Applications of Two-Photon Fluorescence Microscopy in Deep Tissue Imaging

C. Y. Dong[a], B. Yu[b], L. Hsu[a], P. Kaplan[c], D. Blankschstein[b], R. Langer[b], and P. T. C. So[a]

[a]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA
[b]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA
[c]Univerlever Edgewater Laboratory, Edgewater, NJ

ABSTRACT

Based on non-linear excitation of fluorescence molecules, two-photon fluorescence microscopy has become a significant new tool for biological imaging. The point-like excitation characteristic of this technique enhances image quality by the virtual elimination of off-focal fluorescence. Furthermore, sample photodamage is greatly reduced because fluorescence excitation is limited to the focal region. For deep tissue imaging, two-photon microscopy has the additional benefit in the greatly improved imaging depth penetration. Since the near-infrared laser sources used in two-photon microscopy scatter less than their UV/glue-green counterparts, in-depth imaging of highly scattering specimen can be greatly improved. In this work, we will present data characterizing both the imaging characteristics (point-spread-functions) and tissue samples (skin) images using this novel technology. In particular, we will demonstrate how blind deconvolution can be used further improve two-photon image quality and how this technique can be used to study mechanisms of chemically-enhanced, transdermal drug delivery.

Keywords: Two-photon, fluorescence, microscopy, deep-tissue, imaging

1. INTRODUCTION

In two-photon fluorescence microscopy, molecular excitation of fluorescent molecules is caused by the absorption of two near-infared (IR) photons. Popularized in 1990 by the Webb group, the technique has become a powerful tool in examining biological specimen[1]. As a novel microscopic imaging technique, two-photon fluorescence microscopy has several significant advantages compared to conventional technology. Since two-photon excitation requires the interaction of two near-IR photons with the fluorescent molecule, high incident photon flux is required for efficient two-photon excitation. As a result, two-photon absorption is only likely to occur near the focal volume of a microscopic objective where the excitation photons are confined spatially to induce molecular absorption. Therefore, fluorescence imaging using this technology results in much superior image contrast since off-focal fluorescence is virtually eliminated. The point-like excitation volume of two-photon (or higher order excitation) fluorescence microscopy also confine excitation-induced photodamage to near the focal volume. Furthermore, since Rayleigh scattering probability is inversely proportional to the fourth power of the wavelength[2], the redder photons used for two-photon excitation can penetrate deeper into multiply scattering samples such as the tissue than the UV, blue, or green photons used for one-photon microscopy. It has been demonstrated, for example, that multiphoton imaging can penetrate biological specimen at least twice deeper than confocal imaging[3]. Finally, since the near-IR light source used for two-photon excitation is well separately spectrally from the fluorescence emission, the entire fluorescence spectrum can be well studied using two-photon excitation.

In this paper, we present two-photon data characterizing the PSF, blind deconvolved skin images, and monitoring of transdermal drug delivery. Our results show that two-photon fluorescence microscopy is

In *Optical Sensing, Imaging, and Manipulation for Biological and Biomedical Applications*, Robert R. Alfano, Ping-Pei Ho, Arthur E. T. Chiou, Editors, Proceedings of SPIE Vol. 4082 (2000) • 0277-786X/00/$15.00

a powerful technique for studying both the physiological structure and transport characteristics of tissue. With further development, two-photon fluorescence microscopy potentially can be developed into an effective medical instrument at the cellular level for non-invasive, in vivo diagnosis of diseases such as skin cancer.

1.1 Two-Photon Excitation of Fluorescent Molecules

One-photon and two-photon excitation processes have different mathematical forms and physical interpretation. The two processes are demonstrated in Fig. 1[4].

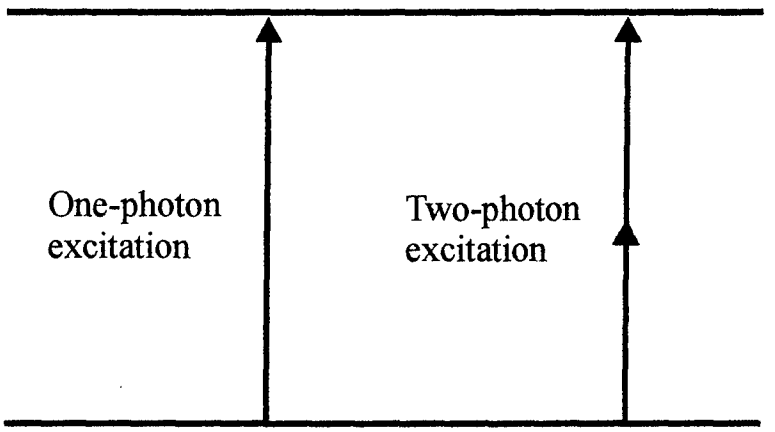


Fig. 1: One- and Two-photon excitation

In one-photon absorption, a molecule absorbs one photon whose energy matches the transition energy between the molecule's ground and excited states. The transition probability is

$$\mathbf{P}_{1\gamma} \sim\sim \mathbf{I}\left|\mathbf{e}\cdot\langle \mathbf{f}|\mathbf{r}|\mathbf{i}\rangle\right|^2 \tag{1}$$

where **r** is the position operator, **e** is the electromagnetic polarization vector, **I** is the excitation intensity, **i** and **f** represent the initial and final states, respectively.

The two-photon absorption process is mathematically represented by

$$\mathbf{P}_{2\gamma} \sim \mathbf{I}^2\left|\sum_{\mathbf{m}}\frac{\mathbf{e}\cdot\langle \mathbf{f}|\mathbf{r}|\mathbf{m}\rangle\langle \mathbf{m}|\mathbf{r}|\mathbf{i}\rangle\cdot\mathbf{e}}{\mathbf{E}_{\mathrm{mi}}-\mathbf{E}}\right|^2 \tag{2}$$

where **m** represents the intermediate state, **E** is the energy of the photon, and $\mathbf{E}_{\mathrm{mi}}$ is the transition energy between the intermediate and initial states. In this mode of interaction, the molecule is interpreted to absorb the two redder photons in sequential steps. One such photon is absorbed and the molecule is taken from the initial state **i** to the intermediate state **m**. At almost the same time, the molecule absorbs the second red photon and reaches the final state **f** from **m**. Since two photons are involved in the absorption process, the excitation probability is proportional to the square of excitation intensity, the origin of the non-linear nature of this process. As to the detection of the intermediate state **m**, one can estimate its lifetime using the uncertainty principle relating the lifetime τ to the energy spread $\Delta\mathbf{E}$ by

$$\Delta\mathbf{E}\tau \sim \hbar \tag{3}$$

where $\hbar$ is Planck's constant. Assuming an uncertainty in the intermediate state energy to be approximately that of a typical fluorescent photon 500 nm in wavelength, the intermediate state only has a lifetime of approximately 0.3 fs, a time too short for realistic detection[5,6].

1.2 Skin as a Deep Tissue Sample

Skin is a tissue sample which two-photon fluorescence microscopy has been applied in studying. The structure of the skin is shown in Fig. 2. In short, the surface of the skin is composed of the epithelium. The basal layer represents the germinating layer from which the epithelial cells are generated. Cells from the basal layers divide and as they divide, the cells migrate toward the skin surface. Structurally, these migrating cells flatten as they approach the skin surface. The outer most layer of the epithelium is the stratrum corneum, a layer of structure which forms the protective layer against the environment. Beneath the epithelium layer is the dermis, composed of filamentous structure[7].

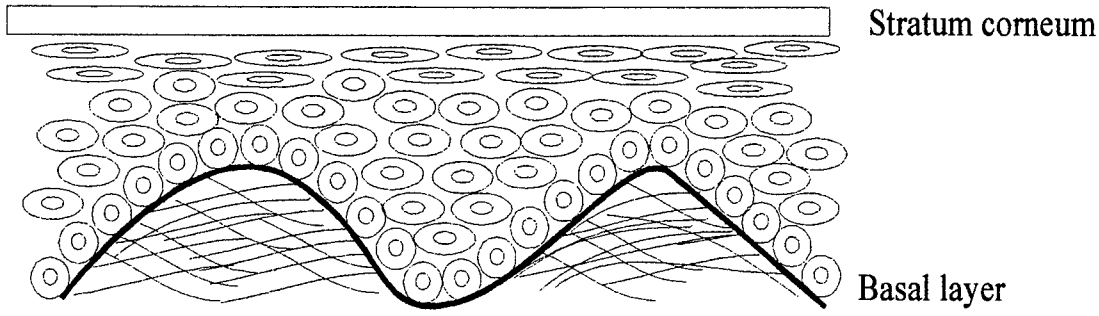


Fig. 2: Structure of the skin

2. EXPERIMENTAL APPARATUS

2.1 Laser Sources for Two-Photon Excitation

For efficient two-photon excitation, photons need to arrive at the sample within a narrow time window. Therefore, lasers with short pulse widths are natural choices for two-photon microscopy. In the commercial market, the titanium-sapphire (ti-sa) laser with pulse duration around 100 fs satisfies this temporal requirement. The titanium-sapphire systems can be pumped by an argon-ion laser (488/514nm) or frequency-doubled, diode pumped Nd-doped crystals (532 nm). These femtosecond sources can generate pulse trains at approximately 80 MHz. In addition, the high lasing bandwidth (700-1000 nm) of the ti-sapphire lasers make them versatile light sources for two-photon microscopy. In addition to the ti-sa lasers, other femtosecond sources such as the Cr:LiSAF and Nd-YLF (pulse compressed) lasers can be used for two-photon excitation[8,9].

In addition, picosecond and continuous-wave (cw) lasers can also be used for two-photon excitation. Mode-locked Nd-YAG with pulse width of 100 ps, dye lasers with pulse duration of around 1 ps, and picosecond ti-sa lasers are possible excitation sources. The 647 nm output of a cw ArKr laser has been used to image DAPI and bisbenzimidazole Hoechst 33342 labeled nuclei[10]. The commonly available 1064 nm output of the cw Nd-YAG laser can also be used for non-linear excitation of fluorescent samples.

2.2 Experimental Set-up of a Two-Photon Fluorescence Microscope

The experimental arrangement for a typical two-photon fluorescence microscope is shown in Fig. 3. A femtosecond ti-sa laser is shown to be the excitation source but other excitation sources discussed in the previous section can also be used for sample excitation.

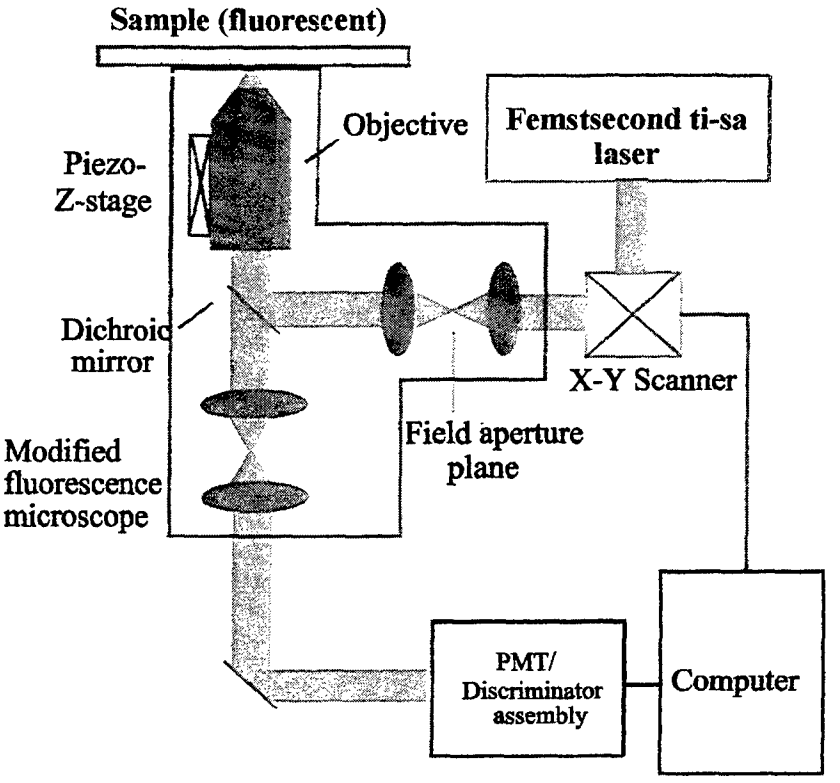


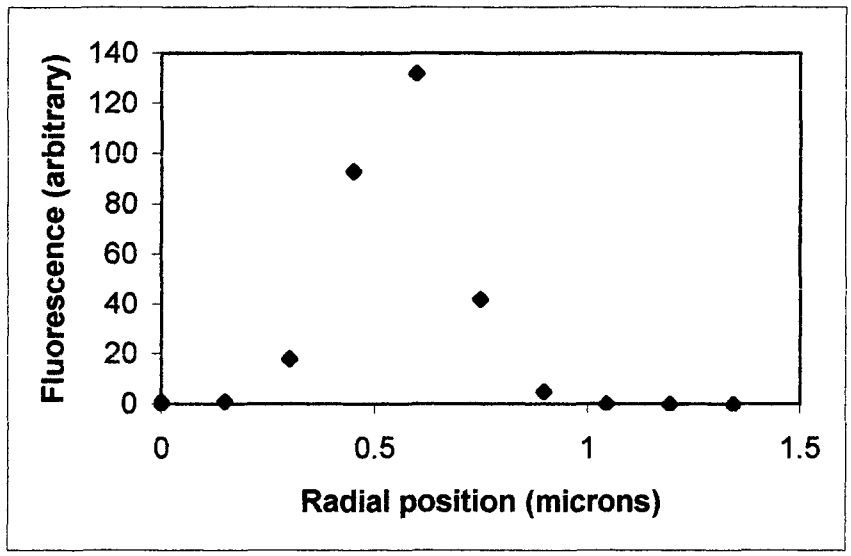
Fig 3: A two-photon fluorescence microscope

The output of the ti-sa laser passes through an x-y scanner prior to entering the modified fluorescence microscope. In out experience, 780 nm output of the ti-sa is sufficient to excite a wide range of fluorophores and is a very useful wavelength. The laser beam then passes a pair of beam expanding lenses where the beam diameter is enlarged to ensure overfilling of the microscope objective's back aperture. To ensure high image resolution, microscope objectives with high numerical aperture (NA) are frequently used. An example is the Zeiss Fluar 40x objective with NA of 1.25. A dichroic reflects the excitation laser into the microscope objective. The angular deviations of the scanning mirrors translate into linear positioning of the focused laser spot on the fluorescent sample. A typical x-y scan is composed of 256x256 pixels. In depth positioning of the focused laser spot on the specimen is achieved by a piezo-driven objective positioner. The fluorescence generated from the two-photon spot is collected by the same microscope objective, passes through the dichroic, and then onto the photomultiplier detector and detection electronics. A commonly used detection scheme involves the use of a discriminator for single photon counting analysis. A computer controls the movement of the laser spot at the sample and also records the incoming fluorescent photons for image analysis.

3. RESULTS

3.1 Two-Photon Point-Spread Functions (PSF)

The spatial resolution in the point-scanning mode of two-photon fluorescence microscopy is determined by the point-spread-function (PSF). Shown in Fig. 4 are the radial and axial PSF's acquired using 0.1 μm fluorescent spheres. The objective used for the PSF measurements was the oil immersion Zeiss 63x Plan Neofluar (NA 1.25).These fluorescent spheres are imbedded in 2% agarose gel and the two-photon microscope is used to acquire a 3-D scan of the spheres.



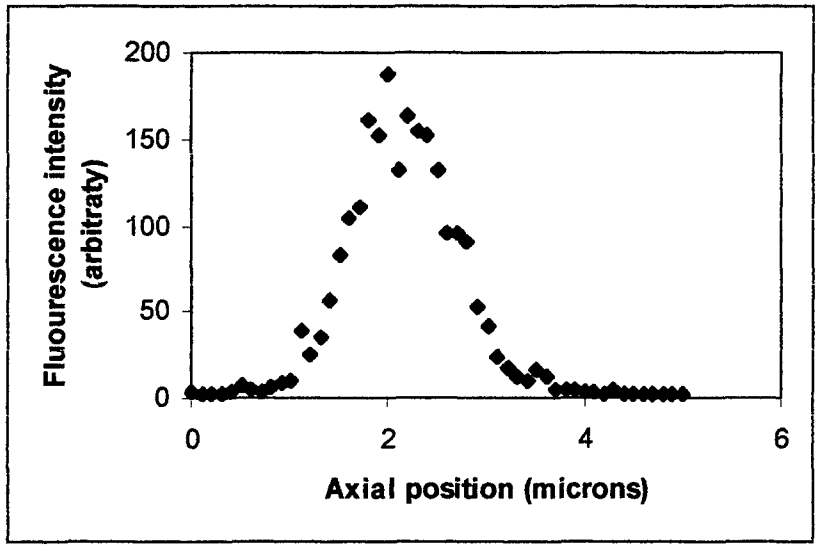


Fig. 4: Radial (top) and axial (bottom) PSF's near the focal plane of the two-photon focal spot, as determined from measuring the intensity of 0.1 micron fluorescent spheres.

From Fig. 4, one can estimate the full width at half maximum (FWHM) of the PSF along the radial and axial coordinates and they turn out to be about 0.3 and 1.2 microns, respectively. Compared to the theoretical results of 0.23 and 1.6 microns, our results compare favorably[11].

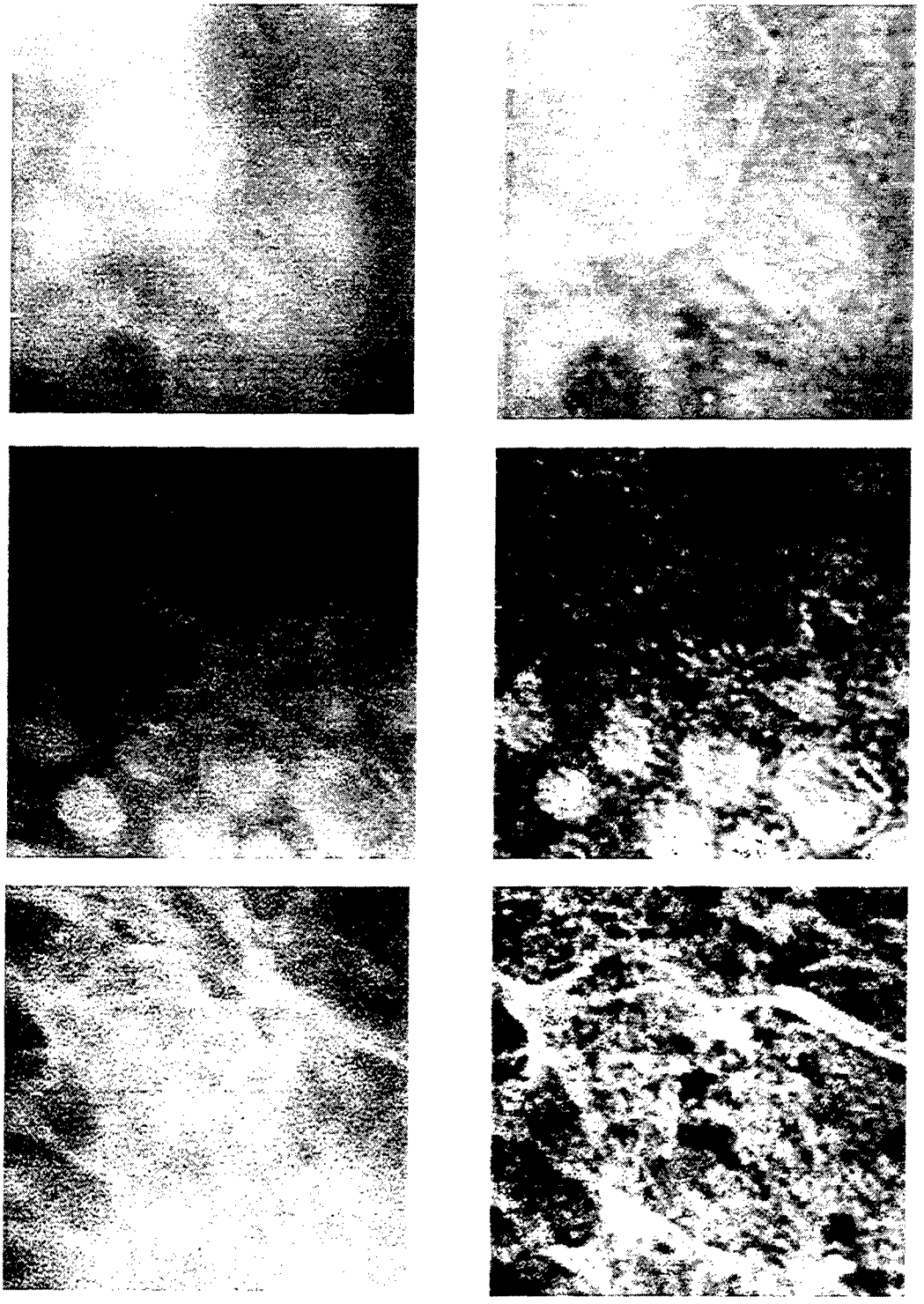


Fig. 5: Raw (left) and post blind deconvolution (right) images of three skin (human) layers. Top: stratum corneum, middle: basal layer, bottom: dermal fiber.

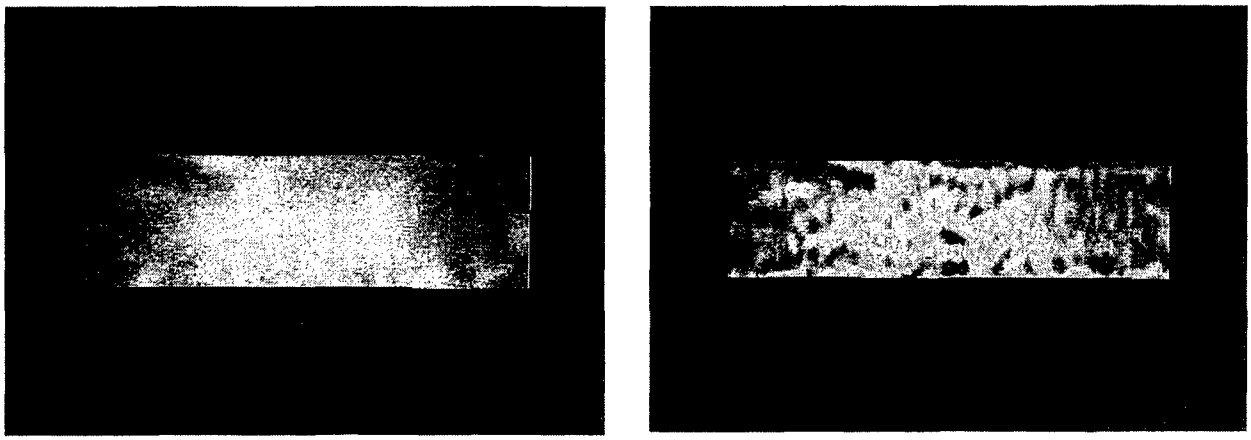


Fig. 6 Raw (left) and blind deconvoluted (right) axial images of the skin (human).

3.2 Skin Image Enhancement by Blind Deconvolution

A common technique used in our lab is to apply blind deconvolution algorithm for further improvement in image resolution. We use the software AutoDeblur™ (AutoQuant, Watervliet, NY) for such image processing. This deconvolution algorithm is based on maximum likelihood approach[12]. Fig. 5 shows both the raw image and deconvoluted images of three different axial planes inside the human skin sample. The surface stratum corneum, the basal layer, and the dermal fibers were all imaged and deconvoluted. In all three cases, the deconvoluted images were sharper and showed finer details than the raw images. For the stratum corneum and the basal layers, the granular nature in the structure is much more apparent. And in fibrous layer, the boundaries of individual fibers were much more apparent. Fig. 6 shows an axial section of the raw and blind deconvoluted results. Once again, structures that were fuzzy in the raw data set show up much sharper after deconvolution.

3.3 Modeling of Transdermal Drug Delivery

Due to the non-invasive nature of two-photon imaging, the technique is ideal for studying the process of transdermal drug delivery. In particular, two-photon microscopy can help to elucidate the method by which the transport pathways are altered under different chemical delivery conditions. To model drugs with different chemical properties delivered through the skin (human), fluorescent dyes with different chemical properties can be used. For example, 1,1'-dioctadecyl-5,5'-diphenyl-3,3,3',3'-tetramethyl indocarbcyanine chloride can be used as a lipoliphilic model drug under different chemical delivery environment. Fig. 7 shows the delivery of the lipophilic drug, in the presence and absence of the chemical enhancer oleic acid. The model drug delivery solution is kept in contact with the skin until equilibrium is reached. When the delivery medium is composed of PBS (buffer) and ethanol, there is low fluorescence counts at the skin surface and the fluorescence gradient across the skin is small. However, when the delivery medium contains 5% oleic acid, the fluorescence counts at the surface is much higher (by about at least 5 times) and fluorescence through the skin depth examined is greater. Furthermore, the fluorescence gradient increased by about at least 3 times near the skin surface (within about 10 μm). Two facts are indicated by these results. First, the generally higher fluorescence throughout the skin treated with oleic acid indicate that oleic acid most likely increased the membrane fluidity of the skin and that permits the dye to get through the skin easier. Secondly, the larger fluorescence gradient, in the presence of oleic acid, indicates that the flux across the skin is higher, and more of the model drug is delivered across the skin. For hydrophilic molecules, the transport mechanism is quite different. Fig. 8 shows the results for a hydrophilic probe sulfonerhodamine bis-(PEG 2000). In this case, the surface fluorescence intensity didn't change much with and without oleic acid but the intensity gradient is much more significant in the presence of oleic acid. This indicates that oleic acid affects the transport pathway and not the fluidization of membrane and it is the enhanced gradient that is responsible for molecular transport.

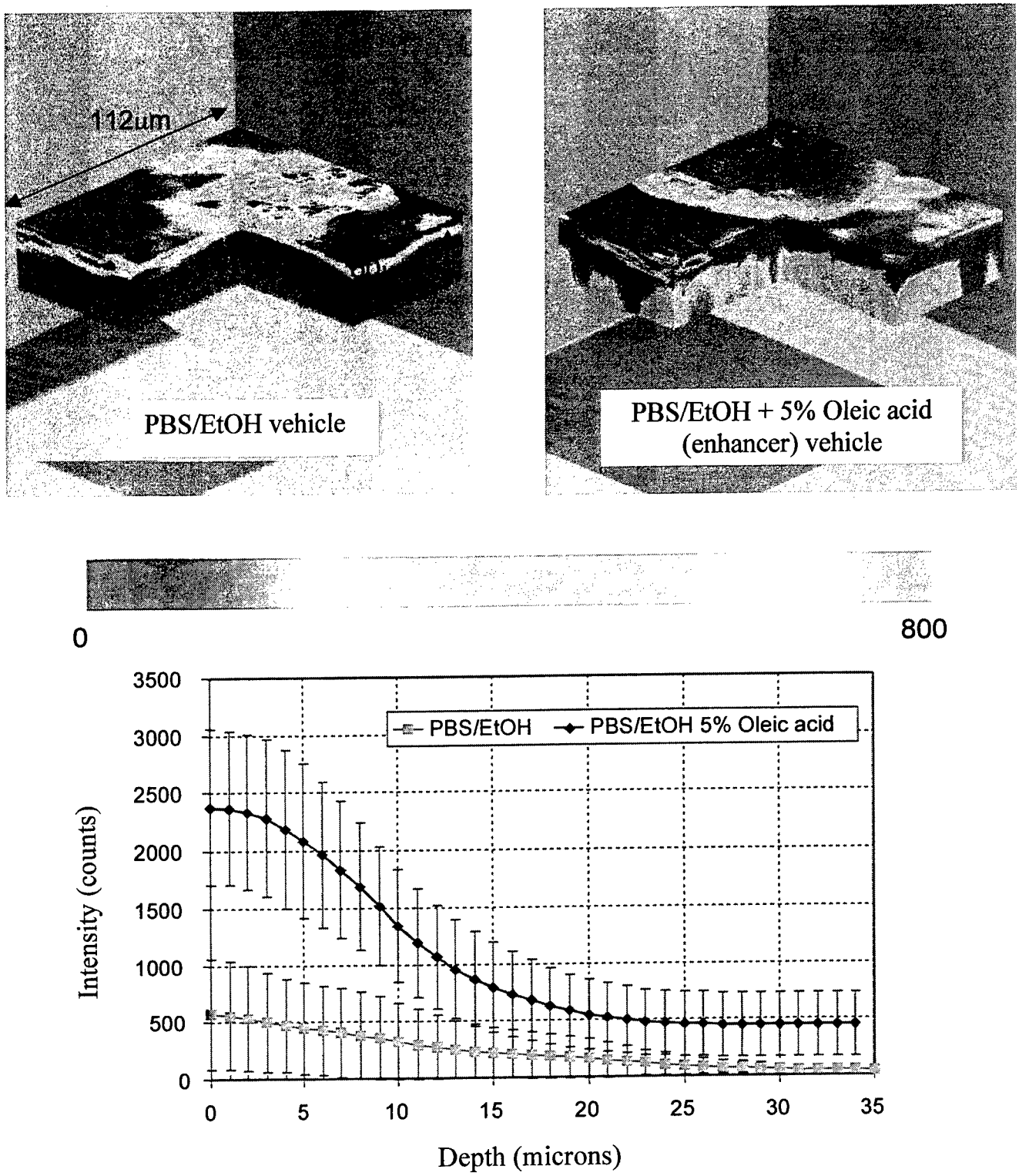
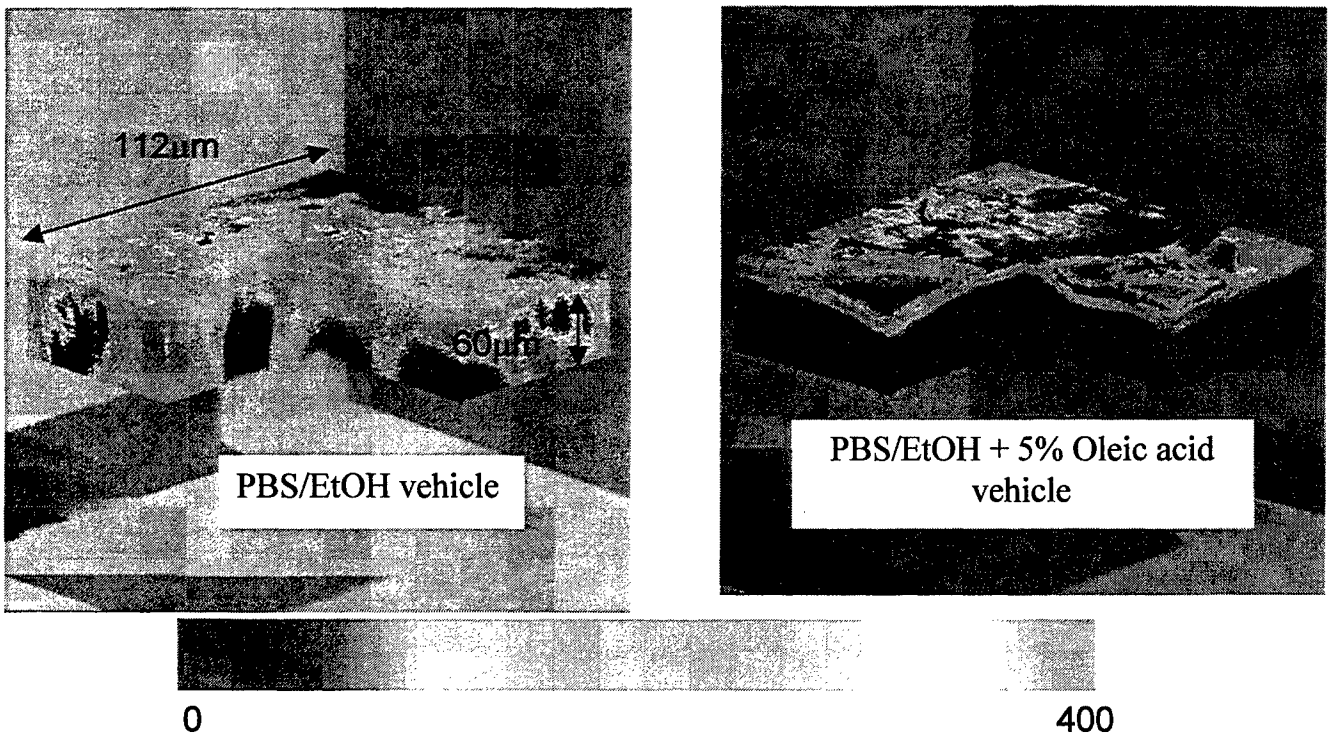


Fig. 7: Effect of chemical enhancer (oleic acid) on the penetration of lipophilic model drug (1,1'-dioctadecyl-5,5'-diphenyl-3,3,3',3'-tetramethyl Indocarbcyanine chloride) across skin (human).



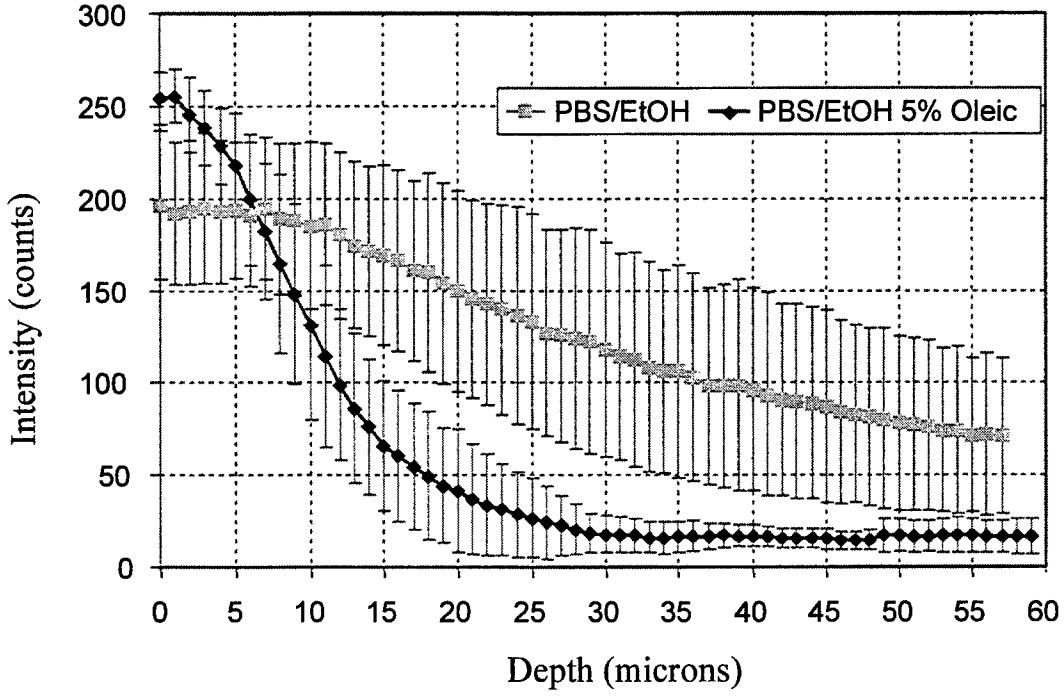


Fig. 8: Effect of chemical enhancer (oleic acid) on the penetration of hydrophilic model drug sulfonerhodamine bis-(PEG 2000) across skin (human).

4. CONCLUSION

In this work, we have demonstrated how two-photon fluorescence microscopy can be used as a powerful experimental tool. Point-like excitation and reduced scattering of excitation light source makes this technique ideal for deep tissue imaging. We have shown that the two-photon PSF obtained experimentally compares favorably with the theoretical predictions. Furthermore, it has been shown that blind deconvolution can be used to furthermore improve image quality of skin at the stratum corneum, basal layer, and fiber level. Finer details in the skin structure boundary separation are revealed after the raw images have been post-processed under blind deconvolution. In addition, two-photon fluorescence microscopy is useful in revealing the effects of chemical enhancer on model drug delivery across the skin. In the presence of oleic acid, both the lipophilic and hydrophilic model drug's gradients across the skin are greater.

Fluorescence microscopy based on two-photon excitation is a useful technique for studying deep tissue process non-invasively. With further development, this technology can become a major diagnostic tool for clinical applications.

5. REFERENCES

1. W. Denk, J. H. Strickler, and W. W. Webb. "Two-photon laser scanning fluorescence microscopy," *Science*, **248**, pp. 73-76, 1990.
2. J. D. Jackson. *Classical Electrodynamics*. John Wiley & Sons, New York. 1975.
3. V. E. Centonze and J. G. White. "Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging," *Biophysical Journal*, **75**, pp. 2015-2024, 1998.
4. J. R. Lakowicz. *Principles of Fluorescence Spectroscopy*. Kluwer Academic/Plenum Publishers, New York, 1999.
5. W. M. McClain and R. A. Harris. "Two-photon spectroscopy in liquids and gases," in *Excited States 3*, E. C. Lim, ed., pp. 1-56, Academic Press, New York, 1977.
6. C. Xu and W. W. Webb. "Multiphoton excitation of molecular fluorophores and nonlinear laser microscopy," in *Topics in Fluorescence Spectroscopy Vol. 5*, J. R. Lakowicz ed., pp. 471-540, Plenum Press, New York, 1997.
7. K. H. Kim, P. T. C. So, I. E. Kochevar, B. R. Maters, ad E. Gratton. "Two-photon fluorescence and confocal reflected light imaging of thick tissue structures," SPIE Proceedings, **3260**, pp. 46-57, 1998.
8. D. L. Wokosin, V. E. Centonze, and J. G. White. "Multi-photon Excitation Imaging with an All-Solid-State Laser, *SPIE Proceedings* **2678**, pp. 38-49, 1996.
9. D. L. Wokosin, V. E. Centonze, J. G. White, D. Armstrong, G. Robertson, and A. I. Ferguson. "All-solid-state ultrafast lasers facilitate laser multiphoton excitation fluorescence imaging," *IEEE Journal of Selected Topics in Quantum Electronics*, **2** (4), pp. 1051-1065, 1996.
10. S. W. Hell, M. Booth, and S. Wilms. "Two-Photon Near- and Far-Field Fluorescence Microscopy with Continuous-Wave Excitation," *Optics Letters*, **23**(25), pp. 1238-1240, 1998.
11. C. Y. Dong, P. T. C. So, C. Buehler, and E. Gratton. "Spatial resolution in pump-probe microscopy," *Optik*, **106**, pp. 7-14, 1997.
12. T. Holmes. "Blind deconvolution of quantum-limited incoherent imagery: maximum-likelihood approach," *J. Opt. Soc. Am. A*, **9** (7), pp. 1052-1061, 1992.