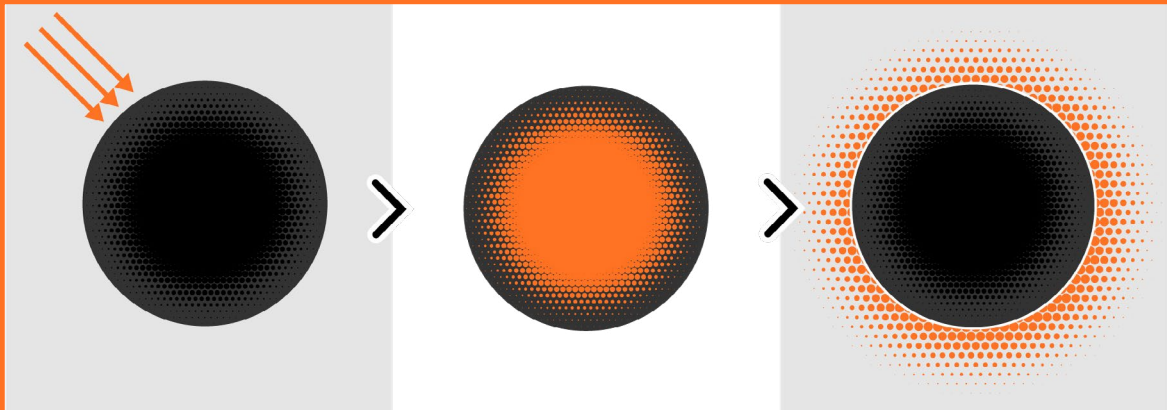


The fluorescence guide

understand fluorescence
and how it enhances
scientific exploration



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Introduction

Fluorophores and fluorescence detection are central to many research applications. Many fixed and live cell imaging techniques rely on immunofluorescence or fluorescent proteins. Fluorescence-based techniques can also be used in many common laboratory assays, including flow cytometry, ELISA, western blot, immunohistochemistry (IHC) and immunocytochemistry (ICC).

Fluorescent detection methods have several advantages over other analytical techniques, including high sensitivity, quantitative analysis, and the potential to carry out multiplex analysis using a range of available lasers, optical filters, and fluorophores.

This guide will enhance your understanding of how to use fluorescence in your research and advise you on choosing the right fluorophores and fluorescent proteins for your experiments. It begins with a basic introduction to fluorescence and how it works, followed by discussions of more advanced fluorescence mechanisms, such as fluorescence resonance energy transfer (FRET) and time-resolved fluorescence (TRF). Finally, the guide covers essential reagents for fluorescence-based experimentation, including fluorophores, tandem dyes, and fluorescent proteins.

What is fluorescence?

Fluorescence is a light signal detected when a chemical compound called fluorophore absorbs energy at a specific wavelength, causing it to become excited. The fluorophore then emits light at a longer wavelength as it relaxes and returns to its ground state.

Fluorescence is a three-stage process, as detailed in Figure 1. In the first step (1), the fluorophore is irradiated with electromagnetic light produced by a laser and passed through optical filters to create a very specific wavelength, matching the signature excitation wavelength for the molecule. The light provides the right amount of energy for an electron in the molecule to jump from its ground state (Gs) to an excited state (Es). In the second step (2), the electron absorbs the light and reaches an excited state.

In the final step (3), the electron undergoes vibrational relaxation to the lowest vibrational state within the excited electronic state, followed by relaxation to its ground state. As the electron returns to the ground state, energy is released in the form of a photon. The amount of energy released and the resulting wavelength of the emitted photon is characteristic of the fluorophore and determines its color and signature emission spectrum. The wavelength of the emitted light is longer than the exciting light because the electron undergoes vibrational relaxation, which emits energy as heat before fluorescence. The difference in wavelength between the exciting light and the emitted light is called the Stokes shift.

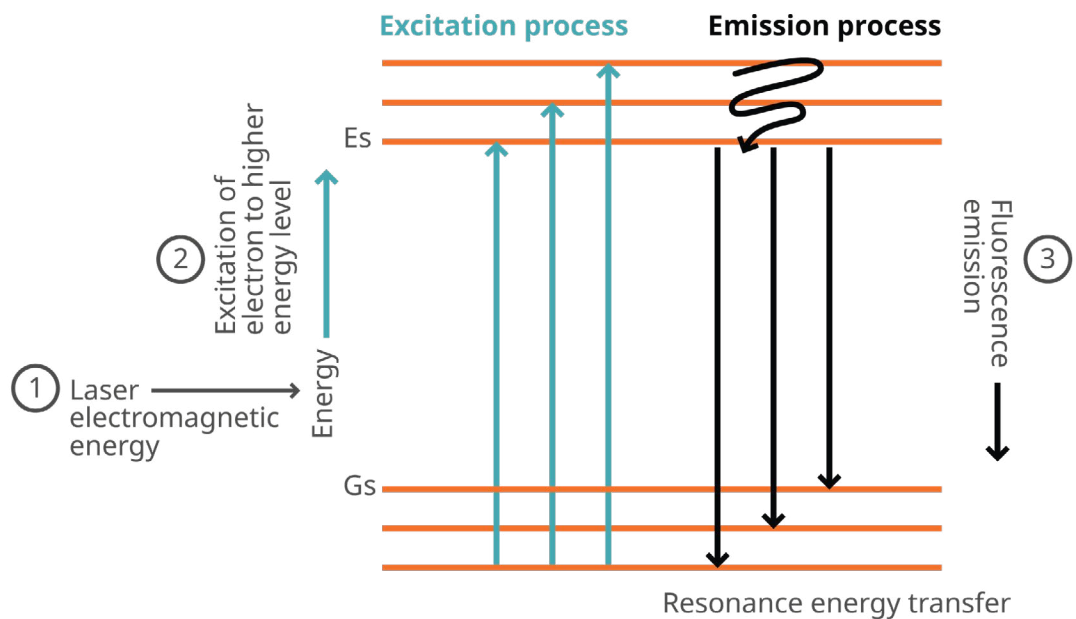


Figure 1. The three stages of the fluorescence event.

What are fluorophores?

Fluorophores or fluorochromes are photoreactive chemicals that absorb and emit energy in a predictable fashion. They re-emit light when excited, making them useful tags for identifying and characterizing cells or molecules in a mixture, visualizing proteins, or quantifying tagged compounds¹.

Fluorophores absorb light energy of a specific wavelength and re-emit it at a longer wavelength. The wavelengths at which the fluorophore absorbs and emits light are the fluorophore's excitation and emission (absorbance) spectra, which collectively make up the fluorescence spectra (Figure 2). Fluorophores differ in the intensity at which they emit light. Their brightness depends on their ability to absorb light and the efficiency of converting the absorbed light into emitted light.

There are many commercially available fluorescent dyes that absorb and emit light at specific wavelengths across the entire visible spectrum, including the infrared region.

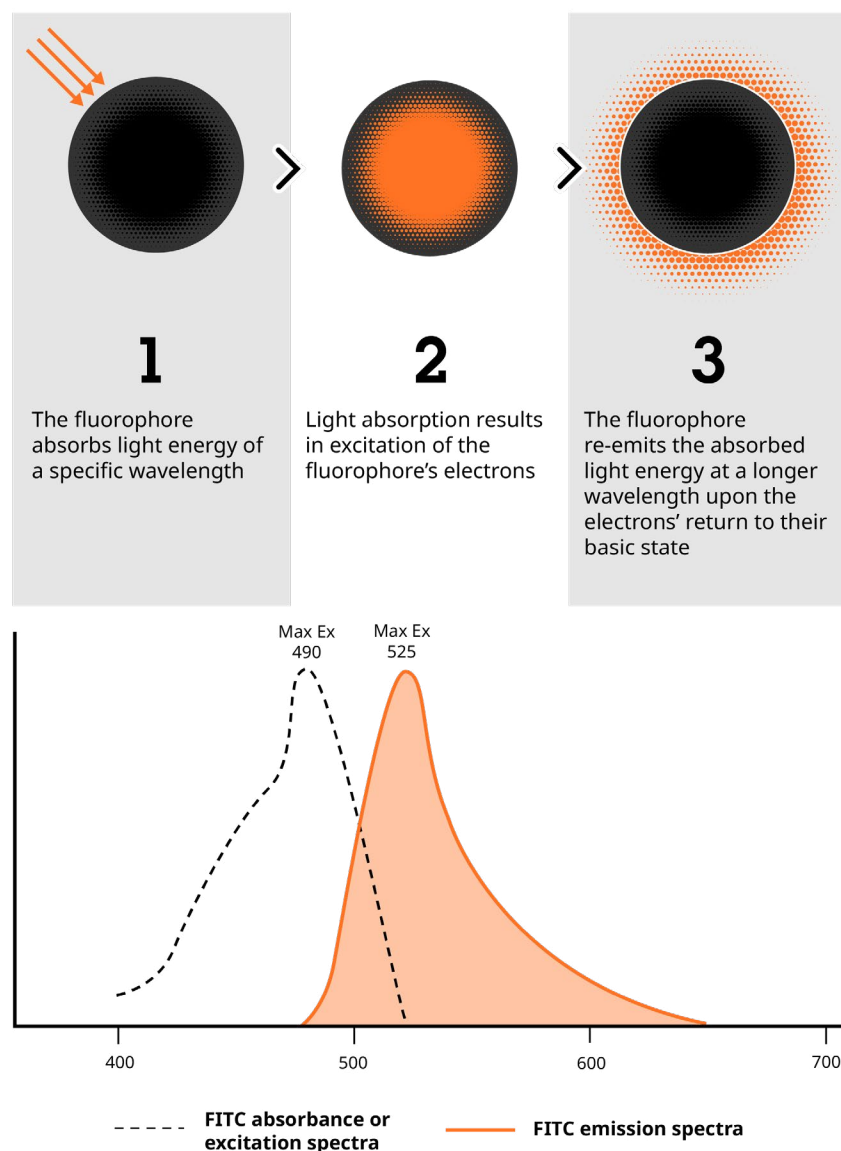


Figure 2. Top: Excitation and emission fundamentals of fluorophores. 1) The fluorophore absorbs light energy of a specific wavelength. 2) Light absorption results in the excitation of the fluorophore's electrons. 3) The fluorophore re-emits the absorbed light energy at a longer wavelength upon the electrons' return to their basic state. Bottom: Excitation and emission spectra of the commonly used fluorophore FITC.

Instruments requiring the use of fluorescent dyes, such as fluorescence microscopes and flow cytometers, are equipped with lasers that produce light at a particular wavelength to excite fluorophores, and sensors that detect various light wavelengths. Some combine multiple lasers and/or optical filters for multiplexed analyses. Light emitted by the fluorophores in the sample is then detected by sensors, providing the fluorescence read-out signal.

Beyond basic fluorescence, several more complicated fluorescence techniques are used in research. The most common are time-resolved fluorescence (TRF) and fluorescence resonance energy transfer (FRET), which are explained in more detail below.

Time-resolved fluorescence

Although conventional fluorophores are extremely popular, they have several limitations that can be overcome using time-resolved fluorescence methods.

Firstly, the simultaneous excitation/emission process from conventional fluorophores can result in high background signal. Secondly, the Stokes shift (the difference between the maximum absorbance and emission wavelengths) of many commercially available fluorophores is relatively small, meaning that these reagents can suffer from self-quenching due to the overlap between their absorption and emission spectra. Thirdly, biological matrices, such as serum or tissue samples, often contain autofluorescent substances, which are excited and emit light simultaneously in the same way as fluorophores and are a source of background signals. Similarly, in high-throughput screening formats, false positives can occur due to the fluorescent nature of certain types of test compounds.

Time-resolved fluorescence (TRF) can help overcome some of these problems. TRF is very similar to standard fluorescence detection, apart from the timing of the excitation and emission processes.

During standard fluorometric detection, excitation and emission are simultaneous with the light emitted. By contrast, TRF relies on using specific fluorescent molecules, called lanthanide chelate labels, which have a long fluorescence lifetime and allow detection of the emitted light after excitation has occurred. The most commonly used lanthanide chelate label is the europium ion (Eu^{3+}).

Find out more about [abcam's time-resolved fluorescence conjugation kits](#) on our website.

Fluorescence resonance energy transfer

During fluorescence resonance energy transfer (FRET), a donor fluorophore is excited by a light source and transfers its energy to a nearby acceptor fluorophore. The acceptor fluorophore absorbs the energy to produce a detectable light emission signal. This process results in the donor's fluorescence loss and the acceptor's fluorescence gain, both of which can be measured.

FRET means we can detect when two molecules are within several nanometers of each other. This distance-dependent event provides information on potential interactions between labeled molecules within a cell.

As a result, FRET is a powerful method for identifying protein-protein interactions and can be used in techniques such as flow cytometry, immunocytochemistry, immunohistochemistry, and ELISA. FRET is also ideally suited to high-throughput screening (HTS) since it is simple, sensitive, and easily automated.

For FRET to occur, several conditions must be met:

- Proximity: The donor and acceptor fluorophores must be close to one another for the FRET process to be efficient. FRET efficiency (E) is defined by the equation $E = R_0^6 / (R_0^6 + r^6)$, where R_0 is the Förster radius, and r is the actual distance between the two fluorophores. The Förster radius is the distance at which 50% of the excitation energy is transferred from the donor to the acceptor, and the R_0 value usually lies between 10-100 Å (1-10 nm). FRET pairs with a R_0 value towards the higher end of this range are often preferred due to the increased likelihood of FRET occurrence.
- Spectral overlap: The donor fluorophore's emission spectrum must overlap the acceptor fluorophore's absorption spectrum. The greater the degree of spectral overlap, the more likely FRET is to occur.
- Dipole orientation: Energy transfer from the donor fluorophore to the acceptor fluorophore occurs via intermolecular dipole-dipole coupling. The spatial relationship between the donor emission dipole moment and the acceptor absorbance dipole moment is described by the orientation factor, κ^2 , which has values ranging from 0 (all dipoles are perpendicular) to 4 (all dipoles are parallel). Dipole orientation between the two fluorophores is typically assumed to be random due to rapid molecular rotation and is taken to equal the statistical average (a value of 2/3) for most calculations of R_0 .

Optimal fluorophore pairs for FRET

FRET relies on the use of high-quality labeled reagents. Depending on the intended assay setup, these could be antibodies, proteins, or peptides. The following fluorophore pairs have been identified as optimal to use in FRET:

- RPE-APC
- RPE-Cy5
- RPE-Cy5.5
- RPE-Cy7
- RPE – DyLight®650
- RPE – APC/Cy5.5
- Fluorescein – RPE
- APC – DyLight750

Find out more about [abcam's labeled reagents for FRET](#) on our website.

Choosing the right fluorophores for your experiment

Selecting the right fluorophore can be difficult. An experiment requiring only one fluorophore is relatively simple, as we only need to consider the molecule's excitation and emission spectra. However, it may take some optimization to find the best fluorophore for the particular application (Table 1).

Table 1. Labels and their most common applications.

Experimental technique	Fluorophores
Fluorescent western blot	Fluorescent dyes
Immunofluorescence	Fluorescent dyes, including Alexa Fluor® dyes
Fluorescent immunohistochemistry (singleplex)	Fluorescent dyes
Fluorescent immunohistochemistry (multiplex)	Fluorescent dyes
Flow cytometry	Fluorescent dyes, including Phycoerythrin (PE), Allophycocyanin (APC), tandem dyes, and Alexa Fluor® dyes
Lateral Flow Assay (LFA)	Fluorescent nanoparticles (Europium)
Fluorescent ELISA	Fluorescent substrate

Designing a multiplex panel with two or more fluorophores can be more challenging as we must consider the spectral overlap between fluorophores, among other factors, to minimize potential problems such as fluorescence spillover and compensation errors.

Fluorescence spillover is a common issue in which fluorescence that should only be detected in one channel is also detected in an adjacent channel. It is caused by an overlap between the emission spectra of the fluorophores being used and can cause inaccurate results.

Compensation is commonly used to correct for fluorescence spillover, but this can also introduce errors. Selecting the right fluorophores is essential for minimizing fluorescence spillover and ensuring accurate results. Using tandem dyes can also help avoid spillover (see next section).

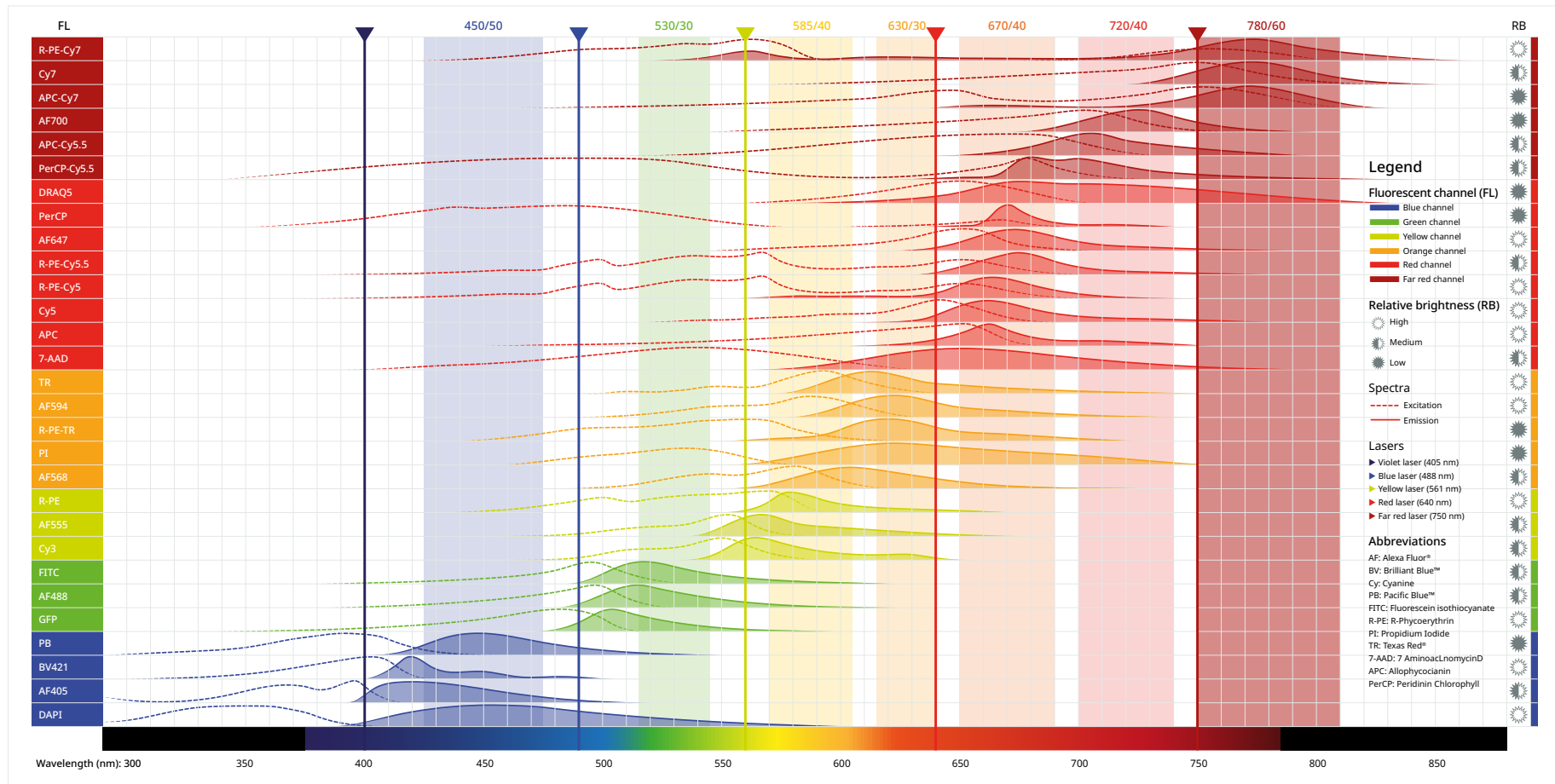
We have put together a fluorochrome chart to guide you through each step involved in selecting a fluorochrome to make the process quick and easy. The chart features the 30 most popular fluorescent labels, allowing you to quickly select the most suitable fluorophores for your next multiplex experiment.

Our abcam fluorophore chart includes:

- Aligned emission and excitation fluorescence spectra for 30 of the most commonly used fluorophores, including tandem dyes.
- Easy visualization of some of the most popular lasers and filters across the fluorescence spectra.
- The fluorescence channel and relative brightness for each of the fluorophores.
- A short guide with examples.

See our abcam fluorophore chart below, to help you choose the most suitable labels for your multicolor panel.

Fluorochrome chart



How to use this chart

1. Check your instrument

Type, number of lasers, filters and detectors dictate the fluorochromes that can be used.
– Try to choose a fluorochrome for each laser excitation range

2. Select bright dyes

It is possible to rank available dyes according to their brightness on a particular instrument.
– Brightest fluorochromes for dim antibodies and vice versa

3. Minimize spillover

The amount of spectral overlap will determine whether compensation is required.
– Sacrifice brightness to avoid spillover
– Avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations

abcam.com/fluorochrome-chart

Examples

Fluorochrome	Target Expression	Lasers	Channels	Brightness	Compensation	Combination
FITC	High	Blue	Green	Medium	Mild	Good
APC	Low	Red	Red	High		
FITC	High	Blue	Green	Medium	Moderate	Medium
PE	Low	Yellow	Yellow	High		
PerCP	High	Blue	Red	Low	Severe	Poor
7-AAD	Low	Blue	Red	Medium		(not recommended)

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Tandem dyes

Tandem fluorescent dyes are conjugated 'dual' fluorescent molecules consisting of two different fluorophores that are covalently attached and close enough for energy to be transferred between them via fluorescence resonance energy transfer (FRET). They are beneficial when designing experiments requiring several different colors. Tandem dyes can also help prevent problems with fluorescence spillover by increasing the separation of the excitation and emission spectra so they do not overlap.

As shown in Figure 3, the fluorophore acting as a donor absorbs and is excited by the light energy of a specific wavelength. Upon excitation of the donor, energy is transferred to the acceptor through FRET due to their proximity. The fluorophore acting as an acceptor then emits the transferred energy as fluorescent light at a characteristic longer wavelength.

For example, in phycoerythrin-Cy7 (PE-Cy7), PE and Cy7 act as the donor and acceptor fluorophores, respectively. The laser excites the donor molecule only (PE) – it is not the correct wavelength to excite the acceptor. The donor molecule's emission energy is the right wavelength to excite the acceptor molecule, Cy7, which then releases energy in the form of a photon at its signature wavelength. Therefore, PE-Cy7 has the excitation wavelength of PE fluorophore, but the emission characteristics of Cy7².

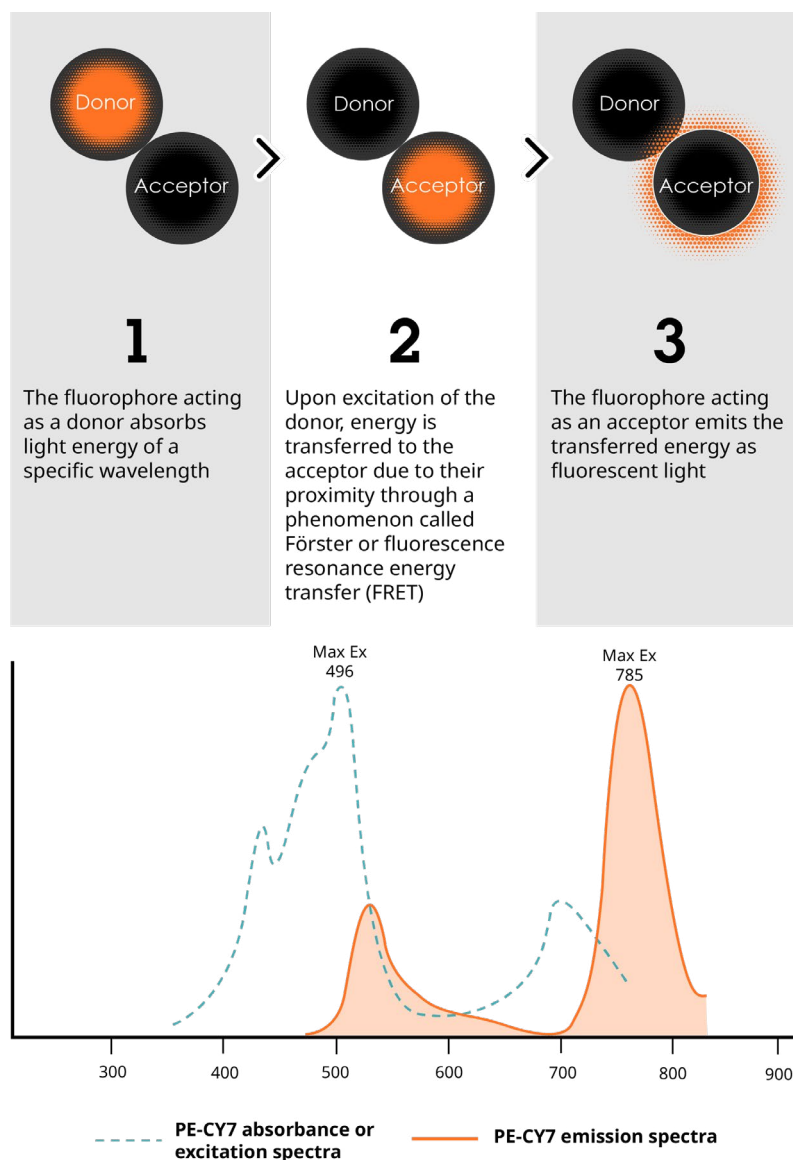


Figure 3. Top: Excitation and emission fundamentals of tandem dyes. Bottom: Excitation and emission spectra of the commonly used tandem dye combination PE-Cy7.

Using tandem dyes can increase your panel size and flexibility. This is because when using tandem dyes, a single laser can excite several fluorophores, which are measured by different detectors. For example, Alexa Fluor® 488, PE, PerCP-Cy5.5, and PE-Cy7 are all excitable with a blue laser (488 nm). However, they will produce green, yellow, purple, and infrared emissions (Figure 4).

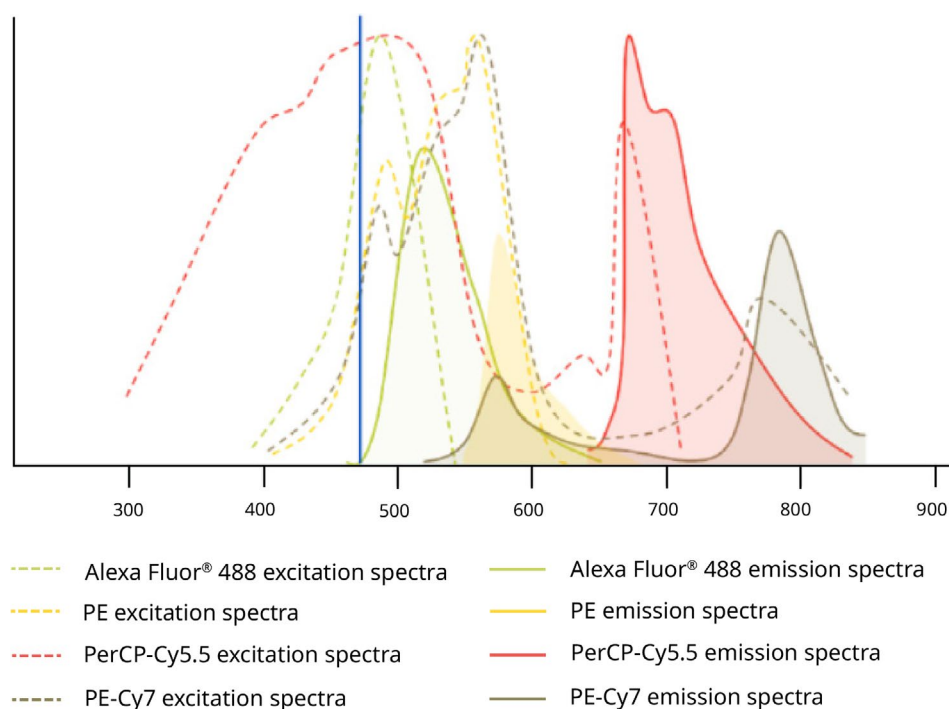


Figure 4. Excitation and emission spectra of Alexa Fluor® 488, PE, PerCP-Cy5.5, and PE-Cy7 following excitation with the blue laser (488 nm).

Hints and tips for handling tandem dyes

- Always protect tandem dyes from light as they are highly susceptible to photobleaching.
- Do not freeze tandem dye antibody conjugates, as this may result in the denaturation of the donor fluorophore.
- Minimize sample fixation or permeabilization as much as possible, as this reduces their brightness.
- Each batch requires optimization due to high batch-to-batch variation.
- Bleed-through emission from the donor may occur as FRET efficiency is not 100%.
- Label cells at 4°C to avoid degradation or decoupling of tandem dyes.

Fluorescent proteins

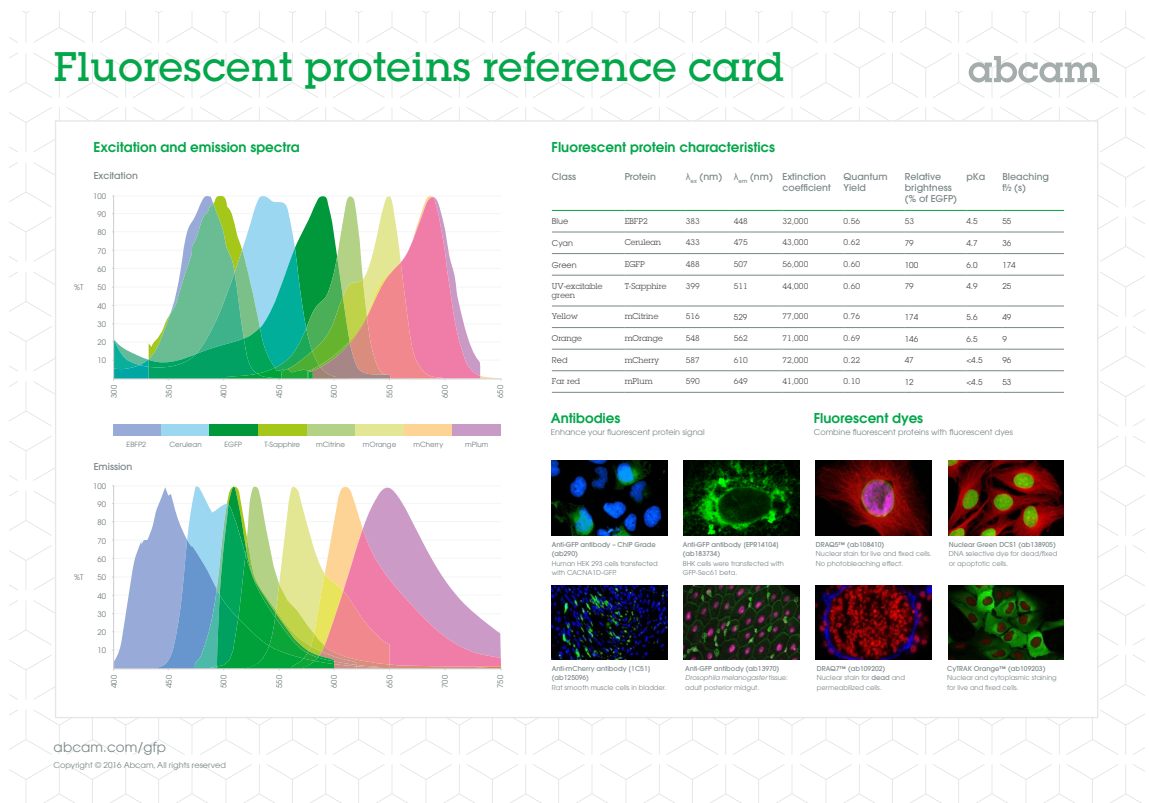
Fluorescent proteins (FPs) are naturally occurring proteins with fluorophores incorporated into their structure. They are commonly used in biological imaging and as biosensors in live cells. FPs can be fused to a protein in transgenic cells or animals, conjugated to an antibody, or even used as a substrate in enzymatic reactions. When combined with FRET, FPs can also provide high-resolution information about the proximity of proteins within cells, overcoming the theoretical resolution limit of optical imaging.

Since the original green fluorescent protein (GFP) gene was cloned from the jellyfish *Aequorea victoria* in 1992³, the variety of fluorescent proteins available for research has increased significantly. In addition to GFP and its variants, other commonly used fluorescent proteins include phycoerythrin (PE), R-phycoerythrin (R-PE), and allophycocyanin (APC), along with naturally occurring and genetically modified fluorescent proteins from a range of species.

How to choose the best fluorescent protein for biological imaging

There are many factors to consider when selecting a fluorescent protein. Below, we have outlined all of the key considerations, discussing why they are important and how they can influence your choice of the fluorescent protein.

Once you understand the properties you need for your specific application, you can choose the best protein using our quick reference card (below), which details some of the properties of the most popular fluorescent proteins.



Choosing C- or N-terminal fluorescent protein fusions

Fusing the fluorescent protein to the C- or N-terminus of your protein of interest largely depends on the protein itself, including how it folds and whether the terminus has a functional role. For example, if the C-terminus is folded inside the protein, you're unlikely to receive any fluorescence signal. If your protein is post-translationally cleaved close to the terminus your fluorescent protein is fused to, it will be removed from the protein of interest.

For a new experiment, it is ideal – if resources are available – to clone both C- and N-terminal tagged constructs to determine the best option. One research group found that more C-terminal fusion proteins localize to the intended subcellular compartment than N-terminal tagged fusion proteins⁴. However, it is important to stress that while C-terminal tagged proteins tend to localize and behave as expected, this is not always possible to predict.

You should confirm fusion protein localization by using an antibody against the native protein in the following ways⁵:

- Immunofluorescence to check that the fusion protein localizes correctly.
- Immunoblotting to confirm the fusion protein is the correct size and expressed at the expected levels.
- Co-immunoprecipitation to assess how the fusion protein interacts with known binding partners.

Excitation, emission, and brightness

If you plan on using multiple fluorescent proteins in the same experiment, choose fluorophores with distinct emission peaks that can all be excited with the lasers available on your equipment. Differentiating the signals can be difficult or even impossible if the emission peaks overlap.

Typically, the brightest FP within your available spectra is best to achieve a clear signal and overcome any background fluorescence. Brightness values are a product of the protein's extinction coefficient, which describes the amount of light it absorbs at a given wavelength, and its photon emission efficiency, known as its quantum yield. However, the resulting number can be difficult to interpret, so an FP's brightness relative to a well-defined fluorescent marker, like EGFP, is a standard alternative measure.

Maturation and bleaching

Maturation defines how long it takes an FP to fold correctly, form the fluorophore, and fluoresce. A short maturation time can be important for time-sensitive events in live cells. Superfolder GFP (sfGFP) can fold in under 10 minutes, while mOrange2 can take over four hours to fold correctly.

Bleaching is a measure of photostability, or how long after excitation, the chromophore loses the ability to emit light. The most common measurement of photostability is bleaching half-life ($t_{1/2}$), the time taken for an initial emission rate (measured in photons per second) to reduce by half. If you plan on conducting lengthy time-lapse experiments, consider an FP with high photostability. T-sapphire has a bleaching $t_{1/2}$ of 25 seconds, but EGFP is much more stable, with a bleaching $t_{1/2}$ of 174 seconds⁶.

Environmental conditions

Like most proteins, FPs are affected by pH, temperature, and oxygen levels. Depending on the environment in which you plan to use your FP, you may need to adjust the conditions slightly or select a more appropriate fluorophore.

The pH can affect excitation and emission peaks, and most FPs are sensitive to acidic conditions. Some FPs can change fluorescent intensity upon pH changes, such as pHTomato, which may be experimentally useful. The pKa value is a good indicator of pH sensitivity, showing the pH at which half of the chromophores are fluorescent.

Temperature and oxygen levels both affect maturation times. Hypoxic conditions tend to delay maturation times, as do temperatures outside the FP's optimal range. However, newer FPs like UnaG, a green fluorescent protein isolated from the Japanese freshwater eel (*Anguilla japonica*), fluoresce even when oxygen levels are low⁷.

Codon optimization

Most FPs are derived from jellyfish or coral proteins rather than the species in which they're most likely to be used experimentally. This can result in poor FP expression and low fluorescence signal due to interspecies differences in amino acid codons usage.

Fortunately, many new versions of FPs have been codon-optimized to reflect mammalian cell preferences. For example, Jürgen Haas and colleagues improved the signal from GFP up to 120-fold by modifying the codon sequence⁸.

If you're using an older plasmid to generate your fusion proteins, it may not contain a mammalian-optimized FP sequence. Check whether your FP sequence has been modified for use in your experimental species.

Avoiding oligomerization of fluorescent proteins

It's essential to determine whether your fluorescent protein is a monomer or dimer and whether this affects your experiment. Many of the early FPs were prone to forming oligomers which can affect the biological function of the fusion protein. EGFP, for example, is a monomer that can form dimers when used in high enough concentrations, potentially distorting subcellular organelles or disrupting experiments like FRET^{9,10}. Monomers are usually denoted by an 'm' prefixing the protein name, such as mCherry, and truly monomeric FPs are recommended in most cases.

These considerations are summarized in Table 2 below.

Table 2. Factors to consider when selecting a fluorescent protein.

FP consideration	Keep in mind
C- or N-terminal fusion	Try both if possible, although C-terminal fusion proteins are generally better. Confirm exact localization with an antibody.
Excitation and emission	Ensure you have the correct lasers to excite your fluorescent protein(s), and that emission peaks do not overlap.
Brightness	Select the brightest FP that also meets your experimental requirements.
Maturation	Select a short maturation for fast/ time-sensitive events.
Bleaching	Choose an FP with high photostability for long-running experiments to reduce photobleaching.
Environmental conditions	Ensure the pH, temperature, and oxygen of your experimental setup are optimized for your FP.
Codon optimization	Check the FP codon sequence is optimized/suitable for your species – especially when using older plasmids.
Oligomerization	Try using a monomeric FP if oligomerization is a problem.

Summary

Understanding the processes and reagents used in fluorescence experiments is essential for successfully using this powerful technology in your research.

This guide has taken you from an outline of the basic mechanisms of fluorescence and what a fluorophore is to understanding common techniques and how to choose the best reagents for your experiments, so you can get the results you need.

For more information, visit our website: [abcam.com](https://www.abcam.com)

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