6 Principles and applications of temporal-focusing wide-field two-photon microscopy

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Abstract: Temporal focusing allows for rapid optically sectioned two-photon wide-field microscopy. Depth sectioning is provided in a wide-field manner, without spatial focusing, by controlling the temporal width of femtosecond laser pulses near the focal plane. This spatial control of the temporal pulse width is achieved by diffracting the light off a grating resulting in spectral component separation and temporal broadening; these spectral components are only recombined at the focal plane to reproduce short, femtosecond pulses. Applications include (i) high speed functional imaging in the brain, (ii) fast FLIM and PLIM, (iii) cell-selective optogenetic excitation, and (iv) temporal focusing photodynamic therapy that may allow selective killing of cancer cells.

6.1 Introduction

The two-photon absorption process was first predicted by Dr. Göppert-Mayer in 1931 [6] and was developed by Sheppard and Webb groups into a powerful microscopic biomedical imaging technique [8–10]. In addition to fluorescence, second (SHG) and higher harmonic generation processes may also be produced via optical interaction with biomolecules with substantial polarizability [11–17]. Multiphoton microscopy has important advantages in 3D depth-resolved image acquisition. First, the quadratic dependence on photon flux results in inherent depth discrimination where over 80% of fluorescent signal is localized to within a 1 μm thick region. Second, photodamage and photobleaching is localized to the same small volume. Third, the multiphoton excitation wavelength lies in the infrared region resulting in significantly lower tissue scattering and absorption and deeper imaging. Fourth, the wide separation of the excitation spectrum and the emission spectra allows more precise spectroscopic measurements.

While lacking depth resolution, conventional fluorescence microscopy, illuminating a sample using wide-field geometry allows 2D images to be acquired at high frame rate. For 3D optical microscopy, such as conventional two-photon laser scanning microscopy (TPLSM), excitation light is focused to a diffraction-limited spot that results in optical sectioning. However, sequential raster scanning of the excitation volume is required to produce a 3D image and data acquisition rate decreases as sample size or resolution increases. Finite fluorophore lifetime and fluorophore concentration ultimately limit fluorescence photon generation rate even with very fast scanning mechanisms, such as an acousto-optical deflectors [23, 24]. Therefore, the most effective way
to improve imaging speed is to parallelize the excitation process [25]. For example, multifocal multiphoton microscopy (MMM) is a successful parallelization approach [26–29]. This chapter focuses on a relatively new multiphoton technique, temporal focusing microscopy (TFM), which can be considered as a method of even higher degree of parallelization (by a factor of $10^2$ to $10^4$) [30–34]. Fig. 6.1 demonstrates the underlying concepts behind spatial focusing and temporal focusing. In Fig. 6.1 (a), an optical pulse is focused laterally in the spatial dimension, traveling along axial direction and its temporal pulse width is constant. The intensity at the focal spot reaches a maximum. Nonlinear optical processes such as two-photon absorption are proportional to the square of the pulse peak power, resulting in optical sectioning. For high numerical aperture (NA) objective, submicron lateral and axial resolution can be achieved. With temporal focusing, the optical pulse travels in axial direction without changing its lateral dimension, unlike spatial focusing (Fig. 6.1 (b)). However, the instantaneous intensity is maximized at the focal plane by manipulating the temporal width of the optical pulse such that it is minimized at the focal plane and is broader in out-of-plane locations. This approach allows wide-field imaging with depth discrimination. TFM controls the spectral dispersion of the ultrafast pulse along the optical path to manipulate pulse width to produce this optical sectioning effect.

![Fig. 6.1](image)

**Fig. 6.1:** (a) Spatial vs (b) temporal focusing multiphoton microscopy.

In this chapter, we will cover the basic principle of TFM and its typical implementation. We will provide both paraxial and vectorial formulation describing image formation for temporal focusing; the limited axial resolution of this approach will be explained and two methods for remedying this limitation will be described. A characterization of penetration depth of TFM for imaging into different tissues and organs will be presented. Finally, this chapter will close with examining several applications where TFM have demonstrated successes. Clearly, it is impossible to cover all application areas that have used TFM and our selection of specific application areas is inherently subjective. However, we believe that TFM excels in application spaces that are characterized by the need for simultaneous, possibly patterned, excitation over
6.2 Invention of temporal focusing two-photon microscopy and basic operating principle

Temporally focusing wide-field two-photon microscopy was introduced almost simultaneously by Oron and Silberberg [30] and by Zhu and Xu [33] about a decade ago. Temporal focusing two-photon microscopy is, in essence, simply a 4-f pulse shaper proposed by Froehly, Colombeau, and Vampouille [35] and Martinez, Gordon, and Fork [36] with the exception that there is no second grating to recombine the pulse. Temporal focusing multiphoton microscopy works because of the time-bandwidth product which states that the product of the pulse width and the spectral content is a constant. In a typical experimental setup (Fig. 6.2), an ultrafast pulse is incident upon a grating. This approach separates the pulse into its constituent wavelengths, each diffracting at an angle governed by the grating equation. A focusing lens converts the wavelength-dependent angular separation of the spectrum into a wavelength-dependent positional offset at the back-focal plane of the objective. The objective collimates and recombines the various spectral components only within the focus of the objective. It is clear that with the exception of the grating surface and the focal plane the pulse

![Diagram of temporal focusing multiphoton microscopy](image)

Fig. 6.2: Implementation of temporal focusing multiphoton microscopy.
width is broadened substantially everywhere in between as a result of the constancy of the time-bandwidth product. This reduces the photon flux everywhere between the grating and the focus. It is only at the focal plane where the spectral components of the pulse overlap and are recombined. As a result of this recombination, the time-bandwidth product dictates that the pulse width is once again minimum everywhere across the plane and photon flux is high enough for nonlinear optical processes to occur.

In terms of instrument design it is clear that a temporal focusing microscope is significantly simpler than that of single focus or multifoci scanning system for two reasons. First, since the whole 2D field is imaged simultaneously, raster scanning mechanism and the associated control electronics are not needed. Second, unlike high sensitivity photomultiplier tubes used in TPLSM requiring custom electronics interface, especially multianode photomultiplier tubes used in MMM [27], temporal focusing system uses commercially readily available CMOS or CCD cameras that significantly simplify data acquisition electronics design. However, for acquiring a 3D data cube, it is still necessary to scan the excitation plane in the axial direction. This axial scan can be accomplished by translating the objective or the specimen as in standard scanning microscopes. Alternatively, given the similarities between a temporal focusing microscope and a 4-f pulse shaper, both positive and negative dispersion may be achieved [36]. This property has been exploited in temporal focusing to shift the position of the focal plane along the z-axis effectively creating an axial scan through optical means [37, 38]. This is done by deliberately up-chirping or down-chirping the beam. This approach is very effective for using a temporal focusing microscope for patterning type applications, but is less effective for imaging type applications since the imaging range is defined by the position of the objective that typically has a relatively short depth of field.

Another important consideration in temporal focusing microscope instrument design lies on the choice of excitation lasers. While temporal focusing systems were first implemented with titanium-sapphire (Ti-Sa) oscillators, it is clear that these low pulse energy oscillators are not optimal for temporal focusing applications. Typically, in a single-point focusing system, efficient two-photon excitation requires pulse energy on the order of 0.1–1 nJ. These oscillators can provide pulse energy about 50 nJ and can at best excite 500 spots simultaneously. Since two-photon excitation is a quadratic process, it is always more efficient for high speed imaging to provide sufficient energy at each location to bring fluorophores to near excitation saturation instead of distributing the energy into more locations. Clearly, high peak power lasers are needed for temporal focusing multiphoton microscopy. Chan and Dong groups first introduced the use of regenerative amplifier as temporal focusing [39]. A typical regenerative amplifier delivers mJ level energy pulses allowing in principle simultaneous excitation of $10^6$ to $10^7$ locations. However, it should be noted that regenerative amplifiers do not provide $10^6$ to $10^7$ level imaging speed improvement because these amplifiers deliver comparable average power as Ti-Sa oscillators (on the order of 10 W level) and the cor-
responding pulse train repetition rate is often reduced to kHz to 10 kHz range. One can readily show that imaging speed improvement effectively scales with average power delivered into the specimen, which is limited mostly by specimen photodamage. The typical damage threshold for point scanning is on the order of 10 mW, while the temporal focusing system has delivered power up to 10 W over approximately a 1 mm² area. Therefore, we expect about two orders of magnitude speed increase when comparing point scanning vs temporal focusing systems. Recent introduction of ultra-high power fiber amplifier with power up to several hundred Watts may increase imaging speed further but the limit imposed by the specimen damage threshold of these lasers remains to be established. While these amplifiers have pulse train profiles very suitable for temporal focusing applications, they are also more limited than Ti:Sa oscillators in terms of fluorophore selectivity. These amplifiers typically have emission wavelengths at about 1050 nm with very narrow or no tuning range. While these wavelengths can excite a broad class of organic fluorophores, they are not well matched to most commonly used fluorescent proteins (with the exception of a few red variants). The conversion of these μJ or mJ pulse lasers to the appropriate wavelengths around 900–1000 nm will require optical parametric amplification and frequency doubling. Both of these processes have low efficiencies that greatly reduce the average power output of the final pulse train negating the imaging speed advantage of the temporal focusing approach. While it is likely that multiple hundred Watt amplifiers may be unsuitable for biomedical imaging applications due to specimen damage, these amplifiers may be combined with parametric amplifiers or parametric oscillators to provide much higher average power pulse train in the wavelength range suitable for fluorescent protein excitation that will greatly broaden the biomedical utility of temporal focusing imaging.

6.3 Image formation theory for temporal focusing microscopes

Image formation in a TFM has been previously analyzed [33, 37, 40, 41]. Most of the prior work assumes that the spectrum of the pulse is taken to be narrow such that \( k \approx k_0 \), where \( k \) is the wave number of the spectral components of the pulse while \( k_0 \) is the mean wave number. These works further make the paraxial approximation where a pencil of rays is assumed and the sines of the angles involved are small and may be approximated to the angle itself. This is clearly not the case for ultrafast pulses and high NA objectives. We have recently published a theoretical extension on the theory of image formation for a temporal focusing microscope using the paraxial and vectorial approaches and compare the validity of both approaches through experiment [21]. We have also determined the 3D optical transfer function (OTF) from numerical calculation and compared with experiment. In this work, we make the assumption that \( k \neq k_0 \), whereas the diffracted angle can be assumed to be small. This is more consistent with most experimental design while being in contrast to some previous
publications [33, 37]. For the vector case, by recasting the problem as a projected pupil function, we were able to apply standard FFT algorithms and the chirp z-transform to minimize the computational process.

Fig. 6.3 illustrates the difference between the FWHM of the intensity temporal focusing axial point spread function (PSF) as calculated from paraxial and vectorial theory. It is clear that the paraxial form and vector theory give similar results, although the drop-off is slower for the vector theory. Paraxial theory gives a smaller axial FWHM of 6.2 μm compared to 7.6 μm from vector theory. This may be attributable to the vector theory accounting for factors such as the apodization, that the spectral content of the ultrafast pulse has a Gaussian intensity profile, and that the wavefront after the objective is spherical and not parabolic. The closeness of the results can be explained if we note that even for 100 fs pulses, the angle of diffraction at the 1/e² wavelength is small enough such that \( \cos \theta_d \approx 1 \). However, the slower fall-off of the axial PSF has implications in poor axial sectioning of this approach.

We now consider the OTF of TFP. Fig. 6.4 shows the cross sections of a cross section of the 3D OTF along \( k_x-k_z \) plane of the 1P wide-field microscope, the 1p confocal microscope with a point detector, the 2P microscope, and the temporal focusing 2P microscope. In Fig. 6.4 (a), it is observed that the 1P wide-field microscope exhibits the well-known ‘missing cone’, indicating that 1P wide-field microscopy is without optical sectioning capability. In Fig. 6.4 (b) and (c), the inclusion of a confocal pinhole or the use of 2P excitation results in optical sectioning. The OTF for a TFM can be seen as a mix between a wide-field and a 2P microscope. The transverse frequency support is similar to that of a 2P microscope. It also misses axial frequency support for the low lateral frequency region but it does not have a true “missing cone” as shown in Fig. 6.5.
Fig. 6.4: (a) Slice view of the OTF for a 1p fluorescence wide-field microscope. The missing cone indicates that no axial information is transmitted. On the other hand, (b) is the slice of the 1P confocal microscope. The missing cone has been filled in and has optical sectioning. A similar OTF is found for (c) a conventional 2P microscope. (d) is the slice OTF of a temporal focusing 2P microscope. The approximate extent of the OTF has been outlined in white as a visual aid [21].

Fig. 6.5: Plotting the transfer function along the $k_z$-axis indicates that the frequency support for temporal focusing 2P is close to half that of the conventional 2P transfer function. The 1P confocal has a frequency support twice that of conventional 2P. The cut-off frequency for the 2P microscope is about twice as large as that of a temporal focusing 2P microscope. In all cases, the excitation wavelength was 790 nm and the emission wavelength of 395 nm [21].
Fig. 6.5 shows a plot along $k_z$-axis from the 3D OTF. The axial frequency support for a 2P microscope is given by $n(1 - \cos \alpha)$. Given that the point spread function of a temporal focusing 2P microscope has an FWHM of 1.8 μm, which is about twice that of a conventional 2P microscope at 0.8 μm, we expect the support for a temporal focusing 2P microscope to be close to half that of the conventional 2P transfer function in the axial direction and is supported by Fig. 6.5. Another key observation is that the drop-off is much quicker for a temporal focusing 2P microscope indicating much faster axial information loss as compared to conventional 2P or 1P confocal microscopes. The axial resolution is therefore much poorer for TFM. We next compared the validity of our high aperture formulation of TFM to experimental measurements. We obtained the 3D OTF (Fig. 6.6 (b)) by taking the 3D Fourier transform of the PSF image (Fig. 6.6 (a)) of a single bead. Since the beads have a peak emission of fluorescence at 485 nm we normalized the spatial frequencies by 485 nm for both cases. It is clear that the theoretically derived PSF and OTF are validated by experiment. A two-photon microscope is worth the complexity and expense because it is able to deliver good optically sectioned images. A TFM has optical sectioning effect that is poorer than that of a 1P confocal or a 2P microscope. We imaged a 0.2 μm fluorescent bead and summed the image over a region equivalent to 2 Airy Units (AU). We found that the FWHM of the imaged bead is 2.1 μm compared to 1.8 μm from theory, while Fig. 6.6 shows the fit.

**Fig. 6.6:** Comparison between the numerically calculated (a) PSF and (b) OTF. (a) Experimental data is represented as blue circles while theory is plot as a red line. (b) Left is OTF from theory and right is experimental. In both cases, the excitation wavelength was 790 nm and the emission wavelength was taken as 485 nm for (a) [21].
Although the image of a single fluorescent bead provides a good estimate of the imaging properties of a microscope, a more common scenario found in imaging is to discriminate between signal that emanates from the in-focus plane and out-of-focus planes. For this purpose, the optical sectioning has been defined to be the response of the microscope to a plane of fluorescence with no transverse variations (i.e., no lateral spatial frequencies). Alternatively, it is the steepness of the gradient of the response due to a sea of fluorescence scanned through the focus. The signal of such an experiment was recorded and graphed in Fig. 6.7(a). The first derivative of the recorded signal, a measure of the optical sectioning capability of a microscope, was obtained and is also presented in the same figure. From the data, we find that the FWHM of the optical response to a sea of fluorescence (given by the dotted red curve) to be 7.6 μm. What this means is that we are only able to locate the interface of an axially extended object with no lateral variation to within 7.6 μm. We further compared the optical response to a sea of fluorescence by measuring the optical sectioning effect, which is obtained by scanning a thin film of fluorescence through the focus. Fig. 6.7(b) illustrates the response of the temporal focusing 2P microscope due to a thin layer of fluorescence. The measured response has an FWHM of 8 μm and is in good agreement with the derived optical sectioning response from a sea of fluorescence as well as the response calculated numerically.

Fig. 6.7: Estimation of optical section of temporal focusing 2P microscope. (a) A thick layer of Rhodamine was scanned through the temporal focus and the recorded signal (blue, solid line) is plotted. The first derivative of the signal was obtained and a curve fitted to it (red, dashed line). (b) A thin layer of fluorescence (red open circles) was scanned through the temporal focus and the captured image was summed over an area corresponding to around 2 Airy Units (AU). This experiment is equivalent to taking the first derivative of the optical response to a sea of fluorescence. The data is compared to vector theory (blue line) and a good fit is obtained between experiment and theory [21].
6.4 Remedying the poor axial resolution of temporal focusing 2P microscopy

TFM suffers from low axial resolution when compared to the standard TPLSM that is partly caused by the under-utilization of the numerical aperture (NA) of the objective [18]. Another problem of TFM, that uses imaging detectors such as CCDs and CMOSs, is that its resolution is based on telecentric mapping of emission photons from the specimen plane to the image plane. For deep imaging, the scattering of emission photons degrades the image contrast obscuring the fine features of the specimen. In contrast, the scattered emission photons of TPLSM are integrated with a large area detector and the spatial structure and resolution of images depend only on the temporal sequence of the scanning process and the excitation point spread function [42]. In addition, it has been reported that the axial extent of the excitation volume is progressively broadened in a scattering medium as the imaging depth increases, which further adds more background noise [43]. Today, besides implementing temporal focusing in a line-scan geometry [31], there are two successful implementations that overcome the resolution limit of standard wide-field temporal focusing 2P microscopy.

![Diagram](image_url)

**Fig. 6.8:** (a) Implementation of multifocal temporal focusing 2P microscope. (b) Pulse splitting and propagation between Echelle and normal grating [18].
Vaziri and co-workers have shown that 0.85 µm FWHM axial resolution can be achieved at the expense of increasing complexity of the system by properly filling the back aperture of the objective [18]. They have termed this technique Multifocal Temporal Focusing (Fig. 6.8). Their implementation is similar to that of Tal and co-workers [31] in implementing line-scan temporal focusing. In the line-scan arrangement, a cylindrical lens first focuses the light in one dimension onto the grating. The tube lens expands the line focus to cover the objective back aperture in one dimension as in other line-focusing scanning microscopes while the dispersion of the grating expands the different spectral components of the ultrafast pulse along the orthogonal direction covering the full back aperture resulting in good axial confinement. The issue with this line-scan approach is that slow mechanical scanning is needed in one direction. Multifocal temporal focusing overcomes this limitation. Instead of using a cylindrical lens to focus light into the grating, an Echelle grating is inserted to reflect the ultrafast light pulse to the normal grating via a 4-f geometry. It is ensured that the grating period of the Echelle grating projected onto the sample via the two 4-f relay is small enough such that it is diffraction limited, thus ensuring the back aperture of the objective is filled along this direction. The grating step size is chosen such that the ultrafast pulses reflected by each step are sufficiently temporally delayed and do not interfere with each other. A more detailed description of this pulse propagation geometry is presented in Fig. 6.8 (b).

Multifocal temporal focusing has very successfully improved the axial confinement of TFM. It formed a 7 µm diameter spot in the specimen with an excellent aspect ratio featuring 0.85 µm confinement in the axial direction. This demonstrated that axial confinement similar to that of the line-scan temporal focusing system can be achieved and thus is comparable to that of traditional TPLSM (Fig. 6.9). The approach has also successfully been applied to improve cellular and tissue imaging resolution (Fig. 6.10).

We have recently demonstrated an alternative approach to overcome the poor axial resolution of temporal focusing by utilizing structured light illumination (SLI) in TFM to effectively reject background scattered emission photons and thereby improve image contrast when imaging in a turbid medium [19]. Equally importantly is that the axial resolution of this system is dictated by the spatial frequency of the structured light thereby allowing an axial resolution that is significantly better than TPLSM even in the absence of scattering. The core idea of this approach is that SLI acts as a virtual pinhole and the low frequency out-of-focus scattered photons beyond the depth of field of the objective can be removed computationally.

A class of depth-resolved imaging techniques based on SLI have been proposed to select a particular imaging plane and to reject out-of-focus background for standard wide-field single-photon microscopy [44, 45] and it has been shown that the axial resolution of these techniques is comparable to that of the confocal microscope [46, 47]. Of these methods, one effective approach we adapted is termed ‘HiLo microscopy’ that combines the in-focus high frequency content extracted from the uniformly illumi-
Fig. 6.9: (a) Axial confinement of multifocal temporal focusing 2P microscope (red line) is comparable with line temporal focusing system (black line). (b) Lateral light distribution corresponding to the measured axial confinement [18].

Fig. 6.10: Comparing multifocal temporal focusing 2P with temporal focusing 2P and wide-field 2P for imaging mouse kidney cells [18].
nated image (UI) and the in-focus low frequency content extracted from the structured light illuminated image (SI) to generate an optically sectioned image [45].

An implementation of structured light TFM is shown in Fig. 6.11 where either a phase grating (generated by a Michelson interferometer) or an intensity granting (generate by a physical grid) can be used. The theoretical axial resolution of SLI can be estimated using the defocused 2D optical transfer function (OTF) derived by Stokseth [48]. Fig. 6.12(a) shows the plot of contrast at the fringe periods of $T_g = 3.42 \mu m$, $1.71 \mu m$, $0.85 \mu m$, $0.43 \mu m$, which correspond to the normalized fringe frequencies of 0.13, 0.26, 0.52, 1.04, respectively. For the fringe period of 1.71 \mu m and higher, FWHM of the axial resolution is expected to be better than that of normal temporal focusing systems reported in the literature [49, 50]. Furthermore, theoretically, SLI has the potential of attaining depth resolution better than TPLSM when the normalized fringe frequency is higher than 0.3 as shown in Fig. 6.12(b). Ideally, the best optical sectioning is achieved when the normalized fringe frequency comes close to 1 but with the trade-off of the reduced signal-to-noise ratio [51]. For this comparison, the intensity of SLI is obtained by normalizing the defocused 2D OTF and the total fluorescence intensity of TPLSM generated at a given z-plane for a uniform specimen is calculated by integrating the two-photon intensity in each z-section [52]. The axial resolution improvement was demonstrated experimentally with a thin layer of Rhodamine solution both in the absence and in the presence of scattering medium. Experimental results show that axial sectioning similar to standard TPLSM can be achieved (Fig. 6.12). We further performed the measurement with a 15 \mu m thick mouse kidney sample under two scattering conditions. First, the experiments were performed without any scattering medium (0% Lipofundin) and then undertaken with 2% Lipofundin as an immersion medium. From Fig. 6.13, it is evident that SLI improves the axial resolution of

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**Fig. 6.11:** Schematic diagram of the temporal focusing structured light illumination setup. Either interferometer or grid is used for the fringe projection or the grid projection. BE: beam expander, RDG: reflective diffraction grating, NPB: nonpolarizing beam splitter, M: mirror, ExTL: excitation tube lens, EmTL: emission tube lens, DM: dichroic mirror, Obj: objective, FP: focal plane [19].
Fig. 6.12: (a) Contrast decay of fringe pattern of the spatial period of \( T_g = 0.43, 0.85, 1.71, 3.42 \) μm as a function of the defocus. (b) Normalized intensity of SLI and TPSM as a function of its distance from the focal plane [19].

Fig. 6.13: Axial resolution of structured light temporal focusing 2P microscope measured with thin layer of Rhodamine solution in the absence and presence of scattering medium. Fringe period of SLI is 1.71 μm. (red) TFM HiLo at 0 % intralipid, (blue) TFM HiLo at 2 % intralipid, (green) TFM at 0 % intralipid, (black) TFM at 2 % intralipid, (orange) TPSM in Fig. 6.2 (b) convoluted with 2 μm thick Rhodamine solution [19].

TFM significantly. At 0 % Lipofundin, TFM’s FWHM is 3.98 μm and TFM HiLo’s FWHM is 2.22 μm. At 2 % intralipid, they are 5.21 μm and 2.42 μm, respectively. This broadening of the axial resolution has been reported in the literature [43]. It is interesting to note that HiLo processed data is not much affected by the scattering medium, which implies that HiLo-based SLI works robustly even in a highly scattering medium. The axial resolution improvement is further demonstrated with a prepared slide of sectioned mouse kidney. Fig. 6.14 shows the xz-section view of a TFM image without SLI and HiLo processed TFM images with fringe period of 3.42 μm, 1.71 μm, 0.85 μm respectively. As the
fringe frequency increases, the sectioning capability of SLI increases and finer features of the specimen becomes visible more clearly. Particularly, the cross-sectional intensity plot along the depth direction indicated by the arrow is also shown on the right side. The weak intensity object in the middle of the sample, which is hidden by the two high intensity objects above and below, is discernible with SLI at $T_g = 0.85 \mu m$. This effect is more clearly visible in the normalized intensity plot where the intensity is normalized with the peak intensity value.

**Fig. 6.14:** $xz$-sections of the glomeruli and convoluted tubules structure in a mouse kidney sample acquired with TFM without SLI, HiLo processed TFM with fringe period of 3.42 $\mu m$, 1.71 $\mu m$, 0.85 $\mu m$, respectively. The thickness of the imaged portion is 14 $\mu m$. Intensity increases from purple to red. The cross-sectional intensity plot along the line indicated by the yellow arrow is also shown on the right-hand side [19].

In summary, both multifoci temporal focusing and structured light TPM can improve axial resolution of images to an extent at least comparable to TPLSMs. The multifoci temporal focusing approach has the advantage that axial confinement improvement is achieved on the excitation side, so it is applicable in patterning type applications and minimizes specimen photodamage. These advantages are traded off with the need for a custom-made Echelle grating. Structured light temporal focusing systems have advantages in simplicity of implementation, potentially improving resolution beyond point scanning, and affording rejection of scattered light background. These advantages are traded off with loss of out-of-plane fluorescence photons and the associated photodamage. Potentially this drawback may be partly alleviated by performing maximum likelihood photon reassignment [53].
6.5 Characterizing performance of temporal focusing 2P microscope for deep tissue imaging

It is expected that TFM can excite samples as deep as, if not deeper than conventional TPSM [54], but because conventional TPSM can make use of the scattered emission photons in the sample (since all the emitted photons are assumed to come from the focal volume), unlike temporal focusing which can only use the ballistic and weakly-scattered photons to form the image, it is expected that in scattering samples, such as biological tissue samples, performance will be worse. We have characterized the extent of this performance degradation in several different tissues [22]. We analyzed the ability of the imaging system to extract information by measuring the modulation transfer function (MTF) of tissue images at different depths. MTF is a property of an imaging system that describes the contrast of a given spatial frequency, placed perfectly in focus in the field of view and imaged through an optical system. As the spatial frequency increases, the contrast (the intensity ratio between the brightest and darkest points) drops. At the point where the MTF drops to zero, that spatial frequency cannot be resolved; any features with this spatial frequency cannot be seen. Consequently, this implies that ‘penetration depth’ is user-specific; users who wish to see large features with low spatial frequencies will likely be able to see further into the sample compared to users who wish to see samples with finer features of interest. It is therefore helpful to try to estimate the MTF as a function of penetration depth. In order to ensure that the features to be discerned in different tissues are comparable, we stained cell nuclei by injecting Hoechst 34580 intraperitoneally and intravenously into a male FVB/NJ mouse (Jackson Laboratories) prior to sacrifice. We chose to target nuclei because they are morphologically similar in tissues tested. Further, these nuclei are small and often have fine features, which provide a high frequency support in the sample. By taking the Fourier transform of an image and taking a ‘radial average’ (i.e., combining all the 2D frequency components with the same frequency, regardless of angle), it is possible to estimate the MTF, assuming that the sample consists of spatial white noise. Clearly this is only a weak approximation, since the images of the stained nuclei will have a particular spatial frequency content, but the estimate is acceptable for determining the maximum spatial frequency that can be observed in the sample; by determining where the MTF reaches the background level, it is possible to define the maximum spatial frequency, and therefore determine the maximum penetration depth for a given desired spatial frequency.

After the animal was sacrificed, organs (kidney, liver, heart, spleen, lung, and white adipose tissue) were extracted and fixed by paraformaldehyde and mounted in a glass-bottomed dish for imaging. We then performed both TFM and TPSM of these organ specimens (Fig. 6.15). Excitation power levels were adjusted for different tissues and different image depths to ensure that the image brightness was comparable for different depths and for different tissues, ensuring that the differences in MTF were not simply due to shot noise or reduced signal. An image processing algorithm was
developed to automatically estimate MTF from these image stacks. First, the image is cropped so that it is square. The 2D FFT is then taken, and the image reshaped so that the low-frequency components lie at the center of the image and the high frequency components are at the edge. A ‘radial average’ is then performed, whereby a ring centered on the zero-frequency component is constructed, with a thickness of one FFT pixel. The ring is normalized such that the sum of all pixels in the ring is equal to 1, and then multiplied elementwise with the FFT image; the sum over the whole result-

**Fig. 6.15:** Representative images acquired by both TFM and TPSM [22].
The resulting plot of estimated MTF vs spatial frequency should have an approximately monotonic decay. The background level is determined by starting from the highest spatial frequency values and working towards the low frequency values. At each new spatial frequency value, it is compared with the mean and standard deviation of all the previous (higher frequency) values, which are all assumed to be equal to the baseline plus an individual noise term. If the value of the new point deviates from the mean by more than three times the standard deviation, it is assumed that it is not a noise term and therefore is the start of the image information; its value represents the highest observable spatial frequency in that image. The entire algorithm is illustrated in Fig. 6.16.

![Fig. 6.16: Image analysis algorithm for extracting tissue imaging depth for TPM and TPSM][22].

Accepting that there are limits to the technique in terms of differences in stain penetration as a function of depth and organ type, it is clear from the data that, depending on the sample, TPSM can achieve approximately twice the penetration depth of TFM when subject to practical issues such as sample damage (Fig. 6.17). It was also possible to compare organs in terms of the achievable penetration depth; the heart consistently demonstrated the lowest penetration depth, around 50 µm for temporal focusing and 120 µm for scanning two-photon microscopy. The lungs, liver and kidneys were all
very similar, with penetration depths of approximately 90 µm for temporal focusing and 150 µm for scanning two-photon microscopy. The spleen is slightly easier to penetrate, at around 100 µm for temporal focusing and 180 µm for scanning two-photon microscopy, but the low absorption and scattering due to the large lipid droplets in white adipose tissue meant that penetration depths of nearly 200 µm for temporal focusing and over 500 µm for scanning two-photon microscopy were possible. The especially large error bars were due to the large variation in measured scanning two-photon penetration depths; values of 190 µm, 635 µm and 695 µm were recorded, and the 190 µm result strongly skewed the results.

![Fig. 6.17: A comparison of achievable imaging depth for different tissues imaged using TFM and TPSM [22].](image)

### 6.6 Application 1: Functional imaging of neuronal network using TFM

One may argue that functional imaging of the neuronal network in a small animal brain in vivo is one of the most suitable applications of TFM, since functional imaging of neuronal communication requires high-speed imaging. For the study of calcium signals, the time constant for sampling of the relevant network structure is on the order of 100 ms. While routine voltage imaging of neuronal network still awaits the development of better voltage sensors, it is clear that sampling a neuronal network with a time constant of several milliseconds is very well suited for TFM-type approaches.

One of the first defining applications of TFM in neuronal network imaging was performed by the Vaziri group; they successfully imaged the calcium dynamics of all the neurons within the head of *C. elegans* [1]. The design of the instrument is fairly straightforward (Fig. 6.18). The neurons in *C. elegans* expressed nuclear-localized GCaMP5K, which simplified the segmentation of functional signals from individual neurons. They acquired data from an image cube with approximately 60 µm lateral dimension and 30 µm axial dimension. Taking an axial step of 2 µm, they achieved
Fig. 6.18: TFM for imaging neuronal network in C. elegans [1].

A volumetric frame rate of about 13 Hz, although the GCaMP5K signal-to-noise level limited the actual imaging speed to about 4–6 Hz. The changes in neuronal calcium levels as a function of ambient oxygen concentration were studied across the whole network in the head region (Fig. 6.19). Importantly, the high speed imaging afforded by TPM allowed many neurons to be simultaneously imaged within the relevant time constant, potentially allowing neuronal communication networks to be deduced by examining the correlation matrix (Fig. 6.19 (a)).

While not strictly speaking wide-field imaging, the implementation of temporal focusing in line-focusing mode is an important extension (Fig. 6.20), removing the need for expensive and complex regenerative amplifiers in order to image large areas. The main limitation of using TFM for neuronal network imaging, especially for green sensors like GCaMPs, is limited laser power. Since commercially available regenerative amplifiers currently only output at around 800 nm, and since the excitation of GCaMP requires excitation in the 900–1000 nm range, the use of optical parametric amplification and frequency doubling is required, resulting in severe power loss as previously discussed. This limitation can be overcome by line-focusing, where power is not distributed over a plane, but only over one line, which is then scanned to acquire the image. Dana, Shoham and co-workers used a modified 150 kHz regenerative amplifier operating at 905 nm with 150 mW power, which proved sufficient for line-focusing application [3, 4]. Importantly, even with the laser operating near its performance margin, they significantly extended the imaging area up to about 500 μm laterally, albeit with a fairly modest lateral and axial resolution of about 2.6 μm and 25 μm respectively. Imaging neuronal cell cultures at a frame rate of about 10 frames per second,
they were able to record calcium signals from neurons expressing GCaMP3. Importantly, they were able to simultaneously ontogenetically stimulate selected neurons using ChR2-mCherry via holographic beam forming (Fig. 6.21).

![Fig. 6.19](image)

**Fig. 6.19:** (a) Correlation of calcium signal across different neurons. (b)–(d) Network calcium signals as a function of ambient oxygen levels [1].
Fig. 6.20: Line-scan TPM developed for large area, high throughput neuronal network imaging [4].
6.7 Application 2: Fluorescence and phosphorescence lifetime imaging using TFM

Fluorescence (FLIM) and phosphorescence (PLIM) lifetime microscopies are information-rich optical spectroscopic techniques [55–61]. A particularly important application of FLIM is in the quantification of fluorescence resonance energy transfer (FRET), the preferred method of quantifying intracellular protein-protein interactions in vivo. For example, FRET measurements have been used to measure intramolecular distances [62], and to observe dynamic conformational changes in proteins [63] and RNA [64]. FLIM has been also applied in disease diagnosis. Koenig and co-workers found that normal skin and cutaneous melanoma can be differentiated by their morphological appearances in combination with their fluorescence lifetime spectroscopic signatures [14]. A clinical trial across multiple centers in Europe is underway to test the utility of multiphoton FLIM in the minimally invasive diagnosis of melanoma [65].

While PLIM is not as widely used as FLIM, this methodology nonetheless has several important potential biomedical applications, due to the availability of phosphorescence-based oxygen sensors. Quenching of phosphorescence by oxygen affects the phosphorescence lifetime of such sensors, which then enables measurement of oxygen partial pressure in vivo in tissues or thick biological samples with high temporal and spatial resolution [66–68]. PLIM-based partial oxygen measurements can be used to quantify the degree of hypoxia in tissues or tumors, a critical physiological parameter of solid tumors that determines tumor growth, gene expression [69], metastatic potential [70], metabolism, prognosis [71–73], and response to therapy [74, 75]. The use of PLIM-based oxygen sensors has also enabled the quantification of oxygen supply and consumption in the brain, which is critical for understanding brain metabolism and cognitive function [76, 77]. Today, PLIM is not widely used mostly due to the associated long lifetime that entails typical image frame rates on the order of minutes to hours.

The implementations of FLIM and PLIM measurements in microscopy have different challenges. For FLIM, the need for picosecond level timing resolution of photon arrival time requires fast electronics; for PLIM, the long lifetime (up to milliseconds) significantly lengthens the required pixel residence time and slows the image frame rate. In general, FLIM and PLIM measurements can be performed either in the time domain or in the frequency domain. In the time domain, a light pulse of short duration excites the sample and the time delay of the emitted photon is measured. A histogram of these time delays provides a direct measurement of the fluorophore’s lifetime. Typical fluorophores exhibit single exponential decay kinetics in a homogeneous microenvironment. However, in practice, even for fluorophores with single exponential decay dynamics, the experimentally measured time delay histogram is a convolution of an

*Fig. 6.21: Combining line-focus TPM imaging with optogenetic excitation [3, 4].
unknown instrumentation response and the exponential decay of the fluorophore. In the presence of more complex environments, multiple exponential decays must be resolved. The frequency domain approach is, mathematically, the Fourier equivalent of the time domain. In the frequency domain, the excitation light is modulated at a frequency $\omega$. The intrinsic fluorescence temporal response acts as a low-pass filter and the emitted fluorescent light is phase-shifted and modulated and modified as a function of the underlying lifetime. In order to obtain accurate phase shift and demodulation measurements, the excitation light modulation frequency $\omega$ has to be of the same order of magnitude as the inverse of the lifetime (i.e., up to $10^8$ Hz for FLIM). Phase shift and demodulation are often measured indirectly by either homodyne or heterodyne approaches. In the homodyne approach, measuring the steady state amplitudes at different phases allows the recovery of the waveform. In the case of heterodyne measurement, the signal is detected by a detector whose gain is modulated sinusoidally at a slightly increased frequency $\omega + \Delta \omega$. This electronic mixing process results in translating the phase and demodulation information to an electronic signal at the cross correlation frequency $\Delta \omega$ that can be readily isolated by low-pass filtering. The relative merits of time domain vs frequency domain approaches and homodyne vs heterodyne detection schemes have been discussed extensively in the literature [78, 79].

We have developed a very fast 3D FLIM and PLIM imaging system based on combining two complementary technologies:

1. temporal focusing wide-field (TFWF) two-photon microscopy, a method for efficiently exciting a single 3D resolved plane in a translucent specimen, and
2. camera-based heterodyne frequency-domain lifetime measurement, a method for highly parallelized wide-field imaging with picosecond lifetime resolution [20].

Fig. 6.22 shows the optical design of the temporal focusing wide-field FLIM/PLIM system.

**Fig. 6.22:** Temporal focusing wide-field FLIM/PLIM design. Electronic subsystem: frequency domain lifetime measurement via heterodyne detection [20].
The accuracy of the developed wide-field 3D lifetime imaging system was evaluated by measuring the fluorescence lifetimes of Rhodamine B solution in different solvents that have been carefully quantified in literature [7]. Fitting the measurements to a single exponential decay model allows the extraction of lifetimes tabulated in Fig. 6.23 (left). The lifetime measurements obtained by the temporal focusing FLIM were in good agreement with literature values within about 0.05–0.1 ns. Note that the two ways to estimate the fluorescence lifetime (either from phase data or from modulation data) provide very similar results, which supports the choice of a single exponential decay model to fit Rhodamine B lifetime data. When plotting the lifetime measurements of each pixel into a single polar plot (a sine and cosine transform of temporal decay information into the spectral domain [80, 81]) the lifetimes of individual pixels cluster around a single location located on the universal circle as expected for single exponential decay processes (Fig. 6.23, right). The locations of these four distributions are consistent with the tabulated lifetimes obtained from averaging over the whole image.

<table>
<thead>
<tr>
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<th>Tau (Ph)</th>
<th>Tau (Mod)</th>
<th>Tau (Magde)</th>
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<tr>
<td>FL</td>
<td>4.01</td>
<td>4.18</td>
<td>4.00</td>
</tr>
<tr>
<td>RhB/ EtOH</td>
<td>2.83</td>
<td>2.84</td>
<td>2.93</td>
</tr>
<tr>
<td>RhB/ MeOH</td>
<td>2.22</td>
<td>2.36</td>
<td>2.46</td>
</tr>
<tr>
<td>RhB/ H2O</td>
<td>1.75</td>
<td>1.79</td>
<td>1.68</td>
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**Fig. 6.23:** Demonstration of accurate measurement of fluorescence lifetime of Rhodamine B solutions in different solvents by TFWF FLIM. Fluorescence in water was used as a reference. (Left) Tabulated results of estimated lifetime values $\tau$ extracted from either phase (Ph) or modulation (Mod) measurements. Literature values are also included as a reference [7]. (Right) Lifetime resolved data for each pixel from the fluorescein and Rhodamine solution images shown in polar plot format [20].

The most important advantage of temporal focusing for FLIM imaging is probably in throughput enhancement. The high data acquisition speed of the developed instrument is demonstrated by FLIM imaging of fixed fibroblasts loaded with conjugate polymer nanoparticles (CPN) of high two-photon cross section (previously measured in excess of 15 000 GM [82]). A representative intensity scaled lifetime image and the associated polar plot of pixel lifetime distribution of the specimen is shown in Fig. 6.24. The nonsymmetric, off universal circle distribution of pixel values in the polar plot indicates nonsingle exponential decay of CPNs. This is consistent with the different mean lifetimes measured from phase and modulation data. It is important to note that consistent pixel fluorescence lifetime measurements can be obtained with integration.
times as short as 5 ms (measurement uncertainty on the order of 0.1–0.2 ns). When the integration time is further reduced to 2 ms, phase-based lifetime measurement still provides a reasonable lifetime estimate (with less than 0.5 ns error) while modulation-based lifetime estimates start to deviate from values obtained with longer average time (> 0.5 ns).

The impact of parallelism inherent in TFM is probably more important for phosphorescence measurements. Phosphorescence lifetime measurement has many uses in biomedical imaging and spectroscopic analysis. One of the most important biomedical uses of molecular phosphorescence is monitoring oxygen concentration in biological systems. Minimally invasive measurements of partial oxygen pressure can be used, for example, to quantify hypoxia in tumors, an important determinant of tumor physiology [69, 70] and its response to therapy [75, 83]. However, 3D PLIM using point scanning confocal or multiphoton excitation is always slow since the required pixel residence time must be substantially longer than the phosphorescence lifetime (as long as a fraction of a millisecond). The estimated necessary duration for high-resolution 3D mapping of oxygen distribution in a tumor can exceed days using palladium-based probes (lifetime approaches one millisecond). In order to demonstrate the accuracy of the developed PLIM TFM system, we quantified oxygen concen-
tration in solutions of 1 mM Tris(2,2′-bipyridyl) dichlororuthenium(II) hexahydrate in PBS equilibrated with several independently calibrated oxygen/nitrogen gas mixtures of O₂ mass fraction of 0, 4, 8, and 21%. The phosphorescence lifetime of the ruthenium solution equilibrated with each mixture was found to be single exponential as indicated by the distribution of the estimated pixel lifetime that lies on the universal circle of the polar plot (Fig. 6.25, left). The measured phosphorescence lifetime ranges from about 400 to 600 ns. There is an inverse dependence of the phosphorescence lifetime on oxygen partial pressure in agreement with a Stern–Volmer relationship (Fig. 6.25, middle, right). We have demonstrated fast sequential 3D-resolved FLIM and PLIM TFM imaging of rhodamine labeled fibroblast in collagen scaffold containing ruthenium for in situ environment oxygen monitoring (Fig. 6.26).

Fig. 6.25: Fast measurement of partial oxygen pressure by TFWF phosphorescence lifetime imaging. (Left) Polar plot of phosphorescence lifetime of 1 mM Tris(2,2′-bipyridyl) dichlororuthenium(II) hexahydrate solutions equilibrated with 0, 4, 8, and 21% PO₂ gas mixtures. Inverse phosphorescence lifetimes (middle, from modulation data; right, from phase data) are plotted against O₂ concentration demonstrating Stern–Volmer dependence with $R^2$ values of 0.99 and 0.95 respectively for linear regression [20].

Fig. 6.26: Fast sequential 3D-resolved TFWF FLIM and PLIM imaging of human dermal fibroblasts seeded in a collagen scaffold. Fluorescence intensity fraction due to Rhodamine (top) and phosphorescence intensity fraction due to ruthenium (bottom) are resolved based on lifetimes [20].
6.8 Application 3: Cell-selective optogenetics using TFM

While TPSM has recently become the method of choice for high resolution in vivo tissue imaging, the importance of multiphoton excitation for inducing localized chemical reaction has been recognized by Denk, Webb and co-workers from almost the very beginning [10]. Several years after demonstrating TPSM, Denk further developed scanning photochemical microscopy to induce localized activation of neuron by using caged neurotransmitters [84]. While their work represented an important milestone in developing technologies to control neuronal circuits, it did not result in any major biological impact for two reasons. First, molecular cages at that time had very low two-photon cross section and the laser power required for uncaging often induced nonspecific photochemical reactions in cells. Second, with the development of optogenetics and photoactivated channels today [85], it is clear that photoactivation of ion channels is much more efficient than releasing neurotransmitters since channel opening provides an “amplification” factor that releasing single transmitter does not [85–87].

The landscape of cell-selective activation of individual neurons in vivo was changed fundamentally with the advent of optogenetics in early 2000s [85–87]. While blue light efficiently activates channel rhodopsin, selective excitation of single neurons in living brain was not possible due to light scattering induced broadening of the activation volume. The groups of Vaziri [5] and Emiliani [2] first conceived the idea of using two-photon excitation to localize the optogenetic process in 2010. Importantly, both of these works utilized temporal focusing geometry for neuron activation for several important reasons. First, it has been observed that the efficient activation of a single neuron requires activating many ion channels and activation with a diffraction-limited point is inefficient for this propose. Second, the study of neuronal circuits often requires simultaneous activation of multiple neurons and patterned excitation can be readily accomplished using temporal focused geometry.

Varizi and co-workers recently demonstrated a high aspect ratio two-photon excitation volume spanning 15 μm in diameter with less than 2 μm thickness enabling single cell excitation in tissues. They further investigated various parameters such as depth discrimination, excitation spot size, power, and duration (Fig. 6.27).

Emiliani and co-workers took temporal focusing-based two-photon optogenetics to the next step by introducing patterned excitation (Fig. 6.28). Since the grating surface is imaged onto the imaging plane of a TPM, pattern generation can be readily accomplished by projecting an appropriate intensity distribution on the grating. This can be accomplished with a photomask or an MEMS mirror. However, these approaches are light inefficient if the illumination area is small relative to the whole field of view. Emiliani and co-workers proposed an approach based on the principle of generalized phase contrast. In this approach, a phase-based spatial light modulator projects a phase pattern via a 4-f relay onto the grating via a phase-contrast filter positioned at the Fourier plane. In the ideal case, the spatial light modulator is a pure phase element and generating a phase-only pattern does not result in light losses,
Fig. 6.27: Quantifying spatial resolution of temporal focusing optogenetics [5].

Fig. 6.28: Temporal focusing optogenetic design with patterning element based on generalized phase contrast [2].
although spatial light modulators suffer significant losses due to design limitations. The phase-contrast filter at the Fourier plane phase retards the low frequency components of the phase image relative to the high frequency components. The interference between the low frequency components with the high frequency components at the grating plane effectively converts the phase-only image at the SLM into an image with intensity contrast at the grating. It can be shown that by careful design of the phase contrast filter, one can maximize the image fidelity and contrast while transmitting higher total intensity than with masks or MEMS mirrors. Emiliani and co-workers have provided a thorough characterization of system parameters in temporal focusing patterned optogenetics and demonstrated simultaneous selective multiple cell activation in brain slices (Fig. 6.29).

![Fig. 6.29: Demonstrating selective multiple cell activation [2].](image_url)
6.9 Application 4: Cell selective photodynamic therapy using TPM

It is broadly accepted that cancer is one of the most important medical challenges in the developed world. One of the primary treatments, chemotherapy, is extremely taxing on the patient due to the toxicity and low selectivity of commonly-used anticancer agents. While cancer cells are preferentially killed, a number of other biochemical systems in the body are negatively impacted, leading to side effects such as cognitive dysfunction, hair loss, and organ damage [88]. Photodynamic therapy (PDT) is a far more selective treatment option, wherein light is used to localize the antineoplastic effect and thereby avoid many of the downsides associated with more systemic treatment. In PDT, a chromophore called a photosensitizer is illuminated with specific wavelengths of light in order to create reactive radical species that generate downstream effects, including apoptosis and necrosis in populations of cells. By controlling the administration of light to select regions of tissue, PDT can be carried out in just the tissue of interest, sparing critical nerves and organs, and preserving functionality where conventional surgery and chemotherapy would cause irreparable damage. In the ultimate limit, light administration could be confined purely to selected cells, avoiding the surrounding tissue entirely. In this case, the photodynamic agent need only be optimized for factors like quantum yield, clearance rate and dark (un-illuminated) toxicity. Such control would enable precise treatment of individual cells within intact tissue for pinpoint anticancer therapy, such as, for example, when tumor cells have extensively infiltrated sensitive organs such as the eye.

The use of multiphoton excitation for photodynamic therapy is not new; Fisher et al. demonstrated two-photon excitation of some psoralen derivatives in 1997 [89], and in 1999, König, Riemann and Fischer described their work on two-photon excitation of photofrin and 5-ALA labeled Chinese Hamster Ovary (CHO) cells [90]. Demonstration of two-photon absorption in verteporfin and photofrin in vitro was performed by Khurana et al. in 2007 [91], and in the same year, two-photon occlusion of blood vessels in a chicken embryo was performed by Samkoe et al. using verteporfin [92]. Blood-vessel occlusion was demonstrated in mice by Collins et al., using a selection of different photosensitizers that were optimized for a high two-photon cross section [93]. The use of different novel photosensitizers was also pursued by Starkey et al., who argued that xenograft tumors could be treated using two-photon excitation from a regenerative amplifier focused through approximately 2 cm of tissue [94, 95].

Though this work is promising, the techniques used above all required the femtosecond laser focus to be scanned in the tissue, a serial process that is inherently slow. We have developed a temporal focusing PDT method by exciting millions of points in parallel, using temporal focusing. Our instrument is designed to project a temporal focusing plane approximately $700 \mu m \times 700 \mu m$ onto a sample. Exposure times are on the order of 10 s to 30 s, which is approaching values that are clinically relevant. As an example application, this exposure duration and illumination area could be suitable for the treatment of residual cancer cells in a resection margin.
OVCAR-5 ovarian cancer cells (Fox Chase Cancer Institute) either grown in 2D on a collagen-coated 35 mm glass-bottomed dish (MatTek, P35GCOL-1.5-14-C) or grown in 3D model tumor nodules [96] were incubated with verteporfin solution for two hours and exposed to light using a TFM system. Patterning was accomplished by projecting the temporal focusing plane onto an intermediate focal plane containing a photomask, before being refocused onto the sample. The efficacy of PDT was evaluated after approximately 8–10 hours using live-dead label consisting of cytoplasmic green Calcein AM (live stain) and nucleic orange Ethidium Homodimer-1 (dead stain).

![Graph showing 50% cell death radius as a function of defocus for different exposure values.](image)

**Fig. 6.30:** 50% cell death radius as a function of defocus for different exposure values.

![Image of mit logo via live-dead stain.](image)

**Fig. 6.31:** Demonstrating patterning of the temporal focusing plane using the MIT logo via a live-dead stain. White lines illustrating the mask boundary are added as a guide to the eye and are not present in the image itself.
We have quantified PDT parameters such as exposure duration and verteporfin concentration. The depth sectioning capability of patterned cell killing was demonstrated, and a depth resolution on the order of 20–30 μm was achieved for an exposure area approximately 300 μm in diameter (Fig. 6.30). We have not pushed the axial resolution limit further as PDT does not require axial resolution beyond the dimensions of a typical cell, which is on the order of several tens of microns. We have further demonstrated patterned cell killing in 2D and 3D cultures (Fig. 6.31).

6.10 Conclusion

In this chapter, the underlying physical principles for TFM are explained in detail. We have further evaluated the depth discrimination capability of TFM, in showing the technique to have relatively poor axial sectioning capability, and have described two more advanced TFM implementations that overcome this limitation. We have provided a survey of the usability of TFM in different tissues and organs. We show that, in general, the achievable penetration depth of TFM is approximately half that of depth as TPSM; the limitation stems from the fact that while infrared excitation light for TFM has similar penetration depth as TPSM, the use of a camera as a detector results in image degradation, due to the scattering of blue-shifted emission photons, resulting in a loss of image signal-to-background ratio. Finally, we describe four typical application areas, divided into two classes, where TFM has found important utility. In the class of rapid imaging, the first application relates to high speed functional imaging of cells and tissues, especially in the brain where rapid sampling of large areas is important. The second application of TFM enables fastest fluorescence and phosphorescence lifetime imaging in cells and tissues due to the high degree of parallelization. It is probably most important in the phosphorescence case where the lifetime is long and complete spectroscopic analysis at each spatial location may take almost one millisecond. The second class of applications utilizes the ability of TFM for depth resolved patterning in thick biological tissues. The most important application in this class is probably cell-selective optogenetic excitation that promises to offer a better understanding of neuronal circuit communications. We have further described an application in temporal focusing photodynamic therapy that may allow selective killing of cancer cells. We foresee applications of patterned temporal focusing as probably the more important direction, as temporal focusing-based imaging is always hampered by the acquired image being blurred by scattered emission photons.

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