Published in final edited form as:

J Microsc. 2006 April ; 222(Pt 1): 58–64.

Multi-photon excitation microscopy in intact animals

Emily C. Rothstein, Michael Nauman, Scott Chesnick, and Robert S. Balaban

Laboratory of Cardiac Energetics, National Heart, Lung and Blood Institute, National Institutes of Health, Department of Human Health Services, Bethesda MD

Abstract

Two-photon excitation fluorescence microscopy (TPEFM) and backscattered-second harmonic generation (B-SHG) microscopy permit the investigation of the subcellular events within living animals, but numerous aspects of these experiments need to be optimized to overcome the traditional microscope geometry, motion, and optical coupling to the subject. This report describes a stable system for supporting a living instrumented mouse or rabbit during endogenous reduced nicotinamide adenine dinucleotide (NAD(P)H) and exogenous dye TPEFM measurements and B-SHG microscopy measurements. The system was a modified inverted Zeiss LSM510 microscope with a rotating periscope that converted the inverted scope in to an upright format, with the objective approximately 15 cm displaced from the center of the microscope base, allowing the easy placement of an instrumented animal. An Olympus 20x water immersion objective was optically coupled to the tissue, without a cover glass, via a saline bath or custom hydrated transparent gel. The instrumented animals were held on a specially designed holder that poised the animal under the objective as well as permitted different ventilation schemes to minimize motion. Using this approach, quality images were routinely collected in living animals from both the peripheral and body cavity organs. The remaining most significant issue for physiological studies using this approach is motion on the micron scale. Several strategies for motion compensation are described and discussed.

Keywords

optical coupling; skeletal muscle; kidney; rabbit; mouse; mitochondria; collagen

INTRODUCTION

Two-photon excitation fluorescence microscopy (TPEFM) and backscattered-second harmonic generation (B-SHG) offers a unique view of intracellular events in living animals which provides a link between macroscopic imaging and molecular biology of subcellular events (Williams, R. M., Zipfel, W. R. *et al.*, 2005);(Cahalan, M. D., Parker, I. *et al.*, 2002), but also requires an stable sample preparation. Previous studies (Dunn, K. W., Sandoval, R. M. *et al.*, 2002);(Larson, D. R., Zipfel, W. R. *et al.*, 2003);(Rudolf, R., Mongillo, M. *et al.*, 2004);(Rothstein, E. C., Carroll, S. *et al.*, 2005), demonstrated the utility of TPEFM in living animals. All of these previous studies focused on small mammals, such as mice or rats, with limited experimental access due to the necessity of operating on the microscope stage. In addition, many required the use of compression cover-slips that could restrict surface blood flow.

The purpose of this study was to create a stable general system for TPEFM and B-SHG in intact animals. We were interested in imaging tissues with minimal disturbance to the normal physiological state with normal blood flow, which meant no pressure applied to the tissue from

a glass cover-slip or any effort to level out the plane of the tissue. Inherent to imaging biological tissues, with its natural curves, was the need for adjustable angles between the microscope and the animal. Therefore, we designed an animal restrainer to have an adjustable angle with various lockable freedoms of motion to adjust the animal placement and reduce gross physiological motion. A periscope for the microscope objective was developed that displaced the objective from the microscope stage and permitted an adjustable angle to impinge on the tissue, thereby increasing the degrees of freedom available for approaches to various tissues. In addition, different anesthesia and ventilation schemes were evaluated for reducing motion. Animal restraining and microscope changes and two simple optical coupling schemes, saline immersion and a custom optical gel, were evaluated.

Using these approaches, quality images were obtained using endogenous and exogenous fluorescent probes, as well as B-SHG of structural elements, in the mouse and rabbit peripheral and body cavity tissues, *in vivo*.

MATERIALS AND METHODS

In vivo mouse saline preparation

For initial induction of anesthesia, each mouse (CD-1 strain) was placed in a mouse anesthesia box (VetEquip, Pleasanton, CA) aired with 2.5% Isoflurane and 30% oxygen. Once anesthetized, the mouse was removed from the box and anesthesia was administered as 2.5% Isoflurane and 30% oxygen through a nose cone to the mouse. Pancuronium (2mg/ml) was given intravenously approximately every 40 minutes at 0.01 mg per kilogram. The tibialis anterior (TA) was exposed by first removing the fur with shaving and treatment with the depilatory Nair (Church and Dwight Co., Princeton, NJ). The bottom of the mouse leg was glued (n-butyl cyanoacrylate) to a cut syringe and allowed to set for 5 minutes. The skin and fascia were resected to expose the TA. The mouse was then transferred to the holding system, which includes an adjustable tilt mouse board with an attached leg restraint made of black Delrin (McMaster-Carr, Dayton, NJ). The end of the cut syringe was placed into a syringe holding device to complete the leg restraint, which held the leg in constant tension to minimize motion. In addition, two small bolts were advanced perpendicular to the length of the leg to hold the knee of the mouse and further reduce motion artifacts. The mouse holder was adjusted to approximately a 65 degree tilt and placed in a fluid reservoir. The fluid reservoir was filled with warmed saline solution until it bathed the leg sufficiently to permit the use of a 20x or 40x water immersion objective. The mouse was placed beside the Zeiss LSM510 microscope on a Zeiss motorized stage with a modified microscope stage support including fluid overflow gutters. A periscope setup (described below) was used to create an inverted optical path to the right of the microscope and above the mouse. Temperature was maintained using a heated saline bath on the lower body of the mouse and with a heating pad under the upper portion of the mouse.

In vivo rabbit saline preparation

For initial induction of anesthesia, each rabbit (New Zealand White) was injected with Ketamine/Acepromazine. Once anesthetized, the rabbit was intubated and anesthesia was administered as 3% Isoflurane and 30% oxygen through a tracheal tube to the rabbit. Pancuronium (2mg/ml) was given intravenously approximately every 40 minutes at 0.01 mg per kilogram. The TA was exposed by first removing the fur with shaving. The skin and fascia were resected to expose the TA. The rabbit was then fixed to the holding system, which includes an adjustable tilt rabbit fiberglass tube with an attached leg restraint made of black Delrin. The right ankle was positioned in a clamp holding device to complete the leg restraint, which held the leg in constant tension to minimize motion during imaging. In addition, a small plastic holder was lowered onto the right knee perpendicular to the length of the leg to hold the knee

of the rabbit and further reduce motion artifacts. The rabbit holder was adjusted to approximately a 65 degree tilt and placed in a fluid reservoir. The fluid reservoir was filled with warmed saline solution until it bathed the leg sufficiently to permit the use of a water immersion objective. The rabbit was placed beside the Zeiss LSM510 microscope on a modified microscope stage support including fluid overflow gutters. A periscope setup (described below) was used to create an inverted optical path to the right of the microscope stage and above the rabbit. Temperature was maintained using a heated saline bath on the lower body of the rabbit and with a heated circulating water pad around the upper portion of the rabbit.

Carbomer gel preparation

D-sorbitol (Sigma-Aldrich, St. Louis, MO) was added to stirred and heated, distilled water to bring the solution to 300 mOsm (approximately 300 mM sorbitol solution) and verified using a calibrated osmometer. The gelling agent, carbomer 940 (Snowdrift Farm, Tucson, Arizona), was added to the heated sorbitol solution under constant stirring at varying concentrations based on weight percent. Once the carbomer is completely dissolved in the solution, the solution pH was adjusted to pH 7.2–7.4 by drop wise addition of triethanolamine. The gel was stored in a sealed container at 4 °C.

***In vivo* mouse gel preparation**

The mouse was placed beside the Zeiss LSM510 microscope on a Zeiss motorized stage with a modified microscope stage support and prepared and fixed to the mouse holding system as described above. The mouse holder was adjusted to approximately a 35 degree tilt. The 0.5% carbomer gel, warmed to room temperature, was applied to the exposed muscle. A periscope setup (described below) with a water immersion objective lens was used to create an inverted optical path to the right of the microscope and above the mouse.

***In vivo* rabbit gel preparation**

The rabbit was placed beside the Zeiss LSM510 microscope on a Zeiss motorized stage with a modified microscope stage support and prepared as above and fixed to the holding system, which includes an adjustable tilt rabbit fiberglass tube and an attached leg restraint made of black Delrin, which were both attached to a flat Delrin fluid retention board that attached to the Zeiss X-Y stage. The right ankle was positioned in a clamp holding device to complete the leg restraint, which held the leg in constant tension to minimize motion during imaging. In addition, the flat head of a metal screw was lowered onto the right knee perpendicular to the length of the leg to hold the knee of the rabbit and further reduce motion artifacts. During kidney imaging, the rabbit was placed in the holding system and a small incision in the abdominal skin and fascia were made to expose the kidney for imaging. The rabbit holder was adjusted to approximately a 35 degree tilt for muscle imaging and no tilt for kidney imaging. The 0.5% carbomer gel was applied to the exposed muscle or tissue. A periscope setup (described below) with a water immersion objective lens was used to create an inverted optical path to the right of the microscope and above the rabbit. Syto Green 24 (Invitrogen, Carlsbad, CA) was injected intravascularly in the rabbit during kidney imaging.

In some cases (noted in figure legends), jet ventilation with a High Frequency Oscillatory Ventilator (SensorMedics, Yorba Linda, CA) was used. Anesthesia and Pancuronium were administered as described above for the rabbit.

TPEFM and B-SHG

In vivo, TPEFM and B-SHG imaging were performed on an inverted Zeiss LSM510 microscope (Carl Zeiss, Inc., Thornwood, NY) with an Olympus 20X/0.95 NA XLUMPlanFI

(Olympus, Melville, NY) water immersion objective connected to the microscope each by an L-shaped periscope device (LSM Technologies Inc., Shrewsbury, PA) that was optimized for that lens. Both periscope devices used were specially designed with a long base and arm in order to reach approximately 6 inches beyond the center of the microscope base and extend to the animal on the peripheral animal holder. The base of the periscope device was fixed by screwing into an objective slot on the Zeiss LSM 510 objective wheel and being held by a black Delrin support. This support was attached to the base of the microscope and had a black Delrin ring which surrounded the periscope base with an adjustable tightening screw for variable resistance. Excitation was provided by a Chameleon extended range laser (Coherent, Santa Clara, CA) with excitation wavelengths from 650 nm to 1100 nm and average output power of 3W. The Zeiss LSM510 stage was removed and relocated for saline and gel mouse and gel rabbit imaging, but not used for saline rabbit imaging. Fluorescence and B-SHG were monitored at specific wavelengths using BGG22 and appropriate longpass, shortpass and bandpass filters. Postprocessing of images was applied to some images using the Zeiss LSM 510 AIM (Carl Zeiss, Inc., Thornwood, NY) and Adobe Photoshop 7.0 (Adobe, Mountain View, CA). Specific experimental, acquisition, and post-processing parameters are provided in the figure legends.

RESULTS AND DISCUSSION

Microscope Adaptation

The periscope system used in this system was analyzed for light loss in both the infrared and visible light. Over a wide range of frequencies, the loss of light was on the order of only 8%–12%, suggesting a minimal compromise in light collection efficiency. The collimation of the expanded beam from the laser microscope is similarly matched in the alignment of the optics in the inverter and the stationary point of the scanning beam at the back aperture is translated proportionally so that the back aperture is illuminated equal to the illumination at the objective revolver. An illustration of the periscope together with the animal holding device is shown in Figure 1. The projection of the microscope objective away from the stage and the one degree of rotation provided an excellent method of increasing the ability to get the objective over various regions of an instrumented anesthetized animal with little difficulty. One compromise associated with the ability to rotate the objective was that the microscope focusing capability, which usually was precise along the vertical (or z axis), was no longer accurate. Thus, to perform true perpendicular stacks of images moving into the tissue with this arrangement, the actual deviation from normal of the objective needs to be determined and appropriate adjustments in animal position in the x and y positions (using the animal holding positioning system) together with z position controlled by the microscope have to be coordinated. The deviation associated with attempting a z-series using the periscope and the normal focus of the microscope is a straightforward geometry problem. The more tilt applied to the lens, the larger the artifact. No rotation of the normal focus generates a normal z-series (no deviation), while at the other hypothetical extreme with the periscope angled 90°, the normal focus direction becomes a translational motion across the tissue rather than into the tissue (complete failure). An expression for this error can be made by assuming that the error can be estimated by calculating the opposite length of the right triangle formed from the true z-stack distance and direction, the adjacent side, with ϕ being the angle of the objective versus the normal focus direction, defined as $\phi = 0$. The hypotenuse becomes the actual focus pathlength. Thus, the error (microns) = $\sin \phi$ focus path length (microns). This equation works at the limits of 0 and 90 degree deflections and predicts with a 30 degree deflection that the error will be 50 microns at 100 microns along the focus path. This is a very significant error for a method with a ~2 micron in plane resolution. By determining ϕ and using this equation, a proper z-series can be constructed by movement of the structure supporting the animal. An alternative solution for creating accurate z-sectioning over a modest field of view with the periscope is the addition of

a piezo device between the periscope and the objective. This device can move the objective in the “z” direction, allowing for control of the focal plane along the axis of the objective without moving the animal.

Optical Coupling

In previous studies, cover-slips on compressed tissue with agarose (Kleinfeld, D., Mitra, P. P. *et al.*, 1998);(Hirase, H., Creso, J. *et al.*, 2004), local saline baths (Rothstein, E. C., Carroll, S. *et al.*, 2005) or optical gels were used to couple the objective to the tissue or cell system for optical imaging. Compressing cover-slips over a tissue is problematical with regard to compressing surface tissues maintaining a fluid phase both between the objective and glass as well as glass and tissue. Finally, the application of this approach to different tissues is limited. The local saline bath we used in a previous study caused difficulty in maintaining a seal between the tissue and saline bath, which was especially a problem when working on a microscope stage, limiting the access to the preparation as well as the tissues for which appropriate local baths could be constructed.

In this study, we took two directions to solve this problem. The first, was to immerse the entire appendage of the animal in heated saline to eliminate the need for tissue to saline seals and permit a wide variation of local tissue structures to be studied. This system was successful but still cumbersome with regard to limited accessibility to the body cavity and the concern that the constant dilution of the extracellular space by the large saline bath would modify the constitution of the extracellular space. A second, more desirable solution was the use of viscous transparent hydrated gels that could be directly applied to the tissue and serve as the optical coupling agent. This gel reduced the dilution of extracellular space by eliminating the need to submerge the tissue of interest in a solution at a depth that allowed for proper working distance between the tissue and objective. A 0.2% Carbomer 980 transparent eye wetting gel (Viscotears Liquid Gel, Ciba Vision, Marcon, Venezia, Italy) had been used in other microscopy studies of living tissue (Mastropasqua, L., Carpineto, P. *et al.*, 2002; Rudolf, R., Mongillo, M. *et al.*, 2004). We found that the commercial products were much too fluid for most of our applications. Therefore, we created and tested osmotically and pH balanced carbomer based gels for optical properties and adhesion to biological tissues. Gels of various weight % of carbomer were tested for optical transmission from 400 to 900 nm along with the refractive indexes. For gels of weight % carbomer of 0.2%, 0.3%, 0.4% and 0.5% the respective refractive indexes were 1.343, 1.342, 1.344, and 1.343, respectively. These refractive indexes are close to that of water (RI 1.33) and to tissue components such as the cytoplasm (RI 1.35) (Charney, E. and Brackett, F. S., 1961), mitochondria (RI 1.40) (Beuthan, J., Minet, O. *et al.*, 1996) or cell membrane (RI 1.46–1.60) (for review, (Johnsen, S. and Widder, E. A., 1999)). It is important to match RI as closely as possible between an objective, connection mediums and sample because inhomogeneities in RI cause the scattering of light. The optical transmission of the gels over a 1 cm pathlength was greater than 95% over the entire bandwidth studied. Assuming that the thickness of the coupling region between the tissue and the objective is much less than 1 cm, it was assumed any optical absorbance or scattering was insignificant with this material. In general, both fluorescent and B-SHG images collected using the gel coupling system were as good or superior to the saline bath approach (Figures 3 and 4). Depending on the application, carbomer gels of varying thickness from 0.3% to 0.5% carbomer were used in this tissue. All variations of the gel ideally maintained the moisture of the tissue and showed no apparent compromising effects on the tissue.

Tissue Motion Restriction

From our experience, we believe that tissue motion remains the major limiting factor in image quality at this time. This is not surprising since with spatial resolution approaching 2 microns *in vivo* that the physiological motions associated with blood flow and respiratory activity would

be significant. Respiratory motion has been identified as one of the major sources of motion at this spatial scale. We found that immobilizing the tissue without restricting blood flow was the most efficient way of limiting respiratory motion in the appendages along with reducing the physical coupling to the body core. For imaging of live animal appendages, the animal holding system we developed held the legs far enough away from the body to reduce respiratory motion coupling. However, even with these adaptations, respiratory motions were detected on the order of 2 to 10 microns in a reproducible manner synchronized to the ventilation (figure 2). This was confirmed by collecting a time series of data and performing a FFT on the time course data from the tibialis anterior of the mouse. This data reveal a dominant respiratory component, while a heart rate component was not detected in most imaging planes. One solution of this problem was to retrospectively gate the image acquisitions, removing those during the active respiratory cycle, or to gate the image acquisition to the respiratory phases. However, to collect the highest resolution images, a breath hold technique was used where the ventilation was held at maximum inspiration for up to one minute. Over this period of time, no changes in blood pressure or heart rate were observed in rabbits or mice under these anesthetized conditions. The breath holding technique was an absolute requirement for imaging organs in the body cavity such as the kidney (Figure 5) and liver (not shown) where the mechanical coupling to the diaphragm resulted in large displacements. This breath hold approach is similar to techniques used in clinical cardiac imaging using MRI or CT modalities.

We also investigated the use of high frequency ventilation schemes (Macintyre, N. R., 2000) that reduce the overall lung displacement but provides adequate gas exchange especially in infants and small animals. However, our initial studies revealed that even though the magnitude of the displacements had been minimized, the respiratory-induced motions could still be detected on the 2 to 3 micron scale based on Fourier analysis. This approach might be most useful if the individual lines of the scan process could be synchronized with the high frequency ventilation permitting a rather rapid respiratory gated image collection scheme.

Even in the skeletal muscle system, which permitted reasonable image quality without respiratory compensation, other motion concerns were observed with physiological perturbations. For example, a simple occlusion of the artery or artery and vein resulted in a large shift of muscle fluid volume that moved the tissue in a complex manner preventing the tracking of the tissue position during such a perturbation. Any perturbation that altered blood flow, systemic pressure or tissue water distributions, let alone contractile activity, results in significant and complex tissue motions on the tenths of a micron scale. This makes tissue tracking a significant issue as we move forward from collecting images to recording physiological events, *in vivo*.

***In Vivo* Imaging Performance**

Once a stable specimen was obtained, we were able to generate high quality blue fluorescence or B-SHG images to a depth of 200 microns with ~25–75 mW of laser power at the back focal plane with either animal model using the periscope-gel animal imaging system. We were able to clearly view cellular structure within the tibialis anterior and kidney of animals such as a rabbit with minimal disturbance to the animal's physiology (figures 3,4, and 5). Imaging at this fine resolution *in vivo* provides a way to examine microvascular flow and its effect on surrounding tissues and allows for delineating different fiber types in muscle as well as the various compartments of mitochondria, including the perivascular, paranuclear and intersarcomeric populations using NAD(P)H TPEFM (Rothstein, E. C., Carroll, S. *et al.*, 2005). In addition, with a stable animal preparation, two-photon excitation B-SHG provides further information regarding the structure of tissues by highlighting myosin and collagen structures, such as is seen in and around skeletal muscle (figure 4). These subcellular

measurements provide the opportunity to monitor the structure and function of tissues such as muscle under various controlled physiological stresses in the living animal.

Summary

The novel design of the animal holding systems, along with the optical gel described in this study, enable the dynamic monitoring of blood flow, tissue structure and subcellular exogenous fluorescence and endogenous mitochondrial metabolic signals such as NAD(P)H in mouse and rabbit skeletal muscle using TPEFM and two-photon excitation B-SHG imaging, *in vivo*. This approach of monitoring fluorescence and B-SHG is valuable for imaging not only the structure and function of skeletal muscle, but is also useful for imaging other physiological systems such as the kidney and liver *in vivo*. The periscope-gel animal imaging system was evaluated and found to be superior because it worked to adapt the imaging tool to natural tissue curvature and to minimize motion and the dilution of the extracellular space and, therefore, facilitated two-photon imaging on most any region of the body. Even with these modifications, motion on the micron scale still remains a significant issue especially when physiological perturbations are attempted that alter blood pressure, tissue volume distribution, or contractile activity. Similar solutions to those used for gating and post-processing other imaging modalities such as MRI and CT continue to be explored because although both fluorescent and B-SHG imaging at depth, in minimally affected tissue, in a living animal, provides a fascinating connection between cellular biology and whole animal physiology, image quality is important for any measurements to be made on the subcellular level.

References

- Beuthan J, Minet O, Helfmann J, Herrig M, Muller G. The spatial variation of the refractive index in biological cells. *Phys Med Biol* 1996;41:369–82. [PubMed: 8778819]
- Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol* 2002;2:872–80. [PubMed: 12415310]
- Charney E, Brackett FS. The spectral dependence of scattering from a spherical alga and its implications for the state of organization of the light-accepting pigments. *Arch Biochem Biophys* 1961;92:1–12. [PubMed: 13692507]
- Dunn KW, Sandoval RM, Kelly KJ, Dagher PC, Tanner GA, Atkinson SJ, Bacallao RL, Molitoris BA. Functional studies of the kidney of living animals using multicolor two-photon microscopy. *Am J Physiol Cell Physiol* 2002;283:C905–16. [PubMed: 12176747]
- Hirase H, Creso J, Buzsaki G. Capillary level imaging of local cerebral blood flow in bicuculline-induced epileptic foci. *Neuroscience* 2004;128:209–16. [PubMed: 15450368]
- Johnsen S, Widder EA. The physical basis of transparency in biological tissue: ultrastructure and the minimization of light scattering. *J Theor Biol* 1999;199:181–98. [PubMed: 10395813]
- Kleinfeld D, Mitra PP, Helmchen F, Denk W. Fluctuations and stimulus-induced changes in blood flow observed in individual capillaries in layers 2 through 4 of rat neocortex. *Proc Natl Acad Sci U S A* 1998;95:15741–6. [PubMed: 9861040]
- Larson DR, Zipfel WR, Williams RM, Clark SW, Bruchez MP, Wise FW, Webb WW. Water-soluble quantum dots for multiphoton fluorescence imaging in vivo. *Science* 2003;300:1434–6. [PubMed: 12775841]
- Macintyre NR. High-frequency ventilation. *Semin Respir Crit Care Med* 2000;21:167–74. [PubMed: 16088729]
- Mastropasqua L, Carpineto P, Ciancaglini M, Nubile M, Doronzo E. In vivo confocal microscopy in primary congenital glaucoma with megalocornea. *J Glaucoma* 2002;11:83–9. [PubMed: 11912354]
- Rothstein EC, Carroll S, Combs CA, Jobsis PD, Balaban RS. Skeletal muscle NAD(P)H two-photon fluorescence microscopy in vivo: topology and optical inner filters. *Biophys J* 2005;88:2165–76. [PubMed: 15596503]
- Rudolf R, Mongillo M, Magalhaes PJ, Pozzan T. In vivo monitoring of Ca(2+) uptake into mitochondria of mouse skeletal muscle during contraction. *J Cell Biol* 2004;166:527–36. [PubMed: 15314066]

Williams RM, Zipfel WR, Webb WW. Interpreting second-harmonic generation images of collagen I fibrils. *Biophys J* 2005;88:1377–86. [PubMed: 15533922]

FIGURE 1.
Rabbit TPEFM imaging system: Periscope with an Olympus 20X/0.95 NA XLUMPlanFI water immersion objective.

FIGURE 2.

Montage of TPEFM images of mouse TA *in vivo* exhibiting the effect of respiratory motion. Endogenous blue fluorescence is seen in the mouse TA *in vivo*. Vasculature and nuclei in the mouse TA is seen as fluorescent voids. An example muscle image montage with motion imaged using 710 nm excitation, 12 bit resolution, 20x magnification with 1.2x zoom and blue emission (emission less than 465 nm) monitored at 512 x 512. Images presented were acquired approximately every 8 seconds and had a scan time of 1.97 seconds. Scale bar of 50 microns is shown.

FIGURE 3.

TPEFM image of mouse TA *in vivo* using a gel optical coupling agent. Endogenous blue fluorescence is seen in the mouse TA *in vivo*. Vasculature in the mouse TA is seen as fluorescent voids. Imaged using 710 nm excitation, 12 bit resolution, 20x magnification and blue emission (emission less than 465 nm) monitored at 1024 x 1024. Scale bar of 50 microns is shown.

FIGURE 4.

B-SHG TPEM images of mouse and rabbit TA *in vivo*. Both skeletal muscle myosin striations and collagen are seen. (A) B-SHG TPEM images of mouse TA *in vivo*. Imaged using 900 nm excitation, 8 bit resolution and blue emission (410–490 nm) monitored. Image cropped from a 512 x 512 image with pixel resolution of 0.299 x 0.299 microns. Scale bar of 10 microns is shown. (B) B-SHG TPEM images of rabbit TA *in vivo*. Imaged using 900 nm excitation, 12 bit resolution, 20x magnification, pixel resolution of 0.45 x 0.45 microns and blue emission (435–485 nm) monitored. Scale bar of 50 microns is shown. (C) Montage of B-SHG TPEM images of rabbit TA *in vivo*. Images presented in 5 micron steps from inside the tissue at a depth of 205 microns from the tissue surface (labeled zero microns) to the tissue surface (labeled 205 microns). Imaged using 900 nm excitation, 12 bit resolution, 20x magnification, pixel resolution of 0.45 x 0.45 microns and blue emission (435–485 nm) monitored. A median (3 x 3) filter was applied. Scale bar of 50 microns is shown.

FIGURE 5.

TPEFM image of rabbit kidney *in vivo* using a gel optical coupling agent. Endogenous and exogenous fluorescence is seen in the rabbit kidney *in vivo* using gel coupled imaging. White cells stained with the nuclear stain SytoGreen24 are visible. The rabbit was maintained on jet ventilation with an imaging ventilation pause. The tissue was imaged using 710 nm excitation, 12 bit resolution, 20x magnification, pixel resolution of 0.90 x 0.90 microns and 515–650 nm emission was monitored at 512 x 512. A dust and scratches noise (radius 1, threshold 21) filter was applied. Scale bar of 50 microns is shown.