TECHNICAL NOTE 10

TECHNICAL

Fluorescence bleed-through: how to reduce or avoid it.

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Terms used:

- Bleed-through (Bleed-thru in USA) – refers to the overlap of emission spectra between two or more fluorescent markers (henceforth called Fluors).
- SOURCE image – the image from which the bleed-through is generated.
- DESTINATION image – the image from which bleed-through needs to be subtracted.
- FRET – Fluorescent Resonance Energy Transfer – whereby excitation of one fluor causes excitation of a second fluor via the transfer of it's emission energy through molecular bonds. This occurs when the two molecules are in very close proximity.
- AOTF – Acousto-Optic Tuneable Filter – this is a device incorporated into many Bio-Rad confocal systems. It enables continuous and independent control of the intensity of each laser line.
- Extinction Coefficient – this is a measure of how well a fluor absorbs light at a given wavelength.
- Quantum Yield – this is an index of the intrinsic brightness of a fluor.

What is bleed-through?

Bleed-through can occur when samples are labelled with more than one fluor and/or when there is significant autofluorescence. It is caused by the fluorescence emission from one fluor overlapping too much with that of another. The result is that the source of the fluorescence (unless specific for a particular structure) cannot be unambiguously determined. Worse, in a merged red/green image, bleed-through can look exactly like co-localisation (especially when one signal is much stronger than another).

Whilst sequential imaging and high cut-on/off band pass emission filters can be used to minimise bleed-through, it is not always a complete solution. This Technical Note makes some suggestions about how to avoid and correct for bleed-through both during image acquisition and post-acquisition using our *LaserPix* processing software.

How do we determine the difference between bleed-through, FRET, co-localisation and non-specific background staining?

The typical example used in this Technical Note is imaging FITC/TRITC with an Argon laser and a Green Helium/Neon laser. This example has been selected because it presents a particularly difficult bleed-through issue.

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Check for bleed-through with a single-labelled sample

Use single-labelled controls eg. stained either with FITC or TRITC then…

1. Excite the FITC sample with 488nm only. Adjust the settings to achieve a good bright FITC image.
2. Then, using the same laser power, filters and PMT settings in the TRITC channel as used for a double-labelled sample, collect any image which appears in the TRITC channel (PMT2 in *Fig 1*). Since there is no TRITC in the sample, this cannot be FRET or co-localisation, but is due to a combination of bleed-through from FITC + any background autofluorescence.

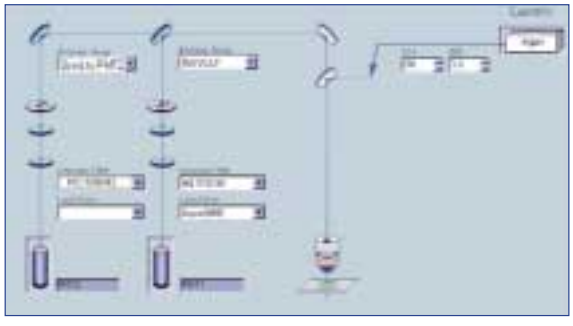

Figure 1 This shows the optical layout and filter configuration for assessing bleed-through from FITC into the TRITC channel with a single FITC- labelled control sample. Excitation is with 488nm only, but the emission paths and filters are set up exactly as they would be for a double-labelled sample.

3. Perform exactly the same procedure with the single-labelled TRITC sample, but this time excite only with the 543 nm line and measure the bleed-through into the FITC channel (PMT1 in *Fig 2*).
4. Now when you image the double-labelled sample, you can use one of the techniques described in this technical note to remove the known amount of bleed-through.
5. Following this correction step, any signal you now see in the TRITC channel has to be entirely due to the presence of TRITC.

Image the double-labelled sample

If you are imaging FITC/TRITC with an Argon laser and a Green Helium/Neon laser with simultaneous 488nm + 543nm excitation, you will get some bleed-through from FITC into the TRITC channel and a little bleed-through from the TRITC channel into the FITC channel – see *Figs 3, 4* and *5*. With simultaneous excitation in the example here, it is not desirable to optimise the emission filter to collect the best TRITC signal because of the consequent increase in the FITC bleed-through. The emission filter shown in *Figs 3* and *5* is an HQ598/40 which means it will collect wavelengths between 578 and 618. TRITC emission is centred around 576, so the filter we have chosen to use for simultaneous double imaging is higher in wavelength than it would be for single labelling.

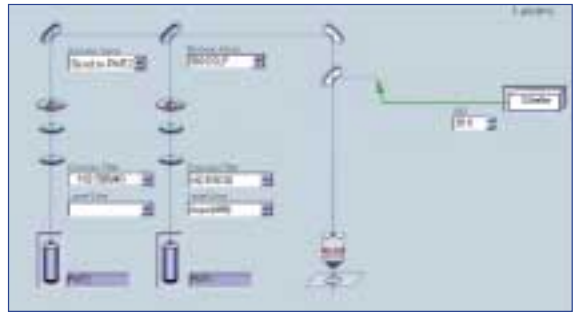

Figure 2 This shows the optical layout and filter configuration to assess bleed-through from TRITC into the FITC channel with a single TRITC-labelled control sample. Excitation is with 543nm only, but the emission paths and filters are set up exactly as they would be for a double-labelled sample.

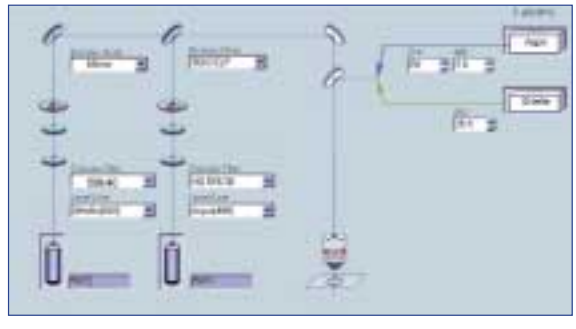

Figure 3 This shows the optical layout and filter configuration which needs to be used for simultaneous imaging of FITC and TRITC with a double-labelled sample. Excitation is with both 488nm and 543nm; the 560LP dichroic will reflect the FITC signal into PMT1 and pass most of the TRITC signal to PMT2. Note the use of the 598/40 emission filter for TRITC. This is not the optimum filter for TRITC, but needs to be used to reduce the amount of bleed-through when imaging simultaneously.

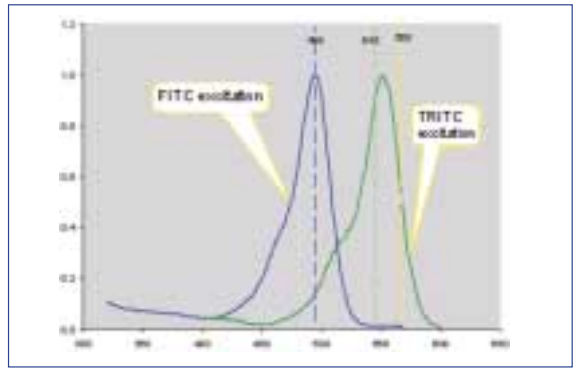

Figure 4 This shows the excitation spectra of FITC and TRITC illustrating the high degree of overlap between them. It also illustrates the high suitability of the 488 line for FITC excitation and of the 543nm line for TRITC excitation.

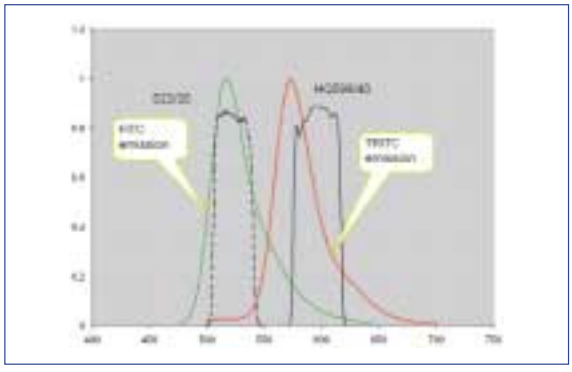

Figure 5 This shows the emission spectra of FITC and TRITC illustrating the high degree of overlap between them. It also illustrates that the 598/40 emission filter used to reduce the bleed-through from FITC into TRITC is not centred around the peak emission of TRITC.

However, if one performs sequential collection ie 488nm (FITC) followed by 543nm (TRITC), the bleed-through will be substantially removed. Moreover, it enables one to select a more efficient emission filter for TRITC – in this case a 580/30.

Therefore, where possible, one should perform sequential (as opposed to simultaneous) excitation of FITC and TRITC, since this reduces bleed-through and at the same time allows the emission filters to be optimised. Sequential imaging is preferred where there is an overlap in either the excitation or emission spectra of the two fluors. To see the advantages of sequential excitation, see *Figs 6, 7, 8*.

The efficiency with which 488nm excites FITC and 543nm excites TRITC are shown in *Figs 9* and *10*. However, the more the excitation spectra of 2 fluors overlap, the more 'non-specific' excitation will occur ie. 488nm can excite TRITC to some extent and 543nm can excite FITC to some extent.

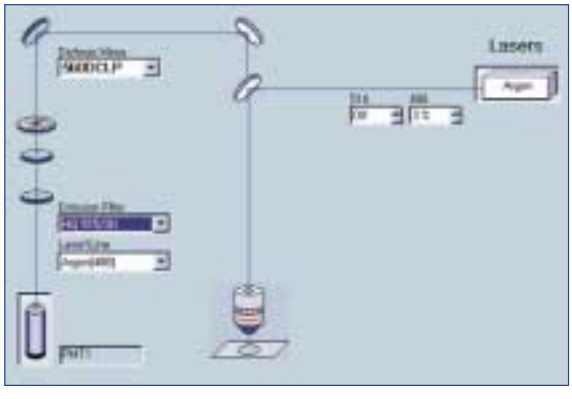

Figure 6 This shows the optical layout which would be used to perform sequential imaging of FITC with a double-labelled sample. Excitation is with 488nm only and the HQ515/30 emission filter is very efficient for collecting FITC signal.

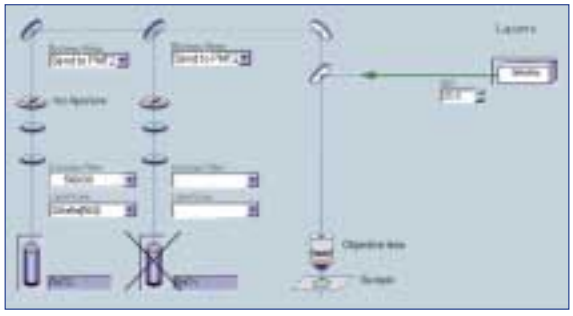

Figure 7 This shows the optical layout which would be used for sequential imaging of TRITC with a double-labelled sample. Excitation is with 543nm only and now the 580/30 emission filter can be used which is much better than having to use the 598/40 shown in *Figs 3* and *5*.

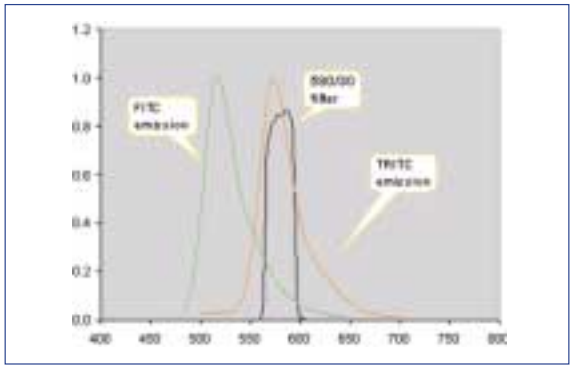

Figure 8 This shows the emission spectra of FITC and TRITC but with the 580/30 instead of the 598/40 emission filter for TRITC. This is much more efficient – compare with *Fig 5*.

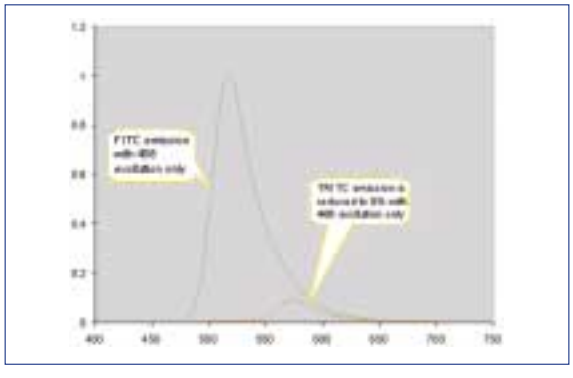

Figure 9 This figure illustrates one advantage of sequential (as opposed to simultaneous) imaging of FITC and TRITC. With 488nm excitation only, FITC is excited very efficiently, but TRITC is only excited at 9% of it's peak value thus reducing the chances of getting TRITC emission in the FITC channel.

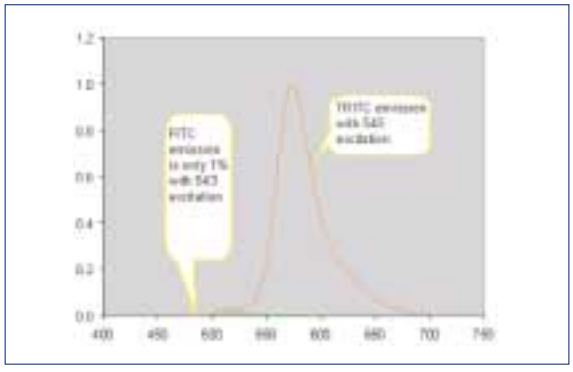

Figure 10 This figure illustrates another advantage of sequential (as opposed to simultaneous) imaging of FITC and TRITC. With 543nm excitation, TRITC is excited very efficiently, but FITC is only excited at 1% of it's peak value thus reducing the chances of getting FITC emission in the TRITC channel.

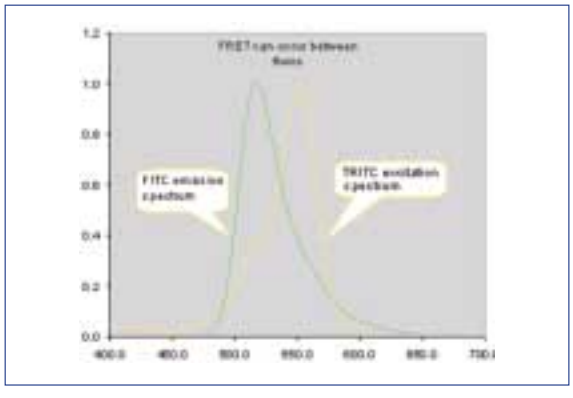

Figure 11 This shows the emission spectrum of FITC and how this has a very high degree of overlap with the excitation spectrum of TRITC. Excitation of FITC would normally lead to FITC emission as fluorescence, but when another fluor such as TRITC is in very close proximity, FRET may occur instead leading to TRITC emission.

Determine if there is any FRET

If the emission spectrum of one fluor significantly overlaps with the excitation spectrum of a second fluor, you may also see FRET (Fluorescent Resonance Energy Transfer) where the two fluors are co-localised. You can tell the difference between bleed-through and FRET by performing control measurements on single-labelled samples.

You can determine if there is any FRET occurring by performing the bleed-though correction and then exciting the double-labelled sample with 488nm only. Any signal now seen in the TRITC channel may well be due to FRET where FITC is the donor and TRITC the acceptor (see *Fig 11*). This will only occur where FITC and TRITC are in extremely close proximity or co-localised. Where FRET occurs, the signal will appear red. Where there is co-localisation, but no FRET, the pixels will appear yellow or orange (see *note*).

NB. *Since the Look up tables used on confocal systems vary, the true colour of the pixels cannot be stipulated here. The statement to which this footnote refers assumes that a green LUT is used for the FITC signal and a red LUT for the TRITC signal.*

Check for Autofluorescence

You can test for autofluorescence by exciting a completely unstained sample using the same instrument parameters.

How do we remove bleed-through?

Using the digital Mixers to remove bleed-through during live image acquisition

Using the LaserSharp software interface, the digital mixers can be set to control the percentage output from each detector or Photomultiplier tube. By default, each digital mixer either displays 100% from PMT1, 2 or 3. See *Fig 12*.

However, the digital mixer can be used to dynamically subtract a proportion/fraction of the bleed-through SOURCE image from the DESTINATION image. See *Fig 13*.

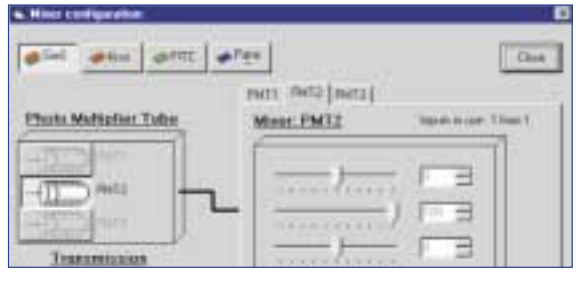

Figure 12 Shows a typical setting for a digital mixer in which the mixer will display 100% of the signal from one PMT and 0% from the others. This is a default setting intended for use with samples which do not display bleed-through.

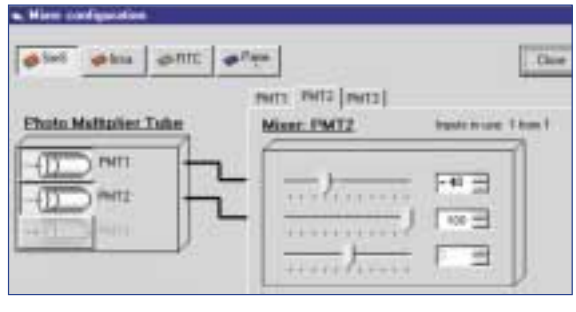

Figure 13 Shows how a mixer can be adjusted to show 100% from one PMT, but a negative percentage from a second PMT, thus compensating for any bleed-through. Mixer adjustments can be made during live imaging of the sample.

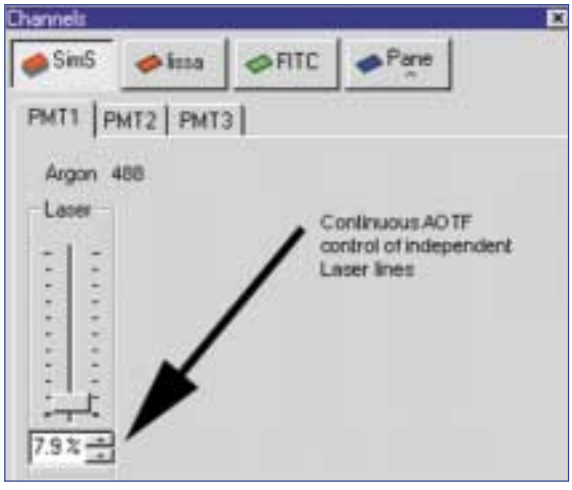

Figure 14 Shows how the AOTF continuous control allows the user to correctly balance the different laser lines so as to achieve the optimum excitation intensities without encouraging the bleed-through which could occur when one fluor is much brighter than the other.

Using the AOTF laser control to reduce bleed-through

The AOTF (Acousto-Optic Tuneable Filter) device enables the user to select the most suitable laser intensity independently for each laser line. This means that if one fluor is much brighter than the other and therefore much more likely to cause bleed-through, the user can reduce the excitation of that fluor, thus balancing the output signal from the two fluors. For each laser line the percentage is selected from a continuous scale from 0 to 100% see *Fig 14.*

Example images of how AOTF control can help remove bleed-through are shown in *Figs 15* and *16*.

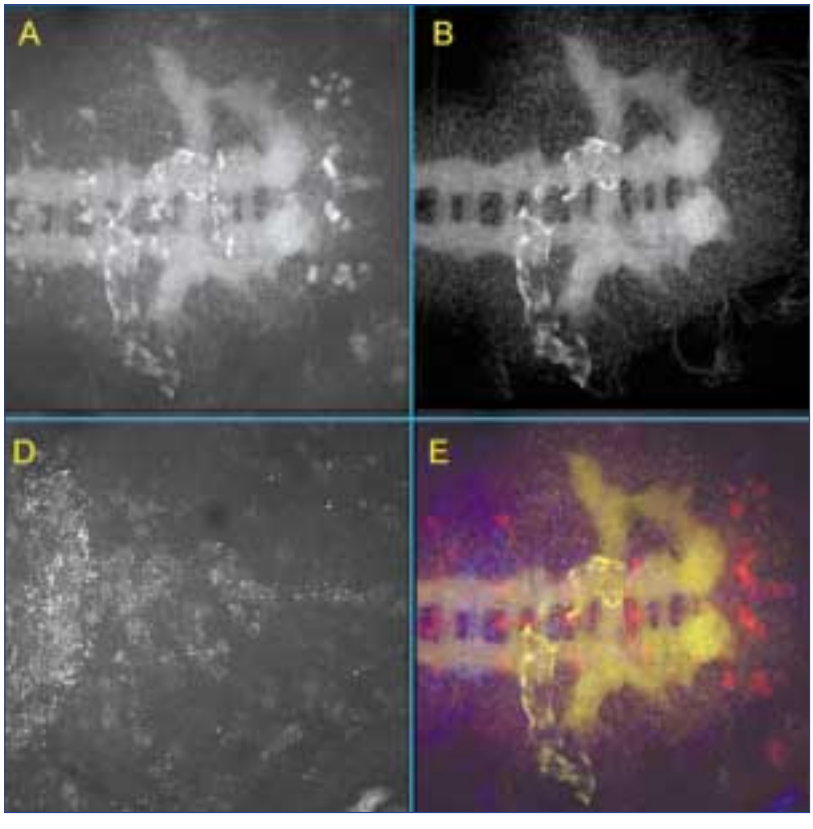

Figure 15 This image shows a triple-labelled Drosophila embryo **Pane A** = red image, **Pane B** = Green image , **Pane C** = Blue image and **Pane D** = merged image. Bleed-through is occurring from the green fluor into the red fluor resulting in the fluorescence appearing yellow in the merged image.

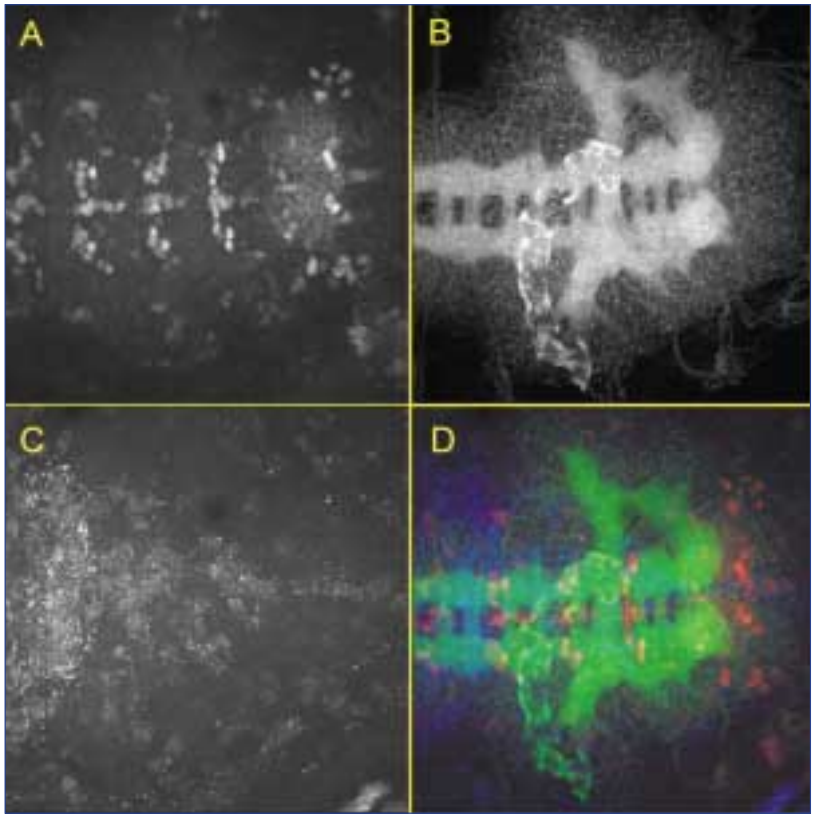

Figure 16 This image shows exactly the same sample as in *Fig 15*, but the AOTF has been used to balance the excitation intensities of the different laser lines. By reducing the intensity of the 488nm excitation with the AOTF, the bleed-through from FITC into TRITC completely disappears so that Pane D now displays the three distinct colours.

Using *LaserPix* to remove bleed-through (post-acquisition)

Where bleed-through occurs, we can call the channel from which the bleed-through is generated, the SOURCE and the channel in which the bleed-through is manifested, the DESTINATION. Provided that the strength of the bleed-through signal from SOURCE image is not greater than the desired signal in the DESTINATION image, it is possible to remove a percentage of the SOURCE image from the DESTINATION image and achieve complete bleed-through correction.

NB. *Bleed-through correction should always be performed prior to co-localisation analysis.*

1. Open the images for correction (either as multi-channel (merged) single section images or as separate single channel zseries files) onto the *LaserPix* application window. See *Fig 17* below.
2. Click on the Bleed-through correction icon. 
3. For merged, multi-channel files, AUTOEXTRACT the channels into separate windows in *LaserPix* (see *Fig 18*).
4. Assign the SOURCE and DESTINATION image windows.
5. Using the Preview button and the % slider, you will see a constant update of your DESTINATION image on the screen. The signal on your DESTINATION image should not reduce in brightness except for where bleed-through occurs.
6. When you assess that all bleed-through is removed, click STOP.
7. When you are sure it is correct, click the CORRECT button to apply the changes to your image data.

Figure 17 Bio-Rad PIC file open dialogue box from *LaserPix*.

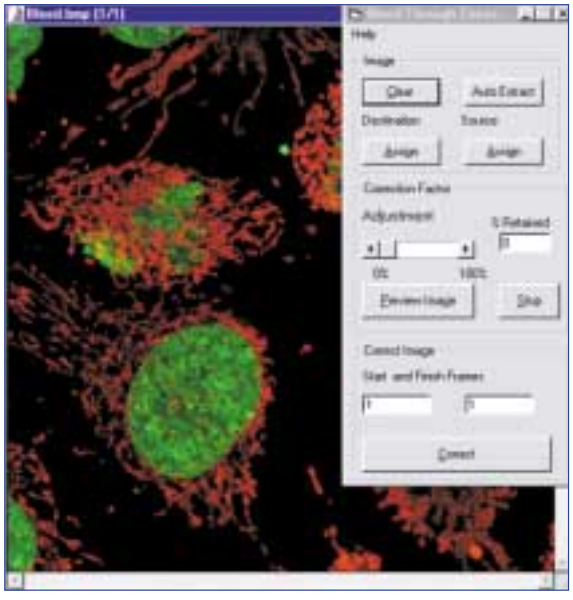

Figure 18 Bleed –through dialogue box and red green merged single section image from *LaserPix*.

8. When you re-merge your corrected DESTINATION image with your original SOURCE image, any co-incident pixels will be due to co-localisation (provided that you have adhered to the protocols outlined in this technical note).

Summary: How to avoid bleed-through

1. Select fluors which can be separately excited with two different laser lines (eg. if you need to use FITC, do not use a second fluor which will also be excited by the 488nm line). A good combination would be Alexa 488 and Cy5™ (see *Fig 19*).

2. Use laser lines which each excite only one fluorescent label. An example is shown in *Fig 19* where Alexa™ 488 is excited only with the 488nm Argon ion laser line and Cy5 by the Kr/Ar laser 647nm line. This combination would be ideal if you need to perform co-localisation studies. If you have a non-ideal choice then you must assess bleed-through using single-labelled controls.

3. Select (where possible) fluors whose emission spectra do not overlap – eg. Alexa 488 and Cy5™ (see *Fig 20*).

4. Ensure that when you do multi-labelling, the fluorescence signals are of approximately the same brightness. The reason for this is if your bleed-through is brighter than the true targeted signal, you will not be able to perform bleed-through correction or subsequent co-localisation analysis. You can use ND filter or AOTF to control and balance laser intensities. This is often enough to remove the bleed-through problem.

5. With reference to fluor brightness, it is worth looking up the Extinction Coefficient (how well the fluor absorbs light) and the Quantum Yield (how brightly the fluor fluoresces). For instance, FITC has a higher Quantum Yield than TRITC, but has a lower Extinction Coefficient. Overall, these two fluors should be about the same brightness when excited at their peak – see 'Suggested further reading' at the end of this Technical Note.

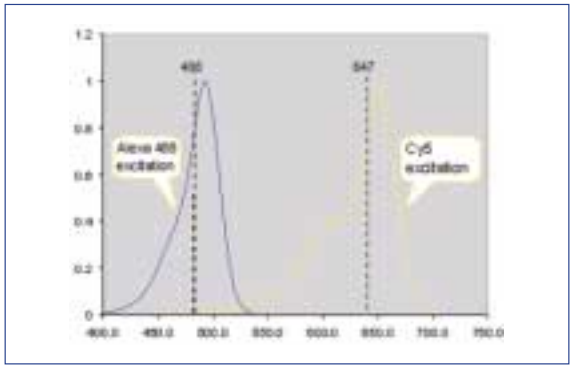

Figure 19 Excitation spectra of Alexa 488 and Cy5™ illustrating how they can be excited entirely separately with the 488 and 647 laser lines during simultaneous acquisition.

6. Use selected band-pass filters which optimise the combination of collection of specific fluorescent signal with emission separation. There is likely to be some compromise between emission separation, specificity (ie. how much bleed-through) and sensitivity (ie. how much signal).

7. Use sequential laser excitation to minimise non-specific fluor excitation.

8. Ensure that signal is brighter than autofluorescence in all channels, since you may need to threshold the autofluorescence out.

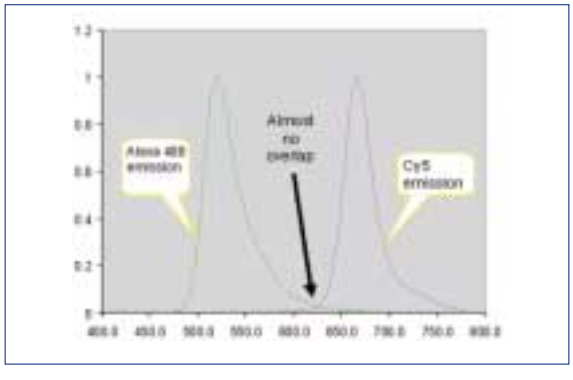

Figure 20 Emission spectra of Alexa 488 and Cy5™ illustrating how their emissions can be entirely separated with emission filters during simultaneous acquisition.

Suggested further reading

Handbook of Biological Confocal Microscopy. (Second Edition) Ed James B Pawley Plenum Press.
ISBN 0-306-44826-2 Chapter 16. 1995.

Cell Biological Applications of Confocal Microscopy (Methods in Cell Biology vol 38) ed Brian Matsumoto. Academic Press.
ISBN 0-12-480430-6 Chapter 4. 1993.

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