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Fluorescence of serotonin in the visible spectrum upon multiphotonic photoconversion

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Abstract: The vital molecule serotonin modulates the functioning of the nervous system. The chemical characteristics of serotonin provide multiple advantages for its study in living or fixed tissue. Serotonin has the capacity to emit fluorescence directly and indirectly through chemical intermediates in response to mono- and multiphoton excitation. However, the fluorescent emissions are multifactorial and their dependence on the concentration, excitation wavelength and laser intensity still need a comprehensive study. Here we studied the fluorescence of serotonin excited multiphotonically with near-infrared light. Experiments were conducted in a custom-made multiphoton microscope coupled to a monochromator and a photomultiplier that collected the emissions. We show that the responses of serotonin to multiphoton stimulation are highly non-linear. The well-known violet emission having a 340 nm peak was accompanied by two other emissions in the visible spectrum. The best excitor wavelength to produce both emissions was 700 nm. A green emission with a ~ 500 nm peak was similar to a previously described fluorescence in response to longer excitation wavelengths. A new blue emission with a ~ 405 nm peak was originated from the photoconversion of serotonin to a relatively stable product. Such a reaction could be reproduced by irradiation of serotonin with high laser power for 30 minutes. The absorbance of the new compound expanded from ~ 315 to ~ 360 nm. Excitation of the irradiated solution monophotonically with 350 nm or biphotonically with 700 nm similarly generated the 405 nm blue emission. Our data are presented quantitatively through the design of a single geometric chart that combines the intensity of each emission in response to the serotonin concentration, excitation wavelengths and laser intensity. The autofluorescence of serotonin in addition to the formation of the two compounds emitting in the visible spectrum provides diverse possibilities for the quantitative study of the dynamics of serotonin in living tissue.

1. Introduction

Serotonin (5-HT), a vital monoamine transmitter and modulator of the nervous system, is widely distributed along phylogeny [1]. Neurons release serotonin from nerve terminals and from virtually all parts of their bodies [2]. Its effects include a wide variety of actions affecting neuronal circuits, from sensory inputs to motor outputs [3,4]. Since its discovery, the chemical

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properties of serotonin have been the matter of numerous studies. For example, the Falck-Hillarp technique founded on the 5-HT propension to produce fluorescent derivatives upon exposure to paraformaldehyde vapors allowed its detection in the central nervous system [5]. With this technique, Fuxe and his colleagues first discovered the serotonin-containing neurons in the brain, and then showed the presence of free extracellular serotonin distant from synaptic contacts [5,6]. Alternatively, the redox properties of serotonin allow its detection by the use of carbon fiber voltammetry or amperometry techniques. Amperometry has permitted to estimate the kinetics of exocytosis from single vesicles [7] and the serotonin vesicular concentration [8]. Voltammetry has permitted to detect serotonin at a distance from its release sites in response to stimulation of serotonergic neurons [9]. In the extracellular space, serotonin becomes diluted, diffuses and exerts actions on from the releasing sites. This type of communication, named volume transmission [5,6,10] permits the modulation of whole neuronal circuits [3]. However, information is still lacking about the mechanism of its release and how it arrives at distant targets in the functioning nervous system. For this reason, the quantitative detection of serotonin in the living nervous tissue is relevant to understand its effects and certain diseases such as depression or post-traumatic stress disorder [11,12].

The fluorescence of serotonin can also be detected in living cells from its fluorescence in response to mono- and multi-photon excitation [13–15]. Like other indoles, serotonin can emit fluorescence in the ultraviolet range. The wide absorption spectrum of serotonin spans from 200-320 nm in the UV region, with absorption peaks at 220, 275 and 295 nm. Excitation generates UV fluorescence that peaks at 340 nm in physiological 7.4 pH [16,17]. As can be predicted, serotonin excitation with three photons at 740 nm also produces the characteristic 340 nm UV fluorescence [15,18]. In addition, under extreme acidic or basic pH, or upon multiphoton excitation, serotonin as other indoles reacts to generate photoproducts that with appropriate excitation emit fluorescence in the visible range [19–21]. For example, excitation of serotonin with four photons at 830 nm induces a photo reaction, which responds to two additional photons to generate a green emission with a ~500 nm peak [14]. An additional blue fluorescence with a ~400 nm peak has been detected when high concentrations of 5-HT are excited with 340 nm, a wavelength out of the UV absorption spectrum of serotonin [22].

The serotonin emissions can be captured from living brain due to the long penetration of infrared light into the tissue and the reduced photodamage and bleaching, that are reduced by the infrared focal excitation [23]. In these conditions, the light penetration of mouse brain can reach ~0.7 mm at a 770 nm and can be enhanced to ~1.4 mm with excitation wavelengths above 1 micron [24,25]. By homogenizing the refractive index of tissues to reduce light scattering [26], clearing of fixed tissues increases the optical penetration by one logarithm unit from ~800 nm to ~800 μm [27–29]. Moreover, studies carried out in the near infrared spectrum ranging from 650 to 1000 nm have shown that the tissue penetration is not significantly different for the whole range of wavelengths [25]. These possibilities permit the study of serotonin storage, release and volume transmission by the use of optical components that operate in the visible range of the electromagnetic spectrum. However, the wide effects of multiphoton excitation on serotonin still require a comprehensive study. On the other hand, the emissions by serotonin photo-derivatives in the visible range promises useful tools for quantitative studies of the serotonin dynamics in living or fixed tissues.

Here we present an extensive study on the effects of multiphoton excitation of serotonin with emphasis on its emissions in the visible range. We studied the fluorescence dependence on the serotonin concentration, the excitation wavelength (λ_{exc}) and laser intensity. A potentially useful emission in the blue range is described here. Therefore, part of the study was aimed to characterize this emission. We also propose a graphic representation of the multivariable multiphoton measurements of serotonin for quantitative purpose.

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2. Materials and methods

2.1. Chemicals and solutions

Serotonin hydrochloride was purchased from Sigma-Aldrich. All solutions were prepared with physiological Ringer (NaCl = 115 mM, $CaCl_2$ = 1.5 mM, KCl = 4 mM, Glucose = 11 mM and HEPES = 10 mM, diluted in tri-distilled water). The pH was adjusted to 7.4. Serotonin solutions were prepared at 270.0, 100.0, 30.0, 10.0, 3.0, 1.0 and 0.3 mM concentrations. Experiments were made on 200 μL drops of serotonin solution.

2.2. Multiphoton excitation and fluorescence collection

The monophotonic absorption and emission spectra of serotonin in the UV range were obtained in a Cary 50 Scan UV Visible Spectrophotometer (Agilent) and a Cary Eclipse Fluorescence Spectrophotometer (Agilent), respectively.

The multiphoton excitation of serotonin was made in a custom-made-upright microscope with a 60x water immersion objective from Nikon (CFI Fluor 60x W, NA = 1.0, W.D. 2.0 mm). The excitation light source was a Ti-sapphire-pulsed laser (Chameleon Ultra II, 140 fs at 80 MHz, Coherent) tunable between 680 and 1080 nm. A scheme of the excitation-collection system is presented in Fig. 1. The laser power was controlled with a custom-made-PID controller previously calibrated with a commercial sensor (S401C, Thorlabs). Transmitted fluorescence was collected with a condenser lens and focused at the entrance of a monochromator SpectraPro-500 (Action Research Corporation). Acquisition of the spectra were made by rotating the diffraction grating (1200 lines/mm 500 nm blaze grating) of the monochromator operating at a 1 nm resolution. Infrared contamination produced by the laser was eliminated by a 2 cm wide liquid filter made with saturated cuprum-sulfate solution [30]. Fluorescence detection was then made with a photomultiplier tube (PMT; H7422-40 Hamamatsu) at the output of the monochromator.

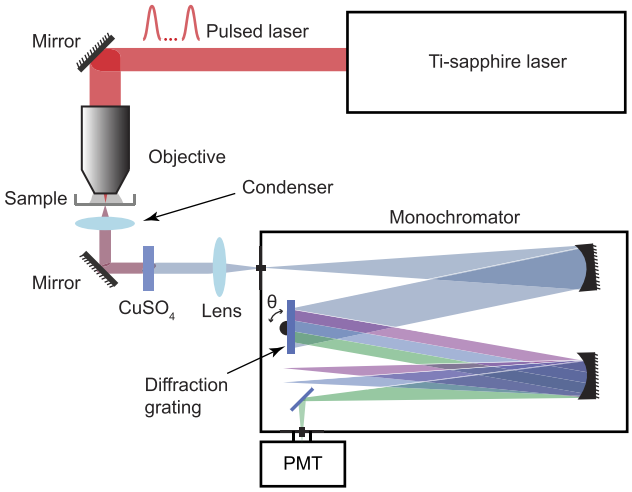

Fig. 1. Diagram of the excitation-acquisition system. A 1.0 numerical aperture objective focuses a femtosecond-pulsed laser on a drop of serotonin solution (Sample). The fluorescence is collected as transmitted light by a condenser lens. Infrared contamination is filtered with a saturated copper-sulfate ($CuSO_4$) filter. Rotation of the diffraction grating of the monochromator permitted to acquire the light emissions with 1 nm resolution. The signal was captured with a high-sensitivity PMT.

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The intensity of illumination (TW/cm^2) at the sample was calculated as:

$$I = \frac{P_{avg}}{f_{rate}\ \tau_{pulse}\ A_{spot}} \tag{1}$$

where P_{avg} is the average laser power, f_{rate} = 80 MHz is the repetition rate of the pulsed laser and τ_{pulse} = 140 fs is the pulse width. The area of the focused spot size is $A_{spot} = \pi \left(\frac{D_{waist}}{2}\right)^2$; the focal gaussian spot diameter is defined as $D_{waist} = \frac{4 f\ \lambda_{exc}}{\pi\ D_L}$, with the focal distance of the objective being f = 3.3 mm and the diameter of the beam at the back aperture objective being D_L = 7.12 mm. Table 1 shows the focal gaussian spot diameter and the area of the focused spot for the different excitation wavelengths used in our experiments. Figure 2 presents the light intensities calculated with Eq. (1) at different laser powers and wavelengths.

Table 1. Area of the focal spot for each excitation wavelength used here

λ_{exc} (nm)	D_{waist} (nm)	A_{spot} (μm^2)
690	407	0.130
700	413	0.134
710	419	0.138
720	425	0.142
730	431	0.146
740	437	0.150
760	449	0.158

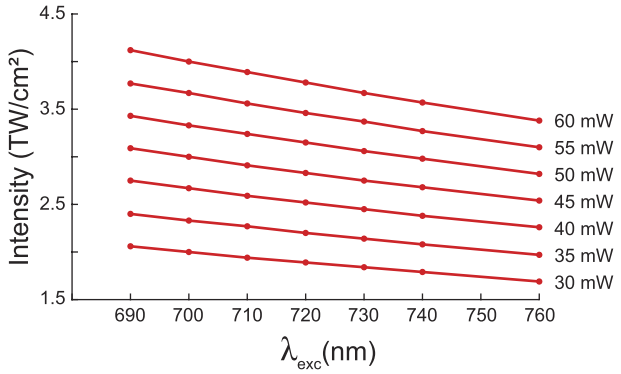

Fig. 2. Light intensity at the sample in TW/cm^2, calculated for each average laser power (right numbers in mW) as a function of the λ_{exc}(nm). The negative slope of the curves occurs because the area of the focal spot increases with λ_{exc}. Therefore, the intensity decreases.

2.3. Estimates of serotonin solubility

The 270 mM high concentration of serotonin was of particular relevance for our study for being equal to the intravesicular concentration that has been quantified in neurons [8]. Since the 270 mM concentration of serotonin is near saturation, we tested its dilution through an absorption versus concentration curve using a 250 ±10 nm UV beam. Figure 3 shows the average data (solid blue line) from four repetitions plotted in logarithmic scale. The absorption between 3 and at 270 mM increases at the same rate, thus confirming the full dilution of serotonin at all the concentrations tested.

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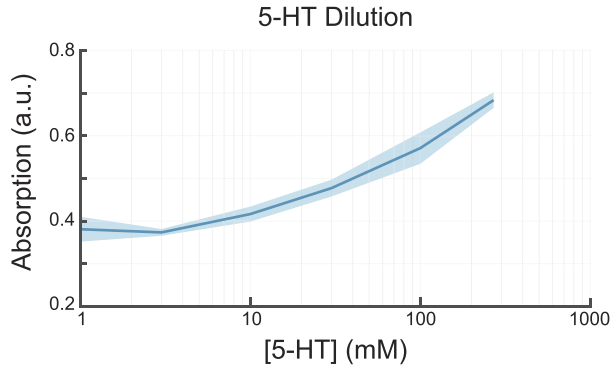

Fig. 3. Absorption of serotonin at increasing concentrations (1.0, 3.0, 10.0, 30.0, 100.0 and 270.0 mM). The absorption wavelength was 250 nm. The plot shows a continuous increase in the absorption rate, thus confirming that even at the high 270 mM concentration, serotonin was well-diluted. The blue line links the average values of four measurements at each concentration; the dim blue background trace is the standard deviation, for informative purposes.

2.4. Data analysis

Spectra were acquired for each concentration using λ_{exc} = 690, 700, 710, 720, 730, 740 and 760 nm. The power of the laser for each wavelength was varied from 30 to 60 mW with 5 mW steps. At least two spectra could be obtained from each drop. Acquisition of fluorescence was made at a ratio of 1 nm/sec. In the end, for each serotonin concentration we obtained 49 spectra. The data were corrected for the grating efficiency and the photomultiplier sensitivity and were then smoothed using the first two components of the Singular Spectrum Analysis (SSA) algorithm [31], as shown in Fig. 4. The peak values of the emissions were calculated from the first derivative of the data smoothed with the SSA algorithm.

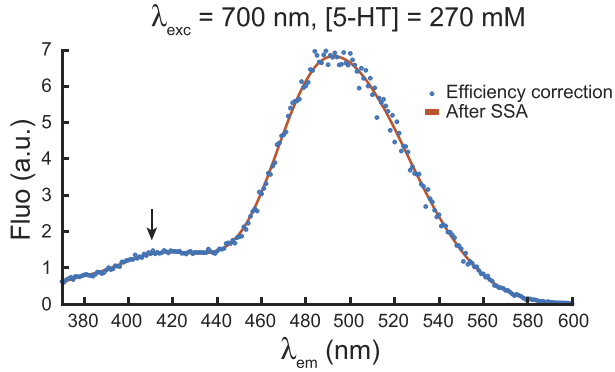

Fig. 4. Processing of data. Fluorescence spectrum (λ_{em}) of serotonin at a 270 mM concentration, λ_{exc} = 700 nm and 60 mW laser power. The blue dots are the data after a correction for the efficiency of the acquisition devices. The orange line shows the data after being smoothed with 2 components of the SSA algorithm. Note the presence of a small fluorescence peak in the plot (arrow).

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2.5. Photoconversion of serotonin

The photoconversion of serotonin was explored in 300 μL of serotonin solution at a 0.3 mM concentration. Samples were irradiated at 700 nm (the most efficient excitation producing the blue fluorescence) for 30 min at 60 mW. In some experiments λ_{exc} of 710 and 720 nm were used to compare the efficiency of the photoconversion. The absorption spectrum was compared with that of non-irradiated similar serotonin solutions. Experiments were repeated three times with solutions prepared a few minutes before the experiment and maintained on ice. After irradiation, monophotonic excitation tests were made using 270, 295, 340, 350, 355 or 360 nm wavelengths.

3. Results

3.1. Fluorescent emissions upon serotonin excitation

To set a baseline for the study of the multiphoton-produced fluorescence, the absorption and emission spectra of serotonin were measured using monophotonic excitation in the ultraviolet range. Figure 5 shows that the absorption spectrum of serotonin expands from below 200 nm to ~325 nm, as has been reported previously [20]. Monophotonic excitation with wavelengths along the absorption spectrum (a, b, c, and d in Fig. 5) produce a fluorescence emission with a peak at 340 nm. However, the amplitude of the fluorescence varies without a correlation with the λ_{exc}.

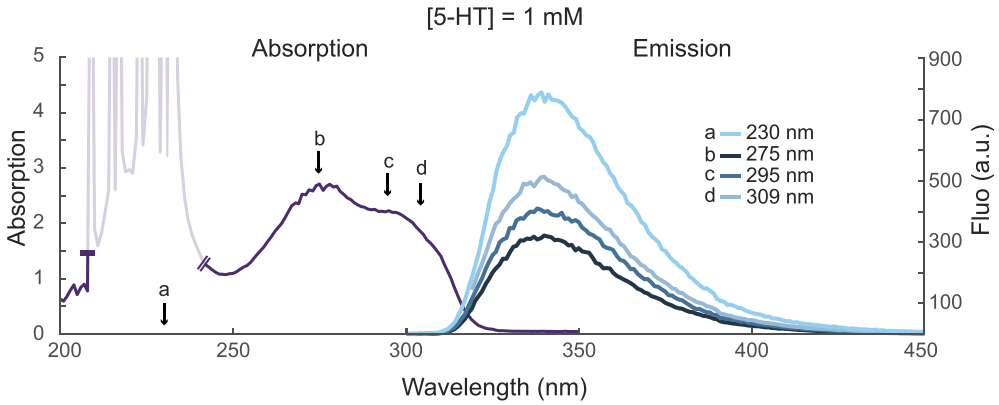

Fig. 5. Spectra of absorption and emission of serotonin upon monophotonic excitation. The serotonin concentration was 1 mM. The purple curve in the low wavelength range (left of the plot) is the absorption spectrum. The peaks in the region above the double bars were produced by saturation of the acquisition device. Illumination of the serotonin solution with wavelengths from 230 to 309 nm (arrows from a to d) produced similar fluorescence emissions (blue traces) with a peak at ~340 nm and amplitudes, determined by the absorption wavelength.

In addition to the well-known UV emission of serotonin upon monophotonic excitation, the multiphoton excitation at wavelengths from 690 to 760 nm produced two other emissions. One appeared in the green range of the spectrum, having a nearly gaussian broad spectrum with a maximum peak by 500 nm, similar to that described by Shear et al. [14]; the other emission had its maximum peak at 405 nm, within the blue range of the spectrum.

Figure 6 gives a full account of the emissions obtained at different excitation wavelengths, power and serotonin concentrations. The excitation wavelength had marked effects on the amounts of the three emissions. The amplitudes of every emission were remarkably larger in response to λ_{exc}=700 nm and their relative amplitudes varied as λ_{exc} was increased. The green emissions had the largest amplitudes at all λ_{exc}. All the emissions decayed in response to λ_{exc}

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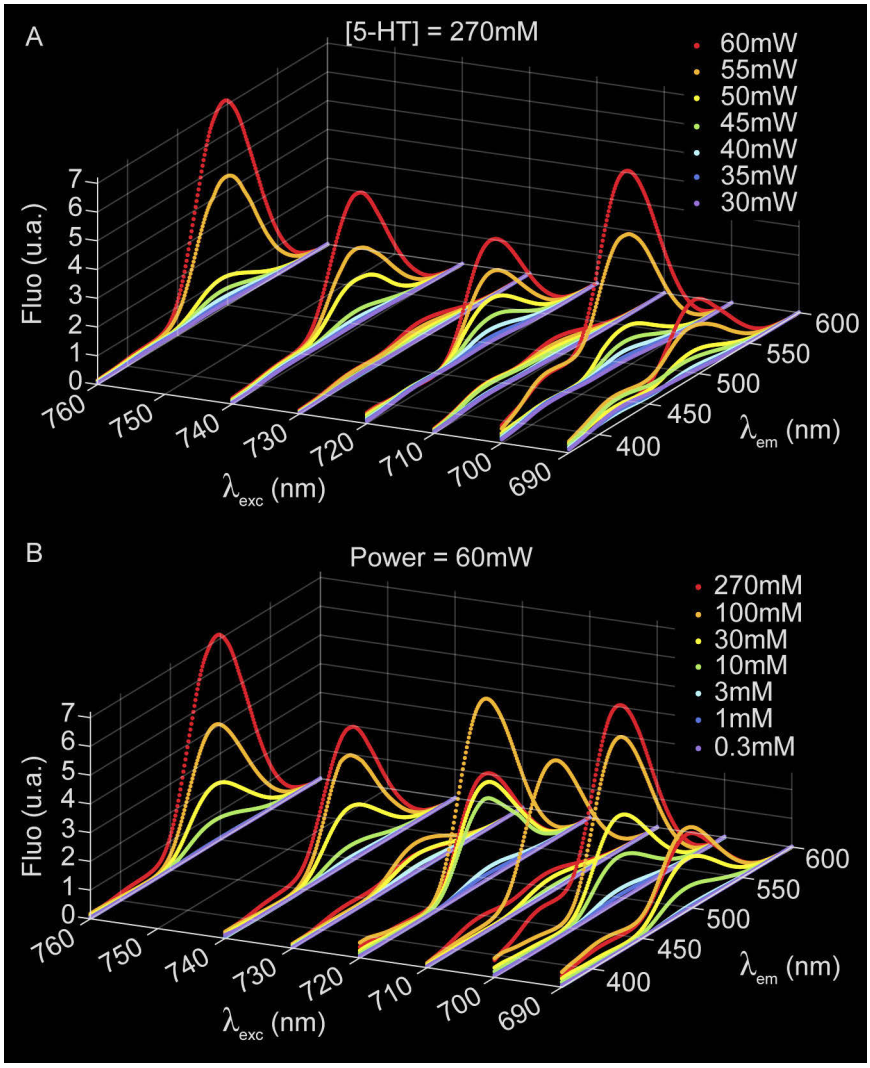

Fig. 6. The fluorescence spectra (λ_{em}) depended on the excitation wavelength (λ_{exc}), power and serotonin concentration. The excitation wavelength is indicated in one of the axis. (A) Laser power-dependence of the emissions. Measurements were made using a high 270 mM serotonin concentration. The excitation powers are indicated in the inset. The violet and blue emissions appeared predominantly in response to λ_{exc} from 690 to 720 nm. Note the reduced levels of green fluorescence obtained with λ_{exc} of 710 and 730 nm. (B) Concentration-dependence of the fluorescent. Spectra were obtained at a fixed 60 mW excitation power. The serotonin concentrations are indicated in the inset. Note the discontinuities in the rate of increase of the spectra as the serotonin concentration was increased. The violet and blue emissions were more prominent at λ_{exc} = 700 nm and concentrations of 100 and 270 mM.

> 760 nm. Although it has been shown that longer wavelengths (i.e. λ_{exc} = 830 nm) produce serotonin fluorescence [14], no further analysis was carried out above 760 nm.

The plot in Fig. 6(A) shows the effect of the laser power on the spectra of a 270 mM serotonin concentration. The intensity of the green fluorescence, which can be clearly identified for

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its larger amplitude, increased at progressively higher rates as the laser power was increased, indicating that it results from a non-linear excitation process [14]. However, increasing the laser power from 55 to 60 mW at λ_{exc}= 690, 700 and 760 nm produced smaller increase rates than the lower power step from 50 to 55 mW, thus suggesting a quenching of the emission. The blue emission can be seen as small deflections, the larger of which appeared in response to 700 nm excitation and decreased as the excitation wavelength was increased or reduced. The violet emission can be detected in response to λ_{exc}= 720 nm and below, as a small tail on the 350 nm region of the emission spectrum. The violet tail corresponds to the UV fluorescence in response to three-photon absorption reported previously [15]. At shorter emission wavelengths the violet emission was lost due to the restrictions of optical systems that operate in the visible spectrum. Spectra in response to λ_{exc}= 710 and 730 nm showed small amplitudes.

Figure 6(B) shows the effect of the serotonin concentration on the fluorescence emissions at a fixed 60 mW laser power for all the excitation wavelengths tested. The non-linearities increased at larger serotonin concentrations. The green emission dominated the spectrum at every serotonin concentration. A visual comparison of the orange and red traces at λ_{exc}= 700 and 740 nm make clear the quenching of the green emission. In addition, at λ_{exc}= 690, 710, 720 and 730 nm the quenching was seen as a reduction in the peak at 270 mM concentration when compared to that obtained at 100 mM. The blue emission was clearly detected at 100 mM and 270 mM concentrations.

Figure 7 compares the spectra of the serotonin emissions in response to λ_{exc}= 700, 720 and 740 nm, which displayed different proportions of the three emissions. Figure 7A presents the emissions as the laser power was increased for a 270 mM concentration. The violet emission could be detected upon λ_{exc} = 700 and was reduced at λ_{exc}= 720 and 740 nm. The blue emission had a reduction similar to that of the violet emission as the excitation wavelength was increased; at the most efficient λ_{exc} =700 nm the amplitude of the emissions was remarkably larger at high power values. Then the amplitudes were similar for 55 and 60 mW powers, again suggesting a quenching effect. At λ_{exc}= 720 we could not record the blue signal and at λ_{exc}= 740 nm it became detectable again. A similar dynamic was seen for the green fluorescence.

The semilogarithmic plots in Fig. 7B show the non-linearities of the emissions as the laser power was increased. These measurements were made at 373 nm (violet), 405 nm (blue) and 494 (green) peak values. Detection of each emission had a different threshold, with green being the largest and blue the smallest. The slopes of the curves (approximated to the next integer value) indicate the number of photons absorbed [32]. The violet emission was produced by a 3 photon absorption, the blue by 2 photon absorption and the green by 5 photon absorption.

Figure 7C compares the fluorescence-dependence of the serotonin concentration at the same selected excitation wavelengths. When λ_{exc}= 700 nm, at low (0.3 to 10 mM) concentrations the fluorescence increases were small; from 10 to 100 mM concentration the fluorescence increases occurred at high rates, and from 100 to 270 mM quenching appeared. The violet emission, which was detected above 3 mM with a one log unit lower intensity, displayed a similar dynamics. The blue emission appeared as a clear 405 nm peak in response to 700 nm excitation, even in a low 30 mM serotonin concentration. The three behaviors already described above for the green emission at λ_{exc}= 700 were similar at a λ_{exc}= 720 and 740 nm. However, at λ_{exc}= 720 nm the quenching of the green fluorescence was increased to the point that its amplitude was smaller than that at the lower 100 mM concentration. The corresponding semilogarithimic plots in Fig. 7D summarize these results graphically.

3.2. Quantitative representation of serotonin fluorescence

A quantitative description of the serotonin fluorescence emissions is presented in Figs. 8 and 9. The plot in Fig. 8 presents data obtained from the 270 mM concentration as a function of the seven excitation wavelengths and the seven laser powers tested. The heptagons in Fig. 9 incorporate

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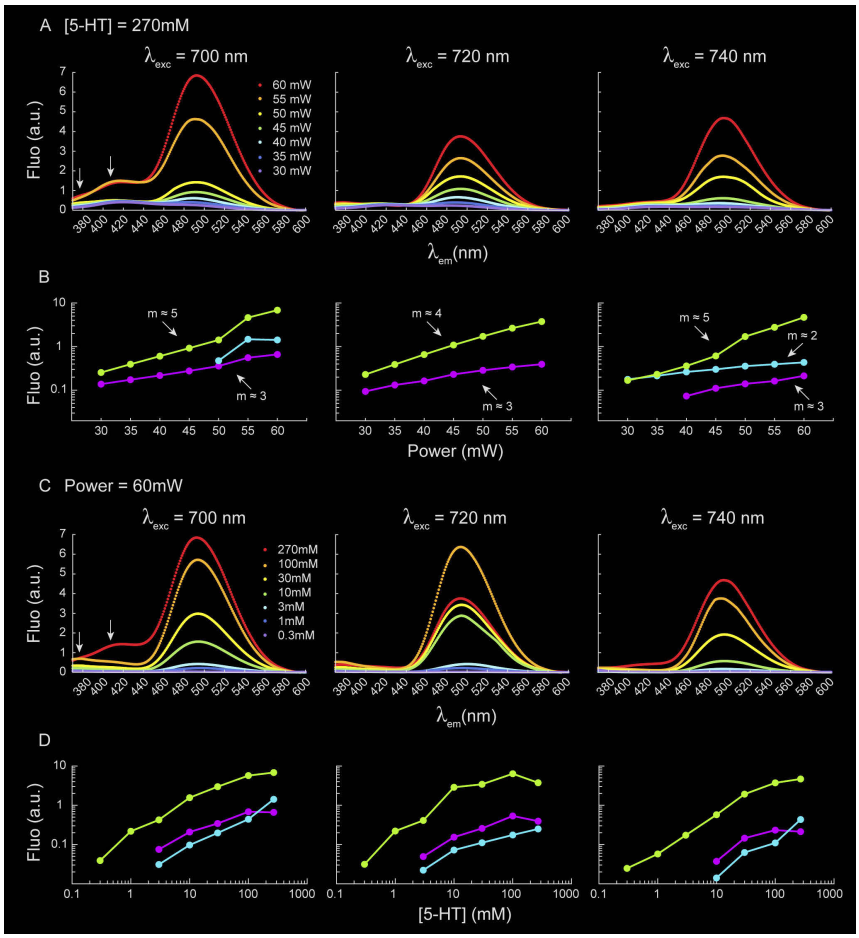

Fig. 7. The serotonin fluorescence depended on the excitation wavelength, laser power and serotonin concentration. A) Fluorescence spectra (λ_{em}) of high (270 mM) serotonin concentration excited with the wavelengths (λ_{exc}) indicated on the top. The colors of the traces correspond to the laser powers in the inset. λ_{exc} = 700 nm produced three emissions. The UV emission, indicated by the first arrow from left to right is partially masked by the blue emission (second arrow) and by the cutoff of the optical system at wavelengths below 350 nm. The largest emission appears in the green range of the spectrum. The fluorescence levels decayed with a λ_{exc} = 720 nm and increased again in response to a λ_{exc} = 740 nm. B) Fluorescence as a function of the laser power for the three excitation wavelengths in A. Each emission follows a different trend. Note that when λ_{exc}= 700 nm the blue emission was detected only at high powers, then was absent when λ_{exc}= 720 nm and reappeared in the whole power range when λ_{exc}= 740 nm. The slopes (m values) were obtained from the whole range of values in each plot. C) At a 60 mW laser power, the variations in the serotonin concentration changed the relative amplitudes of the three emissions. Note the non-linearities in the peak amplitudes of the emissions as the serotonin concentration was increased. The peak amplitudes of the green and violet emissions follow similar trends with quenching at high concentrations, while the blue emissions has an increase at this same 100 and 270 mM serotonin concentrations. Note also that the emissions at λ_{exc}= 700 and 740 nm are qualitatively similar although with different amplitudes. At the intermediate λ_{exc}= 720 nm the quenching of the green and violet emissions was enhanced while the increase in the blue emission was reduced. D) Maximum fluorescence as a function of the serotonin concentration at the three excitation wavelengths shown in C. Note the reduction of the rate of increase of the green and violet emissions as the serotonin concentration increases from 100 to 270 mM. By contrast, the blue emission was increased at those same concentrations.

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the whole set of data for the seven concentrations studied. Each concentration is presented in a triangle that follows the format of Fig. 8. The concentrations increase in a clockwise order indicated by the arrow. The color of each heptagon indicates the emission wavelength.

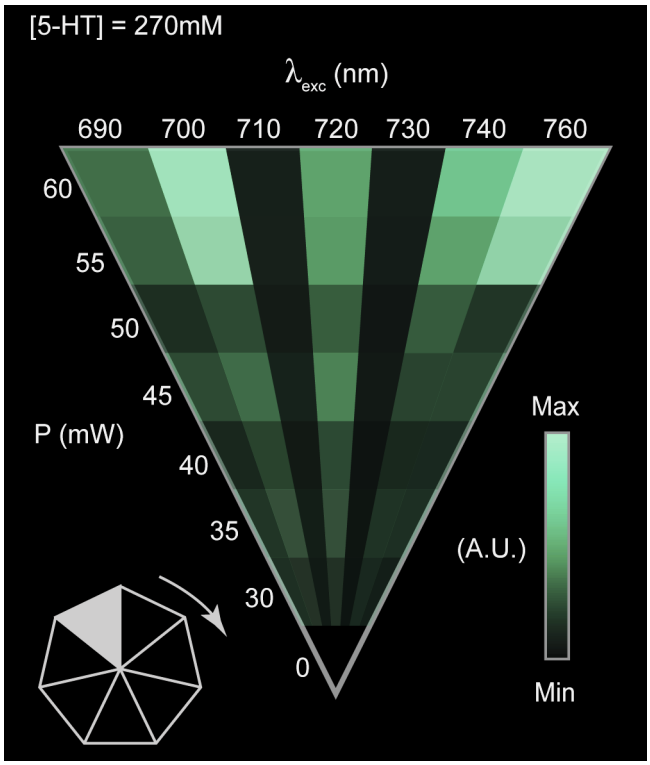

Fig. 8. Quantitative representation of the fluorescence of serotonin as a dual function of the laser power and excitation wavelength. The triangle contains data from all at a emissions from a 270 mM concentration. The green levels are proportional to the peak amplitude of the spectrum in arbitrary units, as indicated in the scale bar on the bottom right. It may be seen that the green emission of serotonin at a 270 mM concentration was more intense in response to λ_{exc} of 700 and 760 nm and at 55 and 60 mW laser powers. λ_{exc} of 710 and 730 nm produced the dimmest emissions. Note that power lasers of 35 and 45 mW produced higher intensity than their immediate higher steps. This plot is the building block of the heptagons in Fig. 9, which are complemented with the information from the six other serotonin concentrations tested arranged in an increasing manner, as indicated by the arrow in the bottom left of this Figure.

The violet heptagon in the left of Fig. 9 shows that the UV fluorescence could be detected from every serotonin concentration, in spite of being only the tail of the emission. The green emission was detected from 10 mM and above, and the blue emission from 30 mM. The three emissions were more efficiently produced by λ_{exc} = 700 nm. It can also be noted that certain laser powers were more effective in producing fluorescence than their subsequent larger values. Such were the cases of the green emission in response to 35 and 45 mW powers, or the blue emissions in response to 40 to 55 mW powers at different excitation wavelengths.

3.3. *Photoconversion of serotonin produces blue emission*

While the violet emission is a direct serotonin response to single UV or multiphoton excitation [15], the green emission seems to result from serotonin photoionization [14]. By contrast, the

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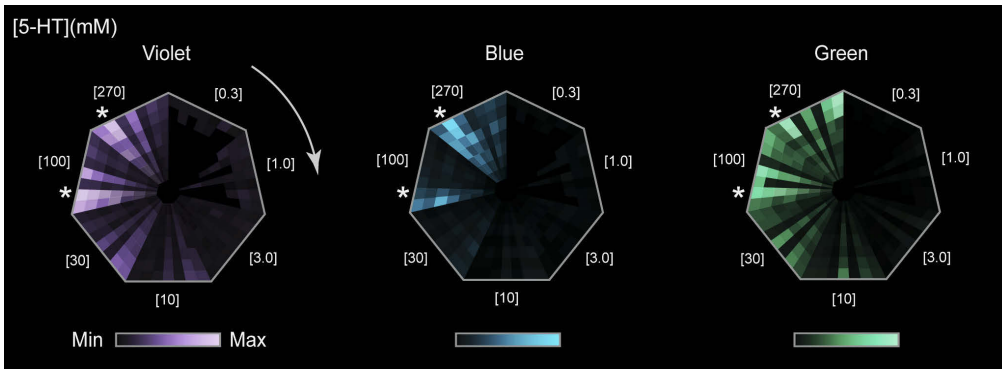

Fig. 9. Quantitative comparison of the three fluorescence emissions of serotonin. Each triangle in the heptagon contains the wavelength- and power-dependence of the fluorescence emission of one serotonin concentration (mM) as indicated in the brackets. The emissions were measured at 373 nm (violet), 405 nm (blue) and 494 nm (green). The arrow in the violet heptagon indicates the increasing order of the serotonin concentrations. The intensity of the color is proportional to the fluorescence intensity, according to the scale bars below each heptagon, which are in arbitrary units. The most efficient excitation wavelength to generate the triple emission was 700 nm (asterisks). It can be seen that due to the non-linearities in the concentration dependence of the emissions, the blue emission in response to a 40 mW laser power could be well-resolved. The lack of fluorescence at concentrations below 30 mM can be attributed to the low sensitivity of our spectroscopic device.

origin of the blue emission was not clear from previous experiments. In this section we explore the origins of the blue emission by comparing the spectra of a pure sample of serotonin at a low 0.3 mM concentration with that of a similar solution that had been irradiated for 30 min with λ_{exc} = 700 nm and 60 mW power, to produce the blue-emitting compound.

Figure 10 shows that the absorption spectrum of the irradiated sample had a tail that spanned from above the 310 nm to ~360 nm, with all the range being above the absorption spectrum of serotonin. A similar effect failed to appear upon illumination of non-irradiated serotonin solutions, which kept their characteristic emission spectrum in response to UV excitation. As shown in Fig. 10B, illumination of the irradiated solution with 350 nm -above the absorption spectrum of serotonin and at half the 700 nm excitation wavelength- produced a blue fluorescence with a maximum peak at 390 nm, similar to the blue peak of the fluorescence produced by the λ_{exc} = 700 nm. Again, such emission was absent from the spectra emitted by non-irradiated serotonin (violet trace in Fig. 10B). The monophotonic blue fluorescence could be detected more than one hour after the end of irradiation, thus suggesting that it originated from a stable photoproduct of serotonin. In these tests the green emission was not detected.

Further evidence that the blue emission upon monophotonic and multiphotonic excitation are produced by the same photoconversion came from an experiment in which the serotonin solutions were irradiated with longer 710 and 720 nm wavelengths, known to be less efficient to produce the blue fluorescence, from the experiments shown in Figs. 6 and 7. Figure 11 shows, after irradiation with 710 nm, illumination of the irradiated solution with 350 nm produced a remarkably dimmer blue fluorescence than that produced after the 700 nm irradiation. Consistently, Fig. 11 also shows that a 720 nm irradiation of the serotonin solution produced an intermediate amount of blue emission in response to the test excitation. The non-irradiated control solutions failed again to generate blue fluorescence, although small amounts of red-shifted fluorescence were present.

A last set of experiments in this series consisted of exciting an irradiated 100 mM serotonin solution with 700 nm to produce a biphotonic excitation of the photocompound. The emission

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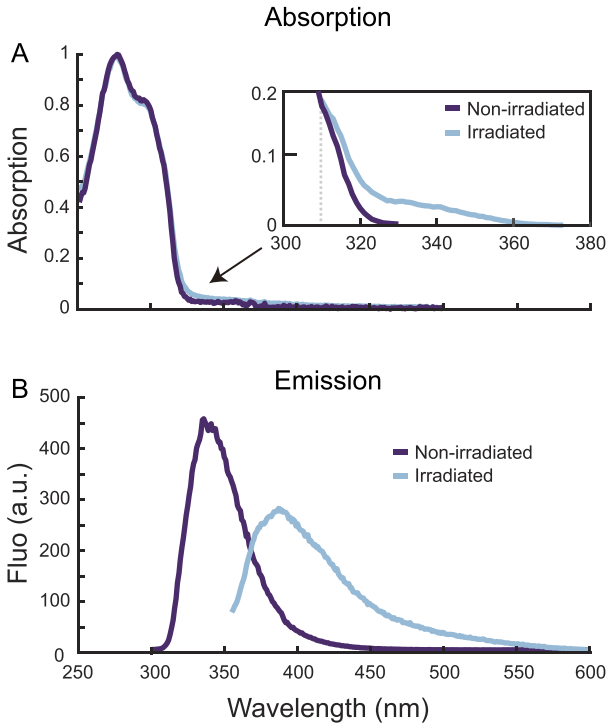

Fig. 10. Absorption and emission spectra of the blue emission in response to photoconversion of serotonin. (A) A sustained 30 minute irradiation of a 0.3 mM serotonin solution with 700 nm at 60 mW produced a tail in the low energy range of the absorption spectrum of the serotonin solution, from ~310 to ~360 nm. The inset shows an amplified segment of the absorption spectrum displaying the tail in the absorption. The dashed line indicates the low value of the absorption. (B) Excitation of the serotonin sample at 350 nm generated blue fluorescence with a ~390 nm peak (blue trace). Such emission was absent from the serotonin emission spectrum generated by a 320 nm UV excitation light (violet trace).

of blue fluorescence supported the idea that it originates from a photoconversion product. The emission spectra of the irradiated and non-irradiated solutions are compared in Fig. 12. Upon multiphotonic excitation a green emission also appeared and this emission increased in solutions that had been irradiated. Altogether, these experiments show that illumination of serotonin with infrared but not UV light produces a photoconversion to a blue-fluorescent molecule.

Figure 13 shows the absorption spectrum of serotonin and the three multiphoton emissions reported in this study. The dotted line is the spectrum added by the irradiation. The plot superimposes the violet emission peaked at 340 produced by monophotonic or three-photon absorption, the blue emission with a ~405 nm peak and the green emission with a ~494 nm peak. The multiphoton excitation and emission wavelengths of the three fluorescence emissions in the visible range are presented in Table 2.

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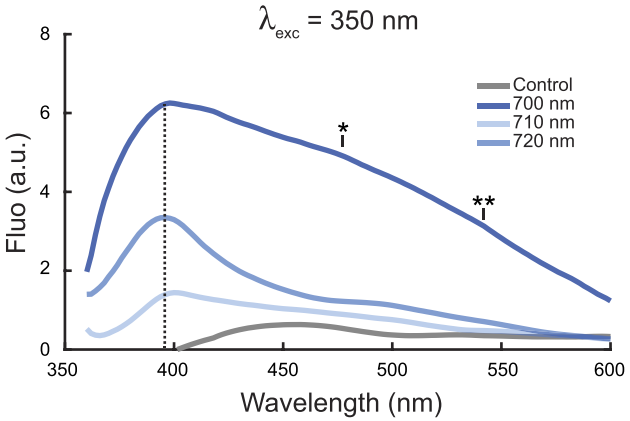

Fig. 11. Excitation-wavelength-dependence of the blue fluorescence. The serotonin sample was irradiated for 30 minutes with 700, 710 or 720 nm. The maximum amplitude of the ~390 peak was proportional to the efficiency of the irradiation wavelength to produce blue fluorescence shown in Fig. 7. The asterisks mark a smaller green emission with a peak by 470 nm (single asterisks) and yet another smaller green-yellow emission with a peak at ~540 nm (double asterisk). Similar green-yellow peaks appeared in the spectrum of the control solution (grey trace at the bottom).

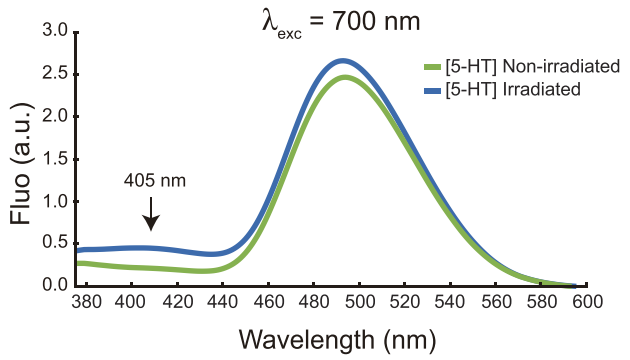

Fig. 12. Biphotonic excitation of irradiated serotonin produces the blue fluorescence. A solution containing 100 mM of 5-HT was irradiated for 30 min with 700 nm at 60 mW. The emission spectrum was tested in response to biphotonic excitation with 700 nm. The green trace is the spectrum of the solution before irradiation and the blue trace is after irradiation. The arrow indicates the 405 nm peak of the blue emission. An increase in the peak of green emission appeared after irradiation.

Table 2. Multiphotonic excitation and emission wavelengths of the blue and green fluorescence serotonin emissions in this study.

	λ_{exc} (nm)	λ_{em} (nm)
Violet	690-760	373 (340-380)
Blue	690-760	405 (388-410)
Green	690-760	494 (494-505)

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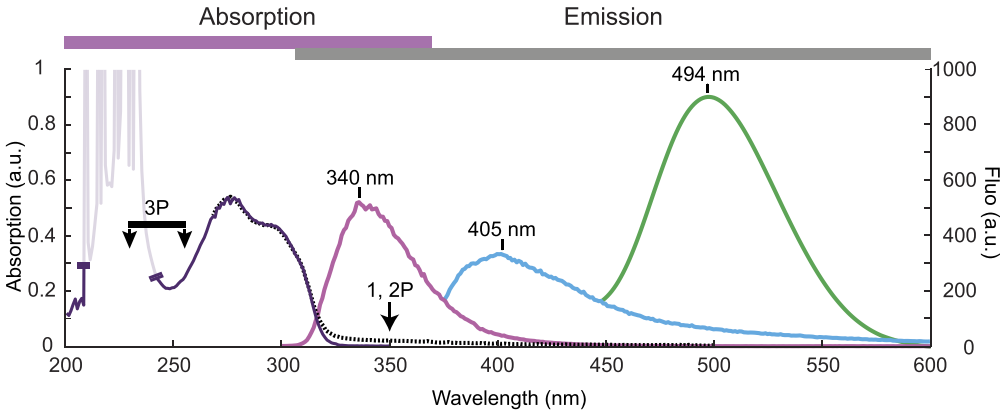

Fig. 13. Direct and indirect emissions of serotonin in response to multiphoton excitation. The excitation spectrum and the three multiphoton emissions of serotonin are superimposed. The absorption spectrum is purple. The bar with the arrows indicates the region of the spectrum excited with three photons in this study. The dim traces above the double bars appear saturated due to the high resolution of the measuring equipment. The violet trace is the UV fluorescence produced by mono or multiphoton absorption. The dotted trace that follows the purple emission is the absorption spectrum of the blue-emitting photoproduct. The arrow indicates the absorption wavelength of the mono-and multiphoton excitation in our experiments. The blue spectrum was obtained from irradiated solutions excited monophotonically. On the right is the green emission spectrum. The approximate peak values are indicated in each spectrum. The amplitude of the spectra are normalized to the 494 nm maximum. Data are in arbitrary units.

4. Discussion

A different process produces each fluorescent emission in response to multiphoton excitation of serotonin. The violet emission is the autofluorescence in response to three-photon excitation, as described by Webb and his colleagues [15]. Such emission has already been applied to detect the release of serotonin from living cells [15,33–37]. The two other emissions have a non-linear origin in the multiphoton illumination of serotonin but are absent from monophotonic illumination. A blue fluorescence similar to that found here has previously been interpreted as a shift of the UV emission occurring at high serotonin concentrations. However, our data gives a fully different interpretation, as a newly described photoconversion product of serotonin upon multiphotonic excitation.

The green fluorescence has been suggested to result from serotonin ionization [17]. However, protonation or oxidation reactions cannot be excluded [20], since, for example, protonation or ionization may convert serotonin into 5-hydroxyindole, a molecule capable to produce green fluorescence [19,20].

The formation of the blue emission has two possible origins. One is that it is a direct photoproduct of serotonin, formed independently from the green emitting compound. The second is that a chain reaction produces the green and blue emitting compounds. Two experimental evidences point to the direct photoconversion of serotonin to form the blue emitting compound. On one hand, at large serotonin concentrations the blue emission increases in parallel to a reduction in the violet (and green) emission (see Fig. 7D). Second, the irradiation experiments produced the blue emitting product with minor generation of green emission. The persistence of the blue emitting photoproduct for dozens of minutes after the laser irradiation is consistent with an irreversible photoconversion of serotonin, in contrast to the instability of the green fluorescence emission, that lasts for tens of nanoseconds [21] and therefore, is absent from our irradiated

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solutions. However, our experiments do not provide any information about the chemical changes taking place in the serotonin molecule or its reaction times.

A difference between ours and previous studies [14,30] is that the use of shorter excitation wavelengths here not only produce an additional and potentially useful blue emission but also the fact that the green emission is produced with three instead of four photons and therefore, a lower laser intensity. This issue may be of relevance for biological studies since increasing the number of exciting photons requires higher laser power, with its concomitant potential damage to the tissue. On the other hand, lower excitation wavelengths contain higher energy, and therefore may produce damage with lower intensities.

The data presented here permit to elaborate multiphotonic protocols for the study of serotonin in living tissues based on its fluorescent emissions in the visible spectrum. The possibility to capture three visible emissions under appropriate experimental conditions is advantageous, since their relative intensities permit to calculate concentrations and analyze dynamics such as those during serotonin release and volume transmission in the nervous system.

A potential source of error in multiphotonic measures of chemical compounds in living neurons comes from the fluorescence of components other than the compounds of interest. In the case of serotonin, contamination can proceed from fluorescence of NADH, which has a similar spectrum than serotonin. Studies done by Maiti and his colleagues have shown that the serotonin UV fluorescence is not contaminated by NADH [38]. However, as it has been shown here, multiphoton excitation can produce molecular photoconversions and generation of new compounds, whose fluorescence properties may interfere with those of the compound of interest. In addition, the autofluorescence of the tissue may be a source of contamination. Experiments combining spectrographic analysis of the tissue and the combined detection of more than one of the serotonin emissions described here may be necessary to reduce the sources of such errors.

A pitfall of our measurements is the existence of second harmonics appearing as 350 nm upon emission by cytoskeletal structures [39]. However, the second harmonics have a thin spectrum that can be eliminated by the use of high quality emission filters. Alternatively, the 760 nm excitation produces a nearly clean green spectrum that increases in proportion to the serotonin concentration and laser power. Although this emission is less accurate for quantitative estimates, it may be combined with other fluorescent markers or with the collection of second harmonics for structural correlations. For these purposes, the quantitative presentation of the data designed for this study permit to design an experimental guide for quantitative experiments under appropriate control of the illumination conditions. Our study was limited by the sensitivity of our acquisition equipment, which is determined by the inherent incoherence of fluorescence added to the long pathway distance of the light due to the monochromator. Once the characteristics of the spectra are known, lower concentrations of serotonin can be detected by the use of filters substituting the monochromator and by placing the photomultipliers closer to the emission source. In such conditions, the capture of light will increase and lower concentrations will be resolved.

A non-explored aspect in this study is whether experimentally-produced photoproducts have a deleterious or pharmacological effects on the living tissue. Preliminary experiments capturing the green fluorescence for periods of illumination up to two hours have shown that neurons remain alive and active [40]. However a similar observation does not exist yet for the blue emission.

5. Conclusions

We studied the fluorescence of serotonin in response to near infrared multiphoton excitation at different serotonin concentrations, excitation wavelengths and laser powers. We found three fluorescence emissions with different excitation wavelength-, concentration- and power-dependencies. The most efficient wavelength to produce these emissions in the same solution is 700 nm. The green broadband emission was markedly larger than the violet and blue emissions and appeared in response to all the excitation wavelengths tested. Its characteristics are such

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of a similar emission reported by Shear et al. [15] in response to longer infrared wavelengths. The blue fluorescence was produced by a serotonin photoconversion to produce a relatively stable compound. Our experiments, made under physiological ionic compositions and pH, were planned to be useful for quantitative studies of serotonin localization and dynamics in living nerve tissue. The simultaneous collection of both emissions may allow accurate quantitation of the serotonin concentration. We present a geometric multivariable display method for the quantitative representation of the data of each emission. Such a procedure allows to select the most appropriate conditions to create experimental tools for the study of serotonin.

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Disclosures

All authors declare that they have no conflicts of interest related to this article.

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40. M. Menz, F. F. De-Miguel, and S. Baccus, Instituto de Fisiología Celular-Neurociencias, Universidad Nacional Autónoma de México. Circuito Exterior, Ciudad Universitaria, 04510, D. F., México. are preparing a manuscript to be called "Release of serotonin from the neuronal soma visualize upon multiphoton excitation".