Two-Photon Imaging of Collagen Remodeling in RAFT Tissue Cultures

Vincent P. Wallace, Mariah L. Coleno, Tatsuro Yomo, Chung-ho Sun and Bruce J. Tromberg

Laser Microbeam and Medical Program (LAMMP), Beckman Laser Institute, University of California, Irvine, CA, 92612, USA

ABSTRACT

Tissue remodeling is associated with both normal and abnormal processes including wound healing, fibrosis and cancer. In skin, abnormal remodeling causes permanent structural changes that can lead to hypertropic scarring and keloid formation. Normal remodeling, although fast and efficient in skin, is still imperfect, and a connective tissue scar remains at the wound site[1]. As a result, methods are needed to optimize tissue remodeling *in vivo* in all cases of wound repair. Since fibroblast-mediated contraction of engineered 3-D collagen based tissues (RAFTs) represents an *in vitro* model of the tissue contraction and collagen remodeling that occurs *in vivo*[2], RAFT tissue contraction studies combined with two-photon microscopy (TPM) studies are used to provide information on ways to improve tissue remodeling *in vivo*. In the RAFT models discussed here, tissue contraction is modulated either by application of exogenous growth factors or photodynamic therapy. During tissue contraction, TPM is used to image changes in Collagen Type I fibers in the RAFT skin models. Tissues are imaged at depth at day 15 after modulation. TPM signal analysis shows that RAFT tissues having the highest collagen density have the fastest rate of decay of fluorescent signal with depth.

Keywords: Two-photon microscopy, collagen, fluorescence, matrix remodeling, photodynamic therapy, wound healing.

1. INTRODUCTION

Wound healing is a biochemical and cellular reaction that is triggered by tissue damage. The damage can be caused by a variety of agents including mechanical trauma, surgical intervention, and chemical and thermal burns. Once a wound has healed, scar-breaking strength only reaches 80% of normal skin, and wound failure remains a clinical problem. For optimal wound healing, infection must be prevented. Rapid wound closure and strong repairs are also essential.

Immediately after injury, a temporary fibrin clot forms to cover the denuded wound surface. Within hours of injury, a non-specific inflammatory response occurs whereby neutrophils and monocytes are recruited to the wound site. Neutrophils infiltrate into the wound site, clear any initial bacteria, and release cytokines that begin to activate local fibroblasts and keratinocytes. After 1-2 days, blood-born monocytes infiltrate into the wound site and differentiate into mature tissue macrophages, which are essential for proper wound healing. After 3-5 days, these macrophages phagocytose any remaining neutrophils. These macrophages also release cytokines that act on other cells at the wound site and orchestrate wound healing through tissue debridement, matrix synthesis and angiogenesis. If macrophage function is impaired, wound healing is severely disturbed. Wound reepithelialization begins several hours after wounding when leading edge keratinocytes crawl forward and cover the denuded wound surface. Reepithelialization is made easier by contraction of the underlying connective tissue, which brings the wound edges together. At this time, dermal fibroblasts at the wound edge begin to proliferate. 3-4 days later, these fibroblasts, which no longer exist in a quiescent state, migrate into the wound matrix, synthesize collagen and contract the wound. Tissue macrophages release growth factors including TGF-β, a multifunctional, multiregulatory ECM protein that activates fibroblasts and promotes fibroplasia (fibroblast migration, proliferation, and matrix production)[1]. Optimal wound contraction leads to smaller scar formation. Once the wound surface is covered by a monolayer of keratinocytes, epidermal migration ceases. The ability of the macrophage to mediate tissue repair suggests that therapeutic strategies used to modulate macrophage functions might effectively augment the repair process. Unfortunately, introducing macrophage derived factors to wounds has had limited success due to inappropriate dosage, delivery, or combination of the two. Stimulating macrophage function within the wound itself using PDT could get around some of these issues.

Multiphoton Microscopy in the Biomedical Sciences, Ammasi Periasamy, Peter T. C. So, Editors,
Proceedings of SPIE Vol. 4262 (2001) © 2001 SPIE · 1605-7422/01/$15.00

Photodynamic therapy (PDT), which utilizes light-excitable photosensitizers (PS) to produce singlet oxygen and free radicals, is commonly used as a cancer treatment[3]. PDT is able to destroy relatively large amounts of malignant tissue with a healing response that produces a good cosmetic result. PDT has also been shown to have effects that range from inactivation to stimulation of macrophage function. Thus when placed in the clinical context of wound repair, macrophage targeted PDT could lead to a variety of responses including stimulation of contraction, enhanced debridement activity, angiogenesis, and suppression of fibrotic tissue formation. Also since photosensitizers can target macrophages specifically, PDT can be used to selectively destroy unwanted tissue without destroying nearby healthy tissue.

2. METHODS

In order to study skin tissue remodeling *in vitro*, an artificial tissue model system, RAFT, was developed. RAFT tissues are made with easy repeatability, and TPM can be used to image RAFT tissue remodeling on a repeatable basis[4,5].

2.1 RAFT Model

The artificial tissue model, RAFT, consists of a basic polymerized collagen gel made of Type I rat-tail collagen (RTTC; Collaborative, Bedford, MA) containing primary human dermal fibroblasts and U937 human tissue macrophages. The collagen gel mix consists of 65% rat tail Type I collagen at a concentration of 4mg/ml in 0.02N glacial acetic acid (Collaborative Research), 20% 5X RPMI (Gibco) and 10% reconstitution buffer (containing 260mM sodium bicarbonate and 200mM HEPES buffer in 0.05N sodium hydroxide). The density of both cell types are adjusted to achieve the desired physiological or pathological condition during wound healing. Aliquots (0.9 ml) of the collagen mixture were pipetted into separate wells of a 24-well tissue culture plate (Costar, Cambridge, MA) with a growth area of 1.5 cm diameter. Collagen gels were allowed to "gel" (cross-link) in a humidified incubator at 37^{o} C with 7.5% CO_2 for 24 hours before the addition of 1ml of fresh RPMI growth medium. Primary human dermal keratinocytes can be applied to the top of the polymerized collagen gel. When the keratinocytes reach 100% confluency, each gel is released from the tissue culture plate and transferred into an imaging petri dish filled with 1ml of culture medium. The polymerized collagen gel containing human dermal fibroblasts, human macrophages and a confluent top layer of human dermal keratinocytes is cultured at the air-liquid interface for 14 days to achieve a full thickness epidermis with keratinocytes at terminally differentiated state on the top layers. The final RAFT tissue is represented in Fig. 1.

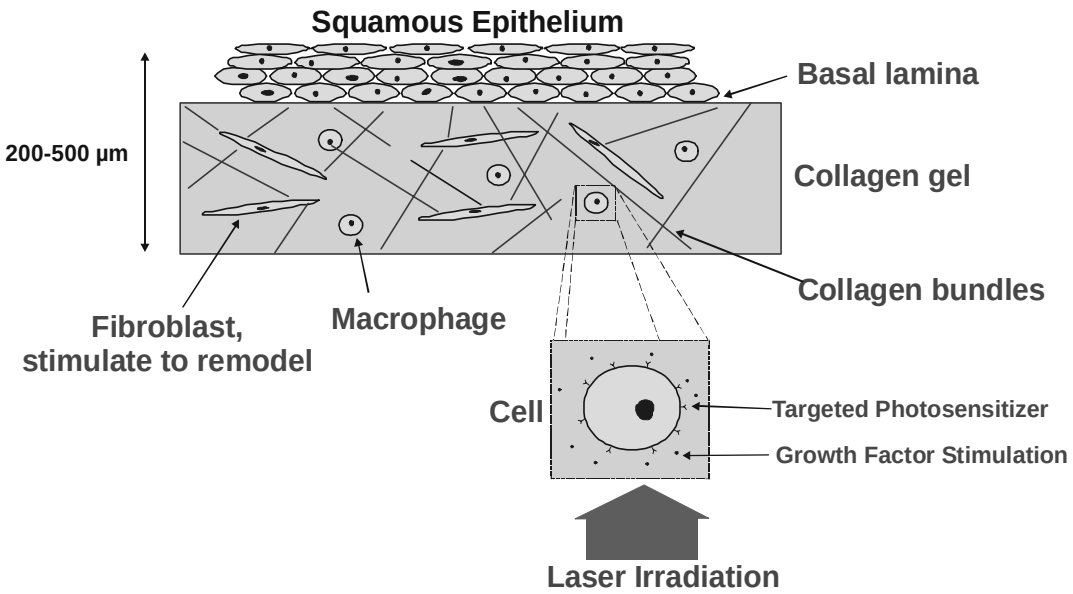

Figure 1. Diagram showing the composition of the artificial skin tissue model (RAFT) and photodynamic targeting of cells.

2.2 Photodynamic therapy

Experiments were conducted to study the effect of photodynamic therapy (PDT) on wound contraction during wound healing. Only RAFT tissue models utilizing a polymerized collagen gel containing human dermal fibroblasts at a concentration of $1X10^6$ cells/ml and human tissue macrophages at a concentration of $3X10^6$ cells/ml were used. Upon releasing each polymerized gel from its corresponding well, a floating experimental collagen gel system was created and used to elucidate the interaction between fibroblast and macrophage cells after PDT at various concentrations of photosensitizer and at different laser parameters.

PDT on the RAFT tissue was performed using the photosensitizer Protoporphyrin IX (PPIX). Initially the tissues were incubated for 3 hours with aminolaevulinic acid (ALA) at a concentration of 100mg/ml. ALA, once transported into the mitochondria of cells is converted to PPIX. After ALA incubation, the unincorporated ALA was removed by washing in phosphate buffered saline. The tissues were irradiated with 635nm CW laser light using an Argon pumped dye laser (Model CR-599, Coherent, Santa Clara, CA). The laser light was delivered through a 40mm multimode fiber, which was terminated with an uncoated microlens (PDT systems, Santa Barbara, CA). The microlens functioned to expand the beam to cover the tissue area (1.5cm diameter). Laser output power was determined using a multifunction optical meter (Model 1835C, Newport, Irvine, CA). The tissues were irradiated using a light dose of 100 J/cm^2. The power density was kept constant at $100mW/cm^2$.

2.3 Two-photon tissue imaging

The RAFT tissue was imaged using a two-photon laser-scanning microscope (TPM). The TPM system consists of a 5W Verdi laser (Coherent, Santa Clara, CA), which is used to pump a Mira 900F Titanium:Sapphire laser (Coherent, Santa Clara, CA). The mode-locked, 150 femtosecond, 76MHz pulse train exiting the Ti:Sapphire laser is used as the two-photon pulsed excitation source. The ultrafast Ti:Sapphire laser was tuned to 850nm, which we found to be the optimal wavelength for efficient two-photon imaging of collagen. 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 pulse train is deflected into the back port of a modified, inverted Zeiss Axiovert S100 2TV microscope (Zeiss, Thornwood, NY) using a PC controlled galvanometer driven X-Y scanner (series 603X, Cambridge Technology, Watertown, MA). Images are generated by raster scanning the x-y mirrors and thus the Ti:Sapphire laser beam across the sample. A custom digital card controls the scanner. Just inside the microscope, a scan lens and tube lens function together to expand and collimate the excitation beam to allow proper over-filling of the back aperture of the microscope objective lens. A Zeiss 63X, 1.2 N.A. water immersion objective having a working distance of 250 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. Two-photon images of the RAFT tissues were acquired on day 15 after PDT at 10 depths within the tissue (~30μm intervals). Each two-photon image was integrated 10 times (~1scan/sec.) and covers an area of 35μm x 35μm. Two-photon autofluorescence images were obtained of collagen using an SBG39 wide pass barrier filter (400-580nm) (CVI, Livermore, CA).

3. RESULTS

In Figure 2, photographs of three different RAFT tissues taken 10 days after treatment. These photographs clearly show differences in tissue contraction with photodynamic modulation and growth factor stimulation. The RAFT tissue containing exogenous TGF-β (left photograph) contracted the most to 20% of its initial area. The control RAFT that did not undergo treatment (center photograph) contracted to 40% of its initial area. The RAFT tissue that underwent PDT treatment (right photograph) contracted the least to 70% of initial area.

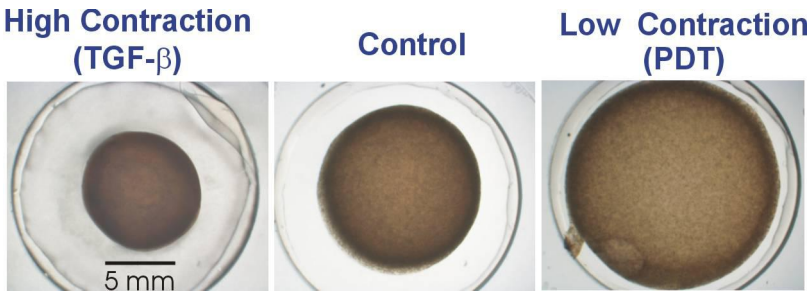

Figure 2. Photograph showing tissue contraction of three different RAFT tissues that have undergone different treatments (indicated above each photo).

Figures 3, 4, and 5 shows two-photon images taken of each of the three RAFT tissues at depth on day 15 after treatment. In each tissue, collagen fibrils are clearly fluorescing. In addition, the collagen autofluorescence intensity is shown to decay with depth with the signal dropping off fastest in the most contracted tissue (RAFT tissue containing TGF-β). For the control RAFT (Fig. 3), the two-photon collagen signal is no longer detectable at 220 um. For the most contracted RAFT, the tissue containing exogenous TGF-β (Fig. 4), the two-photon collagen signal is no longer detectable at 130 um. For the least contracted RAFT, the tissue that underwent PDT treatment (Fig. 5), the two-photon collagen signal is still detectable at 250 um, the working distance of the objective used. Each set of images also show clearly the different density of collagen fibers. By quantifying the density of fibers using image analysis it is possible to determine the effects of tissue modulation[5].

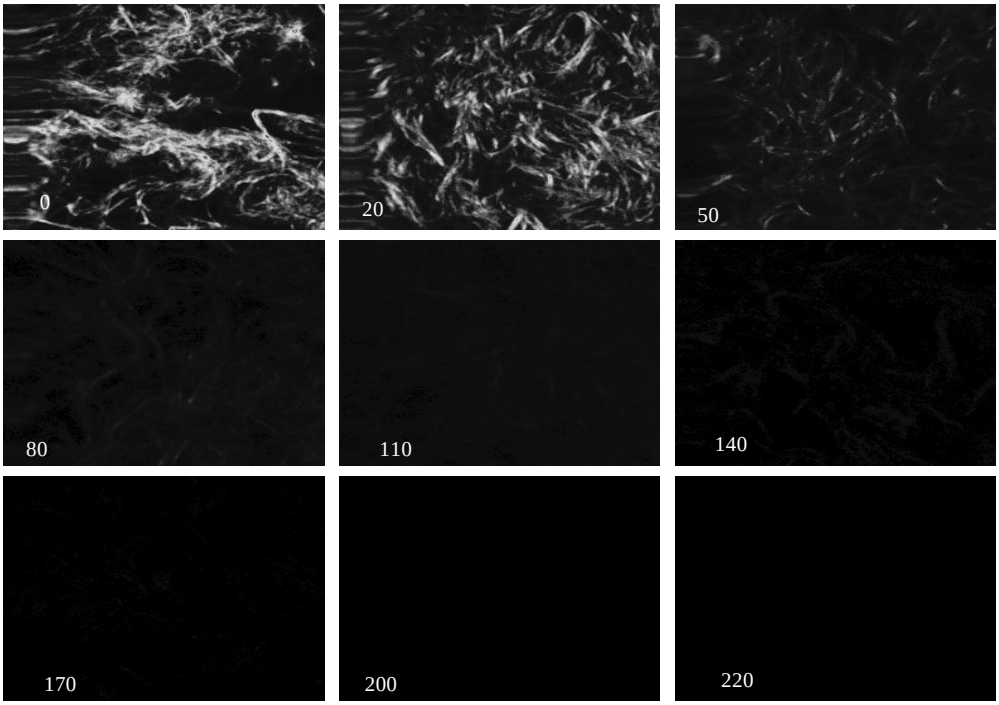

Figure 3. Two-photon fluorescence images of the control RAFT (no treatment) taken on day 15. Image depth in microns is indicated in the bottom left hand corner of each image. (Magnification: 63X. Image size: 35μm X 35μm).

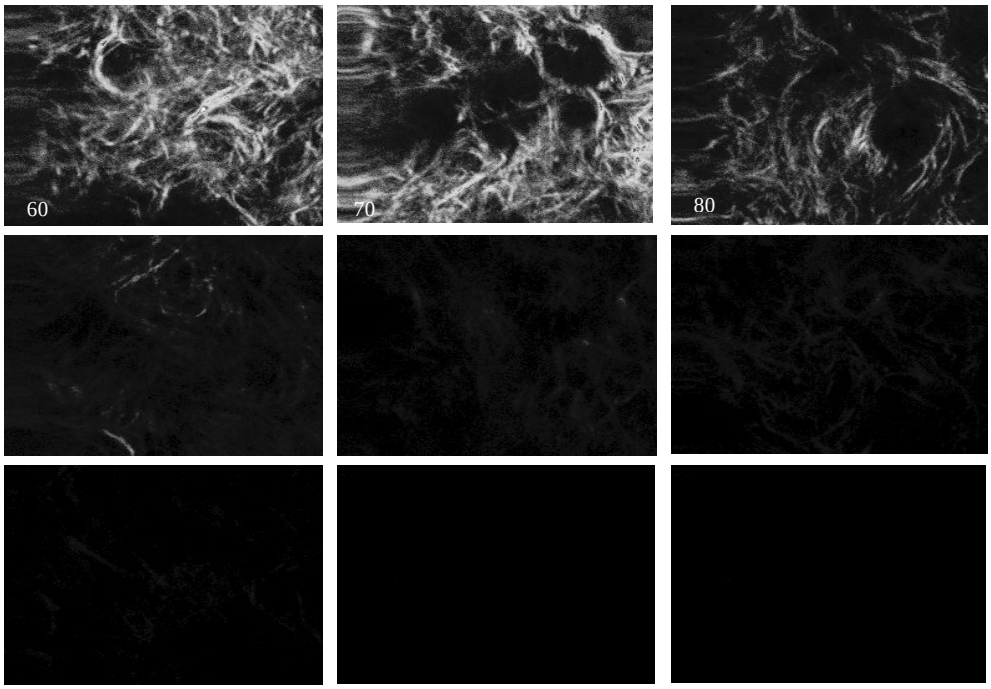

Figure 4. Two-photon fluorescence images of the RAFT tissue containing TGF-β at a concentration of 5ng/ml on day 15 after treatment. Image depth in microns is indicted in the bottom left hand corner of each image. (Magnification: 63X. Image size: 35μm X 35μm).

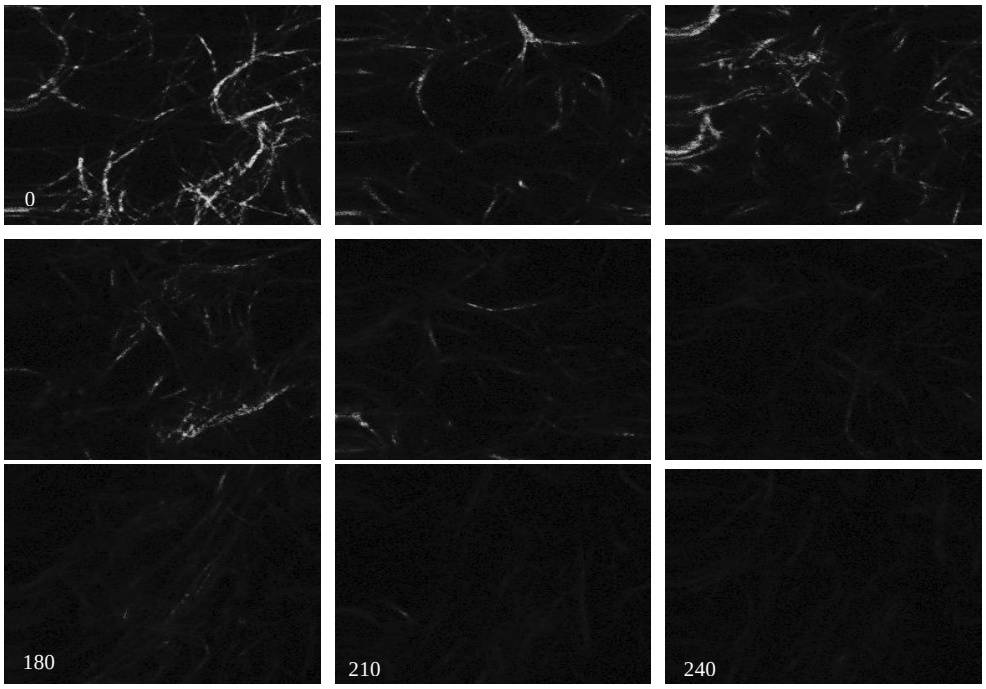

Figure 5. Two-photon fluorescence images of the RAFT tissue which underwent PDT taken on day 15 after treatment. Image depth in microns is indicted in the bottom left hand corner of each image. (Magnification: 63X. Image size: 35μm X 35μm).

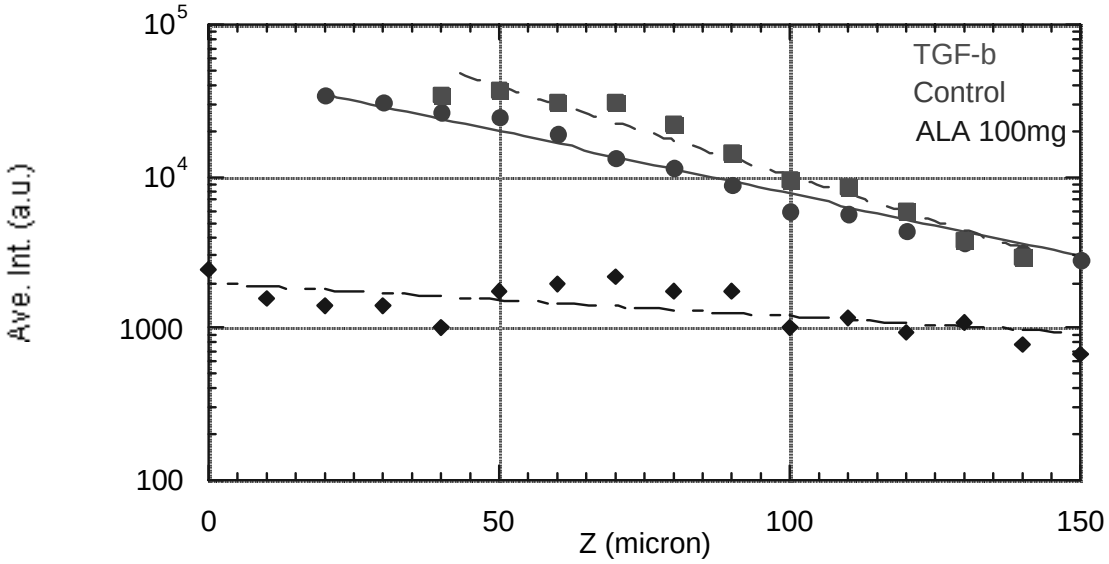

Figure 6. Graph showing the fluorescence exponential decay profiles for three different RAFT tissues: TGF-β RAFT (squares), control RAFT (circles), and PDT RAFT (diamonds).

In Figure 6 the collagen fluorescence intensity is plotted against depth for each RAFT tissue. This figure clearly shows that the RAFT tissue containing exogenous TGF-β (squares) has a steeper decay rate than the control RAFT (circles). The PDT RAFT tissue (diamonds) shows a decay rate, which is much less than the control. In all cases, the fluorescence intensity is shown to decay exponentially with depth, and the gradient of each line is fitted by least squares (Table 1). The fluorescent intensity decay with depth has been shown to be a function of the tissue optical properties[6].

Raft Treatment	**Decay Constant (cm^{-1})**
Control	18.5±0.5
PDT (ALA 100mg/ml)	17.0±2.5
TGF-β 5ng/ml	27.0±6.0

Table 1. Table showing the calculated exponential decay constants for four different RAFT tissues. All values were calculated using least squares analysis.

4. DISCUSSION

Fibroblast mediated contraction of an artificial tissue model, RAFT, represents an *in vitro* model of tissue contraction and extracellular matrix remodeling[2], processes that are vital to normal wound healing. Disruption of this process can result in impaired wound repair and changes to tissue architecture that lead to hypertropic scar formation. The RAFTs studied here were 3-D cultured models of basic human dermis. We were able to impede tissue contraction using PDT. We have utilized the unique abilities of two-photon microscopy to study these RAFTs by imaging autofluorescence at depth with submicron resolution. Two-photon microscopy also showed distinct differences in collagen density between each RAFT type. These results were consistent with the contraction of the RAFTs. It has been shown that the fluorescence signal decays more

rapidly the more contracted the RAFT tissue is with a correlation of r=0.97. This decay has been shown to be a function of the optical properties of the tissue being imaged[6]. The major contributor to the attenuation of the fluorescence signal is scattering, which is due to the collagen fibrils in the RAFT. Scattering is a direct result of tissue structure, hence the decay constants may be used to provide information on collagen remodeling and wound strength. The absorption bands of collagen are situated in the deep UV; evidence that 850nm or greater wavelengths are preferred to view collagen fibers under two-photon excitation suggests that there are other higher order effects contributing to the signal.

Future experiments should include the monitoring of the effects of PDT on incisional wounds with different widths and depths into tissue with or without the epidermis. The effect on wound closure will be monitored by studying diameter changes in rafts and the increased fluorescence of collagen bundles. The possible facilitated migration and proliferation of basal layer keratinocytes into the closed wound surface will also be monitored

5. CONCLUSIONS

TPM has been shown to be useful for imaging cells and collagen in tissue matrix; the signal decay can be used to quantitatively assess collagen density. PDT delays contraction, suppresses TGF-β release which leads to reduced collagen synthesis and could be potentially applied to the reduction of scar formation

6. ACKNOWLEDGMENTS

This work was supported by the Laser Microbeam and Medical Program (LAMMP) funded by the National Institutes of Health (P41RR-01192), the Office of Naval Research (N00014-91-C-0134), and Department of Energy (#DE-FG03-91ER61227).

7. REFERENCES

1. P. Martin. “Wound healing - aiming for perfect skin regeneration” *Science* **276** pp 75-81 (1997).
2. R. Montesano, and L. Orci, "Transforming growth factor β stimulates collagen-matrix contraction by fibroblasts: Implications for wound healing," *Proc. Natl. Academy of Sciences, USA*, p. 4894-4897, July 1988.
3. B. Henderson and T. Dougherty. “Photodynamic therapy: Basic principles and clinical applications. New York: Marcel Dekker, 1992.
4. M. Coleno, V. Wallace, C. Sun, A. Dunn, M. Berns, B. Tromberg. “Two-Photon Excited Imaging of Photosensitizers in Tissues.” SPIE Proceedings **3604**: p. 67-73. 1999.
5. A. Agarwal, V. Wallace, M. Coleno, W. Wu, C. Sun, B. Tromberg, and S. George, "Two-photon laser scanning microscopy of epithelial cell-modulated collagen density in engineered human lung tissue," Tissue Engineering, In press.
6. A. Dunn, V. Wallace, M. Coleno, B. Tromberg. “Influence of Optical Properties on Two-Photon Fluorescence Imaging in Turbid Samples” *Applied Optics* **39** (7) pp 1194-120.12000.