmitted light spectrum, broken up into its constituent parts. It is a visualization of the wavelengths of light that the material failed to attenuate (reflect or absorb).

Transmission, reflection and absorption are all measurable properties and are used to quantify the ways materials interact with light. The illustration above visually highlights the basic principles of transmission, reflection and absorption.

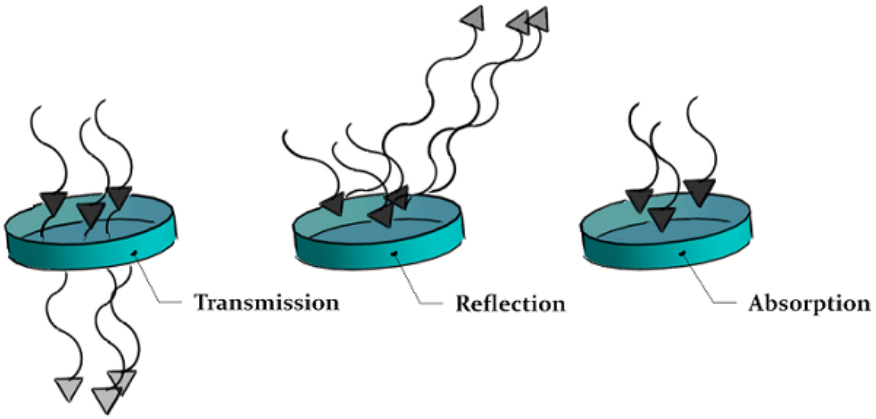

Figure 8: Illustration showing the principles of light interaction with materials: transmission (light passing through), reflection (light bouncing off), and absorption (light being absorbed).

Percent Transmission

Percent transmission (%T) is the most common unit used to quantitatively express how an optical filter transmits light. Percent transmission is measured at a particular wavelength (or wavelength range) and is the ratio of transmitted light intensity (I) to incident light intensity (Io), expressed as a percentage:

%Tλ = I/Io * 100

The diagram below illustrates the terminology typically used when discussing spectra expressed in %T. This figure is explained further in the **Filter Performance Considerations** section.

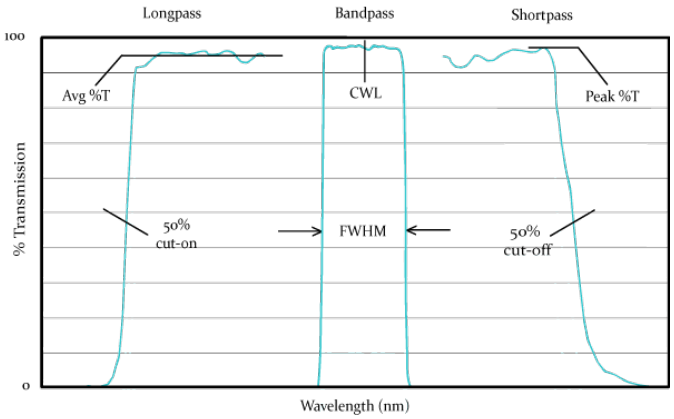

Figure 9: Transmission Nomeclature

The attenuation level (blocking level) and attenuation range (blocking range) of filters are normally defined in units of optical density (OD). Optical density uses the same units as the quantity absorbance, and is expressed as the negative log of the transmission. It should be noted that one should not try to infer optical density from a transmission plot, a common mistake made by those working with fluorescence. Expressed mathematically:

OD= -log Tλ = -log [(%Tλ) / 100]
Example: OD 4.5 = 3 × 10^{-5} (0.003 %T)

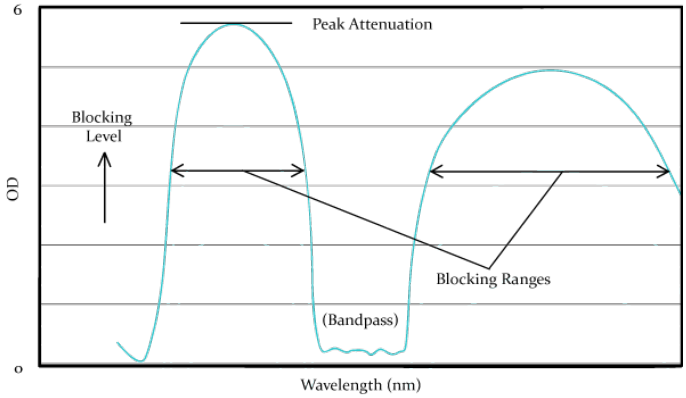

Figure 6: Attenuation Nomeclature

Optical Density

The attenuation level (blocking level) and attenuation range (blocking range) of filters are normally defined in units of optical density (OD). Optical density uses the same units as the quantity of absorbance, and is expressed as the negative log of the transmission.

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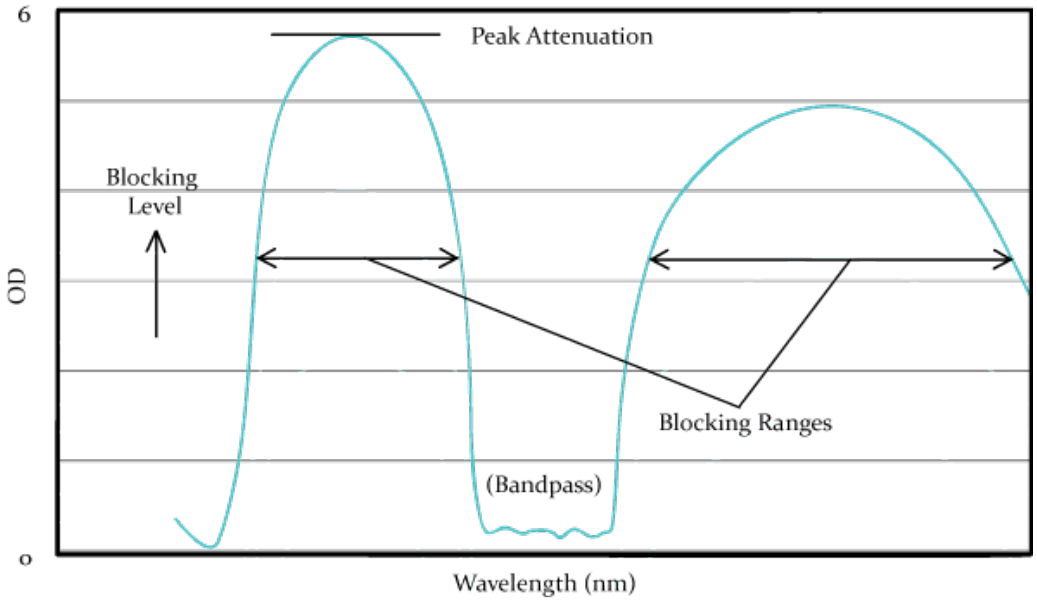
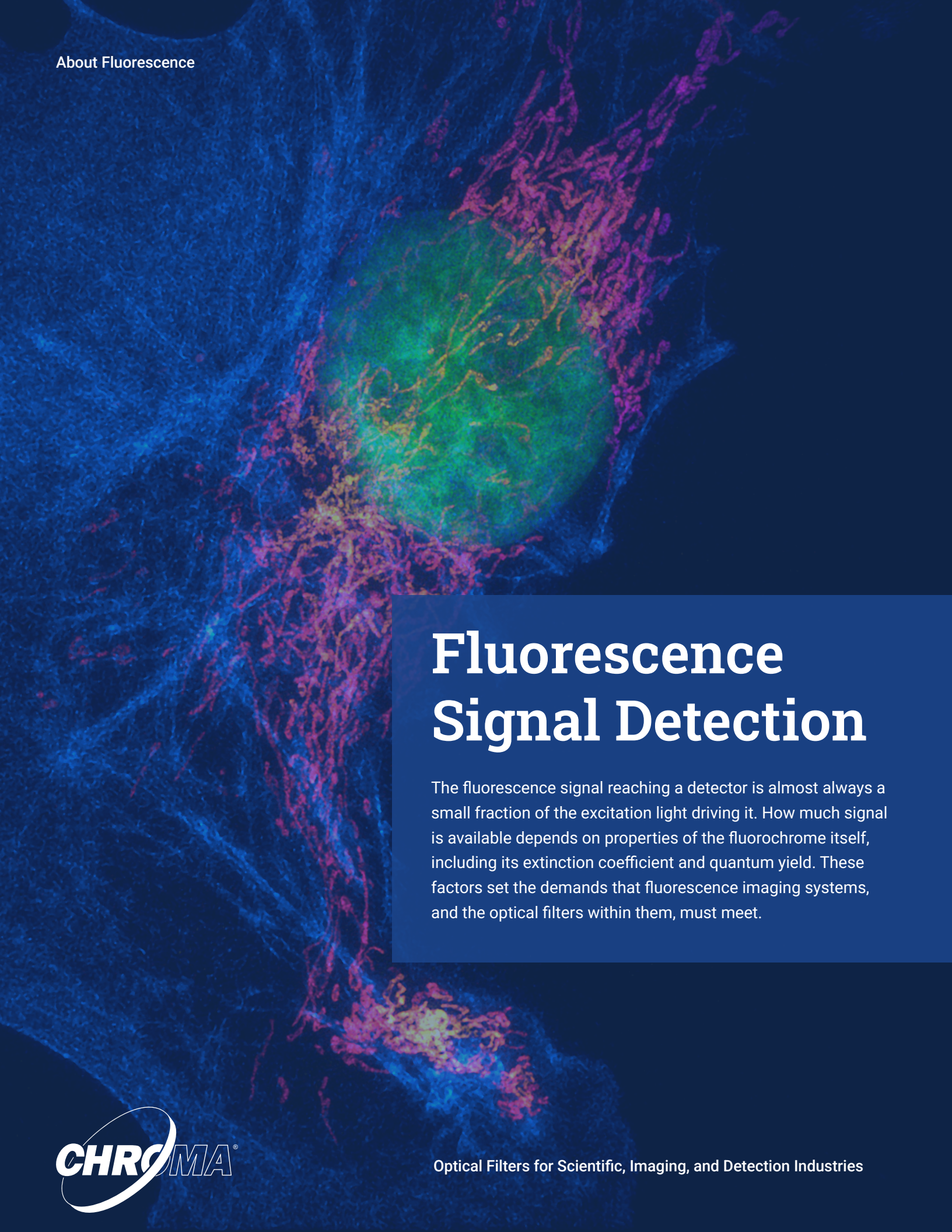


Figure 6: Attenuation Nomeclature



Fluorescence Signal Detection

The fluorescence signal reaching a detector is almost always a small fraction of the excitation light driving it. How much signal is available depends on properties of the fluorochrome itself, including its extinction coefficient and quantum yield. These factors set the demands that fluorescence imaging systems, and the optical filters within them, must meet.

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Factors Affecting Signal Brightness

Several factors influence the amount of fluorescence emitted by a stained specimen with a given amount of excitation intensity. These include:

1. The dye concentration within stained sections of the specimen and the thickness of the specimen;

2. The extinction coefficient of the dye;

3. The quantum efficiency of the dye; and,

4. The amount of stained material actually present within the field of view of the microscope.

Extinction Coefficient

The extinction coefficient tells us how much of the incident excitation light will be absorbed by a given dye, and mirrors the wavelength-dependent absorption characteristics indicated by the excitation spectrum of the fluorochrome. Emission is increased with higher incident light absorption, meaning that fluorochromes with greater extinction coefficients tend to emit more intensely, and require less energy to adequately excite them.

Although many fluorochromes have high extinction coefficients at peak excitation wavelengths, practical sample preparation techniques often limit the maximum concentration allowed in the sample, thus reducing the overall amount of light actually absorbed by the stained specimen.

Experimentally, the benefit of fluorochromes with high extinction coefficients is the ability to use reasonable amounts of excitation light, while avoiding negative quenching processes such as photobleaching or phototoxic damage to the specimen.

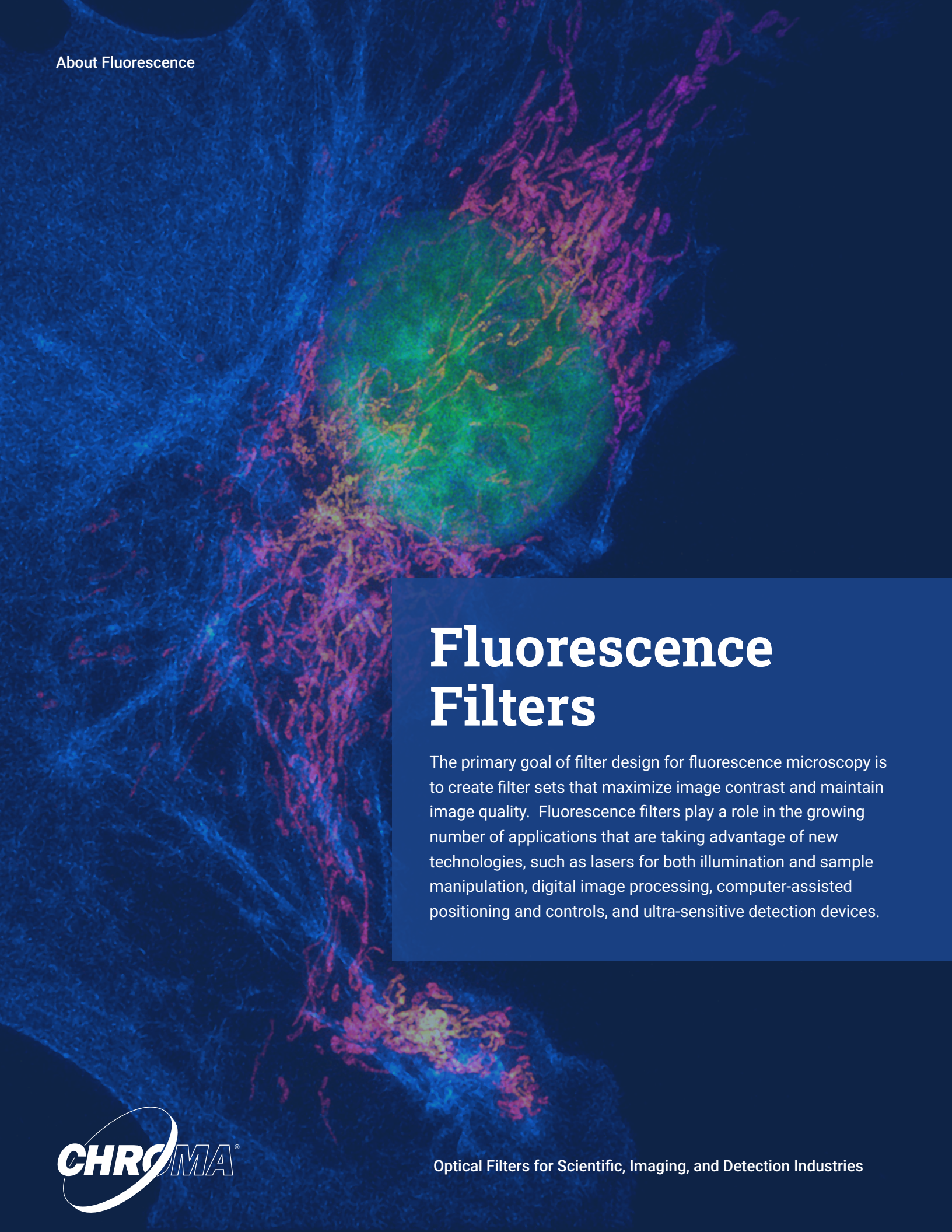
Quantum Yield

Quantum yield (QY) is the ratio of light energy absorbed to fluorescence emitted and determines how much of this absorbed light energy will be converted to fluorescence. Today's most commonly-used fluorochromes have quantum yields of approximately 0.3-0.6 (equalling 30-60% of excitation photons being converted into emitted fluorescent photons), but the actual value can be reduced by quenching.

The product of these factors, in addition to the fact that many specimens have very small amounts of stained material in the observed field of view, gives a ratio of emitted fluorescence intensity to excitation light intensity in a typical application of between 1/10,000 (10^{-4}) for very highly fluorescent samples and 1/1,000,000 (10^{-6})) for dimly fluorescent samples.

Current techniques (e.g. fluorescence in situ hybridization or FISH), which utilize minute amounts of fluorescent material, might have ratios as low as 10^{-9} or 10^{-10}. Thus, in order to see the fluorescent image with adequate contrast, the fluorescence microscope must be able to attenuate the excitation light by as much as 10^{-11} (for very weak fluorescence) without diminishing the fluorescence signal.

These large differences between excitation intensity and emitted fluorescence make optical filters essential components of fluorescence microscopy. The next section explains how filter sets isolate weak fluorescence signals while suppressing unwanted excitation light.



Fluorescence Filters

The primary goal of filter design for fluorescence microscopy is to create filter sets that maximize image contrast and maintain image quality. Fluorescence filters play a role in the growing number of applications that are taking advantage of new technologies, such as lasers for both illumination and sample manipulation, digital image processing, computer-assisted positioning and controls, and ultra-sensitive detection devices.

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Overview of Typical Filter Sets

The primary filtering element in an epifluorescence microscope is the set of three filters housed in a fluorescence filter cube (or filter block): the excitation filter, the emission filter, and the dichroic beamsplitter.

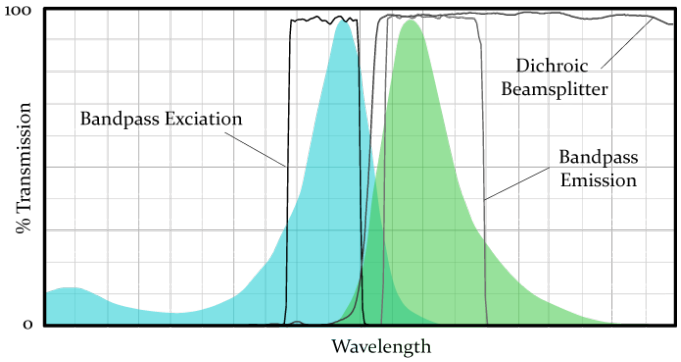


Figure 11: "Ideal" fluorescence set, including an exciter, dichroic, and emitter overlaid on example absorption (excitation) and emission spectra.

The **excitation filter** (also called the exciter) transmits only those wavelengths of the illumination light that efficiently excite a specific dye. Although shortpass filter designs were used in the past, bandpass filter designs are now used almost exclusively.

The **emission filter** (aka barrier filter or emitter) attenuates all of the light transmitted by the excitation filter and very efficiently transmits any fluorescence emitted by the specimen. This emitted light is almost always of longer wavelength (more to the red) than the excitation color in typical Stokes shift-dependent fluorochromes. Emission filters can be either bandpass or longpass filters.

The **dichroic beamsplitter** (aka dichroic mirror or dichromatic beamsplitter) is a thin piece of coated glass set at a 45° angle to the optical path of the microscope. This coating has the unique ability to reflect one color (the excitation light), but transmit another color (the emitted fluorescence). Current dichroic beamsplitters achieve this with great efficiency (i.e., with greater than 90% reflectivity of the excitation wavelengths along with approximately 90% transmission of the emission wavelengths). This marks a great improvement over the traditional half-silvered mirror, which reflects only 50% and transmits only 50%, giving only about 25% total efficiency across a broad range of wavelengths.

The spectral characteristics of a particular fluorochrome can be overlaid with the spectral profile of a fluorescence filter set to aid in the selection of the most appropriate filter set for any given application. Figure 11 illustrates what the spectral profile of an "ideal" filter set might look like for the given fluorochrome's excitation and emission spectra. As you can see, the excitation filter transmits light spanning the peak of the absorption spectrum, and the emission filter transmits a band of wavelengths spanning the peak emission of the fluorochrome. The dichroic attenuates the excitation light to a small

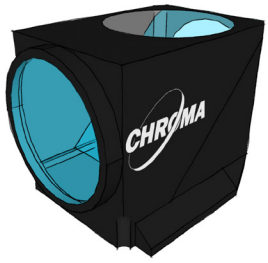


Figure 12: Schematic of a Typical Filter Cube

degree and transmits only that from the fluorescence emission.

Light blocking/attenuation is mostly a function of the excitation and barrier/emission filters in most instruments. Most commonly used perpendicular to the beampaths (or 0° angle-of-incidence, AOI), these filters can achieve blocking/attenuation values of OD6 or greater “out-of-band”. Comparatively, dichroic mirrors (most often used at 45° AOI in epifluorescent microscopes) can achieve OD1.0 - 3.0 in their reflection ranges. Since OD is measured on a logarithmic scale, the majority of blocking/attenuation of unwanted excitation or illumination wavelengths is done by the excitation filter, while blocking/attenuation of the excitation light is done by the barrier/emission filter (and is the origin of the name “barrier filter”).

Once an individual exciter, emitter, and dichroic are chosen for the application, they are mounted in a filter cube and are ready for use in a microscope. The schematic of a typical filter cube for an inverted microscope is illustrated in figure 12 above. Most microscopes have a slider or turret that can hold from two to four individual filter cubes or more. It must be noted that the filters in each cube are a matched set, and one should avoid mixing filters and beamsplitters unless the complete spectral characteristics of each filter component (including OD) are known.

Historically, filter blocks were named after the type of excitation filter present in the filter set: UV or U (Ultraviolet excitation for dyes such as DAPI and Hoechst 33342), B (Blue excitation for FITC and related dyes), and G (Green excitation for TRITC, Texas Red®, etc.). Common barrier filter colors are blue or pale yellow in the U-block, green or deep yellow in the B-block, and orange or red in the G-block. In early filter sets, these were often simple doped absorption glass. Modern filter sets consist of interference-coated optics with high durability, much better transmission efficiencies and deep blocking/attenuation properties.

How Fluorescence Filters Work Together

To better understand how fluorescence filters function as a system, it is helpful to follow the path of light through a typical microscope while imaging a fluorescent specimen. Each component in the filter set plays a specific and coordinated role in isolating a weak fluorescence signal from a much stronger excitation source.

Consider a specimen labeled with a common fluorochrome such as green fluorescent protein (GFP).

Step 1 – Excitation Filtering

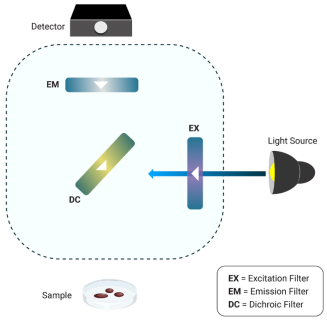


Figure 13: Excitation filter transmitting light at wavelengths associated with the excitation band of the fluorochrome.

Light from the illumination source first passes through the excitation filter. This filter transmits only a specific band of wavelengths that efficiently excite the fluorochrome. For GFP, this typically corresponds to blue light in the range of approximately 450–490nm. All other wavelengths are attenuated to prevent unnecessary illumination and reduce background signal.

Step 2 – Dichroic Reflection

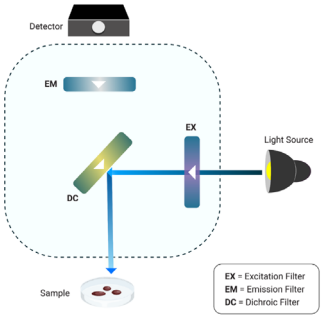


Figure 14: Dichroic filter reflecting the wavelengths of light transmitted by excitation filter towards the sample.

The filtered excitation light then encounters the dichroic beamsplitter, which is positioned at a 45° angle to the optical path. The dichroic is designed to reflect the excitation wavelengths while transmitting longer wavelengths. As a result, the excitation light is reflected down through the objective and onto the specimen.

Step 3 – Fluorescence Emission

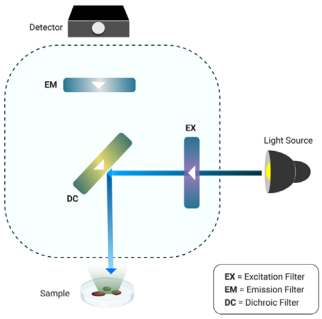


Figure 15: The fluorochrome absorbs the excitation light and fluoresces at a longer wavelength.

When the fluorochrome absorbs this excitation light, it emits fluorescence at a longer wavelength due to the Stokes shift. In the case of GFP, the emitted light peaks around ~510nm (green). This emitted fluorescence travels back up through the objective along the same optical path.

Step 4 – Dichroic Transmission

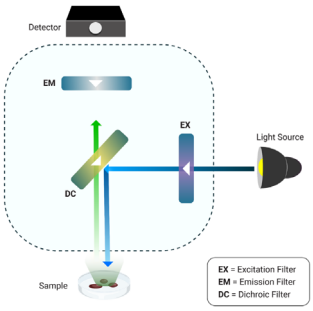


Figure 16: The dichroic filter transmits the wavelengths of emitted light.

As the emitted light reaches the dichroic beamsplitter, it is now within the transmission range of the coating. Instead of being reflected, the longer-wavelength fluorescence passes through the dichroic and continues toward the detector.

Step 5 – Emission Filtering

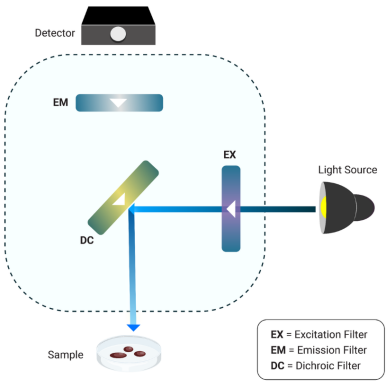


Figure 17: The emission filter transmits only the wavelengths of light associated with fluorescence emission. All other wavelengths are blocked.

Before reaching the detector or eyepiece, the light passes through the emission filter. This filter is designed to block any residual excitation light that may have been scattered or reflected within the system, while transmitting only the desired fluorescence emission. This step is critical for achieving high contrast, as even a small amount of excitation light can overwhelm the relatively weak fluorescence signal.

Result – Isolated Fluorescence Signal

After passing through all three components—the excitation filter, dichroic beamsplitter, and emission filter—the light reaching the detector consists almost entirely of fluorescence from the specimen. This coordinated filtering process enables the visualization of signals that may be orders of magnitude weaker than the original excitation light.

The precise spectral alignment of these components with the excitation and emission properties of the fluorochrome is essential. Even small mismatches can reduce signal intensity or increase background, underscoring the importance of carefully designed and matched filter sets in fluorescence microscopy.

While this example describes a single fluorochrome, many applications require imaging multiple fluorochromes simultaneously, which introduces additional complexity in filter design.

Filter Types

Bandpass Filters:

Bandpass filters are designed to transmit a specific range of wavelengths and block light on either side of that range. These filters are denoted by their center wavelength and bandwidth. The center wavelength (CWL) is the arithmetic mean of the wavelengths at 50% of peak transmission. The full width at half maximum (FWHM) is the bandwidth at 50% of peak transmission.

Longpass & Shortpass Filters (LP & SP):

LP and SP filters are denoted by their cut-on or cut-off wavelengths at 50% of peak transmission. LP or SP filters that have a very sharp slope and are often called edge filters. The average transmission is calculated over the useful transmission region of the filter, rather than over the entire spectrum. (Please note that the use of terms “highpass” and “lowpass” are discouraged because they more accurately describe frequency rather than wavelength.)

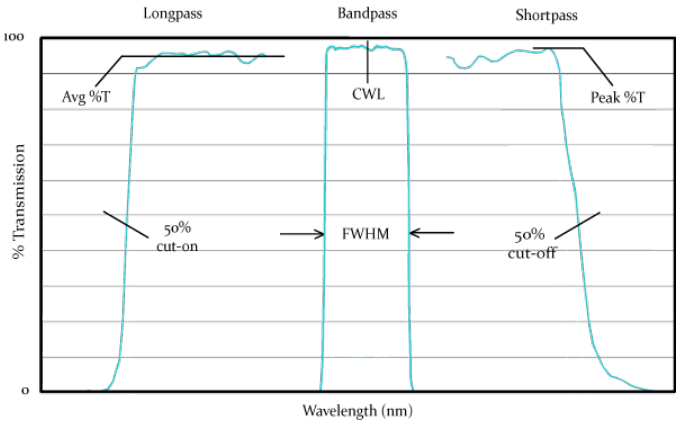


Figure 18: Nomenclature Transmission

Additional Filters Used in Fluorescence Microscopy

Other optical filters can also be found in fluorescence microscopes:

Heat Filters

Also called a hot mirror, this filter is incorporated into the illuminator collector optics of most, but not all, microscopes. It attenuates infrared light (typically wavelengths longer than 800nm) but transmits most of the visible light.

Neutral Density Filters

Commonly called a grey filter, a neutral density (ND) filter blocks all wavelengths of light evenly. For example, a particular ND filter might transmit 2% of the entire visible spectrum, while another (Figure 19) might transmit 50% of light in the same range. ND filters are typically intended for a specific range and do tend to fall off on either end of that range, therefore it is important to specify your application when ordering.

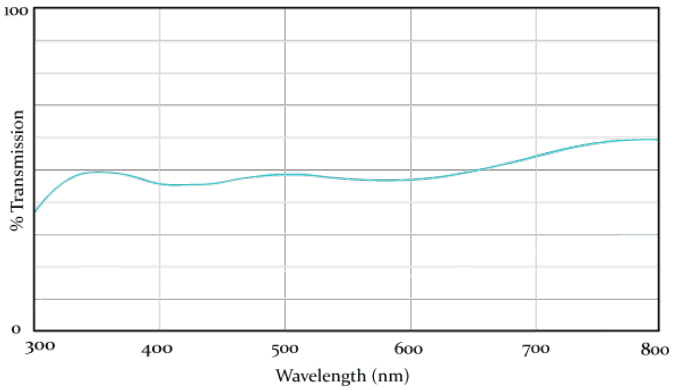


Figure 19: An example of an ND0.3 filter with ~50%T across the “visible”

ND filters are usually housed in a filter slider or filter wheel between the collector and the aperture diaphragm and are used to control the intensity of illumination.

Specialty Filters

This category includes filters used for techniques other than fluorescence, such as color filters for transmitted-light microscopy and linear polarizing filters for polarized-light microscopy.

Multiband Filter Sets

The use of multiple fluorochromes was previously limited by issues arising from the need to switch filter cubes between each single-color image. A 'pixel shift' (caused by optical inconsistencies between the individual filter sets) contributed to artifacts in the digitally-combined image. Additionally, the time it took to switch cubes prevented high speed imaging of live cells and the demand for a new way to image specimens with multiple fluorochromes grew larger. As a result, multiband filter sets were soon introduced, and three distinct filter configurations emerged; each allowing simultaneous or sequential imaging of multiple fluorochromes in the same sample.

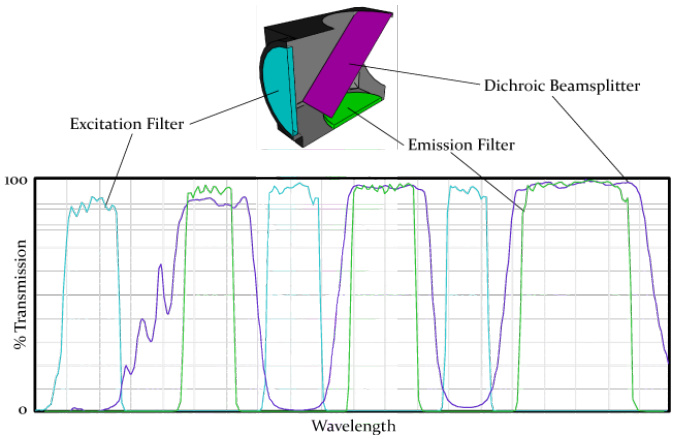


Figure 20: Spectra of a triple-band filter set designed for the dyes DAPI, FITC, and Texas Red® with color-coded cube schematic

One filter configuration is a set including a multiband exciter, multiband dichroic, and multiband emission filter. This configuration is referred to as "full multiband." Figure 20 illustrates the concept of a full multiband set; each filter can be created to selectively transmit and block multiple bands of wavelengths. This configuration requires the user to use a color CCD camera or naked eye and will simultaneously capture multiple fluorescences present in a sample in a single image. However, this ability comes with the costs of being unable to use a monochrome CCD or other detector, and having decreased optical separation of individual fluorochrome signals (called signal bleed-through), due to overlapping excitation and emission spectra between the fluorochromes. An example spectrum of a full multiband set is shown in figure 20 with the corresponding filters color-coded in the cube schematic.

The other configurations, known colloquially as "Sedat" and "Pinkel" configurations (after Drs. Sedat and Pinkel for whom these configurations were first produced by Chroma), both incorporate a multiband dichroic, but differ in the combination of excitation and emission filters used. The "Sedat" filter configuration uses both single-band exciters and single-band emitters, while the "Pinkel" configuration uses single-band excitation filters and a multiband emitter. The signal-to-noise ratio (S/N) achieved while using a Pinkel set is potentially higher than when using a full multiband configuration, although when comparing multiband filter sets, the Sedat configuration will, in most cases, give the highest S/N. As illustrated in figure 21, it is common to use a monochrome camera to individually capture the emission from each fluorochrome (in this case there are three), apply a false color digitally, and overlay them to get a final image when using any multiband filter set.

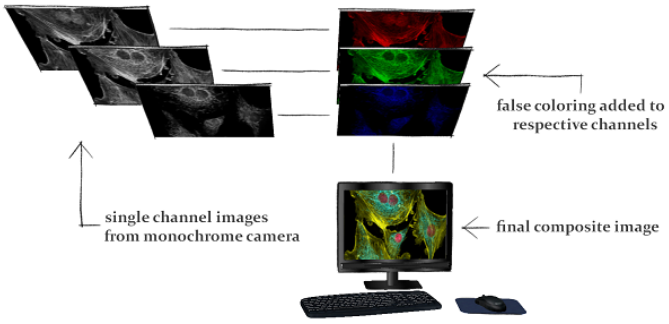
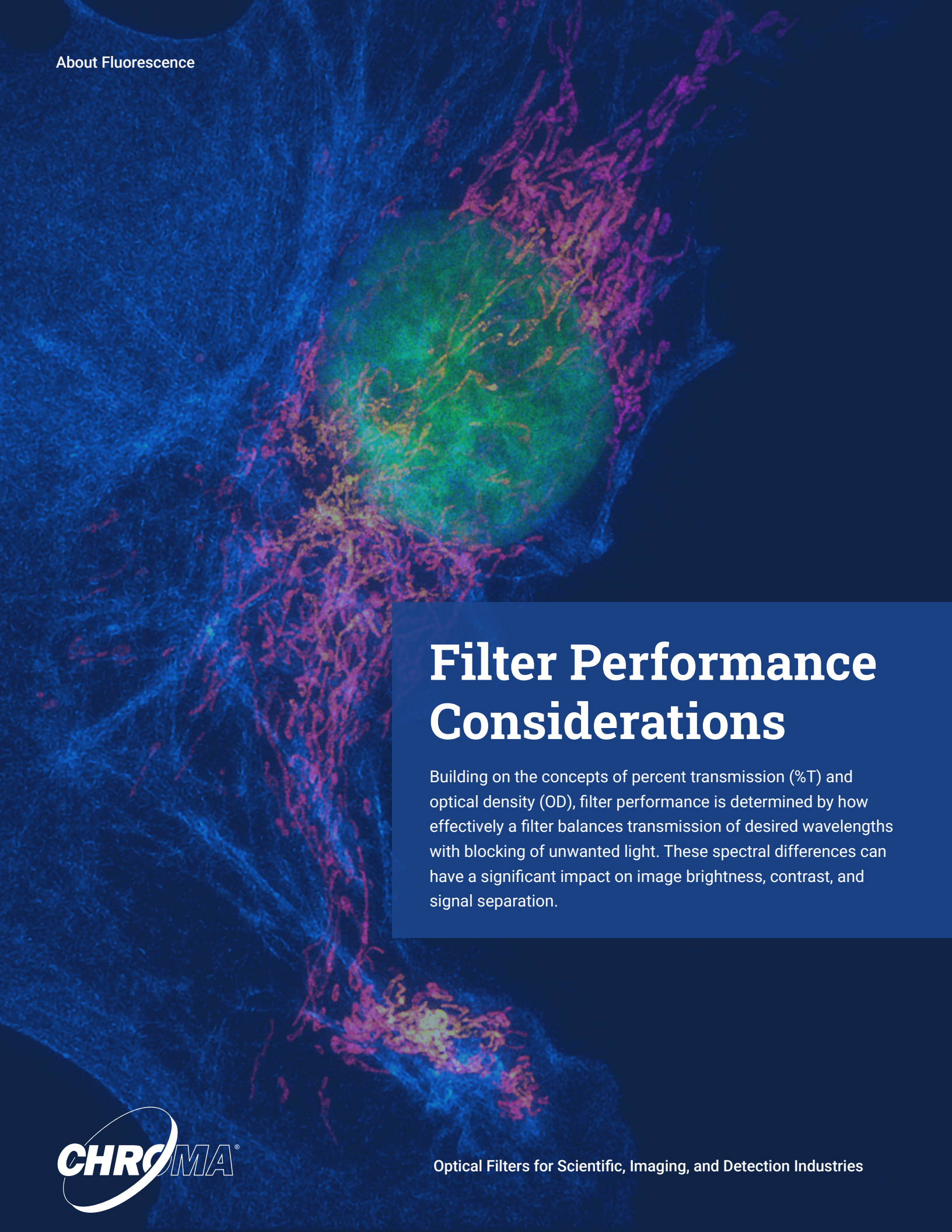


Figure 21: Steps in creating a multiband image using a monochrome camera and digital processing

Multicolor Imaging and Registration

When making a multiple-exposure photograph or multiple electronic images of specimens stained with several fluorochromes using separate filter cubes in a standard microscope, there will be unavoidable registration shifts between exposures. Varying amounts of wedge in the emission filter and beamsplitter, variations in thickness and alignment of the beamsplitter, and mechanical vibration that occurs when the cubes are switched all contribute to this shift. While these effects can be reduced to acceptable levels for some applications, many others require more sophisticated filter designs and optical apparatus.



Filter Performance Considerations

Building on the concepts of percent transmission (%T) and optical density (OD), filter performance is determined by how effectively a filter balances transmission of desired wavelengths with blocking of unwanted light. These spectral differences can have a significant impact on image brightness, contrast, and signal separation.

CHROMA®

Bandpass Shape & Edge Steepness

Bandpass filters are typically constructed using thin-film interference coatings applied to optical substrates. The design of these coatings determines the shape of the transmission band and how sharply the filter transitions between transmission and blocking regions.

Two key aspects define bandpass performance:

- Bandwidth (FWHM) – The width of the transmission band at 50% of peak transmission
- Edge steepness (slope) – How quickly the filter transitions from blocking to transmission (and vice versa)

Filters with steeper edges can more precisely separate excitation and emission wavelengths. This is particularly important when working with fluorochromes that have small Stokes shifts, where excitation and emission spectra are closely spaced.

A steeper transition allows:

- Wider usable transmission bands (more signal)
- Better rejection of unwanted wavelengths (less background)
- Improved separation between fluorochromes in multicolor experiments

In contrast, filters with gradual transitions require greater spectral separation and may result in reduced signal or increased bleed-through.

Transmission & Blocking in Practice

Effective fluorescence filtering requires both high transmission within the desired band and strong blocking outside of it.

- High transmission (%T) increases signal brightness and improves detection sensitivity
- High optical density (OD) ensures that unwanted excitation light is sufficiently attenuated

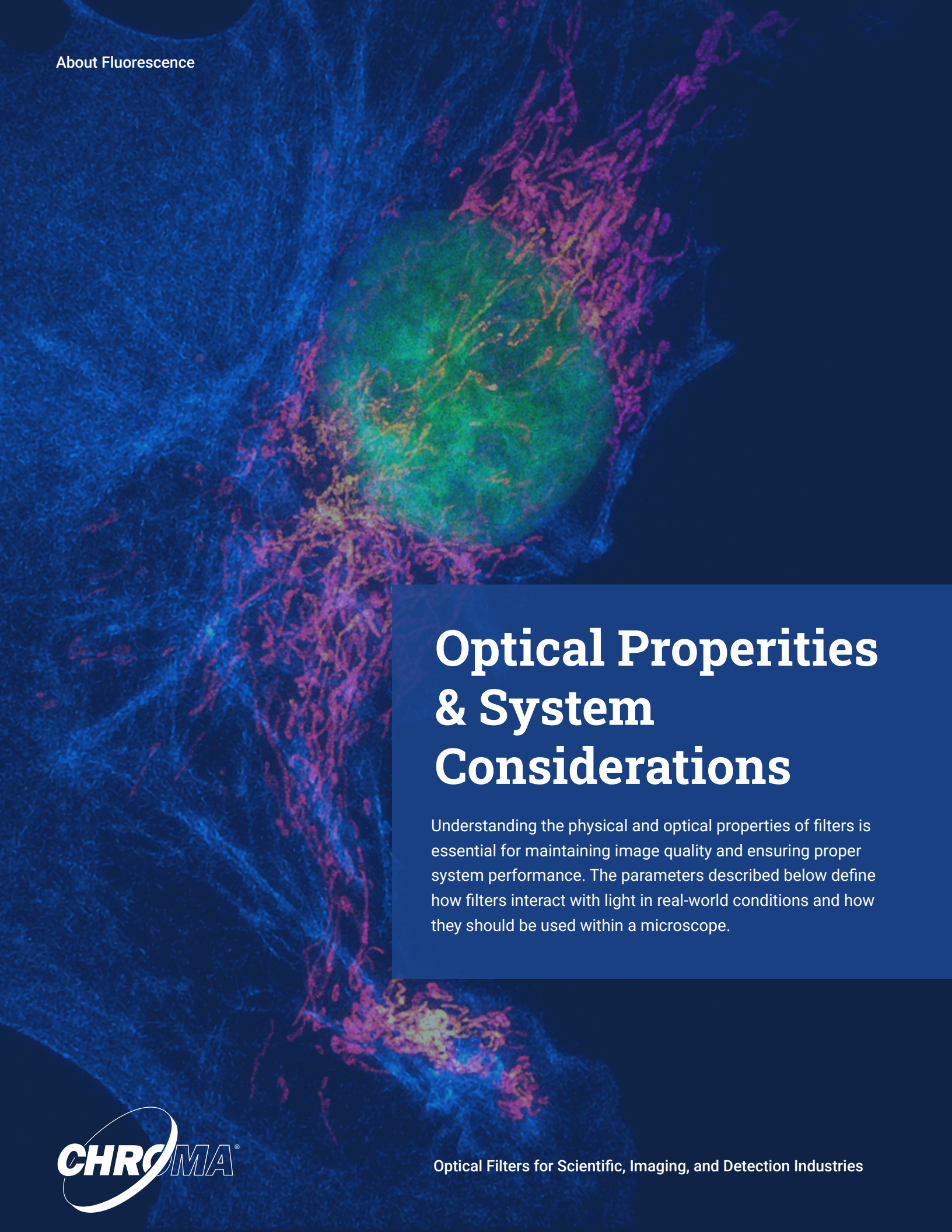
These two properties are independent and must be considered together. A filter may exhibit high transmission within its passband but still provide inadequate blocking outside of it if not properly designed.

In fluorescence microscopy, where emitted signals may be several orders of magnitude weaker than excitation light, strong blocking is critical. Even small amounts of residual excitation light can significantly reduce image contrast.

The most effective filter designs balance these factors by:

- Maximizing transmission across the emission or excitation band
- Providing deep blocking outside the band (often OD6 or greater)
- Maintaining steep spectral transitions between regions

Careful selection of filters based on these characteristics is essential for optimizing signal-to-noise ratio and overall imaging performance.



Optical Properities & System Considerations

Understanding the physical and optical properties of filters is essential for maintaining image quality and ensuring proper system performance. The parameters described below define how filters interact with light in real-world conditions and how they should be used within a microscope.

Optical Quality & Image Performance

The following is a list of important parameters used to define the overall image quality of a filter or beamsplitter. The first three parameters are illustrated in figures 22, 23, and 24.

Surface Flatness

A measure of the deviation of the surface of an optical element from a perfect plane, usually measured in fractions or multiples of a wavelength of visible light (usually 550 or 630nm, but sometimes using the CWL for a bandpass filter). The actual wavefront distortion that a plane wave of light undergoes when reflected from the surface is twice the value of the surface flatness.* The wavefront distortion of light reflected off a beamsplitter or mirror is solely determined by the surface flatness of the reflecting surface, usually the front surface.

*This is strictly true only for light reflected at normal incidence. The value for light reflected at non-normal incidence is modified by a cosine factor. For example, the reflected wavefront distortion at 45 degrees angle of incidence is approximately .4 times the surface flatness.

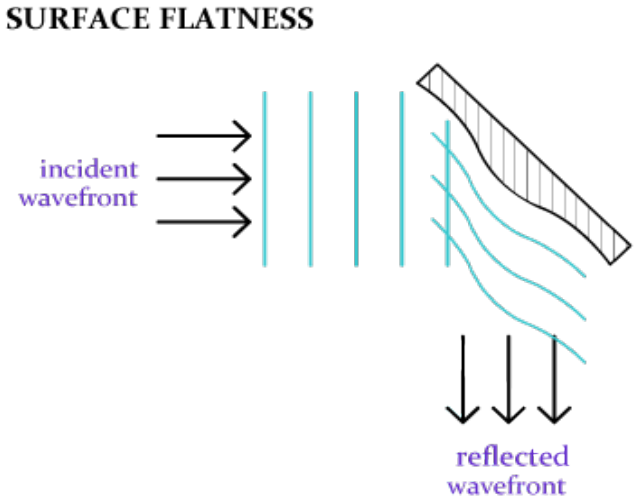


Figure 22: Generalized function of surface flatness in a typical beampath

Transmitted Wavefront Distortion (TWD)

A measure of the distortion a plane wave of light undergoes when transmitted through an optical element is also measured in fractions or multiples of a wavelength of light, the same as for surface flatness described above. The surface flatness of the outer surfaces of the element and, to a lesser degree, internal structures that cause inhomogeneity of the refractive index, combine to make up the overall TWD of the optical element.

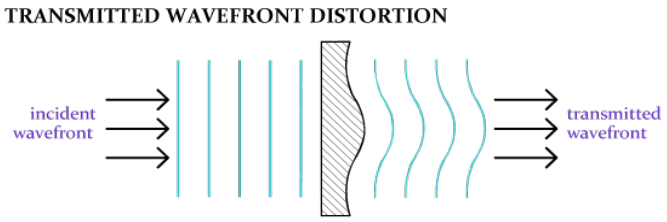


Figure 23: Generalized function of transmitted wavefront distortion in a typical beampath

Wedge

Sometimes called parallelism, wedge is a measure of how parallel are the outer surfaces of an optical element. Wedge is usually measured in arc-minutes or arc-seconds of angle. The main effect of wedge is to induce an angular deviation in the direction of a light beam, causing, for example, image shifting. The amount of angular deviation is about one-half the wedge angle for a typical filter.* The wedge of internal coating surfaces, while not contributing greatly to beam deviation, can cause noticeable ghost images as a result of off-axis internal reflections.

*For small angles of incidence, the angular deviation = (N – α), where N is the refractive index of the glass in the filter, and α is the wedge angle. Most filters use optical glass with a refractive index of approximately ~1.45-1.55.

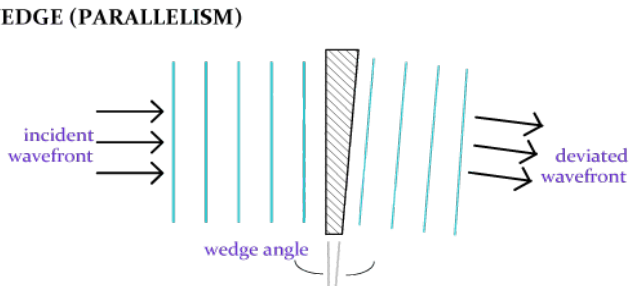


Figure 24: Generalized function of wedge in a typical beampath

Surface Quality & Defects

Surface imperfections can affect both performance and durability, although their impact varies depending on the application.

Scratches & Digs

A set of specifications exist for defining the maximum allowable size and number of scratches and digs on an optical surface. The term digs includes such things as particles and small bubbles embedded inside a filter and macroscopic inclusions in exposed coatings. The scratch/dig values (e.g., 80/50) specify the scratch width in microns and the dig diameter in tens of microns, respectively. Although extensive evaluation procedures exist if rigorous standards must be maintained (military specification MIL-F-48616, for example), a qualitative visual assessment of the scratches and digs usually suffices.

Pinholes

Small breaks in the coating of an interference filter, pinholes are usually caused by the presence of dust particles on the substrate during coating. Pinhole size must be measured against standard maximum-tolerance pinholes under specific conditions using a high-intensity illuminator.

Clear Aperture

The surface area of an optical filter which is free of any defects or obstructions. On interference filters the clear aperture is often delimited by an annulus of metal or opaque material around the outside edge of the filter. The clear aperture of optical filters should not restrict the overall aperture of the microscope. It is also of critical importance that there is no leakage of unfiltered light around the edge of the clear aperture.

Angular Effects & Polarization

Angle of Incidence (AOI)

The angle of incidence (AOI) is the angle between the optical axis of the incident light and the axis normal to the surface of the filter. Most filters are designed to be used at zero degrees angle of incidence, called normal incidence, but for beamsplitter coatings the usual angle is 45 degrees. It should be noted that most types of filters, including thin-film interference coatings, are "angle-sensitive," which means that the characteristic performance changes with angle. If a filter or beamsplitter is to be used at any angle other than the usual zero- or 45-degree angle, it must be specified explicitly.

Polarization

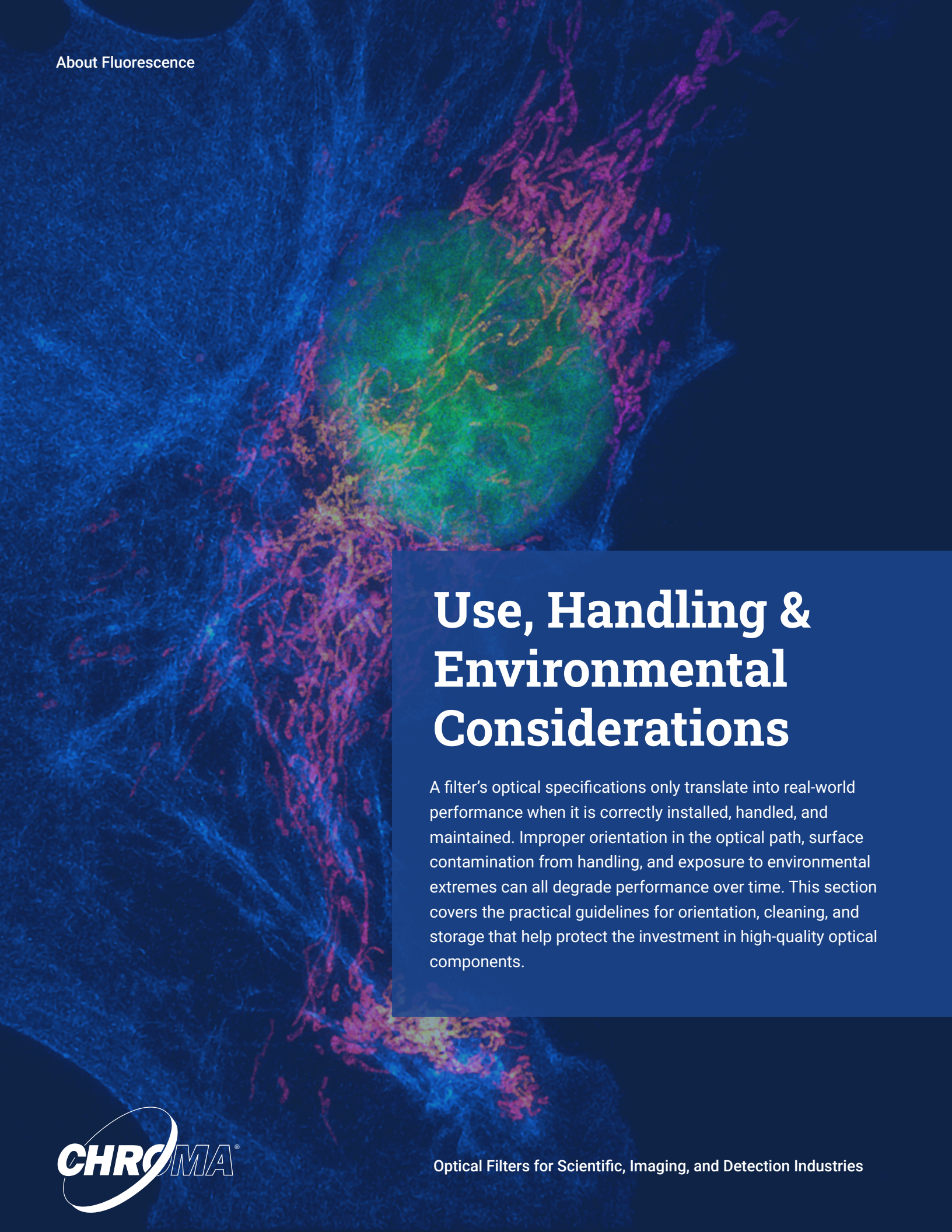
Polarization indicates the vibrational orientation and phase of the electric and magnetic components of a light wave as it propagates in space. These vibrations are transverse to the direction of propagation of the light, and can be oriented at some angle around the axis of propagation. When the orientation and the phase of the vibrations change rapidly and randomly in time, the light is said to be unpolarized. When the vibrations are restricted to one particular orientation angle over an extended length of time, the light is said to be plane-polarized. Light can be partially, as well as totally, plane-polarized.

When light strikes a specular surface at non-normal incidence, the component of the electric field vibrations parallel to the plane of incidence of the surface (P-plane) behaves differently than the component perpendicular to the plane of incidence (S-plane). This causes a polarizing effect that is aligned orthogonally to the orientation of the surface. Dichroic beamsplitters (and, in fact, any thin-film interference coating that is used at non-normal angles-of-incidence) will cause some amount of polarization, the precise effect varying greatly with wavelength and with the particular coating design.

Physical Compatibility & Integration

Filter Dimensions

Filter dimensions vary greatly from microscope to microscope. Chroma's most common filter size is 25mm diameter (~1"), whereas our most common dichroics are 25.5x36mm or 26x38mm. We can, however, produce optics of almost any physical specification that is required. Chroma also offers a wide range of custom sizes and formats to match specific instrument requirements.



Use, Handling & Environmental Considerations

A filter's optical specifications only translate into real-world performance when it is correctly installed, handled, and maintained. Improper orientation in the optical path, surface contamination from handling, and exposure to environmental extremes can all degrade performance over time. This section covers the practical guidelines for orientation, cleaning, and storage that help protect the investment in high-quality optical components.

Orientation

The performance of a filter is highly dependent upon its orientation in a microscope. In addition to being angle sensitive, fluorescence filters must be aligned and oriented correctly to be effective. Proper orientation of the filter is necessary in order to minimize autofluorescence and maximize performance. There is a caret (arrow) located on the edge of each filter in order to aid orientation. Excitation (x) filters should be positioned with the arrow pointing toward the specimen, toward the inside of the cube, and away from the light source. Emission (m) filters should be placed with the arrow pointing toward the specimen, toward the inside of the cube, and away from the detector/eye. Dichroic mirrors should be mounted with the coated surface toward the light source, excitation filters, and the specimen. The dichroics either have an arrow on the side pointing to the coated side, or they are beveled on the coated side. The beveled side is the smaller surface.

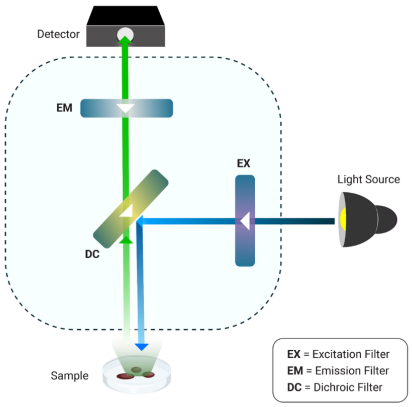


Figure 25: Typical filter orientation in a standard fluorescence microscopy workflow.

Cleaning & Handling

Handling Guidelines

Always handle filters by their edges. Avoid contact with optical surfaces, as fingerprints and oils can degrade performance. When possible, wear clean gloves (such as nitrile) to prevent contamination. If filters must be set down, place them only on clean, soft, lint-free surfaces.

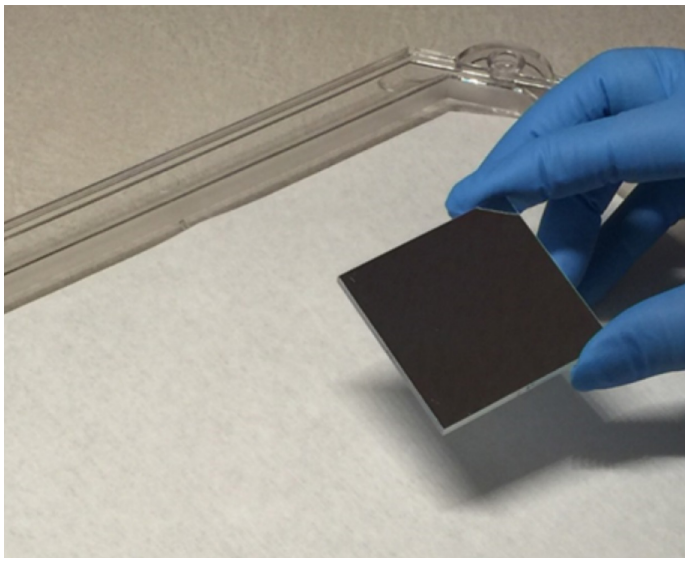


Figure 26: User holding an optical filter by the edges using clean gloves to minimize potential damage.

Cleaning Guidelines

Filters should only be cleaned when necessary. Unnecessary cleaning can introduce dust or cause surface damage.

To clean a filter:

- First, remove loose dust and debris using a bulb puffer or filtered, pressurized air (or dry nitrogen). Never blow on the surface with your mouth.
- If wiping is required, use reagent-grade anhydrous alcohol (or equivalent) and lint-free wipes or swabs.
- Apply minimal pressure and wipe gently in a smooth, continuous motion from the inside out. Use a fresh surface of the wipe for each pass to avoid reintroducing contaminants.

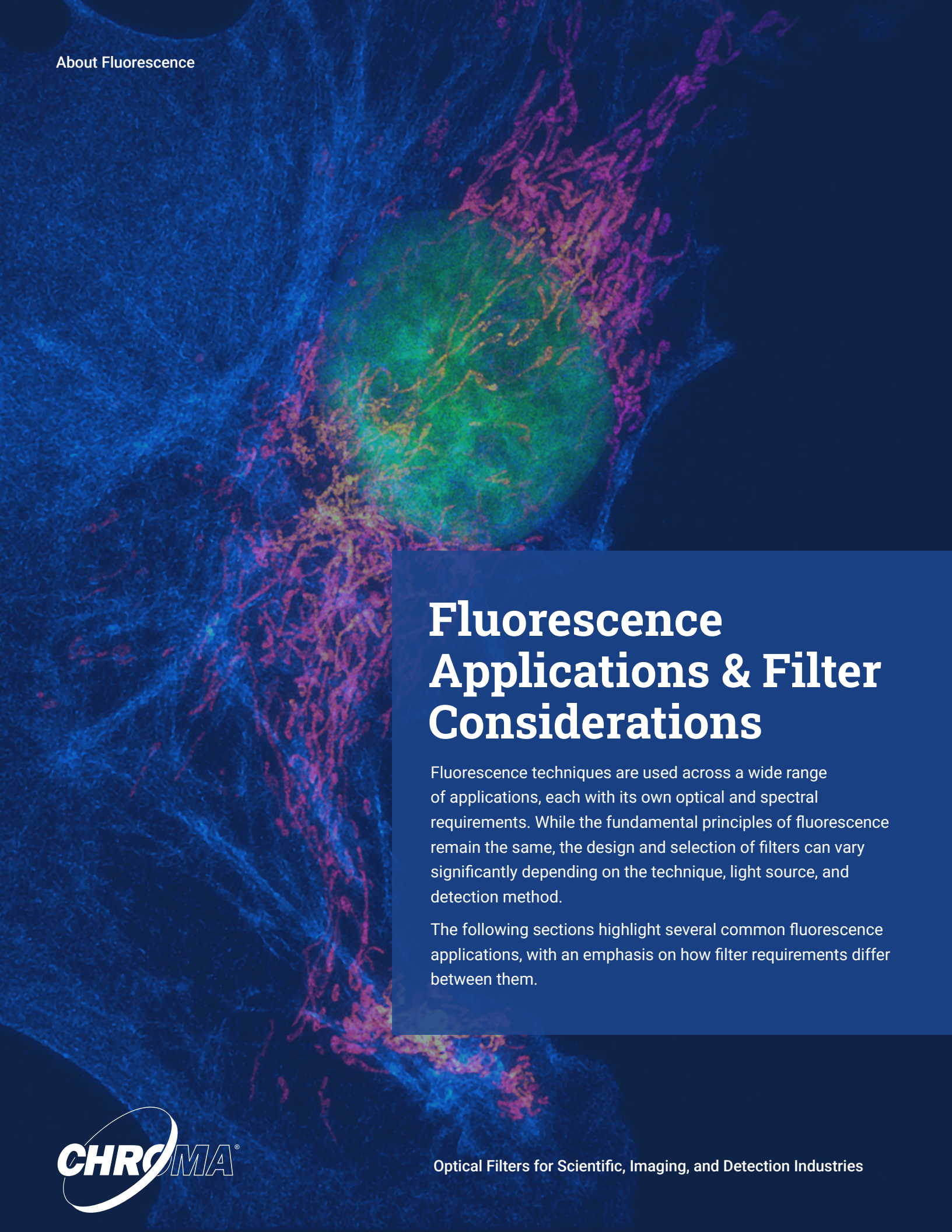
Important Precautions:

- Avoid touching or wiping anti-reflection (A/R) coated or metal mirror surfaces unless absolutely necessary.
- Never handle exposed coatings with bare fingers.
- Careful adherence to these practices will help preserve optical performance and extend the usable life of the filters.

Environmental Factors

The two most important environmental factors to consider when dealing with optical filters are temperature and humidity. Sharp-cut longpass filter glasses have a shift in cut-on of approximately 0.1 to 0.5nm per °C temperature change and some types of filter glass can be affected by high humidity or even unusual environments such as intense UV radiation ("solarization"). (Schott Glasswerke catalogue.)

Additional guidance on filter orientation and handling may be found in Chroma's **Technical Library**.



Fluorescence Applications & Filter Considerations

Fluorescence techniques are used across a wide range of applications, each with its own optical and spectral requirements. While the fundamental principles of fluorescence remain the same, the design and selection of filters can vary significantly depending on the technique, light source, and detection method.

The following sections highlight several common fluorescence applications, with an emphasis on how filter requirements differ between them.

Ratiometry

There is a class of fluorochromes that are intended to measure changes in the intracellular milieu, known collectively as ratiometric dyes. These dyes, such as fura, BCECF and beta-lactamase, are dual-excitation and/or dual-emission fluorochromes that have multiple absorption (excitation)/emission characteristics.

In order to accurately measure changes in fluorescence, the use of filter wheels is often imperative, since switching between single-band filter sets would entail a risk of shifting the sample, and subsequent misalignment of the final images. As an example, fura-2 is often used to measure changes in intracellular calcium concentrations ($[Ca^{2+}]_i$). Fura is a dual-excitation fluorochrome, and absorbs differentially depending on its binding to Ca^{2+}. When bound to Ca^{2+}, fura exhibits an absorption peak at ~340nm, whereas in a calcium-unbound form, it absorbs at a peak of ~380nm.

Typical Fura-2 experiments involve the use of an excitation filter wheel to alternate between 340 and 380 excitation filters. Modern, fast-switching LED light sources are also a viable alternative for the use of filter wheels in many cases. Fluorescence measurements are taken by using a 470-550nm bandpass emission filter mounted within the microscope. Post hoc image analysis is then used to compare 340/380 ratios (or calcium-bound vs. calcium-free levels of fura), which give the user an indication of $[Ca2+]_i$ changes within the cells. One specialized ratiometric application is FRET (Forster Resonance Energy Transfer) microscopy, detailed below.

Filter Considerations for Ratiometry

Like all fluorescence microscopy applications, filter selection should follow the fluorescence properties of the probe being used for both excitation and emission ranges. Given the increase in popularity of fast-switchable LED-based light sources, consideration must be paid to the spectral output of the light source being used to ensure that the ratiometric probe is excited appropriately, presuming it is a dual-excitation probe. In cases like this, a dual-band excitation filter may be used, but in cases of true “white-light” sources (either arc lamps or LEDs), the former approach of using separate single-band excitation filters must be used.

For dual-emission-based ratiometric probes, fluorescence emissions must be separated by either using separate single-band emission filters for each fluorescence peak or using an external beamsplitter device to separate the emissions into separate channels or portions of a detector chip. In the latter case, a dual-band barrier/emission filter may be used, along with a dichroic mirror to separate the two bands, but many find the achieve better S/N using both a dual-band emission filter inside the filtercube (which also allows for visualization with the eye), as well as single-band emission filters downstream nearer to the detector.

FRET Microscopy

"Fluorescence resonance energy transfer", or FRET (sometimes called "Forster resonance energy transfer"), is a phenomenon whereby an "excited" donor fluorophore molecule transfers energy in a non-radiative process to a suitable acceptor fluorophore molecule. This transfer of energy is termed "non-radiative" because it occurs without the emission of a photon from the donor fluorophore. Instead, it involves the direct transfer of energy of a donor molecule in the excited state when its fluorescence emission spectrum overlaps with the excitation spectrum of an acceptor fluorophore. The resulting FRET signal from the acceptor is often referred to as "sensitized emission".

FRET occurs between donor and acceptor molecules only after several conditions are met. First, the molecules must be sufficiently close – typically <10nm, but more often a closer apposition is required to successfully demonstrate FRET. This is the property of FRET which makes it such a powerful tool because such close apposition between molecules is often evidence that the two molecules are interacting. A FRET signal is direct empirical evidence of this close physical relationship, independent of the resolution of the imaging system and independent of conventional colocalization techniques. The Forster distance is the distance at which the efficiency of energy transfer is 50% – typically between 4-7nm, depending on the particular donor/acceptor pair, and is typically used to characterize the potential usefulness of a fluorophore pair. The efficiency of energy transfer increases exponentially as molecules become closer, and is exquisitely sensitive to the distance of separation.

Second, as mentioned above, the excitation spectrum of the acceptor must overlap with the emission spectrum of the donor. Greater spectral overlap generally results in a greater likelihood of FRET, but also complicates the data correction/analysis because this increases the likelihood of false positive FRET as a result of direct excitation of acceptor molecules by donor excitation wavelengths.

Third, the donor and acceptor molecule dipoles must be oriented favorably in order for FRET to occur. Not only must the molecules be sufficiently close, but the dipoles must be sufficiently aligned. Most often this condition is met randomly with large populations of molecules and a standard value for dipole orientation is used in most FRET calculations.

These requirements make FRET particularly sensitive to spectral overlap and signal separation, placing strict demands on filter selection.

Filter Considerations for FRET

FRET is an imaging technique which can benefit greatly by the appropriate choice of filters. In widefield imaging applications, donor excitation filters should be chosen to avoid direct excitation of the acceptor molecule as much as possible. While it's never possible to entirely avoid direct excitation of an acceptor fluorophore, carefully chosen exciters will best exploit the difference between the excitation spectra of the donor and acceptor. For applications involving laser excitation, such as confocal and TIRF imaging, the most appropriate laser wavelengths should be used with appropriate laser clean-up filters.

Likewise, emission filters should be chosen to best separate the donor /acceptor emission spectra. For

example, in some cases it may be best to red-shift the acceptor bandpass to minimize the detection of donor fluorescence emission, while in other cases this will limit detection of the acceptor emission too much and will be counter-productive.

Finally, FRET is a computation-intensive technique involving much image processing and image correction. It is not a simple technique by any means, and is best performed using systems with external filter wheels which afford independent control of excitation and emission wavelengths, or image-splitting systems where donor and FRET signals may be acquired simultaneously in live cell imaging. Temporal resolution may be critical in FRET, depending on the dynamics of the model system. Both approaches will require the use of polychroic mirrors which reflect multiple excitation bands and transmit multiple emission bands.

Additional techniques such as multiphoton excitation microscopy and fluorescence lifetime imaging microscopy (FLIM) are also used in FRET applications, each with their own advantages and disadvantages.

Confocal Microscopy

Confocal microscopes use a unique arrangement of apertures to produce an image at the detector that is a thin optical slice or section of the specimen. A sample that might be several millimeters thick can be reduced to a focal field of less than one micron in the z-axis, for instance.

The field of illumination in the confocal is restricted by an aperture, typically called the pinhole. The field of view is also restricted by a pinhole, which is placed at the image plane conjugate to the illumination point and the first aperture. This 'confocal' configuration results in less out-of-focus light reaching the detector, which increases the signal-to-noise ratio by greatly reducing the imaging volume.

Modern scanning confocal microscopes use a laser, or combination of lasers, for the illumination source. The scan is done by carefully controlled galvanometer mirrors that move the diffraction limited spot of illumination across the field in a raster motion, similar to a CRT television set, controlled by the computer. The scattered, reflected fluorescence light from the sample is sent to a photomultiplier tube (PMT), while the image is formed onto a computer screen one point (picture element, or pixel) at a time.

One of the most important features of confocal microscopy is its ability to optically section specimens. Confocal's optical sectioning characteristic allows users to image thick tissue without the skill of microtomy and to image living cells, tissues, and organisms with very high resolution. This optical sectioning ability also means that the individual slices/images can be saved in the computer and then reconstructed to show the three dimensional portrait of the sample.

This system places some unusual requirements upon the optical elements used for fluorescent imaging. A major misconception is that the laser light output from a specific laser produces only one wavelength. In reality, almost all lasers produce harmonics and/or scatter of other wavelengths. While these secondary lines may be very weak in intensity compared to the primary line, they can, nonetheless, greatly reduce the signal-to-noise ratio. If the fluorescence emission happens to be in the same wavelength region as the harmonic (or other noise from the laser), the fluorescent signal may be completely masked, rendering it invisible.

Filter Considerations for Confocal Systems

The first optical element in the excitation path is typically a laser clean-up filter, which ensures that only the desired laser line reaches the specimen. These filters must provide high transmission at the laser wavelength while blocking unwanted spectral components across a wide range (often from the UV through the near-IR). Clean-up filters are typically narrow bandpass filters (~10nm FWHM), though slightly wider designs (20–25nm) may be used for diode lasers due to wavelength drift.

Because of the collimated and coherent nature of laser light, clean-up filters must also meet strict optical quality requirements. These include low wavefront distortion and minimal wedge to prevent beam deviation and misalignment.

Dichroic beamsplitters in confocal systems are selected for high flatness to maintain beam quality and minimize wavefront distortion. In some cases, thicker

substrates are used to meet transmitted wavefront requirements. These optics must also withstand high laser power without degradation.

Emission filters in confocal systems are primarily responsible for blocking excitation light while transmitting fluorescence. Because laser lines can be extremely intense, blocking requirements may exceed OD8 at specific wavelengths. Although the spectral range of blocking is often narrower than in widefield fluorescence, the required attenuation at the laser wavelength is significantly higher.

Filter sets for confocal systems are typically designed around the available laser lines rather than the peak excitation of the fluorochrome. As a result, fluorophore selection is often constrained by the laser wavelengths available. Polychroic beamsplitters may be used to accommodate multiple laser lines in multi-color imaging systems.

TIRF Microscopy

Total Internal Reflection Fluorescence (Microscopy) is a technique used to restrict the background fluorescence and increase the signal-to-noise ratio (S/N) in the resultant images. This is accomplished in TIRF by using the ability of light to create an evanescent wave (or field) at a very limited range within the sample beyond an interface of two substrates differing in refractive index.

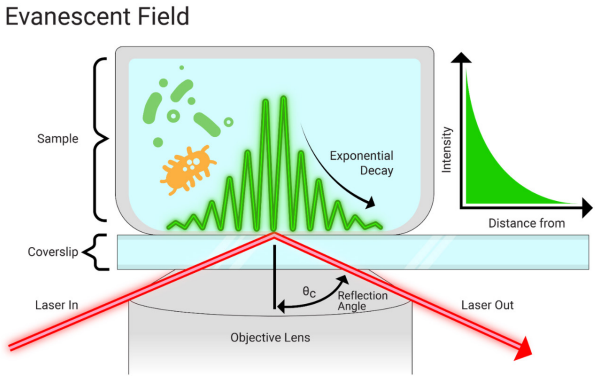


Figure 27: Evanescent field generated by total internal reflection, showing exponential intensity decay.

In practice, this involves imaging a specimen that is in direct contact with a glass slide or tissue chamber. If the angle of the light is greater than the critical angle, this refractive index mismatch will create a field/wave with properties that are identical in frequency to the light. In other words, a fluorescent molecule that would normally absorb light at 488nm can be 'excited' by the electromagnetic field created by a 488nm laser (or other monochromatic source) which is reflected off of the lower refractive index material.

Since this field will decay in intensity exponentially with distance, the resultant fluorescence signal will occur in less than 100 nanometers of the surface. This greatly reduces the z-axis signal and, as a result, significantly increases the S/N of the sample. As a result this means the molecules of interest must be within that limited range of the evanescent field.

Modern TIRF systems commonly use a through-the-objective configuration, where excitation light is delivered and reflected through a high numerical aperture objective. In this configuration, reflected excitation light is collected back through the optical path, placing increased demands on downstream optical components.

Filter Considerations for TIRF

TIRF systems place exceptionally high demands on filter performance due to the fact that the reflected beam is collected by the objective lens and follows the beampath backwards. This means that the emission/blocking optics not only have to deal with huge amounts of excitation energy, but also that this light can be at high angles due to scatter and reflections.

A typical laser emission filter must block the excitation wavelengths to OD6, but in TIRF, this blocking must exceed OD8 in most applications (note that many modern spectrometers are incapable of measuring to OD8 across the full visible spectrum). Chroma addresses this requirement with specialized blocking mechanisms and optics capable of achieving OD8, even at the higher angles of these systems.

In addition to the added blocking requirements, the dichroic mirrors in these applications must be

incredibly stable in reflected wavefront characteristics and flatness. Where a standard laser scanning confocal might require a reflected wavefront distortion (RWD) of less than 2 waves per inch (2λ), TIRF applications require less than ½ wave distortion from the reflected surface. This reflected wavefront demand is after substrate coating as the coating process always exerts torque stresses on the fused silica substrates. This makes producing dichroics that meet these flatness requirements nearly impossible for some coating techniques and for some physical requirements of the substrates.

Chroma addresses these requirements using modified magnetron sputtering processes, proprietary stress-relief methods, and thicker substrates with high initial flatness, enabling reflected wavefront distortion to be controlled to approximately 1/2 wave (λ/2). These requirements have also led to the development of custom cubes, mounts, and holders for certain commercial TIRF systems (e.g. Chroma filtercube # 91032, U.S. Patent 8,488,238).

Multiphoton Microscopy

As mentioned previously, pulsed laser light sources are required for a group of applications known collectively as multiphoton microscopy. Given the extremely brief duration of each laser pulse that these sources are capable of generating (often in the picosecond to femtosecond range), it is possible to deliver multiple photons of light to a target very close to simultaneously in time and space. These photons can generate fluorescence from a fluorochrome in much the same way that "1photon" microscopy can (in laser-scanning confocal imaging, for example), but with the additional benefits of using a lower total amount of power to do so, and with the ability to penetrate tissue samples more deeply.

Tunable pulsed lasers are typically used to generate a multiphoton event and these lasers often range from 700-1100, -1350 or even -1500nm. By directing extremely brief pulses into the sample, these lasers can effectively excite fluorochromes by striking the fluorescent molecules virtually simultaneously with multiple photons. In 2photon microscopy, a theoretical fluorochrome with an absorption/excitation peak at 470nm can be excited by a 2p laser tuned to 940nm or a 3p laser tuned to 1410nm, as an example. The fluorescence generated by that excited fluorochrome would peak at the typical "1p" emission peak published for that fluorochrome and can be measured or imaged as such.

Filter Considerations for Multiphoton Microscopy

As in laser-scanning confocal microscopy, the dichroic mirror used in multiphoton microscopy applications must adhere to fairly tight flatness characteristics to ensure coherence of the beam being reflected off of it. We generally recommend 0.5 waves/inch (0.5λ), though some applications and systems appear to require even "flatter" dichroics. Since this application requires excitation wavelengths in the "far-red/near-IR" region, shortpass dichroics are most often used. These will reflect the "far-red/near-IR" light for effective multiphoton excitation, while transmitting the "visible" wavelength range corresponding to the emission properties of most fluorochromes.

Barrier/emission filters for multiphoton are similar in their blocking requirements to those in confocal or TIRF (OD6 minimum, OD8 or more preferred), though given the nature of the pulsed lasers used in these applications, the highest order of blocking should occur within the "far-red/near-IR" spectral region corresponding to the laser being used. Barrier/emission filters can be either bandpass or shortpass in nature, and often both are used in series to increase/improve blocking of the laser within the detection/imaging pathway. For instance, an ET750SP-2p8 shortpass barrier/emission filter can be used in the common emission/detection pathway before the fluorescence emerges from the microscope itself. Then, an additional bandpass barrier/emission filter (i.e. ET525/50m-2p) may be used to both limit the fluorescence being detected to a narrow band AND improve upon the blocking of the laser at the detector.

Because the lasers being used for these applications are tunable through a wide range (i.e. 700-1100nm), excitation/clean-up filters are generally not used in the excitation/illumination path of the microscope. Historically, there have been instances where a multiphoton laser appears to emit light in the "visible" range (i.e. 680nm), as well as its more-typical "far-red/near-IR" range, and in those cases a longpass filter which can block "visible" and transmit "far-red/near-IR" has been used.

Raman Spectroscopy

Raman spectroscopy is an application used to study the rotational, vibrational and other low-frequency modes in a system. This technique relies on the Raman scattering (inelastic) of monochromatic light, typically from a laser source. The laser light will interact with the specific bonds and electron clouds of a molecule to create a virtual energy state. The laser light interactions with these phonons (molecular vibrations) in the sample will result in the photon's energy being shifted up or down. These shifts in the energy level (different rotational/vibrational states exhibited as either longer or shorter wavelength photons upon relaxation from the virtual energy state) give information about the phonon mode and therefore information about the sample.

The Raman scatter is so weak that the major challenge is to block both the intense laser light illuminating the sample and the Rayleigh scatter close to the laser in wavelength. Until recently this could only be accomplished with holographic devices using multiple steps of dispersion with the final signal going to a photomultiplier tube. This was difficult to align, expensive and slow. With the development of more powerful and less expensive lasers and the achievements in interference filter designs and better CCD cameras, the newer Raman spectroscopy devices are both less expensive and easier to operate.

While Raman spectroscopy may be primarily used for chemical analysis (by recognizing the unique signal from different molecules and specific chemical bonds), it is also used in a wide variety of applications from materials science, crystallography and mineralogy to microscopy (also called microspectroscopy).

Filter Considerations for Raman Spectroscopy

A major optical challenge in Raman applications is suppressing direct and scattered excitation light before it reaches the detector. This happens to be true of all fluorescence applications but is even more important here, because the primary job of the emission filter is not to transmit the emission but rather to block the excitation load.

It is also very important in this application, as it is in all applications involving lasers, that the laser source itself be 'clean' by restricting the output since many lasers have emissions well outside their stated emission points. A laser clean-up filter in the 10–20nm FWHM range is often used, together with an emission filter that blocks this band to greater than OD6. The dichroic mirrors in these applications must meet the requirements of the standard laser configurations listed elsewhere.

Qdots

Qdots (or quantum dots) are fluorescent crystalline constructions that are most strongly absorbent in the UV to violet region of the spectrum, but emit, depending on their size, through the rest of the visible and near-IR. Chroma worked specifically with the original Qdot Corporation during the initial development of Qdots to generate filter sets that were optimal for their detection.

Chroma carries a number of Qdot-specific filter sets that differ in the width of excitation and emission light transmitted through the various optics, depending on the needs of the user.

Qdots are commercially available from several manufacturers and can also be engineered for specific emission properties depending on the application.

Filter Considerations for Qdots

The most common Qdot filter sets contain a shortpass excitation filter that transmits ~340-460nm and a 40 nm-wide bandpass emission filter centered on the peak emission of the individual Qdot (e.g. 565/40 or 565+/-20nm for Qdot 565).

In some applications, narrower excitation bands (e.g. 405/90 or 405+/-45nm, or 420/40 or 420+/-20nm) are used in order to minimize the impact of UV excitation light-driven phototoxicity in live cell imaging, or in instances where UV excitation is not desired.

Flow Cytometry & Plate Readers

Flow Cytometry

Flow cytometry is a high-throughput application that allows for the detection of multiple fluorochromes in a sample consisting of thousands to millions of cells in an extremely rapid fashion. These systems utilize very sensitive PMT-based detectors and often use comparatively narrow bandpass emission filters to capture the multiple fluorescences.

Filter Considerations for Flow Cytometry

All flow cytometers use multiple lasers to drive the individual fluorescences, as well as emission filters to block/attenuate the lasers while transmitting the appropriate emission wavelength ranges. However, these systems can differ in their overall optical configuration.

Some flow models are linear in their configuration, which allows the operator to use common dichroic mirrors and emission filters designed for use at 45 and 0° incidence (AOI), respectively. Other systems require the use of dichroic mirrors designed for use at other AOI (e.g. 11.25°), while still using common 0° AOI emission filters in front of the detectors.

Plate Readers

Fluorescence plate readers are non-imaging instruments used to measure bulk fluorescence signals from samples, typically contained in multi-well plates. These systems are commonly used in assays involving large numbers of samples, such as biochemical measurements, drug screening, and protein quantification.

Unlike microscopy-based techniques, plate readers measure overall fluorescence intensity rather than spatial information. As a result, signal accuracy and consistency are the primary considerations.

Filter Considerations for Plate Readers

Plate readers typically use excitation and emission filters to define the spectral range of illumination and detection. Narrow bandpass filters are often preferred, as they improve signal specificity and reduce background by limiting spectral overlap.

Because these systems measure total signal intensity rather than images, careful control of excitation and emission bandwidth helps prevent detector saturation and improves measurement reproducibility.

In addition to fluorescence measurements, plate readers are often used for absorbance-based assays. In these cases, excitation filters (with or without emission filters) are used to define the wavelength range, and signal is determined by comparing transmitted light intensity between reference and sample wells.

Appendices

CHROMA®

Optical Filters for Scientific, Imaging, and Detection Industries

Appendix A: Chroma Filter Series Overview

Chroma offers a range of fluorescence filter sets designed to meet different performance requirements, imaging modalities, and application needs. The series below are grouped by performance level and intended use.

ET Filter Series — Highest Performance

ET (Enhanced Transmission) filters are manufactured using a modified magnetron sputtering process, producing durable coatings with high transmission (typically 93–97%) and steep spectral edges. These filters are optimized for maximum signal throughput, minimal spectral overlap, and demanding imaging applications. All ET sets are sub-pixel image registered by design.

Single-Band Filter Sets

49000 Series — ET Single-Band (Non-laser)
High-transmission, steep-edge filter sets for general fluorescence imaging. Optimized for modern fluorochromes with short Stokes shifts and applications requiring maximum signal intensity and contrast.

49300 Series — ET Single-Band (FISH / Chromosome Painting)
Spectrally matched to common FISH probes and chromosome painting applications. Designed for use with non-laser light sources.

49900 Series — ET Single-Band (Laser)
Designed for laser-based systems. Includes laser clean-up filters, emission filters, and high-flatness dichroics for precise spectral control.

TRF49900 Series — ET Single-Band (Laser TIRF)
TIRF-optimized sets with enhanced blocking (OD8+) and ultra-flat dichroics. Includes additional emission filtering for improved signal-to-noise in high-background environments.

Multi-Band Filter Sets

59000 Series — ET Dual-Band
Dual-band emission filters for imaging two fluorochromes. Available with either dual-band excitation (simultaneous imaging) or single-band exciters (sequential imaging).

59900 Series — ET Dual-Band (Laser)
Laser-compatible dual-band sets with high-performance dichroics and spectral separation for two laser lines.

TRF59900 Series — ET Dual-Band (Laser TIRF)
Dual-band TIRF sets with enhanced laser blocking and options for additional emission filtering to maximize signal-to-noise.

69000 Series — ET Triple-Band
Triple-band filter sets for imaging three fluorochromes. Configurations available for simultaneous or sequential excitation.

79000 Series — ET Ratiometric
Designed for ratiometric dyes (e.g., Ca^{2+}, pH indicators). Supports quantitative imaging using multiple excitation or emission wavelengths.

89000 Series — ET Multi-Band (Single-Band Components)
Uses single-band excitation and emission filters with a multiband dichroic. Ideal for co-localization, FRET, and live-cell imaging with image-splitting systems.

89900 Series — ET Quad-Band (Laser)
Designed for systems using four laser lines. Provides efficient spectral separation with high transmission and blocking performance.

TRF89900 Series — ET Quad-Band (Laser TIRF)
Quad-band TIRF sets with enhanced blocking and ultra-flat dichroics for high-precision imaging applications.
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AT Filter Series — General Performance

AT filters provide durable sputtered coatings with >90% transmission and excellent blocking performance, offering a cost-effective solution for routine fluorescence applications.

39000 Series — AT Single-Band
General-purpose fluorescence filter sets for routine imaging and assays. Suitable for most applications requiring reliable performance and good spectral separation.

19000 Series — AT Longpass
Longpass filter sets for applications requiring high signal intensity and flexibility across multiple fluorochromes. Not recommended for multi-color separation.

39100 Series — AT Qdot
Designed for quantum dot imaging. Uses a common excitation band with emission filters matched to specific Qdot emission wavelengths.

Supporting Components and Accessories

91000 Series — Filter Cubes / Holders
Filter cubes and mounting hardware compatible with major microscope platforms (Zeiss, Nikon, Leica, Olympus/Evident).

92000 Series — Diagnostic Tools
Tools for evaluating and troubleshooting optical system performance.

Additional Optical Components (General Use)

21000 Series — Mirrors and Beamsplitters
General-purpose mirrors, beamsplitters, and polarizers.

22000 Series — Neutral Density Filters
Uniform attenuation across a spectral range for controlling light intensity.

23000 Series — Miscellaneous Filters
Specialty filters for various optical applications.

33000 Series — Brightfield Filter Sets
Filters designed for transmitted-light (non-fluorescence) microscopy.

Notes on Performance & Selection

- ET series filters are recommended for applications requiring maximum signal, steep spectral separation, and advanced imaging techniques.
- AT series filters provide reliable, cost-effective performance for routine fluorescence imaging.
- Laser-based applications and techniques such as TIRF, confocal, and multiphoton microscopy require filters specifically designed for those illumination conditions.
- Multiband configurations trade spectral separation for speed and convenience, while single-band systems provide the highest signal-to-noise performance.

Appendix B: Chroma Filter Naming Conventions

Reading Typical Part Numbers

Understanding Chroma filter part numbers allows you to quickly identify key specifications such as center wavelength, bandwidth, filter type, and coating family.

- **Excitation filter (e.g., ET480/40x)** – For this example, the center wavelength is at 480nm; full bandwidth is 40nm (±20nm). In some cases where the bandwidth is not specified, the letter "x" is used to define the filter as an excitation filter. This is generally used for narrow band UV excitation filters (e.g., ET340x).
- **Dichroic beamsplitter (e.g., T495LPXR)** – The cut-on wavelength where transmission is approximately 50% is 495nm for this dichroic longpass mirror.
- **Emission filter (e.g., ET535/50m)** – The center wavelength is at 535nm; full bandwidth is 50nm (±25nm).

Prefixes & Coating Families

Code	Description
AT	Magnetron-sputtered exciter, emitter, or dichroic, typically 45° AOI; recommended for only basic, visual applications (such as cell culture) (basic performance)
D	Obsolete parts; early-generation bandpass filter (thermal/"soft" coating)
ET	Magnetron-sputtered exciter or emitter, typically normal incidence (0° AOI) for maximum performance
HQ	Obsolete parts; improved soft-coated bandpass filter
T	Magnetron-sputtered dichroic/polychroic, typically 45° AOI
ZET	Magnetron sputtered exciter or emitter specifically designed for laser use
ZT	Magnetron-sputtered dichroic/polychroic designed for laser use, typically 11–45° AOI

Suffixes and Spectral Function Codes

Code	Description
BS	Beamsplitter or dichroic mirror
DC	Shorthand for “dichroic”, typically 45° AOI
LP	Indicates a longpass filter or dichroic mirror which transmits wavelengths longer than the cut-on and blocks or reflects shorter wavelengths respectively
M	Emission filter
PC	Polychroic beamsplitter. Reflects and transmits more than two bands of light, typically 45° AOI
SP	Indicates a shortpass filter or dichroic mirror which transmits wavelengths shorter than the cut-on and blocks or reflects longer wavelengths respectively
X	Excitation filter
XR	Extended reflection, typically 45° AOI
XRU	Extended reflection including the UV, typically 45° AOI

Glass Type Abbreviations

Code	Description
GG	Green Glass. Longpass absorption glass from Schott Glassworks with cut-on wavelengths in the violet and blue-green regions
OG	Orange Glass. Longpass absorption glass from Schott Glassworks with cut-on wavelengths in the green, yellow, and orange regions
RG	Red Glass. Longpass absorption glass from Schott Glassworks with cut-on wavelengths in the red and far red regions

Appendix C: Glossary of Optical Terms

Here you will find a comprehensive guide to the intricate language of optical filters. In the dynamic realm of optics, filters play a pivotal role in shaping and manipulating light to meet diverse needs across industries. Whether you are a seasoned researcher, an astrophotographer, or a curious enthusiast, this glossary aims to demystify the terminology associated with optical filters.

From fundamental principles like wavelength selectivity and spectral transmittance to advanced concepts such as dichroic coatings and bandpass filters, this compilation provides concise explanations to foster a deeper understanding of optical filter technology.

Acousto-optical tunable filter (AOTF)

An active crystal device that works by setting up radio-frequency acoustical vibrations in the crystal and creating, in effect, a bulk transmission diffraction grating. By varying the frequency, one can rapidly tune the filter to diffract out a desired wavelength of light and transmit this wavelength out of the device.

Angle of incidence (AOI)

The angle between the optical axis of the light incident on the surface of a filter and the axis normal to this surface.

Angular deviation

A shift in the direction of a light beam from the true optical axis of the system, measured in units of angle such as arc-minutes (1/60 of a degree) or arc-seconds (1/60 of an arc-minute).

Aperture diaphragm

An adjustable diaphragm located in the illumination optics controls the numerical aperture of the illuminating beam and affects the brightness of the beam.

Attenuation level

Also Blocking level. A measure of the out-of-band attenuation of an optical filter over an extended range of the spectrum. The attenuation level is often defined in units of optical density (see optical density).

Autofluorescence

In fluorescence microscopy, any fluorescence from substances other than fluorochromes, including primary fluorescence from the specimen, fluorescence from immersion oils and other media, and fluorescence from glass optical components within the microscope.

Average transmission

The average calculated over the useful transmission region of a filter rather than over the entire spectrum. For a bandpass filter, this region spans the FWHM of the transmission band.

Background

Any detectable light that is not a desired primary or indirect fluorescent emission. Sources of background include cross-talk between excitation and emission filters, light leaking through pinholes in filters, and electronic noise in cameras, as well as autofluorescence.

Bandpass

An optical filter that has a well-defined short wavelength cut-on and long wavelength cut-off. Bandpass filters are denoted by their center wavelength and bandwidth.

Bandwidth

Also FWHM (full width at half of maximum transmission). For optical bandpass filters, typically the separation between the cut-on and cut-off wavelengths at 50% of peak transmission, though sometimes calculated as a bandwidth at 10% of peak transmission is specified, as an example.

Barrier filter

See Emission filter.

Blocker

A thin-film interference coating that is incorporated into a bandpass interference filter to extend the blocking range of the primary coating in the filter. A blocker is usually a very wide-band bandpass filter having high transmission in the band of the primary filter.

Blocking level

See Attenuation level.

Blocking range

The range of wavelengths over which an optical filter maintains a specified attenuation level.

Brightfield

A kind of diascopic illumination in which the field of view is illuminated directly. Also, the type of condenser used for this kind of illumination.

Center wavelength (CWL)

For optical bandpass filters, the arithmetic mean of the cut-on and cut-off wavelengths at 50% of peak transmission.

Clear aperture

The surface area of an optical filter which is free of any defects or obstructions. With interference filters the clear aperture is often delimited by an annulus of metal or opaque material.

Critical illumination

A type of illumination optics in which the image of the light source is focused onto the specimen plane, in contrast to Köhler illumination optics. See also Köhler illumination.

Cross-talk

The minimum attenuation level (over a specified wavelength range) of two filters placed together in series. The transmission spectrum of the beamsplitter is sometimes included in this evaluation.

Cut-off wavelength

The edge produced when transitioning between transmission and blocking. The point at which a filter transmits 50% and longer wavelengths are blocked.

Cut-on wavelength

The edge produced when transitioning between blocking and transmission. The point at which a filter transmits 50% and longer wavelengths are transmitted.

Darkfield

A kind of diascopic illumination in which the specimen is illuminated obliquely, i.e., at angles that will not enter the objective directly. Also, the type of condenser used for this kind of illumination.

Diascopic illumination

Illumination uses light transmitted through the specimen, using a condenser to focus the light.

Dichroic beamsplitter

Also Dichroic mirror or Dichromatic beamsplitter. A special mirror housed in the filter cube that selectively reflects the excitation light filtered by the exciter and transmits the emitted fluorescence. Dichroic beamsplitters can also be found in any other part of a microscope system where light needs to be split or combined by wavelength.

Edge filter

Another term for either a shortpass or longpass optical filter. The term usually denotes a filter with a very sharp cut-on or cut-off.

Emission filter

Also Barrier filter or Emitter. A color filter that attenuates all of the light transmitted by the excitation filter and very efficiently transmits any fluorescence emitted by the specimen.

Emitter

See Emission filter.

Epifluorescence microscope

A fluorescence microscope that illuminates the specimen episcopically (i.e., with light reflected onto the specimen).

Episcopic illumination

Also Incident-light illumination. Illumination with light reflected onto the specimen rather than transmitted through the specimen. The illuminating light is reflected through the objective by means of a beamsplitter that is either partially reflective or a dichroic.

Excitation and emission

See Fluorescence.

Excitation filter

Also Exciter. A color filter that transmits only those wavelengths of the illumination light that efficiently excites a specific dye or dyes. See Emission filter.

Exciter

See Excitation filter.

Extinction coefficient

A measure of the absorption characteristics of a fluorochrome.

Fading

See Photobleaching.

Field diaphragm

An adjustable diaphragm located in the illumination optics which controls the area of illumination on the specimen.

Filter block

See Filter cube.

Filter cube

A removable cube-shaped unit that holds a matched set of fluorescence filters. This set usually includes an excitation filter and emission filter, but always includes a dichroic beamsplitter.

Filter glass

Also Absorption glass. Colored glass that is manufactured for technical and scientific applications. The most common types of filter glass used in fluorescence microscopy are UV-transmitting “black glass” filters; IR-absorbing heat filters; and yellow, orange, and red sharp-cut longpass filters. Filter glass selectively attenuates light by absorption, so the spectral performance is dependent on the thickness of the glass. The blocking properties of filter glass are also independent of the angle-of-incidence of the incoming light.

Fluorescence

A molecular phenomenon in which a substance absorbs light (as a photon), then radiates part of this absorbed energy as light of another color, one of lower energy and thus longer wavelength. This process is known as excitation and emission. Fluorescence is distinguishable from other types of luminescence in that the process of excitation and emission occurs nearly instantaneously (i.e., on the order of nanoseconds).

Fluorescent probe

Also Fluorophore. A fluorochrome that has been conjugated to an active substance, such as a protein, antibody, or nucleic acid, in order to selectively stain a targeted substance within the specimen.

Fluorochrome

A fluorescent dye used either directly as a specimen stain or conjugated to an active substance to make a fluorescent probe.

Fluorophore

See Fluorescent probe.

Front surface

The side of a beamsplitter that is designed to face the incident light. In a filter cube, this is the side that faces both the light source and the specimen. Beamsplitters generally perform better when oriented correctly.

FWHM

See Bandwidth.

Group-delay dispersion (GDD)

A measure of the wavelength-dependent time delay introduced by phase shifts and optical path length differences upon reflection from, or transmission through, a thin-film interference coating or other optical component. The usual unit of measure for GDD is fsec^2. The GDD of a coating may be an important consideration when using ultra-fast pulsed lasers.

Half-cone angle (HCA)

The angle between the most oblique ray of a convergent or divergent light beam and the optical axis of the beam. See also Numerical aperture.

Heat filter

An optical filter that attenuates infrared radiation, but transmits the visible. Attenuation is achieved by means of absorption (using filter glass), reflection (using a thin-film interference coating, often called a hot mirror), or a combination of the two.

Hot mirror

See Heat filter.

Indirect fluorescence

Also Secondary fluorescence. In fluorescence microscopy, fluorescence emitted by fluorochromes introduced into a specimen as a stain or probe. See Primary fluorescence.

Infinity-corrected optics

An optical configuration employed by some microscopes in which the objective forms an image at infinity, and a secondary tube lens forms an image at the intermediate image plane. (This intermediate image is focused in turn by the ocular.) This configuration allows for a flexible distance between the objective and ocular because the distance between the objective and the tube lens can be varied without affecting the image-forming characteristics of the microscope. Note that objectives designed for infinity-corrected optics are not interchangeable with objectives designed for standard tube-length optics.

Interference filter

See Thin-film interference coating.

Köhler illumination

A type of illumination optics usually used in wide-field epifluorescence microscopes, in which the image of the light source is completely out of focus at the specimen plane.

Longpass (LP)

An optical filter that attenuates shorter wavelengths and transmits longer wavelengths over the active range of the spectrum (which depends on the specific application). LP filters are denoted by the cut-on wavelength at 50% of peak transmission.

Longwave infrared (LWIR)

The region of the electromagnetic spectrum ranging in wavelength from approximately 8 to 14 microns. This range generally represents the atmospheric transmission window used for thermal detection and imaging of lower temperature sources.

Luminance

See Radiance.

Midwave infrared (MWIR)

The region of the electromagnetic spectrum ranging in wavelength from approximately 3 to 8 microns. This range generally represents the atmospheric transmission window used for thermal detection and imaging of higher temperature sources, as well as molecular absorption detection and spectroscopy.

Nanometer (nm)

A unit of length commonly used for measuring the wavelength of light: 1nm = 10 angstroms (A) = 10^{-9} meters; and 1000nm = 10^{-6} meters.

Near infrared (NIR)

The region of the electromagnetic spectrum ranging in wavelength from approximately 750 to 2500 nanometers.

Neutral-density (ND) filter

An optical filter that attenuates light by an amount independent of the wavelength within the useful range of the filter. Metal-coated filters typically have a wider neutral range than glass filters and are more heat-tolerant.

Normal incidence

An angle of incidence of 0°.

Numerical aperture (NA)

In the microscope, a measure of the effective cone-angle of light focused onto the specimen (NA of the condenser) or light captured by the objective (NA of the objective). A high value of NA improves both the brightness and the resolution of the image. (NA = N sin(θ), where N is the refractive index of the medium surrounding the specimen and θ is the half-cone angle of the light.)

Optical density (OD)

A logarithmic unit of transmission: OD = -log (T), where T is the transmission ($0 < T \leq 1$). In terms of %T, OD = -log (%T/100).

P-plane

See Polarization.

Parallelism

See Wedge.

Phase

When light is represented as a sinusoidal vibration of the electric field, the fraction of a full wave cycle at a particular point in space and time, defined in units of degrees or radians. Usually the point of interest is at the surface of a substrate or a thin-film coating, where an instantaneous phase shift can occur upon transmission or reflection of the incident wave.

Phase difference

The difference in phase shift between the S-plane and P-plane components of light when reflected from, or transmitted through, the surface of a substrate or a thin-film interference coating. The phase difference generally varies with the wavelength of the incident light. At wavelengths where there is zero phase difference, the surface or coating will not alter the polarization state of the incident light.

Phase shift

The instantaneous change in the phase of the incident light when light is reflected from, or transmitted through, the surface of a substrate or a thin-film coating. For a dielectric material such as glass, the phase change of the reflected light will be either 0 or 180°, depending on three factors: 1) the refractive indices of the incident and emergent media, 2) the angle of incidence, and 3) the polarization state of the incident light. For a thin-film interference coating, the value generally will be other than 0 or 180°, and generally will vary with the wavelength of the incident light.

Photobleaching

Also Fading. A photochemical reaction that causes the intensity of fluorescence to decrease with time.

Pinholes

Small breaks in the coating of an interference filter, usually caused by the presence of dust particles on the substrate during coating.

Polarization

The orientation of the electric field vibrations of propagating light. These vibrations are transverse to the direction of propagation of the light and can be oriented at some angle around the axis of propagation. When the orientation of the vibrations changes rapidly and randomly in time, the light is said to be unpolarized. When the vibrations are restricted to one particular orientation angle over an extended length of time, the light is said to be plane-polarized. (Light can be partially as well as totally plane-polarized.) Elliptical polarization is a special case in which the orientation rotates around the direction of propagation over an extended length of time, at the same frequency as the propagation light. (If the amplitude of the vibrations are equal for all orientations, it is said to be circularly polarized.)

When light strikes a specular surface at non-normal incidence, the component of the electric field vibrations parallel to the plane of incidence of the surface (P-plane) generally behaves differently than the component perpendicular to the plane of incidence (S-plane). This causes a polarizing effect that is aligned orthogonally to the orientation of the surface.

Polychroic

A name for a dichroic beamsplitter that has multiple reflection bands and transmission regions.

Primary fluorescence

In fluorescence microscopy, fluorescent emissions from the specimen itself rather than emissions from any fluorochromes present in the specimen. See also Autofluorescence.

Quantum efficiency

A measure of how efficiently a fluorochrome converts absorbed radiation into emitted fluorescence.

Quenching

Any chemical process that reduces the quantum efficiency of a fluorochrome in situ.

Radiance

A measure of the radiometric brightness of a light source. Technically, the radiance is the radiant flux emitted per unit solid angle per unit area of the light source. A common unit is watts per steradian per square centimeter. Luminance is a measure of the brightness of a light source as perceived by the human eye (i.e., a photometric measure), commonly measured in candelas per square centimeter.

Reflected wavefront distortion (RWD)

A measure of the distortion a plane wave of light experiences when reflected off of a surface, measured in fractions or multiples of a wavelength of visible light (usually 546 or 633nm). In general the RWD will be different than the surface flatness for non-normal angles of incidence. For optical components with both transmitted and reflected light paths, such as cube beamsplitters, the RWD represents the total wavefront distortion of the reflected light path through the component.

Registration shift

A shift in the apparent position of the specimen occurs when an optical element is inserted or removed, adjusted, or switched with another element. This shift can occur in X/Y and/or Z/focus planes, depending on the nature and specifications of the optical element.

S-plane

See Polarization.

Scratch/dig

A set of specifications for defining the maximum allowable size and number of scratches and digs on an optical surface. The scratch/dig values (e.g., 80/50) specify the scratch width in microns and the dig diameter in tens of microns, respectively. Although extensive evaluation procedures exist if rigorous standards must be maintained (military specification MIL-F-48616, for example), a qualitative visual assessment of the scratches and digs usually suffices.

Secondary fluorescence

See Indirect fluorescence.

Shortpass (SP)

An optical filter that attenuates longer wavelengths and transmits shorter wavelengths over the active range of the spectrum (which depends on the specific application). SP filters are denoted by the cut-off wavelength at 50% of peak transmission.

Shortwave infrared (SWIR)

The region of the electromagnetic spectrum ranging in wavelength from approximately 1.4 to 3 microns, representing the longer-wavelength band of the NIR. This range includes the primary optical telecommunications bands. With the appropriate photodetector, imaging and analysis based on the unique infrared absorption spectra of molecules can be achieved using standard optical systems. See Applications for SWIR

Signal-to-noise (S/N)

A measure of the brightness of the desired fluorescence (signal) relative to the brightness of the background (noise).

Slope

A measure of the sharpness of the transition from the transmitting region to the blocking region of a color filter.

Spectrofluorimeter

An instrument used for measuring the excitation and emission spectra of a fluorescent substance.

Standard tube-length optics

An optical configuration employed by most microscopes in which the objective forms an image at the intermediate image plane. This intermediate image is focused in turn by the ocular. The distance between the nosepiece that holds the objective and the barrel that holds the ocular is fixed at a standard length of 160nm so that objectives can be interchangeable between microscopes. See also Infinity-corrected.

Stokes shift

The shift in wavelength between the peak excitation intensity and peak emission intensity of a fluorochrome.

Substrate

The ground and polished piece of optical glass that is used as a base for the beamsplitter coating.

Surface flatness

A measure of the deviation of the surface of an optical element from a perfect plane, usually measured in fractions or multiples of a wavelength of visible light (usually 546 or 633nm).

TE-mode

Another term for S-plane polarization (short for “transverse-electric” mode). See Polarization.

Thin-film interference coating

A type of optical coating composed of a stack of microscopically thin layers of material. Although each material is intrinsically colorless, the reflections created at each interface between the layers combine through wave interference to selectively reflect some wavelengths of light and transmit others. Thin-film interference coatings are the main component of interference filters, which consist of one or more coatings separated by glass substrates and frequently one or more layers of filter glass.

TM-mode

Another term for P-plane polarization (short for “transverse-magnetic” mode). See Polarization.

Transmitted wavefront distortion (TWD)

A measure of the distortion a plane wave of light undergoes when transmitted through an optical element, measured in fractions or multiples of a wavelength of visible light (usually 546 or 633nm).

Ultraviolet (UV)

The region of the electromagnetic spectrum ranges in wavelength from approximately 100 to 400 nanometers. Three distinct bands are: 1) near-UV, from 320 to 400nm; 2) mid-UV, from 190 to 320nm; and 3) vacuum-UV (VUV), below 190nm. The terms UV-A and UV-B denote bands with distinct physiological effects: 320 to 380nm and 280 to 320nm, respectively.

Wedge

Also Parallelism. A measure of the deviation of the outer surfaces of an optical element from perfect parallelism, usually measured in arc-minutes or arcseconds of angle.

Wide-field

An epifluorescence microscope in which the full field of view is illuminated, in contrast to a confocal epifluorescence microscope. The term brightfield is also used, but this might be confused with traditional diascopic brightfield illumination.

“The sensitive plate, the gas which is ionised, the fluorescent screen, are in reality receivers, into another kind of energy, chemical energy, ionic energy... luminous energy.”

— Marie Curie

US Corporate Headquarters
10 Imtec Lane
Bellows Falls, Vermont 05101 US
info@chroma.com
Tel: +1-800-824-7662
+1-802-428-2500

Chroma Technology GmbH
Maximilianstrasse 33
D-82140 Olching, Germany
europe@chroma.com
Tel: +49-8142-2847525

Chroma Technology Japan
8F Yokohama Onoecho Building
4-57 Onoecho Naka-ku
Yokohama 231-0015 Japan
Phone: +81 (0) 45 285 1583
Fax: +81 (0) 45 285 1501
japan@chroma.com

Chroma China
Room 803, Building 23, Harmony Times Square
No. 268, Wangdun Road, SIP, Suzhou City
Jiangsu Province, 215028 China
Phone: +86-0512-66051705
china@cn.chroma.com

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