High speed multiphoton axial scanning through an optical fiber in a remotely scanned temporal focusing setup

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Abstract: Simultaneous spatial and temporal focusing is used to acquire high speed (200Hz), chemically specific axial scans of mouse skin through a single-mode fiber. The temporal focus is remotely scanned by modulating the group delay dispersion (GDD) at the proximal end of the fiber. No moving parts or electronics are required at the distal end. A novel GDD modulation technique is implemented using a piezo bimorph mirror in a folded grating pair to achieve a large GDD tuning range at high speed.

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1. Introduction

Depth-resolved microscopy shows great promise in the fields of biology and medicine. The ability to optically scan through layers of cells makes available histological information that would otherwise be obtained only through tissue biopsy. Experiments in depth-resolved tissue spectroscopy and imaging have shown that confocal and multiphoton fluorescence and harmonic generation microscopy techniques can effectively be used in cancer detection and diagnostics [1–3]. Efforts have been made to incorporate these techniques into fiber probes for clinical application [4–7].

Scan speed is an important factor in *in vivo* microscopic imaging to reduce motion artifacts introduced by the sample movement. In a typical point-scanning depth-resolved microscope, the lateral dimensions are scanned using galvanometer mirror scanners at line rates of several kHz. Miniaturized MEMS scanners and piezo-electric scanners have been used for lateral scanning in miniature, flexible fiber probes [5–7]. The axial dimension in a typical microscope is scanned either by moving the sample relative to the output optics of the system or by moving a lens within the optical system. Because of the necessary movement of a bulk object, axial scanning is typically much slower than lateral scanning in a conventional microscope.

There are currently several techniques for higher speed depth-resolved axial scanning. For table-top multiphoton microscopy, a second, identical objective focused onto a small mirror may be placed optically conjugate to the main objective and sample. The mirror may be translated axially at high speed due to its small size, resulting in an axial scan of the focus on the sample [8]. Another method is to place a deformable mirror or spatial light modulator conjugate to the back focal plane of the imaging objective for arbitrary beam pointing and focusing [9]. These techniques are effective, although expensive and complicated. More importantly, since electronics and mechanical moving parts are required near the sample, they cannot be readily miniaturized or incorporated into a flexible fiber probe. While high speed axial scanning is routinely achieved in optical coherence tomography (OCT) fiber probes [10], OCT does not provide the chemical specificity and spectral information offered by confocal and multiphoton techniques.

The temporal focus in a simultaneous spatial and temporal focusing (SSTF) setup can be remotely scanned by modulating the group delay dispersion (GDD) of the excitation pulse at the proximal end of a delivery fiber [11–13]. No moving parts or electronics are required at the distal end. However, previous demonstrations of axial scanning with SSTF are either at too slow a scanning speed or too small an axial scanning range for practical application. In Durst *et al.* [13], GDD tuning is achieved by moving bulk glass prisms on translation stages, severely limiting the maximum scanning speed. In Du *et al.* [14], acousto-optic deflectors are

used to achieve high-speed GDD tuning. However, the small GDD tuning range limits the axial scanning range to only a small fraction of the axial resolution (i.e., axial full width half maximum). In this paper, we present high speed, chemically specific, large range axial scans of mouse tissue using GDD tuning in an SSTF setup. The technique we report is enabled by a novel dispersion modulation device based on a folded grating pair and a piezo-bimorph mirror. The large GDD tuning range (over $5 \times 10^5 \text{ fs}^2$) provided by the dispersion modulation device allows for a large axial scan range, with over 16 independently resolvable depth sections at a scan speed of 200Hz. The excitation pulses are delivered through a single-mode fiber, demonstrating the potential for future integration in a miniaturized fiber probe.

Fig. 1. Experimental setup for remote axial scanning. The group delay dispersion (GDD) of a femtosecond pulse is modulated by (a) a piezo-bimorph mirror in a folded 4-f grating pair setup. The GDD modulated pulse is coupled into a single-mode optical fiber. The output of the fiber undergoes (b) simultaneous spatial and temporal focusing (SSTF), in which a grating is imaged onto the sample forming a wide-field temporal focus. The sample emission is collected by the PMT detector assembly and correlated with the piezo-drive voltage.

2. Simultaneous spatial and temporal focusing

SSTF is a multiphoton technique in which a diffraction grating is imaged onto the sample, creating a wide-field, temporal focus (Fig. 1b) [11,12]. The grating separates the spectral components of the pulse in space, causing the pulse width to vary as a function of axial position. For a transform-limited pulse, the minimum pulse width is recovered at the image plane of the system. Away from the image plane the pulse width broadens quickly. Because two-photon response is inversely proportional to the pulse width, an effective temporal focus is created at the image plane, providing axial confinement of the signal.

Axial scanning of the temporal focus by changing the GDD of the excitation pulse was experimentally shown and theoretically explained by Durst *et al.* [13]. The axial two-photon response of a SSTF system is given approximately by:

$$TPE(z) = \frac{C}{\left[1 + \left(\frac{f - z + \beta\Omega^2 z_R}{z_R}\right)^2\right]^{1/2}} \quad (1)$$

with

$$z_R = \frac{2f^2}{k(s^2 + \alpha^2\Omega^2)} \quad (2)$$

where C is a constant, f is the focal length of the objective lens, 2β is the GDD of the excitation, $\sqrt{2\ln 2}\Omega$ is the full width half maximum (FWHM) of a Gaussian excitation spectrum, k is the mean magnitude of the excitation wave vector, and s and $\alpha\Omega$ are the monochromatic and full-spectrum spot sizes at the back aperture of the objective lens. The two-photon signal is axially confined, with a FWHM given by $2\sqrt{3}z_R$.

The position of the two-photon signal peak varies with the GDD. It can be shown from Eq. (1) that the displacement of the peak, Δz , is given by

$$\Delta z = \beta\Omega^2 z_R \quad (3)$$

By modulating the GDD of the excitation pulse, the temporal focus may be scanned over a range determined by the optical parameters of the system and the total available GDD.

3. Group delay dispersion modulation with a piezo bimorph mirror

To effectively use SSTF in an axial scanning setup, a method of modulating GDD over a large range at high speed is required. Typical methods for dispersion tuning include prism pairs and grating pairs [15,16], but their tuning speed is limited by the mechanical translation of these bulky optical elements. Electronically adjustable devices, such as acousto-optic modulators (AOMs) [17], spatial light modulators (SLMs) [18], and deformable mirrors [19,20], can achieve programmable and high-speed dispersion tuning. These programmable devices can generate spectral phase of nearly arbitrary shape but at a high cost and with a limited GDD tuning range. In addition, their ability to generate higher-order dispersion is unnecessary, since tuning of the second-order group-delay dispersion is sufficient for remote axial scanning.

We demonstrate a GDD tuning device using a single piezo-bimorph mirror at the Fourier plane of a 4-f grating pair setup. Piezo bimorphs assume a quadratic shape with an applied voltage, allowing for large curvatures with high-speed electronic control [21,22]. While pulse shaping using a piezo bimorph with multiple electrodes to independently address multiple sectors of the actuator has been demonstrated in the past [23], the use of a single piezo bimorph not only makes the device cost-effective but also provides large quadratic curvatures (i.e., GDD tuning range), avoiding both fabrication complexity and stroke limitations of multi-element deformable mirrors. Our device is low cost, robust (high damage threshold), high speed, and has a large GDD tuning range of more than 10^5 fs^2 over a broad range of wavelengths.

The experimental setup of the GDD tuning device is shown in Fig. 1 (a). A ruled diffraction grating (1800 lines/mm) spectrally separates the beam from a mode-locked Ti:Sapphire laser ($\lambda_0 = 775 \text{ nm}$, $\Delta\lambda = 8 \text{ nm}$), which is then collimated by a spherical lens with a focal length of 30 cm. At the focal plane of the collimating lens, we place a piezo bimorph (T220-A4-503X, Piezo Systems, Inc.). The center of the piezo bimorph is fixed, allowing it to bend both forwards and backwards. A 380-micron thick pre-polished silicon wafer (University Wafer) is coated with gold to serve as a reflecting mirror, and it is mounted onto the piezo bimorph with double-sided Scotch tape as spacers. The piezo bimorph is driven by a

function generator combined with a linear amplifier. The bending of the piezo bimorph can be described by [21,22,24]:

$$y = -\frac{3d_{31}}{2T^2}Vx^2 \quad (4)$$

where y is the axial displacement of the piezo, x is the lateral position of the piezo with respect to the optical axis, V is the applied voltage, d_{31} is the piezoelectric coefficient, and T is the total thickness of the piezo. The difference in path length traveled by an individual monochromatic component is $2y$ due to the double pass configuration. The grating maps the individual frequency components, ω , to distinct lateral positions, such that for small spectral bandwidth, $x = \alpha_D \omega$. The quadratic path length difference is then mapped to quadratic spectral phase, or GDD:

$$2ky = \frac{1}{2}\text{GDD} \omega^2 = \frac{1}{2}\text{GDD} \left(\frac{x}{\alpha_D} \right)^2 \quad (5)$$

where k is the wavevector corresponding to the center wavelength λ_0 . From Eqs. (4) and 5, the GDD at a driving voltage V is then:

$$\text{GDD} = -\frac{12\pi}{\lambda_0} \frac{d_{31}\alpha_D^2}{T^2}V \quad (6)$$

Equation (6) shows that both normal and anomalous dispersions can be obtained in the same setup by changing the sign of the drive voltage. For example, for typical values $V = 100$ V, $d_{31} = 1.9 \times 10^{-10}$ m/V, $T = 0.51$ mm, $\alpha_D = 2.84 \times 10^{-16}$ m/Hz, and $\lambda_0 = 775$ nm, the corresponding GDD will be 2.8×10^5 fs².

Fig. 2. (a) Plot of measured GDD versus piezo-drive voltage. (b)-(d) Second-order interferometric autocorrelation traces of a 100 fs pulse at piezo drive voltages of (b) 100 V, (c) -10 V, (d) -100 V.

To determine the GDD imposed on the pulse experimentally, we measure the pulse width of the beam exiting the folded grating pair using an interferometric second-order autocorrelator. The nonlinear element is a GaAsP photodiode. The beam is coupled out of the GDD device with a 50/50 beam splitter. A 100-fs pulse can be broadened to 4.2 ps by varying

the drive voltage between $\pm 100\text{V}$ (Fig. 2). Assuming a sech^2 shape of the spectrum, the piezo-bimorph mirror produces a GDD of $\pm 2.5 \times 10^5 \text{ fs}^2$, varying approximately linearly with voltage, and agreeing well with theory (Eq. (6)). The device can be modulated at rates up to 100 Hz with no reduction in tuning range. The tuning range increases beyond 100 Hz as the piezo-bimorph mirror approaches its mechanical resonance (160 Hz), though we did not operate the device in this regime in order to prevent mechanical damage.

4. Axial scanning SSTF through a fiber

We use the GDD tuning device described above to perform remote axial scanning through a single mode fiber with SSTF. The experimental setup is shown in Fig. 1. The Ti:Sapphire laser is tuned to 755nm with spectral bandwidth of 8.5nm (full width half maximum). After the dispersion tuning device (Fig. 1a) the pulse is coupled into an air-core photonic bandgap fiber (Crystal Fibre, AIR-6-800), which has a nominal zero-dispersion wavelength of 754nm. The output of the fiber is collimated and incident on a 1200 lines per mm diffraction grating at 65 degrees such that the first-order diffraction angle of the center wavelength is normal to the plane of the grating. The diffracted beam is collimated by a spherical lens with a 40-cm focal length, and refocused onto the sample with an objective lens.

Emission from the sample is reflected by a dichroic mirror and detected by a photomultiplier tube (PMT). A function generator provides a sinusoidal signal used to modulate the piezo-bimorph mirror and to provide a trigger for photon counting of the PMT signal. The PMT signal is then correlated with the piezo-drive voltage, which is proportional to the change of GDD in the system and thereby the axial displacement of the temporal focus (Eqs. (3) and (6)). Because the temporal focus scans both forward and backward in one voltage cycle, the axial scan rate is twice the modulation rate of the GDD. The triggering setup allows multiple axial scans to be averaged in real time to improve the signal-to-noise ratio.

Fig. 3. a) GDD axial scans of a Rhodamine B film of $0.5\mu\text{m}$ thickness. Two-photon fluorescence signal is plotted against the piezo-drive voltage. The film is moved $50\mu\text{m}$ between scans. b) Mapping of temporal focus position and relative two-photon signal strength to piezo-drive voltage.

A cover slip coated with a 0.5-micron film of fluorescent dye (Rhodamine B) is used as a test sample to calibrate the system. The sample is placed on a translation stage, and can be stepped axially through the temporal focus. The two-photon fluorescence signal is measured vs. the piezo-drive voltage for a number of fixed sample positions (Fig. 3a). Since the peak of each GDD scan corresponds to the sample position, the axial scan traces in Fig. 3 allow us to generate a mapping between the piezo-drive voltage and the position of the temporal focus setup (Fig. 3b). From this mapping we also extract the range and resolution of the remote

scanning. We were able to extend the voltage range to ± 160 V before two-photon signal strength dropped by more than a factor of two. This loss in signal is due largely to the range limit of remote axial scanning using SSTF, which was predicted in previous theoretical work [13], as well as variation in coupling efficiency into the single-mode fiber, measured to have a root-mean-square variation of about 10% over this voltage range.

The data shown in Fig. 3 was taken with an objective lens with a nominal focal length of 8 mm. We measure an axial scan range of $360\text{ }\mu\text{m}$ with a resolution of $22\text{ }\mu\text{m}$ (FWHM). These results show that approximately 16 independently resolvable axial sections can be obtained in our setup. As shown in previous theoretical work, larger or smaller ranges, and correspondingly higher or lower resolutions, may be obtained by changing the focal length of the objective lens [13]. For example, with a nominally 18-mm focal length objective lens, we measure an axial scan range of $1450\text{ }\mu\text{m}$ with a resolution of $75\text{ }\mu\text{m}$. With a nominally 3-mm focal length objective lens, we measure an axial scan range of $60\text{ }\mu\text{m}$ with a resolution of $3.8\text{ }\mu\text{m}$.

Fig. 4. Axial scans of mouse skin. Axial scan taken by moving the sample through the temporal focus with a translation stage is shown in (a). Axial scans by GDD modulation at 10 Hz and 200 Hz are shown in (b) and (c). The 500 nm to 650 nm channel shown is in red. The peak is produced by autofluorescence in the epidermis. The 355 nm to 400 nm channel is shown in blue. The signal is strongest in the deeper, fibrous tissue of the dermis, producing second harmonic generation.

To demonstrate feasibility in a biological sample, we perform axial scans of excised mouse skin. The multiphoton spectral signal of mouse skin is well characterized by Palero and Gerritson *et al.* [25]. The first fifteen to twenty microns of tissue comprise the epidermis, where multiphoton signal is dominated by intrinsic autofluorescence of cellular molecules, such as keratin and NADPH, in the 450 nm to 600 nm range. Beneath the epidermis is the dermis, which is primarily composed of structural proteins such as collagen and elastin fibers which give a strong second harmonic signal at one half the excitation wavelength (377.5 nm for excitation at 755 nm).

In this experiment we use a nominally 2.7-mm focal length water-immersion objective. The excitation wavelength is 755 nm and the pulse width is approximately 100 fs. The incident average power is 72 mW. We collect the signal with two separate PMT channels, one covering the 355 nm – 400 nm range targeting second harmonic generation, and the other

covering the 500 nm – 650 nm range targeting autofluorescence. Remote axial scanning is performed by leaving the sample stationary and scanning the temporal focus by modulating the GDD. The thin film characterization data is used to generate a position axis from the piezo-drive voltage and to correct for variations in the two-photon response at different positions of the temporal focus. For comparison, we also acquire an axial scan of the tissue by mechanically stepping the sample through a stationary temporal focus with a translation stage.

The results are shown in Fig. 4. An axial scanning speed of 200 Hz is achieved (Fig. 4c), sufficient for overcoming motion artifacts for future *in vivo* imaging of live tissues. The epidermis and dermis are clearly seen in the autofluorescence and second harmonic channels respectively, separated by about 20 microns. The GDD scans faithfully reproduce the stage scan (Fig. 4a), demonstrating that remote axial scanning using GDD tuning in an SSTF setup is equivalent to a mechanical sample scan.

5. Discussion and conclusion

In this experiment, the axial scan speed was limited to 200 Hz by the mechanical resonance of the piezo bimorph, measured to be 160 Hz. The resonant frequency can be increased significantly by reducing the size of the piezo-bimorph mirror, allowing for faster scan speeds. The ultimate limitation on the scan speed is imposed by the brightness of the sample, i.e., the desired signal-to-noise ratio of the axial scan trace. As shown in our results, an adequate signal-to-noise ratio can be obtained with an axial scan rate between 10 and 200 Hz.

Because a wide-field focus is necessary for SSTF to achieve a large axial scan range, the two-photon response is significantly smaller for a given excitation pulse than that for a conventional point focus. The reduction in signal generation rate is partially made up for by the increased integration time. A typical point-scanning system has a pixel dwell time on the order of 10 μ s or less, whereas a SSTF system with 10 axially resolved points scanned at 100 Hz has a pixel dwell time of 1 ms, one hundred times larger. Additionally, because the focal spot in SSTF is much larger than that of a point focus, more excitation power may be delivered to the sample without causing nonlinear photodamage. Therefore, the average excitation power may be increased to further improve the signal-to-noise ratio of the axial scan. We note that one-photon absorption in the near IR (e.g., 800 nm) is typically small in biological samples. For example, optical trappings over several seconds with approximately 100 mW average power are routinely used with minimal sample damage [26,27]. In contrast, a tenth of a second or less is sufficient for one axial scan. In special circumstances where linear absorption is a concern, the signal-to-noise ratio can be improved by increasing the excitation pulse energy without increasing the average excitation power (i.e., by reducing the repetition rate).

The lateral width of the wide-field focus is dependent upon the spot size of the excitation beam on the diffraction grating and its demagnification onto the sample. A larger wide-field focus results in greater sample coverage and a longer available axial scan range, but results in decreased two-photon excitation. A smaller wide-field focus increases the two-photon excited signal over a narrower, more specific region of the sample, but reduces the available axial scan range before significant reduction in signal occurs. A more complete discussion of the impact of the lateral width can be found in Durst *et al.* [13]. In this experiment, the spot size on the diffraction grating was kept near 3 mm, resulting in wide-field foci of approximately 20 μ m, 60 μ m, and 140 μ m on the sample for the objective lenses used. This choice is a trade-off between signal strength and the available axial scanning range.

We believe that the demonstrated remote axial scanning technique is best suited in a passive, flexible fiber probe for multiphoton excitation of axially resolved fluorescence and harmonic generation, bridging the gap between imaging optical endoscopes and non-imaging fiber optic probes. It has all the desirable attributes of a passive fiber probe but with added axial sectioning capability. As shown previously in depth-resolved tissue spectroscopy and imaging [1–3], the addition of axial sectioning to a conventional fiber probe will undoubtedly

improve its diagnostic capability. The collection efficiency of such a probe can be enhanced through the use of large mode area or double-clad fibers [6], allowing for high collection efficiency throughout the depth of focus as the focal plane is scanned. A dichroic mirror placed at an appropriate angle after the grating can redirect the two-photon signal into such a fiber without affecting the excitation beam.

In summary, we have demonstrated high speed, large range, chemically specific multiphoton axial scanning of excised mouse skin through a single-mode fiber using SSTF. Because scanning is achieved by modulating GDD on the proximal end of the fiber, this technique may be implemented in a passive multiphoton fiber probe capable of producing high speed axial scans *in vivo* with no moving parts or electronics at the distal end. Such a remote scanning multiphoton probe can be an excellent compliment to existing OCT probes. An integrated SSTF and OCT passive fiber probe can be used to obtain both one-photon reflectance and two-photon fluorescence and harmonic generation signal, potentially enhancing its diagnostic capability.

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