Application Note

Imaging of Fluorescent Proteins

Table of Contents

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1. Introduction

The imaging of fluorescent proteins (FP) has become a major element in life science research and many derivatives of the original fluorescent proteins have been created, providing a huge selection of colours. Because fluorescent proteins can be genetically combined with a cell's own proteins, each copy of a cell that expresses a fluorescent protein will, in general, also express that fluorescent protein, making it relatively easy to track particular components within a cell, or groups of cells, as the cells, tissue or organism develops.
Samples may contain just a single FP or may contain a combination of two or more FPs, where each FP labels a different component within the sample and this has a significant impact for the configuration of our imaging systems as discussed in Sections 2 and 3.

1.1. Requirements for imaging Fluorescent Proteins

Fluorescent proteins are just like any other fluorescent label in that they require a suitable excitation source, a dichroic to separate the excitation and emission optical paths and an emission filter to transmit the emission wavelength range while blocking the excitation wavelength range. The imaging of samples containing just a single FP therefore requires an imaging system that contains a laser excitation wavelength that matches efficiently to the excitation spectra of the FP, an emission (barrier) filter that transmits the bulk of the emission spectra of the FP but which also blocks any excitation light from reaching the detector, and a dichroic mirror to separate the excitation optical path from the emission optical path.

The following example illustrates the principles involved using GFP (green fluorescent protein).

Fig 1 GFP spectra

(from Invitrogen's Fluorescence Spectra Viewer)

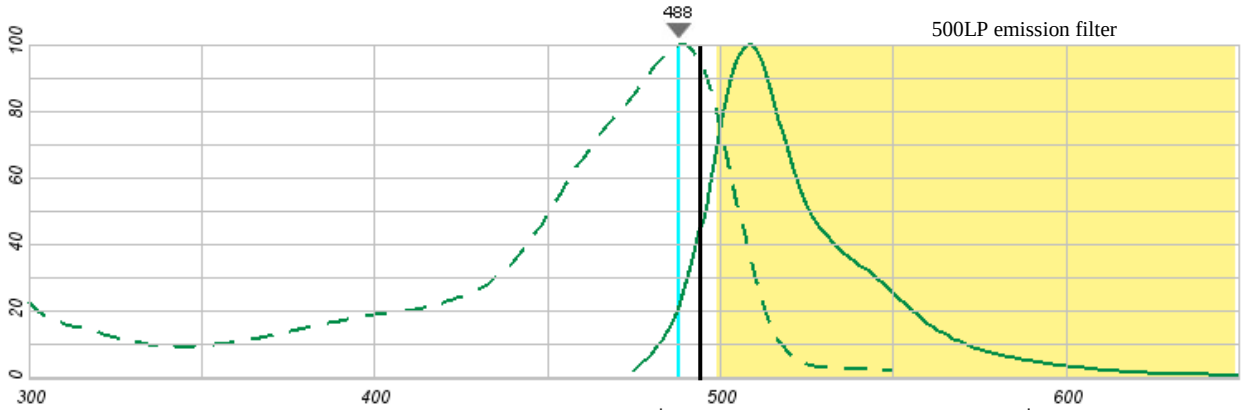


Fig 1 shows the spectral characteristics of GFP. The dotted line represents the excitation spectra while the solid line represents the emission spectra. GFP is typically excited at a wavelength of 488nm (the cyan coloured line labelled 488). A suitable dichroic and 500LP (long pass) emission filter are represented by the black line and the yellow shading respectively. The dichroic must transition between reflection and transmission (or

transmission and reflection depending on the instrument's optical path) in the wavelength range between the excitation wavelength and the emission filter.

Whenever dual wavelength labelling of biological specimens is required, two pairs of proteins have been commonly used: the CFP/YFP and GFP/RFP pairs. In these paired combinations they are also sometimes used as FRET pairs, where the emission of the shorter wavelength (donor) protein becomes the excitation energy for the longer wavelength (acceptor) protein when the two proteins are in very close proximity, typically <10nm.

When using fluorescent proteins in combination, there are some spectral conflict issues that need to be considered. With almost all combinations there is a potential for crosstalk (bleed through) between the emission signals of the fluorescent proteins. Suitable choice of emission filters can minimise crosstalk and is critical when configuring systems. Refer to Section 2.2 to see how this is dealt with.

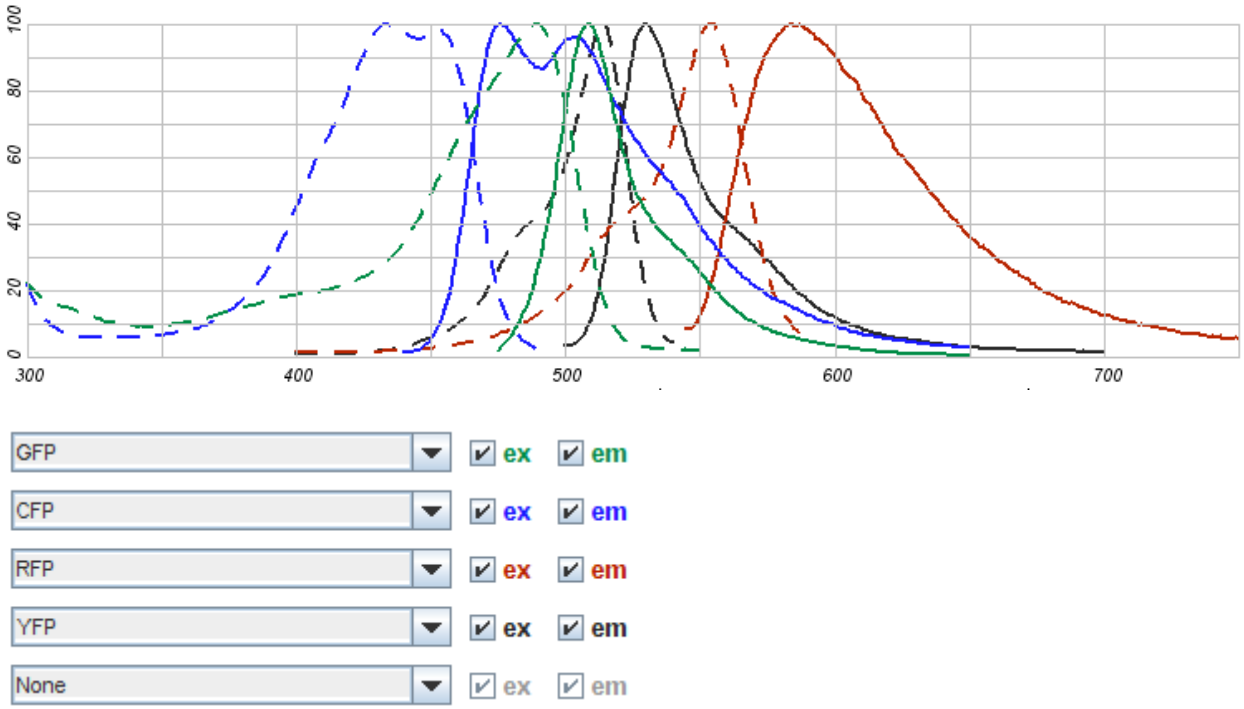
2. Fluorescent Protein Imaging

2.1 Samples containing single fluorescent proteins

With samples containing only a single fluorescent protein, excitation wavelengths, dichroics and emission filters can be configured for each FP as described in the Introduction (Section 1) and for four frequently used FPs are shown in Table 1 with their excitation and emission spectra in Fig 2.

Fig 2 Spectral plots of CFP/GFP/YFP/RFP

(from Invitrogen's Fluorescence SpectraViewer)



The dotted lines are the excitation spectra, the solid lines are the emission spectra.

Table 1 Parameters for imaging FPs

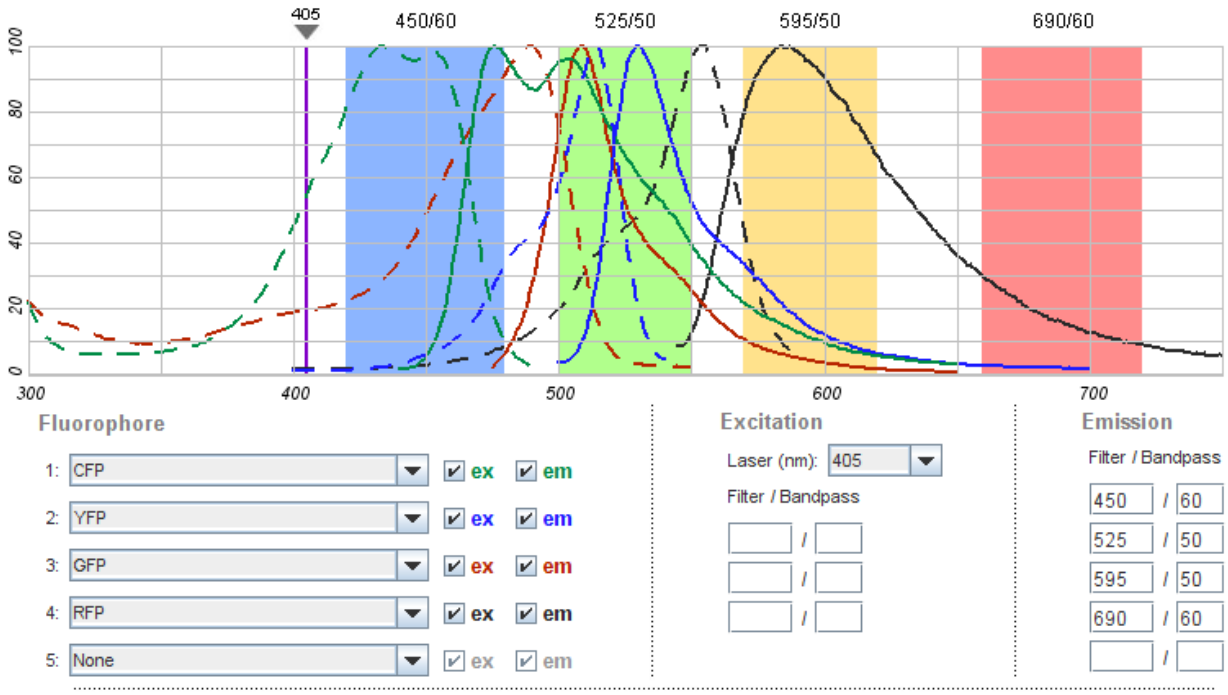
Fluorescent protein	Excitation wavelength	Dichroic transition	Emission filter
CFP	442nm	450nm	460LP
YFP	514nm	520nm	525LP
GFP	488nm	500nm	500LP
RFP	561nm	575nm	575LP

Samples labelled with a single FP can often use a long pass emission filter to maximise the collected signal at the detector, however, some samples may also exhibit auto-fluorescence and therefore the choice of emission filter should be modified to eliminate the wavelengths within the spectra of the auto-fluorescence.

For single labelled samples that may use a variety of different FPs the necessity to change filter sets (dichroic and emission filter) when there is a change of the FP may not always be convenient and therefore multiband filter sets are commonly used. Multiband filter sets provide for the use of appropriate excitation wavelengths, and emission transmission bands so that it is only necessary to switch the excitation wavelength when switching between samples labelled with different FPs. This is simply achieved via software control of the AOTF in VisiTech's laser merge module (refer to Section 3 for more detail).

Fig 3 Example of a multiband filter set

(from Invitrogen's Spectra Viewer)



In this example the four coloured bands represent the transmission bands of a multiband emission filter which also blocks the wavelengths outside of these bands and most importantly has high rejection of the specific excitation wavelengths used. Note that in this example the CFP is excited at 405nm and not at 442nm, (442nm is more frequently used). The multiband emission filter is designed to block each of the excitation wavelengths, i.e. 405nm, 488nm, 561nm and 642nm while transmitting as much as possible in the intervening wavelengths.

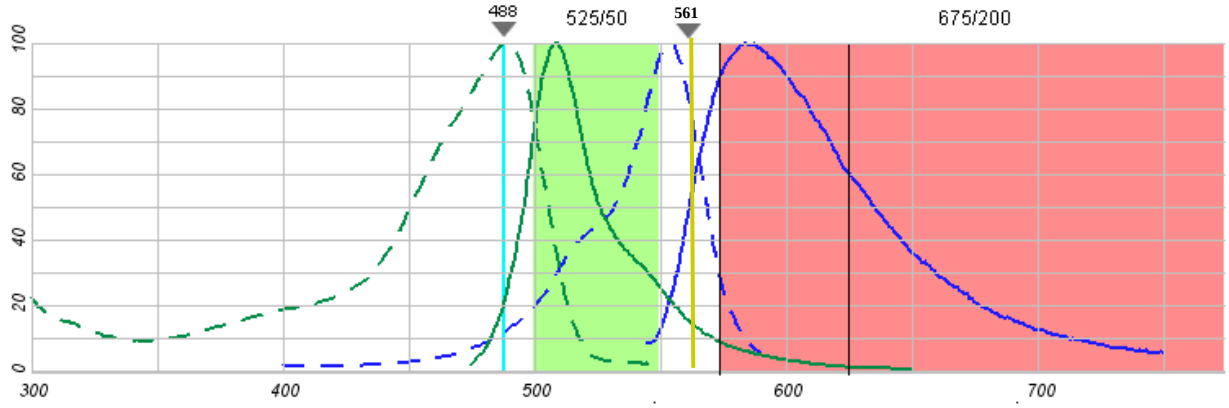
2.2 Samples containing multiple fluorescent proteins

In imaging of individual FPs, the emission filter may be a long pass type, however, when FPs are used in combination it is obvious that only the longest wavelength FP may use a long pass filter. The FP's having the shorter wavelengths require band pass filters for several reasons which are explored below.

From the spectral plots of the FPs (Fig 2) it is obvious that there are considerable overlaps of both excitation and emission spectra. The overlapped spectra have the potential to introduce crosstalk (or bleed through) of one FP's signal into another. When choosing to combine two, or more, FPs in the same sample the degree of overlap needs to be considered and suitable measures taken to eliminate, or at least drastically reduce, the potential for crosstalk. Two of the most frequently used pairs of FPs are used as examples in Fig 4 and Fig 5.

Fig 4 GFP/RFP Excitation/Emission spectra

(from Invitrogen's SpectraViewer)



GFP Excitation spectra (excited at 488nm)
GFP Emission spectra (with 525/50nm band pass filter)
RFP Excitation spectra (excited at 561nm)
RFP Emission spectra (with 575nm long pass filter)

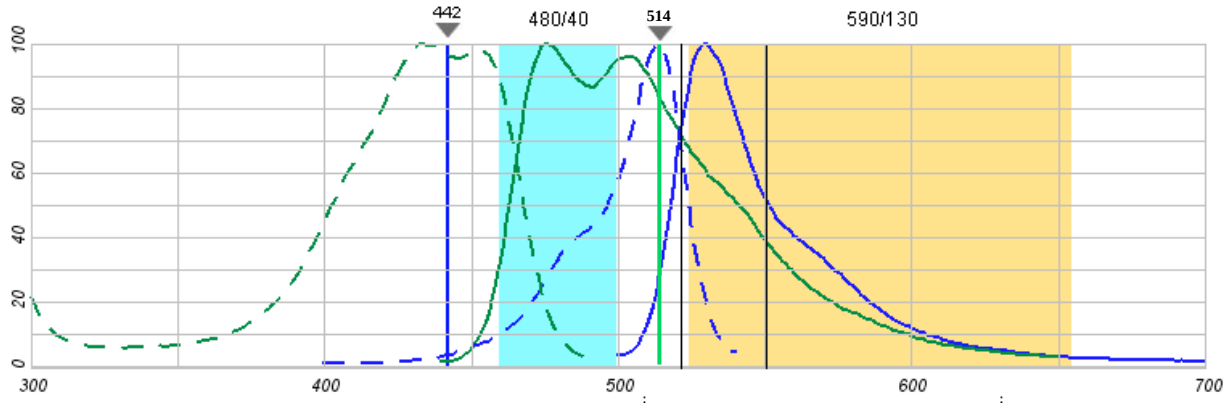
NOTE: For high auto-fluorescence samples the 575nm long pass filter should be replaced with a 600/50nm band pass filter (represented by the two black vertical lines.

Figure 4 shows the spectra of the GFP/RFP combination of fluorescent proteins. The excitation wavelengths and typical barrier filter options are superimposed. When exciting the GFP at 488nm, the RFP is also partially excited due to the significant overlap of the two excitation spectra. To prevent the emission of the RFP contributing to the imaged GFP signal, the detector is filtered (typically with a 525/50nm emission filter) to allow as much as possible of the GFP signal to reach it and to block as much as possible of the RFP signal.

When the RFP is excited at 561nm, the GFP does not respond and therefore the RFP emission can be a long pass filter that blocks the excitation wavelength. However, some samples may exhibit strong auto-fluorescence; in this case the emission filter for the RFP must be a band pass filter chosen to maximise the useful RFP signal and to block the auto-fluorescence. The band pass filter will reduce the signal intensity but improve the contrast when strong auto-fluorescence is present.

Fig 5 CFP/YFP Excitation/Emission spectra

(from Invitrogen's SpectraViewer)



CFP Excitation spectra (excited at 442nm)
CFP Emission spectra (with 480/40nm band pass filter)
YFP Excitation spectra (excited at 514nm)
YFP Emission spectra (with 590/130nm band pass filter)

NOTE: For high auto-fluorescence samples the 590/130nm wide band filter should be replaced with a 535/30nm band pass filter (represented by the two black vertical lines).

Figure 5 shows the spectra of the CFP/YFP combination of fluorescent proteins. The excitation wavelengths and typical barrier filter options are superimposed. When exciting the CFP at 442nm, the YFP is marginally excited due to the small overlap of the two excitation spectra. To prevent the emission of the YFP contributing to the imaged CFP signal, the detector is filtered (typically with a 480/40nm emission filter) to allow as much as possible of the CFP signal to reach it and to block as much as possible of the YFP signal.

When the YFP is excited at 514nm, the CFP does not respond and therefore the YFP emission can be a long pass filter that blocks the excitation wavelength. However, some samples may exhibit strong auto-fluorescence; in this case the emission filter for the YFP must be a band pass filter chosen to maximise the useful YFP signal and to block the auto-fluorescence. The band pass filter will reduce the signal intensity but improve the contrast when strong auto-fluorescence is present.

In both paired FP cases discussed above, the dichroic in the confocal head must either be a dual band dichroic, permitting both excitation wavelengths to reach the sample, and maximising the fluorescence bandwidth that reaches the detector, or two single band dichroics that are quickly interchangeable, for example on a slider or a rotating wheel. The drawback of using separate dichroics is that they must be precisely aligned to each other or the images from each FP will not register on a pixel to pixel basis. Using a single dual band dichroic overcomes this requirement.

3. System configurations for imaging Fluorescent Proteins

In laser confocal imaging systems it has become usual practice to employ solid state lasers since they now provide all of the commonly used wavelengths, for example, 405nm (violet), 442nm (blue), 488nm (cyan), 515nm (green), 561nm (yellow), and 642nm (red).

When more than one laser line is required, the individual lasers are mounted in a merge module. This is a box that encloses a small optical table on which the laser heads are mounted together with mirrors and dichroics in such a way that the laser outputs are combined into a single collinear beam path. This beam path passes through an Acousto Optical Tuneable Filter (AOTF) crystal, which controls the deflection angle of each wavelength beam such that none, or any one or more of the wavelength beams can be deflected to an optical fibre output port, while the remainder are deflected into a beam dump. The beam dump is a block of metal that absorbs the energy from the currently unused laser lines.

The AOTF also controls the intensity of each beam that reaches the optical fibre output port and due to its ability to deflect the beams in a few microseconds it acts as if it were also a high speed shutter for the laser light. When the detector requires an image, the AOTF (through software control) allows the sample to be illuminated and turns the illumination off as soon as the detector has acquired the image, thus reducing the potential for photo-bleaching of the FPs and reducing the creation of photo-toxins.

An optical fibre carries the selected laser light to the confocal head, where a dichroic mirror separates the excitation and emission optical paths. The confocal head uses the laser light to scan the sample via the microscope c-mount port and the fluorescent light from the sample returns through the same port back to the confocal system. (Samples are mounted on the microscope in the conventional manner.) After separating the excitation and emission optical paths, the dichroic mirror in the confocal head directs the fluorescent light to the detector (a camera or a photomultiplier depending on the model of confocal) which generates the signal that forms the image seen on the screen of the imaging system.

3.1 Configuration of a system for imaging samples labelled with a single FP

When imaging samples that contain only a single FP, the configuration of the system will include:

- one or more laser sources in a laser merge module
- an AOTF which controls the laser line selection and intensity, and also provides the high speed shuttering that reduces photo-bleaching and its associated artefacts
- an optical fibre to carry laser light from the laser merge module to the confocal head
- a confocal head and controller
- suitable single band dichroics and single band emission filters and/or multiband dichroic and multiband emission filter as described in Section 2.1
- a high quality microscope connected to the confocal head (no filter cubes are required in the microscope since the optical path separation is inside the confocal head)
- a suitably sensitive detector (camera or photomultiplier) on the output of the confocal head
- Control software and computer for the combined confocal and imaging system

3.2 Configuration of systems for imaging samples labelled with multiple FPs

When two or more FPs are present in the same sample then care needs to be taken to minimise crosstalk. As illustrated in the cases of GFP/RFP (Fig 4) and CFP/YFP (Fig 5), the only way to drastically reduce the crosstalk between the FPs is to ensure that the corresponding single band emission filter is present in front of the detector when the excitation wavelength for a particular FP is selected.

There are two configurations that can achieve this. The most economical is to place a filter wheel in the optical path between the confocal head and the detector so that the software can synchronise the correct emission filter / excitation wavelength combination to the image capture (refer to Section 3.3). The alternative (refer to Section 3.4) is to use two or more detectors and to split the emission bands into two, or more, separate optical paths using secondary dichroic mirrors (the primary dichroic mirror being the one inside the confocal head that separates the excitation from the emission optical path). VisiTech offer dual camera port modules for this purpose. These modules fit on the output of the confocal head and provide the means to install secondary dichroic mirrors and emission filters. Depending on the confocal system, the user may be able to select from manually exchangeable dichroic /emission filter cubes, manually exchangeable individual dichroics and emission filters, or filter wheels in each emission filter position.

Consideration must also be given to the dichroic in the confocal head, it must either be a dual band dichroic, permitting both excitation wavelengths to reach the sample, and maximising the fluorescence bandwidth that reaches the detector, or two single band dichroics that must be quickly interchangeable, for example on a slider or a rotating wheel. The drawback of using separate dichroics is that they must be precisely aligned to each other, because if not, the images from each FP will not register on a pixel to pixel basis. Using a single dual band dichroic ensures that no alignment is necessary.

3.3 Configuration using a single detector with filter wheel

- one or more laser sources in a laser merge module
- an AOTF which controls the laser line selection and intensity, and also provides the high speed shuttering that reduces photo-bleaching and its associated artefacts
- an optical fibre to carry laser light from the laser merge module to the confocal head
- a confocal head and controller
- suitable single or multiband dichroics as described in Section 2.2
- a high quality microscope connected to the confocal head (no filter cubes are required in the microscope since the optical path separation is inside the confocal head)
- an emission filter wheel mounted between the confocal head and the detector
- a suitably sensitive detector (camera or photomultiplier)
- Control software and computer for the combined confocal and imaging system

3.4 Dual detectors with Dual Camera Port

The alternative configuration is to use dual detectors with a dichroic to separate the two emissions bands into two separate optical paths. This permits sequential imaging of different fluorescent proteins without the delays inherent in rotating a filter wheel to a new position. In situations where the fluorescent proteins have sufficient spectral separation, this

configuration also offers simultaneous image capture of the emissions, which is an important consideration for FRET experiments. Channel separation dichroics are normally long pass types with a transition from reflection to transmission around 560nm for use with GFP/RFP and around 515nm for use with CFP/YFP.

3.5 Configuration with multiple detectors

- one or more laser sources in a laser merge module
- an AOTF which controls the laser line selection and intensity, and also provides the high speed shuttering that reduces photo-bleaching and its associated artefacts
- an optical fibre to carry laser light from the laser merge module to the confocal head
- a confocal head and controller
- suitable single or multiband dichroics as described in Section 2.2
- a high quality microscope connected to the confocal head (no filter cubes are required in the microscope since the optical path separation is inside the confocal head)
- a dual camera port module (with exchangeable secondary dichroic mirrors)
- suitable single band emission filters for each camera port
- suitably sensitive dual detectors (cameras or photomultipliers)

3.6 Other solutions

Third party add-on solutions exist where two, or more, channels can be imaged onto individual sub regions of a single camera chip. The emissions are split into two or more channels by one, or more, dichroic mirrors placed in the emission path and each channel is provided with its own emission filter. The compromise made in these solutions is the reduction in the field of view of the image.

4. Conclusion

The importance of fluorescent proteins cannot be underestimated; however, imaging of samples containing more than one fluorescent protein needs careful consideration to prevent errors arising from crosstalk between the FP emissions.

Samples containing just a single fluorescent protein may be imaged using a single detector imaging system fitted with exchangeable single band filter sets for optimum performance or a multi-band filter set for convenience, since crosstalk is not an issue.

For samples containing multiple labels, users must decide if the delays inherent in filter wheels moving from one filter to another are compatible with the dynamics of the phenomena that they are studying. If the answer is yes, then the use of a single detector system with an emission filter wheel is an economical solution. If the answer is no, then some form of multiple emission channel imaging system is required. Multiple channel systems can be of two types, those that use a detector per channel (which maintains both resolution and field size when compared with a single channel system) and those that partition the area of a detector into sub regions, one per channel (these restrict the image field size).