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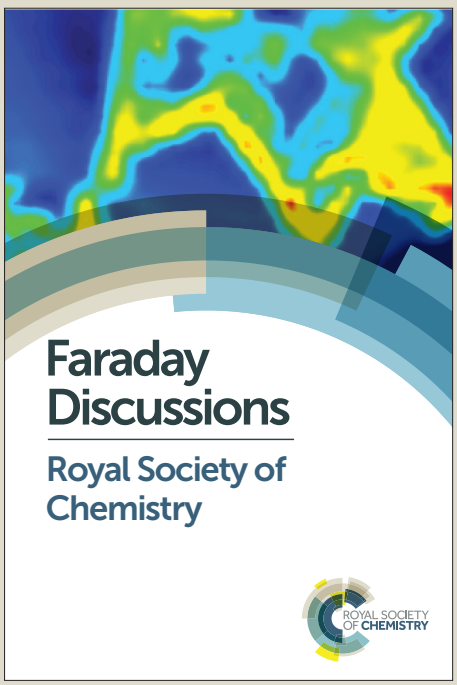

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Resolution Enhancement Through Microscopic Spatiotemporal Control

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Operating at biologically benign conditions, multi-photon fluorescence imaging microscopy has benefitted immensely from recent developments in microscopic resolution enhancement. Fluorescence microscopy continues to be the best choice for experiments on live specimens, however, multi-photon fluorescence imaging often suffers from overlapping fluorescence of typical dyes used in microscopy, limiting its scope. This limitation has been the focus of our research where we show that by making simple modifications in the laser pulse structure, it is possible to resolve the overlapping fluorescence complications. Specifically, by using pairs of femtosecond pulses with variable delay in place of the single pulse excitation, we show controlled fluorescence excitation or suppression of one of the fluorophores over the other through wave-packet interferometry. Such an effect prevails even after the fluorophore coherence timescale, which effectively results in a higher spatial resolution. Here we extend the effect of our pulse-pair technique to the microscopic axial resolution experiments and show that such pairs of pulses can also 'enhance' the axial resolution.

1 Introduction

Fluorescence microscopy continues to be the best choice for monitoring live specimen in spite of the resolution advantage of electron microscopes as the energy deposited in electron microscopy adversely affects the viability of live specimens. This practical compromise implicitly sets resolution enhancement, as one of the most important developmental aspects in fluorescence microscopy.[1] Demonstrating better depth resolution through multi-photon excitation in laser scanning microscopy is thus an important achievement. However, the broad spectral window of an ultrafast laser pulse required for two-photon fluorescence (TPF) microscopy and the overlapping multi-photon absorption spectra of common fluorophores lead to the simultaneous excitation of many different fluorophores. Consequently, selective enhancement or suppression of fluorescence is important for developing better resolution in TPF microscopy. In this context, ultrafast laser pulse-shaping[2] approaches to quantum control that have shown promise in discriminating nearly identical fluorophores[3-5] can be very effectively applied to microscopy.[4-9] Manipulation of excited state population and the corresponding spontaneous emission (and/or fluorescence) through coherent quantum interference is possible through precise control over inter-pulse delay and phase in a pulse pair[10,11] or pulse train[12] excitation. Previously, this approach of fluorophore discrimination was demonstrated in the solution phase.[13] On the other hand, pulse-pair fluorophore excitation can also occur by the manipulation of excited state photo-physics, i.e., incoherent population dynamics, where the only "control factor" is the time delay between the pulse pairs. We have earlier reported selective TPF

suppression using a pulse-pair excitation scheme, which was explained on the basis of selective stimulated emission by a time delayed second pulse following the excitation pulse.[14] In our approaches, though simultaneous two-photon absorption (TPA) of the two different fluorophores occurred, the ensuing one-photon stimulated emission was selective to only one fluorophore, which resulted in the observed discrimination.[15] Thereafter, we also demonstrated selective fluorescence suppression by a train of excitation pulses, where, instead of two time-delayed identical pulses, many pulses with gradually decreasing pulse intensities (in geometric progression) and controllable delay between the successive pulses, were employed.[16] In the multi-pulse scheme, the first pulse induced TPA while the subsequent successive pulses resulted in one-photon stimulated emission in preference over TPA. The effect of varying polarization of the individual pulses used under such incoherent schemes was also explored, which, as expected, was found to be only minimal unlike the coherent case.[1] Thus, using either a simple pair of femtosecond pulses with variable delay or a train of laser pulses at 20-50 Giga-Hertz excitation, we have developed controlled fluorescence excitation or suppression of one of the fluorophores with respect to the other. Since this is an incoherent process, the effect prevails even after the coherence lifetime of the fluorophore and can be used in both the single-photon as well as in the multi-photon excitation conditions to result in an effective higher image contrast resolution. Higher image contrast is achieved as fluorescence from one of the two fluorophores with respect to the other is enhanced as a function of the relative time delay between the pulse pair. Such high image contrast with pulsed broadband excitation can be of immense benefit to multi-photon microscopy.

Use of pairs of pulses in place of single pulse in microscopy begets the natural question on whether such modified pulses would affect the axial resolution of the microscope as well. Here, we address this particular aspect of femtosecond pulse pair with variable delay on microscopic axial resolution for the first time and relate it to our contrast resolution enhancement results in the image plane. In order to make a valid correlation, we compare the enhanced contrast resolution situation in the image plane with that of the axial plane under identical experimental conditions. Comparisons have been drawn from several experiments performed under appropriate conditions with different laser and/or sample characteristics.

2 Experimental

Figure 1shows our experimental setup. The laser system used in our experiments is a mode-locked Ti:Sapphire oscillator (Mira900-F pumped by Verdi5, Coherent Inc., USA) producing femtosecond pulses (pulse-width ranging 120-180 fs depending on the center wavelength) at 76 MHz repetition rate and wavelength tunable in 720–980 nm. The pulse pair was generated using a collinear Mach-Zehnder-type interferometer where the laser beam was separated into two parts by using a beam-splitter and was recombined using another beam-splitter. One of the split beams from the first beam-splitter was passed through a movable retro-reflecting mirror mounted on a mechanical stage (UE1724SR driven by ESP300, Newport) while the other one was routed through a fixed retro-reflecting mirror. The delay stage was interfaced with a computer controlled GPIB card (National Instruments) to provide precise delay steps. The separation between the pulse pair depended on the precise delay of the movable arm in the Mach-Zehnder interferometer. The laser pulse pair thus generated from the Mach-Zehnder interferometer was measured just before it enters the microscope with

a commercial autocorrelator (Model: Pulse Check, A.P.E GmbH). Representative spectra measured with a fiber coupled monochromator (HR2000) as well as the autocorrelation trace for such pulse pairs are shown in Figure 2. Depending on the sample used in the specific experiment, the center wavelength of the laser as well the corresponding pulsewidth varied. The collinearly propagating beams were sent into our confocal-ready multi-photon microscope system (FV300 scan-head coupled with IX71 inverted microscope, Olympus, Japan). The laser beam was focused on the sample using a high numerical aperture (we used used two different cases: either the UPlanApoN 40X 1.30NA or the UPlanApoN 60X 1.42NA, both from Olympus) objective lens. We used an immersion oil of refractive index 1.518 between the objective lens and coverslip. Two mirrors before the scan head were used for fine alignment of the laser beam into the scan-head and for maximizing the signal. For imaging as well as for the axial resolution measurements, the total average power of the laser beam entering the scan-head was ~30 mW. A pair of lenses was used in the detection path to collimate the beam for getting the optimum fluorescence signal from the detector (PMT, Hamamatsu Inc.). Finally an infra-red (IR) cut-off filter was introduced in the fluorescence collection path for blocking any un-wanted back-scattered IR light.

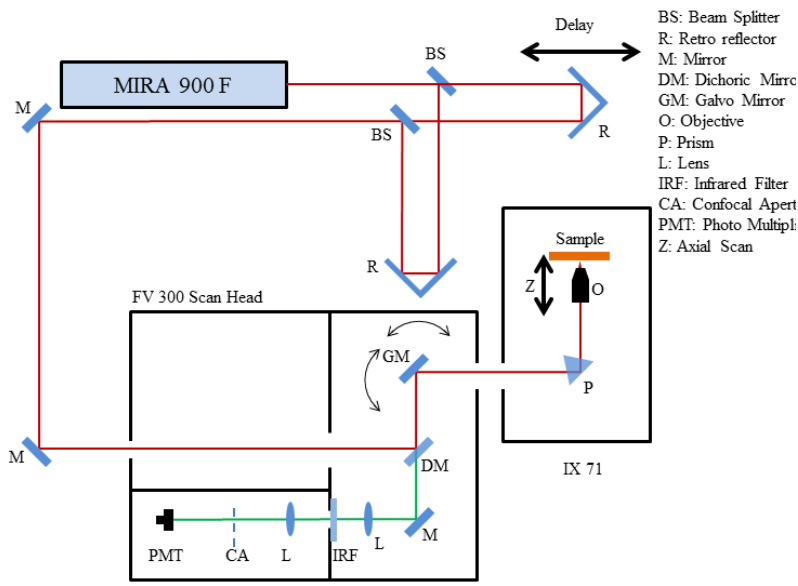
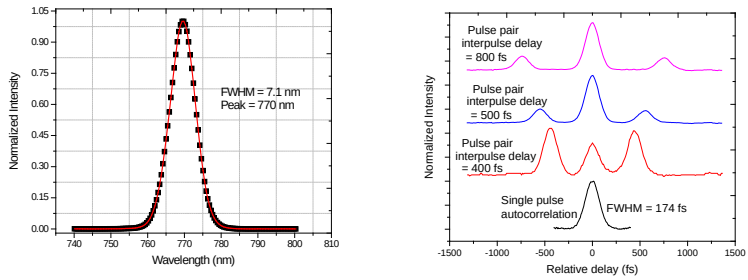


Figure 1: Overall experimental setup used in our experiments. The abbreviations used are expanded in the legend on the side.

2.1 Enhanced Contrast Fluorescence Imaging Microscopy

For pulse pair imaging microscopy, slides of bovine pulmonary artery endothelial (BPAE) cells having nuclei stained with DAPI (blue fluorophore staining nuclei) and Texas Red-X phalloidin (red fluorophore staining mitochondria) were used that are

commercially available (Molecular Probes, Invitrogen, USA). At ~730 nm (pulse width = 180 fs) both these dyes are simultaneously excited through two-photon excitation, which makes these fluorophore labeled samples a perfect choice for our selective excitation experiments. However the fluorescence from Texas Red extends up to ~750 nm, which suggests the possibility of its fluorescence suppression through stimulated emission in contrast to the fluorescence from DAPI that almost dies out at ~650 nm. The image acquisition and intensity counts were performed using FLUOVIEW 300 software (Olympus, Japan). The image resolution for each image collected at different time delays between the pulse-pair was set to 512×512 pixels.

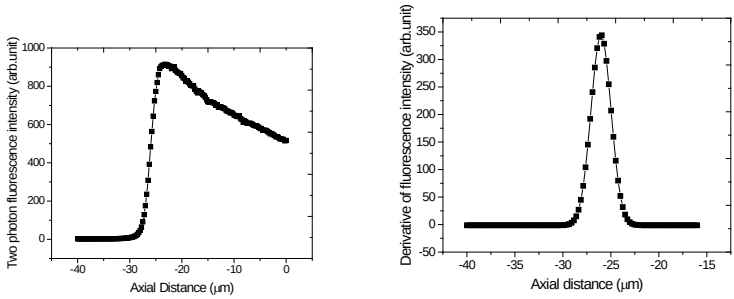


(a) (b)

Figure 2: (a) Laser pulse spectrum as well as (b) representative autocorrelation trace for pulse pairs at a few different inter-pulse delays are shown. These measurements are made before the pulses enter the microscope.

2.2 Measuring the Axial Resolution in Fluorescence Microscopy

For measuring axial resolution, we first used a millimolar (10^{-3}M) methanolic solution of Rhodamine-6G (R6G) taken in a culture dish (35 mm Glass Bottom Dish No. 0 (MatTek Co., USA)). In these experiments, the center wavelength of the Ti:Sapphire excitation laser (Mira900-F) was tuned to 770 nm (pulse-width ~120 fs). The confocal aperture was kept fully open at 300 µm. We performed the axial scan (along z-axis) of the R6G solution to generate the axial edge response as shown in Figure 3a. The z-axis piezo-stage of the microscope objective was moved at a step-size of 0.25 µm during this data acquisition. This axial edge response data is approximated to a sigmoidal (shown by a dotted line in Figure 3a), whose derivative was fitted to a Gaussian function (point-spread function, Figure 3b).[26-30] The full-width-half-maximum (FWHM) of this Gaussian fit is taken as the measure of the axial resolution of the microscope for a specific pulse pair delay. We collected such axial resolution data at different inter-pulse delay for the pulse pair and compared that to the single pulse data. We used two different focussing conditions by using the two objective lenses of the microscope. Next we collected similar axial scan images for the BPAE fixed cells stained with DAPI at specific pulse pair delays at 750 nm (pulse-width ~130 fs) using the UPlanApoN 40X 1.30NA objective. Such axial resolution data from the scanned images of BPAE at different inter-pulse delay for the pulse pair were collected and compared to that of the single pulse data. Thus we were able to generate three different cases of series of axial resolution measurements for various pulse pair delays that were compared to the single pulse data.



(a) (b)

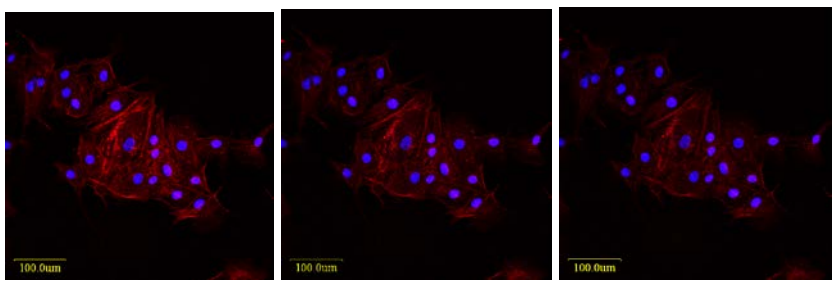
Figure 3: (a) Axial edge response function corresponding to the change in fluorescence intensity during axial scan and (b) its derivative fitted to a Gaussian function.

3 RESULTS AND DISCUSSION

As discussed at the very outset, each of our experiments is performed at one specific center wavelength and as such is one-color experiment. This is different from the typical pump-probe experiments[21,22] where two different colors are used: one for pump and the second color for probe. The pump pulse excites a part of the ground state population to the excited state where it rapidly relaxes to the lowest vibrational level of the excited state where from the fluorescence emission occurs. However, if a time-delayed probe pulse that is wavelength-tuned to the red edge of fluorescence sends the population back to the ground electronic state by stimulated emission in competition with the fluorescence process, fluorescence suppression is observed. Fluorescence, being an incoherent emission, is omnidirectional. Stimulated emission, on the other hand, is a coherent process and, as such, photons generated by stimulated emission travel in the direction of the stimulating beam. So, if we collect only the back-scattered fluorescence (epi-fluorescence as in typical confocal microscopy) there is suppression in fluorescence signal as discussed.[21] However, under the same experimental condition, if one collects the forward scattered photons, a gain in total measured signal was observed due to the additional stimulated emission which could even transform weakly fluorescent molecules into suitable candidates for microscopy applications.[22]

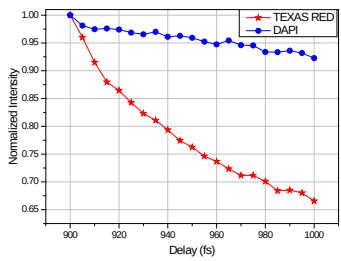
Our pulse-pair microscopy, however, focuses on experiments that are of practical relevance to two photon fluorescence (TPF) laser scanning microscopy and only restricted to one-color excitation scheme.[23] It is important that in this form of identical replica pulse being delayed with respect to the original, it is important that is beyond the FWHM of the pulse otherwise the interference between the pulses are most overwhelming. For image contrast enhancements based on fluorophore discrimination, in our version of one color pulse-pair excitation, we find interesting control strategies that materialized even within a time window of 100 fs as shown in the region of 0.9 ps to 1.0 ps at a step-size of 5 fs, as is seen from the suppression of Texas Red fluorescence in comparison to that of DAPI fluorescence in BPAE cell (Figure 4a and 4b). In this case, the first pulse causes a two photon absorption for both DAPI and Texas Red. The time-delayed second pulse causes

similar two-photon absorption as in case of the first pulse but, in addition, could dump the population by (one-photon) stimulated emission if the red edge of fluorescence coincides with the excitation wavelength (as is the case for Texas Red) (Figure 4c). Thus a distinction is seen between DAPI and Texas-Red in the two-photon fluorescence image. This contrast between the fluorophores at ~1 ps time delay between the pulse pair can be critical in distinguishing the overlapping fluorophores.

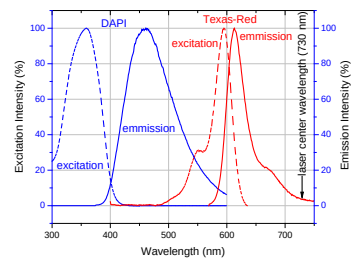


900fs 950fs 1000fs

(a)



(b)

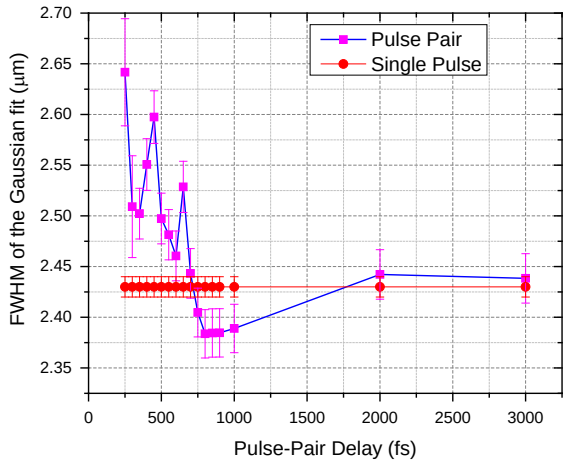


(c)

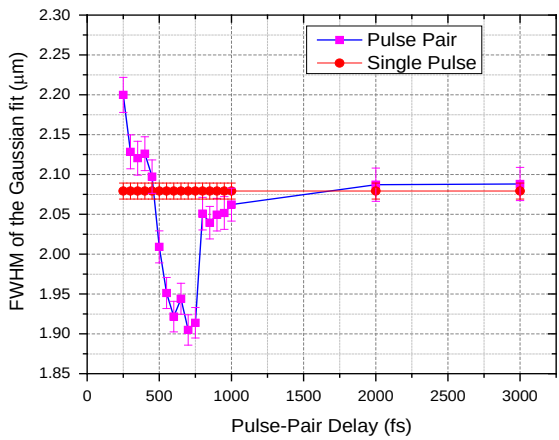
Figure 4: Change in the relative fluorescence intensity of (a) DAPI versus Texas Red, (b) Corresponding fluorescence suppression image of Texas Red relative to DAPI as a function of time delay between pulses under one-color (730 nm) pulse-pair excitation, and (c) Corresponding excitation (broken line) and emission (solid line) spectra of Texas-Red and DAPI relative to the excitation laser wavelength (730 nm) position.

In our axial resolution studies with R6G solution, data at every 50 fs inter-pulse delay for the pulse pair is shown in Figure 5, which is compared to the single pulse data for two different magnification and numerical aperture (NA) cases. As discussed earlier, each data point corresponds to the width of the point spread function (Figure 3b). Several measurements at each inter-pulse delay was collected, which enabled used to report the error bars for each of the plotted data points. At very near to the overlapped condition between the pairs of pulses (i.e., close to zero delay), the axial resolution (as defined by the FWHM of Gaussian fit to the derivative of the edge response function) shows oscillatory nature with a large error bar. The oscillatory nature could be attributed to the interference pattern of the nearly time-overlapped pulse pair. However, it is important to note that the best possible resolution under such closely overlapping pulse pair are always

lesser than the single pulse case. As the pulse separation increases between the pulse pair for both the microscope objective cases (Figure 5a and 5b), until about 1 ps, the axial resolution as per our definition keeps getting better and better with lesser and lesser error-bars (Figure 5). Effect of NA is also quite obvious between these two cases. Interestingly enough, this resolution enhancement starts to fall off for inter-pulse separations beyond 1000 fs to reach the axial resolution comparable to that of a single pulse case.

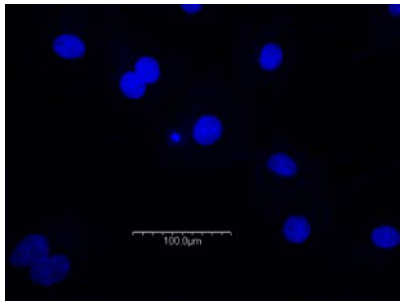


(a)

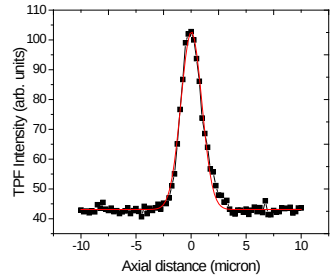


(b)

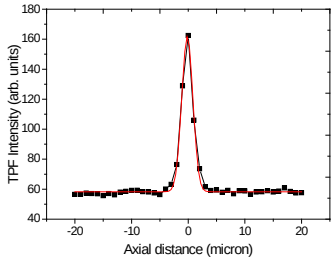
Figure 5. Change in axial resolution as a function of the relative delay between the pulses in a pulse pair excitation scheme for Rh-6G dye solution in methanol. This change is compared to the traditional microscopy case of a single pulse excitation condition. Error-bars for each experimental data point is also provided. Two different magnification cases have been used: (a) 40X 1.30NA objective and (b) 60X 1.42NA objective.



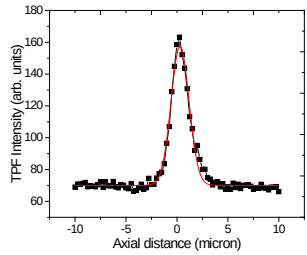
(a) XY image of DAPI stained BPAE cell taken with 40X(1.30 NA)



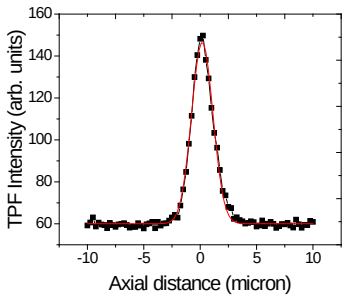
(b) single beam



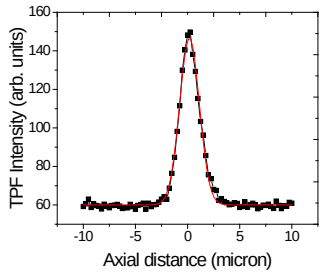
(c) 232 fs



(d) 400 fs



(e) 1000 fs



(f) 2000 fs

Figure 6. Axial Two Photon Fluorescence intensity profile along with Gaussian fit of DAPI stained BPAE Cell at different inter-pulse delay (b-f) during axial scan of XY-image (shown in (a)) along the Z-axis. The FWHM of Gaussian fit is taken as a measure of axial resolution.

The TPF intensity profile of DAPI stained BPAE cell at different inter-pulse delay during the axial scan along z-axis of the XY-image (Figure 6) were fitted with the Gaussian function. The FWHM of Gaussian fit is taken as a measure of the axial resolution for these images. Figure 7 shows the axial resolution of the BPAE cell at different inter-pulse delay for the pulse pair as well as for the single pulse data. Comparing Figures 5 and 7, we find that typically, for pulse separation of ~2 ps and above, only a single pulse character is observed in the axial resolution, while for pulse separation of ~1 ps there is the maximum effect of one pulse on the resolution of the other in resulting the best possible axial

resolution. Increased fluorophore image contrast at ~1 ps (Figure 4b) for the BPAE cells also correlates well with this study showing the coupled impact of both the pulses in the pulse pair.

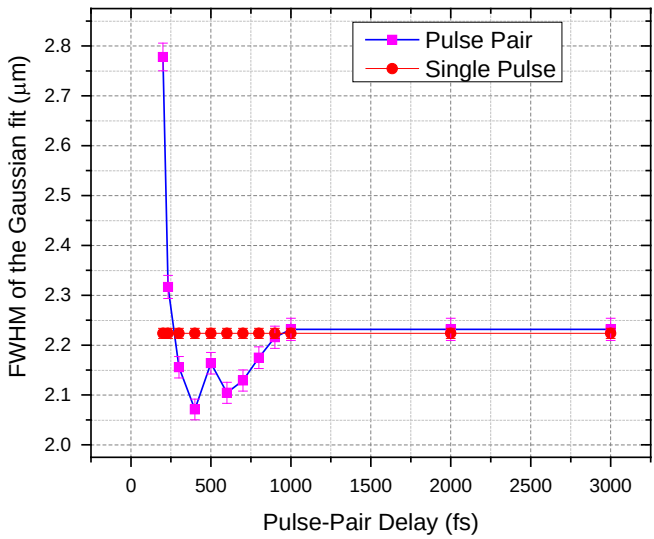


Figure 7. Change in axial resolution as a function of the relative delay between the pulses in a pulse pair excitation scheme for DAPI stained BPAE Cell. The change is compared to the traditional microscopy case of a single pulse excitation condition.

4 CONCLUSION

We have shown how innovative use of relative delay between pulses in pulsed laser illumination can be utilized for resolution enhancement in fluorescence microscopy. Our experiments on pulse-pair control mediated by stimulated emission have interesting applications in microscopy. The control method is based on competition between fluorescence and stimulated emission and has been demonstrated for fluorophore discrimination. We extend our effect of our pulse-pair technique to the microscopic axial resolution experiments. Such experiments show that the pairs of pulses can also 'enhance' the axial resolution as long as one pulse can feel the impact of the other beyond the pulse temporal interference zone. Within errors of the experiments performed on different dyes, there is a strong indication of axial resolution enhancement at ~1 ps delay between the femtosecond pulse pair. This observed enhancement might have an important effect on microscopic resolution.

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