Confocal Application Notes
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Removing Crosstalk Online

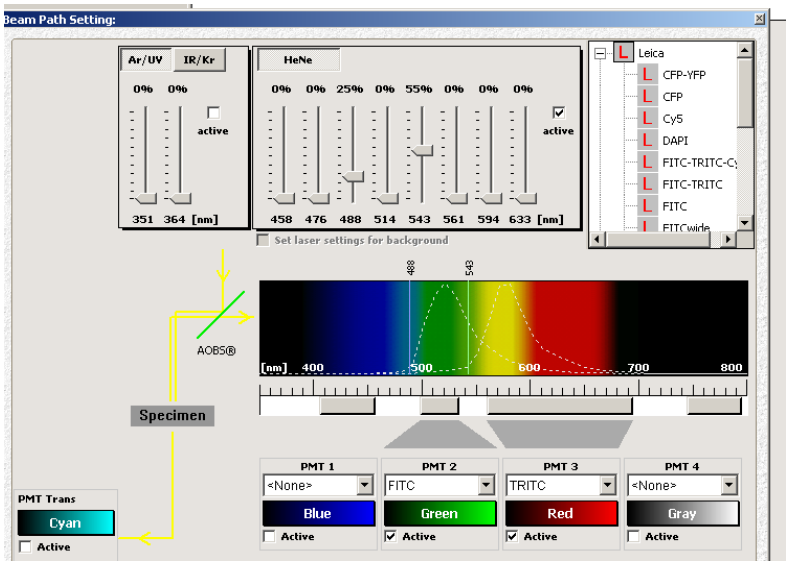
One of the most frequently asked questions from our confocal users is how to correct for crosstalk during simultaneous image acquisition. A precondition to correct for crosstalk is a confocal microscope equipped with an AOTF (Acousto Optical Tunable Filter) and a spectral detection system (TCS SP or TCS SP2).

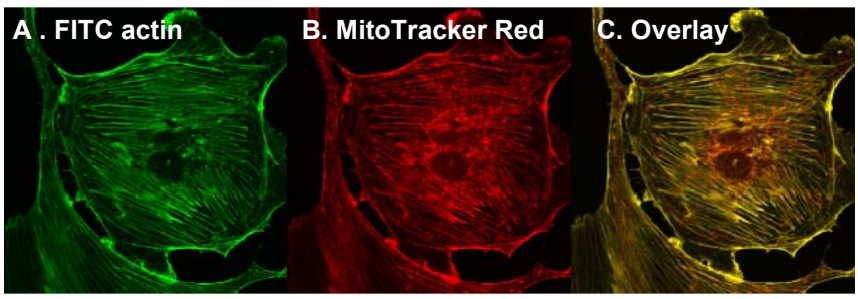
The AOTF is necessary to attenuate every individual laser line separately from 0%-100% and the spectral detection system enables you to create your own detection filter settings (detection slit width) optimized for crosstalk removal.

This application note will demonstrate how to reduce or eliminate crosstalk using the two fluorophores FITC and MitoTracker Red. This protocol can be used to help eliminate crosstalk between virtually any two or three fluorophores. With the Leica SP or SP2 systems these settings can be saved for future use to optimally separate the two fluorophores.

PROTOCOL:

1. To verify whether you do have crosstalk or not in a double or triple labeled specimen e.g. FITC/TRITC you should begin with the simultaneous detection of both labels.





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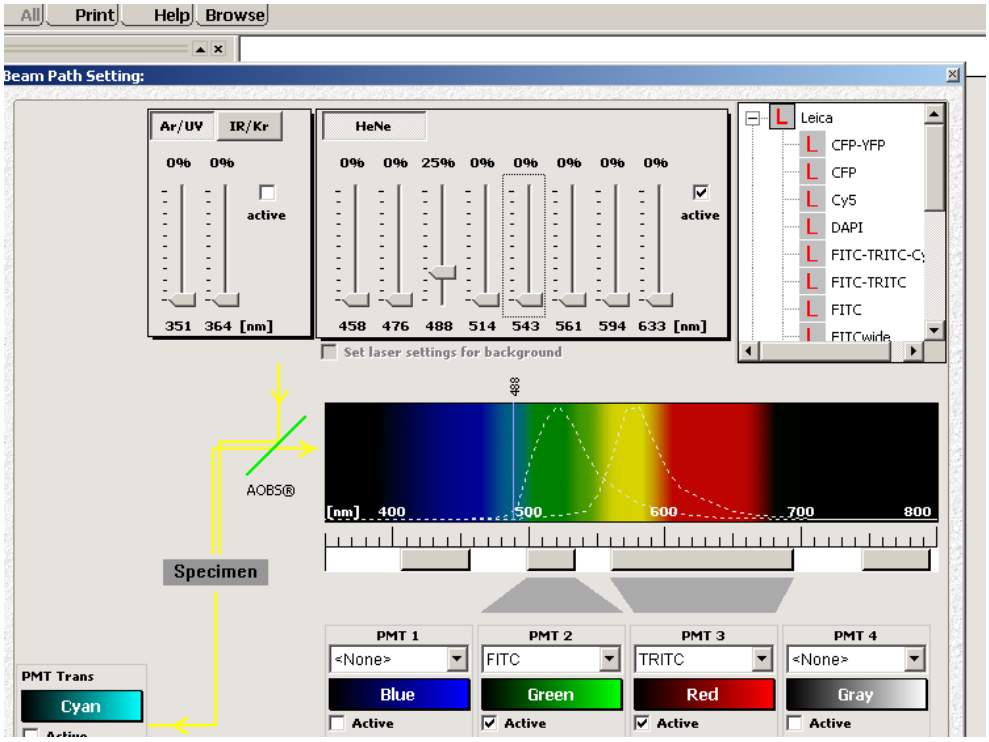
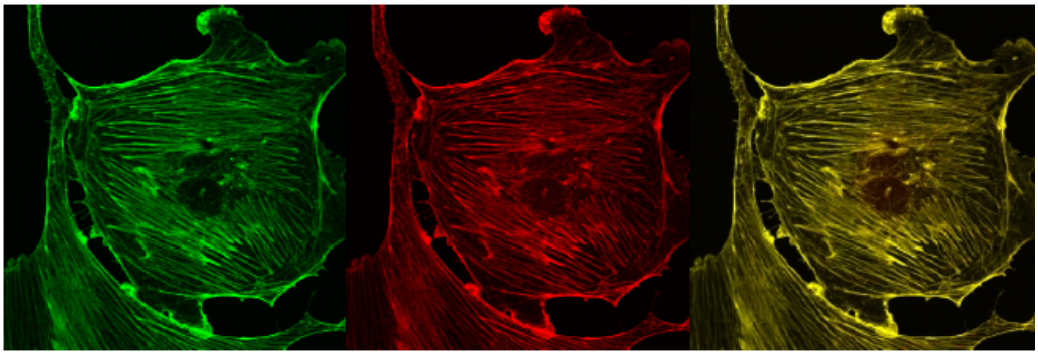
2. Now reduce the excitation light source of the MitoTracker Red label (red channel) down to 0%. Any structure that you still see in the red channel does not arise from the MitoTracker Red label but is the red fraction of the fluorescein emission and can be categorized as crosstalk.





(Crosstalk from FITC into the MitoTracker Red channel)

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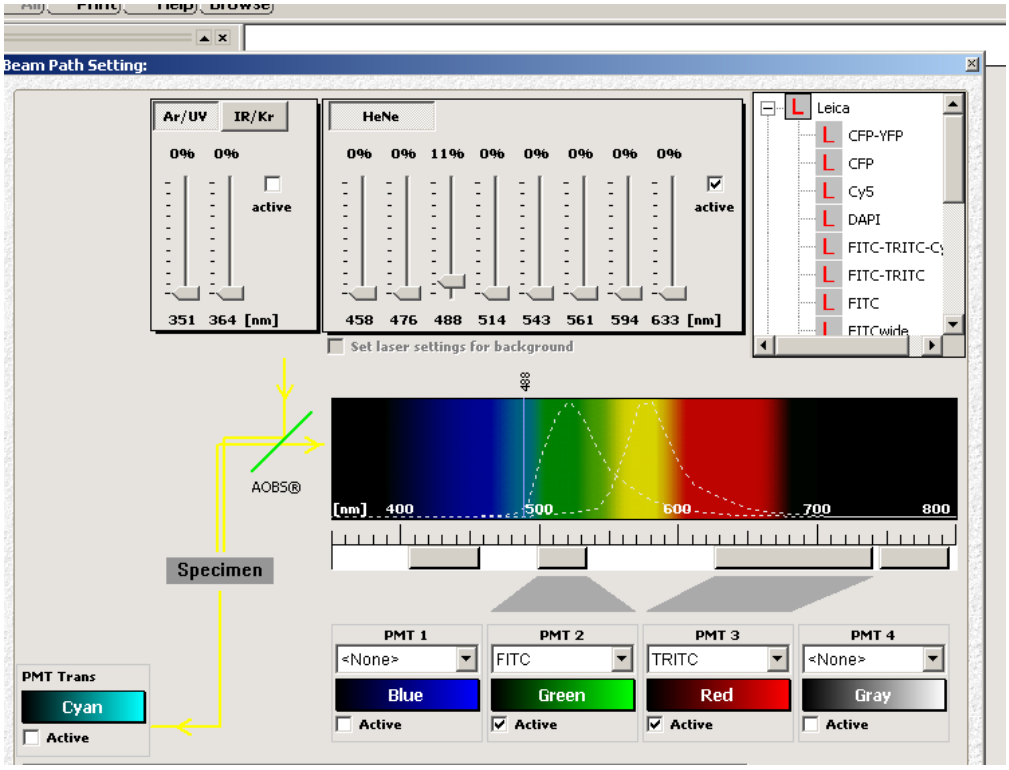
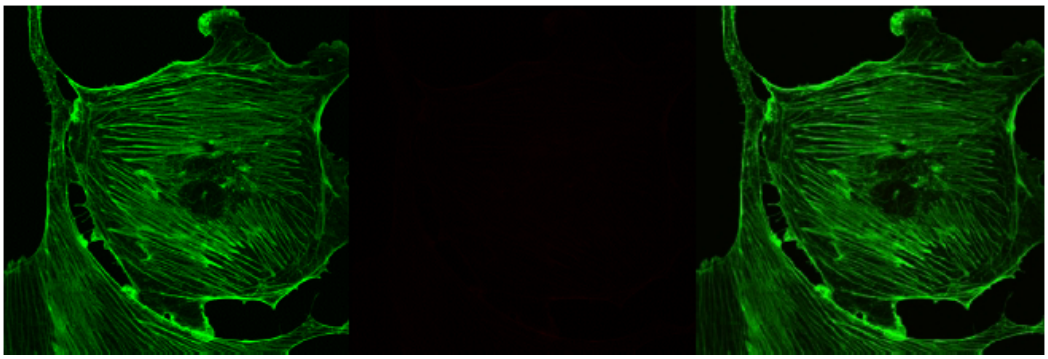
3. To eliminate these effects (i.e. the crosstalk) you must reduce the intensity of excitation light for FITC (488 nm) **and** move the detection slits of the MitoTracker Red detector (PMT3) away from the emission tail of FITC until the red signal disappears. Minor adjustments to the gain/offset of FITC may need to be made.





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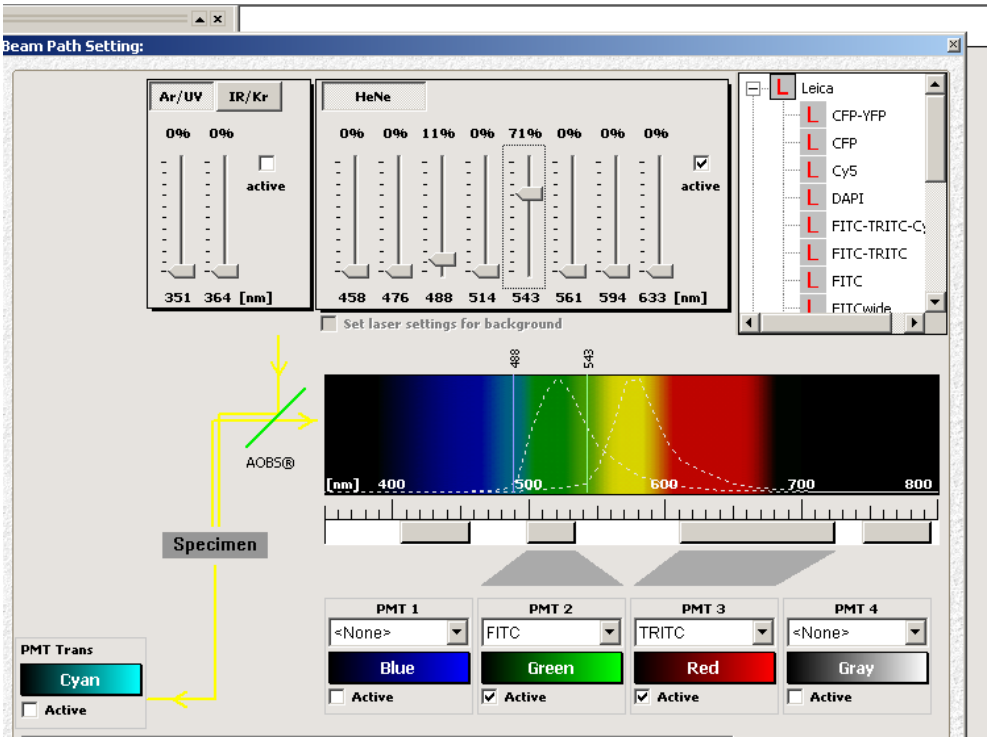
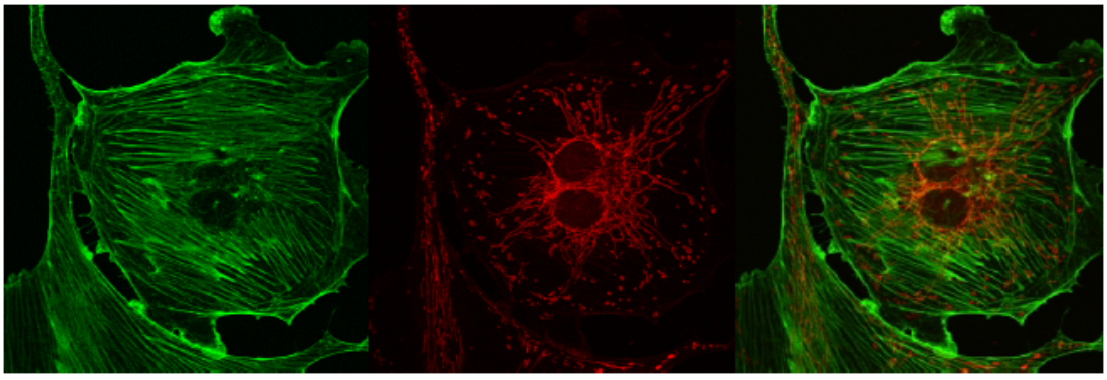
4. Once you have found a satisfactory setting of the 488 nm line i.e. good or acceptable signal-to-noise ratio for the FITC signal and little or no signal remaining in the red you can now again excite MitoTracker Red with the 543 nm line. Adjust the intensity of the 543 nm line until you have a good signal-to-noise ratio.





5. These settings can now be saved for future use when using these fluorophores.

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