Pulse-shaping multiphoton FRET microscopy

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ABSTRACT

Fluorescence Resonance Energy Transfer (FRET) microscopy is a commonly-used technique to study problems in biophysics that range from uncovering cellular signaling pathways to detecting conformational changes in single biomolecules. Unfortunately, excitation and emission spectral overlap between the fluorophores create challenges in quantitative FRET studies. It has been shown previously that quantitative FRET stoichiometry can be performed by selective excitation of donor and acceptor fluorophores. Extending this approach to two-photon FRET applications is difficult when conventional femtosecond laser sources are used due to their limited bandwidth and slow tuning response time. Extremely broadband titanium:sapphire lasers enable the simultaneous excitation of both donor and acceptor for two-photon FRET, but do so without selectivity. Here we present a novel two-photon FRET microscopy technique that employs pulse-shaping to perform selective excitation of fluorophores in live cells and detect FRET between them. Pulse-shaping via multiphoton intrapulse interference can tailor the excitation pulses to achieve selective excitation. This technique overcomes the limitation of conventional femtosecond lasers to allow rapid switching between selective excitation of the donor and acceptor fluorophores. We apply the method to live cells expressing the fluorescent proteins mCerulean and mCherry, demonstrating selective excitation of fluorophores via pulse-shaping and the detection of two-photon FRET. This work paves the way for two-photon FRET stoichiometry.

Keywords: Multiphoton microscopy, FRET, pulse-shaping

1. INTRODUCTION

Fluorescence Resonance Energy Transfer (FRET) microscopy is a familiar technique that has been used extensively in biological studies.[1] Its chief utility is as a "Spectroscopic Ruler," [2] where its sensitive nanometer-scale distance dependence makes it easy to obtain information about the relative locations of fluorophores within a labeled molecule.[1,3,4] FRET has been used for several other purposes as well, including investigating DNA hybridization states, rates of diffusion, and protein interactions at cell surfaces.[1,3,4] Unfortunately, FRET has two key weaknesses which limit its usefulness. First, it can be difficult to obtain quantitative information from FRET studies, due to complications in the placement and orientation of the dye molecules.[1] Second, the required spectral overlap between the dye molecules in FRET confounds separation of the enhanced acceptor emission due to directly excited donor and acceptor fluorescence in the acceptor emission channel.[5] FRET stoichiometry has been demonstrated to correct for the spectral overlap, providing quantitative measures including the relative concentrations of donor- and acceptor- labeled molecules and the fractions of each in complex.[5]

While quantitative one photon FRET methods are relatively mature, quantitative multiphoton FRET methods are still under development. Thus the benefits of multiphoton approaches, including automatic sectioning, increased penetration depth in tissues, decreased photobleaching, and decreased photodamage,[6] have not been fully realized for FRET applications. While multiphoton FRET based on Fluorescence Lifetime Imaging Microscopy (FLIM-FRET) has

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been previously demonstrated, FLIM-FRET often requires several minutes for image acquisition,[7,8] limiting its utility for observing rapid dynamics of cellular processes. Multiphoton FRET stoichiometry has not yet been implemented, largely due to the challenges of performing rapid, multicolor multiphoton imaging: typical femtosecond laser sources must be tuned to excite multiple fluorophores, and the tuning process is slow (~seconds). An attractive alternative is to use broadband lasers, with bandwidths of >100nm. While large bandwidths enable the simultaneous excitation of multiple fluorophores, the lack of selectivity for separate excitation of donor and acceptor species complicates the implementation of FRET stoichiometry. Here we present laser pulse-shaping as a method to resolve the problem of spectral overlap, enabling future applications of multiphoton FRET stoichiometry.

Pulse-shaping methods have previously been used for selective excitation of fluorescent proteins over autofluorescence[9,10] and for the selective enhancement of fluorescence from particular fluorophores.[11–13] Isobe *et al.*[14] have recently used pulse-shaping methods to image a FRET-based calcium indicator. Using pulse-shaping, we tailor the excitation pulse to selectively excite particular fluorophores, which permits multicolor imaging while avoiding the problem of exciting multiple fluorophores simultaneously. Such selective excitation reduces background in FRET measurements caused by direct excitation of the acceptor. Pulse-shaping resolves these problems and provides improved time resolution in addition to selective excitation, as pulse shapes can be changed quickly (~10ms). [15] Here, we demonstrate the use of pulse-shaping for selective excitation of donor (mCerulean) and acceptor (mCherry) fluorescent proteins in live COS-7 cells. For selective excitation of mCherry, we take advantage of the large two-photon excitation cross-section for S_0-S_n excitation[16,17]. We discuss the implications of this excitation scheme to FRET stoichiometry.

2. ULTRAFAST PULSE-SHAPING

Ultrafast pulse-shaping is a growing field - numerous methods have been developed and implemented in areas ranging from optical communications to quantum control.[15] A popular technique is to spatially disperse the pulse spectrum and use an amplitude and/or phase mask to manipulate the pulse characteristics. The advent of computer-controlled spatial light modulators (SLMs) has made this method particularly useful and accessible.[15] Here we implement phase-shaping via a technique called Multiphoton Intrapulse Interference (MII)[18,19] which manipulates two-photon processes.[13]

In two-photon excitation, there are many combinations of frequencies that yield the desired total energy required for excitation. MII pulse-shaping adjusts the relative phase of the individual frequencies in the pulse, creating constructive and destructive interferences between them. This directly affects the probability of two-photon excitation.[18–20] To understand this, consider the fluorescence signal S produced upon two-photon excitation:

$$S \propto g(\omega)\left|E^{(2)}(\omega)\right|^2 \tag{1}$$

where $g(\omega)$ is the two-photon excitation spectrum of the fluorophore.[10,18,19] $\left|E^{(2)}(\omega)\right|^2$ is the second harmonic power spectrum of the laser pulse, [9,10,18–20] which is expressed as

$$E^{(2)}(\omega) = \int_{-\infty}^{\infty} d\omega' \left|E(\omega')\right| \left|E(\omega-\omega')\right| \exp\left\{i\left[\varphi(\omega') + \varphi(\omega-\omega')\right]\right\} \tag{2}$$

A clear dependence on spectral phase can be seen in the interference term of this equation. Consequently, by manipulating the spectral phase function $\varphi(\omega)$, the two-photon excitation signal is directly influenced.

The goal of phase-based pulse-shaping for selective excitation is to set the phase function such that the second harmonic power spectrum closely resembles the fluorophore's two-photon excitation spectrum.[21] Various phase functions have been used, including sinusoidal functions and, to achieve very narrowband shaped spectra, binary functions. [18,19,22] The key property considered when choosing the phase function to apply is symmetry. For a spectral phase that is antisymmetric with respect to a frequency ω', the phase term in equation 2 will go to zero and $E^{(2)}(2\omega')$ reaches its maximum.[10] This maximum is also reached for the case of transform-limited pulses, when $\varphi(\omega) = 0$. For other frequencies, destructive interferences cause $E^{(2)}(\omega)$ to decrease. [10,22,23] Thus, it is possible to choose a phase function that is antisymmetric around a specific frequency, leading to a narrow peak around the desired frequency in the second harmonic power spectrum and permitting selective two-photon excitation.

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As mentioned above, it is possible to achieve very narrowband pulses, with high second harmonic intensity at the desired frequency and little second harmonic signal elsewhere, using a version of MII called binary phase shaping (BPS).[22] This technique relies on the same basic principles as MII, but with the observation that dealing with just two values for the phase, 0 and π, can simplify the process.[22,24–26] This binary phase shaping technique is employed here to achieve selective excitation of fluorophores and detection of two-photon FRET.

3. EXPERIMENTAL METHODS

Figure 1 shows a diagram of the microscopy setup used in this experiment. Pulses from a 75 MHz titanium:sapphire oscillator (Femtolasers Synergy), with 110nm bandwidth centered at 780nm, were phase-shaped in a transmissive 4f pulse-shaper setup. The beam was spatially dispersed by a grating and sent through a CRi phase-only, 640 pixel SLM controlled with Matlab. The shaped beam was then recombined and aligned into the scan head of a Prairie Technologies modified Olympus BX51WI upright microscope for imaging. An Olympus UPlanApo 60x, 1.2NA water-immersion microscope objective was used to focus onto the sample and collect fluorescence signal. Excitation power at the sample was approximately 5.5mW. The signal was then separated from the excitation light and split into two PMT channels for multicolor detection. Multiphoton Intrapulse Interference Phase Scan (MIIPS)[26–29] was used to pre-compensate for dispersion caused by the microscope optics and achieve a transform-limited pulse prior to application of the desired phase mask.

Images were acquired from two sets of live COS-7 cells expressing mCerulean as the donor fluorophore and mCherry as the acceptor fluorophore. The "unlinked" sample was co-transfected with the two individual fluorophores such that cells would express both proteins but have minimal likelihood of FRET between them. The "linked" sample was transfected with a construct of mCerulean and mCherry separated by a linker of 27 amino acids with a measured FRET efficiency of 22.19% by FLIM-FRET.[7,8] COS-7 cells were plated on 35mm tissue culture dishes. Twenty-four hours later, cells were transfected using FuGene HD and allowed 24 hours for transient expression prior to imaging. Just before imaging, the media was removed from the dishes and replaced with pre-warmed Ringer's Buffer. The cells were kept at 37 °C during imaging with a heated stage, and the water-immersion objective was submerged directly into the buffer for image acquisition.

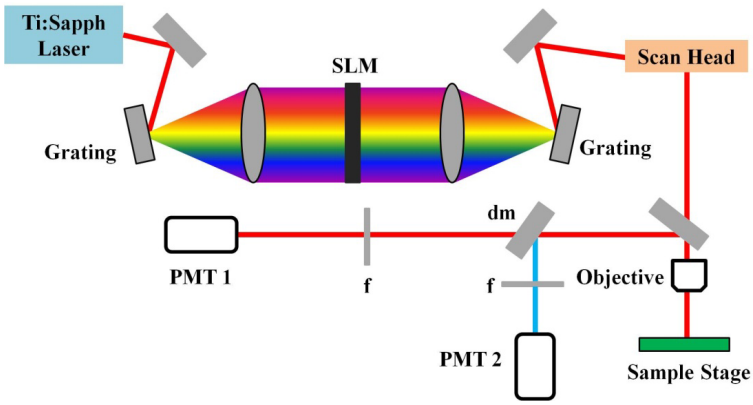

Figure 1: Diagram of the experimental setup. f = filter; dm = dichroic mirror; SLM = spatial light modulator.

Pulse shapes for selective excitation were chosen based on knowledge of the two-photon absorption spectra of mCerulean and mCherry[17,30] and are shown in Figure 2. The mCherry pulse in particular was chosen to take advantage of its strong transition to an excited state higher than S_1, hence its position at shorter wavelengths than the mCerulean pulse. The phase masks were made using binary phase shaping, with central flat-phase sections of 40nm and a random choice of 0 or pi elsewhere. The masks were symmetric about the center of the flat-phase section. Further optimization of the pulse shapes is under active investigation. The shaped pulses, as well as an unshaped transform-limited pulse, were applied to multiple cells in each sample and images were collected using Prairie Technologies' PrairieView software. Final images are averages of 128 scans across the region of interest with pixel dwell times of 4μs. Image analysis was performed in Matlab. The images were also masked so that calculations were performed only on the central portions of the cells.

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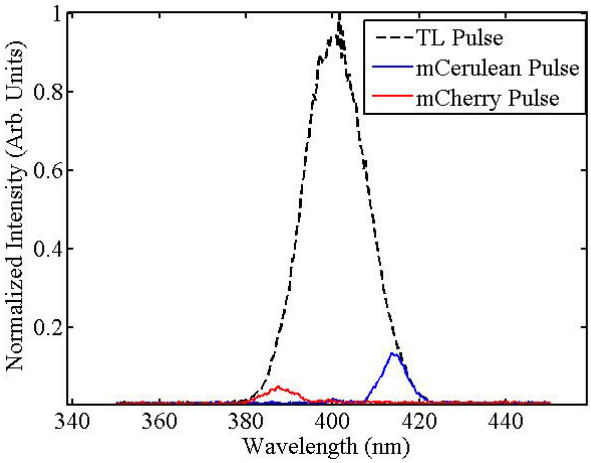

Figure 2: Second harmonic intensity for the transform-limited (TL) and shaped pulses used to selectively excite mCerulean and mCherry. Note that the mCherry pulse is centered at shorter wavelengths than the mCerulean pulse to better excite the higher transition of mCherry.

4. RESULTS

Figure 3 shows imaging results from the "No FRET" condition, which consisted of cells co-transfected with free mCerulean and mCherry. The left column shows the mCherry fluorescence, and the right column shows the mCerulean fluorescence. The top row shows a cell excited with the unshaped transform-limited (TL) pulse. As the figure shows, using the TL pulse provides the most fluorescence signal, but it does not discriminate between fluorophores. Differences in brightness between the mCerulean and mCherry signal here are due to differences in the fluorophores' inherent brightness and how well the fluorescent proteins were expressed.

The middle and bottom rows are images taken when the same cell was excited by shaped pulses designed to excite mCerulean and mCherry, respectively. These images have been normalized to the TL result in each channel to account for differences in brightness and transfection efficiency. They have also been masked so that the central part of the cell is used to calculate contrast. Blue contrast is defined as (mCerulean fluorescence – mCherry fluorescence)/mCerulean fluorescence when a shape designed to preferentially excite mCerulean is used. Similarly, when a shape designed to preferentially excite mCherry is used, red contrast is defined as (mCherry fluorescence – mCerulean fluorescence)/mCherry fluorescence. The middle row shows that when a shape tuned to preferentially excite mCerulean was used, blue contrast of 73% was achieved. When using a shaped tuned to preferentially excite mCherry, as shown in the bottom row, red contrast of 49% was achieved. These results were consistent across multiple cells and multiple applications of the pulse shapes (i.e., the shapes did not change when removed and reapplied).

We next consider the "FRET" condition, which consisted of cells transfected with the linked mCerulean-mCherry complex. Here, we expect that when the donor is excited, some of its energy will be transferred to the acceptor, appearing as an increase in mCherry fluorescence and a decrease in mCerulean fluorescence. Consequently, when a shape tuned to preferentially excite mCerulean is used, we expect to see a decrease in blue contrast. This is due to the fact that energy is transferring to the acceptor and appearing as mCherry fluorescence rather than mCerulean fluorescence. Conversely, when the mCherry shape is used, we expect to see an increase in red contrast. This is caused by the fact that even when the mCherry pulse is used, the donor fluorophore is still excited, although weakly. The excited donor can transfer energy to the acceptor regardless of the excitation wavelength used, leading to an increase in mCherry fluorescence.

The results shown in Figure 4 support these expectations. Again, the top row shows the two color channels (mCherry fluorescence on the left, mCerulean fluorescence on the right) from a cell excited by the TL pulse. As before, we see that both fluorophores are excited. Images in the middle and bottom rows are again normalized to the TL image intensity and masked. The middle row shows images taken with a pulse designed to excite mCerulean , and we see the expected decrease in blue contrast: 53% compared to the 73% seen in the No FRET condition. The bottom row shows images taken with a pulse shaped to excite mCherry, and the contrast increases as expected, from 49% in the No FRET

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condition to 69% in the FRET condition. These results were again consistent over multiple cells and multiple applications of the pulse shapes. Thus, we have demonstrated selective excitation of donor and acceptor in live cells, and the ability to detect two-photon FRET.

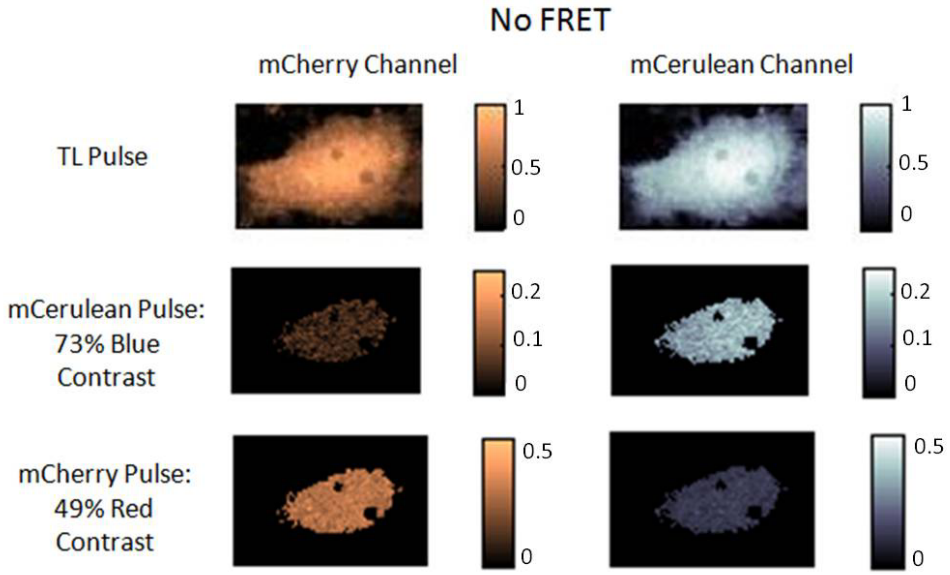

Figure 3: Image data taken from cells in the No FRET condition. The use of pulse shapes tuned for preferential excitation leads to contrast of one fluorophore over the other. The columns show mCherry fluorescence on the left and mCerulean fluorescence on the right. The top row is fluorescence from a transform-limited pulse, the middle row is fluorescence from a pulse shaped to excite mCerulean, and the bottom row is fluorescence from a pulse shaped to excite mCherry.

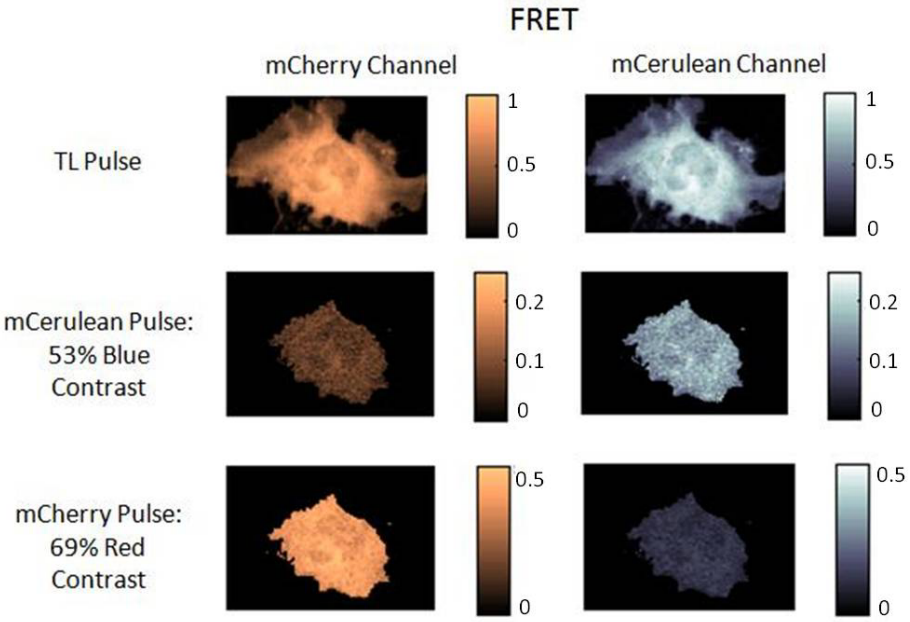

Figure 4: Image data taken from cells in the FRET condition. Here, the same pulse shapes are used, but the FRET process changes the contrast achieved. Blue contrast is decreased because as energy transfers to the acceptor, blue fluorescence decreases. Red contrast is increased because the transferred energy appears as red fluorescence. Columns and rows are the same as in Figure 3.

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5. SUMMARY

Here, we have demonstrated the use of laser pulse-shaping for selective two-photon excitation of donor and acceptor fluorophores in live cells, in addition to the detection of FRET between those fluorophores. This method has several advantages over other two-photon FRET microscopy techniques, including improved temporal resolution. The SLM can change phase masks in approximately 10ms, much faster than a laser can be tuned. The method demonstrated here lays the foundation for two-photon FRET stoichiometry measurements. To extend the one-photon FRET stoichiometry approach of Hoppe *et al.*,[5] some changes need to be made to the assumptions underlying the theory. In particular, one-photon FRET stoichiometry assumed that the donor fluorophore cannot be excited by the same wavelengths used to excite the acceptor directly. This assumption is essential to correct for accidental direct excitation and crosstalk between channels, and does not necessarily hold for two-photon excitation – particularly when the acceptor fluorophore is excited via its S_0-S_n transition. Eliminating this assumption leads to changes in correction factors, as well as the creation of new ones to account for the accidental excitation of the donor and FRET that can arise from that. The next step in the process of developing a quantitative two-photon FRET microscopy technique will be to combine such corrections with the methods presented here.

6. ACKNOWLEDGEMENTS

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