Fluorescence lifetime imaging of serotonin and other intracellular molecules

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Introduction

Fluorescence microscopy of cellular systems using multiphoton near-infrared excitation with sub-picosecond pulses provides several advantages over UV excitation [1]. These include reduced phototoxicity, the ability to scan the focal spot of the focussed beam in three dimensions as in confocal microscopy, and the use of standard microscope optics. In addition the pulsed nature of the excitation immediately enables time-correlated detection of photons and implementation of Fluorescence Lifetime Imaging (FLIM). A particular advantage of FLIM is that it enables quantification of images through correction for dynamic quenching of intensities arising from the Stern-Volmer relation between unquenched and quenched intensities (I_0, I) and lifetimes (τ_0, τ) (equation 1).

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q] \qquad (1)$$

As well as providing excitation of fluorescence, the peak light intensities of focussed laser pulses enables photochemical activation such as uncaging of biomolecules and the generation of reactive species [2]. In the extreme case, dielectric breakdown in water may directly generate species such as hydroxyl radicals [3,4].

Results and Discussion

The neurotransmitter serotonin has been imaged in cells by imaging of UV (340 nm) fluorescence by three photon excitation at 700 – 750 nm using the direct tuneable output of a Ti:sapphire laser [5,6]. We have now developed two-photon excitation using the 630 nm output of a femtosecond OPO laser, and have shown it to be more selective than the 3-photon method (vide infra). In the brain, reuptake of neurotransmitters such as serotonin (5HT) and norepinephrine is inhibited by pharmacological agents such as propranolol (a beta-blocker) and fluoxetine (an antidepressant) [7]. There is therefore considerable interest in imaging neurotransmitter uptake and release in suitable cell systems. In the course of our experiments we have found that propranolol may be excited using the same combination of two-photon excitation at 630 nm and emission at 340 nm as used for serotonin (Figure 1), allowing for intracellular imaging of propranolol. The quantum yield for propranolol fluorescence (0.5) is higher than that of serotonin (0.3). Importantly for FLIM, propranolol has a characteristic fluorescence lifetime of 9.6 ns that is considerably longer than for serotonin (3.8 ns) and cellular autofluorescence (ca 2-3 ns). However serotonin and propranolol have almost identical rates of dynamic self-quenching of fluorescence (2.5×10^9 M^{-1} s^{-1}).

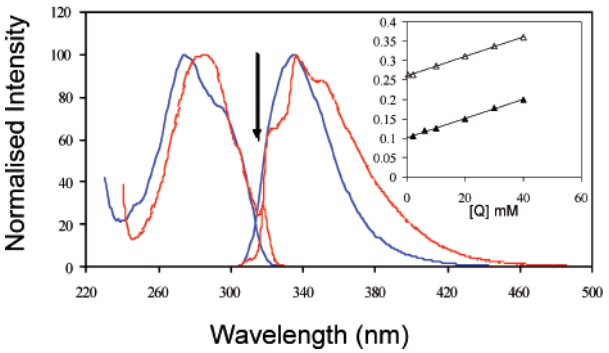

Figure 1. Fluorescence excitation (left) and emission (right) spectra of serotonin (blue) and propranolol (red) in phosphate-buffered saline (pH 7.3). The arrow indicates the 315 nm wavelength equivalent to selective two-photon excitation of 5HT at 630 nm. *Inset*: Stern-Volmer plots of reciprocal lifetime (τ) versus solute concentration (Q) for self-quenching of serotonin (△) and propranolol (▲).

We have now imaged serotonin and propranol using 630 nm two-photon excitation. Figure 2 shows fluorescence intensities from equimolar solutions of tryptophan and 5HT plotted against laser power at both 630 nm and 590 nm.

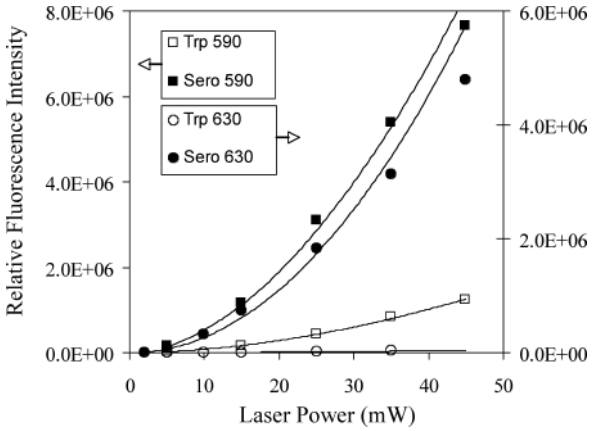

Figure 2. Effect of laser power on fluorescence intensity from equimolar solutions of 5HT and tryptophan excited at either 590 or 630 nm as indicated.

Since cells are rich in proteins containing tryptophan residues, it is evident that the autofluorescence background from this source will be considerably reduced and 5HT imaged more selectively using 630 nm 2-photon excitation. Overall the degree of selectivity using excitation at 630 nm of 5HT over that of tryptophan is improved by a factor of

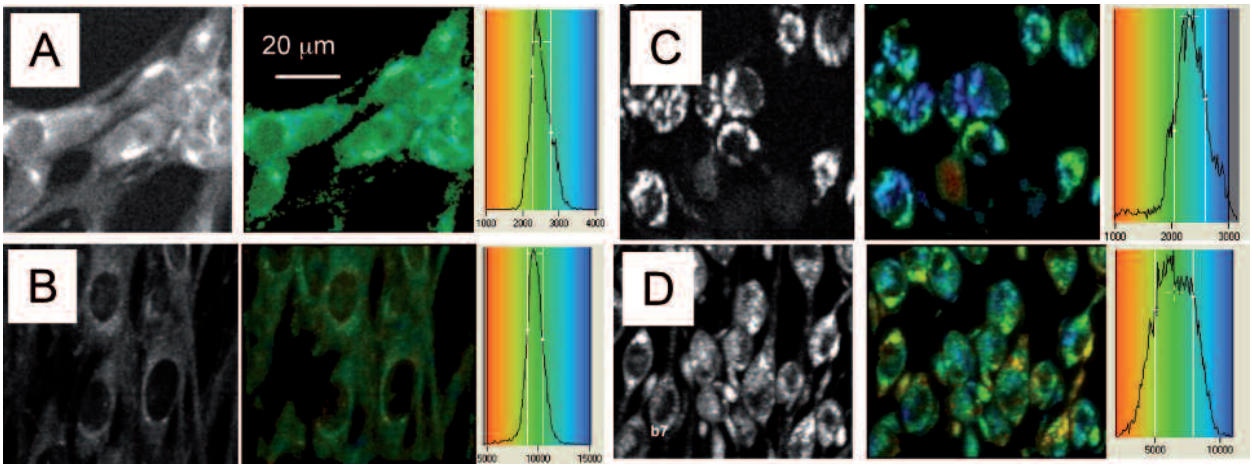

Figure 3. Intracellular molecular imaging of 5HT and propranolol. A and B show results from C6 glial cells; C and D show images from RBL-2H3 mast cells. The cells have been incubated either with serotonin (A and C) or propranolol (B and D). In each set the left hand side fluorescence intensity image is compared with the lifetime image (centre) and intensity-weighted lifetime distribution map (right).

20 compared with 590 nm. Log-log plots of the data in Figure 2 (not shown) have slopes of 2.0 ± 0.2 confirming the 2-photon process, although there is evidence of saturation at the higher laser powers used to obtain the data in Figure 2.

Images of intracellular 5HT and propranolol in rat leukaemic mast (RBL-2H3) cells and C6 glial cells are shown in Figure 3. In each case the molecules are taken up by the cells, resulting in intracellular concentrations between 2 and 3 orders of magnitude that of the solute in the suspending medium. The RBL-2H3 mast cells possess intracellular vesicles or granules for the storage of biogenic amines including 5HT and histamine. The presence of 5HT in such storage vesicles is clearly observed in Figure 3C. The lifetime map shows that the 5HT lifetime is considerably quenched from the solution value of 3.8 ns to between 1.9 to 2.5 ns in these granules. According to the self quenching data, a reduction in lifetime from 3.8 ns in solution to 2.0 ns in solution would reduce the intensity by nearly 50%. Calibration with standard 5HT solutions and correction of intensity data using equation (1) shows that the 5HT concentration within granules ranges up to

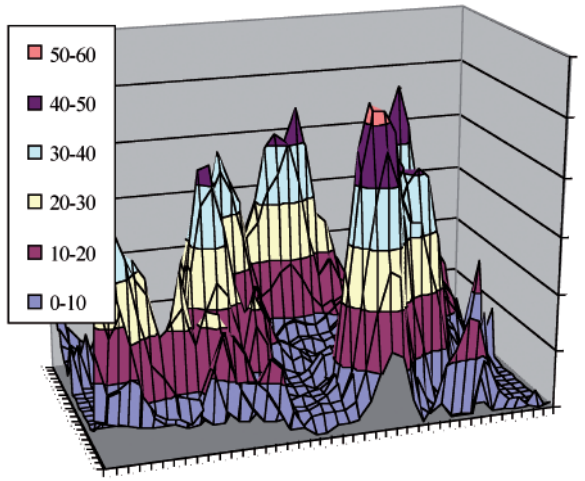

Figure 4. Surface plot of 5HT concentration in a single RBL-2H3 cell obtained from both intensity and lifetime data.

60 mM. A surface plot of 5HT concentration in a single mast cell is (Figure 4) shows the distribution of the storage granules in the cytoplasm around the cell nucleus. Figure 3D shows that propranolol appears to be taken up by the same type of granule within RBL-2H3 cells and the fluorescence lifetime is reduced from the solution value of 9.6 ns to the range 5-8 ns. In contrast, propranolol and 5HT are relatively uniformly distributed within the cytoplasm of glial cells and are excluded from the nucleus. The lifetime of cytoplasmic 5HT in glial cells is in the region of 2.5 ns, indicating some degree of quenching, most probably through binding to cellular macromolecules. The fluorescence lifetime of propranolol in the glial cell cytoplasm is the same as in solution. The results obtained here show that FLIM may be used to generate additional contrast, in that within the cell cytoplasm and storage granule of the cells studied there are significant differences in fluorescence lifetime. This may in part be due to self-quenching, especially of 5HT in mast cell granules, but there are other interactions to be discovered that generate this contrast. Future work in this area will concentrate on the kinetics of drug uptake and release and the effects of inhibitors of transport systems in the cell plasma membrane.

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