Uses of fluorescence spectroscopy and confocal fluorescence microscopy when looking at fluorophore-stained root cells

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ABSTRACT: When utilizing a scanning confocal microscope, better resolution and image contrast is achieved when compared to traditional methods. Thin optical sections of living specimens, 100 micrometers thick can be imaged with such devices, which are capable of examining fluorescence emission between 400nm and 750nm. By using laser light of wavelength 470nm as an excitation source, a root cell sample stained with two different fluorescent dyes was examined. Two distinct peaks were noted at 643.98nm (red) and 537.72nm (green). The fluorophores used were deemed to be monolignol (green) and Peridinin Chlorophyll (red) as they matched the ratio of excitation/emission wavelength detected in the experiment.

1. Introduction

Fluorescence microscopy is arguably the most common technique used for imaging in biophysics. It is able to detect single molecules, while not exhibiting invasive characteristics of other imaging methods; it can be used ‘in vivo’ and its quantitative nature allows it to measure molecular concentrations and dynamics. However, one of the most striking uses of Fluorescence microscopy lies in its ability to specifically detect and localize fluorophores in 3D at sub-micron resolution. The Physics behind fluorescence microscopy can be summarized in a Jablonski energy diagram, as seen in figure 1.

In a Jablonski diagram, the straight lines represent the transition between a photon and an electron energy whereas the curved lines represent transitions between electron energies without photon interaction. A different emission wavelength is witnessed depending on which energy pathway is undertaken.

The electronic energy eigenstates are represented by thick lines and within each of these energy eigenstates, there are various vibrational energy levels, and looking in even greater detail, each of these vibrational energy states can be subdivided into rotational energy levels. The number of electronic, vibrational and rotational energy levels depends on the nature of the molecule under scrutiny. When a photon of a specific energy is absorbed by the molecule under investigation, an electron is excited from a lower energy eigenstate to a higher energy eigenstate as the photon energy is transferred to the electron. Due to the discrete nature of the system, only certain very well defined wavelengths will allow absorbance to occur.

2. Fluorescence

There are many different pathways for the molecule to deal with the absorption of a photon, the one pertinent to fluorescence occurs when the molecule emits a photon of its own. In figure 1 this phenomenon can be seen as the red line traversing the energy eigenstates. Fluorescence takes $10^{-9}$ to $10^{-7}$ seconds to occur, this is a relatively long time, and hence, the probability of fluorescence occurring at electronic energy eigenstates much higher
than the first excited state is much lower. At higher energy eigenstates, it is more likely for the energy to be dissipated through vibrational relaxation and internal conversion.

![Figure 2: Fluorescence occurring after the initial absorbance and internal conversion steps have been completed.](image)

The emitted photon's energy is equal to the energy difference between the transition eigenstates, in the case of fluorescent photons however, this energy is usually lower due to the fact that some energy is lost in the internal conversion and vibrational relaxation processes. The Stoke shift is the difference in wavelength between the excitation wavelength and the emitted wavelength, from the above argument, the latter is always larger than the former. The emission is distributed over a range of wavelengths because of the existence of a large number of vibrational energy levels. Molecules which exhibit fluorescent character are called fluorophores.

3. Fluorescence microscopy
To be able to obtain information from fluorophores and thus of the structure which they pervade, a laser scanning Confocal microscope is used:

![Figure 3: The optical pathway and principal components in a laser scanning confocal microscope.](image)

The light emitted by the source is coherent and passes through a pinhole aperture found in the confocal plane whereas the other pinhole aperture is positioned in front of the detector. The laser is reflected by the dichromatic mirror and scans across the sample along a well-defined focal plane. Fluorescence from the points on the sample passes through the dichromatic mirror once more but due to their longer wavelength now pass straight through and focus at a confocal point on the detector. In the confocal geometry, a point is illuminated and scanned across the sample as opposed to wide field illumination.

![Figure 4: Wide field vs. confocal microscopy illumination, demonstrating the difference in size between point scanning and wide field excitation light beams.](image)

Although fluorescence occurs above and below the sample, all these emitters are not confocal with the pinhole aperture, a very small fraction of an unfocused fluorescence manages to pass through the pinhole, whereas the vast majority will not be picked up by the photomultiplier, thus will not have an effect on the final image. The advantages of using confocal point as opposed to wide-field illumination are diverse, in the latter, the whole specimen is intensely illuminated by an incoherent source, the unwanted result of which is to detect a non-negligible signal also from background light and auto fluorescence from areas above and below the focal plane. This leads to an important reduction in resolution and image contrast. If one wanted to increase the gain, also the noise would increase, it is therefore vital to balance all these factors to obtain the desired image.

When preparing the sample it is important to not over-label it as this might cause fluorescence quenching which leads to a loss in fluorescence emission or a decrease in solubility. The emission wavelength and intensity are influenced by pH, polar and non-polar interactions and temperature as well as positioning of the fluorophore in the sample.

When exposed to an excitation wavelength the sample might undergo photo bleaching, which alters the dye in such a way as to permanently make it unable to fluoresce. Controlled photo bleaching however, does allow for reduced auto fluorescence, improving the noise to signal ratio.
**Analysis**

The aim of the experiment was to understand the nature of the fluorescent dye used, and possibly if more than one dye was used to stain a sample of root cells. The following sets of images were taken:

![Figure 5 - images showing stained root cell tissue, the excitation wavelength used was 470nm. The image is a 60x magnification of the original sample.](image)

From figure 5 it can be understood where these two dyes are situated in the sample. The fluorophore which predominantly emits green light is present in the filament-like structure clearly highlighted in the green image, whereas the second dye can be considered as having pervaded the whole structure.

The decrease in intensity of the red emitted wavelength in the filament-like section of the image is due to the fact that the intensity of the green light emitting fluorophore is non-negligible, hence, the relative intensity of the red light emitting fluorophore will be lower in these regions. It is important to note that in figure 5 both fluorophores are still emitting light, the only difference lies in the observation window. Confusion must not be made in believing that one fluorophore is ‘switched off’.

The green coloured image appears dimmer than the red tainted one because green light is diffracted more than red light, hence the intensity will decrease more for the green wavelength light than for the red wavelength light when travelling over the same distance. The lateral resolution of the microscope is limited by diffraction to be:

$$ R = \frac{0.611}{NA} $$

Where NA is the size of the objective lens. Furthermore, the green light emitting dye could have a lower quantum yield (ratio of the number of emitted photons to absorbed photons) than the red light emitting dye. When looking at figure 5, it is interesting to notice that the dye is mostly present on the outskirts of the cells making up the tissue. This gives further clues as to the nature of the dye used, as it seems that this dye primarily targets the root’s cell wall or cell membrane.

**Figure 6 - From the emission spectrum it is clear that there are two peak wavelengths. The y-axis shows the relative intensity of each dye as a ratio of the region of interest.**

In the case of the ROI 3 plot, there appears to be only one bright peak, this was due to the fact that a small region of interest was looked at specific to the green channel.

From the graph it is clear that two wavelengths of light are emitted as one maxima is found at: 643.98nm (red wavelength) whereas the other is at: 537.72nm (green wavelength). Since two wavelengths of light are emitted from one excitation wavelength, this gives an indication as to the number of dyes in the sample. If there was only one dye, only one wavelength of light would be emitted, corresponding to the energy transition experienced by that specific dye’s electrons being excited by the excitation wavelength. However, some dyes do exhibit non-singular fluorescence, i.e. they emit more than one wavelength according to the value of the excitation wavelength; in this case though, there is only one excitation wavelength and all other factors, such as pH, temperature and concentration are kept constant, thus, there must be two dyes staining the root cells.

Had the pinhole been wide open there could have been the risk of having a cut off peak in figure 6 due to saturation of the detector, therefore having a range of acceptable outcome values for the emission wavelength. Identifying which kind of dyes were used would have been very difficult. However, a bigger aperture leads to a better contrast, but also to a lower depth resolution.
Most plant cells are covered by an extracellular cell wall composed primarily of cellulose and a low quantity of lignin. Since the interior of the cells does not appear to be stained, the nature of the green light emitting fluorophore is very likely to be monolignol as it has an excitation/emission value of: 488nm/500-650nm, thus accounting for the excitation wavelength of 470nm and for the green light emission wavelength of 537.25nm. The dye's identity can further be proven by looking at a similar stain performed by Tobimatsu et Al. where the same dye caused a very similar image to be formed.

There are a number of possible candidates which could have been used as the red light emitting dye, however, it is interesting to point out how there are very few red wavelength emitting dyes which are excited around 470nm, a dye which exhibits a very large Stoke shift has been used. A possible candidate is Peridinin Chlorophyll (PerCP) a fluorescent protein which has an excitation wavelength of 483nm and an emission wavelength of 676nm.

Fluorescence microscopy allows to scan 3d structures as well. This is done by rotating the sample 360° through its vertical axis and allowing the electron beam to scan the sample creating one image for a specific angle, the combination of all these images reconstructs the sample.

**Conclusion**

Lignin is localized to the xylem of vascular bundles, hence monolignol seems to have been the dye used for the green wavelength emission, whereas Peridinin Chlorophyll was probably used for the red wavelength emission due to its very large Stoke shift. Further analysis could include utilizing a dye to stain the interior of the cell, so as to have a more detailed representation of the whole cell and not just its skeletal outer cell wall/membrane representation.

**REFERENCES**


Websites visited:
Fluorophore.org, tsienlab.com