Greater signal, increased depth, and less photobleaching in two-photon microscopy with 10 fs pulses

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Abstract

The fundamental advantages to using ultrafast (<100 fs) laser pulses in two-photon microscopy for biomedical imaging are seldom realized due to chromatic dispersion introduced by the required high numerical aperture microscope objective. Dispersion is eliminated here by using the multiphoton intrapulse interference phase scan (MIIPS) method on pulses with a bandwidth greater than 100 nm full width at half maximum. Higher fluorescence intensity, deeper sample penetration, and improved signal-to-noise ratio are demonstrated quantitatively and qualitatively. Due to the higher signal intensity obtained after MIIPS compensation, lower laser power is required, which decreases photobleaching. The observed advantages are not realized if group delay dispersion is compensated for while higher-order dispersion is not. By using a pulse shaper and taking advantage of the broad spectrum of the ultrafast laser, selective excitation of different cell organelles is achieved due to the difference in nonlinear optical susceptibility of different chromophores without requiring an emission filter wheel. Experiments on biological specimens, such as HeLa cells and mouse kidney tissue samples, illustrate the advantages to using sub-10 fs pulses with MIIPS compensation in the field of two-photon microscopy for biomedical imaging.

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1. Introduction

Two-photon excitation processes were first proposed by Göppert-Mayer in 1931 [1]. In 1990, this modality was introduced to biological microscopy by Denk and Webb, who took advantage of the newly available femtosecond laser technology with ~100 fs pulse duration [2]. The high peak power density at the focus of a femtosecond laser greatly increases the probability for two-photon absorption to an excited state for which fluorescence is observed. This nonlinear optical process is dubbed two-photon excitation fluorescence (TPEF). Similarly, second-harmonic generation (SHG) signal can be generated from the non-centrosymmetric structure of a biological sample, such as the cell membrane and extracellular collagen fibers. The advantages of multiphoton imaging include the inherent optical sectioning capability [3,4], deep penetration depth [5], and minimal photo-damage to the tissue [6]. Here we report on the quantitative and qualitative assessment of multiple advantages that arise from the use of sub-10 fs laser pulses for nonlinear optical imaging, which include reduced photobleaching, higher signal-to-noise ratio, deeper penetration depth and the possibility of selective excitation without laser tuning [7,8]. Although most current biological dyes have a large cross-section for two-photon excitation [9], some of the two-photon laser systems can provide spectral tuning capability for selective excitation to optimize the excitation efficiency for certain dyes [10]. Selective excitation without laser tuning, as shown in this work, ensures registration between subsequent images.
In theory, the TPEF efficiency is expected to be inversely proportional to the pulse duration. However, current multiphoton microscopy still utilizes ~100 fs laser pulses, even though femtosecond technology has reached single digit pulse durations [11–13]. The major barrier to the development of the application of shorter pulses for TPEF imaging lies on the fact that shorter pulses have a broader spectral width, and thus suffer from higher chromatic phase dispersion when passing through thick optical media, such as the required high numerical aperture (NA) microscope objective. Previous efforts for compensating chromatic dispersion using a pair of prisms have been reported [14,15]. Unfortunately, prism pairs correct only second-order dispersion (SOD), also known as group delay dispersion (GDD), but add substantial higher-order dispersion (HOD). If left uncompensated for, these dispersions prevent one from realizing possible advantages from pulses shorter than 100 fs [16]. Here, we demonstrate several distinct advantages for biomedical imaging with dispersion free ultrashort pulses.

The compensation of higher-order dispersion from high numerical aperture objectives up to a 60×1.45 NA objective was demonstrated using multiphoton intrapulse interference phase scan (MIIPS) method on a ~10 fs Ti:Sapphire laser system [17]. MIIPS is a novel method for measuring and eliminating chromatic dispersion (SOD and HOD) [18–20]. MIIPS uses a pulse shaper to introduce a series of calibrated spectral phase functions to the pulses. The SHG response to those functions reveals the unknown chromatic dispersion of the laser at the focus of the microscope objective. Once the phase distortions are measured, the pulse shaper introduces a spectral phase that effectively cancels the distortions. With MIIPS, SOD and HOD are eliminated within minutes, thus realizing the various advantages of ultrashort pulses, as shown here.

One of the principal advantages of two-photon microscopy over confocal single-photon microscopy is the ability to excite multiple fluorescent dyes with a single laser system using fluorescent filters to distinguish between different chromophores [2,9]. In certain cases, however, fluorescence filters are not sufficient to correctly determine colocalization of these chromophores and one would like the flexibility to achieve selective excitation. For this purpose, tunable femtosecond lasers have been used [10]. Here we show that ultrabroad bandwidth laser pulses allow one to achieve selective excitation without the need to tune the laser system.

2. Methods and materials

The schematic configuration of the laser system and microscope is shown in Fig. 1. An ultrashort oscillator with 110 nm bandwidth, sub-10 fs pulse duration and repetition rate of 86 MHz was used as the excitation source (K&M Labs). The beam was expanded spatially before entering the pulse shaper. Inside the pulse shaper, a 300-line/mm grating and a spherical mirror with a 350 mm focal length were used. A phase-only liquid-crystal spatial light modulator (SLM) (P-640-CRI) was used to introduce phase retardation. A pair of SF-11 prisms was used to introduce negative dispersion in order to minimize the SOD of the system.

The two-photon microscopy system used was based on a Nikon TE200 inverted microscope. After the prism pair, the beam was reflected through a dichroic filter (DC) (700DCSPXR, Chroma Technologies) onto a pair of galvanometers. The galvanometers scanned the beam through a 3X telescope system to the microscope objective. The telescope was composed of one \( f = 100 \) mm scan lens on the galvanometer side and one \( f = 300 \) mm tube lens on the objective side to collimate and expand the beam from 8 mm to 24 mm to overfill the back aperture of the Nikon Plan Apo TIRF oil-immersion 60×1.45 NA objective. Between the telescope lenses, a periscope was installed to raise the beam height to the mercury lamp port, which was used to introduce the laser beam with no additional optical media.

The two-photon fluorescence signal was collected by the same excitation objective in epi-fluorescence mode and was de-scanned by the galvanometer. The DC reflects the TPEF

![Fig. 1. Microscope setup diagram for two-photon laser scanning microscopy with shaped ultrashort laser pulses.](image-url)
signal to the PMT detector. After a short-pass filter, which was used to reject any remaining laser light, a fiber collimator with a 400 μm core fiber was used to collect the fluorescence signal. The optical fiber then guided the fluorescence to an amplified photomultiplier tube (PMT).

The driving signal for the galvanometers was provided by a National Instruments PCI 6251 Data Acquisition (DAQ) system, which also functions as the A/D converter to digitize the analog PMT signal to a 16-bit digital signal for imaging. Origin (Originlab) and ImageJ (NIH) were employed jointly to analyze the images and process the data.

It is widely accepted to measure the point spread function (PSF) by using fluorescent microbeads with a diameter smaller than the FWHM of the PSF [21]. The measurement is a convolution of the bead size with the PSF. Assuming a uniform bead size, the simulation result shows that there is a 10% enlargement of the measured PSF for a bead size 1/2 of the PSF. To measure the PSF of our microscope, Fluo- resbrite® carboxylate beads (Invitrogen) with 100 nm diameter were imaged. The lateral and axial FWHM were measured to be 0.29 μm and 0.90 μm with the 60× 1.45 NA objective, as shown in Fig. 2.

A thin (0.05 mm) BBO crystal (CASTECH) was placed at the focal plane of the microscope objective to generate the SHG signal required for MIIPS. A f = 75 mm lens (Edmund Optics) was used directly above the objective to collect the SHG with an optical fiber. A spectrometer (Ocean Optics, USB4000) was used to obtain the SHG spectrum. The MIIPS measurement and compensation routine was performed as described in literature [17,19].

In our experiment, MIIPS was employed to obtain pulses that satisfy Δt/Δt_{TL} = 1.01, where Δt is the duration of the pulse and Δt_{TL} is the calculated duration of the TL pulse based on the pulse spectrum. In Fig. 3, we show a typical spectrum of the laser used in this work. We also show the measured phase of the pulses after GDD-compensation by a prism pair. Notice that once SOD was compensated for by the prism pair, the measured phase reflects the third order dispersion (TOD) present. The upper insert corresponds to the residual phase after MIIPS measures and compensates for HOD.

### 3. Results and discussions

#### 3.1. SHG and fluorescence intensity

Femtosecond lasers are ideally suited for inducing nonlinear optical processes because their short pulse duration results in high peak power. For two-photon processes (such as TPEF and SHG), the signal intensity \( I^{(2)} \) is linearly proportional to the inverse of the pulse duration \( \Delta t \); equivalently it is linearly proportional to the spectral bandwidth \( \Delta \lambda \). For a Gaussian transform-limited (TL) pulse centered at 800 nm, \( \Delta \lambda \Delta t \approx 940 \) nm fs.

After transmission through thick optical media, such as a multi-element high NA objective, the phase dispersions become dominant, and the pulses are broadened well beyond TL. Moreover, because the dispersion is directly related to \( \Delta \lambda \), shorter pulses endure more severe broadening from dispersion. It is, therefore, a must to compensate the dispersion in order to fully attain the benefits of ultra-short laser pulses. Without compensation, a 10 fs pulse can be easily broadened to a picosecond pulse after a high NA objective.

The component of the electromagnetic field responsible for TPEF or SHG is given by \( |E^{(2)}(2\omega)| \), therefore, the two-photon excitation probability can be written as [19]
\[ I^{(2)}(2\omega) = |E^{(2)}(2\omega)|^2 \]

\[ \propto | \int |E(\omega + \Omega)E(\omega - \Omega)| \exp \{ i[\phi(\omega + \Omega) + \phi(\omega - \Omega)] \} d\Omega|^2. \]  

(1)

The signal intensity is proportional to the integral of the product of the amplitude of the field times a complex quantity that is dependent upon the spectral phase of the laser pulses \( \phi(\omega) \). Here, \( \Omega \) is a dummy integration variable that plays the role of spectral de-tuning away from the fundamental frequency, \( \omega \).

For TL pulses, the exponential term in Eq. (1) is maximized; therefore, nonlinear signal is optimized. The presence of a quadratic phase (linear chirp in the time domain), given by \( \phi(\omega + \Omega) = 1/2\phi''(\omega)\Omega^2 \), leads to a decrease in \( I^{(2)}(2\omega) \), which affects only the amplitude [17]. This case is illustrated in Fig. 4, where the quadratic phase is shown to cause a decrease in the signal. The presence of a cubic phase, given by \( \phi(\omega + \Omega) = 1/6\phi'''(\omega)\Omega^3 \), causes a significant change in \( I^{(2)}(2\omega) \), which goes beyond simple attenuation. In Fig. 4B and D, we show the effects caused by SOD and HOD (in this case it is third-order dispersion).

Fig. 4. Effects of SOD and TOD to a Gaussian pulse with central wavelength of 800 nm and FWHM of 110 nm. (A) 200 fs\(^2\) SOD (shown in thick line) added onto a Gaussian pulse and (B) spectra of the TL versus SOD-added (shown in thick line) SHG signal; (C) –2000 fs\(^3\) third-order dispersion (TOD, shown in thick line) added onto a Gaussian pulse and (D) spectra of the TL versus TOD-added pulse.

Fig. 5. The effect of increasing bandwidth on TPEF (A) and SHG signal (B) excited with MIIPS compensated pulses and GDD-only compensated pulses.
on the pulse spectrum. The amount of SOD and HOD introduced by a high NA microscope objective can be as much as one order of magnitude greater than the values used to illustrate their effects in Fig. 4. Clearly, HOD must be eliminated in order to achieve reproducible results in TPEF microscopy.

In Fig. 5, we show the intensity of TPEF and SHG for laser pulses as a function of bandwidth. The measurements are performed under two conditions: TL and GDD-only compensated pulses. For TL pulses, high-order phase distortions are eliminated using MIIPS and the pulse duration is inversely proportional to the bandwidth. For GDD-only compensated pulses, GDD is eliminated using a pair of prisms (the same prism pair that is used in the MIIPS setup), but HOD is not compensated for. In both experiments, the excitation intensities were kept constant for all bandwidths using a linear attenuator so that the excitation intensity on the sample was constant at 2.28 mW. For TPEF generation, a red fluorescence standard slide (Chroma Technologies) was employed. The TPEF signal was collected with the PMT detector of the two-photon microscopy system. In addition, we measured the intensity of SHG signal generated by the BBO crystal. The TPEF signal intensity initially increases with increasing bandwidth as expected for TL pulses (solid squares). As the...
bandwidth increases from 10 nm to 80 nm, TPEF signal increases by a factor of ~8. For GDD-only compensated pulses (open triangles) TPEF increases until a 30 nm bandwidth is attained; thereafter, no further signal gain is realized. For broad bandwidths (>30 nm), HOD becomes dominant, causing pulse broadening and decreasing the TPEF efficiency. The results of TPEF are confirmed by measuring the intensity of SHG resulting from the same laser parameters; see Fig. 5B.

We confirmed the two-photon dependence of the signal following excitation with 110 nm FWHM bandwidth pulses. We find, as shown in Fig. 6, that both TL pulses and GDD-only compensated pulses have a power dependence of 2.10 and 2.02, respectively; which confirms that the excitation of the sample is a two-photon process and is far from saturation.

To illustrate the difference found between MIIPS compensated and GDD-only compensated pulses for TPEF microscopy, a mouse kidney slide (Molecular Probes, F-24630) was imaged and the results are presented in Fig. 7. The intensity with GDD-only compensated pulses was amplified 2× in Fig. 7A. The average intensity of image of Fig. 7B, taken with MIIPS compensated pulses, is 4.7 times that of Fig. 7A, which was taken using GDD-only compensated pulses. By taking a three line average from the image (see dotted line), we show in Fig. 7C the improvement in signal intensity between GDD-only and MIIPS compensated pulses.

### 3.2. Penetration depth

To study the penetration depth capability in a biological sample, we imaged a thick mouse kidney tissue section (over 100 μm) stained with DAPI (cell nuclei), Mitotracker-488 (mitochondria), and Phalloidin-568 (actin). A series of up to 100 optical sections (section distance of 1 μm each) were recorded (512 × 512 pixels per image, scanning speed of 1 frame per second, 8 images were averaged for each depth), in a sequential mode. The objective used was Nikon 60× 1.45 NA, with working distance of 130 μm. We used a focus drive (TOFRA, Inc.) to facilitate the depth scanning. The scattering length of the sample used was approximately 65 μm.

The results shown here demonstrate increased penetration depth when MIIPS is applied, compared to GDD-only compensation (Fig. 8). The images show the collagen wall...
components of a blood vessel in the mouse kidney at a depth of 40 μm. The collection duct region above it, at a depth of 50 μm, is seen only when MIIPS is applied.

To better assess image quality, we also applied signal-to-noise ratio (SNR) calculations. The SNR of the images can be estimated as

$$SNR = \frac{\text{Max}[\text{Median}(I)]}{2\sigma_{\text{noise}}},$$

where $\text{Median}(I)$ denotes the median filtering of the image $I$; $\text{Max}()$ calculates the maximum intensity; and $\sigma_{\text{noise}}$ is the standard deviation of the noise. To calculate the SNR of the image, an objective determination of "pure noise" image was obtained without sample. As the signal for biological samples is usually continuous to its adjacent pixels, the median filtering can effectively remove the shot noise while maintaining the intensity of the signal [22]. Here the radius of the median filter was chosen to be 1.0 pixel. The $2\sigma_{\text{noise}}$ range covers 95% of the distribution of the noise. In Fig. 8B, we plot the SNR at each depth. The SNR values are significantly higher for MIIPS than for GDD-only compensated pulses. If a given SNR value of 10 is selected as the lower limit of image quality, in this case, MIIPS compensated pulses can penetrate from 3 to 70 μm (total 67 μm), whereas GDD-only can resolve from 10 μm to 42 μm (total 32 μm), with decreased image quality (Fig. 8 A).

### 3.3. Photobleaching

The green fluorescence standard (Chroma Technologies) was used to study photobleaching effects because it provided a homogenous medium with fixed chromophores. The laser was focused using the Nikon 60 × 1.45 NA objective. For GDD-only compensation, the excitation power was 9 mW. The excitation power was kept the same as that of when GDD-only compensated pulses were used, as seen in Fig. 9A, to evaluate the difference at same excitation power; and was attenuated to 3 mW in Fig. 9B for MIIPS compensated pulses to keep the fluorescence intensity the same as that of GDD-only compensation. For these experiments, we continuously scanned a 5 μm line on the sample at a 4 ms/line rate. The data were fit with a four-exponential decay model,

$$P_i = A_i \exp[-t/t_i],$$

where $t_i$ represent the decay timescale from short to long, and $A_i$ represents the components for each timescale. We fixed the time factor $t_1$ and $t_4$ at 5 and 10,000 s, respectively; we find that $t_1$ does not change significantly for the three cases, and the experiment is not long enough to accurately measure the long time-component $t_4$. The parameters of the photobleaching curves are listed in Table 1. We find that when the initial fluorescence intensity is kept constant, for MIIPS compensated pulses the timescale is always longer than that of GDD-only compensation, and the components with longer time scales have a larger value than that of GDD-only compensation. When the same excitation power is used, MIIPS compensated pulses give greater signal than those of GDD-only compensation even after an extended period of time, which makes the signal with MIIPS compensation always greater than that of GDD-only excitation.

![Fig. 9. Photobleaching curves for MIIPS compensated and GDD-only compensated pulses: (A) at the same excitation power of 9 mW; (B) at the same initial fluorescence intensity (3 mW for MIIPS compensated pulse and 9 mW for GDD-only compensated pulse).](image-url)

<table>
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<th>Table 1: Photobleaching parameters for MIIPS compensation and GDD-only compensation</th>
<th>$A_1$</th>
<th>$t_1$</th>
<th>$A_2$</th>
<th>$t_2$</th>
<th>$A_3$</th>
<th>$t_3$</th>
<th>$A_4$</th>
<th>$t_4$</th>
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<tr>
<td>MIIPS (9 mW)</td>
<td>0.95</td>
<td>5</td>
<td>0.90</td>
<td>34.04</td>
<td>1.42</td>
<td>700.03</td>
<td>0.63</td>
<td>10,000</td>
</tr>
<tr>
<td>MIIPS (3 mW)</td>
<td>0.22</td>
<td>5</td>
<td>0.26</td>
<td>54.72</td>
<td>0.17</td>
<td>620.75</td>
<td>0.34</td>
<td>10,000</td>
</tr>
<tr>
<td>GDD-only (9 mW)</td>
<td>0.32</td>
<td>5</td>
<td>0.24</td>
<td>53.69</td>
<td>0.15</td>
<td>461.70</td>
<td>0.27</td>
<td>10,000</td>
</tr>
</tbody>
</table>

* $t_1$ and $t_4$ were fixed during fitting process.
3.4. Selective excitation

By taking advantage of the broad spectral bandwidth of ultrashort pulses, one can realize selective excitation without tuning the laser or using an emission filter wheel. Our group has previously shown the ability to control selective excitation using phase modulation [8]. Now, this same technique has been applied to biological samples with subcellular features stained with fluorescent dyes that absorb at different wavelengths.

The spectra of the SHG of the two different pulses that were used in order to achieve selective excitation, as well as the TL pulse as shown in Fig. 10. The pulses used for selective excitation were obtained by using the lower or the higher spectral frequency sides of the pulses. In brief, here we call them blue or red pulses and the SHG spectra are shown in Fig. 10. These pulses were generated with amplitude spectral shaping at the pulse shaper without further adjustments. First, control experiments ensured that only one dye is excited via two-photon absorption by the blue or red pulses. The controls consisted of slides in which only

![Fig. 10. SHG spectra of the pulses used to obtain selective excitation. TL: the spectrum generated with TL pulses. Blue: the SHG spectrum generated with <800 nm excitation. Red: the SHG spectrum generated with >800 nm excitation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)

![Fig. 11. Actin filaments of HeLa cells stained with Phalloidin-350 and mitochondria of HeLa cells stained with MitoTracker-488 imaged with TL, blue, and red pulses. Scale bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)

![Fig. 12. Two-photon selective excitation of HeLa cells: (A) the actin filaments stained with Phalloidin-350 and excited using blue pulses, (B) shows the mitochondria of the HeLa cells stained with MitoTracker-488 excited with red pulses, and (C) the merged image. Scale bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)
one subcellular structure was stained with a single dye. The upper row in Fig. 11 shows the ability to excite actin filaments in HeLa cells stained with Phalloidin-350 using TL and blue pulses. We find that the Phalloidin-350 can be excited with blue or TL pulses, but no visible signal is presented when red pulses are used. On the lower row, we show that the MitoTracker-488 can be excited with either red or TL pulses, but little signal is seen with blue pulses. This shows that using different pulses makes it possible to choose whether or not to excite a particular dye.

Once it was determined that selective excitation was possible via the dyes chosen in the controls, the technique was tested on samples that had subcellular structures stained with different dyes that absorbed at different wavelengths. Selective excitation of actin filaments in HeLa cells as seen in Fig. 12A. Actin was stained with Phalloidin-350 and excited using the blue pulse. In Fig. 12B, the red pulse was used to excite the mitochondria, which were dyed with MitoTracker-488. It is clear in each image that only the actin (Fig. 12A) or the mitochondria (Fig. 12B) are excited and not both. This shows that by using ultrafast laser pulses and using correct shaping across the broad bandwidth, selective excitation of dyes is possible. These two images were merged in Fig. 12C to show their subcellular localization.

Our work prompts the question: what is the optimal pulse duration for TPEF microscopy? In principle, the pulses should be short enough that their bandwidths overlap the two-photon absorption spectrum of the chromophores used for labeling. Typical bandwidths of two-photon absorption spectra are in the 50–100 nm range, corresponding to 20–10 fs pulse durations, respectively. Once the entire absorption spectrum of the chromophore has been encompassed, a broader laser bandwidth would not result in greater signal, however, it can still be used for selective excitation. In that sense, the optimal pulse duration would be one that results in a laser pulse that encompasses the absorption spectra of the labeling chromophores. In our lab, we have a 4.6 fs laser that allows us to do selective excitation from 625–1050 nm [13]. In addition to imaging, this laser system allows us to measure chromatic dispersion of materials and two-photon excitation spectra of fluorescent dyes [23].

4. Conclusion

During the first decade of multiphoton microscopy, the majority of the efforts towards pulse compression were centered on elimination of the group delay dispersion from the microscope objective using a pair of prisms. Here, we find that the higher-order dispersion from the objective and the prisms themselves plays a very significant role in signal degradation and has been responsible for preventing the use of ultrashort pulses in multiphoton microscopy. We find that correction of the higher-order dispersion leads to a significant increase in the signal intensity and ensures reproducibility of the results through the delivery of the shortest possible pulses at the sample. In addition, we found deeper penetration depth, reduced photobleaching, and higher signal-to-noise ratio can be achieved using MIIPS compensated pulses. Selective two-photon excitation of subcellular components becomes possible when ultrashort pulses are used for imaging because of their broad spectral bandwidth.

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