High Throughput 3D Optical Microscopy: from Image Cytometry to Endomicroscopy

by

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Abstract

Optical microscopy is an imaging technique that allows morphological mapping of intracellular structures with submicron resolution. More importantly, optical microscopy is a technique that can readily provide images with biochemical contrast based on different spectroscopic modalities such as fluorescence spectrum and lifetime, Raman spectrum, optical polarization and phase. Although, optical microscopy can provide superior resolution over many other medical imaging modalities such as MRI, CT or ultrasound, the relatively low throughput limits its range of biomedical application.

In this thesis, high throughput 3D optical imaging instruments have been developed for medical and biological applications based on widefield 3D resolved techniques. First, we developed a high throughput depth resolved widefield image cytometer based on the structured light illumination and the high speed remote depth scanning. This system improves imaging throughput by an order of magnitude over the current technology and can potentially be applied to image cytometry investigation to study cultured cell morphologies with statistical significance comparable to the flow cytometer. The statistical accuracy of this instrument is verified by quantitatively measuring the rare cell populations. Hyperspectral imaging is also possible based on the use of an interferometric full field spectrometer. Second, we developed a depth resolved widefield two photon endomicroscope for the medical diagnosis based on the temporally focused widefield two photon microscopy. The developed instrument can parallelize the image acquisition process over the whole field of view without the need for any scanning mechanism at the distal end of the optical fiber. The method for delivering high peak power laser pulses
through the optical fiber has been proposed to improve the signal to noise ratio which in turn improves the imaging throughput. A structured light illumination method have also been proposed and demonstrated for improving depth resolution. In addition, this temporally focused widefield two photon microscopy has been applied to the high throughput depth resolved measurement of fluorescence and phosphorescence lifetime with millisecond level frame rate.

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Chapter 1

Introduction to 3D optical microscopy

Optical microscopy is an imaging technique that allows morphological mapping of intracellular structures with submicron resolution. More importantly, optical microscopy can provides images with biochemical contrast based on different spectroscopic modalities such as fluorescence spectrum and lifetime, Raman spectrum, optical polarization and phase. Of these, fluorescence microscopy by its nature has several advantages for biomedical applications since fluorescence imaging is specific to particular chemical and biological features. Biological structures can often be identified by their endogenous emission characteristics, but, more importantly, the abundance of contrast agents developed in the past several decades allow specific labeling of biological specimens. It is also very important that the Stokes shift between the excitation and emission wavelengths allows highly sensitive detection. The detection of single protein tagged by a single fluorophore is routine today. Importantly, the location of single fluorophore can be determined much below the diffraction limit.

Wide-field fluorescence microscopy is very useful for imaging thin sample. However, for a thicker sample where the scattering of the light blurs the image, wide-field microscopy can only provide limited depth information, meaning that it cannot be used for studying 3D structures of many biological systems. There are now a variety of methods to extract depth information from biological specimens. In this chapter, we describe some of the 3D fluorescence imaging techniques that are relevant for the biomedical applications.

1.1 Confocal laser scanning microscopy (CLSM)

In 1961, Minsky filed the first patent on confocal microscopy where conjugated pinholes were used to reject out of focal plane background while point scanning was used for mapping volumetric information [1]. Conceptually, this form of 3D microscopy works by placing a spatial filter (pinhole) in the confocal plane of the objective so that only light emanating from the focal volume can efficiently pass through the pinhole. Out-of-focus illuminated objects form a
defocused spot at the pinhole and consequently only contribute weakly to the signal. This optical sectioning effect allows imaging in three-dimensions based on the confocal principle. Confocal microscopes [2-4] may be based on reflected light (similar to white light modalities of wide-field microscopy) and fluorescence signals. The image contrast in reflected light confocal microscopes is generated by index of refraction mismatch in the specimen [5] and the image contrast in fluorescence light confocal microscopy is based on the non-uniform distribution of endogenous fluorophores.

Confocal microscopy generates images at a single point at a time. Therefore, in order to map out the 3D fluorophore distribution, raster scanning of either the specimen or the light beam must be performed. Typically, confocal microscopes acquire a 2D image by scanning the beam, where the light is deflected by mirrors to focus at different lateral locations. Typical confocal microscopes use a galvanometer system consisting of two synchronized scanners that move in tandem to produce an x-y raster scanning pattern. The axial position of the excitation light is typically accomplished by either piezo-driven or mechanically driven objective positioners. The emission light is focused down and filtered through a pinhole to accomplish the confocal detection.

Fig.1.1 Typical configuration of the confocal microscope. A pinhole that is positioned at the conjugate plane of the focal plane of the objective blocks out-of-focus light for 3D resolved imaging.
1.2 Two photon laser scanning microscopy (TPLSM)

Denk, Webb and co-workers in 1990 introduced two-photon fluorescence microscopy [6]. Fluorophores can be excited by the simultaneous absorption of two photons, each having half the energy needed for the excitation transition. Since the two-photon excitation probability is significantly less than the one-photon probability, two-photon excitation occurs only at appreciable rates in regions of high temporal and spatial photon concentration. The high spatial concentration of photons can be achieved by focusing the laser beam with a high numerical aperture objective to a diffraction-limited spot. The high temporal concentration of photons is made possible by the availability of high peak power mode-locked lasers. In general, two-photon excitation allows 3-D biological structures to be imaged with resolution comparable to confocal microscopes but with a number of significant advantages: (1) Conventional confocal techniques obtain 3-D resolution by using a detection pinhole to reject out of focal plane fluorescence. In contrast, two-photon excitation achieves a similar effect by limiting the excitation region to a sub-micron volume at the focal point. This capability of limiting the region of excitation instead of the region of detection is critical. Photo-damage of biological specimens is restricted to the focal point. Since out-of-plane chromophores are not excited, they are not subject to photobleaching. (2) Two-photon excitation wavelengths are typically red-shifted to about twice the one-photon excitation wavelengths. The significantly lower absorption and scattering coefficients at these longer wavelengths ensure deeper tissue penetration. (3) The wide separation between the excitation and emission spectra ensures that the excitation light and any Raman scattering can be rejected without filtering out any of the fluorescence photons. This sensitivity enhancement improves the detection signal to background ratio.

![Fluorescence signal generated near the focal region of the objective](image)

Fig.1.2 Fluorescence signal generated near the focal region of the objective (a) one photon excitation (b) two photon excitation. The figure is adapted from the paper by Zipel et al [7].

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1.3 Temporally focused widefield two photon microscopy (TFM)

In TPLSM, the high concentration of photons is generated by spatially focusing the excitation pulsed laser to a small spot with high NA objective while the pulse width of the beam is maintained constant during the propagation through the focal region of the objective. In TFM, the high concentration of photons is generated by temporally focusing the excitation pulsed laser while the spatial size of the beam is maintained constant. The advantage of TFM over TPLSM is that it can parallelize the image acquisition process in 2D without beam scanning and achieve higher imaging rate.

\[
\text{Total TPE signal per plane} = \int TPE(z) dA \propto \frac{1}{A \cdot \tau}
\]

Fig. 1.4 In spatial focusing, the pulse width (\(\tau\)) is fixed while the illumination area (A) is minimal at focus. In temporal focusing, the illumination area (A) is fixed and the temporal pulse width (\(\tau\)) is minimal at focus. The figure is adapted from the PhD thesis by Daekeun Kim [8].
Figure 1.5 shows one realization of TFM. The pulse width is controlled by first spectrally dispersing the excitation pulse with grating and recombines them only at the focal plane of the objective so that the original short input pulse width is only recovered at the focal plane of the objective and the pulse width is broadened above and below the focal plane. Thus, the depth resolved widefield images can be obtained.

![Ultrafast Optical Pulse from Ti:Sapphire Laser](image)

Fig. 1.5 Widefield TFM

1.4 Light Sheet Microscopy (LSM)

High-resolution imaging methods such as confocal and multiphoton microscopy provide optical sectioned imaging of specimens with relatively small dimensions (<1 mm). However, 3D imaging of specimens with dimensions of several millimetres is not practical with highly time consuming point scanning based methods such as CLSM and TPLSM. LSM typically have slightly lower resolution than CLSM and TPLSM but provide deeper imaging in relative transparent specimens, such as embryos. However, the most important advantage of LSM is its high throughput.

In LSM, the specimen is illuminated by a thin sheet of light, perpendicular to the imaging axis, defining the optical sectioning of this technique. In an ideal case, fluorescence signal should be only generated at the plane of excitation at the specimen and the emission fluorescence can be collected at either side of the light sheet. Imaging is performed using the conventional wide-field microscopy system that determines its lateral resolution. As the thickness of the sheet is tailored to be thin, it is possible to achieve excellent optical sectioning of the specimen and suppression
of out-of-focus light. Compared to confocal microscopy, LSM provides significantly less photobleaching because the excitation occurs only at one plane, at a time, of the specimen perpendicular to the imaging axis.

Figure 1.6 shows the experimental schematic of LSM. A laser beam is passed through a cylindrical lens to generate the laser “sheet” at the focal plane of collection objective, which penetrates into the sample. Thus, only the fluorophores in the vicinity of the focal plane of the collection objective are excited. Fluorescence emission is passed through an emission filter for fluorescence detection and is collected by the camera. LSM was demonstrated for various biological applications such as optically sectioned imaging deep inside live embryos [9], 3D visualization of neuronal network in whole mouse brain [10], single molecule imaging in cells [11], deep and fast imaging living specimens using two-photon LSM [12].

Fig.1.6 Optical system arrangement of LSM. A cylindrical lens used to create a sheet of light that penetrates the sample. Inset shows the close look of the illumination and selection mechanism in LSM [9]

1.5 Organization of the thesis

In this thesis, we have developed high throughput optical imaging instruments based on the widefield depth resolved fluorescence imaging techniques such as the structured light illumination microscopy and the temporally focused widefield two photon microscopy.

The thesis is organized as follows:
Chapter 2 describes the high 3D image cytometer based on widefield structured light microscopy and high-speed remote depth scanning. Chapter 3 describes the depth resolved hyperspectral imaging cytometer based on structured light illumination and Fourier transform interferometry. Chapter 4 describes the two photon point scanning endomicroscopy for clinical diagnosis. Chapter 5 describes the widefield two photon endomicroscope based on the temporally focused two photon microscopy for higher throughput imaging. Chapter 6 describes a method for improving axial resolution and contrast with structured light illumination in temporally focused widefield two-photon microscopy. Chapter 7 describes the 3D-resolved fluorescence and phosphorescence lifetime imaging using temporally focused wide-field two photon excitation. Chapter 8 summarizes the thesis and describes the contributions of this thesis work.

References

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Chapter 2

Three-dimensional image cytometer based on widefield structured light microscopy and high-speed remote depth scanning

2.1 Introduction

Flow cytometry is a powerful tool for gathering statistical data on large population of cells to correlate multiple parameters such as size, internal complexity, phenotype within a cell population [1]. In addition, flow cytometry can characterize the biochemical states of individual cells, such as whether particular proteins are expressed, based on fluorometric assays. The key strength of flow cytometry is speed that can routinely process 10,000 cell/sec; however, processing speed up to 100,000 cell/sec has been demonstrated previously [2]. A drawback of flow cytometry is that it provides little morphological information of these cells which is important to complement biochemical information to fully determine the physiological condition of cells. Therefore, there is a clear need to develop new cytometry methods that can obtain cellular morphological information while maintaining comparable throughput as conventional flow cytometry.

Image cytometry was developed to address this need by providing high content assays at high throughput although the current throughput is not quite close to that of the flow cytometry. Imaging provides morphological context for understanding physiological change. As demonstrated by Bakal et al. morphological analysis of cell shape allows association of protein expression with mechanistic steps of cell migration [3]. In addition, Perlman et al have demonstrated that morphological responses of cells allow the classification of drugs and to identify mechanisms of new drugs [4].

A number of 2D image cytometer has been introduced with different instrument complexity and information content. While they share common features such as automated cell positioning and focusing to achieve high throughput imaging rate, these system are slightly
different in terms of target specimen preparation, image acquisition method, and autofocus mechanism [5]. Most of the commercially available system deals with adherent cell types that are plated either on the slide glass or multi-well plate. On the contrary, imaging flow cytometry captures two dimensional images of floating cells as they are transported through the fluidic channel similar to the flow cytometry. The state of the art system can achieve 1000 cell/sec imaging rate in bright field, dark field and fluorescence simultaneously by imaging multiple cells with extended depth of field in widefield imaging mode [6]. Imaging flow cytometer has been extended to in vivo detection of rare circulating cells [7].

However, 3D imaging capability is required for better quantify the morphological features in 2D cell culture environment. For example, accurately quantifying the foci of H2AX protein in nuclei is an important criteria for accessing the DNA double stand break that is related to the initiation of the cancer [8]. These H2AX foci are distributed in 3D within nuclei and sometimes hard to distinguish the overlapped foci with 2D widefield imaging methods. In addition, optically sectioned 3D imaging is a more suitable imaging method for studying the differentiation process induced by the interaction of the stem cells with the feeder cells in a co-culture environment [9]. Other than those applications, there are numerous cases where 3D imaging can provide more accurate measures on the morphological features such as cell size, shape and volume for the physiological study of cells [10].

Not only in 2D cell culture environment, 3D resolved images can better represent cells in 3D tissue environment. Since cells in tissue interact with and are regulated by the extra cellular matrix for their physiological and pathological behavior, cells cultured in 3D matrix can better mimic the behavior of cells in tissue than cells cultured in two dimensional well plate or slide glass. For example, cell-matrix interaction has proven to be an important factor for the outcome of the nerve regeneration [11]. Therefore, image cytometer with 3D imaging capability can better quantify cellular behavior in their natural condition. 3D imaging or in other words optically sectioned imaging is typically achieved with the laser scanning microscopy such as confocal or two photon microscopy. Either spinning disk or line scanning confocal microscopy have been utilized to increase frame rate in most commercially available image based high throughput high content imaging system [12]. Multiphoton microscopy is better suited for deep imaging in a scattering opaque tissue specimen. High throughput multiphoton imaging has been achieved by scanning multiple foci in parallel or by using high speed polygonal mirror scanner, which was
applied in the high speed 3D deep tissue cytometer [13]. Recent advances in optical clearing technology have opened the door for studying a whole organ in single cellular level [14-16].

In this chapter, we introduce a new type of high throughput cell-based 3D cytometer based on the optically sectioned widefield fluorescence imaging method. For a 80% confluent cells, this system can image at about 800 cell/sec in 3D at submicron resolution, which corresponds to about 20 min imaging time for 1 million cells. Throughput of the developed system is orders of magnitude faster than the state of the art commercially available high throughput image cytometer in terms of the resolvable pixel/sec. The large field of view, high numerical aperture objective and the large pixilation, high frame rate sCMOS camera can capture a large number of cells simultaneously in submicron resolution. Widefield optically sectioned image is acquired by the structured light illumination. In addition, high speed depth scanning has been made possible by the remote depth scanning. Motorized stage scan the sample automatically during the image acquisition process, which enables a large area sample imaging. This system could be useful for the 3D image based cell assay for the drug screening and the image informatics study to infer the role of signaling pathway components of the cellular system.

2.2 Design of the overall system

Fig.2.1 shows the schematic layout of the depth-resolved image cytometer. Three diode-pumped solid states lasers (CNI laser) with emission wavelength of 473nm, 561nm and 660nm respectively were used as light sources that can excite broad range of fluorophores. The three beams are combined into one collinear beam using the laser beam multiplexers (LM01-503-25, LM01-613-25, Semrock). Photobleaching effect is minimized by controlling passage of the excitation light with the optical shutter (Ch-61, EOPC) which operates in synchrony with the exposure status signal of the camera so that the sample is exposed to the excitation light only when the camera is recording the emission light. The diffractive optical element (DS-033-Q-Y-A, HOLO/OR) split the beam into +1 and -1 order and minimize the power loss by 0th order and other higher diffractive order beam. Since the beams split by DOE reach the focal plane of the objective via common path, they form a stable fringe pattern for SLI. The optical shutter (Ch-61, EOPC) placed in one of the diffracted beam path blocks the beam for a uniform illumination (UI)
and unblock the beam for a structured illumination (SI) which is formed by the interference of the two beams. The optical shutter is triggered by the external TTL signal and enables high speed switching between UI and SI. A quad-band dichroic mirror (FF410/504/582/669 -Di01-25x36, Semrock) was used to separate the excitation beam from the emitted fluorescence signal. A quad-band emission filter (FF01-440/521/607/700-25, Semrock) right in front of the detector was used to block any stray excitation light reflected from the intermediate optical elements. The z-scanning of the sample is performed remotely by first forming the perfect 3D image of the sample in the focal space of the remote focusing objective and then by scanning the mirror in axial direction with piezo actuator. The cytometer can be functional both as high throughput imaging mode and hyperspectral imaging mode. For high throughput imaging mode, the intermediate image formed at the focal plane of L8 is relayed to the imaging detector through the two relay mirrors. Different color channel can be selected by a filter wheel with three band pass filters (FF01-520/35, FF01-609/54, FF01-692/40, Semrock). For hyperspectral imaging mode, the Sagnac interferometer is utilized to record the interferogram which is subsequently processed to build the spectral cube of every pixels in two dimensional field of view [17]. sCMOS camera (PCO.EDGE 5.5, PCO) records a field of view with size of 420 μm x 350 μm at maximum 100 frame per second at 2560 x 2160 pixelation. When the bit depth of a pixel is 16 bit, the total data rate becomes approximately 1 GB/sec. Since the writing speed of the currently available hard drive is limited to 400 MB/sec even in the case of the state of the art solid state drive, multiple hard drives are configured as a RAID 0 to record the data in parallel which effectively increases the writing speed and storage capacity. After z stack images for a field of view are acquired for SI first and then for UI, the motorized stage (SCAN IM 120x100-2mm, Marzhauser ) translates the sample to the next field of view and the same sequence of imaging is repeated. The data acquisition procedures are fully automated and controlled by the custom-made control software written in C# programming language.
2.3 Structured light illumination (SLI) for depth-resolved widefield imaging

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 [18, 19]. Of these methods, one effective approach we adapted is termed ‘HiLo microscopy’ which generates an optically sectioned image by post-processing the uniformly illuminated image (UI) and the structured light illuminated image (SI) [19]. This algorithm is based on an assumption that 2D image can be divided into low frequency and high frequency contents. Since the high frequency contents are in-focus by its nature, the goal of using SLI is to encode the in-focus low frequency contents especially laterally zero frequency component with high frequency SLI. More specifically, the in-focused high frequency contents are extracted by high-pass filtering UI with a Gaussian shaped high-pass filter. The in-focus low frequency contents are extracted by low-pass filtering the absolute of UI subtracted by SI with the complementary low pass filter to the high pass filter. The cutoff frequency of the Gaussian
filter is determined by the sinusoidal spatial frequency of the structured illumination. Subsequently, the optically sectioned image is obtained by combining the two with an adjusting factor so that the transition from low to high frequencies occur smoothly [20]. HiLo microscopy has been widely used in the context of the background rejection for light-sheet microscopy [20, 21], the temporally focused widefield two photon microscopy [22] and the depth-resolved microrheology [23]. Compared to the phase shifted SLI which requires at least three images taken at every $\pi/3$ phase of the fringe illumination that is used by some of the commercial high content image cytometer [24], HiLo based SLI requires only two images for a depth resolved image. This is a great advantage for high throughput imaging since the switching between SI and UI can be performed in high speed by shuttering one of the two beams with an optical shutter. In addition, HiLo method is insensitive to the motion artifact of a sample since the precise phase control is not required. Fig.2.2 shows one example how the HiLo method can be used for rejecting the background signal. The fringe pattern projected on the in-focused part of the sample is clearly visible as shown in Fig.2.2(b) and the HiLo processing remove the common background from out-of-focal regions and produce much clear image as shown in Fig.2.2(c).

![Fig.2.2 Background rejection with HiLo algorithm](image)

(a) Uniformly illuminated image (b) Structurally illuminated image (c) HiLo processed image

2.4 Axial resolution of the SLI measured with a thin Rhodamine solution.

Theoretical axial resolution of SLI can be estimated using the defocused 2D optical transfer function (OTF) derived by Stokseth [25].

$$C(u,m) = f(m) \left\{ \frac{2J_1 \left( \frac{um \left[ 1 - m/2 \right] }{um \left[ 1 - m/2 \right]} \right) }{um \left[ 1 - m/2 \right]} \right\}$$

where $f(m) = 1 - 0.69m + 0.076m^2 + 0.043m^3$, $m$ is the normalized fringe frequency and is related to the real fringe period $T_g$ via $m = \lambda/(T_g \cdot NA)$, $NA = n \sin(\alpha)$ and $u$ is the normalized
defocus and is related to the actual defocus $z$ via $u = 4kz\sin^2(\alpha/2)$, $k = 2\pi/\lambda$. In incoherent detection, OTF represents the contrast of the fringe detected at the image plane and is equivalent to the signal generated when a thin sheet of fluorescence is scanned through the focus. Ideally, the best optical sectioning is achieved when the normalized fringe frequency comes close to 1 [26]. However, as the fringe frequency is increased the modulation depth is reduced which in turn reduces the signal to noise ratio.

Fig.2.3 shows the radially integrated fluorescence intensity at each depth in the vicinity of the focal plane of the objective for a thin sheet of fluorescent solution for both widefield (WF) and SLI imaging condition. In case of WF, the integrated fluorescence from each $z$ plane is constant, which implies that there is no depth discrimination for a laterally zero frequency component. This effect is caused by a "missing cone" of OTF. Practically, this corresponds to the uniform background comes from the defocused objects. In confocal microscopy, the pin hole conjugated to the focal point filters out this background. In SLI, the SI acts as a virtual pinhole and filter out computationally the low frequency out-of-focus background beyond the depth of field of the objective. In case of fringe period of 1.3μm, the expected axial resolution in terms of the intensity full width at half maximum (FWHM) is 1.28 μm and the experimentally measured value is FWHM is $1.84 \pm 0.05\mu m$.

The thin fluorescent solution is prepared by first dissolving Rhodamine power (Rhodamine 6G, Sigma-Aldrich) in alcohol and diluted to 300μM concentration. Then, a drop of Rhodamine solution is placed on a slide glass and covered with a cover slip and squeezed to form a meniscus around the edge of the cover slip. The volume of a drop of Rhodamine solution is determined by multiplying the area of the cover slip with the desired thickness that needs to be less than the axial resolution.

![Total fluorescence from z planes](image)

Fig.2.3 Optical sectioning measured with thin Rhodamine solution. WF: Widefield, HiLo, Theory
2.5 High speed remote depth scanning

Typically, the microscope objective is scanned in depth direction with piezo-scanner to acquire the volume image of 3D object. However, direct objective scanning is relatively slow for the purpose of high throughput imaging because of the inertia of the objective. Although 10Hz volume imaging rate has been demonstrated by resonantly driving the objective piezo scanner [27], this can also potentially resonate the immersion medium and induce motion of sample. One of the way to improve the scanning speed without sample disturbance is to form perfect 3D image of object remotely and sequentially scanning the remotely formed 3D object in the axial direction with a small light-weight mirror driven by a piezo actuator [28]. The perfect 3D imaging can be achieved by designing the imaging system such that it satisfies both the sine and the Herschel condition. As a consequence of these constraints, the lateral and the longitudinal magnifications of the imaging system becomes equal to the ratio of the immersion media refractive indices of object and image space [29]. As proposed by Botcherby et al [28], the perfect 3D imaging system can be constructed by ensuring that the intermediate optical elements satisfy Eq.(2.2) which can be derived from the perfect imaging condition.

\[
\frac{f_2}{f_1} = \frac{n_2 M_1 F_2}{n_1 M_2 F_1}
\]  

(2.2)

where \(f_1\) and \(f_2\) are the focal length of the imaging and the remote focusing objective respectively. \(n_1\) and \(n_2\) are the refractive index of the immersion medium in object and image space respectively. \(M_1\) and \(M_2\) are the magnification of the objectives. \(F_1\) and \(F_2\) are the nominal focal length of the objectives. Practically, it is important that the pupil plane of the imaging objective (W Plan-Apochromat 20x 1.0 N.A. water, Zeiss) is imaged onto the pupil plane of the remote focusing objective (CFI Plan Apochromat Lambda 20x 0.75 N.A. air, Nikon) by the 4-f system consisting of the Zeiss tube lens with the focal length of 164.5mm (58-452, Edmund Optics) and the achromatic doublet with the focal length of 150mm (AC504-150-A, Thorlabs) so that the phase distortion induced by the imaging objective is cancelled out by the equal amount of negative phase distortion induced by the remote focusing objective and consequently the distortion-free 3D image is formed with isotropic magnification in all direction [30]. The axial scanning is performed by moving the mirror (NT68-321, Edmund Optics) attached to the piezo actuator (P-830.10, Physik Instrumente). The travel range of the current piezo actuator is 15μm, which corresponds to 22.5μm scanning range in the object space by the combined effects of the
magnification from object to image space and the mirror scanning in the image space. Since an
air immersion objective (ROBJ, see Fig. 2.1) can be chosen for remote focusing, sample
disturbance does not occur even for high speed scanning. As ROBJ is designed for imaging
through a 170μm coverslip, a coverslip is placed in between ROBJ and the remote focusing
mirror to prevent additional spherical aberration. To improve the detection efficiency of the
unpolarized fluorescence emission light, a λ/4 plate (2-APW-L/4-018A, Altechna) and a
broadband polarizing cube beamsplitter (10FC16PB.3, Newport) are used as shown in Fig.2.1.

The point spread function (PSF) of the remote focusing is measured by imaging a 0.5 μm
fluorescent bead immersed in 2% Agarose gel at axially different locations through the focal
region of the imaging objective (OBJ, see Fig. 2.1). After a bead is placed at the focal plane of
the objective, the position of the bead relative to the focal plane of the objective is varied by
moving the objective manually which is mounted on the micrometer driven translation stage
(SM1Z, Thorlabs). The positive direction means the objective is moved away from the bead and
the negative direction means the objective is displaced close to the bead. At each position of the
objective, the remote focusing mirror is scanned axially for a uniformly illuminated excitation
beam so that the full diffraction pattern of the single bead is recorded. Fig.2.4 shows the PSFs
from -100 μm to 100 μm at 20 μm interval. Asymmetric pattern of PSF at the focal plane
represents some of the residual spherical aberration. It may partly originate from the index
mismatch of the immersion medium of the imaging objective and the bead immersed in the
agarose gel or it may partly originate that the phase distortion induced by the imaging objective
is not perfectly canceled out by the remote focusing objective. In addition, as the bead is located
further away from the focal plane the PSF is more strongly elongated which is a typical pattern
of the spherical aberration. Since the remote focusing theory is based on the linear approximation
that the point source is near the focus of the imaging objective, the measured PSF shows more
amount of spherical aberration as the bead is displaced away from the focal plane of imaging
objective [30].
2.6 Application of the high throughput 3D image cytometer

HiLo based SLI can effectively reject the out-of-focus background and enable high speed 3D resolved imaging. Fig.2.5 shows the 3D image stacks of mouse kidney section (F-24630, Molecular Probe) obtained with the uniform illumination (Fig.2.5(a)) and the same section after the HiLo processing (Fig.2.5(b)). Elements of the glomeruli and convoluted tubules labeled with Alexa Fluor® 488 wheat germ agglutinin is excited with the 473nm laser line and the emission light is detected with 520nm/35nm band pass filter (FF01-520/35, Semrock). The filamentous actin prevalent in glomeruli and the brush border labeled with red-fluorescent Alexa Fluor® 568 phalloidin is excited with the 561nm laser line and the emission light is detected with the 609nm/54nm band pass filter (FF01-609/54, Semrock). The out-of-focus background that blurs images is removed in the HiLo processed images and consequently the contrast is improved.

![Fig.2.5 3D image stack of mouse kidney obtained with (a) Uniform illumination (b) after HiLo processing. Green color represents elements of the glomeruli and convoluted tubules labeled with Alexa Fluor® 488 wheat germ agglutinin. Red color represents the filamentous actin prevalent in glomeruli and the brush border labeled with red-fluorescent Alexa Fluor® 568 phalloidin.](image)

The developed system enables image informatics based on high content high throughput imaging. Morphological features such as nuclear size, perimeter of the cytoplasm, 3D distribution of the protein can be extracted and statistically processed to provide a morphological context of the physiological changes. As shown in Fig.2.6(a), the 3D map of the mitochondria can be measured to study the metabolic states of the cells. Another example where high throughput 3D image cytometer could be applicable is the image informatics study to infer the
role of signaling pathway components of the cellular system. For example, Fig.2.6(b) shows the fibroblast activated by TGFβ which is important factor that determines the outcome of the tissue regeneration. In short term, TGFβ activation causes SMAD protein to enter the nucleus and in longer term, SMAD protein exit the nucleus and it causes the morphological changes on the cells such that cell shape is elongated and more actin fiber are generated.

![Fig.2.6](image)

Fig.2.6. (a) 3D image of the muntjac skin fibroblast cells (F36925, Invitrogen). The green color represents filamentous actin labeled with green fluorescent Alexa Fluor 488 phalloidin. The red color represents mitochondria labeled with an anti-OxPhos Complex V inhibitor protein mouse monoclonal antibody in conjunction with orange fluorescent Alexa Fluor® 555 goat anti-mouse IgG (b) TGFβ activated fibroblast cells. Green color represents actin filament labeled with rhodamine phalloidin and blue color represents SMAD protein labeled with Alexa Fluor 488

The developed 3D image cytometer can also acquire a large sample size at submicron resolution where the size limit is only constrained by the scanning range of the motorized stage. Fig.2.7 shows a section of the whole mouse kidney that was imaged with 473nm laser line and 520nm/35nm band pass filter. Total 16x16 FOVs are stitched together in the post processing. This clearly demonstrates that the developed image cytometer can achieve a high resolution and a large area sample imaging at the same time.
2.7 Rare Cell Detection in 2D cell culture

The performance of the 3D cell cytometer was characterized by quantifying rare cell subpopulations in 2D specimens in vitro [13]. The ability to detect a rare cell can be an important criteria for the cancer diagnosis. For example, there are about 1-10 circulating tumor cell (CTC) out of 1 million white blood cells [31]. As the cancer progresses, the number of CTC in the blood stream increases. The ability to detect CTC in early stage of cancer requires a high throughput system that can screen out CTC in a short time scale.

2.7.1 2D cell culture preparation

A549 human alveolar adenocarcinoma cells transfected with histone H2B-mCherry cDNA for nuclear staining were maintained in two T75 flasks (Nunclon A Surface) in Dulbecco’s modified Eagle medium (Gibco/Invitrogen 12100) supplemented with 10% fetal bovine serum (Gibco/Invitrogen) and 1% penicillin-streptomycin. Puromycin 0.01% was added for the selection of mCherry-expressing cells with the medium change every 36 to 48 h. To prepare rare cells with cytoplasm staining prior to the imaging, one of the flasks of the grown cells were
labeled with 5-chloromethylfluorescein diacetate (CMFDA Cell Tracker™ Green, Invitrogen) and incubated for 20 minutes, and then replaced with serum free culture medium for another 20 minutes. For passage, cells at 70–80% confluency were detached from both flasks by treating with 0.05% trypsin–ethylenedinitriletetraacetic acid (Trypsin-EDTA) at 37 °C in an incubator for 3 minutes. Medium was then added to inhibit the enzymatic reaction of Trypsin-EDTA. Subculture seeding density was kept at 2-3 × 10^4 cells/ml.

In one group of cells, nuclei were expressing with mCherry with emission peak at 560nm. In the other group of cells, cytoplasm were labeled with CMFDA with emission peak at 520nm in addition to the nuclei staining. The latter group of cells with both nuclei and cytoplasm labeling played the role of the rare cells. The two group of cells, detached using the aforementioned procedure for passage, were resuspended to two cell suspensions of 10^4 cells/μl. Imaging samples were prepared using the two cell suspensions with a range of mixing ratios: 1:1, 1:10, 1:10^2, 1:10^3, 10^4 and 1:10^5. The mixed populations of cells were plated in a 8 well chamber and incubated for about 6 hours, with each well chamber contains about 5 × 10^4 cells. It should be noted that at least 20 rare cells were used to generate the specified ratios to achieve reasonable statistical accuracy. For example, in the sample of 1:10^5 ratio, the total number of cell was 2 millions and the number of rare cells was about 20. All cell samples were fixed using 4% Paraformaldehyde (PFA) and maintained in PBS to be used for long-term imaging.

2.7.2 Data acquisition and analysis

Generally, the image acquisition procedure of the 3D cell cytometer is as follows. For a single field of view with size of 420 μm x 350 μm, SI image stack is first acquired with the SLI generated by the interference of the two beams with shutter (OS2) open and then for the same FOV the UI image stack is acquired with the uniform illumination by blocking one of the beam with shutter (OS2) closed. The motorized stage translate the sample to the next FOV and the same sequence for acquiring UI and SI image stack is repeated until the whole sample size is covered. For the multichannel imaging, above procedure is repeated for each detection color selected by the band pass filter wheel. To maintain the immersion medium in place during the stage scanning the water chamber is constructed around the imaging objective lens and filled up with immersion medium. Representative 3D image of rare cell sample at 1:1 mixing ratio is shown in Fig.2.8.
For the rare cell detection experiment, the prepared samples of two group of cells mixed at different ratios are imaged in 2D with uniform illumination. Before starting the image acquisition, the tilting of the sample holder is adjusted to maintain the whole imaging area in focus over the required scanning range of the motorized stage. Cytoplasm labeled with Cell Tracker Green is excited with the 473nm laser line and the emission light is imaged with 520nm/35nm band pass filter (FF01-520/35, Semrock). Nucleus labeled with m-Cherry is excited with the 561nm laser line and the emission light is imaged with the 609nm/54nm band pass filter (FF01-609/54, Semrock). Fig.2.9 shows the representative image of 1:10^4 sample where the nucleus is labeled with m-Cherry (represented as blue color) and the cytoplasm labeling with Cell Tracker Green (represented as green color). The acquired image is processed to count the number of cell in each population. The cell counting algorithm was implemented in MATLAB (MathWorks, Natick, MA) and run by the GPU processor. The detailed image processing algorithm is described in detail in the next section.
Fig. 2.9 Representative image of 1:10^4 ratio sample where the nucleus is labeled with m-Cherry (represented as blue color) and the cytoplasm labeling with Cell Tracker Green (represented as green color). The size of single FOV is 420 μm x 350 μm and the image shown is part of the 1:10^4 sample where 20x15 FOVs are stitched together.

Fig. 2.10 shows the rare cell counting result. The expected ratio and the corresponding measured ratio is plotted in log scale in both x and y axis. The accurate correlation between the expected ratio and the measured ratio can be confirmed from the slope (0.9299) and the goodness-of-fit (R^2 = 0.9964) of the linear regression line, which demonstrate the capability of the developed system for detecting a rare cells down to 1:10^5 ratio.

\[ y = 1.8656x^{0.9299} \]

\[ R - square : 0.9964 \]

Fig. 2.10 Rare cell subpopulation counting result
2.7.3 Image processing procedure

Flat field correction

The raw images acquired by the cytometer system are generally affected by the field inequality of the illumination field of view (FOV) caused by the non-uniform intensity distribution of the excitation laser beam profile. Therefore, B-spline based technique is employed to fit for the excitation pattern and normalize the intensity distribution over the FOV before image mosaicing. Since the high throughput imaging often involves sample size on the order of hundreds of millimeters which is a $10^6$ times of a FOV, a completely data driven approach is employed to determine the background as well as signal pattern and used a combined additive and multiplicative model to normalize the FOV. Firstly, the background is approximated by taking the minimum projection greater than a predefined noise level of the detector over all the images of the sample ($I_{BG}$). Secondly, the illumination pattern was approximated by the mean projection of the same image set after capping the pixels with photon count greater than a predefined threshold in order to account for the significantly brighter regions ($I_s$). Then the flat-field correction was estimated for each image $I$ (for each FOV) using Eq.(2.3).

$$I = \frac{I - I_{BG}}{I_B - I_{BG}}$$  \hspace{1cm} (2.3)

where the $I_{BG}$ is the 2D B-spline fitted to $I_{BG}$ and $I_B$ is the 2D B-spline fitted to $I_s$.

Image mosaicing

The flat field corrected images were then stitched together using a maximum cross correlation based algorithm [32] to generate an image mosaic for each sample. The images were first arranged in a 2D matrix according to the scan pattern of the motorized stage and the neighboring images were overlapped according to the extent of overlap introduced during image acquisition. Due to the limited accuracy of the stage positioning there are slight changes in the overlap in the different regions of the image mosaic. Hence for each boundary between two FOVs in the image matrix the size of overlap was changed in a sequence of steps in both X and Y directions in a predefined range and the cross correlation coefficient was calculated for each step. The XY position, which results the maximum cross correlation coefficient, was chosen as the best overlap for the particular boundary between the particular FOVs. This was repeated for all the
boundaries between all the images (FOVs) in the mosaic to generate the final stitched image of the sample.

**Segmentation and cell counting algorithm**

The cell counting algorithm was designed according to the labeling of the culture. All the cells contain the nucleus label and hence, it was used to detect and count the total number of cells in the sample. The rare cells contain the cytoplasm label, which was imaged in a separate channel, and it was used in conjunction with the nucleus to separate the rare cells from the normal ones.

**Nuclei counting**

The nuclei of the cells labeled using mCherry were exited using a 561nm laser line and were imaged in one channel (nucleus channel). Firstly the stitched image of the nucleus channel was applied with a maximum cutoff intensity, i.e. all the pixels with greater photon count than the cutoff was assigned the cutoff photon count. Secondly the resulting image was spatially filtered using a circular averaging filter to smooth the image. Then morphological opening [33] using a circular structuring element was applied on the image. These steps preprocess the original image to help identify local intensity peaks, which are used to count the number of nuclei in the sample. Then the extended maxima transform, which is the recognition of local maxima on the h-maxima transform [34] was performed to identify the intensity peaks, which represents the nuclei. Then the number of connected components of the resulting binary image were counted to determine the number of nuclei, and hence the number of cells, in the sample. The nuclei counting pipeline is shown in Fig.2.11(a).

**Rare cell counting**

In order to count the number of cytoplasm labeled cells first the stitched image form the cytoplasm channel was segmented using a predefined threshold and then morphological opening was performed on the resulting binary image. This threshold was kept in a low enough value to include all the labeled cells since the counting accuracy should be high enough to detect the rare cell to normal cell ratio for all the samples with extreme mixing ratios such as $1:10^4$ and $1:10^5$. However this means inclusion of the false positives and hence further refining is necessary to separate true positives from false positives. Therefore we characterized the true positive, i.e.
cytoplasm and nucleus labeled cells using multiple image features of two channels. The image features used were, cell area (region enclosing from the cell boundary and is a measurement of cell volume), mean photon count of the cell area in cytoplasm channel, maximum photon count of the cell area in cytoplasm channel, the degree of overlap of the corresponding nucleus (nuclei for cell clusters), the area ratio of the corresponding nucleus and the cytoplasm. Measuring the above features requires proper cytoplasm and nucleus segmentation and therefore we applied a level-sets algorithm [35] to determine the correct segmentation boundary of the cytoplasm and the nucleus. The binary images from the nuclei counting and the cytoplasm thresholding were used as the starting points for the level-sets. For the clustered regions of the sample the above step determines the cluster boundary.

In order to separate the cells in the cell clusters we applied watershed [36] on the distance transform of a combined binary images resulting from the extended maxima transform and the level-sets of the nucleus channel according to the following equations. (Fig.2.11(b))

\[
D_{EX} = \text{distanceTransform}(BW_{EX})
\]

\[
D_{LS} = \text{distanceTransform}(BW_{LS})
\]

\[
D = \frac{D_{EX}}{(D_{EX} + D_{LS})}
\]

\[
BW_N = BW_{LS} \& \text{waterShead}(D)
\]

Here \(D_{EX}\) and \(D_{LS}\) are respectively distance transforms of resulting binary image, \(BW_{EX}\) from nuclei counting step and resulting binary image, \(BW_{LS}\) from nucleus segmentation using level sets. \(D\) is the combined distance image and \(BW_N\) is the final nuclei segmentation after cluster splitting using watershed on \(D\). Finally the correctly segmented nucleus and cytoplasm channels were used to measure the previously explained image features for each cytoplasm labeled segment and this multi-dimensional feature vector was thresholded to filter out the false positives and count the number of nuclei that includes label in the corresponding cytoplasm channel. The entire rare cell counting pipeline is shown in Fig.2.11(b).
2.8 Conclusions

This paper presented a high throughput cell-based 3D cytometer based on the optically sectioned SLI widefield fluorescence imaging and the high speed remote depth scanning with fully automated sample scanning by a motorized stage. The developed system enables image cytometry investigation to study cultured cell morphologies with statistical significance comparable to the flow cytometer. For a highly confluent cells, this system can image at about 800 cell/sec in 3D at submicron resolution. Compared to the commercially available high throughput high contents imaging systems which are either based on spinning disk or line scanning confocal microscopy and the three phase shifted structured light illumination microscopy, the developed system does not need any scanning and requires only two images of UI and SI for optically sectioned image. This feature combined with an optimized system design using high NA, large FOV objective and large pixilation, high frame rate sCMOS camera enables an order of magnitude faster throughput in terms of the number of resolvable point per second over the commercially available systems. Whereas the commercially available 3D high contents imaging systems is mostly focused on the background rejected single layer imaging of
cells, the developed system can be applicable for a 3D imaging of cells in 2D cell culture environment. The statistical accuracy of the high throughput imaging capability of this instrument is verified by quantitatively measuring the rare cell populations from 1:10 down to 1:10^5.

Literatures

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Chapter 3

Depth resolved hyperspectral imaging cytometer based on structured light illumination and Fourier transform interferometry

3.1 Introduction

Spectral resolved imaging is a very powerful method to quantify cellular biochemical environment identifying chemical species and quantifying their concentration [1]. By labeling cellular or tissue constituents with different fluorescent labels, multicolor imaging allow more precise determination of specimen morphology and composition; an example is the karyotyping of human chromosomes [2]. The availability of many fluorescence probes with emission spectra sensitive to their chemical environment further allows intracellular mapping of biochemical states including ion concentration, pH, and voltage. Spectral resolved imaging was also shown to be an effective tool for studying protein-protein interactions based on resonance energy transfer measurement [3]. Spectral resolved imaging is also valuable for quantitatively identifying spectral signatures of endogenous biochemical species and to measure their concentrations in vivo. In clinics, spectroscopic measurement of cellular biochemistry can often provide information complementary to morphological analysis affording more accurate diagnosis, more precise demarcation of surgical margin, and more reliable evaluation of treatment outcome [4-6]. Spectral resolved imaging of course does not have to be based on fluorescent contrast, many non-fluorescent analytes can be quantified based on their vibronic spectra using Raman imaging [7].

While bandpass filter wheel based spectral imaging is useful in applications where a few wavelength bands are sufficient for analysis, more sophisticated applications requires higher spectral resolution and fast temporal resolution. The goal of the spectrally resolved image is to record a 3D spectral cube which is composed of the pixels in 2D spatial dimension with its full spectral signature in spectral dimension.

Currently available hyperspectral imaging methods can be classified as scanning and non-scanning type. The scanning type methods can be further classified into three categories of
spatial, spectral and optical path difference (OPD) scanning depending on the domains in which the scanning is performed. In the first case, the full spectrum is recorded for a point or a line with spectrally dispersive elements such as prism or grating and with 2D detector array. 4D data cube is sequentially recorded by scanning in 3D spatial locations [1]. In the second case, the full spatial 2D field of view is imaged with 2D detector simultaneously and a single wavelength 2D image is sequentially recorded by scanning the wavelength with the tunable bandpass filters such as the acousto-optical tunable filter (AOTF), the liquid crystal tunable filter (LCTF), Fabry-Perot tunable filter and the angle tuned thin film bandpass filter [8]. In the third case, spectral data is inferred after taking a sequence of 2D image data by scanning OPD between the two emission beam paths in the interferometer. This category is termed as Fourier transform spectroscopy (FTS). There are different types of FTS depending on how OPD is scanned in the interferometer. OPD scanning can be implemented in various ways but the most commonly adapted mechanisms perform OPD scanning by either translating the mirror in one of the beam path in Michelson interferometer [7, 9] or rotating the whole Sagnac interferometer including the beam splitter and the two reflective mirrors with respect to the incoming beam [10] or translating the Wollaston prism in the birefringent FTS [11]. Among these, Sagnac and birefringent type FTS have the advantages in stability because of its common path geometry.

The non-scanning type FTS acquires 3D \((x, y, \lambda)\) data cube in a single exposure without scanning at all. This types of spectrometer have the advantage in terms of temporal resolution but typically there is a tradeoff between spectral and spatial resolution and often they require extensive computation in post-processing. For example, the image mapping spectrometer (IMS) records 3D \((x, y, \lambda)\) data cube in single shot by sharing the CCD detector for recording both spatial and spectral data simultaneously. However, the spatial resolution (or the field of view) is reduced as the spectral resolution increases [12].

For the depth resolved spectral imaging, the 3D imaging techniques such as the point scanning confocal microscopy or the two photon microscopy can be combined with spectrometer based on dispersive elements [1]. Alternatively, the depth resolved widefield technique such as the temporally focused widefield two photon microscopy [13, 14] or the light sheet fluorescence microscopy [15] can be combined with the wavelength scanning spectrometer. Another approach for the depth-resolved widefield imaging especially for single photon fluorescence imaging is to use a structured light illumination (SLI). SLI approach has been demonstrated in the Sagnac interferometer based imaging FTS where the excitation light passing through FTS forms a SLI
which encodes the depths information and is used to reject the out-of-focus photons from the emission light which pass through the same FTS [16]. Also, SLI images based on phase-shifted sinusoidal illumination has been adapted to reject out-of-focus light in IMS [17].

In this chapter, we describe a new implementation of depth-resolved hyperspectral imaging cytometer based on SLI and FTS. Hyperspectral data cube is acquired with the Sagnac interferometer type FTS and the signals from out-of-focal planes are rejected by SLI based on HiLo microscopy. The current configuration has the advantage of achieving both high spatial and spectral resolution images. The overall system configuration is described in Section 3.2. The general working principle of the FTS based on Sagnac interferometer is described in Section 3.3. The proposed method of depth resolved FTS is mathematically derived in the Section 3.4. In section 3.5, the spectral background rejection with SLI is experimentally demonstrated with the fluorescent bead immersed in the uniform background. The depth-resolved hyperspectral 3D imaging is demonstrated with a kidney tissue section and densely aggregated cells labeled with fluorophores with two different colors.

3.2 Overall instrument design of the imaging Fourier transform spectrometer

Fig. 3.1 shows the schematic layout of the depth-resolved hyperspectral image cytometer. Three diode-pumped solid states lasers (CNI laser) with emission wavelength of 473nm, 561nm and 660nm respectively were used as light sources that can excite broad range of fluorophores. The three beams are combined into one collinear beam using the laser beam multiplexers (LM01-503-25, LM01-613-25, Semrock). Photobleaching effect is minimized by controlling passage of the excitation light with the optical shutter (Ch-61, EOPC) which operates in synchrony with the exposure status signal of the camera so that the sample is exposed to the excitation light only when the camera is recording the emission light. The diffractive optical element (DS-033-Q-Y-A, HOLO/OR) split the beam into +1 and -1 order and minimize the power loss by 0th order and other higher diffractive order beam. Since the beams spitted by DOE reaches to the focal plane of the objective via common path, they form a stable fringe pattern for SLI. The optical shutter (Ch-61, EOPC) placed in one of the diffracted beam path blocks the beam for a uniform illumination (UI) and unblock the beam for a structured illumination (SI) which is formed by the interference of the two beams. The optical shutter is triggered by the external TTL signal and enables high speed switching between UI and SI. The dichroic mirror was used to separate the excitation beam from the emitted fluorescence signal and the emission
filter right in front of the detector was used to block any stray excitation light reflected from the intermediate optical elements. The z-scanning of the sample is performed remotely by first forming the perfect 3D image of the sample in the focal space of the remote focusing objective and then by scanning the mirror in axial direction with piezo actuator [18]. The hyperspectral image is acquired by recording the interferogram formed by the Sagnac interferometer which is subsequently processed to build the spectral cube of every pixels in two dimensional field of view.

![Schematic diagram of the high throughput depth-resolved imaging Fourier transform spectrometer. Enclosed by dotted line is the Sagnac interferometer. M: mirror, DM: dichroic mirror, DOE: diffractive optical element, OS: optical shutter, PBS: polarizing beam cube splitter, BS: 50R/50T plate beam splitter, QWP: quarter-wave plate, EmF: emission filter, PA: piezo actuator, FFP: front focal plane, OBJ: objective lens (Zeiss 20x Water NA1.0), ROBJ: remote focusing objective lens (Nikon 20x Air NA0.75)](image)

3.3 Principle of imaging Fourier transform spectrometer based on Sagnac interferometer

In imaging Fourier transform spectrometry, each pixel of the 2D detector records simultaneously the spectrum of the source at the spatial location that is the conjugate point of the pixel. The emission light from the object first forms an intermediate image which is relayed to the 2D detector by the telescope. The interferometer placed in between the telescope split the emission light into two paths and induce phase delay (OPD) in one of the beam path. Then, the
two beams that are phase delayed recombined at the 2D detector which records the time average intensity of the two electric fields that are phase delayed by the interferometer. Mathematically, these processes can be described as follows. The incident electric field upon the interferometer can be expressed as

\[ E(x, y, \nu) = A(x, y, \nu) \exp[i(2\pi \nu t + \phi(x, y, \nu))] \]  

where \( \nu = c/\lambda \), \( c \) is the speed of light, \( \lambda \) is the wavelength of the emission light, \( x \) and \( y \) are the spatial location in the focal plane, \( A \) is the amplitude of the electric field. When OPD of the two beam paths in the interferometer differs by \( \delta \), the intensity pattern recorded at the detector is the time average of the sum of the electric fields from path 1 and 2.

\[ E_1 + E_2 = A(x, y, \nu) \exp[i(2\pi \nu t + \phi(x, y, \nu))](1 + \exp[i(2\pi \nu/c)\delta]) \]  

\[ I(x, y, \delta) = 2|A(x, y, \nu)|^2 \left(1 + \cos \left(\frac{2\pi \nu/c}{\delta}\right)\right) \]  

For a broad band source, it is the sum of all individual frequency components.

\[ I(x, y, \delta) = 2\int |A(x, y, \nu)|^2 \left(1 + \cos \left(\frac{2\pi \nu/c}{\delta}\right)\right) d\nu \]  

The detected intensity can be measured as a function of OPD between the two beam paths, which is called the interferogram. In Eq.(3.4), the first term is the dc term and the second term contains the spectral information. The intensity of source spectra, \( |A(x, y, \nu)|^2 \), can be readily recovered by cosine transform (the real part of the Fourier transform) of the interferogram. Mathematically, the interferogram is the autocorrelation function of the electric field. The inverse Fourier transform of the autocorrelation function of the electric field amplitude as a function of position is the spectrum, i.e., flux density as a function of wave number. This is known as the Wiener-Khinchine theorem [19].

Fig.3.2 shows the imaging Fourier transform spectrometer based on Sagnac interferometer. The basic configuration of the interferometer design is based on the commercial device (SpectraCube300, Applied Spectral Imaging). As shown in Fig.3.2(a), the emission light from the intermediate image plane is collimated by the first lens and split into two paths which corresponds to the transmitted beam and the reflected beam at the beam splitter respectively. Then, the two beams that are phase delayed are focused to the sCMOS camera (PCO.EDGE 5.5, PCO) by the second lens. OPD originates from the fact that the two beam paths in the Sagnac interferometer are not parallel within the beam splitter and have different optical path length for a nonzero rotation angle of the Sagnac interferometer with respect to the position where the normal to the beam splitter in the Sagnac interferometer makes a 45° with respect to the optical
axis [10]. Optical path difference between the two beam paths can be express in Eq. (3.5) which is a function of the thickness (t) and the refractive index (n) of the beam splitter, the angle of the optical axis of the telescope on the beam splitter (β) and the rotation angle of the Sagnac interferometer (θ) with respect to the central position as shown in Fig. 3.2(a) [20].

\[
OPD(\beta, \theta, t, n) = t \left[ \left( n^2 - \sin^2 (\beta + \theta) \right)^{0.5} - \left( n^2 - \sin^2 (\beta - \theta) \right)^{0.5} + 2 \sin \beta \sin \theta \right] 
\]  

(3.5)

In case of small θ near the θ=0, the OPD can be approximated as a linear function of θ as expressed in Eq. (3.6).

\[
OPD(\beta, \theta, t, n) = 2t \sin \beta \left[ 1 - \left( n^2 - \sin^2 \beta \right)^{0.5} \cos \beta \right] \theta 
\]  

(3.6)

Fig.3.2(c) shows the OPD scanning range for the spatial point on the optical axis. Since the spatial points off the optical axis have a non-zero input angle with respect to the optical axis after the first lens, the origin of the OPD scanning range shifts in proportion to the distance of the spatial point from the optical axis. Thus, each pixel along the horizontal direction experiences a OPD span that are shifted in proportional to the distance off the optical axis for the same rotational angle of the Sagnac interferometer.

![Fig. 3.2 (a) Ray tracing from the intermediate image plane to the detector at central position of the Sagnac interferometer. Color represents the spatial field points. (b) Photograph of the setup installed in the emission beam path of the image cytometer. (c) OPD as a function of the rotational angle of the Sagnac interferometer for the spatial field point on the optical axis (the rays represented in green color in (a) with the following parameters: β = 45°, t = 2.93mm, n = 1.517 (Refractive index of N-BK7)](image)

The sampling process is performed in two steps. First, the 2D detector array capture the interferogram across the field of view at a given angle of θ. As shown in Fig.3.3, OPD has a linear variation along the x direction due to the non-zero input angle of the ray from the off-axis spatial points while the OPD along the y direction is identical. Second, the zero OPD line is scanned across the field of view along x direction by rotating the Sagnac interferometer so that each pixel observes at least the single-sided interferogram. The double sided interferogram can
provide a higher signal to noise ratio and induce minimal phase distortion compared to single sided interferogram at the expense of increased data acquisition time. For every pixel to observe the double sided interferogram the zero OPD line has to be scanned more than half the width of the double sided interferogram from the right hand edge of the field of view and finish more than half the width of the double sided interferogram from the left hand edge of the field of view. The sampling periods in space and time are determined by the pixel size of the 2D detector array and the stepping size of the OPD shift respectively [10]. To satisfy the Nyquist sampling theorem, a period of the fringe has to be sampled at least by two pixels of the 2D detector and the OPD stepping size has to be smaller than the half the wavelength. Therefore, the minimum detectable wavelength can be expressed as Eq. (3.7).

$$\lambda_{\text{min}} (\text{or } 1/\sigma_{\text{max}}) = 2\Delta OPD$$  \hspace{1cm} (3.7)

where the sampling frequency, $\sigma_s = 1/\Delta OPD$. The spectral resolution is determined by the total OPD span due to the Fourier transforming relation between the interferogram and the spectrum and can be expressed in Eq. (3.8).

$$\Delta \sigma = \frac{1}{OPD_{\text{max}}}$$  \hspace{1cm} (3.8)

Fig.3.3. Sampling of the interferogram (a) Fringe pattern of a white light LED at $\theta = 0^\circ$ sampled by the sCMOS camera. The dotted yellow vertical line represents a zero OPD which shifts to the left as the interferometer rotates. Pixels in each column see the same OPD and each column see a linearly varying OPD (b) Sampled interferogram observed by the yellow pixel at $\Delta \theta = 0.005^\circ$ (2 pixel or 170nm in OPD space) (c) Recovered spectrum of LED after quad band emission filter with minimum detectable wavelength of 340nm.

The raw interferogram is first band pass filtered by digital FIR filter with pass band between 400nm and 700nm. In case of biological sample, the photo bleaching is observed during the long range of OPD scanning and corrected by modeling the decaying dc values of
interferogram with exponentially decaying function and subsequently dividing the raw interferogram with the exponentially decaying function. Digital bandpass filtering also has similar effect of correcting the photon bleaching since the low frequency of exponential decay is filtered out by the band pass filter. Afterward, the interferogram is phase corrected [21-23], apodized with hanning filter, zero padded and then Fourier transformed by fast Fourier transform algorithm and then taken the absolute value.

The actual OPD generated by the interferometer deviates from the theoretical estimation given by Eq. (3.5) in a number of reasons. First of all, since the OPD is a function of the refractive index of the beam splitter, each wavelength see a different range of OPD depending on the dispersion property of the beam splitter material. In addition, OPD is sensitive to the beam splitter angle and slight misalignment generate a huge error in the OPD estimation. To compensate these errors, the spectrometer is first calibrated with three color lasers at 473nm, 561nm, 660nm wavelength. The interferogram first recorded with each color laser as a light source and then the wavelength is obtained using the OPD calculated using Eq.(3.5). Then the calibration factor is calculated by dividing the expected wavelength by the measured wavelength for each lasers. Then, the OPD is rescaled by multiplying the calculated OPD with the average of the three calibration factors. This procedures can be summarized as follows.

\[
CF_1 = \frac{473}{\lambda_m} , \ CF_2 = \frac{561}{\lambda_m} , \ CF_3 = \frac{660}{\lambda_m} \\
CF = (CF_1 + CF_2 + CF_3) / 3 \\
OPD_{rescaled} = CF \cdot OPD_{calculated}
\]

After the initial calibration, the spectrum of the known emission wavelength fluorophores are measured and compared with their reference values. Fig. 3.4 shows the raw interferogram and the corresponding spectrums of a 6μm Yellow-Green fluorescent bead (F-8859, Molecular Probe), Rhodamine 6G solution (Sigma-Aldrich), a 4μm Red fluorescent bead (F-8858, Molecular Probe). The samples are excited with the 473nm laser and the emission light is detected with the long pass dichroic mirror (FF495-Di02-25x36) and the long pass emission filter (BLP01-488R-25) for measuring the full emission spectrum.
Fig. 3.4. Interferogram of 6μm yellow green fluorescent bead (a), Rhodamine 6G solution (b), 4μm red fluorescent bead (c) and its the processed spectrum are (d), (e) and (f). Green curve in spectral graph is the reference value from http://www.lifetechologies.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

3.4 Depth-resolved imaging Fourier transform spectrometry

Since the fringe formed by the interferometer is only determined by the light distribution in the input aperture of the interferometer, they form interferogram with the same modulation depth whether the light is from in- or out-of-focus plane. Therefore, the recorded interferogram contains both in-focus and the out-of-focus spectra information and the post-processed spectral cube from these raw interferogram would not provide depth-resolved spectral information. One of the methods for rejecting the spectral background signal is to use a structured light illumination (SLI) to encode the depth information in the (x, y, λ) spectral cube. The degree of modulation depth in the λ-plane contains the depth information.

So far, 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 [24, 25]. Of these methods, one effective approach we adapted is termed ‘HiLo microscopy’ which generates an optically sectioned image by post-processing the uniformly illuminated image (UI) and the structured light illuminated image (SI) [25]. This algorithm is based on an assumption that 2D image can be divided into low frequency and high
frequency contents. Since the high frequency contents are in-focus by its nature, the goal of using SLI is to encode the in-focus low frequency contents especially laterally zero frequency component with high frequency SLI. More specifically, the in-focused high frequency contents are extracted by high-pass filtering UI with a Gaussian shaped high-pass filter. The in-focus low frequency contents are extracted by low-pass filtering the absolute of UI subtracted by SI with the complementary low pass filter to the high pass filter. The cutoff frequency of the Gaussian filter is determined by the sinusoidal spatial frequency of the structured illumination. Subsequently, the optically sectioned image is obtained by combining the two with an adjusting factor so that the transition from low to high frequencies occur smoothly [26]. HiLo microscopy has been widely used in the context of the background rejection for light-sheet microscopy [26, 27], the temporally focused widefield two photon microscopy [28] and the depth-resolved micro rheology [29].

HiLo method can be applied to the spectral background rejection by first constructing the \((x, y, \lambda)\) spectral cubes for both UI and SI and then post-processing each \(\lambda\)-planes from UI and SI with HiLo algorithm. Following the similar approach by Heintzmann et al.[16], we derived the mathematical formulation behind the spectral background rejection using SLI. The fringe pattern is imposed along the \(x\) direction by the interference of the two plane wave, which can be expressed as:

\[
I_{\text{ref}}(k_{\text{ref}}, x) = 2(1 + \cos(k_{\text{ref}}, x)) I_0(k_{\text{ref}})
\]

where \(x\) is the spatial dimension, \(I_0\) is the intensity of one of the beam. The emission spectrum of a fluorophore at \(x\) can be expressed as

\[
I_{\text{em}}(k_{\text{ref}}, k_{\text{em}}, x) = m_{\text{SLI}} I_{\text{ref}}(k_{\text{ref}}, x) S_{\text{em}}(k_{\text{em}}, x) \rho(x) S_{\text{em}}(k_{\text{em}}, x)
\]

where \(\rho(x)\) is the local fluorophore distribution, \(S_{\text{ex,em}}(k_{\text{ex,em}}, x)\) is the excitation and emission spectrum of the fluorophore, \(m_{\text{SLI}}\) is the degree of modulation of the emission light excited by the structured illumination which decreases as the object is defocused. According to Eq.(3.4), the interferogram observed by a pixel in the detector as the OPD is scanned can be expressed as

\[
I_{\text{ds}}(\delta, k_{\text{ref}}, x) = \int_0^\infty (1 + m_{\text{bs}} \cos(k_{\text{em}}, \delta)) I_{\text{em}}(k_{\text{ref}}, k_{\text{em}}, x) dk_{\text{em}}
\]

\[
= \left[m_{\text{SLI}} I_{\text{ref}}(k_{\text{ref}}, x) \rho(x) S_{\text{em}}(k_{\text{em}}, x)\right] \int_0^\infty (1 + m_{\text{bs}} \cos(k_{\text{em}}, \delta)) S_{\text{em}}(k_{\text{em}}, x) dk_{\text{em}}
\]

where \(\delta\) is the optical path difference, \(m_{\text{bs}}\) is the degree of modulation of the two interfering emission light, which can vary depending on the beam splitting ratio. From the Fourier transform of Eq. (3.11), the spectral cube of SI can be obtained as
\[
S_{SI}(k_{em}, x) = \int_{\delta_{max}}^{+\delta_{max}} I_{sl}(\delta, k_{ex}, x) e^{-j\delta k_{x}} d\delta
= \left[ m_{SI}/I_{ex}(k_{ex}, x) \right] m_{s} \rho(x) S_{ex}(k_{ex}, x) S_{em}(k_{em}, x)
\]  \hspace{1cm} (3.12)

First term of Eq. (3.12) implies that each \( \lambda \) plane of the spectral cube preserves the depth information encoded by the structured light illumination. Now the situation is similar to the intensity background rejection by SLI.

3.5 Experimental validation of spectral background rejection

3.5.1 Hyperspectral imaging

The hyperspectral imaging is demonstrated with the prepared slide containing the muntjac skin fibroblast cells (F36925, Invitrogen) stained with three different color fluorescent dyes. The filamentous actin is labeled with green fluorescent Alexa Fluor 488 phalloidin with emission peak at 519nm. Mitochondria are labeled with an anti-OxPhos Complex V inhibitor protein mouse monoclonal antibody in conjunction with orange fluorescent Alexa Fluor® 555 goat anti–mouse IgG with emission peak at 571nm. Nuclei are labeled with the red fluorescent TO-PRO-3 nucleic acid stain with emission peak at 661nm. The spectral images is first acquired with the band pass filter as shown in Fig. 3.5(a) and then the hyperspectral image is taken for the area marked in red box in Fig. 3.5(a) as shown in Fig. 3.5(b).
Fig. 3.5 (a) Two color image of the muntjac skin fibroblast cells (F36925, Invitrogen). The green color represents filamentous actin imaged with the 525nm/35nm bandpass filter (FF01-520/35, Semrock) and the orange color represents mitochondria imaged with 609nm/54nm band pass filter (FF01-609/54, Semrock). (b) \( \lambda \) plane images of the region marked in red box in (a) at selected wavelengths.

3.5.2 Spectral background rejection by SLI

We first demonstrate the spectral background rejection in a situation where a 6 \( \mu \text{m} \) yellow-green fluorescent bead is placed in the focal plane and a uniform Rhodamine solution is placed in the out-of-focal plane. The sample is excited with both 473nm and 561nm laser to equalize the emission light from both colored samples. The emission light is detected with the quad-band dichroic mirror (FF410/504/582/669 -Di01-25x36, Semrock) and the quad-band emission filter (FF01-440/521/607/700-25, Semrock). Fig. 3.6(a) shows the spectral images for UI, SI and after the HiLo processing. In UI, the spectra from both the in-focused bead and the out-of-focus Rhodamine solution are detected, which demonstrates that there is no depth
discrimination. In SI, the fringe pattern is visible only on the in-focused bead. After the HiLo processing the spectrum from the out-of-focus Rhodamine is rejected. Fig. 3.6(b) shows the spectra of a point in the center of the bead. After HiLo processing, the out-of-focus spectrum is rejected.

![Fig. 3.6](image)

(a) Spectral images from UI, SI and after HiLo processing at \( \lambda = 521 \text{ nm} \) and \( \lambda = 608 \text{ nm} \) (b) Spectra for UI and after HiLo processing. The spectrum from the out-of-focus Rhodamine solution is rejected after the HiLo processing.

Next, we obtained a depth resolved hyperspectral images with the same prepared cell sample used for Fig. 3.5 and the uniform Rhodamine solution. As in the previous case, the cells are in-focus and the Rhodamine solution is out-of-focus. The samples are excited with the 473nm laser and the emission light is detected with the long pass dichroic mirror (FF495-Di02-25x36) and the long pass emission filter (BLP01-488R-25) for measuring the full emission spectrum. As shown in Fig. 3.7, the background signal from the Rhodamine is present over the broad emission wavelength range from 525nm to over 600nm with strongest contribution around 550nm. The HiLo processing removes this background and improves the contrast. The mitochondria which has similar emission wavelength with the Rhodamine is more effectively represented by rejecting the background noise.
Fig. 3.7 λ plane images of the muntjac skin fibroblast cells (F36925, Invitrogen) with Rhodamine background for UI and after HiLo processing.

We further demonstrate the depth-resolved hyperspectral imaging with a prepared slide of sectioned mouse kidney (F24630, Invitrogen). The sample is imaged with the same imaging condition for Fig. 3.6. Each spectral channel of the hyperspectral images are combined to produce a true color image as shown in Fig. 3.8. The thickness of the sample is 15μm and the very top surface was put in focus during data acquisition. The contrast of the HiLo processed images improves over the UI and the weak fluorescent features are more clearly visible.
Fig. 3.8 True color images of mouse kidney (F24630, Invitrogen) for UI and after HiLo processing. Green colored feature represents the elements of the glomeruli and convoluted tubules labeled with Alexa Fluor 488. Orange colored feature represents the filamentous actin and the brush border labeled with Alexa fluor 568.

3.6. Conclusion

This paper presented a depth resolved widefield hyperspectral imaging cytometer based on the imaging Fourier transform spectrometer and the structured light illumination. While the Sagnac interferometer in the emission beam path does not have a depth resolving power, the structured light illumination generated in the excitation beam path has been demonstrated to encode depth information in the recorded interferogram and the spectral background has been rejected with HiLo algorithm.

Since the OPD has a linear distribution across the field of view at any given instance in the Sagnac type imaging FTS, the zero OPD line has to be scanned across the entire field of view for each pixel at least to observe the single-sided interferogram. Currently, sampling interferogram is performed according to the Nyquist sampling criteria. Therefore, the total data acquisition time increases in proportion to the width of the field of view and can induce a significant photobleaching in case of biological sample. A recent development in the signal processing such as fast orthogonal search algorithm or compressive sensing technique may
allow optical spectrum to be inferred with significantly fewer sampling of the interferogram while keeping the same spectral resolution [30, 31].

References
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Chapter 4

Two photon point scanning endomicroscopy for clinical diagnosis

Endoscopy operates on the same resolution and depth penetration regime as a typical laboratory microscope but its miniaturization enables clinical applications. Endoscopy allows noninvasive examination of the gastrointestinal (GI) track and the epithelial surface of other internal organs of a patient. Since many cancer types occur on the epithelial surface of interior body cavities, endoscopes, and endomicroscopes (a term that we will use interchangeably), are routinely used in clinical diagnosis and treatment today.

The importance of developing more effective endoscopes can be seen from the incidence rate of some of these diseases. For example, the incidences of colorectal, esophageal, and cervical cancers are over 12,000, 150,000, and 15,000 new cases per year in USA, respectively. To further illustrate the use of this new technology for cancer detection, we will examine the situation with cervical cancer in greater depth [1-6]. The onset of cervical cancers has been associated with a sexually transmitted human papillomavirus (HPV) infection; an infection which affects over 40 million people. Approximately one million new cases of HPV are detected each year. Some forms of HPV have been identified as the primary cause of cervical intraepithelial neoplasia. HPV is often detected because of either the observation of genital warts or from an abnormal Pap smear. The common Pap smear cannot localize the lesions. Patients with an abnormal Pap smear are often referred to colposcopy, a conventional microscopic imaging method of the cervix. Colposcopy has had false positive rates as high as 40%-50%. Furthermore, high-grade disease may be misclassified as low grade. Punch biopsy, another common technique, is subject to false negative results through incomplete sampling. This is an area where endomicroscopy may be applied to identify the most critical biopsy sites and provide more informative diagnosis, since endomicroscopy can provide subcellular level images similar to histopathology.

In addition to the initial diagnosis, endomicroscopy may also provide a benefit of long-term, periodic monitoring of premalignant conditions. For example, Barrett’s esophagus is very
common and is the result of acid reflux. Barrett’s esophagus has a high chance of developing into cancer and must be carefully monitored clinically [7, 8]. Endomicroscopy may permit more accurate noninvasive monitoring of the potential progress of the disease at an organ site where frequent and high-coverage excisional biopsy is highly undesirable. In the case of cervical cancer, genital warts often involve surgical intervention, with all the associated risks thereof. Low-grade lesions may be topically treated with trichloroacetic acid or 20% podophyllin solution. More extensive cases are treated with cryosurgery, laser vaporization, or loop electrosurgical excision. Since early stage genital warts often regress and excision procedures may compromise patient fertility, a conservative management scheme has often been suggested. The success of conservative management schemes depends on the existence of reliable clinical markers for cancer progression, and imaging techniques to monitor them. Endomicroscopy may allow a more accurate, noninvasive determination of the propensity for Barrett’s esophagus and cervical lesions to progress into a more invasive form.

4.1. Review of the endoscopic microscopy

4.1.1. Non-depth resolved Endomicroscopes

Conventional endoscopes operate in a wide-field imaging mode and most often at low resolution. Endoscopes come in different formats but typically consist of a light source for illumination, a lens for image formation, a fiber bundle for relaying the image to the image sensor in the proximal end and an instrumentation channel for other treatments such as excisional biopsy. Currently, endoscopy is routinely used in medical procedures for the diagnosis and treatment of some diseases. For example, the bronchoscope, the colonoscope and the hysteroscope are used for the inspection of the trachea and bronchi, the colon and large intestine and the inside of the uterus, respectively [9]. Recently, the capsule type endoscope has been introduced that takes pictures as it travels through the GI tract by the bowel movement, and sends the images wirelessly to the receiver [10]. Patients swallow the pill type capsule and it can acquire images in either from a head-on view (PillCam, Given Imaging [11]) or from a side view (Sayaka, RF [12]). The advantage of this type of endoscope is that it can examine the whole GI tract, which is especially useful for imaging the small intestine where upper endoscopy and colonoscopy cannot easily reach. The capsule type endoscope is also compatible with examination on an outpatient basis, providing patient convenience and minimizing medical cost.
However, capsule type devices typically do not provide as high quality images as conventional endoscopy.

In addition to generating image contrast based on scattered light, other image contrast mechanisms such as tissue auto-fluorescence, spectroscopy, Raman scattering and fluorescence lifetime can increase the endoscopic diagnostic accuracy [13]. Richards-Kortum and coworkers have demonstrated that a high-resolution microendoscopy of intact oral mucosa has similar diagnostic accuracy to the histopathological diagnosis result [14]. However, these techniques are still confined to the surface imaging of the tissues and subject to limited utility for other types of diseases, such as cancer occurring in the epithelial layer of tissue.

4.1.2. Depth resolved endomicroscopes

Since wide-field endomicroscope imaging can only provide tissue surface information, it cannot replace traditional histopathological diagnosis where microscopic examination of histological tissue sections provides morphological information on individual cells and their organization within tissues. Therefore, wide-field microendoscopy is typically used to identify suspicious tissue sites where an excisional biopsy will be taken so that a definitive clinical diagnosis based on histopathological analysis can be performed. However, these procedures are clearly invasive. More importantly, excisional biopsy is time consuming, involving tissue excision, fixation and subsequent examination by trained pathologists; the long cycle time of this process often extends the time required to keep a patient in the operating room when excision biopsy is being used for surgical margin determination. With the advent of high resolution 3D endoscopic imaging technologies with the potential for providing images comparable with excisional histopathology, there is a clear opportunity today to significantly extend the 3D, depth resolved imaging capability of endoscopes and laparoscopes in clinical diagnosis. The three classes of high-resolution endomicroscopes that have been developed today are based on optical coherence tomography (OCT), confocal microscopy, and nonlinear optical (NLO) microscopy.

4.1.2.1 OCT endoscope

Optical coherence tomography is based on the optical ranging of reflected light pulses from surfaces based on precise timing of femtosecond pulses interferometrically. Among the three 3D endomicroscope approaches, endoscopes based on OCT are the most advanced because
the miniaturization of this technology is easier for two important reasons. Firstly, OCT systems inherently obtain depth information based on the timing of the low-coherence optical pulses such that no mechanical scanning in the depth direction is required. 2D sagittal or coronal sections of the tissue can be generated by only mechanically scanning the light ray along a single direction either radially or axially. Since scanning along only one direction is required, this greatly simplifies micro-scanner design. Secondly, OCT automatically provides depth-resolved information when the imaging optics have a large depth of field, i.e., a relatively low numerical aperture. This longer depth of field, and hence longer working distance, significantly reduces the complexity involved in assembling the micro-optical components in the distal end of the endoscope at the expense of lower resolution. OCT endoscopes have entered into a number of clinical trials including the identification of Barrett's esophagus [8, 15-23] and colonic polyps [18, 23, 24]. OCT endoscope development is advanced enough that devices with an outer diameter less than 2 mm have been fabricated and applied to the evaluation of intracoronary stenting and the study of atherosclerotic plaques in patients [25-30]. While OCT endoscopes are finding important clinical applications, the combination of using low NA optics, the relatively narrow bandwidth of light sources, and the inherently low contrast of the OCT signal in tissues limits the lateral and axial resolutions of these systems to about 5-7 μm [31]. Therefore, OCT endoscopes often do not provide images with a resolution comparable to standard histopathology.

4.1.2.2 Confocal endoscope

Today, endoscopes with subcellular resolution are based on confocal detection or non-linear optical (NLO) excitation principles. Unlike in an OCT system, confocal and NLO endomicroscopes obtain information from a single 3D-resolved location in the tissues. Scanning along two directions must be performed to generate a 2D image. The need to incorporate scanning along at least two directions presents a challenge in endoscope design. Nevertheless, significant progress has been made in confocal based systems. Today, some confocal endomicroscopes are becoming commercially available and preliminary clinical tests using these devices have started [32]. Three classes of confocal endomicroscopes have been developed. The most popular approach for confocal endomicroscopes utilizes high density, flexible fiber optical bundles and performs scanning at the proximal end of the device [33-36]. The advantage of this
class of endomicroscope is its simplicity. Since scanning is performed at the proximal end, outside the patient, the scanning mechanism does not require miniaturization, but the resolution of these systems are limited by the fiber bundle pitch size and the static pattern noise caused by imperfections fiber bundle fabrication. The second class of device incorporates micro-scanning devices that scan micro-optic components, such as the tip of a fiber optic, which are integrated in the distal end of the endomicroscope [37]. This class of device is very promising as high resolution images have been demonstrated. However, the state-of-the-art device is still fairly large with over 6 mm outer diameter and relatively modest resolution (0.7 mm lateral and 7 mm axial). Significant progress was also made in a third class of devices that is based on MEMS mirrors or a MEMS mirror array integrated at the distal end of an endomicroscope [38-40]. While MEMS mirror based confocal endoscopes have shown potential for clinical imaging at an early date and with good promise, there are few significant clinical instrument breakthroughs along this direction recently due to the difficulty and cost of MEMS mirror fabrication.

4.1.2.3 Nonlinear optical (NLO) endoscope

Endomicroscopy based on NLO excitation provides complementary information to confocal systems. NLO endomicroscopes can better utilize molecular level contrast. Fluorescence and second harmonic generation are efficient contrast mechanisms allowing molecular imaging and metabolic imaging, although preliminary work is underway using gold nanoparticles as contrast agent in reflected light confocal endomicroscopes [41]. NLO microendoscopy has comparable excitation penetration as reflected light confocal endomicroscopes when operated in the infrared wavelengths. For fluorescence imaging, a confocal endomicroscope has shallower penetration depth due to the use of shorter excitation wavelengths. Furthermore, the use of a detection aperture in confocal microendoscopy results in significant rejection of scattered photons that can be retained in nonlinear optical endomicroscopes allowing deeper imaging with higher signal to noise ratio in principle. Finally, NLO endomicroscopy produces less tissue photodamage as compared to a fluorescence confocal endomicroscope, due to the inherent localization of the excitation volume. Given these potential advantages, a number of NLO endomicroscopes have been developed despite the greater technical challenges in constructing these systems.
Endoscope imaging started with preliminary work on human subjects using laboratory format microscopes [42]. These instruments were ultimately unsuitable for clinical work. The most significant progress in clinical applications using laboratory-scale NLO microscopes is the work of Konig, Kaatz, and coworkers on dermal lesion diagnosis [43]. This group of investigators obtained, for the first time, regulatory approval to evaluate the clinical utility of NLO microscopy in the diagnosis of skin disorders with a substantial population of patients.

The extension of these systems for clinical examination of internal organs faces three major technical challenges. Firstly, the efficiency of second order non-linear optical excitation is a linear function of laser pulse width. Due to non-linear optical effects, such as self-phase modulation, that occur during transmission of high intensity light through a typical silica core optical fiber, femtosecond pulses can be significantly broadened in a fiber endomicroscope, which greatly decreases the excitation efficiency of these systems. Secondly, due to pulse dispersion considerations, the successful confocal endomicroscope designs that utilize distal scanning through a fiber optics bundle are not feasible for NLO excitation. The results from initial experiments are not optimal [44]. Therefore, micro-scanning mechanisms must be packaged in the proximal end of the device while the instrument outer diameter must be kept within a few millimeters. Thirdly, fluorescence and second harmonic signals are weak. Typically, each pixel of a video rate image has a maximum photon count between hundreds and thousands of photons when the sample is labeled with a good fluorophore such as Rhodamine. However, the photon count can be as few as tens to hundreds when imaging is based on tissue endogenous fluorophores, such as NAD(P)H. Therefore, unlike a reflected light confocal system where a strong optical signal can be obtained, a NLO endomicroscope must be designed to maximize light collection efficiency.

Significant progress has been made in overcoming the first challenge as photonic bandgap crystal technology is coming of age, allowing the creation of ultra-broadband, omnireflective mirrors and waveguides [45, 46]. Photonic bandgap crystal technology is poised to produce novel optical switches and multiplexers [47-49]. Most importantly for the future of endomicroscopy, photonic bandgap crystal technology allows the creation of novel waveguides for femtosecond pulses with controllable dispersion [50, 51].

Initial design of the nonlinear endomicroscope used the conventional bench top multiphoton microscope and scanning mechanism with a long gradient index lens (GRIN) for
relaying the excitation light. Practically, the depth of the NLO microscopy is ultimately limited by the scattering of the excitation light by tissues. For example, the typical penetration depth is about 500µm in brain tissue which corresponds to about 2 to 3 scattering mean free path lengths [52]. Rigid type endomicroscopes have been used to image deep in the tissues such as green fluorescent protein (GFP) expressing neurons in the hippocampus of the mice brain [53, 54] and to image the long term tumor progression in the mice colon [55] that are not easily accessible with the conventional technique. Initial clinical studies have been conducted by Konig and his coworkers for the deep tissue skin imaging with second harmonic generation (SHG) and autofluorescence as contrast mechanism [56]. While these devices allow cutting-edge study of neurobiology and tumor biology in small animals, they are not feasible for clinical human use other than the skin due to their length limitation and their rigidity.

Significant progress has been made in overcoming the need for multiple axis scanning mechanisms. Fiber optic based nonlinear endomicroscopes started with miniature, handheld two-photon systems using Lissajous resonant scanning of a short length single mode fiber first developed by Helmchen and coworkers [57]. This system was used to study the vasculature structure and dendritic calcium dynamics of a freely moving rat. Schnitzer and coworkers developed a similar system but with much lighter weight that can be directly mountable on the mice brain. The unique feature of this device is that the miniaturized objective lens at the tip of a long relay lens was inserted into the mice brain and could image hippocampal vessels as well as vessels near the neocortical surface [58, 59]. These kinds of head mountable multi-photon endomicroscope can provide a useful tool for the neuroscientist to study the intact living mammalian brain without constraining the motion of the mice.

More clinically applicable endomicroscope started with a number of parallel developments, including further miniaturization of the Helmchen design by resonantly scanning a dual core photonic crystal fiber instead of single mode fiber for more compact design of the imaging probe by the groups of Gu and Li [60-62]. The major advance here involves the use of a dual core fiber with single mode, low dispersion transmission of excitation light and multimode, higher efficiency collection of the emitted signal. The addition of an axial translation capability with shape memory alloy actuator in a recent design enabled 3D scanning NLO endomicroscope [63]. As opposed to Li's design performs spiral scanning using tube type piezo actuator, the recent development by Xu and coworker performs raster scanning which generates more uniform
illumination pattern [64]. A parallel development by Chen and coworkers utilizing 2D-scanning MEMS mirrors has resulted in another class of NLO endomicroscopes [65, 66]. In addition to actuation of the scanning algorithm, distal scanning endoscopes are limited by a number of other optical constraints, such as the NA of miniaturized lenses. Recent advances in coupling spherical lens to the tip of GRIN lens has first resulted in NLO endomicroscopes with objective numerical apertures approaching 0.8 [67].

For compactness and simplicity, most NLO endomicroscopes uses the same optical fiber for excitation and detection. Since the excitation and emission wavelengths in nonlinear microscope imaging are widely separated, the endomicroscope optics optimized for excitation is in general very suboptimal for emission detection due to chromatic aberration. In case of using the single fiber for the excitation beam delivery and the emission beam collection, it results in significant loss in collection efficiency. Li group have partially solved this problem by splicing the tip of the dual core fiber with multimode fiber which maintains the single mode excitation beam delivery and effectively increases the signal collection area. By further introducing an effective achromatic triplet as the objective, the chromatic misalignment problem is now largely overcome [68].

The main contrast mechanisms implemented in most NLO endomicroscopes are based on two-photon fluorescence and second harmonic generation. A system has been recently designed based on third harmonic generation and has been applied to study the dermal fibrosis and hyperkeratosis [69]. In addition, an exciting new system has been developed by Ben-Yakar and coworkers that combines imaging and microsurgery capability in the same compact system [70]. While normal NLO endomicroscopes produce a 3D image stack by scanning the focal spot in 3D, the nonlinear excitation based on the principle of spatio-temporal focusing pioneered by Silberberg and Xu groups greatly simplifies instrumentation design by producing a depth resolved 2D section without using any scanning mechanisms [71, 72]. Depth scanning can be accomplished by tuning the group velocity dispersion at the proximal end as proposed and demonstrated by Durst at el [73].

4.2 Side-view 3D point scanning two photon endomicroscope

The currently developed point scanning two photon endomicroscope is either head-on view which cannot be directly applied to examine the side wall of the GI track or limited only for
2D scanning without depth scanning capability. In this section, we introduce a novel 3D two photon endomicroscope that can better examine the side wall of the GI track with all 3D scanning mechanism integrated in the distal end.

4.2.1 Design of the two photon endomicroscope

Fig. 4.1(a) shows the optical design of the TPE endomicroscope system. A ultrafast optical pulses from a Ti:Sapphire laser with 100fs pulse width, 80MHz repetition rate at the center wavelength of 780nm (MaiTai, Spectra-Physics) was used as the excitation light source.
Excitation light is delivered to the distal end through the double clad photonic crystal fiber (DCPCF) (DC-165-16-Passive, Crystal Fibre A/S). A DCPCF has a dual core structure where the excitation light is delivered through the single mode core and the emission light delivered back through the multimode inner clad and the core to the photomultiplier tube (PMT). A pre-chirping unit was used to pre-compensate the linear pulse dispersion induced by DCPCF and maximize the two-photon excitation efficiency, which is inversely proportional to the pulse width of the excitation beam. After propagating through the DCPCF, the excitation beam was guided to the MEMS actuator, going through a prism and lastly a gradient index (GRIN) lens as the objective (GT-LFRL-100-017-0.5NA, GRINTECH ). The emission from the focal spot in the sample then traveled back through the optical train, and was guided to a photomultiplier tube (PMT) detector via a dichroic mirror (DM).

3D volume imaging is obtained by scanning the focal point of GRIN objective in three axes by using the fiber resonator and MEMS actuator which are all assembled in the endoscope housing as shown in Fig. 4.1(e). The fiber resonator resonates the tip of the DCPCF at its first resonance frequency to scan in the fast axis (X) and the prism assembled to the MEMS actuator oscillate along the X axis guided by the rotary flexural bearing to scan in the slow axis (Y). The combination of the fiber and prism motion generates a raster-scanning pattern. GRIN lens assembled to the shuttle of MEMS actuator moves linearly guided by the translational flexural bearing to scan in the depth direction (Z). The principle of MEMS actuator is based on the thermomechanical actuation where the current flow induces the Joule heating and thus subsequently induced the elongation of the beam. This small motion is amplified and guided by the flexure mechanism [74]. The scanning speed can be variable depending on the length of the fiber tip. For example, when the length of the fiber tip is 19.6mm, its first resonance frequency and the frame rate become 700Hz and 3.5Hz, respectively. The total scanning volume of the current design is 100um × 100um × 100um. The endoscope housing is designed to hold the fiber resonator and the MEMS actuator and to allow fine adjustment for the alignment between optical components.
4.2.2. Characterization of the DCPCF for the two photon excitation

4.2.2.1. Single mode excitation beam delivery through the inner core of the DCPCF

DCPCF has a dual core structure. For the multi-photon endoscope application, it has unique feature of delivering excitation beam through its inner core and emission beam through its core and inner clad. Thus, it can significantly simplify the design of the endoscope structure[75]. We choose the double clad photonic crystal fiber(DCPCF) (DC-165-16-Passive, Crystal Fibre A/S) for this application. The use of a single fiber simplifies the endomicroscope design and makes the device more compact. This fiber delivers single mode illumination beam in the near infrared (NIR) spectrum and collects the visible emission light as a multimode beam. Fig. 4.2(a) shows the far field image of the inner clad of the DCPCF and the single mode beam from its core. The triangular shaped bright beam is the single mode beam from the core and the honey comb structure surrounding the core is the inner clad.

As pointed out by Helmchen et al. it’s important to maintain single mode beam for the efficient two photon excitation [76]. To confirm the single mode property of the beam from the core we measured the intensity distribution and wavefront of the beam from the core. As can be seen in Fig. 4.2(b) the intensity profile of the beam from the core has a Gaussian distribution. In addition, the wavefront measured with the Shack-Hartman wavefront sensor (HASO, Imagine Optic) shows that the wavefront aberration of the beam from the core has a root mean square (RMS) value of 0.01λ as shown in Fig. 4.2(c). These measurements confirm that the beam delivered from the core is truly a single mode beam.

![Figure 4.2](image)

Fig. 4.2 (a) Far field image of the cross section of DCPCF. Single mode beam in core is shown as bright triangular shape. Inner clad structure surrounds the core and used for emission beam delivery. (b) Gaussian intensity profile of single mode beam from the core. (c) The wavefront of the single mode beam from the core, RMS = 0.01 λ.
4.2.2.2. Effect of the pulse dispersion during the beam delivery through DCPCF

Typically, the pulse width of the input pulse is broadened during the propagation through the solid core region of the fiber which is caused by the group velocity dispersion (GVD). The common solution for this problem is to prechirp the laser beam either with grating or prism pair. However, these methods can only compensate the linear pulse broadening due to GVD and cannot compensate the nonlinear pulse broadening effect caused by self-phase modulation (SPM). To characterize how the grating pair prechirping unit can compensate the linear pulse broadening, we measured the pulse width of the single mode beam from the core with an autocorrelator by varying the distance between the grating pair as shown in Fig. 4.4. The length of the DCPCF is 172cm. Original pulse width of the air delivered Ti-Sapphire pulsed beam is 110 fsec. Without prechirping the pulse width is 2027 fsec and we could achieve the 205 fsec when the distance between the grating pair is 48cm. From this result, the GVD parameter of DCPCF of solid core is 234 fsec^2/cm.

![Experimental setup for characterizing the dispersion of DCPCF. The diagram is adapted from Kim et al [77].](image-url)
Fig. 4.4 Temporal pulse width measurement by varying the distance between the grating pair

The prechirped single mode beam after the fiber is delivered to the normal two photon microscope with 40x oil immersion objective using Fluorescein solution as a sample. Fig. 4.5 shows the TPE measured with different amount of GVD induced by the grating pair pre-chirping unit. The slope of each curve is close to 2 demonstrating that the signal is generated by the two photon excitation process. The intercept of the linearly interpolated curve with the y axis is related to the TPE, which is inversely proportional to the pulse width of the excitation beam.

Fig. 4.5 Two photon efficiency measured with Fluorescein. The slope of each curve is close to 2, which demonstrates that the signal is generated by the two photon excitation process. The intercept of the linearly interpolated curve with the y axis is related to the two photon excitation efficiency, which is inversely proportional to the pulse width of the excitation beam.
As shown in the Fig. 4.6, TPE is getting close to the air delivered TPE as the grating distance increases. Due to the 3rd order pulse dispersion effect, the prechirping unit cannot restore the temporal pulse width to the air delivered value. At the optimum prechirping distance of 48cm we could measure a factor of 2 lower TPE compared with that of the air delivery.

![Graph showing the ratio plot of TPE and pulse width vs. grating distance](image)

**Fig. 4.6** Ratio plot of the TPE and the pulse width by varying the distance between the grating pair. 0cm grating distance means no prechirping. TPE ratio = \( \frac{\text{TPE}_{\text{Air}}}{\text{Photon Count}_{\text{DCPCF}}} \), Pulse Width ratio = \( \frac{\text{FWHM}_{\text{DCPCF}}}{\text{FWHM}_{\text{Air}}} \)

### 4.2.3 Imaging experiments and preliminary results

Custom-developed data acquisition software and control electronics were constructed to synchronize the different scanning axes with the photon counting circuitry monitoring the optical signal from the photomultiplier tube. To demonstrate 3D optically sectioned imaging capability of the TPE endomicroscope system a stack of 15 micron fluorescent beads was used for preliminary imaging experiments. Fig. 4.7 shows the bead cross-sectional image after correcting the artifact induced by the resonance scanning scheme [78]. The input current is the half the value for the full motion range and the measured field of view is 50umx60um.

![Stack of fluorescent beads images](image)

**Fig. 4.7** A stack of fluorescent beads images with a field of view of 50 x 60 microns. Each bead is of 15 micron in diameter and each frame is 3 micron apart in Z-axis. The images is acquired at 3.5 frame/sec
References


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Chapter 5

Improving femtosecond laser pulse delivery through a hollow core photonic crystal fiber for temporally focused two-photon endomicroscopy

5.1 Introduction

In the last two decades, a number of new biomedical imaging technologies have emerged that fundamentally changed the procedures of disease diagnosis and expanded the frontier of the biological research that were not accessible previously. For example, two photon microscopy has now become an indispensable tool for the study of the brain function and disease based on the morphological map of the neuronal network [1]. Optical coherence tomography is routinely used in the diagnosis of retinal diseases such as glaucoma and age related macular degeneration [2]. More recently, photoacoustic tomography has shown its great potential in in-vivo monitoring of the tumor angiogenesis [3].

Among the other imaging modalities, multiphoton microscopy can provide both morphological and spectroscopic information on the state of the disease from the endogenous fluorophores in tissue. For example, cancer cells show the distinctive morphological changes in their carcinogenic progression such as the increase of nuclear to cytoplasmic ratio. In addition, the decrease in the redox ratio of the metabolic coenzyme FAD and NADH has been observed [4, 5]. Multiphoton microscopy can also utilize second harmonic generation (SHG) to monitor collagen state in the extracellular matrix. Recent study has shown that SHG can be useful diagnostic marker for the muscular dystrophy and ovarian cancer [6]. In addition, we have also recently shown that SHG from abnormally increased collagen fiber content is correlated with liver fibrosis progression [7]. For these reasons, multiphoton microscopy in endoscopic format has great potential as an optical biopsy tool for diagnosing diseases state such as colorectal, esophageal, and cervical cancer which occurs on epithelial surface of interior body cavities that cannot be reached without miniaturizing the physical size of the device.
Although multiphoton endomicroscopy is a promising technology, it is technically challenging for miniaturizing the device in that it requires the 3D beam scanning mechanism for the volume imaging at the distal end of the optical fiber that are used to deliver the excitation beam to the remote site [8-12]. Also, a point scanning makes the image acquisition process inherently slow. These problems can be circumvented by utilizing the temporally focused widefield two photon microscopy (TFM) that can greatly reduce instrument complexity and offer the potential for higher speed imaging based on parallelization by producing a depth resolved images without the need for scanning mechanisms at the distal end [13, 14]. Axial scanning can also be performed remotely by chirping excitation pulses in the proximal end [15]. However, it has been demonstrated that high peak power pulses from the regenerative amplifier is required to take full advantage of this widefield imaging method [16]. The use of high energy pulses poses a great challenge in the delivery of the excitation beam since the fiber can be easily damaged by the extremely high intensity focused beam at the inlet of the fiber.

In this paper, we propose a strategy, based on manipulating laser pulse energy and repetition rate with a compact pulse splitter and laser group velocity dispersion with a pulse compressor, for delivering femtosecond, high energy pulses from a regeneratively amplified Ti-sapphire laser through a hollow core photonic crystal fiber (HCPCF). We demonstrate that the excitation efficiency of a temporally focused widefield two photon endomicroscope (TFEM) can be substantially improved.

5.2 Improving the two photon excited fluorescence signal using pulse splitter

Light guidance by the standard step index fiber is based on the total internal reflection with core having higher refractive index than the surrounding cladding. However, this solid core fiber is not optimum for delivering ultrafast high peak power pulses because of the distortion of the temporal shape of the input pulses induced by the intense nonlinear interaction of the core material with the high peak power pulses such as self-phase modulation and group velocity dispersion (GVD). On the contrary, light guidance of a HCPCF is based on the photonic band gap effect where the light of the certain wavelength band is trapped inside the hollow fiber core surrounded by the periodic wavelength scale lattice of air holes in the cladding [17]. Since the light propagates through the air, the temporal distortion of the input pulse is minimized [18].
Because of this unique properties, HCPCF have been utilized for delivering high peak power pulses for various applications including two photon fluorescence microscopy [19], laser micromachining [20] and laser surgery [21].

While the two photon laser scanning microscopy (TPLSM) requires 0.01-0.1nJ pulse energy from a Ti-sapphire laser for the optimum excitation efficiency, TFM requires $10^4 - 10^5$ times higher pulse energy to optimally excite the whole field of view. Considering the power loss during beam delivery through the optical fiber and the intermediate optical elements, even the full power from Ti-sapphire laser cannot provide sufficient peak power for the optimum two photon excitation at the sample. Although it has been demonstrated that the high peak power from the regeneratively amplified Ti-sapphire laser could be used for recording even the Brownian motion of the fluorescent bead in a TFM, it cannot be directly applied in the TFEM since such a high peak power pulse could damage the fiber either by the thermal or the ionization effect.

Fig. 5.1 shows a flat cleaved surface of HCPCF (HC-800-01, NKT photonics) and the damaged surface by the high peak power pulses from the regeneratively amplified Ti-sapphire laser at 800nm wavelength with 130 fsec pulse width and 10 kHz repetition rate (Legend Elite, Coherent). The damage of HCPCF mostly occurs at the inlet of the fiber when the focused beam spot size and shape does not match with the mode field diameter of HCPCF. The maximum transmittable power is closely related to the coupling efficiency. If the fiber coupling is not performed at its optimum condition, the fiber is damaged before it reaches the absolute maximum value set by the damage threshold.

Fig. 5.1 Far field image of the end surface of HC-800-01 fiber (NKT photonics) (a) clean surface (b) damaged surface. These images are acquired by illuminating the proximal end of the fiber with a white light LED and imaging the distal end with a 40x air objective and a CCD camera. The bright dot shown is the photonic lattice structure where the light from LED propagate through these structures via total internal reflection (c) intensity pattern of the output beam from the hollow core fiber.
There are several factors that determine the coupling efficiency such as the flatness of the cleaved surface, the input beam diameter, the numerical aperture (NA) of the coupling lens, NA of the fiber and the size of the mode diameter of the fiber. The rule of thumb of maximizing the coupling efficiency is that the NA of the focused beam should be less than the NA of the fiber. At the same time, the focused beam spot size should be less than the size of the core diameter. The HCPCF (HC-800-01, NKT photonics) used for delivering the pulses from the regenerative amplifier has NA of 0.2, a mode field diameter (1/e² width) of 8.8 μm and the zero dispersion wavelength near 805 nm.

The damage threshold of the fiber is measured by increasing the input pulse energy to the fiber at every 20 nJ until the damage occurs in the fiber. The pulse energy from the regenerative amplifier is controlled by using a half wave plate (10RP02-800, Newport) and a polarizer (GL15-B, Thorlabs). First, the output beam diameter from the regenerative amplifier is reduced to the aperture size of the coupling lens using the telescope and then it is coupled to the fiber using the aspheric lens with NA of 0.16 (C260TME-B, Thorlabs). The optimum coupling lens has been selected after trying several coupling lens with NA varying from 0.15 to 0.25. For 0.16 NA coupling lens, 90% coupling efficiency have been achieved. The damage in the fiber occurs sequentially. First, the constant coupling efficiency is maintained up to 360 nJ input pulse energy and then starts to drop slowly as cracks are started to appear in the core. Subsequently, the power drops suddenly with the total destruction of the core as shown in Fig. 5.2. The fiber damage by the ionization effect can be avoided by coupling in and out of the fiber within the vacuum chamber. In this condition, high energy femtosecond pulses of up to 1.8 μJ was transmitted through HCPCF without fiber damage [22]. However, this strategy is difficult for endoscopic application especially at the distal end.
For a given FOV, there is a required pulse energy to optimize excitation efficiency. However, this pulse energy often cannot be used due to the lower damage threshold of the fiber. To better utilize the remaining pulse energy available from the regenerative amplifier, it is better to split each regenerative amplifier pulse into multiple pulses instead of just attenuating the laser. This approach increases the number of available pulses for two photon excitation with pulse energy less than the damage threshold of the fiber. Theoretically, two photon absorption (TPA) per fluorophore per second can be expressed as Eq. (5.1).

\[
TPA = \delta \left( \frac{\lambda}{hc} \right)^2 \frac{1}{FOV^2} P_{peak}^2 f = \delta \left( \frac{\lambda}{hc} \right)^2 \frac{1}{FOV^2} \frac{e_{pulse}^2}{\tau} f
\]  

(5.1)

where \(\delta\) is TPA cross-section, \(\lambda\) is the wavelength, \(c\) is the speed of light, \(h\) is the Plank constant, \(P_{peak}\) is the peak power of pulse, \(e_{pulse}\) is the pulse energy, \(f\) is the pulse repetition rate, \(\tau\) is the pulse width and \(FOV\) is the illumination field of view [1]. Eq.(5.1) implies that the signal in TFEM can be maximized by setting the pulse energy (peak power) below the fiber damage threshold and by increasing the number of pulses as many as possible. In a sense, this is similar to an optimization problem where the objective function is TPA and the optimization parameters are pulse energy and repetition rate with the fiber damage threshold as a constraint. Our solution is to implement a pulse splitter that multiplies the incoming pulse to many identical pulses while keeping the pulse energy below the damage threshold.
Fig. 5.3 shows the pulse splitter that multiplies an incoming pulse to 64 identical pulses and the timing diagram showing the pulses at the intermediate steps. The detail design is adapted from the work by Ji et al [23]. To summarize the sequence of pulse multiplication steps, the pulse is split into two by non-polarizing beam splitter (BS₁). One of them goes to PS₁ directly and the other is delayed by delay line (l₁) and goes to the other input port of PS₁. Each input pulse to PS₁ is multiplied to 4 pulses by the 4x pulse splitter where the two pulses from each output ports are time delayed by 74 ps. Pulses from one of the output ports of PS₁ is delayed by l₂ and recombined with pulses from the other output ports of PS₁ at BS₂. BS₂ copies these pulses to two sets of same pulses. One group of pulses goes directly to PS₂ and the other group of pulses go to DL which add about 2.5ns time delay before entering the other input port of PS₂. Each input pulse to PS₂ is multiplied to 4 pulses by the 4x pulse splitter where the two pulses from each output ports are time delayed by 37 ps. Pulses from one of the output ports of PS₂ is delayed by l₃ and recombined with pulses from the other output ports of PS₂ using half-wave plate (HWP) and polarizing beam cube splitter (BS₃). The time delayed pulses in PS₁ and PS₂ are produced by splitting the input pulses at the interface of the two different refractive index materials so that each pulse travels different optical path length and recombined at the beam splitting interface in such a way that the recombined pulses from the output ports of pulse splitter propagate collinearly. After pulse splitting, the pulses are prechirped by the single prism type pulse compressor to compensate for the group velocity dispersion that may be induced by the intermediate optics and the fiber. Since the separation between the pulses are much smaller than the lifetime of the fluorophores which is typically in nanoseconds range, the benefit of increasing the repetition rate can only be meaningful when two photon excitation efficiency is low enough so that the ground state depletion does not occur as in many cases during temporally focused wide field two photon imaging. In addition, increasing the repetition rate while decreasing the pulse intensity quadratically can sometimes reduce photodamage and photobleaching while maintaining the same signal level [23].
5.3 Increase the damage threshold by stretching the pulse

The damage of HCPCF typically occurs at the inlet of the fiber where the lattice structure of the air hole and silica surrounding the core is fractured by the strong reaction of the ionized air molecules with the silica in the cladding generated by the extremely high peak power focused beam. Thus, reducing peak power by stretching the pulse would allow more power to be transmitted before damaging the fiber. Although this method has not been tried for femtosecond pulses, it has already been applied in delivering the high energy nanosecond pulse [24].
Pulse width can be controlled by adding negative group delay dispersion (GDD) using a pulse compressor. As shown in Fig. 5.4, the amount of GDD induced by the pulse compressor is linearly proportional to the distance between the prism and the corner cube mirror (CCM). The pulse width is measured with the single shot autocorrelator (Delta single shot autocorrelator, Minioptic Technology). From the measured pulse width, GDD can be calculated assuming that the shape of the input pulse is in a transform limited Gaussian form using Eq.(5.2) [25]. Input pulse width was 130 fsec\(^2\).

\[ GDD = \frac{r_{in}}{4\ln 2} \sqrt{\frac{r_{out}^2 - r_{in}^2}{fsec^2}} \]  

(5.2)

Fig. 5.4 (a) Schematic drawing of single prism pulse compressor adapted from [26]. Large tuning range of GDD is achieved by using a prism made of highly dispersive glass, SF66. GDD is tuned by changing the distance between the prism and the corner cube mirror. (b) Measured pulse width as a function of the corner cube mirror position. (blue curve). GDD is calculated using Eq.(5.2) from the measured pulse width. Input pulse width was 130 fsec\(^2\).

Damage threshold of HCPCF (HC-800-02, NKT photonics) is measured with the configuration shown in Fig. 5.3. HC-800-02 has a NA of 0.2, a mode field diameter (1/e\(^2\) width) of 5.5 \(\mu\)m and the zero dispersion wavelength near 778nm. Damage threshold is measured first without pulse stretching and then at four different CCM positions each at 20 cm, 30 cm, 40 cm, 50 cm. The pulse width at these conditions correspond to 150 fsec, 187 fsec, 359 fsec, 632 fsec, 922 fsec respectively. At each measurement conditions, the input power is increased at 5mW increment until the coupling efficiency starts to degrade from the initial value more than 10%. The damage threshold is defined as the power level at which the coupling efficiency drops 10% from that of the non-damaged fiber. At each conditions, the measurements were repeated five
times. Typical coupling efficiency was 80% which is lower than that without the pulse compressor because the beam shape is slightly elongated horizontally while the beam propagate through the pulse compressor. According to Fig. 5.5, the damage threshold increased about factor of two while the input pulse width is increased or the peak power is decreased about a factor of six. The damage threshold does not linearly increase with an increase in pulse width or a decrease in peak power. This may be caused by the thermal stress built up by the pulses with picosecond time separation whereas the time constant for the thermal dissipation of HCPCF is nanosecond range. Currently, the input pulses from the 10 kHz regenerative amplifier are separated by 100 msec time delay and this single input pulse is copied to 64 pulses with 37 psec time delay. This thermal stress can be alleviated by separating the pulses more uniformly in between the pulses of the 10 kHz pulses. However, this would increase the overall footprint of the pulse splitter.

![Graph](image)

**Fig. 5.5** Damage threshold of HC-800-02 (NKT photonics) measured without pulse stretching (150 fsec) and CCM positions at 20 cm (187 fsec), 30 cm (359 fsec), 40 cm (632 fsec), 50 cm (922 fsec).

Although stretching pulse may allow more power to be delivered through the fiber, it decreases the two photon absorption as can be predicted by Eq.(5.1). Ideally, the pulse is first stretched with the negative dispersion provided by the pulse compressor to reduce the ionization effect and then the pulse width is recovered to the transform-limited pulse width with the positive dispersion provided by HCPCF. For 800nm working wavelength, HC-800-01 has a positive GDD and HC-800-02 has a negative GDD. Since the prism type pulse compressor can add a large negative GDD, HC-800-01 model is better suited for the currently proposed pulse delivery scheme. Once the beam is coupled to HCPCF, over 95% of the optical power is
confined in the air core. Therefore, the material dispersion is minimal and the waveguide dispersion is the dominant factor for the group velocity dispersion induced in the HCPCF [27]. GDD of HCPCF (HC-800-01) is calculated from the pulse widths measured before and after the fiber. The input pulse width to the fiber was 130fsec and the pulse width after propagating 80cm long fiber was 200fsec. From this, GDD can be calculated to be 7000 fsec$^2$. The negative GDD of the fiber can be cancelled out by the same amount of positive GDD of the pulse compressor which can be obtained when CCM of pulse compressor is positioned at 20cm as shown in Fig. 5.6.

We combine both ideas of pulse splitting and pulse stretching to improve the high peak power pulse delivery through the fiber. After 64x pulse multiplication, the damage threshold increased to 60mW. After the pulse stretching it further increased to 75mW. The reason the damage threshold does not increase 64 times more compared to that of without pulse multiplication (4mW) could be the thermal effect as explained in Fig. 5.5. Since the pulse width increases to 187fsec at 20cm CCM position, it increases the damage threshold and allows more power to be transmitted through the fiber as shown in Fig. 5.7. As shown in Fig. 5.5, a factor 2 more power can be delivered through the fiber while recovering the original input pulse width by using long enough length of the fiber so that its positive GDD is matched with the negative GDD from the pulse compressor.
As shown in Fig. 5.2, the fiber damage occurs at 360nJ pulse energy. We expected that the 64x pulse splitting would enable to deliver 64x pulses with 360nJ before damaging fiber. However, it turns out that the fiber is damaged at much lower pulse energy of about 94nJ (average power of 60mW at 640 kHz) after 64x pulse splitting. This implies that increasing repetition rate using the current scheme may not guarantee to improve TPA since the linear increase of TPA by the increased repetition rate would be caught up by the quadratic decrease of TPA by decreased pulse energy. Ultimately, it is desirable to have a variable repetition rate regenerative amplifier that can provide more flexibility in tuning the pulse energy keeping it below fiber damage threshold and tuning the repetition rate to generate as many pulses as possible within the total power limit of the light source or until the occurrence of ground depletion state of the fluorophore.

5.4 Design of a temporally focused widefield two photon endomicroscope (TFEM)

Endoscopic implementation of the TFM requires a design strategy for implementing the size of the probe small enough to be applicable for the GI track imaging while achieving a reasonable field of view and resolution at the same time. This section describes the design principles for a TFEM based on the previous literatures for optimizing the performance of a TFM [28, 29].
The form factor of TPE require the use of micro-lenses as objectives. Currently, the NA of the available micro-lenses is relatively small compared to normal objectives. For example, the highest NA of commercially available microlenses is 0.8 that have been achieved by the compound lens made of a GRIN lens and a plano-convex lens [30]. This limits the achievable depth resolution of a TFEM. To utilize the full NA of the objective the focal length of the tube lens has to be such that the full spectrum of the excitation beam just fills the back aperture of the objective. At the same time, the excitation field of view is demagnified by the focal length ratio of the tube lens and the objective. Therefore, the focal length of the focusing lens has to be selected such that both the depth resolution and the field of view are satisfied at the same time. The angular spread $\Delta \beta$ of a spectrum with spectral width $\Delta \lambda$ of first order diffraction can be obtained by differentiating the grating equation, assuming the incidence angle is constant [31].

$$\Delta \beta = G \sec \beta \Delta \lambda \quad (5.3)$$

where $\beta$ is the first order diffraction angle, $G$ is the groove frequency. Assuming the paraxial approximation, the width of the focused spot size at the back aperture of the objective is $f \Delta \beta$, where $f$ is the focal length of the tube lens. Therefore, to utilize the full NA of the objective, each design parameters have to be selected to satisfy the following relation

$$D = fG \sec \beta \Delta \lambda \quad (5.4)$$

where $D$ is the diameter of the back aperture of the objective.

Fig. 5.8 shows the ray tracing of both excitation and emission beam path of TFE. Excitation beam is delivered through HCPCF (HC-800-01, NKT photonics) and collimated by plano-convex lens (NT65-308, Edmund Optics, $f=2.54$ mm, $\theta$2mm) and reflected by the prism (NT45-524, Edmund Optics ) with the custom gold mirror coating (Evaporated Coatings) to a reflective grating (1200 line/mm, 2 mm x 2mm, LightSmyth). The grating is custom-made so that the first order diffraction is maximized for the excitation wavelength at 800nm. The input pulses are spectrally dispersed by the grating and focused by a plan-convex lens (Edmund Optics, NT45-964, $f=12$ mm, $\theta$3mm ) at the back aperture of custom-made GRIN objective (NA 0.8, GRINTECH) that collimates each spectral component and recombines them at the focal plane of the objective to restore original input pulse width. The image from the focal plane is magnified and relayed through custom-made GRIN lens and beam cube splitter block (GRINTECH) and detected by a 1/10" size CCD camera at the distal end directly or can be relayed to the high sensitivity detectors such as EMCCD camera through the imaging fiber bundle located at the
proximal end. Depth scanning can be accomplished by tuning the group velocity dispersion at the proximal end as proposed and demonstrated by Durst at el[15]. In this way, all the scanning actuators at the distal end can be removed, which makes the endomicroscope structure more compact and robust.

![Diagram of optical components](image)

Fig. 5.8 (top) Ray trace of the excitation beam path. Color represents wavelength. (bottom) Ray trace of the emission beam path. Color represents spatial field. Excitation field of view (FOV): 30 x 60 um, Working distance of objective: 180um, NA of objective: 0.8. All ray tracing is performed by commercial software (Zemax, Radiant Zemax)

The optical components are assembled in the custom-made endoscope housing as shown in Fig. 5.9.

![3D modeling and photo](image)

Fig. 5.9 (a) 3D modeling of the assembled TFEM. (b) Photo showing the assembled TPE. Size: 8mm (diameter), 35mm (length)

5.5 Experimental validation of the pulse delivery methods

The proposed pulse delivery method for TFEM imaging is validated experimentally by comparing the two photon excited fluorescence signal of the fluorescent beads in three different
cases. In the first case, pulses from 10kHz regenerative amplifier is delivered directly without pulse multiplication and dispersion compensation. In the second case, a pulse is multiplied 64 times without dispersion compensation. In the third case, a pulse is multiplied 64 times with dispersion compensation. The excitation beam for these conditions are delivered through the fiber (HC-800-01) to the temporally focused widefield two photon endoscope probe. The power levels after the fiber delivery are 2.5mW, 50mW, 50mW respectively. These values are obtained when the input power to the fiber are set to about 70% of the damage threshold to prevent the fiber damage during long-term imaging process. The emission signal is detected in transmission mode with 40x air objective and the EMCCD camera.

![Normalized two photon signal](image)

Fig. 5.10 (a) Normalized two photon signal for three cases. 1. No pulse multiplication without dispersion compensation 2. With 64x pulse multiplication and without dispersion compensation 3. With 64x pulse multiplication and dispersion compensation (b), (c), (d) are 2μm bead images for case 1, 2 and 3 respectively.

The two photon signal is compared with the densely packed 1μm fluorescent beads (F-13081, Molecular Probes) sandwiched by the two cover slips. The image is acquired at 100 msec exposure time and repeated 10 times for each condition. Fig. 5.10(a) shows the normalized total fluorescence signal summed for the whole field of view. Compared to case 1, the signal level increases a factor of 7.5 in case 2 and a factor of 11.5 in case 3. In case 2, while the repetition rate is increased to 64 times, the peak power of the pulse is reduced to 1/3 compared to case 1. Since the two photon fluorescence signal per second has a quadratic dependence on the peak power and a linear dependence on the repetition rate, about 7 fold increase can be expected. Also,
GDD of the fiber stretches the pulse width from 130fsec to 187fsec and the original pulse width was recovered as shown in Fig. 5.6. Since the two photon absorption (TPA) is inversely proportional to the pulse width, about 1.4 fold increase in two photon signal can be expected. Therefore, by using the pulse splitter and dispersion compensation (case 3) 11.5 fold increase in two photon signal have been achieved. This effect can also be verified in Fig.10(b)-(d) where 2μm fluorescent bead (F-8827, Molecular Probes) is imaged at each conditions. The field view is quite elongated vertically because the input angle of the excitation beam to the grating surface is 73 degree. The FOV can be made square by expanding the beam horizontally with two cylindrical lens pair before impinging on the grating.

5.6 Conclusion

This paper presented an improved delivery of high peak power pulses from the regenerative amplifier through the hollow core photonic crystal fiber for maximizing the two photon excited fluorescence signal in the temporally focused wide-field two photon endomicroscopy.

Signal improvement has been demonstrated by using the pulse splitter that multiplies a pulse into 64 identical pulses with peak power below the damage threshold. Effective increase in damage threshold of the fiber was achieve by stretching the pulses with negative GDD by pulse compressor. At the same time, the negative GDD compensates the positive GDD by the fiber and recover the original pulse width after the fiber. By employing the pulse multiplication and the dispersion compensation in TFEM, 11fold signal increase have been demonstrated.

If our assumption that thermal stress plays a role, variable repetition rate regenerative amplifiers would provide more flexibility in finding the optimum pulse energy and the repetition rate that can maximize the signal without damaging the fiber. The damage of the fiber occurs in most cases at the inlet of the fiber where the delicate lattice structure around the air core is fractured by the high intensity focused beam. Recent development in fiber end treatment technology has a great potential that can increase the damage threshold by collapsing the end face and hermetically sealing the fiber [32]. In this way, the lattice structured does not in contact with air directly and reduce the possibility of the damage by the ionization effect. Also, since the input beam is guided to the core by refraction when passing through collapsed area, the beam
size at the end face can be made larger such that the damage threshold increases due to the reduced intensity of the beam at the end face.

There are also many rooms for improvement in the endoscope design. The currently available high NA GRIN lens is not well corrected for the chromatic aberration, which causes each spectral component to be focused at different position in axial direction and broadens the pulse width of the recombined beam at the focal plane and could lower the efficiency of the two photon excitation. The power loss within the endoscope is mostly occurs at the grating. To reduce the length of the endoscope size, a high groove density grating with 1200 line/mm was used in the current design. The power loss is significant and only 15% of input power is diffracted to the first order beam. The current design of the endoscope is 8mm in diameter. A more compact design can be achieved by using a GRISM such that the excitation beam path can be made straight in-line without the need for beam steering mirror [33, 34]. Finally, the GDD is tuned by manually translating the corner cube mirror in the pulse compressor. This could be made in high speed by tuning the GDD using a piezo bimorph mirror for both dispersion compensation and for the remote z scanning [35].

References

32. "Photonic crystal fiber end-sealing," (NKT Photonics).
Chapter 6

Improvement of axial resolution and contrast in temporally focused widefield two-photon microscopy with structured light illumination

6.1 Introduction

Temporally focused wide-field two-photon microscopy (TFM) of ultrafast optical pulses is an imaging technique by which depth-resolved wide-field two-photon images can be acquired without the need for raster scanning of the focal spot [1, 2]. Because of its simplicity and high-speed image acquisition capability, TFM has been adapted to various biological imaging applications such as 3D super-resolution imaging [3], cellular dynamics imaging [4] and the depth-resolved fluorescence and phosphorescence lifetime imaging [5]. In addition, TFM has been applied to single cell precision optogenetic control of neuronal activity [6, 7] and high throughput microfabrication [8, 9]. Recently, it has been also shown that TFM can maintain a uniform illumination field of view even in a highly scattering medium [10].

However, TFM suffers from a broader axial resolution when compared to the standard two-photon laser point scanning microscopy (TPLSM), which is caused by the under-utilization of the numerical aperture (NA) of the objective [11]. The FWHM axial resolution achieved to date is 1.6μm through optimizing the spectral width of the ultrafast pulse and the parameters of the intermediate optical elements which is still worse than TPLSM by a factor of two [3, 8]. Further improvement in axial resolution down to 0.85μm FWHM was achieved by filling the back aperture of the objective with the spectrum of an ultrafast pulse that is dispersed in two dimensions at the expense of increasing complexity of the system [11]. More significant problem is that the axial extent of the excitation volume is progressively broadened in a turbid medium as the imaging depth increases, which further increases the background noise [12]. Recently, it has been shown that this effect can be partly avoided by using the line focused TFM which maintains axial resolution even at 2.5 scattering mean free path (MFP) [13].
Apart from a reduced axial resolution TFM uses imaging detectors such as a CCD or a CMOS. The resolution is based on the 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 image depends only on the temporal sequence of the scanning process and the excitation point-spread function [14].

In this paper, we demonstrate that the use of structured light illumination (SLI) in TFM can effectively reject background scattered emission photons and thereby improve image contrast when imaging in a turbid medium. 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 TFM 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 removable computationally.

6.2 Methods of generating structured light illumination in TFM

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 [15, 16] and it has been shown that the axial resolution of these techniques is comparable to that of the confocal microscope [17, 18]. Of these methods, one effective approach we adapted is termed ‘HiLo microscopy’ which generates an optically sectioned image by post-processing the uniformly illuminated image (UI) and the structured light illuminated image (SI) [16]. More specifically, the in-focused high frequency contents are extracted by high-pass filtering UI with a Gaussian shaped high-pass filter. The in-focus low frequency contents are extracted by low-pass filtering the absolute of UI subtracted by SI with the complementary low pass filter to the high pass filter. The cutoff frequency of the Gaussian filter is determined by the sinusoidal spatial frequency of the structured illumination. Subsequently, the optically sectioned image is obtained by combining the two with an adjusting factor so that the transition from low to high frequencies occur smoothly [19]. HiLo microscopy has been widely used in the
context of background rejection for light-sheet microscopy [19, 20], TFM [21, 22] and depth-resolved microrheology [23].

Fig. 6.1 shows the schematic diagram of an SLI TFM based on a Michelson interferometer. Ultrafast optical pulses from Ti:Sapphire laser with 100fs pulse width, 80MHz repetition rate at the center wavelength of 780nm (MaiTai, Spectra-Physics, Mountain View, CA) are diffracted off the reflective diffraction grating with the groove frequency of 600 grooves/mm (53004BK02-35IR, Richardson Grating Lab, Rochester, NY) and each spectral component of the beam is focused at the back focal plane of the objective (Zeiss C-Apochromat 40x, NA 1.2 water immersion, Zeiss MicroImaging, Thornwood, NY) that collimates each spectral components and recombine them at the focal plane of the objective to restore the original input pulse width.

Fig. 6.1. Generating SLI is possible through either an interferometer or a grid. The interferometric setup shown here is much simplified for grid projection where the components in the dotted box are bypassed and the light goes directly from RDG through the f=200mm and f=100mm lenses before passing through the grid and onwards. BE: beam expander, RDG: reflective diffraction grating, NPB: non-polarizing beam splitter, M: mirror, ExTL: excitation tube lens, EmTL: emission tube lens, DM: dichroic mirror, Obj: objective, BFP: back focal plane, FFP: front focal plane, GP: grid projection, FP: fringe projection

There are two possible methods of generating SLI. First, it can be generated by projecting a fringe (Fringe Projection (FP)) where the incoming beam diffracted off a grating is split by a beam splitter and travels down two separate arms. The angle of mirrors 1 and 2 are adjusted so that two parallel strips of pulse spectrum are focused at the back focal plane of the objective and they interfere at the front focal plane of the objective resulting in fringe pattern. The optical path
length of the two arms is matched to maximize the contrast of SLI. The other method is projecting a grid (Grid Projection (GP)) where the grid is placed at a plane conjugate to both the grating and the front focal plane of the objective. The entire interferometric arm may be bypassed so that the incoming beam off the grating passes directly through the $f = 200\text{mm}$ lens onto the $f = 100\text{mm}$ lens before passing through the grid and the rest of the optical setup. In GP, there are three strips of focused spectrum at the back focal plane each corresponding to 0th and ±1st order diffracted beam from the grid. The interference of these three orders generates the fringe pattern at the focal plane of the objective. SI and UI are obtained sequentially by manually blocking and unblocking one of the beam path in the Michelson interferometer for the case of FP. However, one could use the optical chopper to automate this process. For the case of GP, we manually move the grid in and out of the optical path at the position indicated in Fig. 6.1. Another way is to use two different beam paths of which one includes a grid and the other without a grid. SLI is accomplished by switching between the two beam paths with a galvanometer mirror as demonstrated by Ford et al. [24]. Finally, one may combine TFM with a spatial light modulator and generates an arbitrary excitation pattern for various applications which need patterned two photon excitation [4, 7, 25].

The intensity at the front focal plane of the objective for FP can be expressed as

$$I_{\text{fringe}} = 1 + \cos(2k \sin \theta_f y)$$

(6.1)

where $k$ is the wave number of the center wavelength and $\theta_f$ is the half angle between the two beams after the objective. Here, we ignore the complexity of the pulsed beam and take only the center wavelength and assume equal intensity for the two interfering beams. Likewise, the intensity for GP can be expressed as

$$I_{\text{grid}} = 1 + 4\alpha \cos(k \sin \theta_g y) \cos \left( k \left( \cos \theta_g - 1 \right) y \right) + 2\alpha^2 \left( 1 + \cos(2k \sin \theta_g y) \right)$$

(6.2)

where $\alpha$ is the relative strength of the first order beam compared to zero order beam and $\theta_g$ is the angle between the zero order beam and the first order beam after the objective. In case of rectangular grid, $\alpha = 2/\pi$. The 3D plots of both Eq. (6.1) and Eq. (6.2) are shown in Fig. 6.2(a) and 6.2(b) respectively. In case of GP the fringe pattern is formed not only laterally (along y-axis) but also axially (along z-axis). This property was utilized to increase the resolution both laterally and axially in a conventional wide-field microscope [26].

The two methods of SLI generation is compared in terms of the fringe contrast and density. The contrast of the sinusoidal fringe pattern is defined as
Contrast = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} + I_{\text{min}}} \quad (6.3)

Instead of using Eq. (6.3), we calculate the contrast of either case in the frequency domain from the recorded fringe patterns. This method is superior to the direct calculation of the contrast using Eq. (6.3) in that it is less affected by the shape of the illumination pattern and the local intensity variation due to the noise. For example, for the Gaussian illumination shape the sinusoidal fringe pattern is expressed as

\[ f(y) = \frac{1}{2} (1 + M \cos(ky)) \cdot \exp \left( -\frac{y^2}{2\sigma^2} \right) \quad (6.4) \]

where \( M \) and \( k \) are the contrast and the spatial frequency of the sinusoidal fringe pattern respectively. The Fourier transform of Eq. (6.4) is

\[ F(m) = \left[ \frac{1}{2} \delta(m) + \frac{1}{4} M \delta(m-k) + \frac{1}{4} M \delta(m+k) \right] \otimes \left[ \sigma\sqrt{2\pi} \exp \left( -\frac{\sigma^2 m^2}{2} \right) \right] \quad (6.5) \]

where \( \otimes \) represents convolution, \( \delta(m) \) is delta function. Therefore, \( M \) can be calculated as follows.

\[ M = 2 \frac{F(m = \pm k)}{F(m = 0)} \quad (6.6) \]

The coherent illumination fringe pattern is recorded by reflecting SLI from a mirror placed at the focal plane of the objective. The emission filter in the detection path is removed to allow detection in the excitation wavelength. The incoherent detection fringe pattern is recorded by illuminating SLI on thin layer of Rhodamine solution. The thin Rhodamine solution is prepared by first dissolving Rhodamine power in alcohol and diluted to 300\( \mu \)M concentration. Then, a drop of Rhodamine solution is placed on the cover slip and covered with the same size cover slip and squeezed to form a meniscus around the edge of the cover slip. The volume of a drop of Rhodamine solution is determined by multiplying the area of the cover slip with the desired thickness that needs to be less than the axial resolution of TFM. The recorded image is a fluorescence image and the emission filter is placed in the detection path.

In principle, the contrast of the illumination fringe pattern for GP and FP should be close to 1 as long as the pulse spectrum is within the pass band of the coherent optical transfer function (OTF) of the excitation path as can be predicted by Eq. (6.1) and Eq. (6.2) and their plot in Fig. 6.2(a) and 6.2(b). The contrasts of the detected fringe pattern of thin Rhodamine solution are attenuated by the incoherent OTF of the detection path. The average and standard deviation of 10
independently measured contrasts for the structured illumination period of Tg = 1.71μm are summarized in Fig. 6.2(c), which shows 0.785 ± 0.002 and 0.778 ± 0.014 for GP and FP respectively in case of illumination and 0.236 ± 0.002 and 0.225 ± 0.001 for GP and FP respectively in case of detection. There are quite differences between the estimated contrast values shown in Fig. 6.3(a) and the measured contrast values. This could be attributable to the broad-band excitation and emission wavelengths. The estimated contrast of fringe pattern is based on the peak wavelength of both excitation and emission band. In case of illumination, the bandwidth of pulsed excitation laser has about 10nm FWHM (20nm full width). The sum of interference pattern by each individual wavelength components has the effect of adding a DC value and this reduces the contrast. In case of detection, this effect is much stronger since the emission band of Rhodamine has about 50nm FWHM (100nm full width). The other reason could be the out of focus background. The estimated thickness of the thin Rhodamine solution is 2μm, which is thicker than the SLI resolution and the signal from out-of-focus background could reduce the contrast.

In terms of the fringe pattern stability from the external disturbances such as mechanical vibration or air flow, GP is more stable than those obtained through FP since GP is essentially a common path geometry whereas FP is formed by the two different beam paths in Michelson interferometer. In instances when signal level is low and longer exposures are required, the contrast of FP is slightly worse than GP since the fringe pattern jitters due and the image of the fringe is smeared and therefore averaged during the exposure time. Practically, the power loss due to reflection from the grid surface is significant and this lowers the excitation efficiency.

The fringe density of the illumination pattern determines the signal to noise and the depth resolution of HiLo processed image. FP can generate higher density pattern than GP for objectives with limited aperture size such as GRIN lens. For example, in case of the fringe period of 1.71μm, θg is about two times larger than θf, which means the distance between +1 and -1 order of the pulse spectrum of GP has to be two times larger than the distance between the two pulse spectrum of FP.
6.3. Depth resolution improvement using SLI in TFM

6.3.1 Theoretical estimation

It has been shown that the integrated two photon excitation (TPE) signal of TFM at an axial plane at position \( z \) decays proportional to \( z^{-1} \) [27] whereas that of the TPLSM has \( z^2 \) dependence. Also, the shape of this integrated TPE curve of TFM is similar to the square root of Lorentzian function [3], which has a long tail on both sides of the peak of the curve. Therefore, the axial confinement of the excitation volume of TFM is worse than TPLSM and the long tails of the excitation volume generate out-of-focus background. Thus, the use of SLI in TFM has the potential of removing such background signal and improving the axial resolution of TFM.

Theoretical axial resolution of SLI can be estimated using the defocused 2D OTF derived by Stokseth [28].

\[
C(u,m) = f(m) \left[ \frac{2 J_1 (um[1-m/2])}{um[1-m/2]} \right]
\]  

(6.7)

where \( f(m) = 1 - 0.69m + 0.076m^2 + 0.043m^3 \), \( m \) is the normalized fringe frequency and is related to the real fringe period \( T_g \) via \( m = \lambda/(T_g \text{ NA}) \), \( \text{NA} = n \sin(\alpha) \) and \( u \) is the normalized defocus and is related to the actual defocus \( z \) via \( u = 4kznsin^2(\alpha/2) \), \( k = 2\pi/\lambda \). In incoherent detection, OTF represents the contrast of the fringe detected at the image plane and is equivalent to the signal generated when a thin sheet of fluorescence is scanned through the focus [29].

Fig. 6.3(a) shows the plot of Eq. (6.7) 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 μm and higher, FWHM of the axial resolution is expected to be better than that of TFM reported in the literatures [3, 8]. 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. 6.3(b). Ideally, the best optical sectioning is achieved when the normalized fringe frequency comes close to 1 but with the tradeoff of the reduced signal to noise ratio [29]. 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 [30].

![Figure 6.3](image.png)

Fig. 6.3. (a) Contrast decay of fringe pattern of the spatial period of Tg = 0.43, 0.85, 1.71, 3.42 μm as a function of the defocus (distance from focal plane). (b) Normalized intensity of SLI and TPLSM as a function of the normalized defocus unit

6.3.2 Experimental verification

A thin sheet of Rhodamine 6G (Sigma-Aldrich, St. Louis, MO) solution is used to measure how much SLI improves the axial resolution in different scattering conditions. A scattering medium is simulated by using, Lipofundin-20 (B. Braun, Germany), as the immersion medium of the objective. This approach allows us to precisely vary the scattering coefficient of the specimen by controlling the concentration of Lipofundin ranging from 2% to 5% v/v. The reduced scattering coefficients (μ’s) of the Lipofundin solutions were measured with a commercial near-infrared spectrometer (OxiplexTS, ISS Inc, Champaign, IL) at 690nm, 830nm and the μ’s at 780nm is estimated by the linear interpolation, which is subsequently used to calculate the scattering MFP (l_s) and anisotropy coefficient, g was estimated to be 0.65 [31]. z
represents the scattering length and \( z/l_s \) is calculated assuming \( z \) is equal to the working distance of the objective which is 280\( \mu \)m. The result of these measurements is summarized in Table 6.1. The 2\% scattering property is similar to the human dermis \cite{32} and has been used for characterizing point spread function of TPLSM in turbid medium \cite{33} and well characterized by previous studies \cite{31,34}.

<table>
<thead>
<tr>
<th>Table 6.1. Reduced scattering coefficients</th>
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<tr>
<td>( \mu'_{s} ) (cm(^{-1})) at 690nm</td>
</tr>
<tr>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>14.6±0.8</td>
</tr>
<tr>
<td>20.1±0.2</td>
</tr>
<tr>
<td>27.2±0.5</td>
</tr>
<tr>
<td>32.6±0.6</td>
</tr>
<tr>
<td>( \mu'_{z} ) (cm(^{-1})) at 830nm</td>
</tr>
<tr>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>12.5</td>
</tr>
<tr>
<td>17.2</td>
</tr>
<tr>
<td>23.4</td>
</tr>
<tr>
<td>28.1</td>
</tr>
<tr>
<td>( l_s ) (( \mu )m) at 780nm</td>
</tr>
<tr>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>1.4</td>
</tr>
<tr>
<td>1.9</td>
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</table>

We performed the measurement under two scattering conditions. First, the experiments are performed without any scattering medium (0\% Lipofundin) and then undertaken with 2\% Lipofundin as an immersion medium. The measurements were repeated 10 times independently at each scattering condition. The FWHM value is estimated by fitting the raw data with a Gaussian curve. From Fig. 6.4, it is evident that SLI improves the axial resolution of TFM significantly. At 0\% Lipofundin, TFM’s FWHM is 3.92 ± 0.05\( \mu \)m and TFM HiLo’s FWHM is 2.19 ± 0.06\( \mu \)m. At 2\% Lipofundin, they are 5.21 ± 0.18 \( \mu \)m and 2.37 ± 0.05\( \mu \)m, respectively. The axial resolution of TFM measured with 0.1\( \mu \)m fluorescent polystyrene beads (F-8803, Invitrogen, Carlsbad, CA) is 2.01 ± 0.10\( \mu \)m. We may deconvolve the measured depth profile of the thin Rhodamine solution using the point spread function measured with 0.1 \( \mu \)m bead and estimate that the thickness of the Rhodamine solution is about 2 \( \mu \)m. As a comparison the theoretical TPLSM response to the thin Rhodamine solution is also shown in Fig. 6.4(a) with FWHM of 2.08 \( \mu \)m. Considering the thickness of the Rhodamine solution is about 2 \( \mu \)m, the axial resolution is broadened about 1.67 times at 1 scattering MFP condition. This broadening of the axial resolution has already been reported in the literature \cite{12}. Although the direct comparison with the previous results might not be possible since the important parameters affecting the axial resolution including the grating groove frequency, the pulse width and the objective (NA, magnification) are not the same as the setup used for this paper, we can make comparison of general trend of broadening effect. According to the Fig. 3 in \cite{12}, at 200 \( \mu \)m
scattering depth which corresponds to about 1 scattering MFP, the FWHM of TFM is broadened about 1.6 times compared to 0μm scattering depth for both 40x, NA=0.8 and 60x, NA=1 imaging conditions. This result agrees well with our result shown in Fig. 6.4. 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 highly scattering medium.

Fig. 6.4. (a) Axial resolution measured with the thin Rhodamine solution. Fringe period of SLI is 1.71μm (red) TFM HiLo at 0% intralipid (blue) TFM HiLo at 2% intralipid (green) TFM at 0% intralipid (black) TFM at 2% intralipid (orange) TPLSM in Fig. 6.3(b) convoluted with 2 μm thick Rhodamine solution. (b) Averages and standard deviations of FWHM of 10 measurements for 0% and 2% scattering condition of both TFM and HiLo TFM

The axial resolution improvement is further demonstrated with a prepared slide of sectioned mouse kidney (F24630, Invitrogen, Carlsbad, CA). At 0% Lipofundin condition, the sample is imaged at 0.5μm step size for a total of 14μm scanning range axially. Fig. 6.5 shows the xz section view of TFM image without SLI and HiLo processed TFM images with fringe period of 3.42μm, 1.71μm, 0.85μm respectively. As the fringe frequency increases the sectioning capability of SLI increases and finer features of the specimen becomes visible more clearly. The intensity profiles along the axial direction at the point indicated by the yellow arrows are also shown on the right side of Fig. 6.5. The weak intensity object in the middle of the sample indicated by green arrows, which is hidden by the two high intensity objects above and below, becomes discernible with SLI at Tg = 0.85μm. This effect is better visualized in the normalized intensity plot where the intensity is normalized with the peak intensity value. Clearly, the SLI approach improves TFM axial resolution.
Fig. 6.5. \(xz\) sections of the fine 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 side. Further details on the sample can be found in http://products.invitrogen.com/ivgn/product/F24630

6.4. Contrast enhancement using SLI in TFM

In addition to the depth resolution improvement, SLI is beneficial to rejecting the out-of-focus scattered emission photons and consequently improves the signal to background ratio or image contrast. To simulate the various scattering conditions the sample was imaged through 280\(\mu m\) of immersion fluid and approximately 5\(\mu m\) into the sample at 0\% (Fig. 6.6(a) and 6.6(b)), 3\% (Fig. 6.6(c) and 6.6(d)), 5\% (Fig. 6.6(e) and 6.6(f)) Lipofundin concentration. The sample is identical as the one used in Fig. 6.5. SI was imaged with the fringe period of 1.71 \(\mu m\). It is clear that the background rejection improves the contrast dramatically and fine structures are more clearly visible in HiLo processed images. However, the fine features are progressively lost as the concentration increases and this lost information cannot be recovered by the background rejection as shown in red box in Fig. 6.6. This can be viewed in two ways. First, the axial confinement of the excitation volume is broadened at the scattering condition as shown in Fig. 6.4 and this contributes to increased out-of-focus background signal. Although this background signal can be removed by the HiLo processing, the shot noise generated by the background signal still survives and the reduced signal to noise ratio ultimately limit the imaging depth in...
turbid medium. Second, the fine features are lost by the scattering of the emission photons in widefield imaging modes and this cannot be recovered by the HiLo processing.

Fig. 6.6. Unprocessed images in scattering conditions (a) 0% v/v, (c) 3% v/v, and (e) 5% v/v Lipofundin-20. Processed images are (b), (d), and (f) for the same scattering conditions, respectively. Each image represents a 90μm x 70μm.

6.5. Conclusion

In conclusion, we have demonstrated the first use of a HiLo based SLI in TFM. While TFM has intrinsic optical sectioning, the presence of excitation and emission photon scattering can compromise this ability. It is therefore useful to implement SLI to counter the broadening of the axial extent of the excitation volume in a scattering medium as well as to reject the scattered background photons, which improves the axial resolution and the contrast of the image. However, the increased shot noise from the background signal in the turbid medium and the information lost due to emission photon scattering cannot be avoided with the use of SLI.

Current implementation of SLI in TFM is not optimized in terms of imaging speed, excitation efficiency and the sample exposure to the excitation light for minimizing the photobleaching and phototoxicity effect. Here, we propose methods to improve the current performance of SLI TFM. First, HiLo TFM is slower than TFM due to the fact that it requires two images per z-section. Thus, the imaging speed can be improved by automating the switching
mechanism between UI and SI. For example, 10Hz imaging speed was demonstrated by using a galvano-mirror to switch between UI and SI in case of GP SLI [24] or one can split the beam into +1 and -1 order using a diffractive optical element and modulate one of the diffracted beam using high speed laser beam shutter in case of FP SLI. This also reduces the power lost in the FP SLI where the interferometer based FP SLI wastes half of the excitation beam power. Second, the excitation efficiency of TFM is limited by the available laser power in a turbid medium. As demonstrated by Cheng et al. [35], high peak regenerative amplifier can be used to increase the excitation efficiency and thereby improving the imaging speed of the TFM. Imaging speed can be further improved by increasing the repetition rate of the pulsed laser source using the pulse multiplier. However, this works only in a regime where the two photon excitation probability is low enough so that increasing repetition rate would not induce the saturation of fluorophores. In addition to the increased signal level, the photo-bleaching effect was also shown to be reduced in this way [36].

Lastly, this technique might be useful for improving the depth resolution of TFM based endoscopy which has poor depth resolution due to limited NA of the GRIN lens.

References


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Chapter 7

3D-resolved fluorescence and phosphorescence lifetime imaging using temporal focusing wide-field two-photon excitation

7.1 Introduction

Fluorescence (FLIM) and phosphorescence (PLIM) lifetime microscopies are information-rich optical spectroscopic techniques [1-7]. A particularly important application of FLIM is in the quantification of Förster 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 [8], and to observe dynamic conformational changes in proteins [9] and RNA [10]. 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 [11]. A clinical trial across multiple centers in Europe is underway to test the utility of multiphoton FLIM in the minimally invasive diagnosis of melanoma [12].

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 [13-15]. PLIM-based partial oxygen measurements can be used to quantify the degree of hypoxic tissues or tumors, a critical physiological parameter of solid tumors that determines tumor growth, gene expression [16], metastatic potential [17], metabolism, prognosis [18-20], and response to therapy [21, 22]. 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 [23, 24]. Today, PLIM is not widely used mostly due to the associated long lifetime which entails typical image frame rate on the order of minutes to hours.
The implementations of FLIM and PLIM measurements in microscopy have different challenges. For FLIM, the need for picoseconds 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 micro-environment. However, in practice, even for fluorophores with single exponential decay dynamics, the experimentally measured time delay histogram is a convolution of an 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 \((\Delta \phi)\) and modulated \((M)\). For single exponential decay fluorophores, the modulation and phase shift are related to the fluorescence lifetime as:

\[
\Delta \phi = \tan(\omega \tau) \quad \text{and} \quad M = \frac{1}{\sqrt{1 + \omega^2 \tau^2}}
\]  

(7.1)

More complex decay mechanisms can be measured by measuring the modulation and phase shift at multiple excitation frequencies of \( \omega \). Importantly, the unknown instrumentation response can be more readily isolated and removed in the frequency domain. In order to obtain accurate phase shift and demodulation measurements, the excitation light modulation frequency of \( \omega \) 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 of \( \omega + \Delta \omega \). This electronic mixing process results in translating the phase and demodulation information to an electronic signal at the cross correlation frequency of \( \Delta \omega \) 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 literatures [25, 26].

High throughput FLIM has been the focus of technology development of several research groups. The developed instruments fall into two categories. In the first category, gated cameras are used for time-resolved full field imaging. These instruments have been implemented with traditional wide-field fluorescence microscopy without depth resolution [27, 28] or with spinning disk confocal microscope for 3D resolved imaging [29, 30]. Spectral-lifetime resolved imaging using this approach has also been implemented [31]. Another approach to achieve 3D imaging is based on using multifocal multiphoton microscopy (MMM), detecting in parallel the signal from each foci using separate time-correlated single photon counting (TCSPC) circuitry [32]. The next-generation instruments described in this report are built on the foundation established by these prior works.

7.2 Temporal focusing wide-field Two-Photon FLIM and PLIM

This paper describes the design of a fast 3D FLIM and PLIM imaging system that is 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. Fig. 7.1 shows the optical and electronic design of the temporal focusing wide-field FLIM/PLIM system. A titanium sapphire femtosecond pulsed laser (Tsunami, Spectra-Physics, Mountain View, CA) pumped by a continuous-wave diode-pumped solid-state laser (Millennia V, Spectra-Physics, Mountain View, CA) is used to provide a 80 MHz train of 100 fs pulses at a center wavelength of 780 nm. The 1/e² beam diameter of the ray is about 1-2 mm. The excitation light is intensity modulated using an acousto-optical modulator (AOM, 1205C-2, ISOMET, Springfield, VA). Excitation light is dispersed by a reflective diffraction grating with groove frequency of 600 grooves/mm (53004BK02-35IR, Richardson Grating Lab, Rochester, NY) and is directed towards a high numerical aperture objective (Fluar, 40X/1.30 NA Oil, Zeiss MicroImaging, Thornwood, NY). The diffraction grating surface is placed at the conjugate plane of the object plane resulting in an excitation area of approximately 100×100 μm². Away from the focal plane,
this arrangement guarantees that the different spectral components dispersed by the grating are separated spatially, resulting in broader pulse width and lower two-photon excitation (TPE) efficiency due to the constancy of the time-bandwidth product. At the focal plane, the different spectral components are recombined, the pulse width is minimized, and TPE efficiency is maximized. Volumetric imaging is accomplished by plane-by-plane excitation by translating the objective using a piezo-positioner (MIPOS500, piezosystem Jena Inc., Hopedale, MA).

The fluorescence or phosphorescence images are acquired using a high speed phase resolved camera (LI2CAM MD connected to a LIFA control unit, Lambert Instruments, Roden, Netherlands) modified for heterodyne detection. The high speed phase resolved camera consists of a high sensitivity proximity focused image intensifier. The gain of the intensifier can be modulated by injecting an external analog gating signal at the intensifier cathode. The phosphor output window of the intensifier is fiber optically coupled to a 1392x1040 pixel CCD camera, used in 2x2 binned mode, to ensure maximum light detection efficiency.

In TFWF FLIM, the femtosecond titanium-sapphire laser pulse train provides the required modulation frequencies at 80MHz and its harmonics. In TFWF PLIM, the AOM, driven by a frequency synthesizer (2024, Marconi Instruments, discontinued, and DS345, Stanford Research Systems, Sunnyvale, CA) and a digital modulation RF driver (522C-2, ISOMET, Springfield, VA), provides modulation frequency of $\omega$ from DC to about 1 MHz. The Lambert Instruments camera has an internal high voltage amplifier that controls the gain of the intensifier; this amplifier is driven by a second frequency synthesizer that provides a sinusoidal driving frequency at the modulation frequency of $\omega$ plus a small cross correlation frequency of $\Delta\omega$. The CCD camera frame acquisition is triggered by a third frequency synthesizer operating at a frequency equal to a quarter of the cross correlation frequency of $\Delta\omega$ allowing the cross correlation signal to be sampled four times per wave. For precise phase resolved measurement, these three frequency synthesizers are phase locked to the output of the titanium laser pulse train at 80 MHz. The typical cross correlation frequency of $\Delta\omega$ is chosen to be 1 Hz since the current version of the Lambert camera is not designed for heterodyne detection and has a maximum frame rate of about 4 Hz. Significantly faster imaging (up to kHz level) should be possible by using cameras of higher frame rate and by ensuring that the phosphor screen of the intensifier has sufficiently fast response time. The development of an improved camera for high speed heterodyne detection is underway under the Lambert Instrument/MIT collaboration.
7.3. Results and analysis

7.3.1 Fluorescence lifetime measurement performance and applications

The accuracy of the developed wide-field 3D lifetime imaging system was evaluated by measuring the fluorescence lifetimes of Rhodamine B (79754, Sigma-Aldrich Products, St. Louis, MO) solution in different solvents that have been carefully quantified in literature [33]. As a lifetime reference, fluorescein (R14782, Invitrogen, Grand Island, NY) was dissolved in 0.1M NaOH pH 8.0 buffer at a concentration of 50 μM. The lifetime of the fluorescein solution is assumed to be a 4.0 ns single exponential. Rhodamine B was dissolved in deionized water or spectroscopic grade methanol, or ethanol at a concentration of 50 μM. All fluorescein and rhodamine solutions were enclosed in a hanging-drop slides covered and sealed with #1.5 cover slip.
3D resolved wide-field images of these solutions were acquired 50 µm away from the cover slip. The modulation frequency and the cross correlation frequency were set to 80 MHz and 1 Hz respectively, while the lifetime camera readout rate was set at 4 Hz providing four amplitude measurements of each cross correlation waveform. Results from twenty cross correlated waveforms were integrated to generate a single dataset resulting in a total image acquisition time of 20 seconds. The phase delay and demodulation of Rhodamine B solutions are then quantified relative to the modulation and phase of a reference fluorescein specimen. Fitting the measurements to a single exponential decay model allows the extraction of lifetimes tabulated in Fig. 7.2 (a). The lifetime measurements obtained by TFWF 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 [34, 35]) the lifetime of individual pixels cluster around a single location located on the universal circle as expected for single exponential decay processes as shown in Fig. 7.2 (b). The locations of these four distributions are consistent with the tabulated lifetimes obtained from averaging over the whole image.

![Table and graph](image)

Fig. 7.2 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. (a) Tabulated results of estimated lifetime values of τ extracted from either phase (Ph) or modulation (Mod) measurements. Literature values are also included as a reference [33]. (b) Lifetime resolved data for each pixel from the fluorescein (FL) and rhodamine solution images shown in polar plot format.

The high data acquisition speed of the developed instrument is demonstrated by FLIM imaging of fixed fibroblasts loaded with conjugated polymer nanoparticles (CPNs) of high two-
photon cross section previously measured in excess of 15,000 GM [36]. Wide-field two-photon lifetime resolved imaging is fundamentally limited by the need to simultaneously and optimally excite fluorophores within a large area plane via two-photon absorption. Optimal excitation corresponds to two-photon absorption probability on the order of 10% per laser pulse. In this case fluorophores are excited without saturation and image resolution degradation [37]. The two-photon absorption probability per fluorophore per excitation photon pair per laser pulse can be expressed as [38]:

\[ pr \propto \frac{\delta}{f^2 \tau} \left( \frac{P}{N} \right)^2 \]  (7.2)

where \( N \) is the number of pixels (assuming diffraction-limited pixel size) measured in parallel, \( \delta \) is the fluorophore two-photon cross section, \( P \) is the average power per image, \( f \) is the laser pulse repetition rate, and \( \tau \) is the laser pulse width. For typical titanium-sapphire femtosecond laser sources and typical fluorophores with approximately 10 GM two-photon cross section, optimal excitation occurs at approximately \( N = 100 \). For imaging at diffraction limited resolution of about 0.5 \( \mu \text{m} \), the maximum field of view is only about \( 10 \times 10 \mu \text{m}^2 \); an area too small for typical biological imaging. A recent study has shown that this limitation can be remedied by the use of high peak power sources such as regenerative amplifiers [39]. This limitation is less critical for fluorophores of high two-photon cross section such as quantum dots or CPNs where image size can be extended to about \( 100 \times 100 \mu \text{m}^2 \). Imaging CPN-labeled specimens by TFWF FLIM was used to demonstrate the data acquisition speed of TFWF FLIM and to ensure that this experiment was not limited by inefficient wide-field two-photon excitation. A representative intensity scaled lifetime image and the associated polar plot of pixel lifetime distribution of the specimen is shown in Fig. 7.3. The non-symmetric, off universal circle distribution of pixel values in the polar plot indicates non-single 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 time 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 (\( > 0.5 \text{ ns} \)).
Fig. 7.3 (a) An intensity scaled mean lifetime image of fixed fibroblasts with vacuoles loaded with endocytosed conjugated polymer nanoparticles of high two-photon absorption cross section. Color scale represents pixel lifetime values corresponding to the color bar with units of seconds. Image brightness represents pixel intensity values. Black regions are ignored in analysis corresponding to locations with intensity below 500 photons that are mostly outside the boundary of this cell. (b) Representative polar plot of pixel lifetime values for 10 ms data acquisition time. (c) Tabulated mean lifetime values and their standard deviations are estimated from the modulation or the phase data for four different image acquisition rates.

The application of temporal focusing wide-field two-photon imaging in lifetime resolved biological imaging is demonstrated in a thin 6μm thick histological cross section of a formalin fixed injured rat sciatic nerve. After transecting the nerve, the two resulting nerve stumps were connected with a collagen tube [40, 41]. In order to quantify the degree of induced regeneration, myelin sheaths surrounding the newly-grown axons were visualized by labeling using FluroMyelin Green (F34651, Grand Island, NY). Lifetime imaging by the TFWF FLIM system was conducted using identical imaging parameters with the ones used in rhodamine solutions imaging. Fig. 7.4 (a) shows a zoom-in cropped image of 20μm×20μm field of view displayed as an intensity scaled lifetime image, where the color of each pixel represents its mean lifetime and the brightness of each pixel is proportional to the number of photons acquired. Pixel lifetime information can also be displayed in a polar plot format as shown in Fig. 7.4 (b). Unlike the rhodamine and fluorescein solutions, the distribution of pixel lifetimes is broader and does not fall directly on the universal circle indicating either that the fluorescence lifetime of FluroMyelin Green is not single-exponential or that its lifetime may be affected by the local tissue.
microenvironment. The broadening of the lifetime distribution may also be caused by the presence of autofluorescence signal emitted by the fixed nerve specimen. Since the fluorescence lifetime of FluoroMyelin Green has not been adequately quantified in the literature, we evaluated the accuracy of our TFWF lifetime measurement by imaging the identical specimen using a point-scanning two-photon microscopy equipped with a Becker and Hickl SPC730 TCSPC module for time-domain lifetime measurement as shown in Fig. 7.4 (c). The total pixel residence time was 4.8 ms and the integration time for a single lifetime resolved image was about 100 sec. The mean lifetimes for the labeled myelin sheath are in good agreement at approximately 2.5 ns. The long lifetime blobs in the TCSPC correspond to nuclei separately labeled with DAPI with a typical lifetime of approximately 4 ns.

![Fluorescence lifetime imaging of an ex vivo histological sample by TFWF FLIM.](image)

Fig. 7.4 Fluorescence lifetime imaging of an ex vivo histological sample by TFWF FLIM. (a) Intensity-scaled fluorescence lifetime-resolved image of a regenerated ex vivo rat sciatic nerve after injury stained with FluroMyelin Green. The image field of view is approximately 20x20 μm² (a) and 100x100 μm² (c). Scale bar has units of seconds. (b) Polar plot representation of the pixel lifetime estimated based on the phase data. (c) A larger view of the same sample acquired using a point scanning two-photon microscope equipped with TCSPC lifetime resolved imaging system. The scale bar has unit of nanoseconds. The lifetime measurements for both systems are in excellent agreement.

Fast lifetime-resolved imaging with 3D resolution is demonstrated by imaging fluorescent beads of 15μm (F8844, Invitrogen Inc, Grand Island, NY) and 4μm diameter (F8858). Fig. 7.5 (a) shows the intensity-scaled lifetime images taken at 5μm axial steps. The depth resolution of the system is on the order of 2μm. The 4μm bead is uniformly labeled with fluorophores while the 15μm is only labeled at the surface while its interior is not impregnated with fluorophore. It is interesting to note that while the 15μm and 4μm beads are emitting in the yellow-green and red spectral ranges respectively, these beads have essentially the same lifetimes. The associated polar plot of pixel lifetimes acquired at z=15μm depth suggests an almost single exponential decay for these fluorophores as shown in Fig. 7.5 (b).
Fig. 7.5 Demonstration of fast 3D-resolved lifetime imaging by TFWF FLIM system. (a) Intensity scaled lifetime images of 15 μm yellow-green beads and 4 μm red beads imaged at different axial plane locations z. Pixel lifetimes were estimated from modulation data. The scale bar represents 5 μm in length. (b) Representative polar plot of pixel lifetime information at z=15 μm.

The 3D resolved imaging ability of the developed system in living biological specimens was demonstrated by imaging live human dermal fibroblasts interacting with a porous collagen scaffold, similar to the one used to induce nerve regeneration in Fig. 7.4 [42, 43]. Fibroblasts were labeled by either Calcein AM (a marker of cytoplasm and nucleus), or Syto 13 (a nucleic acid marker) or both. 3D-resolved lifetime imaging was performed using the same imaging parameters as described in Fig. 7.3-5. A representative image of a cell labeled with both markers is shown in Fig. 7.6 (a). The lifetime of Calcein AM was estimated to be 3.5 ns and 3.1 ns using phase or modulation data respectively. These two lifetime rate estimates are in good agreement with the published TCSPC measurements [44]. The fluorescence lifetime of Syto 13 was estimated to be 2.9 ns and 1.8 ns using phase and modulation data respectively. The large difference between phase and modulation results indicates the non-single exponential nature of the decay. Similar non-single exponential decay has been observed when Syto 13 was used in bacterial cultures, where a similarly broad distribution of lifetime from 1.5-2.5 ns were observed suggesting different lifetimes depending on whether the dye binds to DNA or RNA [45]. The polar plot of the pixel lifetimes for specimens labeled only with Calcein AM shows a tight distribution of pixel lifetimes close to the unit circle, which indicates a single exponential decay (Fig. 7.6 (b)). On the contrary, the polar plot for specimen labeled with only Syto 13 shows broad distribution of pixel lifetime rates that suggests a non-single exponential nature of fluorescence decay (Fig. 7.6 (d)). As expected, the polar plot in specimens labeled with both
markers has a pixel distribution at the algebraic mean between the tight distribution of Calcein AM close to the unit circle and the broad distribution of Syto 13 (Fig. 7.6 (c)).

Fig. 7.6 Fluorescence lifetime imaging of human fibroblasts seeded inside a collagen scaffold double-stained with Calcein AM and Syto 13 by TFWF FLIM. (a) Intensity scaled lifetime images. (b) Polar plot of fibroblasts labeled only with Calcein AM. (c) Polar plot of fibroblasts labeled with both Calcein AM and Syto 13. (d) Polar plot of fibroblasts labeled only with Syto 13.

7.3.2 Demonstration of 3D phosphorescence and fluorescence lifetime-resolved imaging in tissue engineering scaffolds

Wide-field lifetime-resolved imaging systems enable not only efficient 3D fluorescence lifetime imaging, but also fast quantification of phosphorescence lifetime. Phosphorescence lifetime measurement has significant applications in biophysics including background-free labeling, and monitoring the binding of high molecular weight proteins. However, the most important biomedical use 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 [16, 17] and its response to therapy [22, 46]. Improved methods to quantify the 3D distribution of molecular oxygen partial pressure pO$_2$ with cellular resolution inside solid tumors in animal models could provide novel information for understanding how cancer physiology affects
therapeutic responses. Oxygen sensors based on phosphorescence quenching offer a powerful method to measure pO2 distribution in vivo. Phosphorescence measurement is necessary since tumor pO2 concentration is low and the chromophores must have an excited state lifetime longer than the characteristic time of chromophore-oxygen interaction. Both phosphorescence intensity and lifetime are sensitive functions of pO2. Intensity-based measurements are undesirable since they depend on the local concentration of the phosphorescence probe in the tissue, which cannot be readily quantified. In contrast, phosphorescence lifetime provides a concentration-independent probe for measuring oxygen quenching rate. The lifetime of typical phosphorescence sensors for pO2 range between hundreds of ns to several hundred μs. In simple biological samples that do not require 3D imaging, single-photon wide field phosphorescence lifetime imaging has been implemented efficiently [47-50]. 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). While these very long lifetime probes are sensitive to oxygen concentration and suitable for hypoxic environments, such as the interior of a solid tumor, their long “turn-around” time greatly limits the speed of PLIM. Importantly, unlike FLIM measurements, PLIM measurement speed cannot be easily improved using conventional speed-enhancement methods such as spinning-disk confocal, since the long phosphorescence lifetime will result in significant signal loss due to the motion of the pinholes unless disk rotation rate is kept sufficiently low. On the other hand, the parallelized nature of the proposed TFWF two-photon lifetime resolved imaging system shortens PLIM acquisition time by several orders of magnitude with frame rate approaching phosphorescence lifetimes.

In order to demonstrate the accuracy of the developed TFWF PLIM system, we quantified oxygen concentration in solutions of 1 mM Tris (2,2'-bipyridyl) dichlororuthenium(II) hexahydrate (TDRT, 544981, Sigma Aldrich Products, St. Louis, MO) 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. 7.7 (a)). 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. 7.7 (b), Fig. 7.7 (c)):

\[
\frac{1}{\tau} \propto pO_2 \tag{7.3}
\]

(a) (b) (c)

Fig. 7.7 Fast measurement of partial oxygen pressure by TFWF phosphorescence lifetime imaging. (a) Polar plot of phosphorescence lifetime of 1 mM Tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate solutions equilibrated with 0, 4, 8, and 21% pO_2 gas mixtures. Inverse phosphorescence lifetimes ((b), from modulation data; (c), from phase data) are plotted against O_2 concentration demonstrating Stern-Volmer dependence with R^2 values of 0.99 and 0.95 respectively for linear regression.

The accuracy of TFWF two-photon PLIM method is a function of integration time of each image frame. Specifically, measurement accuracy comparable to the one shown in Fig. 7.7 can be obtained when the integration time is on the order of several seconds. It is important to note that this relatively long integration time is due to the relatively low two-photon cross section of TDRT, the limited number of phosphorescence photons generated, and the limited excitation laser power (about 3W maximum output power for typical Ti-Sapphire laser sources) since the power of about 0.7W is distributed over a 100x100 µm^2 area. The uncertainty of ruthenium phosphorescence lifetime measurements at integration times of 1.2, 1.5, 2.4, 3.0, 3.6, 5.5 and 6.0 seconds are shown in Fig. 7.8 (a) and the representative polar plots at the integration times of 1.5, 3.0 and 6.0 seconds are shown in Fig. 7.8 (b). It is clear that as integration time decreases, the distribution of the polar plot broadens indicating greater uncertainty of lifetime measurement. The uncertainty in the pixel lifetime measurements of an image for given integration time, as quantified by the full-width-at-half-maximum of the pixel lifetime values in the polar plot, can be related to the average number of photons per pixel. When plotted in a log-log format, linear regression gives a slope of approximately 0.5 indicating that the lifetime measurement error is dominated by photon shot noise.
Fig. 7.8 Uncertainty in estimating the lifetime of 1mM TDRT solutions depends on the image integration time. (a) TDRT phosphorescence lifetime measurement uncertainties (FWHM) for images acquired with different integration times are plotted against the average number of photons contained in these images. (b) Representative polar plots of TDRT solution pixel phosphorescence lifetime measurements at integration times of 1.5, 3, and 6 sec.

The application of TFWF imaging in fast sequential phosphorescence and fluorescence lifetime 3D imaging is demonstrated in an in vitro 3D cell-matrix system that can be used to study how oxygenation affects cell-matrix interactions inside tissue engineering devices. The system consists of live human dermal fibroblasts stained with Rhodamine DHPE (the dye is soon endocytosed and primarily localized in intracellular vesicles) seeded inside a porous collagen scaffold similar to Fig. 7.6. In situ monitoring of the oxygenation state of cells was accomplished by adding 1mM TDRT in the cell culture buffer (PBS). 3D-resolved fluorescence and phosphorescence lifetime imaging of the cell-seeded scaffolds was acquired sequentially using modulation frequency (ω) of 80 MHz and 300 kHz respectively. Representative images are shown in Fig. 7.9. In PLIM imaging cells can be distinguished based on the increased lifetime (less oxygen) observed intracellularly compared to the surrounding medium pixels. Representative intensity scaled lifetime-resolved images are shown in Fig. 7.10. At each pixel, the relative intensity contributions from fluorescence and phosphorescence components can be
readily separated based on the pixel location within the polar plot. The distributions of the fluorescence and phosphorescence components are shown in Fig. 7.11. As expected, the fluorescence emission of Rhodamine DHPE is localized in cellular vesicles, while the phosphorescence emission of TDRT is predominately localized in the medium surrounding the cells and the scaffold.

Fig. 7.9 Fast 3D-resolved TFWF PLIM in sample consisting of human fibroblasts stained with Rhodamine DHPE, seeded inside a collagen matrix and treated, in PBS buffer containing 1 mM TDRT ruthenium-based oxygen sensor. PLIM Images were acquired at 300 kHz modulation frequency. In sequence from left to right: intensity image, phosphorescence lifetime-resolved image from phase data, phosphorescence lifetime-resolved image from modulation data. On the far right is the polar plot of the pixel phosphorescence lifetime-resolved measurements. The pixel lifetime data in the polar plot distributes between the ruthenium phosphorescence lifetime that lies close to the universal circle and the rhodamine fluorescence lifetime at the lower right hand corner corresponding to an effective zero lifetime for 300 kHz modulation frequency. PLIM Movie sequences corresponding to phase and modulation lifetime measurements throughout a 3D matrix are included (Supplementary material Mov 1, Mov 2).

Fig. 7.10 Representative single plane intensity scaled lifetime-resolved image measured at a modulation frequency of 300 kHz.
7.4 Conclusion

We have developed an efficient fluorescence and phosphorescence lifetime resolved imaging system. The system combines temporal focusing two-photon excitation for 3D resolved wide-field excitation, and highly parallelized frequency domain lifetime resolved measurement using a nanosecond gated imager. There are two primary limitations of the proposed system.

The first limitation stems from the fact that the peak power of typical titanium-sapphire lasers is not sufficient to optimally excite many commonly available fluorophores over a large area, therefore reducing the efficiency of this approach. This low excitation efficiency can be compensated by using fluorophores of higher two-photon cross section (such as CPNs demonstrated here). However, the most generalizable approach is using high peak power light sources (such as regenerative amplifiers) for more efficient wide-field excitation. In general, sufficient laser flux should be provided to ensure fluorophores are effectively excited by each laser pulse without excitation saturation, corresponding to two-photon excitation probability per fluorophore per pulse of about 10%. Mathematically, two-photon excitation probability can be expressed as in Eq.(6.2). This equation can be rewritten as to estimate the number of pixels that can be simultaneously excited per excitation pulse:
We can further estimate imaging efficiency in terms of the number of pixels that can be excited per unit time:

\[ N \propto \sqrt{\frac{\delta}{\text{Pr} \tau} p_0 f} \]  

For given fluorophore cross section, laser average power and pulse width, the number of pixel that can be simultaneously excited scales inversely with the laser repetition rate. This is the reason that higher peak power, lower repetition rate regenerative amplifiers are superior for simultaneous wide field imaging compared with lower peak power, higher repetition rate oscillators. However, does wide field imaging (imaging many pixels simultaneously) provide an imaging speed advantage compared with point scanning or multi-foci scanning methods? Since \( r_N \) is independent of repetition rate, it would appear that it is just as efficient to image a single pixel at a time as imaging many pixels at the same time. However, this conclusion must be modified by taking into account of at least three additional time constants of the imaging system. One of these time constants is the excited state lifetime of the chromophores. For efficient excitation, the inverse of the laser repetition rate must be much longer in duration than the fluorophore lifetime. Otherwise, depleting the molecular ground state will not only results in a reduction of the efficiencies of the subsequent pulses but will also cause resolution degradation. Given the nanosecond lifetime of most fluorescence probes, this is not a concern for fluorescence lifetime-resolved imaging. However, for phosphorescence molecules with microsecond to millisecond lifetimes, the laser repetition rate should be chosen to be sufficiently slow such that these phosphorescence molecules can be relaxed prior to the next excitation pulse. Therefore, wide field imaging is almost always more efficient for phosphorescence lifetime-resolved imaging. Another time constant is the detector “dead time.” Photon detectors, especially lifetime resolved detectors, have a finite “dead time” between subsequent measurements. Depending on the principles underlying these detectors, the “dead time” is typically on the order of microseconds. The other relevant time constant is the scan speed of the imaging system. Depending on the scanning mechanism, stage, galvanometric scanner, acousto-optical scanner, scan speed quantifies how fast laser light can be directed across individual pixels (point scanning) or a group of pixels (multi-foci scanning). Scan speed typically ranges from
microsecond to millisecond time scale. In general, image efficiency can be increased if pixel integration time is made substantially longer than the detector “dead time” and the scan speed. Taking these three time constants into account, more efficient imaging can often be achieved with wide field imaging mode using a high peak power amplifier for both fluorescence and phosphorescence modes.

The second major limitation of the current system is that the read-out rate of the camera is limited to four frames per second. While we have demonstrated that lifetime-resolved images can be acquired with integration time as short as several milliseconds, the slow read-out rate limits the actual frame rate to about 4 Hz. This limitation results from the fact that the camera used in this experiment is not specifically designed for frequency domain heterodyne lifetime measurement but was custom modified for this experiment. Today, CCD or CMOS cameras can be readily read out at a rate in excess of 1 GHz. By coupling a high speed gated microchannel plate with a high frame rate, low noise camera will produce an ideal system that can perform fluorescence lifetime-resolved measurements in excess of kHz. For phosphorescence imaging, the integration time must be at least over an order of magnitude longer than the phosphorescence lifetime of the probe. Nonetheless, phosphorescence lifetime imaging with sub-second frame rate should be possible for even millisecond lifetime phosphorescence probes.

Finally, wide-field two-photon imaging uses significantly higher power than point scanning; possible specimen photodamage should be considered. In the absence of one-photon absorber and thermal damage, the two main mechanisms for photodamage are multiphoton ionization and oxidative damage. The probability of multiphoton ionization is proportional to laser flux. With a 3W titanium-sapphire laser, approximately 1W of power is eventually delivered upon a $100 \times 100$ μm$^2$ specimen surface. The laser flux is approximately 0.1 mW/m$^2$. As a comparison, the laser flux of typical single focus scanning is on the order of 10-100 mW/m$^2$. As discussed previously, a regenerative amplifier is a better laser source for wide-field two-photon imaging providing higher laser flux such that the two-photon excitation probability can reach about 10%. Since both wide-field and point-scanning approaches reach the same excitation probability level at the same flux, they have the same potential for photodamage. In terms of oxidative damage, since the amount of reactive oxygen generation is proportional to the total fluorescence signal, the total dosage of reactive oxygen species generated is also the same for these two approaches. Of course, since wide-field imaging has higher frame rate, the dose
rate is higher for the wide-field case. A priori, it is impossible to determine whether higher dose rate results in significantly greater photodamage. Future studies are needed to carefully compare the oxidative damage potentials of two-photon wide field imaging and point-scanning for different biological systems under study.

References


Chapter 8

Conclusion

8.1 Thesis summary

In this thesis, we developed novel 3D optical microscope systems that greatly improved the throughput of high volume biological assays. First, high throughput 3D cell based image cytometer have been developed based on the widefield structured light illumination and the high speed remote depth scanning. The sequence of the image acquisition processes are all automated and enables the large area sample imaging at submicron resolution. Assuming that an 80% confluency cell culture is imaged, this system can image adherent cells on the slide glass at 800 cell/sec in 3D. The statistical accuracy of this instrument is verified by quantitatively measuring the rare cell populations down to $1:10^5$ ratio. The developed system can potentially be applied to image cytometry investigation to study cultured cell morphologies with statistical significance comparable to the flow cytometer. Hyperspectral imaging is also possible based on the use of an interferometric full field spectrometer, which enables the full spectrum measurement over the whole field of view simultaneously. Spectral background rejection algorithm is mathematically formulated and experimentally demonstrated. The depth resolved hyperspectral imaging would allows 4D ($x, y, z, \lambda$) imaging and is expected to provide a more accurate spectral signature of the biological system.

Second, we developed a depth resolved widefield two photon endomicroscope for the medical diagnosis based on the temporally focused widefield two photon microscopy. We proposed an improved method for delivering a high energy pulse through the hollow core photonic crystal fiber to increase two photon excitation efficiency. We have demonstrated that the combined strategy of increasing the repetition rate with pulse splitter and reducing the peak power increase excitation efficiency through the endomicroscope by eleven folds. A structured illumination method for improving poor depth resolution caused by the low NA microlens used in these endomicroscopes has demonstrated with resulting resolution comparable to conventional point scanning multiphoton microscope. In addition, temporally focused widefield two photon
microscopy has been developed for high throughput depth resolved fluorescence and phosphorescence measurement with millisecond level integration time.

8.2 Thesis contribution

Current methods of optical microscopy have limitations in terms of imaging deep into the tissue, achievable resolution and its throughput. The imaging depth is fundamentally limited by tissue scattering and the best achievable imaging depth is a couple of millimeter at best. The resolution is limited by the diffraction of the light and the best achievable resolution is about half the wavelength of light. The imaging throughput of optical microscopy is much lower compared to many other medical imaging modalities such as MRI, CT or ultrasound although it provides much higher resolution.

The current research in optical microscopy are conducted to overcome these limitations. Tissue scattering problem is partly solved by using optical clearing, adaptive optics and solving mathematically the light scattering problem in turbid tissue. Optical resolution limit set by the diffraction of light is now mostly overcome by using various techniques such as stimulated emission depletion microscopy (STED), photo activated localization microscopy (PALM), statistical optical reconstruction microscopy (STORM). The throughput of the optical microscopy especially for depth-resolved imaging has been increased by parallelizing the imaging process using such techniques as multifocal multiphoton microscopy (MMM), spinning disk confocal microscopy, structured light illumination microscopy and temporally focused widefield microscopy. Along with these exciting research directions in the field, this thesis contributed to improve the throughput of the optical imaging instruments by utilizing the structured light illumination and the temporally focused microscopy and expanded the frontiers of the biomedical applications.