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Control of the blue fluorescent protein with advanced evolutionary pulse shaping

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Abstract

We demonstrate optical coherent control of the two-photon fluorescence of the blue fluorescent protein (BFP), which is of interest in investigations of protein-protein interactions. In addition to biological relevance, BFP represents an interesting target for coherent control from a chemical perspective due to its many components of highly nonexponential fluorescence decay and low quantum yield resulting from excited state isomerization. Using a genetic algorithm with a multiplicative (rather than ratiometric) fitness parameter, we are able to control the ratio of BFP fluorescence to second harmonic generation without a considerable drop in the maximized signal. The importance of linear chirp and power scaling on the discrimination process is investigated in detail.

Keywords

BFP; GFP; two-photon; coherent control; pulse shaping; ultrafast; FRET; multiphoton; genetic algorithm

INTRODUCTION

Through its role as a fluorescence resonance energy transfer (FRET) donor for the green fluorescent protein (GFP), biologists have used the blue fluorescent protein (BFP) to gain deep insight into fundamental processes including protease cleavage, calcium signaling, phosphorylation, and programmed cell death [1]. However, due to its low quantum yield and high rate of photobleaching, most investigators now favor the red-shifted pair of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) despite the fact that the better emission separation of a BFP-YFP pairing gives the greatest decrease in the YFP emission

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upon cleavage of donor from acceptor [2]. It has already been shown that shaping of two-photon excitation pulses is able to reduce the photobleaching rate of various GFP variants [3]. If it would be possible to control the fluorescence of BFP in this manner without a reduction of its already weak signal, this could restore the role of BFP in the field of cell biology. Experiments [4] and calculations [5;6] have shown that the efficient quenching of the excited state of BFP stems from the excitation-induced isomerization of the chromophore, which makes BFP an interesting target for coherent control. Ideally, control of the evolution of the excited state wavepacket could steer the BFP chromophore away from undesirable isomerization pathways in favor of fluorescence.

Here we control the fluorescence of BFP by shaped femtosecond excitation pulses. At low pulse energies, adaptive learning algorithms seeking to maximize quantum yield converge on transform-limited pulses, consistent with results of dyes investigated previously [7]. Thus, we follow the tradition of employing an additional two-photon process, second harmonic generation (SHG), in the fitness parameter of the search [8]. This proves to be a difficult control problem, and a ratiometric fitness parameter of two-photon fluorescence / SHG has already been shown to be incapable of discriminating the two second-order processes in BFP [3]. However, employing a multiplicative fitness parameter and controlling for factors such as power-scaling differences, dispersion mismatches in different arms of the experiment, and photobleaching, we are able to differentiate the two processes to nearly a factor of three.

EXPERIMENTAL SETUP

Samples

BFP was purchased from QBiogene (“superglo” sgBFP, Montreal, Canada), thawed, and immediately used in its original concentration of 0.5 $\mu\text{g}/\mu\text{L}$ in TE buffer, pH 8.0.

Optical layout

The experimental setup is shown in Figure 1. The laser system consists of a regeneratively amplified Ti:sapphire system (RegA, Coherent, Santa Clara, CA) providing up to 4 μJ , 50 fs pulses at 250 kHz with a center wavelength of 800 nm and 27 nm FWHM bandwidth. The “Dazzler,” an acousto-optic programmable dispersive filter (AOPDF) (Fastlite, Palaiseau, France), is used to shape the laser pulses [9]. Diffracted power and polynomial phase to fourth order are the most basic control parameters. Additionally, arbitrary amplitude and phase modulation can be accomplished by binning the pulse spectrum into numerous (typically 20 to 100) frequency bins and giving each bin an arbitrary amplitude and phase.

A glass coverslip reflects part of the laser beam from a onto a photodiode to give a reference value for incident power I_{ref} . Normalization to this value measures the efficiency of each two-photon process signal S as the ratio S/I_{ref} . Another beamsplitter divides the beam into two arms. The first arm is focused into the cuvette holding the dye sample, and the fluorescence signal I_{flr} is collected in the perpendicular direction by a photomultiplier tube (PMT). In the other arm SHG from a beta barium borate (BBO) crystal gives the signal I_{SHG} . All three signals I_{ref} , I_{flr} , and I_{SHG} are read in parallel by a DAQ board synchronized by the Dazzler trigger.

After measuring fitness with a specific individually shaped pulse, the adaptive learning algorithm programs the next individual, closing the loop for the iterative improvement.

After the experiment, optimal pulse time-frequency characteristics were measured with a commercial SHG-FROG unit [10] (Grenouille 8–50 from SwampOptics, Atlanta, GA).

Time-domain two-photon excitation cross-section measurement

Using the same experimental setup, we implemented a time-domain measurement of the two-photon excitation cross-section spectrum of BFP in the region of interest. This was performed analogously to the description by Ogilvie and colleagues [11], except a virtual Michelson interferometer was used. A delay of t_0 between pulses is achieved with a cosine amplitude mask $\cos(\omega t_0/2)$ in the frequency domain, with ω the laser carrier frequency.

Experimental considerations for adaptive learning convergence - power and chirp scaling

Before each experiment, the optical system and laser power were adjusted to ensure quadratic scaling of discriminated two-photon processes with I_{ref} . Scattered excitation light reaching the fluorescence-detecting PMT, for example, would result in subquadratic scaling. As another example, if I_{flr} is approaching saturation while I_{SHG} is still in the quadratic region, then this can trivialize the convergence to mere pulse stretching. With proper attenuation, typical diffracted pulse energies after the Dazzler used during experiments were estimated to be 2 nJ. Assuming a diffraction-limited beam waist, we estimate a typical fluence on the sample at the laser focus on the order of 0.5 mJ/cm² (peak intensity on the order of a GW/cm² transform-limited).

Even if the incident laser power is unvarying in the course of the adaptive learning algorithm, true control possibilities could still be masked by uninteresting convergences with simple linear chirp (second order polynomial phase) adjustments. For example, two-photon fluorescence in the saturation region would not decrease under increasing chirp, while the increased pulse duration would diminish the unsaturated SHG signal. If the two-photon fluorescence emission has a short enough lifetime to be saturated by peak laser intensity rather than the pulse fluence, the two-photon signal could actually increase with the increased group delay dispersion. Another case in which linear chirp could mask control is when the dispersion in the different materials in the two optical paths is not exactly matched. In this case the optimal pulse shape would be found simply by tuning to compensate dispersion differences.

Algorithmic considerations for adaptive learning convergence

A genetic learning algorithm (GA) is used to find the optimal pulse shape. Amplitude and phase settings for the Dazzler define are used as “genes” to define a specific individual laser pulse. The algorithm usually is set to place physical constraints on the allowable phases, i.e. the derivative of phase with respect to frequency is limited so the length of the acoustic wave is shorter than the crystal length. At the beginning, the algorithm generates a random population of 50 individuals. This population is ranked via a fitness parameter, and the best 10 individuals are passed unmodified to the next generation. Remaining individuals of the new generation are created by mating and mutation of fitness-preferenced individuals of the preceding generation. Our implementation relies on two-point crossover and mutation operators similar to those already described by Pearson *et al.* [12]. We additionally scale the mutation rate by the homogeneity of the preceding generation to help avoid local minima and use a circular chromosome to avoid edge effects. Including measurements and creation of the subsequent generation, a 50 individual generation takes on the order of 25 seconds to run. Almost all of this is calculation and implementation time of the novel acoustic waveform, typically in excess of 400 ms. For imaging applications this delay would be removed by precalculation of the optimal waveform.

To use pulse shaping to discriminate different two-photon processes, many investigators use as a fitness parameter the ratio of two second-order processes A/B, for example with A as two-photon fluorescence and B as SHG [3;8]. As the two-photon signals can drop significantly for example with changing pulse width, as affected by chirp, this approach can easily lead to low signal levels with degree of discrimination dictated by the arbitrarily set low-signal limit. To

avoid bias towards the noise-dominated low-signal regime, we maximize a ratio A/B with the fitness parameter $(A-c)(c^{-1}-B)$, where values of A and B are normalized to the unshaped pulse measurement. We have found that setting c to 0.8 yields better convergence relative to the original implementation of unity for c [13]. If both multiplicands are negative, an infinite negative fitness is given to the pulse. If either multiplicand alone falls below 0, the inverse of the other is taken. This will ensure for example that if B exceeds c^{-1} by a fixed amount, decreases in the maximized signal A towards c result in a decreased (more negative) fitness. As a negative fitness will result whenever the maximized signal A falls below c , the search is strongly biased to enable the maximized signal to surpass that of an unshaped pulse.

RESULTS

With experimental and algorithmic considerations above, we control two-photon fluorescence of BFP relative to SHG. Figure 2 shows the convergence of the GA for the parallel maximization and minimization experiments. Only polynomial phase to 4th order was adjusted until generation 20. Most of the improvement in discrimination occurs only after this, when arbitrary phase (and no amplitude) adjustments were permitted to the adaptive learning algorithm, with 20 frequency bins over 60 nm of bandwidth. The phase manipulation was constrained for delays in the TeO₂ crystal between 1000 and 3000 fs. The highest achieved ratio of the two-photon processes (normalized to the unshaped pulse) is 1.42 whereas the minimum in the parallel experiment is 0.44. Figure 2C confirms consistency of the $I_{\text{flr}}/I_{\text{SHG}}$ ratio with linear chirp adjustment. Excluding the points adjacent to 0 chirp (with almost no diffracted power), the ratio can be controlled at most by a factor of 1.45 over the full range of this one-parameter scan. Arbitrary phase shaping by the genetic algorithm, however, was able to control the ratio of two-photon fluorescence from BFP to SHG to a factor of 3.27 (maximum to minimum).

We also wanted to understand how applicable and reproducible our method of excitation might be after several experiments on the same BFP sample at room temperature. Thus we repeated the initial experiment and variations thereof for almost continuous excitation over the course of more than 24 hours. A comparable degree of control (maxima and minimum $I_{\text{flr}}/I_{\text{SHG}}$ 1.38 and 0.49 for a total control factor of 2.83) was still achieved in the repetition of the original experiment two days later, despite realignment of the system causing slight differences in the power-law and chirp-dependences (maxima and minima 1.383 and 0.489 for a total control factor of 2.83). Thus we conclude that our control attempts are very reproducible, both from the standpoint of the pulse search convergence, but also from the standpoint of possible physical changes in BFP during extended investigations.

We tried many variations of the GA to see how details of our implementation influence the result. The most complicated aspect of the GA programming is the constraint on the control parameters of the Dazzler, which ensures that the acoustic wave envelope does not exceed the crystal dimensions. An immediate repetition of the initial experiment in the same alignment condition without imposing physical constraints on the phase weakened the result noticeably (maximum and minimum $I_{\text{flr}}/I_{\text{SHG}}$ 1.31 and 0.54 for a control factor of 2.43). Immediate repetition with the original (physically constrained) algorithm and with the added ability for amplitude modulation had a somewhat smaller but also appreciable negative effect on the final result (ratios 1.42 and 0.57 for a control factor of 2.49). We note that adding amplitude modulation doubles the search space of the GA. The most deleterious effect to control was seen by maintaining the optimal experimental conditions of Figure 2, but quintupling the GA search space by increasing only the number of frequency bins of phase control from 20 to 100 over the 60 nm programmed bandwidth. This brought the control factor down to 2.166. Thus we conclude that the most important factor in our success was keeping the lowest possible search space for the algorithm consistent with the physical process responsible for the control.

As further evidence of the low density of optimal solutions to the BFP control problem, Figure 3A shows the results of the GA search experiment with algorithm almost identical to that for Figure 2. Slightly more scattered excitation light from the cuvette results in a drop in the power scaling factor of the BFP signal I_{flr} relative to I_{ref} from 1.63 to 1.51. As a result, the control is eliminated. The best control factor of 1.57 (1.14 and 0.73 maximizing and minimizing pulse $I_{\text{flr}}/I_{\text{SHG}}$ ratios, respectively) at the end of the run are almost the same as that seen in the first generation. In the failed experimental alignment, linear chirp adjustment made the optimal minimizing pulse's $I_{\text{flr}}/I_{\text{SHG}}$ ratio identical to that of the maximizing pulse. For control problems with a robust solution, the increased scattering does not, however, hurt convergence. Immediately following the failed experiment, the SHG arm was routed via a flip mirror to focus tightly in a cuvette of DiI, with a power-law scaling of two-photon fluorescence to I_{ref} at 1.81, not far from the 1.83 power law scaling in our SHG detector. As shown in Figure 3B, maximization of the ratio of two-photon fluorescences of BFP to DiI yielded a factor of three discrimination for this robust discrimination problem (1.21 vs 0.41). As seen in the first 30 generations of the experiment, this problem does not map to polynomial phase modulation.

Figure 4 shows the time-domain measured two-photon excitation cross-section spectra of BFP and DiI as well as the second harmonic power spectra of the optimal pulses for maximization and minimization of the ratio of BFP two-photon fluorescence to SHG.

DISCUSSION

Maintaining a minimal search space for the adaptive learning algorithm was the most important factor in successful discrimination of BFP from SHG. This observation, consistent with our investigations in other systems, underscores the difficulty of the control problem in BFP. The control landscape in the presence of noise is evidently dominated by suboptimal solutions, and most modifications to the search algorithm spread the parameter space to include more regions of inferior control. Pulse shapes not physically confined within the crystal have little probability of successful control. Phase adjustments are known to offer better control of the second harmonic spectrum of the laser pulse than amplitude modulation [14], which coincides with decreased control with the addition of the latter. Finally, high frequency resolution in the two-photon fluorescence problem at hand does not offer benefit sufficient to outweigh the disadvantages of the expanded phase space.

Notably, the multiplicative fitness parameter ensures that the signal of the favored two-photon process is comparable to, or significantly higher than that with the unshaped pulse. For example, in Figure 2B, for the maximization of BFP / SHG signal, the value of BFP two-photon fluorescence efficiency $I_{\text{flr}}/I_{\text{ref}}$ normalized to the unshaped pulse converges on values above one (closed green triangles). In the parallel minimization experiment, however, $I_{\text{SHG}}/I_{\text{ref}}$ exceeds one when thus normalized (open blue circles). This is important for practical applications where signal-to-noise ratio (SNR) is a typical limiting factor. The large generational variations in the signal level of the best pulse (Figure 2B) concurrently with a consistent trend in the ratio of the discriminated processes (Figure 2A) emphasize the independence of the GA search result from signal strength. We were unable to elicit control of BFP with identical experimental conditions when we substituted the ratiometric for the multiplicative fitness parameter. In those experiments, the maximized signal dropped well below that of the unshaped pulse, consistent with previous findings [7]. This distinction underscores the value of the multiplicative fitness parameter for practical SNR-limited applications.

Second harmonic spectral tuning accounts for control of BFP

The mechanism of control of BFP becomes evident by considering the relation of the second-harmonic spectrum $S_2(\omega)$ of the shaped pulse to the spectrum of the two-photon excitation

cross-section $\sigma^{(2)}(\omega)$ of BFP (Figure 4). Assuming no intermediate resonant levels between the ground and excited state, the two-photon fluorescent signal will be proportional to the overlap integral of $\sigma^{(2)}(\omega)$ and $S_2(\omega)$ [8]. Using the overlap integral, Ogilvie and colleagues demonstrated excellent quantitative agreement between the time-domain measurement of $\sigma^{(2)}(\omega)$ for coumarin and the fitness converged on in a GA-led discrimination experiment [11].

Clearly, maximization tunes $S_2(\omega)$ to regions where the BFP cross-section ratio is maximal, whereas the minimization algorithm converges in the opposite direction. As second-harmonic tuning is best performed with arbitrary, rather than mere 4th order polynomial phase control, it becomes clear why the GA result improves drastically at the algorithmic switch at generation 20 (Figure 2A). The $\sigma^{(2)}(\omega)$ interpretation also explains the robustness of the control of the BFP / DiI two-photon fluorescent ratio. The two-photon excitation cross-section spectrum for DiI shows a much sharper spectral feature, almost a cliff, near the 400 nm second harmonic. Thus, tuning $S_2(\omega)$ will affect the DiI two-photon fluorescence more significantly than for a typical fluorophore.

That second-harmonic spectral tuning accounts for our result implies that the problem can be reduced to second-order perturbation analysis [8], and photon-photon interferences are the fundamental mechanism of control. Despite control of two-photon fluorescence from BFP, at our laser fluences near 0.5 mJ/cm² (2×10^{15} 800 nm photons / cm²), control of the two-photon excited state wave packet of BFP probably does not contribute significantly. This is consistent with the observations of Bardeen and colleagues in YFP, another GFP mutant. Specifically, they found that they could control two-photon fluorescence via reduced stimulated emission by appropriately chirping the pulse, but only with order of magnitude higher photon fluence than we employ [15]. Indeed, it is to be expected that such a pump-dump scheme can only meet with success in far from linear fluorescence, in the saturation region we avoid in our experiments.

Practical significance

This work implies that shaped two-photon excitation could be a valuable tool to enable BFP-based FRET imaging. Due to reduced peak intensity, success in controlling the ratio of two-photon fluorescence to SHG with shaped pulses is sufficient to reduce photobleaching. Shaped pulses therefore have the potential to moderate this shortcoming of BFP. The extent of improvement is difficult to predict theoretically, but a 4-fold increase in fluorescence lifetime was observed with similarly optimized shaped pulses in a different GFP variant [3].

While moving from the single to the multiphoton regime offers benefit for photobleaching, it does not detract the primary advantage of BFP over CFP as a FRET donor for YFP, namely the greater separation of excitation spectra. The two-photon cross-section of YFP is relatively small and flat near our 400 nm second-harmonic wavelength [16]. Therefore its limited fluorescence yield will parallel the yield of SHG. Shaped pulses that optimize the BFP fluorescence to SHG ratio will also selectively excite BFP over YFP. CFP, however, shows no improvement from one-to two-photon excitation in degree of spectral overlap [16].

Finally, energies commonly available from standard femtosecond oscillators are sufficient to create the photon-photon interferences that we have found to control BFP fluorescence. Thus, the same shaping of our kilohertz amplified pulses will enable image acquisition rapid enough for live FRET images with an 80 MHz laser system.

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Figure 1.
Experimental Setup. Lenses are depicted as ovals.

Figure 2.

(A) Control of ratio of BFP fluorescence efficiency to SHG efficiency. Measurements for each generation's best individuals in the maximization are shown with filled markers, whereas the parallel minimization experiment is shown with open markers. All measurements are normalized to the unshaped pulse. Black squares show the ratio $I_{\text{flr}} / I_{\text{SHG}}$. (B) For the same experiment, green triangles show the BFP fluorescence efficiency $I_{\text{flr}} / I_{\text{ref}}$ whereas blue circles mark the SHG efficiency $I_{\text{SHG}} / I_{\text{ref}}$. (C) Linear chirp dependence of two-photon signals in the same experiment. Blue circles show I_{SHG} , green triangles show the Texas Red fluorescent signal I_{ref} , red dots show I_{ref} , and black squares show the dependence of the ratio $I_{\text{flr}} / I_{\text{SHG}}$ ratio on the chirp. Note the drop in diffraction efficiency of the Dazzler when little second-order polynomial phase is applied (0 chirp).

Figure 3.

(A) Failed control of ratio of BFP fluorescence efficiency to SHG efficiency. The ratio of BFP fluorescence efficiency / SHG efficiency is plotted, normalized to the unshaped pulse.

Measurements for each generation's best individuals in the maximization are shown with filled markers, whereas the parallel minimization experiment is shown with open markers. (B) Same experimental conditions, with I_{SHG} replaced by two-photon fluorescent signal from DiI.

Figure 4.

Time-domain measured two-photon excitation cross-section spectra of BFP (cyan line) and DiI (red line) with second harmonic power spectra of the optimal pulses for maximization (black line) and minimization (blue asterixes) of the ratio of BFP two-photon fluorescence to SHG.