Functional Studies in Living Animals Using Multiphoton Microscopy

Kenneth William Dunn and Timothy Alan Sutton

Abstract

In vivo microscopy is a powerful method for studying fundamental issues of physiology and pathophysiology. The recent development of multiphoton fluorescence microscopy has extended the reach of in vivo microscopy, supporting high-resolution imaging deep into the tissues and organs of living animals. As compared with other in vivo imaging techniques, multiphoton microscopy is uniquely capable of providing a window into cellular and subcellular processes in the context of the intact, functioning animal. In addition, the ability to collect multiple colors of fluorescence from the same sample makes in vivo microscopy uniquely capable of characterizing up to three parameters from the same volume, supporting powerful correlative analyses. Since its invention in 1990, multiphoton microscopy has been increasingly applied to numerous areas of medical investigation, providing invaluable insights into cell physiology and pathology. However, researchers have only begun to realize the true potential of this powerful technology as it has proliferated beyond the laboratories of a relatively few pioneers. In this article we present an overview of the advantages and limitations of multiphoton microscopy as applied to in vivo imaging. We also review specific examples of the application of in vivo multiphoton microscopy to studies of physiology and pathology in a variety of organs including the brain, skin, skeletal muscle, tumors, immune cells, and visceral organs.

Key Words: fluorescence microscopy; in vivo imaging; in vivo microscopy; multiphoton microscopy; two-photon microscopy

Introduction

Most biomedical research involves studies of cultured cells. However, scientists are increasingly aware that these simple, tractable experimental systems necessarily lack biological relevance for many questions. In the process of immortalization, cultured cells

lose many of their in vivo characteristics. In addition, it is impossible to reproduce the complex cellular and systemic influences that determine cellular function in vivo. For these reasons, researchers increasingly turn to studies of laboratory animals.

As described elsewhere in this issue, in vivo imaging techniques such as magnetic resonance imaging (MRI), positron emission tomography (PET), and computed tomography (CT), all typically associated with clinical applications, have been adapted for small animal imaging and, along with whole body optical techniques, have been used to address fundamental issues of cell and organ physiology. Pharmaceutical companies are also increasingly turning to these technologies to evaluate the pharmacokinetics and physiological effects of drugs under development (Massoud and Gambhir 2003; Rudin and Weissleder 2003; Sinskey et al. 2004a,b,c). When combined with specially engineered imaging probes, these techniques are capable of characterizing physiological processes ("functional imaging") and the tissue distribution of drugs and their targets ("molecular imaging").

However, the limited sensitivity and spatial resolution of these techniques limit their ability to resolve critical events that occur at cellular and subcellular levels. For example, a PET signal derived from a 1-mm diameter volume may reflect the behaviors of perhaps half a million cells of various types. This level of resolution is insufficient to define and explain drug effects that are specific to particular types of target cells. If we take the example of a drug with renal toxicity, the mechanism of toxicity will be different depending on whether the drug accumulates in cells of the vascular system, glomerulus, proximal tubule, or distal tubule. A PET image will not be able to resolve this distribution. Indeed, to the degree that a drug accumulates in just one of these cell types, its renal accumulation may not be detectable at all. Similarly, MRI is frequently applied to studies of vascular function but cannot characterize the variations in vascular permeability that occur at the level of single cells (Sutton et al. 2003), nor the variations in blood flow that occur at the level of single capillaries (Sutton et al. 2003; Yamamoto et al. 2002). The limited resolution of conventional forms of in vivo imaging renders them incapable of characterizing many of the important biological processes that are mediated at the scale of cells and organelles. In the context of preclinical drug development, these techniques are incapable of resolving the localized distribution and effects of drugs that are frequently critical to understanding their physiological actions and toxic side effects.

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In vivo fluorescence microscopy is an effective way to address many of the compromises of studies of cultured cells on one hand and studies of animals on the other. While the volume of tissue that can be imaged by in vivo fluorescence microscopy is not as large as that obtained by conventional in vivo imaging methods, the capability of in vivo fluorescence microscopy to provide images in living animals at subcellular resolution in 1 second or less is a unique advantage. Furthermore, the simultaneous imaging of multiple fluorophores makes fluorescence microscopy uniquely capable of multiparameter imaging, supporting powerful correlative analyses. Additionally, similar to the conventional forms of in vivo imaging, in vivo fluorescence microscopy can be used to image an animal longitudinally (i.e., before and after an intervention), which enables the collection of within-animal control data and reduces the number of animals needed for a given analysis.

Scientists have performed in vivo microscopy for nearly 200 years (Cohnheim 1889; Ghiron 1912; Steinhausen and Tanner 1976; Wagner 1839), but its capabilities have significantly advanced with the recent development of multiphoton microscopy (Denk et al. 1990; and as reviewed in Denk and Svoboda 1997; Dunn and Young 2006; Zipfel et al. 2003b). This technique, which depends on the simultaneous absorption of two infrared photons by a fluorophore, resulting in spatially localized fluorescence excitation, is capable of collecting high-resolution (0.4 microns) fluorescence images hundreds of microns into tissues (Centonze and White 1998; Theer et al. 2003). In addition to providing better penetration and resolution than traditional methods of microscopy, the use of infrared light also makes multiphoton microscopy significantly less toxic to mammalian cells (Squirrell et al. 1999).

In providing the capability for collecting fluorescence images of living animals at a subcellular resolution, in vivo multiphoton microscopy has revolutionized the way that biomedical researchers can study physiology, pathophysiology, and drug action. While some cases require minor surgery to gain access (for example, to internal organs), in many cases it is possible to conduct the imaging from outside the body, with little or no surgical manipulation. In the next section we present a review of applications of this technology in biomedical research.

Applications of in Vivo Multiphoton Microscopy

Brain Function

Understanding the function of the brain is possible only in the context of the intact organ, including the vascular system and the network of intercellular connections. Thus it is not surprising that neuroscientists have been some of the earliest adopters of multiphoton microscopy for in vivo imaging (reviewed in Svoboda and Yasuda 2006). Indeed, Winfried Denk, one of the inventors of multiphoton micros-

copy, participated in the first example of in vivo multiphoton microscopy (Svoboda et al. 1997). In this study, investigators labeled layer 2/3 pyramidal neurons with a calcium-sensitive fluorescent probe, and conducted multiphoton microscopy through a cover glass attached to the brain of an anesthetized rat, following a craniotomy. These studies elegantly demonstrated a close correlation between neuronal calcium transients and sodium-dependent action potentials, as detected with intracellular voltage measurements. This group described a similar approach in a second publication (Helmchen et al. 1999), demonstrating the presence of dendritic calcium transients in deep-layer pyramidal neurons.

More recently, in vivo multiphoton microscopy has been an effective way to identify distinct calcium dynamics in astroglia and neuronal networks (Nimmerjahn et al. 2004) as well as in astrocytes that correlate with neural activity, suggesting neuron-glia communication (Hirase et al. 2004b; Wang et al. 2006). Stosiek and colleagues (2003) developed a method for labeling large numbers of neurons in vivo and used it to characterize entire networks of neurons. This study demonstrated that whisker stimulation resulted in exciting a subset of layer 2/3 neurons in mice.

The injection of bulk fluorescent tracers into the vasculature facilitates the monitoring of blood flow, which is critical to brain function. The dynamic regulation of blood flow was suggested by the studies of Kleinfeld and colleagues (1998), who found cortical blood flow to be highly variable, even reversing in direction in some capillaries, and sensitive to stimulation. Chaigneau and colleagues (2003) obtained similar results in studies of the olfactory bulb, where capillary blood flow varied according to odor stimulation, in fact mapping closely to regions of synaptic activation. Takano and colleagues (2006) elegantly demonstrated the potential role of astrocytes in this process. These investigators found that the release of calcium in astrocytes, via photolysis of caged calcium, decreased both the diameter and blood flow in the associated blood vessels, suggesting that astrocytes may control local blood flow by mediating vasodilation.

In addition to providing better penetration into tissues, multiphoton microscopy can be much less injurious to biological tissues than other forms of fluorescence microscopy (Squirrell et al. 1999). Accordingly, multiphoton microscopy is useful for repeatedly collecting images from the same living tissue over periods of hours and even for supporting longitudinal studies of animals over a period of days to months. This capability has been widely utilized to analyze the dynamics of neurons, typically labeled by transgenic expression of green fluorescent protein (GFP), over multiple time scales.

A reorganization of neural networks, involving a net decline in the number of synapses, generally accompanies neural development. In vivo multiphoton microscopy studies have associated this decline with age-dependent differences in dendritic spine dynamics. Grutzendler and colleagues (2002) observed that dendritic spines are highly

dynamic in layer 5 pyramidal neurons of the visual cortex of young mice, with the majority of these dynamic events resulting in spine loss. In contrast, extended longitudinal studies of adult mice (over periods of up to 4 months) demonstrated that the great majority of spines in adult mice are stable, with a half-life of over 13 months. These studies suggest that dendritic spine turnover underlies the remodeling in the visual cortex during development. Holtmaat and colleagues (2005) obtained similar results in studies of the somatosensory cortex of mice, although these authors also identified significant spine turnover even in adult mice, suggesting that neurons of the somatosensory cortex may retain their plasticity longer than those of the visual cortex.

Protracted studies, involving repeated imaging of the same tissues over a period of months, have demonstrated that while neuronal branching patterns are relatively stable in adult animals, dendritic growth and remodeling continue, involving a process of extension and retraction of existing branches that occurs on a time scale of days (Lee et al. 2006). Similarly, axonal remodeling continues. Stettler and colleagues (2006) imaged axons in the primary visual cortex over a period of several weeks and found that new branches appeared and disappeared each week, suggesting an ongoing process of synaptic remodeling. De Paola and colleagues (2006) presented similar results, and also determined that axonal remodeling varied with cell type.

A number of studies have evaluated the role of experience in neural remodeling. Whereas, as described above, brain development is normally associated with a net decline in the number of synapses, Zuo and colleagues (2005) observed that sensory deprivation, accomplished by trimming the whiskers of mice, reduced the rate of spine elimination, thus blocking the normal decline in dendritic spine density in mouse barrel cortex neurons during development. Lendvai and colleagues (2000) demonstrated that dendritic spines are highly dynamic, at a temporal scale of minutes, in rat barrel cortex. However, spine motility was significantly reduced in sensory-deprived rats, suggesting that sensory experience may influence dendritic plasticity and thus neural circuit reorganization. There is also evidence that sensory deprivation increases turnover of dendritic spines in mouse barrel cortex (Trachtenberg et al. 2002). In contrast to these studies, Mizrahi and Katz (2003) demonstrated that apical dendrites of olfactory bulb macrophage/primary T (M/T) cells are highly stable over periods of days to weeks, even under a variety of different olfactory conditions, and suggested that stability may be important for maintaining a structural scaffold of the neural circuitry.

Multiple studies investigating the consequences of different kinds of injury to the brain have applied in vivo multiphoton microscopy. Research indicates that experimentally induced epileptic foci increase local blood blow (Hirase et al. 2004a), but that even extended protracted neocortical seizures have undetectable effects on dendritic morphology (Rensing et al. 2005). Two simultaneously published studies (Davalos et al. 2005; Nimmerjahn et al.

2005) demonstrated the role of microglia in immune surveillance in the brain, noting that microglia, while highly active under normal conditions, rapidly deploy to sites of injury. Davalos and colleagues (2005) also demonstrated that adenosine triphosphate (ATP) mediates this migration, and found that the injury-induced microglial migration could be reproduced by local addition of ATP, and blocked in animals treated locally with an ATPase. A similar study conducted by Kim and Dustin (2006) found that whereas injury outside the blood-brain barrier resulted in leukocyte infiltration, injury inside the blood-brain barrier did not, unless the vasculature itself was damaged. Instead, microglia and astrocytes cooperatively mounted an immune response inside the blood-brain barrier.

An especially fruitful application of in vivo multiphoton microscopy to neural research has been in the study of Alzheimer's disease, pioneered by Bradley Hyman (2001). Combining transgenic models of Alzheimer's disease with novel methods for fluorescent labeling of amyloid plaques, researchers have been able to exploit the ability of in vivo microscopy to evaluate functional and morphological consequences of amyloid deposition (see review in Skoch et al. 2005).

Using a transgenic mouse model that expresses the human amyloid precursor protein, Kimchi and colleagues (2001) labeled amyloid deposits with thioflavine S and evaluated their effect on the vasculature of the brain, labeled with fluorescent dextran. These studies demonstrated that amyloid angiopathy initiates adjacent to branch points in the vasculature, with vessel dilation associated with amyloid deposition. Longitudinal studies of individual animals over periods of up to 5 months demonstrated that amyloid plaques, once formed, are remarkably stable, suggesting that growth is regulated by a feedback mechanism (Christie et al. 2001).

Tsai and colleagues (2004) used multicolor multiphoton microscopy of mice whose neurons were labeled with either 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) or yellow fluorescent protein (YFP) to demonstrate that amyloid deposits have toxic effects on nearby dendrites and axons. In a similar study Spires and colleagues (2005) evaluated the effect of amyloid plaques, labeled via intravenous injection of methoxy-X04 (Klunk et al. 2002), on neurons expressing GFP. Extended imaging over periods of weeks demonstrated that amyloid plaques disrupted neurites and reduced the density of dendritic spines, even in dendrites not in contact with plaques.

Longitudinal studies have been used to evaluate the effect of immunotherapy on amyloid plaques. Using a transgenic mouse expressing YFP in neurons, Brendza and colleagues (2005) demonstrated that treatment of mice with an antiamyloid antibody reduced neurite dystrophy over a period of 3 days. A similar study by Prada and colleagues (2007) verified that antibody treatment decreased the size of arterial plaques, but found that chronic administration was necessary for a robust, lasting effect.

Skin Structure and Function

Easy accessibility to the skin makes it a logical target for in vivo imaging by multiphoton microscopy. Consequently, many of the earliest applications of multiphoton microscopy to in vivo imaging focused on studies of skin structure and physiology (Masters et al. 1997, 1998). Autofluorescence from endogenous compounds in the skin, including nicotinamide adenine dinucleotide phosphate (NADPH) and elastin, coupled with second harmonic generation from collagen fibers allows the examination of skin structure by multiphoton microscopy without exogenously administered fluorescent probes (Chen et al. 2006; Laiho et al. 2005; Masters et al. 1997). On the other hand, a potential drawback of utilizing multiphoton microscopy in the examination of living skin is that absorption of single-photon infrared light by melanin granules present at the dermal-epidermal junction can cause dermal-epidermal cavitation and injury to the skin. However, refinements in the delivery of the excitation laser pulses have been demonstrated to mitigate this potential injury (Masters et al. 2004). Multiphoton microscopy is therefore well suited to provide a noninvasive "optical biopsy" of the skin to examine alterations in skin structure and function.

Despite the ease of accessibility to the skin, most published studies employing multiphoton imaging have utilized ex vivo skin preparations, skin tissue models, or fixed biopsy specimens. Included among the many research applications in this setting are examinations of skin injury and wound healing (Hanson and Clegg 2005; Torkian et al. 2004; Werrlein and Madren-Whalley 2003), the biological response and efficacy of needle-free delivery systems for vaccines, genetic material, and pharmaceuticals (Mulholland et al. 2004, 2006; Raju et al. 2006; Stracke et al. 2006), and the transdermal diffusion of pharmaceuticals (Sun et al. 2004; Yu et al. 2002, 2003a,b). But these applications are just as well suited for true in vivo imaging of the skin by multiphoton microscopy. Indeed, researchers have applied multiphoton microscopy noninvasively in mice to examine wound healing (Navarro et al. 2004) and cell migration in the skin (Flesken-Nikitin et al. 2005). Furthermore, the potential utility of in vivo examination of the skin by multiphoton microscopy has inspired commercial development of a device for the diagnosis and assessment of dermatological conditions in the clinical setting (Konig and Riemann 2003). The real-time application of this technology may prove to be an important adjunct to the screening, diagnosis, and therapy of a variety of dermatological conditions such as skin cancer (Lin et al. 2006).

Skeletal Muscle Structure and Function

The exposure of skeletal muscle through a simple incision in the skin provides excellent accessibility for in vivo imaging by multiphoton microscopy. Several studies have utilized a preparation exposing the tibialis anterior muscle in mice. This preparation has been effective for examining skeletal muscle morphology and mitochondrial metabolic function (Rothstein et al. 2005), localizing Ca^{2+} uptake transients during contraction (Rudolf et al. 2004), and studying the shuttling of nuclear factor of activated T cells (NFAT) in skeletal muscle (Tothova et al. 2006). The latter two studies employed transient transfection of the tibialis anterior muscle to introduce fluorescent probes into the muscle for examination of cellular processes by multiphoton microscopy. Stockholm and colleagues (2005) transfected the same muscle with fluorescent calpain sensors and combined this with in vivo multiphoton microscopy and fluorescence resonance energy transfer (FRET) imaging to examine the kinetics of calpain activation at the cellular level in living mouse muscle.

Tumor Biology

The study of tumor biology is especially well suited to in vivo microscopy. Tumors can be implanted in subcutaneous locations that are easily accessible for microscopy after an incision in the overlying skin. Using fluorescent probes, it is easy to label both tumor cells and the cells that interact with them. Whereas early studies using wide-field microscopy were limited to relatively thin samples (e.g., Wood 1958), multiphoton microscopy has enabled imaging deep into tumors, supporting evaluations of angiogenesis, perfusion, drug delivery, and the behaviors of cancer cells and their interactions with immune cells (reviewed in Brown et al. 2001; Condeelis and Segall 2003).

The laboratory of John Condeelis has made extensive use of in vivo microscopy, first using confocal microscopy (Farina et al. 1998; Wyckoff et al. 2000) and later using multiphoton microscopy (Wang et al. 2002; Wyckoff et al. 2004, 2007; Xue et al. 2006), to gain seminal insights into the behavior of cancer cells during metastasis. In each of these studies, microscopic observation of individual cells has been uniquely capable of revealing the cellular processes underlying the entry of metastatic tumor cells into the vasculature. Wang and colleagues (2002) determined that metastatic tumor cells are characterized by more directed motility than nonmetastatic cells, and that this motility frequently involves migration along collagen fibers and blood vessels. Wyckoff and colleagues (2004) determined that metastatic cell motility and entry into the vasculature appears to be based on directional movement of tumor cells toward growth factors. Wyckoff and colleagues (2004, 2007) demonstrated that migration of tumor cells into the vasculature is facilitated by interactions with macrophages.

In vivo multiphoton microscopy has provided crucial insights into the dynamic in vivo interactions between immune cells and tumor cells. Mrass and colleagues (2006) observed that tumor-infiltrating lymphocytes migrate randomly within subcutaneous tumors in mice, but form long-lasting contacts with antigen-containing tumor cells. These lymphocytes were also observed to physically interact with

macrophages, suggesting the possibility of antigen cross-presentation between lymphocytes and macrophages. Bois-annas and colleagues (2007) extensively characterized the interactions between cytotoxic T lymphocytes and antigen-bearing tumor cells in subcutaneous tumors of mice. Cyto-toxic T lymphocytes migrate within tumors until they encounter living, antigen-expressing tumor cells, at which point they arrest in contact with the tumor cell.

Immune Cell Biology

In vivo microscopy has been used since the 19th century to study leukocyte behavior in experimental animals (Cohnheim 1889; Wagner 1839). More recently, studies conducted over the last 30 years have resulted in a model of immune cell trafficking based on selective adhesion and migration into target tissues (reviewed in Mempel et al. 2004). Multiphoton microscopy has extended the reach of these studies, permitting analyses of immune cell trafficking and interactions deep within tissues, particularly lymph nodes (reviewed in Cahalan and Parker 2005; Germain et al. 2005).

While most studies of immune cell trafficking have involved analyses of explanted lymph nodes, a number have used intact, living animals in which lymph nodes are surgically exposed. The first use of multiphoton microscopy to analyze immune cell trafficking in lymph nodes in living animals was presented by Miller and colleagues (2003), whose analysis of 3-dimensional T-cell migration in mouse lymph nodes suggested a stochastic rather than directed search process. Mempel and colleagues (2004) used in vivo multiphoton microscopy to identify three sequential stages of T-cell behaviors after entering mouse lymph nodes, beginning with multiple, brief encounters with dendritic cells, followed by formation of persistent contacts with dendritic cells, culminating in a final stage of rapid motility and proliferation. Similar studies by Shakhar and colleagues (2005) likewise detected extended contacts between dendritic cells and newly arrived T cells, which are seen as a critical determinant of T-cell activation in vivo. In contrast to T cells, natural killer cells showed limited motility in the lymph nodes of mice, and interacted with dendritic cells even in the absence of an immune response (Bajenoff et al. 2006).

Two recent studies have used in vivo multiphoton microscopy to elucidate the effects of regulatory T cells, which modulate the immune response, on T-cell behavior in lymph nodes. Mempel and colleagues (2006) found that the presence of regulatory T cells reduces the killing of antigen-presenting target cells by cytotoxic T lymphocytes. Studies by Tadokoro and colleagues (2006) suggest that reduced killing may result from the effect of regulatory T cells on interactions between T cells and dendritic cells. In vivo multiphoton microscopy of mouse lymph nodes demonstrated that the presence of regulatory T cells increased the motility of cytotoxic T cells and shortened the period of contact between T cells and antigen-containing dendritic

cells. Consequently, cytotoxic T cells have diminished opportunity to contact and kill targeted cells.

Most in vivo studies of immune cell behavior have involved analyses of cellular behaviors and interactions in lymph nodes. However, Cavanagh and colleagues (2005) have conducted in vivo studies of bone marrow, demonstrating that dendritic cells home to bone marrow cavities, where they form antigen-dependent contacts with central memory T cells.

Visceral Organ Structure and Function

While multiphoton microscopy has been utilized to examine organ structure and physiology in a variety of visceral organs ex vivo, the vast majority of in vivo imaging of visceral organs by multiphoton microscopy has been performed in studies of the kidney. Progress in applying this technology to imaging the kidney is in part due to (1) the relative ease of creating a surgical window to obtain access for presentation of the objective lens to the kidney and (2) the ability to largely isolate the kidney from excessive motion artifact. Barriers in getting the objective lens to deep visceral organs like the pancreas or in overcoming motion artifact in organs like the lung and heart have impeded rapid advances in the application of multiphoton microscopy for in vivo imaging of those organs as compared to the kidney. Nevertheless, these barriers are not insurmountable: progress in the design of objectives, fiber-optical devices, and techniques for synchronizing image collection with biological functions will expand the application of multiphoton microscopy for in vivo imaging.

The following sections focus on studies that have used multiphoton microscopy for the in vivo imaging of visceral organs. However, in the case of visceral organs for which such studies are sparse, we cite selected studies that have utilized multiphoton microscopy for imaging ex vivo organ preparations, to give an appreciation of the potential utility of multiphoton microscopy.

Kidney

Studies by Dunn and colleagues (2002) and later by Kang and colleagues (2006) have demonstrated the broad application of multiphoton microscopy for imaging kidney morphology and physiology in living animals. Furthermore, the development of technology to engineer and express fluorescent protein chimeras in animals has dramatically increased the potential for the application of in vivo multiphoton microscopy to evaluate the function and responses of particular proteins in the kidney. This application has been exploited in transgenic animals, which stably express fluorescent protein chimeras, and in the use of micropuncture techniques to introduce vectors containing constructs of fluorescent protein chimeras for transient expression (Tanner et al. 2005).

More specific applications of multiphoton micros-

copy—to examine the dynamic flow in the juxtaglomerular interstitium (Rosivall et al. 2006), to quantify the relative rates of glomerular filtration and tubular reabsorption in a single nephron (Yu et al. 2005, 2007), and to analyze the transport of organic anions (Tanner et al. 2004)—have provided key insights into the underlying physiology of the kidney. In these studies, in vivo multiphoton microscopy provided cellular and subcellular resolution in a dynamic setting that was critical to understanding the underlying process at a level of analysis that could not be achieved by other current imaging methods.

In vivo imaging by multiphoton microscopy has also facilitated progress toward understanding the pathophysiology of kidney injury in various animal models. Ischemic and septic models of acute kidney injury, with the use of in vivo multiphoton microscopy, have enabled fundamental insights into alterations of tubular function (Gupta et al. 2007), cellular apoptosis and necrosis (Kelly et al. 2003), trafficking of leukocytes and mesenchymal stem cells (Gupta et al. 2007; Kelly et al. 2004; Sutton et al. 2005; Togel et al. 2005), tubular cell uptake of endotoxin (El-Achkar et al. 2006), and microvascular blood flow and permeability (Gupta et al. 2007; Sutton et al. 2003, 2005). Multiphoton microscopy has also been exploited to provide important contributions to understanding folate uptake and trafficking in the kidney (Sandoval et al. 2004), as well as cellular and tissue responses to *Escherichia coli* urinary tract infection in living animals (Mansson et al. 2007). A recent study challenged the current understanding of glomerular filtration and tubular uptake of albumin by utilizing in vivo multiphoton microscopy to reveal a 50-fold greater value for the glomerular sieving coefficient of albumin than what has been accepted based on more invasive studies (Russo et al. 2007).

Other Visceral Organs

Like the kidney, the liver is readily accessible for in vivo imaging. However, given the liver's subdiaphragmatic location, isolation from motion artifact caused by respiration requires particular attention. Liu and colleagues (2007) utilized multiphoton microscopy to monitor hepatocyte uptake and biliary excretion of the organic anion 6-carboxyfluorescein diacetate, demonstrating the power of this technique to examine in vivo hepatobiliary physiology and metabolism. Multiphoton microscopy has also been applied to ex vivo preparations of liver to examine fibrosis following chemical injury (Lee et al. 2004) and to demonstrate the importance of myosin II and Gal-GalNAc lectin on the pathogenicity of *Entamoeba histolytica* during hepatic abscess formation (Coudrier et al. 2005). Additionally, multiphoton microscopy coupled with third harmonic generation has been effective in examinations of lipid metabolism in liver sections isolated from regenerating livers after partial hepatectomy (Debarre et al. 2006).

Using simple surgical techniques, the intestine is also easily accessible for in vivo imaging. However, many of the biological processes of interest in the intestine take place on the luminal side of the intestine and thus are beyond the working distance of multiphoton microscopy when an approach is taken from the serosal side of the intact intestine. Watson and coworkers (2005) bypassed this obstacle by opening a tiny segment of the small intestine in order to present the mucosal side of the intestine to the objective lens. They used this preparation to study the mechanisms involved in epithelial shedding and permeability barrier maintenance during epithelial cell shedding.

The pancreas, like the kidney, has a retroperitoneal location. However, unlike the kidney, it is difficult to create a retroperitoneal approach (or an abdominal approach) to the pancreas for imaging. Consequently, the application of multiphoton microscopy to examine dynamic processes in the pancreas has generally been limited to ex vivo preparations of exocrine pancreatic acini and islets. Nonetheless, researchers have used multiphoton microscopy to make important contributions toward understanding the physiology of insulin secretion by pancreatic islets (Bennett et al. 1996; Fukui et al. 2005; Hatakeyama et al. 2006; Kasai et al. 2005; Miura et al. 2006; Piston et al. 1999; Takahashi et al. 2002, 2004) and exocytosis by pancreatic acini (Dolman et al. 2005; Nemoto et al. 2001). Bertera and colleagues (2003) have developed an interesting approach to this problem by transplanting islet cells under the kidney capsule to facilitate in vivo imaging by multiphoton microscopy.

As mentioned above, the normal movements of the heart and lungs in a living animal have been an impediment to the application of multiphoton microscopy as a tool for the in vivo imaging of these organs. But the procedure has been a useful tool for examining mitochondrial function, physiological coupling, and Ca^{2+} signaling transients of cardiomyocytes in nonbeating, Langendorff-perfused hearts (Matsumoto-Ida et al. 2006; Rubart et al. 2003a,b). In ex vivo preparations of lung, multiphoton microscopy has also provided key insights into Ca^{2+} signaling of bronchial smooth muscle cells (Bai et al. 2007; Bergner et al. 2006), matrix structure (Debarre et al. 2006), matrix remodeling during fibrosis (Pena et al. 2007), and metastatic tumor cell extravasation (Voura et al. 2004).

Limitations to in Vivo Multiphoton Microscopy

As described above, in vivo multiphoton microscopy has made substantial contributions to our understanding of multiple physiological and disease processes. However, the technology of this research approach is still relatively immature and several technical challenges hamper the realization of its full potential.

Introduction of Fluorescent Probes into Cells in Animals

The first challenge for in vivo fluorescence microscopy concerns the need to generate contrast through fluorescence.

While investigators have made productive use of the fluorescence of endogenous proteins, such as NAD(P)H (Masters et al. 1998; Piston et al. 1995; Rothstein et al. 2005; Zipfel et al. 2003a; Zoumi et al. 2002) and elastin (Konig and Riemann 2003; Zoumi et al. 2004), the utility of endogenous fluorescence is limited by the small number of molecules with significant fluorescence. Thus in vivo fluorescence microscopy generally requires fluorescent labeling of specific cells or compartments. In some cases, for example labeling the blood volume with fluorescent dextran, this can be accomplished by simple intravenous injection of probes (e.g., Brown et al. 2001; Dunn et al. 2002; Kleinfeld et al. 1998). Intravenously injected Hoechst dye 33342 efficiently labels cells throughout the body, and rhodamine 6G labels circulating leukocytes and endothelial cells (Dunn et al. 2002). The direct injection of probes into individual cells is a laborious process best suited to small numbers of cells, but it has nonetheless been useful for measuring calcium dynamics in neurons (Helmchen et al. 1999; Svoboda et al. 1997). Local administration of membrane-permeant acetoxymethyl esters enables the labeling of a larger number of cells and has also been used to introduce calcium-sensitive probes into neurons (Garaschuk et al. 2006; Hirase et al. 2004b; Nimmerjahn et al. 2004; Stosiek et al. 2003). However, the instability of acetoxymethyl esters in the serum after intravenous injection (Jobsis et al. 2007) limits their in vivo utility.

In general, the specific labeling of cells in vivo with exogenous probes is challenging. One solution to the problem is to use transgenic animals that express fluorescent proteins in specific types of cells (e.g., Brendza et al. 2005; Nimmerjahn et al. 2004; Wyckoff et al. 2004), or to study animals transplanted with cells that express fluorescent proteins (e.g., Mempel et al. 2006). Viral infection (Dittgen et al. 2004; Tanner et al. 2005) and in vivo transfection are also effective methods of accomplishing the expression of fluorescent proteins in experimental animals, although these techniques require special methods to ensure the stability and efficient expression of the gene. The potential of fluorescent protein technology in in vivo microscopy remains to be fully realized, as new methods for introducing and expressing genes are devised, and new fluorescent indicators are developed and expressed in animals.

While we have focused in this review on multiphoton fluorescence microscopy, we note that alternative nonlinear processes are also effective for generating contrast in in vivo microscopy, including second harmonic imaging (Brown et al. 2003; Zipfel et al. 2003a; Zoumi et al. 2002) and coherent anti-Stokes Raman scattering (CARS) microscopy (Evans et al. 2005). Significantly, both of these techniques generate contrast using signals from endogenous molecules, without the need for targeted probes. However, much like the use of endogenous autofluorescence, these techniques are limited by the number of structures that are capable of generating sufficient signal. For example, collagen provides one of the only molecules whose structures are sufficiently ordered for second harmonic imaging, and the spatial heterogeneity and Raman response of lipids make them much easier to image than proteins or nucleic acids (Evans et al. 2005).

Depth of Imaging by Multiphoton Microscopy

Under the best circumstances, the working distance of the microscope objective limits the depth to which images may be obtained by multiphoton microscopy. However, despite the fact that multiphoton microscopy is better suited to deep-tissue imaging than any other form of optical microscopy, signals nonetheless attenuate with depth. Thus, the effective reach of multiphoton microscopy is typically no more than 150 microns in the living kidney (our unpublished observations), and no more than 1,000 microns in the case of brain tissue (Theer et al. 2003).

Some of the signal lost at depth results from absorbence and scattering of fluorescence emissions. Scattering is particularly significant in the imaging of biological tissues (Cheong et al. 1990). For instance, calculations indicate that the light-scattering properties of the brain result in the scattering of nearly all emissions arising from a point 100 to 200 microns into brain tissue (Helmchen and Denk 2005). Under these conditions the objective collects light from what is essentially a diffusely glowing surface of the tissue. The effects of scattering are somewhat mitigated by the use of large-area detectors, in some cases placed on either side of the sample, to collect fluorescence emissions in multiphoton microscopy. The efficient collection of scattered fluorescence is one of the major advantages of multiphoton microscopy.

The attenuation of signal with depth also results from scattering and absorbence of the laser illumination. Although this attenuation is reduced by the use of near-infrared light that falls in the range of the "optical window" of biological tissue, in which both absorbence and scattering are minimized, the effect of illumination attenuation is magnified by the fact that fluorescence is quadratically related to illumination; thus a halving of illumination reduces fluorescence fourfold. These losses can be reclaimed by increasing laser power with depth. However, the effect of the increased illumination on the surface of the sample limits the efficacy of this solution. It is not useful to increase illumination to the point that it either induces damage at the surface of tissue or excites sufficient fluorescence at the surface to obscure fluorescence at the focal point (Theer and Denk 2006).

Finally, the quadratic dependence of fluorescence excitation on illumination makes multiphoton microscopy especially sensitive to factors that distort the shape of the focal spot. A primary factor here is spherical aberration. Since the refractive index of biological tissue generally differs from that of the immersion medium of the objective lens, the peripheral and paraxial rays of illumination focus to differ-

ent points in the sample. This effect, which increases with depth, broadens the focal spot, thus reducing fluorescence excitation. An adaptive optics system can reduce the effects of this distortion (Booth et al. 2002; Sherman et al. 2002), but this solution is not yet widely available.

Speed of Imaging by Multiphoton Microscopy

The ability to collect images from living animals raises the question of the capability of multiphoton microscopy for dynamic imaging. Rapid image collection is a necessity for in vivo microscopy as it can minimize the effects of movement, an inevitable consequence of in vivo microscopy that can seriously compromise image quality (Rothstein et al. 2006). Image formation in multiphoton microscopy, like confocal microscopy, is a laser scanning imaging technique, in which the fluorescence image emerges as the laser scans across the sample. Most multiphoton microscope systems scan a single point of illumination across the sample; for a 512×512 pixel image, this scanning results in image capture rates of one to two frames per second. Higher-speed systems exist (Fan et al. 1999; Kim et al. 1999; Nguyen et al. 2001; Padera et al. 2002), but because they increase speed by shortening the interval of fluorescence collection at each point, their utility is limited to samples with strong fluorescence.

High-speed laser scanning microscopy can also be accomplished by "parallelizing" the illumination, such that multiple points of illumination simultaneously stimulate fluorescence from multiple points in the sample. This "tandem scanning" approach can increase speed in proportion to the number of illumination foci. But tandem scanning microscope systems are generally unsuited to thick tissues, as the adjacent beams can interact with each other away from the focus, resulting in the generation of background fluorescence. In multiphoton microscopy, the temporal offsetting of the illumination beams by 250 to 1,000 femtoseconds resolves this problem by effectively eliminating the interaction of the beams (Andressen et al. 2001; Egner and Hell 2000; Nielsen et al. 2001).

A larger problem is that fluorescence emissions are collected onto an imaging detector, such as a charge-coupled device (CCD). As discussed above, a primary advantage of multiphoton microscopy is the ability to collect scattered fluorescence photons with multiple detectors and to map the scattered photons to a single point of fluorescence excitation in the sample. This advantage vanishes when collecting onto an imaging detector because scattered emissions no longer contribute to the image of the fluorescent structure but instead generate a diffuse background. In essence, the scattered photons reduce the resolution and contrast of the resulting image. Given the contribution of scattered photons to image collection at depth, this presents a serious limit for the utility of tandem scanning multiphoton microscopes for in vivo imaging.

Future Prospects for in Vivo Multiphoton Microscopy

In vivo multiphoton microscopy is a powerful technique that brings the resolution previously associated only with studies of cultured cells into the relevant context of the whole organism. But the use of multiphoton microscopy, although growing exponentially (Zipfel et al. 2003b), is still largely driven by relatively few laboratories. Accordingly, the foci of active application of in vivo multiphoton microscopy still have their roots in the research interests of the original developers and "early adopters" of multiphoton microscopy. In the past few years, the technology of multiphoton microscopy has developed to the point that commercial systems are available that are as simple to use as confocal microscopes. Thus it is reasonable to expect that the next 10 years will see the proliferation of in vivo multiphoton microscopy to many more laboratories, and an accelerated implementation of this powerful technology to many more novel research questions. In addition, the development of new methods for fluorescent labeling of cells in animals, and new approaches to increase the depth and speed of multiphoton fluorescence imaging, will further spur the increased use of in vivo multiphoton microscopy.

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