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Intracavity enhancement of GFP fluorescence induced by femtosecond laser pulses

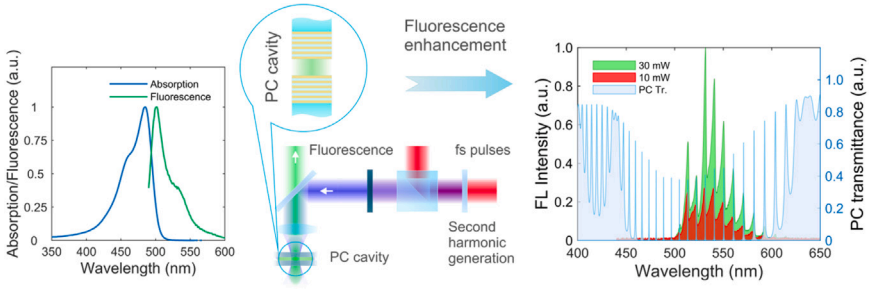
Sofiya A. Vyunisheva [a,b,*], Sergey A. Myslivets [a,c], Nikolay N. Davletshin [a,c], Elena V. Eremeeva [d,1], Eugene S. Vysotski [d,1], Igor N. Pavlov [b,e], Andrey M. Vyunishev [a,c,**]

[a] Kirensky Institute of Physics, Federal Research Center KSC SB RAS, Akademgorodok 50, Bldg. 38, Krasnoyarsk, 660036, Russia
[b] Sukachev Institute of Forest, Federal Research Center KSC SB RAS, Akademgorodok 50, Bldg. 28, Krasnoyarsk, 660036, Russia
[c] Institute of Engineering Physics and Radio Electronics, Siberian Federal University, Academician Kirensky Str. 26, Krasnoyarsk, 660074, Russia
[d] Institute of Biophysics, Federal Research Center KSC SB RAS, Akademgorodok 50, Bldg. 50, Krasnoyarsk, 660036, Russia
[e] Department of Chemical Technology of Wood and Biotechnology, Reshetnev Siberian State University of Science and Technology, Mira Ave. 82, Krasnoyarsk, 660049, Russia

HIGHLIGHTS

- GFP provides high biocompatibility and bioabsorbability.
- Laser-induced fluorescence allows decreasing the detection limit of bioassays.
- Use of photonic crystal microcavity leads to fluorescence enhancement.
- Femtosecond pulses avoid thermal effects and preserve the viability of biosamples.
- Combining these approaches expands traditional methods of laser-induced fluorescence.

GRAPHICAL ABSTRACT



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ABSTRACT

The phenomenon of fluorescence is widely used in molecular biology for studying the interaction of light with biological objects. In this article, we present an experimental investigation of the enhancement of laser-induced fluorescence of *Clytia gregaria* green fluorescent protein. The laser-induced fluorescence method applied in our work combines the advantages of femtosecond laser pulses and a photonic crystal cavity, with the time dependence of the fluorescence signal studied. It is shown that a green fluorescent protein solution placed in a microcavity and excited by femtosecond laser pulses leads to an increase in fluorescence on the microcavity modes, which can be estimated by two orders of magnitude. The dependences of fluorescence signal saturation on the average integrated optical pump power are demonstrated and analyzed. The results obtained are of interest for the development of potential applications of biophotonics and extension of convenient methods of laser-induced fluorescence.

1. Introduction

Fluorescence has found vast application in various applied biological and biomedical research [1–4]. Laboratory methods of analysis based on the phenomenon of fluorescence are characterized by high sensitivity and biological compatibility with living organisms, which allows their use in molecular diagnostics and studies of transcription

* Corresponding author at: Kirensky Institute of Physics, Federal Research Center KSC SB RAS, Akademgorodok 50, Bldg. 38, Krasnoyarsk, 660036, Russia.
** Corresponding author.
E-mail addresses: s.vyunisheva@iph.krasn.ru (S.A. Vyunisheva), sam@iph.krasn.ru (S.A. Myslivets), kdavletshin@iph.krasn.ru (N.N. Davletshin), l_eremeeva@mail.ru (E.V. Eremeeva), eugene_vysotski@ibp.ru (E.S. Vysotski), forester24@mail.ru (I.N. Pavlov), vyunishev@iph.krasn.ru (A.M. Vyunishev).
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and translation mechanisms [2,5,6]. Owing to the advanced optical microscopy methods of analysis, such as confocal [1] and multiphoton ones [7,8], possibility has come to study many protein cellular structures with high spatial resolution. The development of new fluorescent labels [2,9,10] based on green fluorescent protein (GFP) and its analogues made it feasible to obtain high-contrast images with controlled depth of field, and the development of fluorescent probing methods paved the path to detailed study of the chemical composition of living cells [11], as well as its changes in time and space, which initiated fluorescent molecular imaging [3,12].

In molecular biology, the green fluorescent protein reporter gene is widely used, the expression of which is manifested by the appearance of a green fluorescent glow. Since the discovery [13] and sequencing [14] of the GFP gene in 1992, GFP and its homologues have become universal luminous markers for studying expression mechanisms [15]. Chimeric proteins encoded by a combination of several genes containing a fluorescent label are used in molecular diagnostics and therapy. The main features of fluorescent proteins are the ability of autocatalytic formation of a fluorophore inside the cell without the participation of external enzymes, resistance to the actions of cellular proteases, and low toxicity, which opens the way to using these proteins in a variety of organisms without affecting the functional activity of living systems [5]. The structure of the protein is a β-barrel, resembling a cylinder, inside of which a fluorophore is hidden [16]. The shell of the cylinder protects the fluorophore from quenching of its fluorescence by the components of the microenvironment. The excellent properties and diverse applications of green fluorescent protein make it an interesting subject for fluorescence enhancement studies using photonic crystal structures.

Laser sources have become a convenient tool for solving urgent problems of biophotonics. Femtosecond laser sources are widely used in bioinspired techniques because of their advantages over continuous wave and nanosecond ones laying in two aspects. Firstly, the duration of the femtosecond pulses results in a higher peak intensity. Laser pulses can also be focused into a micrometer spot, achieving extremely high radiation power density. Secondly, the most significant property of ultrashort optical pulses while operating with biological systems is the absence of undesirable thermal effects, since for such a short time of pulse exposure, the biological sample does not undergo a noticeable thermal effect in the volume, which positively affects the viability of biosamples. The latter circumstance is of critical importance while working with biological objects.

In photonics, periodic structures called photonic crystals (PCs) are of great use [17,18]. The characteristics of photonic crystals are very sensitive to changes in their material parameters and their microenvironment, which determines their wide use in biosensorics. A microcavity based on such structures provides spatial localization of light and a sufficiently high Q-factor, in some cases reaching 10^6.

Of great interest is a method of laser-induced fluorescence for biological objects to be studied. At present the laser-induced fluorescence method of studying organisms with weak luminescent properties and samples with insufficient fluorescent signal owing to low fluorophore concentration is made use of. Thus, enhancement of the fluorescent signal in various bioassays will reduce the limits of detection of biomarkers with low concentrations in the sample. The amplification of fluorescence arising in the microcavity under the action of laser pulses provides the gain of the useful signal. With the properties of the PC microcavity varied, the number of the fluorophores used is not limited only to fluorescent proteins, but also synthetic organic fluorophores with different spectral characteristics. Using PC, one can increase the photon yield due to the mechanism of selective excitation. Combined with the simplicity of microcavity assembly and compatibility with existing biodetection tools, the microcavity laser-induced fluorescence method is expected to find wide applications in early biomarker detection, biosensing and lab-on-chip engineering.

In this work, we report on the enhancement of femtosecond laser-induced GFP fluorescence in a Fabry–Perot microcavity, which can lead to the sensitivity improvement of biosensors and the development of biophotonic applications.

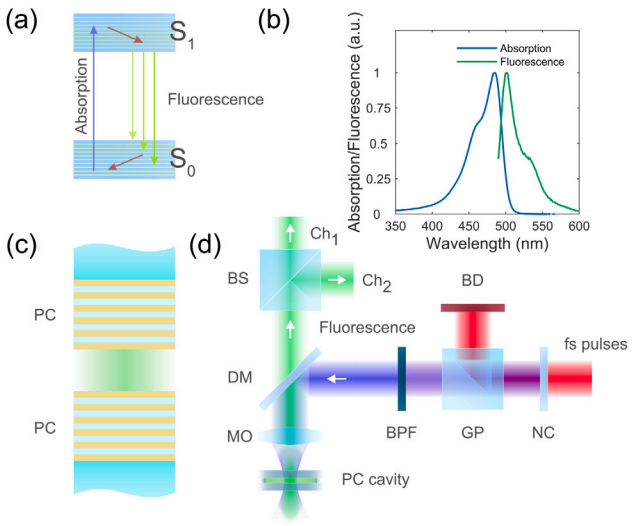

Fig. 1. (a) Expected energy level system of GFP; (b) Absorption and fluorescence emission (excitation at 488 nm) spectra of GFP solution; (c) Design of PC microcavity consisting of two PC mirrors separated by polystyrene microspheres; (d) Experimental setup for excitation and detection of fluorescence from GFP solution embedded into PC cavity. MO, microscope objective; DM, dichroic mirror; BS, beam splitter; Ch, channel; BPF, band pass filter; BD, beam dump; GP, Glan prism; NC, nonlinear crystal.

Materials and methods

GFP solution

Recombinant GFP from *Clytia gregaria*, which is an obligate homodimer [19], was used in all experiment. For GFP production, *Escherichia coli* cells XL1-Blue carrying the pQEcgreGFP25 plasmid [20] were cultivated at 37 °C in vigorously shaken ampicillin-containing LB medium until an OD_{600} of 0.8 was reached. To induce GFP synthesis, 0.5 mM IPTG was added and cultivation was continued overnight at 20 °C in order to obtain a fully maturated protein. GFP was purified from supernatant as described elsewhere using affinity chromatography [20]. Afterwards GFP was transferred in 50 mM phosphate buffer pH 7.0, concentration was 37 μM.

When optically pumped, the GFP solution is likely to form a quasi-four-level energy system (Fig. 1(a)), in which the ratio between radiative and non-radiative processes defines the fluorescence enhancement in the microcavity. In contrast to the wild-type protein, isolated from jellyfish *Aequorea victoria* and having two absorption peaks at 395 nm and 475 nm due to the neutral and ionized forms of the chromophore, respectively, the *cgre*GFP is a natural variant of the GFP, with a significant shift to the longer wavelength region of the spectrum. The *cgre*GFP has an absorption peak at 488 nm owing to only the ionized form of the chromophore and a maximum fluorescence yield in the range of 510–525 nm as shown in Fig. 1(b). Media molecules can be in two types of states: singlet (S_0, S_1 and S_2) and triplet (T_1 and T_2) [21]. To note, singlet–singlet and triplet–triplet transitions are processes of greater probability than singlet–triplet and triplet–singlet ones, that is determined by the value of the total spin of all excited molecules. The difference in photon energy resulting from the spontaneous transition of an electron from an excited singlet state S_1 to the upper sublevels of the ground state S_0 (so-called Stokes shift) is spent on non-radiative relaxation processes, turning into heat. As a result, a photon is emitted with a lower energy and a shift to a longer wavelength region of the spectrum. Nevertheless, at a high pump power, feasibility of transitions to the S_2 level and non-radiative relaxation from the S_1 level to the T_1 level exists, leading to lengthening the decay time of fluorescence.

PC microcavity

The photonic crystals used in our work are one-dimensional structure with a periodically changing refractive index, which allows to obtain photonic band gaps (PBG) for incident light, that is, the frequency range in which a PC effectively reflects incident light. A system of two plane-parallel mirrors forms a microcavity which optical axis is orthogonal to the surface of the mirrors as shown in Fig. 1(c). Photonic crystal under study consist of five bi-layers from (TiO_2-SiO_2) on BK9 glass substrate, covered with additional TiO_2 layer. The thicknesses of the layers are 53 and 98 nm for TiO_2 and SiO_2, respectively. It results in photonic band gap with the central wavelength of 520 nm and the full bandwidth at 50% transmittance of 170 nm (Fig. 2). To ensure the required gap between the resonator mirrors, 10-μm polystyrene microspheres (Polymer Latex, Russia) are placed as spacers. The microcavity made provides the possibility of fine adjustment directly during the experiment. If the cavity has been well adjusted, then fluorescence peaks have appeared corresponding to the microcavity modes. It is microcavity modes appeared in resonator that give the ability to accumulate a fluorescence signal, thereby amplifying it as it propagates along the optical axis of the cavity. Microcavity modes provide amplification of the fluorescence signal at certain wavelengths within the fluorescence band of the fluorophore. The spectral width of the modes lets to roughly estimate the quality factor of the cavity. In our experiment, a solution of green fluorescent protein, the source of the fluorescent signal, is placed in the cavity in order to amplify the fluorescence signal with the help of the resonator.

Experimental layout

As an optical pump source, we use the second harmonic of a femtosecond laser (Tsunami, Spectra-Physics) (wavelength 400 nm, full width at half maximum (FWHM) 4 nm, 140 fs, 80 MHz) generated in a beta barium borate (BBO) crystal cut in the phase matching direction for ooe-type interaction. The scheme of experimental setup is presented in Fig. 1(d). The radiation power of the second harmonic is varied from 1 to 40 mW using a Glan prism and a polarizer. The residual radiation at a wavelength of 800 nm is filtered out using a band pass filter and, as a consequence, processes induced by two-photon excitation are completely excluded. This radiation is directed using a dichroic mirror through a 20× objective with a numerical aperture 0.42 (Mitutoyo) to the microcavity in such a way that the focal spot is inside the microcavity in the area filled with GFP solution. This is confirmed by the smallest diameter of the fluorescence spot. The generated GFP fluorescence signal propagates in the opposite direction with respect to the incident signal and is collected by the same objective. Then the fluorescence signal is sent through a dichroic mirror and a filter with transmission in the long wavelength region to the registration unit, which is a two-channel system. The fluorescence signal, passing through the beam splitter, is recorded by a CCD array, which allows us to observe and monitor the object under study. The second channel provides the possibility of installing a photodetector for measuring the energy and time characteristics of the signal, or a collimator of optical fiber coupled with spectrometer (HR4000, Ocean Optics Inc.) to register the spectral characteristics.

2. Results and discussion

In our work, we used two modes of operation (i) a focused beam into the focal plane located inside the microcavity and (ii) a defocused one. We have done most of the work under conditions of a defocused beam, allowing us to record the energy characteristics and individual spectra. Moreover, the defocused beam mode avoids the unwanted effects of photobleaching in the absence of the ability to reduce the high pulse repetition rate of the excitation radiation (up to about 10^8 pulses per second). Fig. 2 shows the fluorescence spectra of GFP in

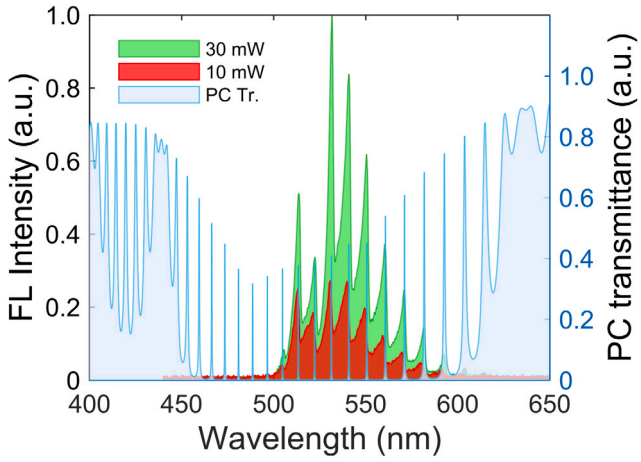

Fig. 2. Measured fluorescence emission spectra of GFP embedded in PC microcavity for specific excitation powers (left axis) and calculated transmittance of PC microcavity (right axis).

a Fabry–Perot cavity at various levels of excitation power. It can be seen that a series of peaks appear in the GFP fluorescence area, the spatial position of which coincides with the spatial position of the cavity modes, which indicates an increase in the fluorescence signal in the system under consideration. For the best agreement between calculated and measured modes the gap width (gap between the cavity mirrors) has been chosen as 10.84 μm. In the calculations the imaginary part of GFP solution refractive index has been assumed accounting for the GFP molar extinction coefficient of $64 \cdot 10^3$ at the wavelength 485 nm. The FWHM of an individual peak allows rough estimation of the quality factor (Q-factor) of the microcavity, which is defined by the simple formula $Q = \lambda/\delta\lambda$ [22]. The FWHM of peak is 3 nm at a wavelength of 530 nm, from these considerations, the quality factor of the cavity can be estimated at ≈177 at specific wavelengths coinciding with the cavity modes. At the same time, the calculated Q-factor of the microcavity is $\approx 4.4 \cdot 10^3$ at the single mode wavelength of 531 nm coinciding with the maximum spectral peak of fluorescence. The discrepancy between calculated and measured Q-factors is explained by broadening of measured peaks of the fluorescence spectra. Moreover, the asymmetry of the measured peaks in the short wavelength range of the spectrum has been observed. In our opinion, this is due to the broadening of the angular spectrum when operating in the out-of-focus mode. This is similar to the case when light incident at an angle on the microcavity results in a blue shift of the microcavity modes.

As mentioned above, the fluorescence enhancement manifested by the peaks has been observed only in a well adjusted microcavity. The mode structure of the microcavity indicates an increase in the fluorescence signal. Furthermore, we have performed a series of experiments with a drop of a GFP solution deposited on a glass substrate and excited by femtosecond laser pulses at a wavelength of 405 nm in the same experimental setup. In this case, the signal has been impossible to register, since it has been very weak. This allows us to conclude that the fluorescent signal is enhanced in PC microcavity.

To realize an essential enhancement of fluorescence, the lifetime of the excited state of the fluorescent medium ought to be greater than the repetition time of following pulses. The fluorescence lifetime is estimated at 2.78 ns [19], and the pulse repetition frequency in our system is 80 MHz, which corresponds to a time interval between pulses of 12.5 ns. The repetition time of following pulses significantly exceeds the lifetime of GFP fluorescence, and, as a result, the next pulse enters the system after the latter relaxes to the ground state. In this regard, necessity comes to increase the lifetime of excited state. The use of a microcavity with a high Q-factor makes it achievable to increase the lifetime of the excited state. The single round trip time of the cavity

can be evaluated by the formula $t = 2dn/c$, where d is the length of the microcavity gap, n is the refractive index of the medium (since the protein concentration in the solution is low and is about 37 μM, the refractive index of the solution is not too different from the refractive index of water and is equal to ≈1.35 for the GFP solution [23]), c is the speed of light. In our case, this time is regarded to be about 100 fs, which is comparable to the duration of a single pulse of 140 fs. In order for the amplification effect to be considerable, multiple passes of the cavity with radiation are required to make the media remain in the excited state.

The measured spectra, shown in Fig. 2, are determined by two factors. On the one hand, the spectra consist of the set of maxima corresponding to cavity modes. Light entering the cavity reflects repeatedly from the mirrors and interferes with the formation of characteristic peaks in the distribution of the light field at specific frequencies. Radiation at other frequencies, suppressed by destructive interference, leaks the cavity. The Fabry–Perot cavity modes follow equidistant in the frequency scale, where the modes located in the vicinity of the edge of the photonic band gap of the photonic crystal have a higher amplitudes for obtaining enhancement of the GFP fluorescence. On the other hand, the GFP fluorescence spectrum, which has a peak at 532 nm (Fig. 2), is determined by the absolute maximum of the fluorescence intensity in this region of the spectrum. What is more, the quality factor of the cavity is considered to be of the greatest value in the center of the photonic band gap, leading to an increase in the fluorescence lifetime and a peak elevation in the spectrum. Therefore, amplification of the fluorescent signal in the cavity mode near the 514 nm is explained by the nearby edges of the photonic band gap, which, as it is said, is the cause of enhancement realization in the system studied. To note, when excited by femtosecond pulses at the wavelength of 400 nm, enhancement occurs at the same spectral region while exciting by 488 nm pulses, which may evidence about absence of the transition to higher singlet states, i.e. $S_0 \rightarrow S_2$, frequency of which lies in a shorter wavelength region. A possible transition of the excited molecules accumulated at the T_1 level to the T_2 level might significantly reduce the gain and lead to a drop in the fluorescent signal. To cut down on the undesirable effects of these processes and the photodegradation of the medium, we are forced to move the microcavity in the focal plane, simulating a flow system with "renewal" and cooling of the medium.

Fig. 3 shows the dependencies of the fluorescence signal intensity on the average integral excitation radiation power. The dependence corresponding to a defocused beam shows a linear fit, smoothly turning into a plateau, indicating saturation of the system. A sharp rise has not been expected to appear, but we could not increase the excitation power to further investigate the process in this range. In the case of a focused beam, one can observe a faster growth of the curve, which also changes with saturation, at a pump power of 10 mW, which corresponds to the peak power density 0.28 GW/cm^2.

The dependence of the intensity of the fluorescence signal on time in the case of a defocused beam is shown on Fig. 4. In the continuous mode of excitation with a power of 36 mW, the signal is approximated according to the bi-exponential law $I(t) = a\exp(-\gamma_1 t) + b\exp(-\gamma_2 t)$, in which $\gamma_{1,2}$ are the rates of decay, a, b are coefficients ($a = 0.4987, b = 0.4411$). The values of γ_1 and γ_2 are the reciprocals of the corresponding decay times, which are $\tau_1 = 2.7$ s and $\tau_2 = 87$ s, respectively. These lifetimes are expected to be addressed to two reasons. Firstly, on account of the high pump power, photobleaching of the fluorophore takes place, with the brightness of the protein luminescence decreasing. Secondly, the accumulation of excited molecules in the triplet state T_1 is possible as a result of the transition $S_1 \rightarrow T_1$, and the transition from the level T_1 is probable both to the higher triplet state T_2 ($T_1 \rightarrow T_2$) and the less possible relaxation to the ground state S_0 ($T_1 \rightarrow S_0$).

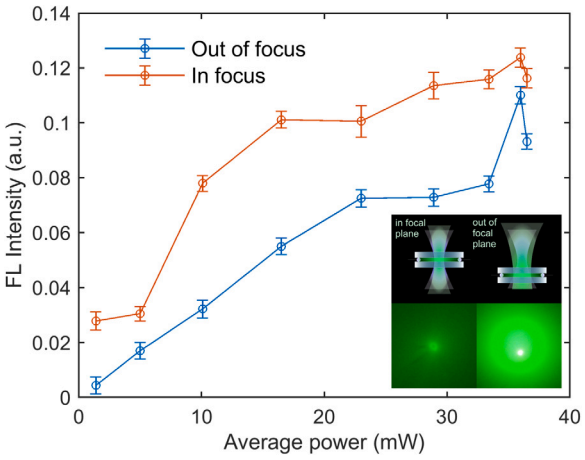

Fig. 3. Integral fluorescence intensity of GFP solution in PC cavity on excitation average power in focus and 240 μm upper GFP solution (out of focus). Inset demonstrates respective configurations and observed images.

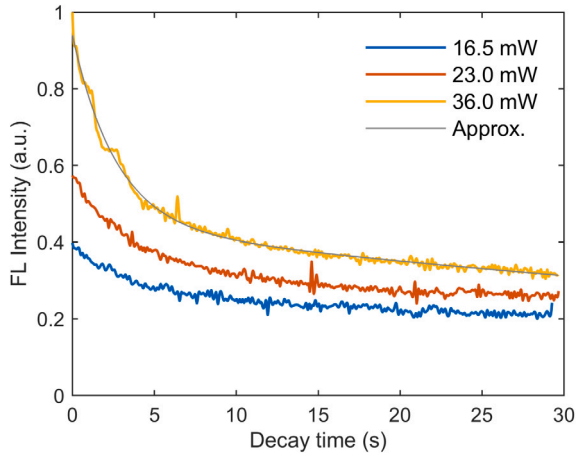

Fig. 4. Temporal dependence of fluorescence intensity of GFP solution in PC cavity for specific excitation average powers.

3. Conclusions

In conclusion, we have demonstrated the enhancement of GFP fluorescence in a microcavity upon excitation by femtosecond laser pulses. Despite of the complex spatially-temporal dynamics of femtosecond laser pulses inside the microcavity, the fluorescence emission spectra of the GFP solution contain a number of peaks corresponding to the increase in fluorescence in microcavity modes. The fluorescence enhancement at the certain wavelengths in this system can be estimated from the measured Q-factor characteristics of the microcavity and reach 177. The efficiency of bioassays can be improved by increasing the signal-to-noise ratio, which, as a result, will lead to the detection of biomarkers at the lowest concentrations. Enhancement of the fluorescence signal at certain wavelengths corresponding to microcavity modes leads to signal accumulation in the microcavity and, consequently, decreasing the detection limit. The spectral information can also be used to spectral separating fluorophores having partially overlapping fluorescence spectra, since the modes generated in the cavity have a smaller peak width than the fluorescence spectral range. There is a great variety of fluorophores which can be used in PC microcavities. For instance, fluorescein derivatives resemble with GFP by having the similar fluorescence spectra, so they can be utilized in

biomedical assays. Microcavities based on photonic crystals are simple, compact and relatively cheap; the high biocompatibility and the special molecular structure of GFP, which provides a high quantum yield and maximum brightness, can be used as an excellent basis for the potential biophotonic applications.

CRediT authorship contribution statement

Sofiya A. Vyunisheva: Experimental study, Data curation, Analysis, Writing – original draft. **Sergey A. Myslivets:** Calculation of PC transmittance, Analysis, Discussion, Editing. **Nikolay N. Davletshin:** Assistance in the experiments, Data curation, Analysis, Discussion. **Elena V. Eremeeva:** Preparation of GFP solution, Analysis, Discussion, Editing. **Eugene S. Vysotski:** Selecting GFP for studies, Analysis, Discussion, Editing. **Igor N. Pavlov:** Discussion, Editing. **Andrey M. Vyunishev:** Conceptualization of study, Project management, Experimental study, Analysis, Discussion, Editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elena V. Eremeeva, Eugene S. Vysotski reports financial support was provided by Russian Foundation for Basic Research.

Data availability

Data will be made available on request.

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