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Fluorescence Lifetime imaging in Bilateral Confocal Microscopy by Double Pulse Excitation

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It has been recognised for some time, that the fluorescence lifetime of a molecule not only depends on the internal structure of the molecule itself, but also on its environment. Recently this environmental dependence of the fluorescence lifetime has been introduced as an important contrast parameter in the field of microscopy. An example is for instance the imaging of free cytosolic Ca^{2+} concentrations (Lakowicz and Szmacinski 1993 and the local pH in biological samples (Draxler et al. 1993, Lippitsch et al. 1992].

The use of the fluorescence lifetime as a contrast parameter in microscopy is, among other applications advantageous when the fluorescence lifetime of a probe molecule differs considerably from that of the background radiation or autofluorescence, thus providing an opportunity to suppress the contributions of the latter to the total signal. Also this technique can provide a straightforward alternative to the technique of ratio imaging based on the excitation at two different wavelengths and detection at one fluorescent wavelength or vice versa excitation at one and detection at two, as have been used for the imaging of ion concentrations like Ca^{+} in biological samples.

The principle of the newly developed method of Double Pulse Fluorescence Lifetime Imaging (DPFLIm) is that the fluorescent output from the specimen is collected with excitation by pairs of short pulses having two different timing conditions between the pulses. Each of these pulses is sufficient to drive the fluorescence into saturation or very close to it. If the two pulses of the pair are separated by a time interval which is short with respect to the fluorescence relaxation time then there will be no difference between the fluorescence produced by a single pulse or by the pulse pair. This is because the saturation induced by the first pulse has the effect that the second one cannot induce any further fluorescence. However if the second pulse of the pair is delayed by an interval which is a substantial fraction of the fluorescence lifetime then at the arrival of the second pulse sufficient fluorophore molecules will have decayed to the ground state that additional fluorescence can be induced by the second pulse. This extra amount of fluorescence radiation will be higher for longer delays between the pulses, thus providing a means to determine the fluorescence lifetime from the relative fluorescence in both pulse conditions. These data are collected by separately collecting, on a CCD with the bilateral confocal imaging technique (Brakenhoff and Visscher 1992, 1993) two separate images being illuminated by a stream of pairs of pulses with the indicated different timing conditions. From these images the fluorescence lifetime image can be calculated with an appropriate algorithm.

A theoretical analysis of this new technique for fluorescence lifetime imaging will be presented. Bilateral confocal microscopy and a (cooled) CCD is used for sensitive signal detection over a large dynamic range as required for the calculation of the lifetimes from the image data The sensitivity of the technique is analysed, taking into account: photo degradation, the effect of the laser repetition rate and the effect of non-steady state excitation and the features of the technique are compared to more conventional methods for fluorescence lifetime determination. It is found that under typically experimentally expected conditions, images can be obtained with contrasts corresponding to fluorescence lifetime differences of 10%, when differences in the DPFLIm signal ratio can be resolved within 5%, something which appears feasible (Brakenhoff et al. 1994). One may thus anticipate, that

the DPFLIm technique is as an fluorescence lifetime imaging technique will offer comparative or possible better life time discrimination in comparison with existing techniques, creating new opportunities for using fluorescence lifetime as a contrast parameter in confocal imaging.

Finally we want to emphasize two aspects. First the technique does not require electronic gating of the signal. Rather it relies on an optical gating technique with a time resolution which, depending only on the pulse duration, can be an order of magnitude shorter resulting in the possibillity of imaging with a correspondingly higher time resolution. Second the use of a cooled CCD-camera in the detection - facilitated by the bilateral confocal scanning approach - is essential in this new approach as it provides both the integrating capabilties and the high dynamic range/ signal-to-noise required for the calculation of fluorescent life-time images. As an additional benefit it facilitates fast image collection thanks to parallel speciem excitation and data collection, a practical proposition when operating in the line mode and high excitation powers (Brakenhoff and Visscher 1993).

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