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Selective Imaging of Multiple Probes Using Fluorescence Lifetime Contrast

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The most common technique in Fluorescence microscopy for discriminating between distinct fluorescent species relies on differences in their emission spectra. Thus experiments using multiple labels utilize spectral filters to produce fluorescent images corresponding to the distribution of each probe. However, in many instances the fluorescent probe molecules which need to be employed exhibit overlapping emission bands and cannot be distinguished spectrally. Nevertheless, a discrimination between them can be achieved by the simple stratagem of exploiting the differences in their intrinsic fluorescence lifetime. Moreover, this contrast mechanism can also be used for suppressing the autofluorescence background from the images. The method effectively imposes a lifetime filter on the intensity image.

Several techniques are currently employed for the measurement of fluorescence lifetimes of fluorophores in bulk samples. The techniques may be subdivided into frequency domain (e.g. phase fluorometry) and time domain methods (time-gating, time correlated single photon counting). Both types of technique have been implemented for fluorescence lifetime imaging in wide field as well as confocal microscopes. Here we shall present applications of time-gated detection combined with confocal imaging for the acquisition of selective images in multilabelling experiments.

We have implemented a time-gated detection scheme in a commercially available confocal laser scanning microscope (CLSM). The setup utilizes a low-power CW Ar-ion laser equipped with an electro-optical chopper producing nanosecond pulses with a repetition rate of up to 25 MHz . The fluorescence emission excited by every light pulse is acquired in two consecutive time windows. In the simplest scheme, the first window is opened straight after the excitation pulse while the second is opened immediately after shutting the first window. Both windows have identical widths (several nanoseconds) and the fluorescence lifetime information is obtained on taking the ratio of the accumulated intensities.

Almost all of the fluorescence is detected in the two time-windows after each light pulse. This makes the method very efficient in terms of the number of photons required to determine the fluorescence lifetime. Only 200 detected fluorescence photons are necessary to achieve an accuracy of 10% in the lifetime. In addition the simultaneous acquisition in the two windows makes the method insensitive for photo bleaching. Experience has shown that this time-gated detection technique enables the measurement of the fluorescence lifetime of a pixel in 40 μs.

For a mono-exponential fluorescence decay, the decay time τ can be calculated from the ration of the intensities recorded in the time windows (I_A/I_B):

$$\tau=\Delta t/\ln(I_A/I_B)$$

with Δt the width of the windows. This equation is only valid for windows of equal width and provided the excitation pulse is short compared to the decay time. The two channel time-gated detection affords the determination of only a single decay time. Consequently, it determines an effective lifetime if the fluorophores exhibit a multi-exponential fluorescence intensity decay. This limitation can be overcome by adding more detection windows.

The potential of fluorescence lifetime contrast for selective imaging in multilabelling experiments will be illustrated using Sertoli cells stained with three different probes: Syto 13, $DiOC_6$ and Nile Red. These vital staining probes are commonly used for

staining the nucleus, the cytoplasm and lipid droplets respectively. The probes are cell permeable and spread through the cell by diffusion. The overlap of the emission spectra of the probes makes it difficult to distinguish these probes by their emission spectra. However, since the probes have different fluorescence lifetimes (the lowest being Nile Red, the middle $DiOC_6$ and the highest Syto 13) it is possible to separate them by their lifetime.

The fluorescence intensity image of a layer of Sertoli cells stained with Syto 13, $DiOC_6$ and nile Red shows a wide distribution of fluorescence intensities, which cannot be related to the morphology of the cells in a straightforward way. On the other hand, the fluorescence lifetime image of the same cells exhibits three distinct lifetime components, each of which corresponds to the individual probes. The image can now be subdivided in three different lifetime ranges by imposing lifetime masks on the image (thresholding), with each mask corresponding to one of the probes.

The maximal number of probes which can be distinguished in this way depends on both the lifetime range and lifetime resolution of the instrument. In principle it should be possible to perform multi-labelling experiments with 10 or more different probe molecules and this number can probably be doubled on combining the lifetime contrast method with spectral filtering techniques.

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