A Short Review of Deep Tissue Imaging Techniques and Applications

J Song¹ and Z Dean²,³

¹ Undergraduate Student, University of Massachusetts at Amherst, MA, USA
² Post-Doctoral Fellow, San Francisco State University, CA, USA
³ Post-Doctoral Fellow, Center for Cellular Construction, CA, USA
Email: ziahdean@sfsu.edu

Abstract. Deep tissue imaging is a fundamental research method in the field of biological imaging. With an intent on obtaining more clear and detailed images deep within tissue, researchers have put much focus on developing deep tissue imaging techniques. In the past few decades, deep tissue imaging techniques have been improved in many aspects. In contrast to increasingly refined techniques, the application of these techniques seems to have not received equally attention. In this review, we will focus on the advanced applications of deep issue imaging that have emerged in recent years.

1. Introduction
In the field of biological imaging, deep issue imaging has always been an essential study tool in peering deep within biological structures and monitoring biological interactions. Deep tissue imaging is made possible with the cooperation of many fields including spectroscopy, advanced imaging, microscopy, optics, and mechanics. In this paper, we focus on three specific deep issue imaging techniques, 2-photon microscopy, adaptive optics microscopy and light sheet microscopy, discussing the benefits and limitations of these approaches. In particular, we will be exploring the biological applications of these techniques and determining whether there is a correspondence between technique and application.

2. Selected deep tissue imaging techniques

2.1. 2-Photon Microscopy
To better understand 2-Photon Microscopy, we need to introduce Confocal Microscopy first. Confocal Microscopy is an imaging technique that takes advantages of a spatial pinhole to eliminate the out-of-focus light signals, which can reduce the influence of unexpected fluorescence light and improve resolution [1]. 2-Photon Microscopy as illustrated in Figure 1, is a technique that is ameliorated from Confocal Microscopy. In traditional imaging techniques, such as Confocal Microscopy, fluorescence dyes are activated by the absorption of single photon. Relatively, in 2-Photon Microscopy, the process of fluorescence excitation is done by 2-photon absorption, which means that two photons arrive at a fluorescence molecule at the same time, usually a time scale within 0.5fs can be recognized as simultaneously, combining their energies to promote the molecule from a ground state to an excited state [2]. 2-Photon Microscopy has some features that make it a stronger tool than Confocal Microscopy for deep tissue imaging purposes. Firstly, 2-Photons of light are capable of penetrating deeper. The absorption wavelength of 2-Photon Microscopy is between about 700-1000nm, which is in the near-infrared range. Light in this range can penetrate deeper and reach more tissues in depth.
Second, 2-Photon Microscopy creates less phototoxicity. Because near-infrared light has lower energy level and lacks significant endogenous absorbers in most tissues, it causes less phototoxicity in sample tissues, which is crucial for longer time dependent imaging [2]. Third, localized excitation of fluorescence dyes. In 2-Photon Microscopy, the excitation of fluorescence dyes and emission of light signals are confined to the region near focus, whereas in Confocal Microscopy, the emission occurs along the whole laser path. The absence of out-of-focus excitation in 2-Photon Microscopy reduces the influence of unexpected signals and further increases the signal to noise ratio, and improving the resolution of deep tissue imaging [2].

![Figure 1](image1.png)

**Figure 1.** Illustration of a simplified 2-Photon Microscope with Laser transmitter, Scan Mirror, Detector, Dichroic, and Objective.

### 2.2 Adaptive Optics Microscopy

![Figure 2](image2.png)

**Figure 2.** A simplified schematic of an Adaptive Optics system with deformable mirror, wavefront sensor and computer analyser. (red lines indicate light path and blue lines indicate wiring path)

When a fluorescence dye is excited by a laser for fluorescent lamp, the dye emits light which is collected by an objective lens. This is the basic principle of observing in fluorescence microscopy. As the light passes through the biological tissue like cell membrane, aberrations can be introduced that distort the wavefronts, blurring the images. To solve this problem, adaptive optics is introduced into microscopy. Adaptive optics (AO) is a technique that used for improving the performance of optical systems by reducing the effect of incoming wavefront distortions by deforming a mirror in order to compensate for the distortion (Booth 2007). Adaptive optics was first used in telescopes, aiming at reducing the aberration caused by the refraction that occurs when light passes the atmosphere. This situation is similar to the aberrations caused by cellular structures in deep tissue imaging. Thus,
adaptive optics was introduced into microscopy, as an auxiliary to Confocal Microscopy or 2-Photon Microscopy. An Adaptive optics system shown in Figure 2, can be divided into three parts, sensing, computing, and correcting. Most prominent methods of sensing wavefront aberration are the Shack–Hartman wavefront sensor and interferometric sensors. These methods require a point-like reference source, such as the guide star used in astronomical systems, to produce a well-defined wavefront. After getting light signal from the fixed “guide star”, a computer will start calculating the aberration data and send it to deformable mirror (DM). Then the DM will deform into certain shapes to neutralize the aberration, recovering the original structure both in 2D and 3D. Adaptive Optics Microscopy is a great tool for enhancing image resolutions, reaching deeper imaging depths, and demonstrating precise biological structures in small scales within cells. Adaptive Optics has tremendous potential in observing within live biological tissues at the cellular level, this is advantageous for a wide range of biological research fields from neural science to developmental biology.

**Figure 3.** A simplified demonstration of a Light Sheet Microscope, here detection is performed by a camera, and the excitation is accomplished by a laser the illuminates a whole sheet, rather than single point.

2.3. **Light Sheet Microscopy**

Light Sheet Microscopy is a fluorescence microscopy technique with an intermediate-to-high optical resolution, and fast imaging rate. In contrast to other fluorescence microscopy techniques which excite fluorescence dyes point by point, Light Sheet Microscopy is capable of activating fluorescence by thin slices, so called “light sheet”. Light sheet microscopy combines two distinct optical paths, (as shown in Figure 3), one for fast wide-field detection and one for illumination with a thin sheet of light, perpendicular to the detection path. The light sheet is aligned with the focal plane of the detection path, and the waist of the sheet is positioned in the center of the field of view (M, M, and J 2014). During the construction of an image, each slice of specimen is excited once, as a result, the photo-toxicity and photo-bleaching is limited to a minimum. In addition, with Light Sheet Microscopy, cameras can record the whole activated sheet in each frame, making it super-fast to collect images. These features make Light Sheet Microscopy an alternative to confocal microscopy because of some superior features such as high-speed full-frame imaging, extremely low photo-toxicity, and multi-dimensional view of sample rotation, meeting the requirement of long time-lapse imaging (M, M, and J 2014). Such as the time-lapse like recording of embryo development a length biological process. Light Sheet Microscopy and 2-Photon Microscopy are very similar, both have low photo-toxicity and good at long-term imaging. But they still have differences and unique features. Because fluorescence in Light Sheet Microscopy is excited by planes and images are taken by cameras, so it can take much more frames in a period than 2-Photon Microscopy. On the other hand, 2-Photon Microscopy can reach smaller structures and provide higher resolution than Light Sheet Microscopy. So, Light Sheet Microscopy is powerful in extra-long time imaging with large scales, like filming the development process of embryo.

3. Applications

Although the technological advancement of these techniques have received a great amount of attention, here we review several applications of each technique in an effort to demonstrate the breadth of practical impact these techniques have made in our understanding of biological processes. Each technique consists of advantages and disadvantages based on a particular type of application. Thus, we
will be able to answer the critical question of whether each technique is broadly applicable or particularly suited for a precise biological application.

3.1. 2-Photon Microscopy

3.1.1 The study of cerebrovascular dynamics in ultrasound-induced blood-brain barrier.
According to Eunice Cho, Jelena Drazic et al, the blood-brain barrier (BBB) can be disrupted by applying ultrasonic and circulating microbubble contrast agent. In their experiment, a single-element, ring-shaped piezoelectric transducer was attached to male Wister rats’ skulls. Within the ring, that part of skull was replaced by a piece of transparent cover glass (as shown in Figure 4), making it possible to directly observe the Texas Red injected into brain vessels through 2-Photon Fluorescence Microscopy. Further, scientists can infer the ultra-sonic disruption rate of BBB by observing the amount and speed of leakage of Texas Red. Finally, they could come up with a conclusion that, by varying acoustic pressure, one can possibly control the opening of blood-brain barrier, causing different kinds of leakage and vessel size.

![Figure 4](image-url)

**Figure 4.** (A) A general structure of the experiment setup. (B) Replace a piece of rat skull with glass cover, ensuring the observation. (C) Real image of experiment setup. [3]

![Figure 5](image-url)

**Figure 5.** (A) Arrow marks a fast leakage. (B) Arrow marks a sustained leakage. (C) Arrow marks a slow leakage. [3]
In this experiment, 2-Photon Microscopy enables Cho and her colleagues to observe the brain vessels in Wister rats for a long period of time, monitoring the status of leakage of Texas Red, a kind of fluorescence dye. Besides, the high image contrast as well as high resolution of 2-Photon Microscopy provided extreme high quality deep tissue images[3](Figure 5).

3.1.2. Phosphorescence lifetime microscopy of retinal capillary plexus oxygenation in mice.

Another research was operated by Ikbal Sencan, Tatiana Esipova et al, who decided to study retinal capillary plexus oxygenation in mice. The impairment of the delivery and consumption of oxygen can be recognized as a pathophysiology symbol of retinal related diseases. However, in present, there is an absence of effective research tools of measuring oxygen concentration and oxygen transportation in retinal tissues. So, scientists designed this experiment to exam the possibility of using phosphorescence lifetime microscopy to address the absolute partial pressures of oxygen (pO2) in the retinal capillary plexus (results shown in Figure 6).

![Figure 6](image)

**Figure 6.** pO2 distribution in three layers of imaging for three depths. [4]

Because retina is sensitive to light, it is crucial to take photo-toxicity into consideration. The low photo-toxicity as well as low photo-bleaching of 2-Photon Microscopy makes it perfectly suitable for this experiment. After the performance of in vivo measurements of this designed system, Ikbal and their team can prove the ability of their setup to detect the change in retinal oxygenation as well as longitudinal gradients from arterioles to capillaries to venules (Sencan et al. 2018).

3.1.3. 2-photon time-lapse microscopy of BODIPY-cholesterol

Cholesterol is an important membrane component, but we do not know much about the transportation of cholesterol in cells. With previous studies, scientists have established a theory that both vesicular transport and non-vesicular transport can be benefit to sterol’s transportation from plasma membrane. The significant photo-bleaching of deep tissue imaging methods, however, restricts the possibilities to study sterol in depth or for a long analyze time. In Frederik Lund’s publishment, he introduced 2-Photon Microscopy into this research area. 2-Photon Microscopy has relatively low photo-bleaching and photo-toxicity, compared with other microscopy methods, for example, Confocal Microscopy. This feature makes 2-Photon Microscopy meet the requirement of this experiment perfectly. Lund found that BODIPY-cholesterol is surprisingly photostable under 2-Photon excitation, it can endure several hundred frames of photographing without significant photo-bleaching. Lund got a conclusion from long-term tracking of BODIPY-cholesterol that, the movement of sterol-containing vesicles for a
short time scale could reflect dynamic rearrangements of the cytoskeleton, while directed transport of sterol vesicles occurs likely along both, microtubules and actin filaments, and spatially varying anomalous diffusion could contribute to fine-tuning and local regulation of intracellular sterol transport. (Lund et al. 2012).

3.2. Adaptive Optics Microscopy

3.2.1. Fast high-resolution miniature microscopy for brain imaging in freely behaving mice.

A target of neuroscience’s study is to understand the principles underlying the neuronal information processed in freely behaving animals in different levels, like subcellular, cellular, circuit, or higher. Developments of microscopy have enabled the observation of brain activities and structural dynamics. However, there still is a challenge to achieve such goals when animals are in self-determined behaviours (Experiment set up shown in Figure 7). Weijian Zong and his team put up with a design and application of a fast high-resolution, miniaturized 2-Photon Microscopy together with Adaptive Optics that accomplishes the challenge of monitoring activities of single dendritic spines in freely behaving animals.

Figure 7. The imaging instrument attached to free behaving rat. [5]

Because the movement of mice is random and chaos, these unpredictable behaviors would largely blur the imaging results of real-time neural system. That is why adaptive optics was introduced into this experiment. With the ability of strongly reducing the influence of aberration and enhancing the resolution of deep tissue images, the unavoidable negative influence originated from animal behaviors could be restrain to a controllable level and get acceptable deep tissue images of single dendritic spines (Zong et al. 2017).

3.2.2. Deep-brain imaging at synaptic resolution over large volumes

Deep-brain high resolution imaging in vivo has always been a great challenge during the past decades. 2-Photon Microscopy is a narrowly approach to this problem, providing a minimally invasive method of imaging deeply buried brain structures. Although the near-infrared light that used in 2-Photon Microscopy has greater penetration depth, its imaging resolution as well as the field of view are limited by aberrations of membranes and lens. Here, a new research performed by Zhongyan Qin and other researchers took advantages of the deep penetration and high resolution of Adaptive Optics to enhance the performance of 2-Photon Microscopy, achieving the challenge of deep-brain imaging at synaptic resolution over large volumes. They developed a new deep tissue imaging system by attaching Adaptive Optics based on direct wavefront sensing to a 2-Photon Microscopy set, which enables recovery of aberration-limited resolution in deep-brain imaging.

After applying Adaptive Optics, the quality of deep-brain images is tremendously increased, both in accuracy and penetration depth (results shown in Figure 8) [6].
3.2.3 Super-resolution structured imaging in the living brain

To fully understand a neurobiological processes, it is essential to study them in vivo and within cell level, as cells act as important components of extended neural networks in the brain. To get clear images in such small scale, within cells, Super-resolution Microscopy is needed. Super-resolution Microscopy is a partial resolution to the problem of cell scale imaging. It can provide structural and functional insights that are not easily accessible with other conventional microscopy technologies. However, when apply Super-resolution Microscopy into vivo brain imaging, there are challenges in 3D imaging if a target tissue is constantly moving. So Raphael Turcotte, Yajie Liang and other researchers added Adaptive Optics to Super-resolution Microscopy system, in order to correct the optical aberrations in Super-resolution structured Illumination Microscopy (SIM) in vivo. They used both fish and mammal as experiment animals and successfully imaged the brains of live zebrafish larvae and mice, observing the dynamics of dendrites and dendritic spines at a extremely small nanoscale [7].

3.3 Light Sheet Microscopy

3.3.1. Quantitative imaging of cell dynamics in mouse embryos

Ryan Udan and his team has reported the first 24-hour time-lapse of post-implantation mouse embryo development images. In Ryan Udan’s experiment, he used Light-Sheet Microscopy to image cell dynamics in mouse embryos. Light-Sheet Microscopy system has several advantages that make it superior to other fluorescence microscopy methods in live and 3D microscopy. Light-Sheet Microscopy systems are valuable for studying embryonic development. In Light-Sheet Microscopy, the geometry of the light path in a form that requires the sample to be fixed in place as well as accessible from multiple sides, so that it can be rotated around the axis. In present, most used methods of preparing samples include hanging specimen from a pin or embedding it in the 1-2% agarose. These methods are specific to handle certain samples. But for post-implantation mouse embryo, it expands significantly in size and are very delicate and sensitive to mounting. To overcome the sample setting problem and to establish a robust strategy for a long-term imaging of E6.5-8.5 mouse embryos, they developed a method that uses hollow agarose cylinders specially designed to accommodate for embryonic growth and can provide boundaries to minimize tissue drift and enable imaging in multiple axis as well. After the experiment, Ryan successfully demonstrates that Light-Sheet imaging can
provide both quantitative data for tracking changes in morphogenesis and reveal new insights into mouse embryogenesis [8].

3.3.2. Reconstruction of Zebrafish Early Embryonic Development
Light-Sheet Microscopy is not only used in mammal study, Philipp Keller and his team uses Light Sheet Microscopy to reconstruction zebrafish’s early embryonic development. They got a goal to map all the cell behaviors during the embryogenesis. So, they developed a Light-Sheet Fluorescence Microscopy recording nuclei localization and movement in two kinds of zebrafish embryos, wild-type and the mutant, over the first 24-hour development. They built up a database consist of images filmed at 1.5 billion voxels per minute, which provides the possibility of reconstruction of “digital embryos,” in another word, that is a comprehensive database of cell positions, divisions, and migratory tracks (as shown in Figure 9).

![Figure 9](image)

**Figure 9.** (A) Embryo state tracking images of 289min, 368min, 599min and 841min. (B) Dividing cells are marked with red, daughter cells are marked with blue. [9]

They further put up with a model of germ layer formation and got a conclusion that the endoderm originated from one-third of total embryo’s cells at a time. To build their digital embryos, 55 million nucleus entries are provided as a resource. [9].

3.3.3. Imaging adult C. elegans
Observing biological activities in the form of living organisms can provide important insights into the dynamics of these activities. However, the complexity of multi-cellular organisms makes it a challenging environment to implement observations through fluorescence microscopy. Because of the out-of-focus signals, confocal or other widefield fluorescence microscopy suffers from low signal-to-background ratio (SBR), photo-toxicity and photo-bleaching of fluorescent probes. In Light-Sheet Microscopy, only the selected plane of the specimen is illuminated, minimizing the out-of-focus fluorescence lights and photobleaching, thereby enhancing SBR. J van Krugten together with his team present a Light-Sheet Microscope with a 1.0-NA detection objective as well as a fast sample-positioning stage that allows four degrees of freedom. By imaging the sensory cilia and neural system of living adult C. elegans, they demonstrated that this set of microscopes is perfectly suited for relatively fast imaging of large living specimen [10].
Figure 10. A) One slice of image obtained by LSM. B) A projection of 115 slices of images in the head region of C.elegan. [10]

4. Discussion

The amazing combination of fluorescence and optics provides us with a powerful tool in biophysics research, the fluorescence microscopy. In decades of improvement, fluorescence microscopy technology has evolved from simple Confocal Microscope to complex, more powerful microscopes, such as the 2-Photon Microscope, Adaptive Microscope and Light-Sheet Microscope.

As these advanced fluorescence microscopes are all based on same underlying techniques, fluorescence, and optics for instance, they naturally have commonalities. All three microscopes have a restricted imaging depth, because of the penetration limitation of excitation laser and outgoing fluorescence light. Besides that, the observation time is also constricted by the photo-bleaching phenomenon and fluorescence protein denaturation.

These advanced fluorescence microscopy systems are built to meet different experiment requirements, they all have irreplaceable features that make them different and unique from each other. In the demand for deeper penetrate depth and longer observation time, 2-Photon Microscope is developed from Confocal Microscope and has improvement in photo-damage and penetration. It uses non-linear infrared laser for excitation rather than ultraviolet laser, which is of less energy and larger wavelength, providing less photo-damage and deeper penetration than Confocal Microscope. As a result, 2-Photon Microscope is good at deep tissue imaging like brain structure observation introduced before.

The structure of Light-Sheet Microscope is far more different from original Confocal Microscope than 2-Photon Microscope. It extremely reduces the photo-toxicity and photo-bleaching, offering a possibility of ultra-long tissue observation but sacrifices imaging resolution. This feature makes it a perfect tool for observing embryo development.

Adaptive Optics is an assistant tool to provide resolution upgrade for Confocal Microscope and 2-Photon Microscope. By applying computer and deformable mirror, it can cancel the aberration caused by cell membrane and give us more precise and clearer deep tissue images.

AO can largely enhance the resolution of Confocal or 2-Photon Microscopy, but it cannot be applied to Light-Sheet Microscope. The recording part of LSM is supported by camera, a high-resolution camera is expensive and not easily accessible by most researchers. So, it will be interesting and useful to find out a way to improve the resolution of LSM, making it even a stronger tool for research.

In evaluating different applications of each technique, we can see that a large driving force for advancement of these techniques is the particular application they are set to accomplish. While new biological problems will necessitate further advancements of these deep tissue imaging techniques, the current technology has given way for solving a great deal of problems that were not possible before. It is therefore of utmost importance that we evaluate these applications to allow for the biological community to be able to determine how they might be able to apply it for their outstanding questions.

Reference

[1] Nwaneshiudu A, Kuschal C, Sakamoto F H, Rox Anderson R, Schwarzenberger K and Young R C 2012 Introduction to confocal microscopy J. Invest. Dermatol. 132 1–5

[2] Helmchen F and Denk W 2005 Deep tissue two-photon microscopy.pdf
[3] Cho E E, Drazic J, Ganguly M, Stefanovic B and Hynynen K 2011 Two-photon fluorescence microscopy study of cerebrovascular dynamics in ultrasound-induced blood-brain barrier opening J. Cereb. Blood Flow Metab. 31 1852–62

[4] Sencan I, Esipova T, Yaseen M, Fu B, Boas D, Vinogradov S, Shahidi M and Sakadzic S 2018 Two-photon phosphorescence lifetime microscopy of retinal capillary plexus oxygenation in mice J. Biomed. Opt. 23 1

[5] Zong W, Wu R, Li M, Hu Y, Li Y, Li J, Rong H, Wu H, Xu Y, Lu Y, Jia H, Fan M, Zhou Z, Zhang Y, Wang A, Chen L and Cheng H 2017 Fast high-resolution miniature two-photon microscopy for brain imaging in freely behaving mice Nat. Methods 14 713–9

[6] Qin Z, Chen C, He S, Wang Y, Tam K F, Ip N Y and Qu J Y 2020 Adaptive optics two-photon endomicroscopy enables deep-brain imaging at synaptic resolution over large volumes Sci. Adv. 6

[7] Turcotte R, Liang Y, Tanimoto M, Zhang Q, Li Z, Koyama M, Betzig E and Ji N 2019 Dynamic super-resolution structured illumination imaging in the living brain Proc. Natl. Acad. Sci. U. S. A. 116 9586–91

[8] Udan R S, Piazza V G, Hsu C W, Hadjantonakis A K and Dickinson M E 2014 Quantitative imaging of cell dynamics in mouse embryos using light-sheet microscopy Dev. 141 4406–14

[9] Keller P J, Schmidt A D, Wittbrodt J and Stelzer E H K 2008 Reconstruction of Zebrafish Early Embryonic Development by Scanned Light Sheet Microscopy Science (80-. ). 322 1065–9

[10] van Krugten J, Taris K K H and Peterman E J G 2021 Imaging adult C. elegans live using light-sheet microscopy J. Microsc. 281 214–23

Acknowledgments
The authors would like to thank the NSF-Center for Cellular Construction (Z.D) and the Genentech Foundation (Z.D.) for their support. Disclaimer: Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of Genentech Foundation or National Science Foundation.