Two-Photon Excited Imaging of Photosensitizers in Tissues

Mariah L. Coleno[1,2], Vincent P. Wallace[1], Chung Ho Sun[1], Andrew K. Dunn[1], Michael W. Berns[1], Bruce J. Tromberg [1,3]
[1]Laser Microbeam and Medical Program (LAMMP), Beckman Laser Institute and Medical Clinic
[2]Department of Chemical and Biochemical Engineering and Materials Science
[3]Department of Physiology and Biophysics
University of California, Irvine, Irvine, CA 92612, USA

ABSTRACT

Two-photon microscopy (TPM) is a non-invasive biological imaging technique that can be used to selectively image cellular activity and photosensitizer (PS) localization within highly scattering epithelial tissues at depths of ~ 200 μm with submicron resolution. The principal objective of this study was to develop a model system for understanding the impact of photodynamic therapy on cellular and extracellular matrix remodeling in biological tissues. An artificial tissue model (RAFT) composed of collagen, embedded fibroblasts, and macrophage cells has been developed for this purpose. TPM is utilized to monitor extracellular matrix remodeling following PDT by imaging collagen/elastin autofluorescence. Selective uptake of photosensitizers by specific cellular components in the matrix can also be visualized by TPM.

Keywords: Two-photon microscopy, fluorescence, matrix remodeling, photodynamic therapy, photosensitizers, collagen, fibroblasts, macrophages, wound healing, tissue imaging

1. INTRODUCTION

The wound healing immune response is mediated by cells of the monocyte/macrophage lineage. In wounded tissue, first neutrophils and then macrophages invade the site of injury and begin engulfing debris. Macrophages after degrading the connective tissue actively contribute to the tissue repair and remodeling process. Although precise regulation of the wound healing process is not well understood, macrophages are thought to be a source of growth factors for fibroblasts which infiltrate into the wound site and other factors which locally control the extent of collagen production by other cells. In adults, macrophage depletion impairs wound healing, while overexpression of macrophage products leads to too much collagen production thus producing chronic inflammatory states, which can lead to excessive tissue scarring. In an attempt to suppress this scar formation during tissue remodeling, we are interested in studying the effects of macrophage targeted in vitro photodynamic therapy (PDT) on cellular and extracellular matrix remodeling. PDT, which utilizes light-activatable photosensitizers to damage cells and tissues via the production of highly reactive oxygen intermediates, can have effects ranging from inactivation to stimulation of macrophage function depending on the light dose utilized. The technique we will use to study collagen structure, photosensitizer localization in tissue and eventually the effect of PDT on wound repair is two-photon microscopy (TPM). TPM utilizes pulsed, near-IR light and is effective for high-resolution imaging of thick tissues (e.g. 100-500μm) due to minimal scattering and absorption at longer wavelengths.

2. EXPERIMENTAL DESIGN

One of our goals is to develop an artificial tissue model that can be used to image cells and extracellular matrix material involved in the wound healing process. In particular, we need a tissue model that enables images of collagen, macrophage and fibroblast structure (autofluorescence) to be obtained at depth. In the same tissue we need to be able to show that photosensitizers (e.g. Protoporphyrin IX) can selectivity target macrophages and fibroblasts involved in the wound healing process (exogenous fluorescence). Once these goals are accomplished, we plan to extend our studies to qualitatively determine how Protoporphyrin IX uptake in the cells and tissue varies with ALA incubation time.

Part of the SPIE Conference on Functional Imaging and Optical Manipulation of Living Cells
San Jose, California • January 1999 SPIE Vol. 3604 • 0277-786X/99/$10.00

3. METHODS

3.1. Cell culture

The artificial tissue model (Fig. 1) that was developed and used for the wound healing studies is the RAFT model, which is an epithelial tissue model grown in culture. This tissue model consists of a top layer of keratinocytes with underlying layers consisting of collagen with macrophages and fibroblasts embedded throughout. The actual tissue used for these studies was ~ 0.5 mm thick and did not have the top layer of keratinocytes. P388 D1 mouse-derived macrophage cells (an established cell line) were used at a concentration of $3X10^5$ cells/ml, and fibroblasts which were taken from a primary human cell culture line from neonatal dermal foreskin were used at a concentration of $1.5X10^5$ cells/ml (unless otherwise stated). The artificial tissue was grown in growth medium containing 10% fetal bovine serum. The tissue was maintained in a humidified incubator at $37^{O}C$ with 7.5% CO_2. For the Protoporphyrin IX images, the RAFT tissue was incubated for 0 (control), 3 and 20 hours with equivalent concentrations of ALA (1 mg/ml). After each ALA incubation cycle, the unincorporated ALA was removed by washing in phosphate buffered saline (PBS). The tissue while in PBS was then imaged on the microscope stage. All incubations were performed on the same tissue sample.

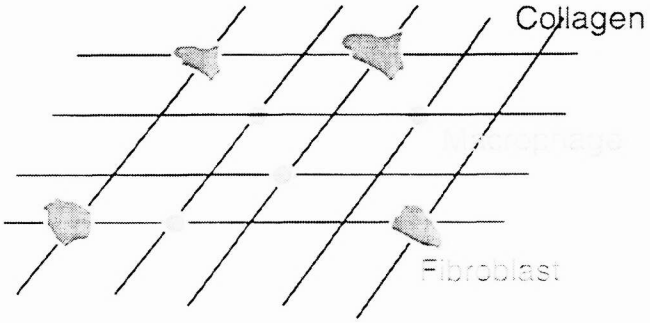


Figure 1: RAFT Model

3.1.1. Two-photon microscope instrumentation

The RAFT tissue was imaged using a two-photon scanning fluorescence microscope system. The two-photon experimental set-up (Fig. 2) is based on the system constructed by So et al at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana, Champaign. The TPM system consists of an Argon Innova 200 Ion laser (Coherent, Santa Clara, CA) which is used to pump a Mira 900F Titanium:Sapphire ($Ti:Al_2O_3$) laser (Coherent, Santa Clara, CA) which is used as the two-photon pulsed excitation source. The ultrafast Ti:Sapphire laser is tunable between 720-810nm thus allowing for two-photon imaging of many UV excitable fluorophores. The average power after the Ti:Sapphire laser is controlled using neutral density filters. Thus low average powers required for tissue and cell survival could be maintained at the sample (5-10mW) while still maintaining sufficient peak power for two-photon excitation to occur. The mode-locked, 100 femtosecond, 76 MHz pulse train exiting the Ti:Sapphire laser is expanded and collimated using two lenses to overfill the back aperture of the microscope objective. The beam is scanned across the sample, which is placed on an inverted Zeiss Axiovert 100 microscope (Zeiss, Thornwood, NY), using a PC controlled X-Y scanner (Series 603X, Cambridge Technology, Inc., Watertown, MA). A custom digital card controls the scanner. A Zeiss 63X, 1.2 N.A. water immersion objective having a working distance of 200 μm was used for these studies (Zeiss, Thornwood, NY). The two-photon fluorescence from the tissue is detected using a single photon counting detection system that consists of two PMTs (Hamamatsu Corp., Bridgewater, N.J.) arranged perpendicularly, one optimized for green light (R7400P), the other for red light (R7400P-01) thus allowing for simultaneous detection of fluorescence in two different wavelength regions.

4. EXPERIMENTAL SET-UP

In skin, endogenous fluorescence (autofluorescence) occurs due to the natural fluorescence of proteins already present in the skin. Exogenous fluorescence occurs due to the addition of a fluorophore or photosensitizer that fluoresces when excited by light but which is not already a component of the tissue. Two photon autofluorescence images were obtained of collagen and NADH localized in the nucleus of the macrophages and fibroblasts, and two photon exogenous fluorescence images were obtained from fibroblast and macrophage cells labeled with ALA. ALA, aminolaevulinic acid, once transported into the mitochondria is converted into Protoporphyrin IX. The excitation/emission maxima for Protoporphyrin IX are 390nm/635nm respectively while collagen and NADH display blue (400-500nm) emission upon UVA (350-390nm) excitation. Thus efficient excitation of both autofluorescence and Protoporphyrin IX fluorescence in the RAFT tissue model is achieved using a Ti:Sapphire laser wavelength of 780nm corresponding to a UV one photon wavelength of 390nm. Autofluorescence images providing detailed structural information of collagen and NADH (400-580nm) are collected using the green channel and an SBG39 wide pass barrier filter (CVI, Livermore, CA), and Protoporphyrin IX fluorescence is collected using the red channel and a 635/50nm barrier filter (Chroma Technology Corp., Brattleboro, VT).

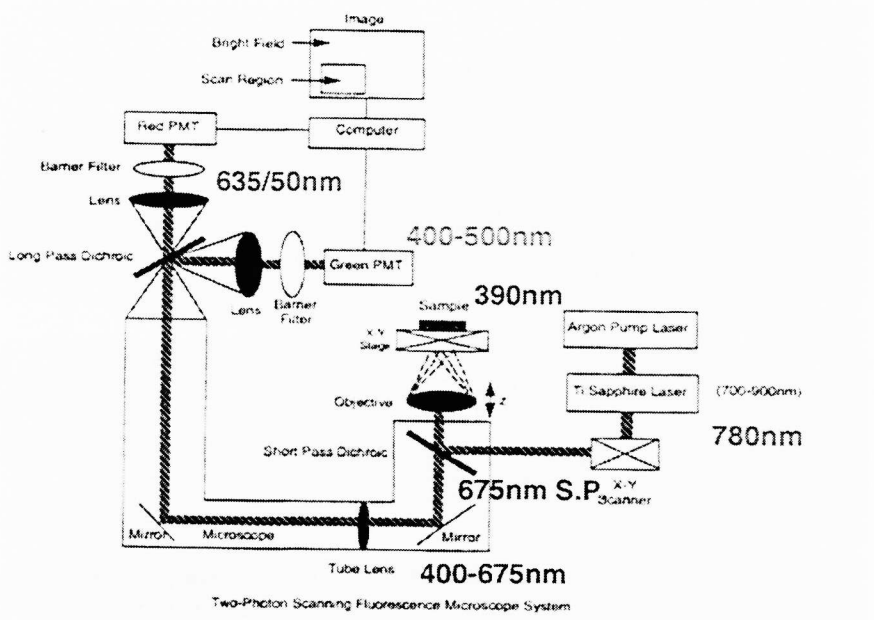
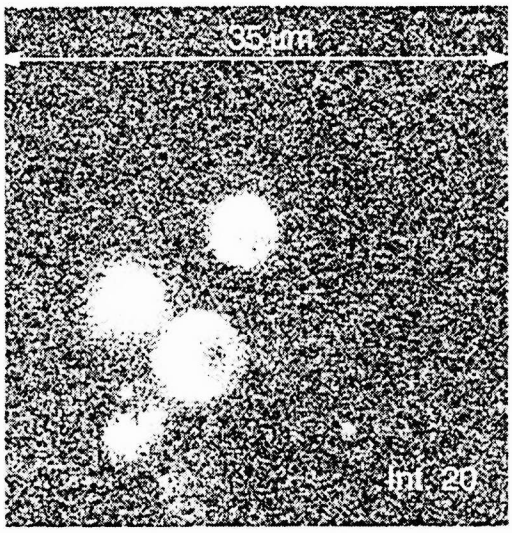


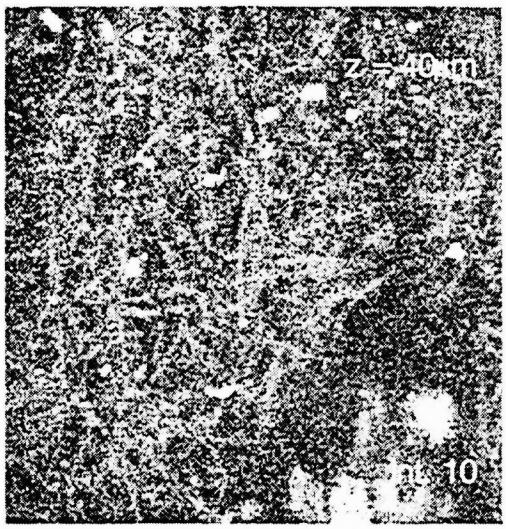
Figure 2: Two-Photon Scanning Fluorescence Microscope System

5. RESULTS AND DISCUSSION

Using freshly made, non-contracted RAFT tissue (approximately 1 cm in diameter), collagen autofluorescence was undetectable even after integrating 20 scans at a frame rate of 2.5 sec/frame (Fig. 3a). In this same tissue, however, NADH autofluorescence was easily seen in the macrophages. We determined that to see collagen structure, the tissue and consequently the collagen needed to contract. By keeping the fibroblast concentration in the RAFT tissue at $1.5X10^5$cells/ml and allowing the tissue to contract for 14 days to about 1/4 cm in size, collagen and NADH autofluorescence was easily seen throughout the entire working distance of the objective (Fig. 3b). Although this image was integrated over 10 scans for clarity, the collagen fluorescence was detectable when scanning only one frame. The scan region for each image taken is 35 μm by 35 μm.



(a) Green channel/autofluorescence

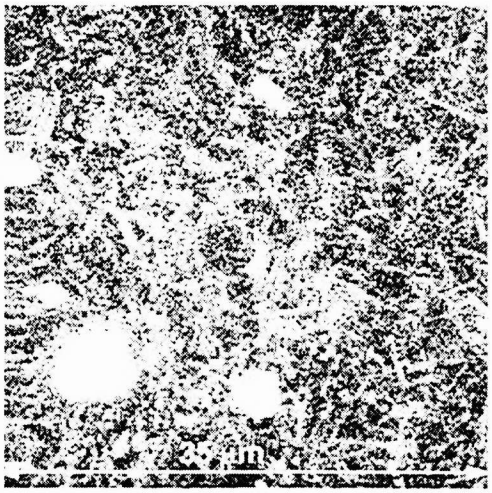


(b) Green channel/autofluorescence

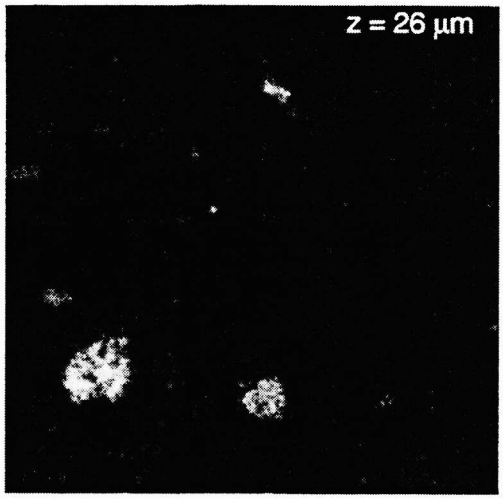
Figure 3: Two-Photon Microscopy autofluorescence images of the artificial RAFT tissue (X-Y section, z = 40 μm): (a) non-contracted RAFT tissue showing NADH autofluorescence in the macrophages but lack of collagen autofluorescence (integrated 20X) (b) contracted RAFT tissue showing NADH autofluorescence localized to the macrophages and collagen autofluorescence (integrated 10X).

Up to this point, the RAFT model was optimized to provide a collagen autofluorescence signal intense enough to study wound healing at depth. We next needed to determine whether Protoporphyrin IX could be selectively targeted to the cells in the RAFT tissue at depth. ALA incubation times of 0 hours (control), 3 hours, and 20 hours were imaged, and all images were integrated over 10 scans. In the following two photon fluorescence images, Protoporphyrin IX distribution was observed in the red channel and collagen and NADH autofluorescence was observed in the green channel. The fibroblast concentration used for all of the following images was doubled from that originally used to $3X10^5$ cells/ml. The macrophage concentration remained the same at $3X10^5$ cells/ml. By doubling the fibroblast concentration, the time for tissue contraction was decreased from 10-14 days to 3 days. For the control (0 hour ALA incubation time), collagen structure and NADH autofluorescence in the macrophages was easily seen in the green channel (image not shown but similar to Fig. 3b) while, as expected, these same macrophages could not be seen in the red channel since the sample did not contain Protoporphyrin IX.

For the same RAFT tissue, but with an ALA incubation time of 3 hours, collagen and NADH autofluorescence is again easily seen in the green channel (Fig. 4a). This time, however, as expected, Protoporphyrin IX fluorescence was easily seen in the red channel (Fig. 4b), and the fluorescence appeared to be localized in the macrophages. Recall that collagen autofluoresces in the green, so as expected, collagen fluorescence was not observed in the red channel.



(a) Green channel/autofluorescence

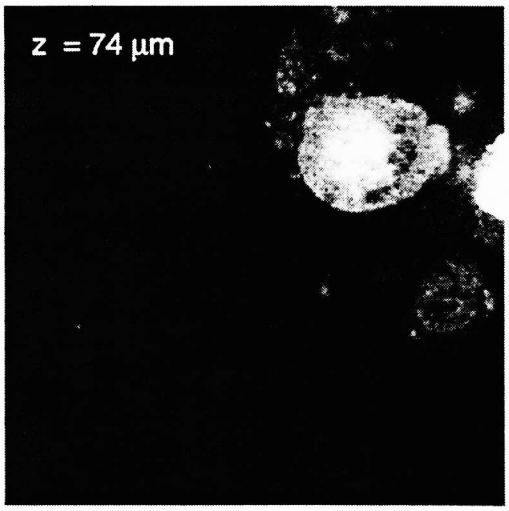


(b) Red channel/Protoporphyrin IX

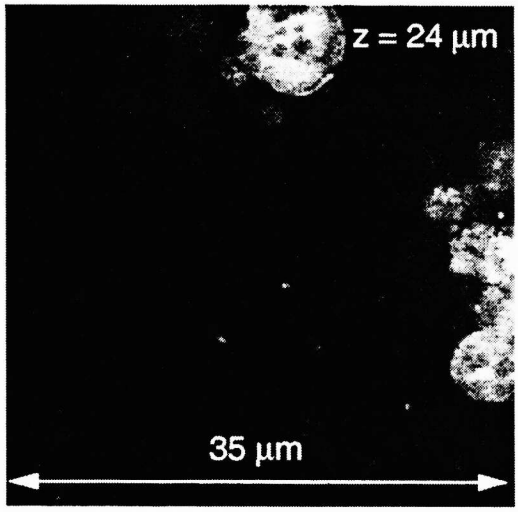
Figure 4: Two-Photon fluorescence images of the contracted RAFT tissue (X-Y section, z = 26 μm) incubated with ALA for 3 hours: (a) autofluorescence image showing collagen and NADH (localized to the macrophages) structure (b) exogenous fluorescence image showing Protoporphyrin IX fluorescence localized to the macrophages but lack of collagen fluorescence.

For the same contracted RAFT tissue but with an ALA incubation time of 20 hours, TPM images of collagen and NADH autofluorescence were again easily seen in the green channel (image not shown). The corresponding Protoporphyrin IX fluorescence images obtained in the red channel (image not shown) reveal that Protoporphyrin IX fluorescence accumulates in the macrophage sites. Again, collagen fluorescence was not observed in the red channel. The 3 hour and 20 hour ALA incubated tissue sections were examined for drug distribution by taking the ratio of the intensities of the red channel to the control and taking the mean value. From this data, we observed that Protoporphyrin IX fluorescence uptake increased in the macrophages with increasing ALA incubation time.

Selective uptake of Protoporphyrin IX by specific subcellular components of the matrix was also visualized at depth utilizing the TPM system (Fig. 5). The two photon images obtained demonstrate the high spatial resolution (~ 0.5μm) obtainable with two photon microscopy (comparable to confocal).



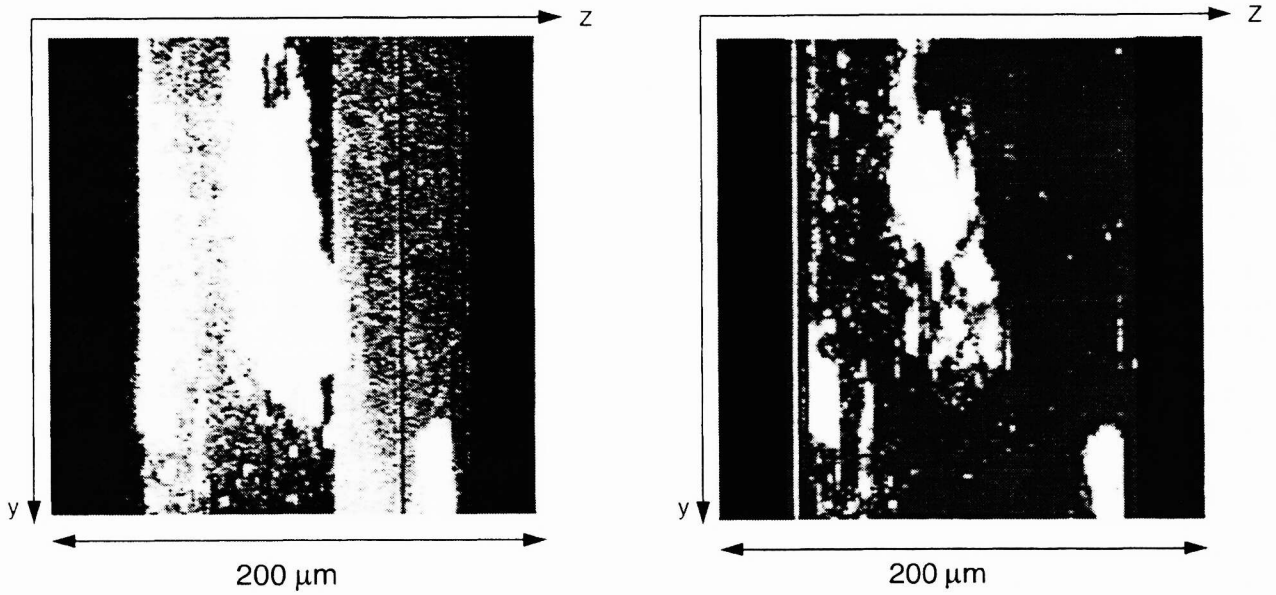
(a) Red channel/Protoporphyrin IX



(b) Red channel/Protoporphyrin IX

Figure 5: Two-Photon fluorescence images of the contracted RAFT tissue (X-Y section) showing subcellular localization of Protoporphyrin IX: (a) 3 hour ALA incubation time (b) 24 hour ALA incubation time.

Although the images shown of collagen, NADH and Protoporphyrin IX in the RAFT tissue model were taken at the z values stated, we were able to obtain similar images throughout the entire working distance of the 1.2 N.A. water immersion objective which was 200 μm. A series of images were taken in 2μm steps, and two 3-D tissue reconstructions of the RAFT tissue were obtained over the full 200μm's. The autofluorescence 3-D reconstruction shows collagen structure and NADH. The NADH is localized in the macrophages and fibroblasts (Fig. 5a). For the exogenous fluorescence 3-D reconstruction, Protoporphyrin IX distribution in the macrophages and fibroblasts was easily seen (Fig. 5b). We should note that 200μm is the limit set by the objective used. To image throughout the entire 0.5 mm thickness of the RAFT tissue, we would need to use a longer working distance objective which will give decreased resolution.



(a) Green channel/autofluorescence

(b) Red channel/Protoporphyrin IX

Figure 5: Two photon fluorescence 3-D reconstruction of the contracted RAFT model (Y-Z section) incubated with ALA for 3 hours (a) 3-D reconstruction showing collagen and NADH autofluorescence. NADH is localized to the macrophages and fibroblasts. (b) 3-D reconstruction showing Protoporphyrin IX fluorescence localized to the macrophages and fibroblasts. Collagen fluorescence was not observed in the red channel.

6. SUMMARY

We have expanded the capabilities of our two-photon microscope system to selectively image cellular activity and photosensitizer localization within highly scattering epithelial tissues at depths of ~ 200μm with submicron resolution. As our goal is to use TPM as a non-invasive imaging technique to study wound healing in living tissues, we developed an artificial tissue model (RAFT) that will ultimately be used to study wound healing. In particular, this RAFT tissue model composed of collagen, embedded fibroblasts, and macrophage cells will be used to understand the impact of photodynamic therapy on cellular and extracellular matrix remodeling in biological tissues. Because we plan to follow the repair of the extracellular matrix following different PDT doses by imaging collagen/elastin autofluorescence, we first needed to optimize the procedure for growing the RAFT tissue so that we could maximize collagen autofluorescence at depth using the TPM. We found that RAFT tissue contraction leads to increased collagen autofluorescence as does increasing the fibroblast concentration by twice that originally used. Using dual wavelength imaging and the RAFT model, we were able to visualize both collagen and NADH (autofluorescence) and photosensitizer (Protoporphyrin IX) localization (exogenous fluorescence) within the RAFT tissue model in vitro to a depth of 200μm. Selective uptake of Protoporphyrin IX by specific cellular components of the matrix was also visualized at depth utilizing the TPM system, and Protoporphyrin IX distribution in the cells was found to increase with ALA incubation time. Using TPM, 3-D reconstructions of both autofluorescence and Protoporphyrin IX distribution in the RAFT tissue were obtainable over the entire working distance of the objective. Having acquired the ability to image both collagen and photosensitizer localization within the RAFT tissue

model at depth using TPM, future experiments will be designed to look at the effects of PDT on cellular and extracellular matrix remodeling in wound tissue.

7. ACKNOWLEDGMENTS

This work was supported by the Laser Microbeam and Medical Program (LAMMP), a National Institutes of Health Biotechnology Resource (P41RR01192, 2R01CA32248-15A1), the Office of Naval Research (ONR N00014-91-C-0134), the Department of Energy (DE-FG03-91ER61227).

8. REFERENCES

1. W. Denk, D. Piston, and W, Webb, "Two-photon molecular excitation in laser-scanning microscopy," Handbook of Biological Confocal Microscopy, New York: 445-458, 1995.
2. W. Denk, J. Strickler, and W. Webb, "Two-photon laser scanning fluorescence microscopy," *Science*, **248**: pp. 73-76, 1990.
3. R. Clark, Cutaneous Wound Repair, The Molecular and Cellular Biology of Wound Repair, New York: 576-601, 1996.
4. P. Hanninen, E. Soini, and S. Hell "Continuous wave excitation two-photon fluorescence microscopy," *Journal of Microscopy* **176**: 222-225, 1994.
5. K. Konig, P. So, W. Mantulin, B. Tromberg, and E. Gratton. "Two-photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress," *Journal of Microscopy* **183**, 197-204, 1996.