TECHNICAL NOTE D04

TECHNICAL

The Use of an AOTF to Achieve High Quality Simultaneous Multiple Label Imaging

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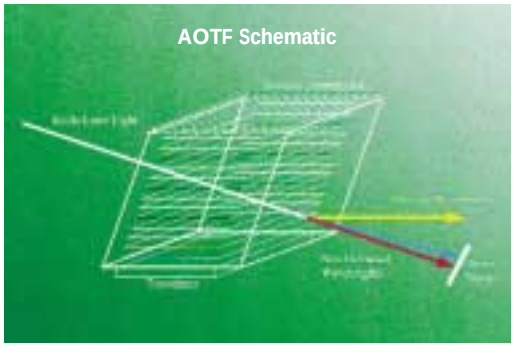
Introduction

An AOTF is now offered as an option with a number of products in Bio-Rad's range of confocal imaging systems. This technical note explains how the device works and its application in confocal fluorescence microscopy.

AOTF – Technical Description

An **Acousto-Optic Tuneable Filter (AOTF)** is an electro-optical device which can be used as an electronically tuneable excitation filter to simultaneously control the intensities of several laser lines from one or more lasers.

It consists of a Tellurium dioxide (TeO_2) crystal to which a transducer is bonded. This transducer emits acoustic (vibrational) waves in response to the application of an oscillating radio frequency (RF) electrical signal. As these acoustic waves pass through the TeO_2, they cause the crystal lattice to be alternately compressed and relaxed. The resultant refractive index variations act like a transmission diffraction grating or Bragg diffracter. Unlike a classical diffraction grating the AOTF only diffracts one specific wavelength of light, so that it acts more like a filter than a diffraction grating. This is a result of the fact that the diffraction takes place over an extended volume, not just at a surface or plane, and that the diffraction pattern is moving in real time.



The wavelength (e) of light selected is a function of the frequency of the RF applied to the crystal as determined by the phase matching condition described in the equation following:

$$e = A n_a i_a / f_a$$

where

n_a is the birefringence of the TeO2 crystal
i_a is the velocity of the acoustic wave
f_a is the frequency of the acoustic wave
A is a complex parameter depending on the design of the AOTF

The amount of light transmitted is proportional to the power of the RF.

Thus, by varying the frequency and power of the RF, the wavelength and intensity of the separated or filtered laser light can be varied.

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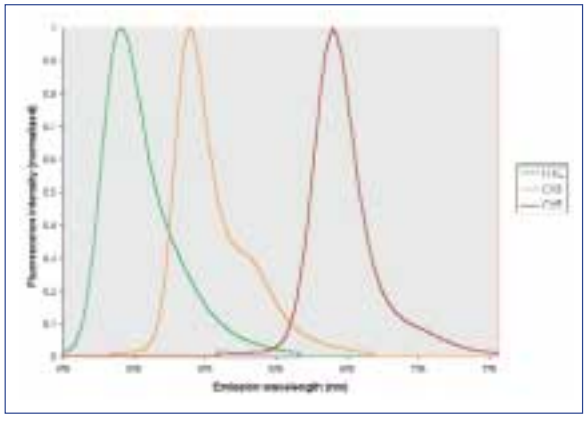
As indicated in the AOTF schematic, the diffracted light intensity is directed into two beams, termed the zero order (non defracted) and first order (selected) beam. To use the AOTF as a tunable filter, a beam stop is used to block the non diffracted light and the selected wavelength(s) is directed into the excitation fibre. The angle between the beams is a function of device design, but is typically a few degrees. The bandwidth of the selected light depends on the device and the wavelength of operation, and can be as narrow as 1nm FWHM (in laser scanning microscopy this offers the possibility of controlling laser lines which are of a very similar wavelength). Transmission efficiencies are typically in excess of 85%. Another useful feature of the AOTF is its inherent property of polarisation preservation which enables high sensitivity simultaneous fluorescence and DIC imaging.

Spectral separation considerations in multi-colour fluorescence imaging



It is well understood that acquisition of multi-colour images which are free from bleedthrough (crosstalk) is best achieved by sequentially, rather than simultaneously, illuminating each of the fluorophores with an appropriate single laser line[1]. This is particularly true when a Krypton Argon laser is used because all three lines are attenuated using a common neutral density filter. The situation is exacerbated by two additional factors:

- FITC is a particularly bright fluorophore and has an extended emission tail as shown in the graph to the left.
- During the life of a Krypton Argon laser it is likely that the power balance between the lines will change.

However, in the following section we demonstrate how the AOTF can be used to significantly reduce the bleedthrough between channels in simulataneous acquisition.

A practical demonstration of the use of an AOTF with a Krypron Argon laser

Equipment

A Bio-Rad MRC-1024ES, using an AOTF controlled 15mW Krypton Argon laser, installed on a Sub-Mount (Keller) Nikon Diaphot 200DV. All imaging was done using a Nikon 60x 1.4 NA PlanApo Oil immersion objective lens.

NB: It should be noted that the MRC-1024ES Real Time Bleedthrough Correction function from the digital mixer control was not applied. All image data acquired was only Kalman averaged for 5 frames. No other image enhancements were used.

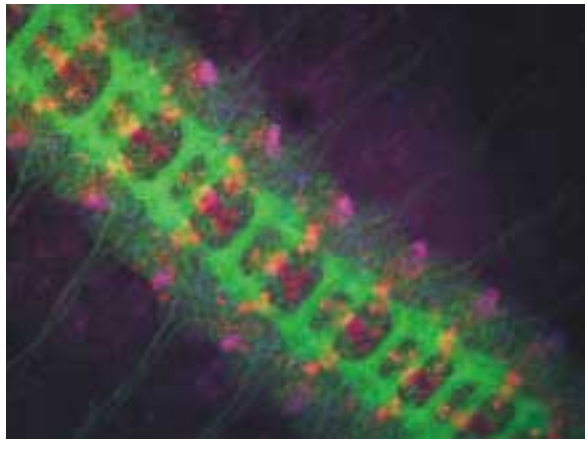
Sample

Triple labelled Drosophila embryos labelled with FITC anti-HRP (green), CY3 anti-ßgal (red) and Cy5 anti-engrailed (blue). An embryo in a late stage of development was chosen because of the clearly visible segmentation.

NB: The same embryo was imaged for both techniques firstly using the "Common ND method" and then using the AOTF.

Demonstration Methodology:

To compare the differences between collecting simultaneous



Multi labelled data using ND (neutral density) control versus AOTF control.

In our sample, the need to ensure sufficiently strong fluorescence signals from the CY3 and CY5 labelled structures was paramount.

Comparison of techniques

Neutral Density (ND)

Due to the relatively weak fluorescence emission from the CY3 and CY5, the determining factor in setting the ND level was the amount of 568nm (yellow) and 647nm (red) light required to satisfactorily excite the fluorophores.

An appropriate neutral density level was chosen to deliver sufficient laser light to the weakest label in our sample, CY5. As a consequence an equally high excitation signal is delivered to the sample for the FITC and CY3. This can be partially compensated for by adjusting the PMT detector gains. However, as a result of this stronger illumination, especially of the FITC, there is an increase in the relative emission intensities, leading to significant bleedthrough of the FITC into the CY3 channel. Ideally we want to minimise this but in order to collect a sufficient signal from the CY3 structure, the PMT detector gain could not be reduced to minimise the FITC bleedthrough.

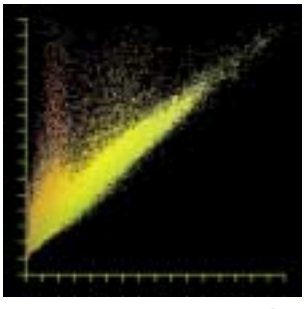


Fig 2

As can be seen in figure 1 top right, a compromise in PMT gain has to be achieved between maintaining a discernible CY3 signal and trying to reduce the FITC bleedthrough. The accompanying Fluorogram (*Fig 2*), generated using Bio-Rad LaserSharp's Co-localisation software, shows very clearly the amount of colocalised signal between the FITC (green) and CY3 (red) detectors, which, in this case is FITC bleedthrough into the CY3 (red) channel. The two axes in the fluorogram represent the two PMT channels; the Y (vertical) axis is the CY3 (red) channel and the X (horizontal) axis the FITC (green) channel. Both axes run from 0 -255 grey level intensities. Hence, the yellow appearance of the merged image in figure 1 and the yellow band at 45° in figure 2, showing similar intensities at the same spatial position in both channels.

AOTF (Acousto-optic Tunable Filter)

As has been described above, the desire is to be able to image the sample with as high a spectral separation of the fluorophores as possible. By using the AOTF to balance the excitation powers required for each of the fluorophores, the relative fluorescence emission intensities can be balanced, resulting in significantly reduced bleedthrough. This was achieved by using the AOTF control software in LaserSharp to ensure a sufficient amount of 568nm (yellow) and 647nm (red) laser light was available to excite the CY3 and CY5 respectively. In addition, because of the relatively high FITC signal, the 488nm (blue) laser light was reduced to a minimum.

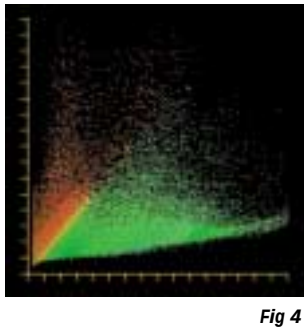


Fig 4

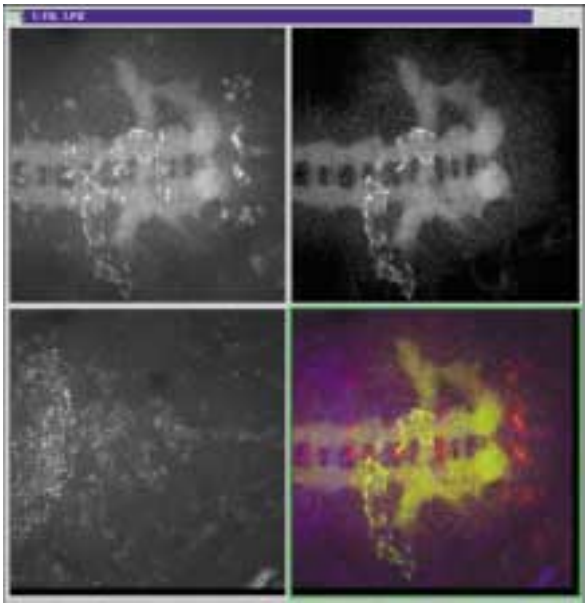


Fig 1 CY3 - Red channel (HQ598/40), FITC - green channel (522/35) and CY5 - blue channel (680/32) and a 24bit merge.

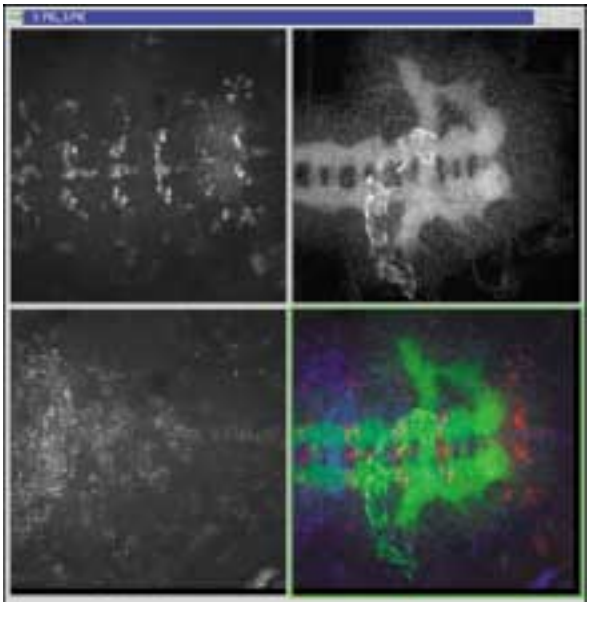


Fig 3 CY3 - Red channel (HQ598/40), FITC - green channel (522/35) and CY5 - blue channel (680/32) and a 24bit merge.

The following sequence was carried out to ensure optimum image collection:

1. The CY5 channel was set by balancing 647nm (red) excitation power and adjusting the PMT detector gain to achieve a good signal to noise ratio (S/N).
2. The same process was used in setting the CY3 channel, i.e. sufficient 568nm (yellow) excitation power and optimised detection (S/N).
3. The 488nm (blue) light was set; this time the critical factor in setting the excitation power was the desire to minimise the bleedthrough into the CY3 channel, while providing sufficient excitation for the FITC. With the FITC concentration being relatively strong and the PMT detectors highly sensitive, the excitation power could be attenuated to reduce the relatively high FITC emission signal, thus eliminating the FITC bleedthough almost completely. The FITC (green) PMT detector gain was adjusted accordingly to maintain a good FITC signal.

As can be seen in figure 3, there is virtually no FITC bleedthough into the CY3 (red) channel.

Again, a fluorogram (*fig 4*) is used to show the amount of colocalised signal between the FITC and CY3 channels. It can be seen that in this data set there is virtually no colocalised information, i.e. virtually no bleedthrough. Hence the stronger green appearance in both the merged image of figure 1 and the fluorogram (*fig 4*).

Conclusion

It can be seen that the use of an AOTF, in simultaneous multi-labelled imaging, can significantly reduce fluorescence emission bleedthrough. By balancing the laser wavelenths, to accommodate any variations in signal strengths, high quality images can be obtained. In summary the clear benefits are:

- Highly optimised signal balancing in multiple labelled samples.
- Continuously variable independent attenuation of all laser lines.
- Significant reduction in bleedthrough of fluorescence emission signals.
- LaserSharp software control.
- Improved productivity through simultaneous multi-labelled data collection.

References

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2. *Measurements of co-localization of objects in dual-colour confocal images*: Journal of Microscopy. Vol. **169**, Pt 3, March 1993, pp 375-382 - MANDERS, E.M.M., VERBEEK, F.J. AND ATEN, J.A.

Acknowledgements

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