

Understanding Fluorescence Imaging

Introduction

Fluorescence imaging is a tool often used to visualize cells and subcellular structures. Since the development of the first fluorescent dye in 1871, its usefulness to life science applications has been demonstrated as biomolecular labels, cellular stains, ionic indicators, and more. Many modern biological and biomedical research applications rely in part on fluorescence imaging to understand cellular structures and events.

Brightfield vs Fluorescence Imaging

In brightfield microscopy, the sample is illuminated with transmitted white light. This creates an even illumination of the sample under the microscope to observe highly contrasted, stained or naturally pigmented samples. However, brightfield is not sufficient to distinguish between transparent/translucent, unstained cells or cellular structures to study processes of interest.

The concept of fluorescence microscopy relies on the use of fluorophore-containing molecules that emit light of a specific visible wavelength when exposed to light of a different wavelength. When these fluorophores are bound to a targeted structure of interest, photons emitted from the fluorophore can be used to visualize this structure of interest. The benefit of fluorescence microscopy is that the targeted structures are illuminated while the undesired areas of the sample have little to no fluorescence, allowing for easy targeting and imaging.¹

The Molecular Basis of Fluorescence

Molecules often respond when exposed to electromagnetic radiation such as visible and ultraviolet light. Some of these molecules have active portions of their molecular structures that absorb, and later release incident light. These molecules, known as fluorophores, have similar structural characteristics that enable photon interactions.

Electrons of a single chemical bond (containing the standard two electrons) have a very high probability of remaining in the space between the two atoms, this is referred to as a sigma bond. When double-bonds are introduced to a molecule, pi bonds overlap the sigma bonds but are less stable, resulting in movement of the electrons across pi-bonds. When a molecule has several regions containing a double bonded structure in an alternating pattern, the electrons within that region delocalize from their original bond and can move along the entire molecule comprising alternating pi-bonds. These destabilized electrons are vulnerable to the environment, namely stimulation by electromagnetic radiation.²

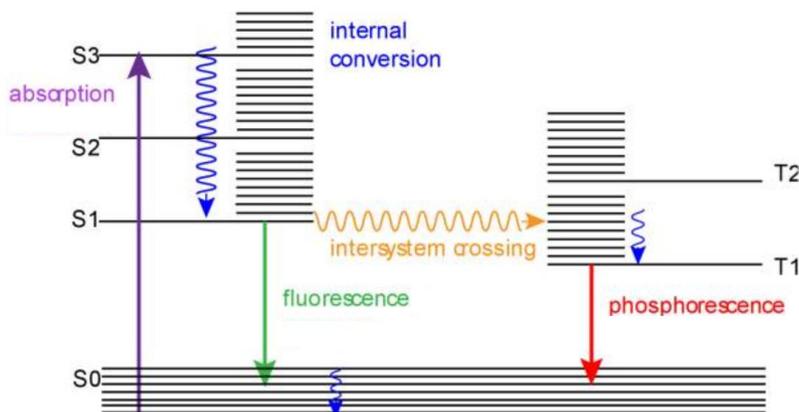


Figure 2: Jablonski diagram describing excitation of an electron from the ground state (purple line, left), fluorescence emission (green line, middle), and phosphorescence (red line, right) and other internal processes (blue line) to return to ground state.⁴

Factors that Determine Detected Fluorescence Intensity

Fluorescence Quantum Yield (ϕ) and Extinction Coefficient (ϵ)

In the emission process, some processes compete with the above-described emission process. Photochemical, non-radiative processes and resorption of emission photons all compete with the number of photons released in fluorescence. The quantum yield (ϕ) of a fluorophore is the ratio of the number of released photons to absorbed (Equation 1). The quantum yield is often expressed as a value from 0-1, which 1 being 100% efficiency of photon conversion. It is also important to note that each fluorophore has a unique pH, ionic strength, and temperature for optimal fluorescence efficiency.

$$\text{Quantum yield } (\phi) = \frac{\text{Number of photons consumed}}{\text{Number of photons absorbed}}$$

Equation 1: Fluorescence quantum yield equation

In addition to the quantum yield, every fluorophore has a different capacity to absorb photons even if they are within an appropriate wavelength range to excite it. Although a fluorophore is exposed to a photon appropriately matching its excitation wavelength, the probability that a photon will be absorbed is a measurable characteristic and known as the extinction coefficient (ϵ). A fluorophore’s quantum yield and extinction coefficient are often displayed together to describe how bright the fluorophore is shown to be in experimental settings.⁵

Fluorescence Lifetime

When a fluorophore electron absorbs a photon it does not immediately release a longer wavelength photon. The release of some energy between excited energy states is known to take different lengths of time. The amount of time that an electron spends in the excited state before releasing a photon and returning to the ground state is a measurement of its fluorescence lifetime. The lifetime of every fluorophore is unique and can be measured experimentally. When using fluorescent dyes experimentally, it is helpful to understand the length of time between stimulation of a target and the onset of fluorescence. In experiments that require fluorescence to occur very shortly after a tagged event, like the quick onset of calcium-induced fluorescence in neurons.⁶

Excitation Wavelength Intensity

Most fluorescent microscopy setups include a source of light that can be tuned to output the desired wavelength range. Many fluorescent light sources can also be adjusted for excitation intensity to increase the number of photons moving through the light path. In a fluorescently-labeled sample that is exposed to its excitation wavelength, every fluorophore will not be activated at the same time. By increasing the excitation intensity and increasing the number of photons reaching the sample, there is a higher probability that more fluorophores will be excited.

Photostability

Photostability is the ability of a molecule or organism to resist damage. In fluorescence microscopy, fluorophores will eventually stop absorbing oncoming photons and go into a permanent dark state. As an organism gathers more fluorophores in the dark state, the appearance of the labeled target reduces, and the sample is said to be photobleaching. In fluorescence microscopy, steps are often taken to reduce the amount of photobleaching experienced during experimentation. Some measures include a reduction in the intensity of light interacting with the sample and the use of specialized fluorescent dyes that do not remain active for as long as other dyes.

Scientific Camera Considerations for Fluorescence Microscopy

Researchers using fluorescence microscopy can use modern imaging devices to tackle challenges previously thought impossible. Advancements in scientific camera sensitivity and speed, combined with the modernization of imaging techniques has created an explosion in the addition of fluorescence images to published data. When considering camera technology for fluorescence applications, some performance specifications can influence the ability to capture desired data. The imaging needs of fluorescence experiments are varied, and this list is not exhaustive of all possible imaging considerations but provides a point of reference for quantitating data obtained from images or movies.

Quantum Efficiency

Camera sensors capture photons released from a sample. The photoactive regions of the sensor convert the incident photons into electrons which can be converted into a digital signal and displayed on a PC. The percentage of photons that are converted to electrons by the camera sensor is referred to as the quantum efficiency (QE). The QE of a sensor varies with wavelength and is stated by the sensor manufacturer. The quantum efficiency of a camera sensor is of high importance to researchers working with a limited photon-budget or those who want to minimize the impact of photodamage and photobleaching or detect the onset of fluorescence of a biological event. Cameras with high quantum efficiency convert a greater number of photons into electrons, enabling improved detection at lower light

levels. Comparing the fluorophore emission wavelength with the quantum efficiency of the fluorophore used in the experiment can ensure that more incident photons will be converted into a digital signal.

Pixel Size

The size of the pixels making up the active array can make a dramatic impact on the quality of the image. As stated, each pixel converts incident photons into electrons that are used to create the digital image. The size of the pixel is directly related to the number of photons collected as larger pixels have a larger surface area for photon collection. Larger numbers of photons detected per pixel is related to better signal detection. Hence, cameras that have larger pixels are more sensitive. Many EMCCD cameras, widely considered to be highly sensitive cameras, usually have a pixel size between 13-16 μm . However, larger pixels are also associated with a reduction in spatial resolution as incident photons received from closely associated structures are more likely to fall on the same pixel and be read as one structure. Microscopists must make a tradeoff between the high sensitivity of a larger pixel and the high spatial resolution of a smaller pixel.

Exposure Time and Speed

Each image taken with a scientific camera is the result of exposing the sensor to incident photons for a limited amount of time, referred to as the exposure time. Longer exposure times are associated with higher signal intensities as there is more time for emitted photons to land on the sensor. However, the exposure time of each frame plays a role in the minimum number of frames that can be collected per second. When successive image frames are taken to create a movie or study a dynamic process, the rate of acquisition of images should be faster than the speed of the fluorescent process being studied. For fluorescent samples with a limited photon budget, higher exposure times are often needed to achieve detection but this may limit the speed of acquisition.

Conclusion

Since the introduction of the first fluorescent dye, fluorescence microscopy has been a heavily used tool to visualize cells and cellular structures with higher specificity than traditional brightfield microscopy techniques. Researchers can manipulate the structure, optical properties, and probe of interest in fluorescence experiments to obtain relevant data. Such flexibility has allowed fluorescence microscopy to be included in many life science experiments.

Depending on the type of sample and fluorophore, a scientific camera should be carefully selected to achieve the best imaging results.

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