

Multiphoton excitation spectroscopy of serotonin

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Introduction

Serotonin is an important neurotransmitter in the brain. Lowered levels of serotonin in the brain and the cerebrospinal fluid are strongly associated with depression and suicidal behaviour^[1]. Real-time monitoring of serotonin levels in the brain or the associated cerebrospinal fluid is therefore of considerable importance^[2] and the imaging of serotonin and other biomolecules using non-linear spectroscopy is of considerable interest^[3]. Webb and co-workers^[4] were the first to report the characteristic 'hyperluminescence' at 500 nm produced by multiphoton excitation of serotonin using femtosecond near infra red (830 nm) laser pulses. Between 750 and 830 nm, the green emission arises from 4-photon excitation of serotonin to produce a photochemical intermediate which is induced to fluoresce by a further two photon process. The overall 6-photon nature of hyperluminescence causes it to have an inherently low cross section. Our earlier investigations of hyperluminescence^[5] of 5-hydroxyindoles are now being directed to applications of this technique.

Experimental

The experiments used the Ti:sapphire laser system (700-950 nm, 200 fs pulses at 75 MHz, Coherent) available in the Confocal Microscope Laboratory at the LSF. Multiphoton excitation was achieved by focussing the laser beam with the $\times 60$, na 1.2 objective of a Nikon TE2000 microscope system. The microscope was linked to spectrometer-CCD combination for determination of emission spectra, whilst nanosecond lifetimes were obtained by time-correlated single photon counting (Becker-Hickl) using a fast photomultiplier (R3809U, Hamamatsu).

Results and discussion

Imaging of hyperluminescence from serotonin granules in mast cells

The incubation conditions required for efficient loading of serotonin into rat leukaemic RB3 mast cell have been studied and degranulation observed at long incubation times (>18 hours). The lifetime map of serotonin in cells has been obtained (Figure 1) and shows a distribution of lifetimes between about 0.7 and 1.2 ns, centred on the solution value of 0.9 ns. This is intriguing because the exact state of serotonin within the granules is not known. However it is believed to involve a complex with acid polymers such as heparin^[6]. Degranulation in response to addition of polymyxin B has also been observed.

Measuring detection limits under various conditions.

The usable range of powers has been studied in solutions over a wide range of concentrations. Shear *et al.*^[4] originally observed a non-linear correlation between hyperluminescence intensity and concentration and showed logarithmic plots indicating a power dependence of 1.3 on

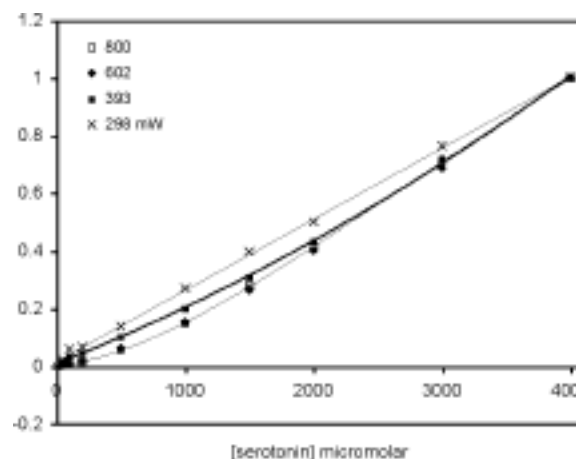


Figure 1. Hyperluminescence intensity versus serotonin concentration in phosphate buffer (pH 7) at 4 laser powers.

serotonin concentration. We have been able to reproduce these effects but further investigation reveals this apparent power dependence to be due to loss of signal at low serotonin concentrations. Our results shown in Figure 2 indicate that at high laser power there is a depletion of signal at low serotonin concentrations (<100 micromolar). Significantly, this effect disappears at lower laser power and must therefore be ascribed to consumption of serotonin by the chemistry occurring within the confocal volume.

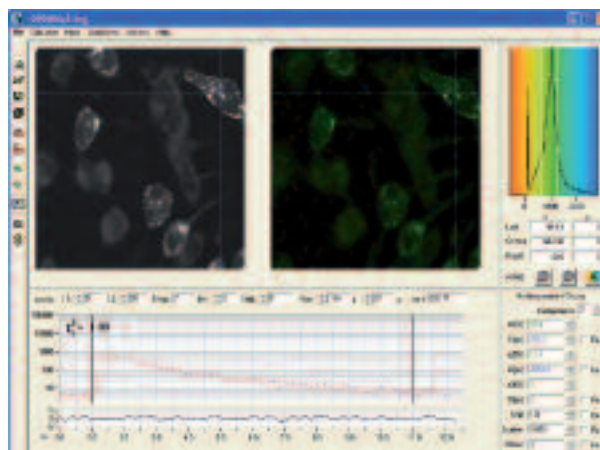


Figure 2. Figure 1. Hyperluminescence (excited at 750 nm) imaged within rat mast cells incubated with serotonin:- a) top left – intensity image; b) top middle – lifetime image; c) top right – lifetime distribution with false colour code for b); d) bottom – nanosecond decay profile at selected pixel in a).

Evaluating the effects of buffers and other solutes.

Shear *et al.*^[4] also reported an enhancement of hyperluminescent intensity in solutions of serotonin at low concentration when buffered by HEPES (4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid). Again we have been able to reproduce this effect, but note that a similar effect can be obtained with the singlet oxygen quencher DABCO (1,4-diazabicyclo[2.2.2]octane, Figure 3) We have found that HEPES is also a singlet oxygen quencher with a rate constant at pH 12 (i.e. for the fully deprotonated form) of $1.7 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ comparable with that for DABCO ($9.1 \times 10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$). These observations suggest that rather than providing additional hyperluminescence, these solutes prevent photochemical depletion of serotonin by oxidative processes within the confocal volume. This relates to our previous observation^[7] that the triplet excited state of 5-hydroxytryptophan forms singlet oxygen with a quantum yield of ~ 0.1 on direct excitation at 308 nm.

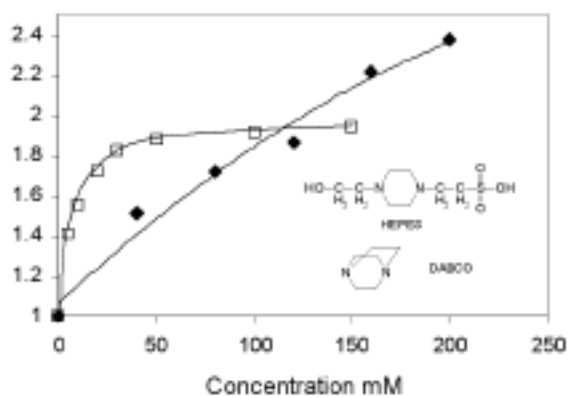


Figure 3. Effect of DABCO (◆) and HEPES (□) on hyperluminescence intensity from serotonin on excitation at 750 nm.

Use of hyperluminescence to detect oxidative stress.

It has previously been reported that hyperluminescence ascribed to 5-hydroxytryptophan is observed from solutions of tryptophan when exposed to laser powers sufficiently high to cause dielectric breakdown in water^[8]. This suggests that hydroxyindole hyperluminescence might be used report on hydroxyl radicals generated in solution by tryptophan used as a scavenger. Figure 4 shows the results of initial experiments using the “Photo-Fenton” reagent, 2-mercaptopyridine-N-oxide (MPNO), which is reported to generate hydroxyl radicals on photolysis^[9]. In solutions of tryptophan alone, the tail of the UV fluorescence (distorted by the CCD spectral response at $<360 \text{ nm}$), excited in a three-photon process, is seen at $<400 \text{ nm}$. Solutions of MPNO also gave weak luminescence at ca 520 nm, presumably due to photoproducts. Photolysis of solutions containing both solutes gave a clearly identifiable peak of 5-hydroxytryptophan hyperluminescence at 500 nm. This appears to show that 2-photon activation of the photo-Fenton reagent gives rise to a hydroxylating species. However further experiments with ethanol and DMSO showed only small effects, suggesting that hydroxyl radical itself is not the reactive species generated by MPNO.

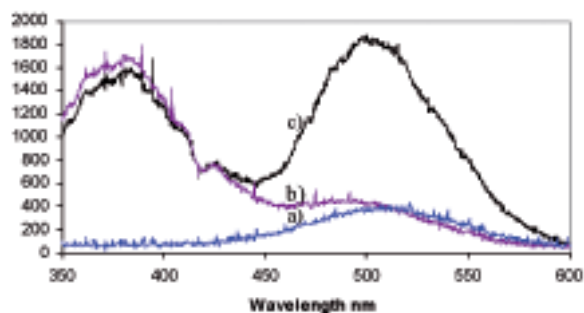


Figure 4. Luminescence spectra observed from multiphoton-excited solutions of a) mercaptopyridine N-oxide (2 mM); b) tryptophan (3.6 mM); and c) mixture of tryptophan (3.6 mM) and 2-mercaptopyridine N-oxide (2 mM). Laser power ca. 40 mW at 750 nm.

Conclusions

The experiments described above are indicating the conditions required for effective detection and quantification of serotonin at low concentrations in biological samples. Hyperluminescence may also have the capacity to be used as a marker for 5-hydroxyindoles arising from oxidative stress in biological systems.

References

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