TECHNICAL NOTE 06

TECHNICAL

The Use of Intensity Windowing to give 2 x 8 Bit Data Acquisition in Confocal Microscopy

Introduction

In the vast majority of confocal fluorescence applications 8 bit data acquisition is more than adequate. In some applications, for example physiology experiments, the ability to cope with signals over an extended dynamic range can, however, be valuable. In these cases the flexibility of Bio-Rad's LaserSharp software and the digital mixers, incorporated in the confocal data acquisition hardware, enable a flexible approach to acquisition of wide dynamic range signals.

Signal Digitisation in Confocal Microscopy

In a typical confocal microscope, light detection is achieved when one or more photons strike the photocathode of the photomultiplier giving rise to photoelectrons. The resulting current is converted to a voltage, amplified and fed to an analog-to-digital converter (ADC), where it is sampled and then converted to a digital number (*see Figure 1*).

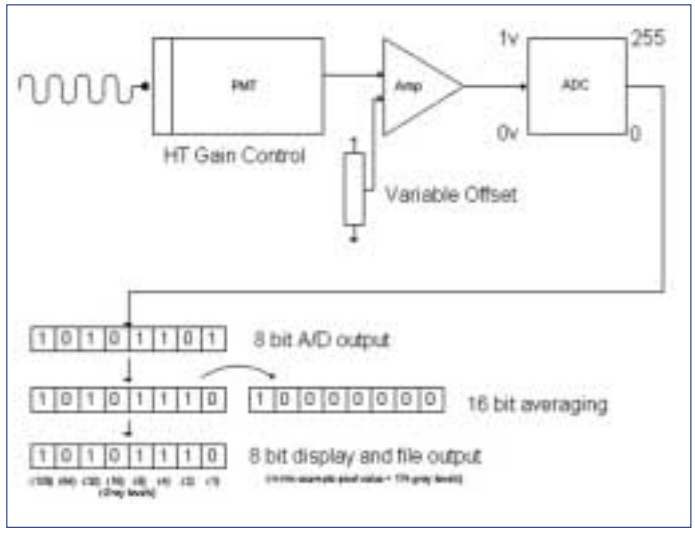

Fig 1

When successive image frames are averaged (for example, using the Kalman filter) the calculations are made to a resolution of 16 bits, to eliminate the possibility of rounding errors occurring. For display and file saving purposes, only the most significant 8 bits (the meaningful data) are used.

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Imaging Light Levels

It is widely accepted (reference: *The Pixelated Image, R. H. Webb & C. K. Dorey - Chapter 4, Handbook of Biological Confocal Microscopy second edition – Ed. J Pawley*) that in confocal fluorescence imaging the range of light levels likely to be encountered will result in a photon flux of between 10 and 1,000 photons per pixel at a pixel dwell time of 2 microseconds. This range extends from extremely low signal levels in live cell applications through to the brightest possible fluorescence. For the purposes of the following discussion a flux of 200 photons per pixel, relating to bright immunofluorescence, is used.

Poisson statistics tells us that the signal to noise ratio is equal to the square root of the number of detected photons. So, for a signal of 200 photons the noise will be 14 photons or 7% of the total signal.

An 8 bit ADC has a digitisation resolution of 1/255 or <0.4% so it is immediately obvious that it has much more than sufficient resolution to faithfully sample signals from typical fluorescence labelled specimens. Indeed, it is worth noting that one would need to detect more than 65,000 photons per pixel to fully utilise the sampling resolution of an 8 bit ADC.

Extending the Dynamic Range

As noted above in most fluorescence imaging applications, 8 bit data acquisition is more than adequate. However, in some specialised applications particularly kinetic experiments an extended dynamic range is desirable. It may be necessary to measure signals in two distinct intensity ranges. Firstly, low level signals need to be monitored when the cells or organism are exhibiting low ion (i.e. calcium) concentrations – usually known as the 'resting state'. Secondly, following stimulation of the cells a dramatic increase in, and possibly fluctuations of, this signal are seen. *Figure 2* shows a typical "Intensity versus Time" graph of a physiology experiment.

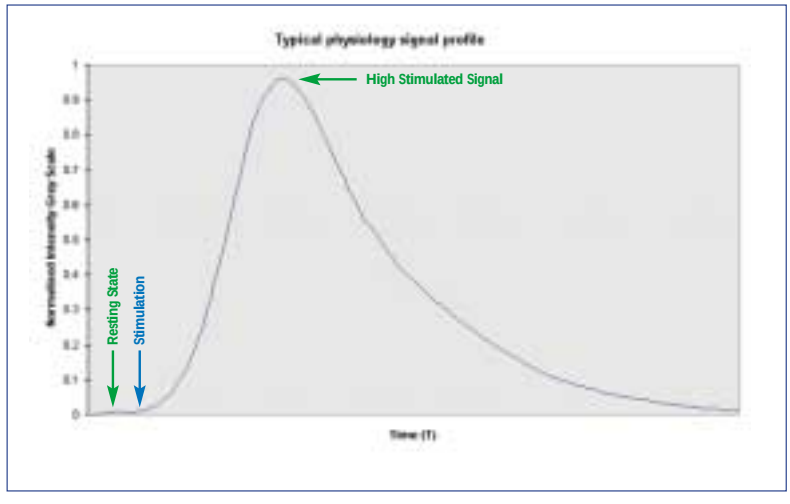
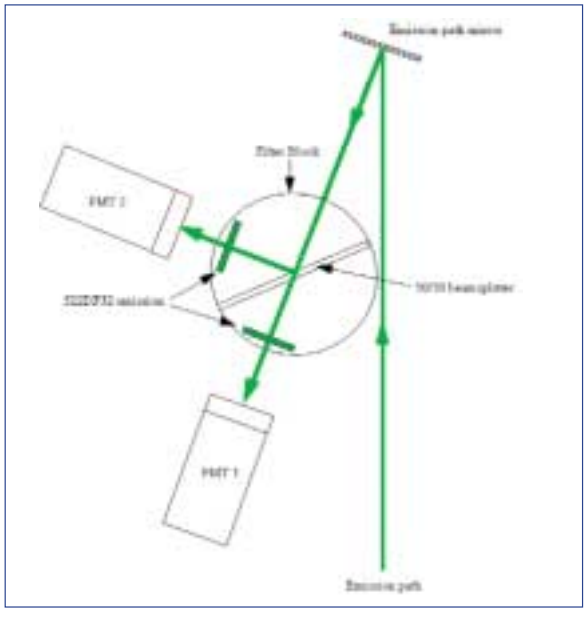


Fig 2



In order to monitor these two very different signals effectively, the unique software and hardware configuration of the Bio-Rad confocal systems, allows the monitoring of the low intensity resting state signal and the high stimulated signal to be assigned to two separate detector channels. Each of these channels can be set optimally to collect their respective signals and the offset between channels can be set up by the user to suit their application. *Figure 3* is a diagram of how the confocal emission path would be utilised.

Fig 3

The full range of the fluorescent signal can then be "dissected", also known as intensity windowing into the 2 PMT's giving 2 x 8 bits of data. This can be set up in a number of ways to suit the specific needs of the experiment (see *Figure 4*).

For example in *Figure 4a*, the PMT offsets are adjusted such that each PMT covers 50% of the signal range. Thus a continuous scale from 0 to maximum signal is set up.

In *Figure 4b,* the offsets and gain are set up to introduce a 'gap' in the scale where the signal is of no interest. In this case therefore the observed dynamic range can be further extended.

In *Figure 4c*, the additional flexibility to operate each PMT at a different gain and offset is illustrated. This further enhances the ability to discriminate signal levels in either the low signal (resting state) or high signal (stimulated) part of the experiment, this effectively allows the user to "intensity zoom" to the area of interest.

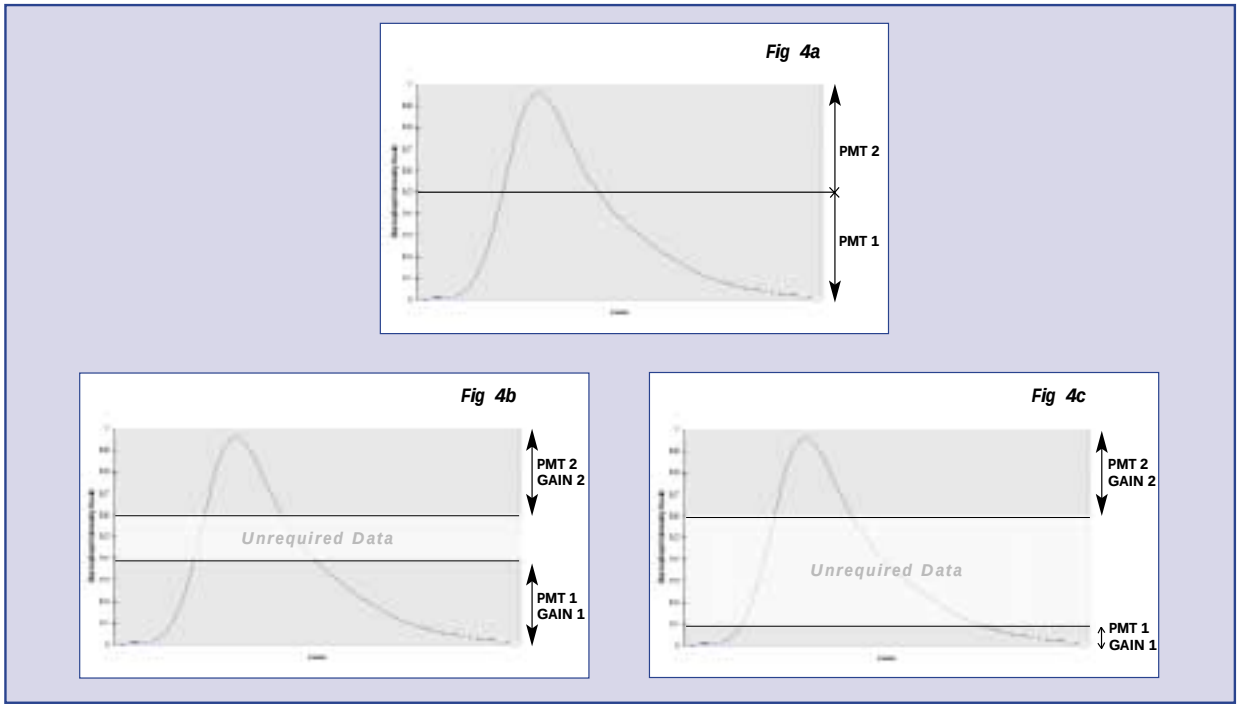


Fig 4

Summary

- In some experiments, notably physiology studies an extended dynamic range is useful.
- The uniquely flexible emission path of Bio-Rad confocal systems enables a number of data acquisition regimes to be configured.
- At each of the extreme signal states: resting and stimulated, the signal range can be assigned a full 8 bit (256 grey levels) pixel resolution. This gives the equivalent of at least 2 x 8 bit data which further more can be displayed, measured and saved; unlike many 12 bit systems.
- The ability to use Intensity Windowing of 2 x 8 bit data acquisition can be flexibly configured to suit the specific needs of the experiment.

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