Simultaneous Optical Coherence and Multiphoton Microscopy of Skin-Equivalent Tissue Models

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ABSTRACT

Three-layer skin-equivalent models (rafts) were created consisting of a collagen/fibroblast layer and an air-exposed keratinocyte layer. Rafts were imaged with a tri-modality microscope including optical coherence (OC), two-photon excited fluorescence (TPEF), and second harmonic generation (SHG) channels. Some rafts were stained with Hoechst 33343 or rhodamine 123, and some were exposed to dimethyl sulfoxide (DMSO). OC microscopy revealed signal in cell cytoplasm and nuclear membranes, and a characteristic texture in the collagen/fibroblast layer. TPEF showed signal in cell cytoplasm and from collagen, and stained specimens revealed cell nuclei or mitochondria. There was little SHG in the keratinocyte layer, but strong signal from collagen bundles. Endogenous signals were severely attenuated in DMSO treated rafts; stained samples revealed shrunken and distorted cell structure. OC, TPEF, and SHG can provide complementary and non-destructive information about raft structure and effect of chemical agents.

Keywords: dimethyl sulfoxide, fluorescence imaging, image contrast, optical coherence tomography, multiphoton microscopy, skin

1. INTRODUCTION

1.1 Skin equivalents

Organotypic skin-equivalent tissue models, or rafts, offer an alternative to *in vivo* systems for studying the effects of chemical compounds and therapies. These models have the same major structural layers as skin: a "dermis" consisting of fibroblasts suspended in a collagen matrix, an "epidermis" consisting of a layer of stratified keratinocytes, and a superficial kertin layer [1]. The thickness of the keratinocyte and keratin layers increases during the maturation process, thus can be controlled to the thickness desired. These rafts are versatile; the cell types can be varied to study pathologies (e.g. keloid formation [2]). They may also be used to study response to insults such as cuts, laser irradiation, and application of irritants. The rafts may be immersed in a test solution or the solution can be applied to the keratinized surface only.

1.2 Skin equivalent imaging with OCT

Ultrahigh resolution optical coherence tomography (OCT) with an 800 nm center wavelength, 83 nm bandwidth femtosecond laser source has previously been used to visualize skin equivalents. Spoler et. al [3] showed that the keratin and keratinocyte layer thicknesses could be measured with high accuracy, and that both had relatively low backscattering. The boundary between the two was seen as a high-signal band, due to changes in the index of refraction of these two layers. The keratin layer itself showed strong variations in signal intensity, attributed to air pockets in the loose structure. The basement membrane zone was evident as a low-signal band. This signal dip was explained as the result of collagen type 4 in the lamina densa, which forms a fine meshwork and may backscatter less efficiently than the fibular collagen 1 of the epidermis and dermis. Additionally, the lamina lucida mainly consists of small-size extracellular glycoproteins and transmembrane glycoproteins which may similarly present low backscattering. The dermal layer was

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relatively highly backscattering. No structures were visible within layers, due to the limited resolution of the system (3 μm axial in tissue x 10 μm lateral), which is too coarse for visualizing cells or collagen bundles.

1.3 Skin equivalent imaging with multiphoton microscopy

Two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) microscopy, and separate, standard-resolution OCT of thermally injured skin equivalents was performed by Yeh et al. [4]. The OCT system used a 1300 nm center wavelength, and had 15 μm axial and 10 μm lateral resolution. The MPM system utilized a 150 fs, wavelength tunable Ti:Sapphire laser source. In native rafts, the keratinocyte layer was not easily distinguishable from the collagen/fibroblast layer on OCT, due to the limited resolution, and perhaps the longer wavelength than in the Spoler study [3]. OCT signal from the dermis was relatively high in native tissue and relatively low in thermally coagulated tissue. MPM signal was nearly absent from the thermally denatured region, except for some cellular debris. The loss of signal is attributed to transition from an ordered array of molecules with a characteristic length comparable to the probe laser wavelength, to randomly oriented collagen molecules with a characteristic length much less than the probe laser wavelength. Between two and seven days post treatment, the investigators visualized fibroblast migration and remodeling of collagen fibers in the injured region, evidenced by return of OCT signal, SHG signal from collagen, and TPEF from fibroblasts. This study showed the complimentary nature of the three modalities. The ability to acquire all types of images simultaneously, in the same plane and with higher resolution in the optical coherence channel, would be desirable.

A tri-modality system similar to that used in this study (OC, TPEF, SHG microscopy) was developed and used to image a dermal model (collagen and fibroblasts only) by Tang et al. [5]. With micron-scale resolution and careful co-registration, the investigators obtained images of cells suspended in the collagen matrix. SHG signal was seen from the matrix but not the cell body. Bright but non-uniform TPEF was seen throughout the cell body but little was seen from the matrix. OC signal was seen from both regions, including from some matrix not visualized with SHG. The OC signal showed complex patterns, which may be due to subcellular features or to speckle.

1.4 Goals of this study

OC, TPEF, and SHG microscopy can provide complementary information about skin equivalent models without the need for destructive processing. In this study, we performed tri-modality imaging of complete skin equivalent models, including both a fibroblast/collagen layer and a stratified keratinocyte layer. The first goal was to characterize the signals obtained at each layer of the model. Secondly, we subjected some models to immersion or topical application of dimethyl sulfoxide (DMSO) to determine the effect of this drug delivery and optical clearing agent on the signal from each of the modalities.

2. MATERIALS AND METHODS

2.1 Skin-equivalent models

Twelve skin-equivalent models (rafts) were created containing a fibroblast/collagen layer and an air-exposed keratinocyte layer. Fibroblasts (10^5/ml) were suspended in a collagen matrix consisting of type I rat tail collagen. One ml of collagen suspension was pipetted into each well of a 24 well plate and incubated for 24 hours. Keratinocytes (10^6/ml) suspended in KGM-2 medium were seeded into the wells (0.5 ml per well). Three days later (after verification of a confluent, adhesive keratinocyte layer), the rafts were lifted onto a stainless steel grid covered with filter paper. The constructs were place in a Petri dish with media up to the level of the filter paper. The air-exposed keratinocytes stratified and differentiated into a full-thickness epithelium for 10 days. All raft models used for this study were created in a single process. A sketch outlining the process is shown in Figure 1.

2.2 Optical coherence/multiphoton microscopy system

A system very similar to that used in this study has been described previously [5]. Briefly, the system consists of 12-fs Ti:Sapphire laser with a spectral bandwidth of 100 nm and a center wavelength of 800 nm (Femtolasers). The laser beam is split into two arms; the sample arm light is raster-scanned by two galvanometer mirrors across the backplane of a 0.95 NA, 63X objective. Backscattered, TPEF, and SHG signals are collected by the same objective. The TPEF and SHG signals are separated with dichroic filters and detected by photomultiplier tubes. The backscattered (OC) light is mixed at a PIN detector with light returned from a piezo-mounted mirror in the reference arm. The measured resolution is

approximately 0.5 μm transverse and 1.5 μm axial for all three modalities. This system differs in several respects from the OCT/MPM system described by Beaurepaire et al. [6]. The short coherence length of this light source eliminated the need for the pinhole described in the Beaurepaire system. Additionally, the reference arm mirror is moved in a linear fashion for a distance of a couple wavelengths, enabling lock-in based signal demodulation at the Doppler frequency.
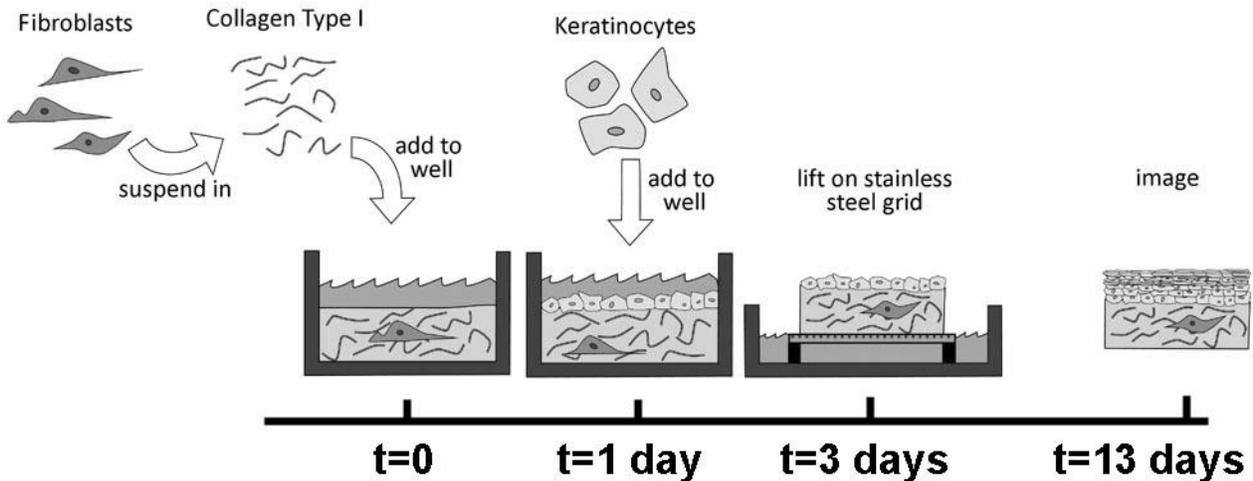

Fig. 1. Process of creating the rafts. Fibroblasts were suspended in collagen type 1 and pipetted into a well. Keratinocytes were added and allowed to form a confluent layer. The raft was lifted, and the air-exposed keratinocytes stratified and formed a keratin layer. Rafts were imaged on day 10 after lifting.

2.3 Imaging and analysis

Eight of the rafts were exposed to Dimethyl Sulfoxide (DMSO) either topically or by immersion. Six rafts were incubated with rhodamine 123, and one with Hoechst 33342 stains. Rhodamine 123 stains mitochondria and enhances signal from extracellular matrix. Hoechst brightly stains the condensed chromatin of apoptotic cells and more dimly stains normal chromatin of live cells. Stacks of en-face images were obtained near the center of the raft, extending from the surface to a depth where the signal became severely attenuated. At the end of imaging, the rafts were fixed in 10% buffered formalin, paraffin embedded and sectioned axially. Sections from approximately the same location as images were photographed. The layer thickness, density of cells, and cellular orientation were compared to OC, TPEF, an SHG images

3. RESULTS AND DISCUSSION

3.1 Skin-equivalent models

Histology revealed uniform rafts with 3-5 layers of keratinocytes and an approximately equally thick superficial layer of keratin. Frequently the keratin layer had embedded thin keratinocytes (parakeratosis). The collagen layer had abundant fibroblasts. Near the interface with the keratinocyte layer, the fibroblasts were frequently oriented with their long axis perpendicular to the surface, whereas they were oriented parallel to the surface in deeper layers. Figure 2 shows an example hematoxylin and eosin stained histology section from the center of a raft.

3.2 Imaging of unstained, untreated rafts

OC, SHG, and TPEF images from the keratinized surface, keratinocyte layer, interface, and collagen/fibroblast layer of a native raft (no stain or DMSO) are shown in Figure 3. Signal is seen from keratin at the top surface. OC and SHG images are similar and appear to show the structure of a flake, and TPEF shows strong keratin fluorescence in the same region. In the keratinocyte layer, little SHG is seen from this primarily cellular layer, but both OC and TPEF images show signal in the cell cytoplasm and to a lesser extent in the intracellular space. In both modalities, signal is absent from the nucleus. Tang et al [7] studied the OCM signals from cells and concluded that signal arises from mitochondria, plasma membranes and associated actin filaments, and the boundary between cytoplasm and nucleus. The images in Figure 3 are consistent with this finding. At the interface between the keratinocyte and collagen/fibroblast layers, the OC signal nearly disappears in agreement with the observations of Spoler [3]. SHG signal becomes strong indicating the

presence of collagen (perhaps of type 4 as suggested by Spoler). Some TPEF signal is seen from collagen. In the collagen/fibroblast layer, strong SHG is seen from the collagen, with hypointense regions that may correspond to the locations of cells. The OC signal has a characteristic texture, and also shows hypointense regions in the same locations as the SHG, possibly corresponding to cell nuclei. The TPEF is very weak, but some cell cytoplasm and collagen signal is present.
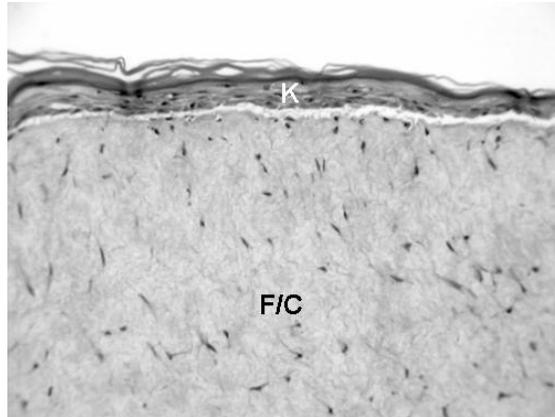

Fig. 2. Micrograph of example histology section of a raft. Fibroblast are suspended in collagen to for the base layer (F/C). Multiple layers of keratinocytes (K) top the base layer. At the most superficial is a layer of keratin. The space between the fibroblast/collagen and the keratinocyte layers is a histologic artifact.
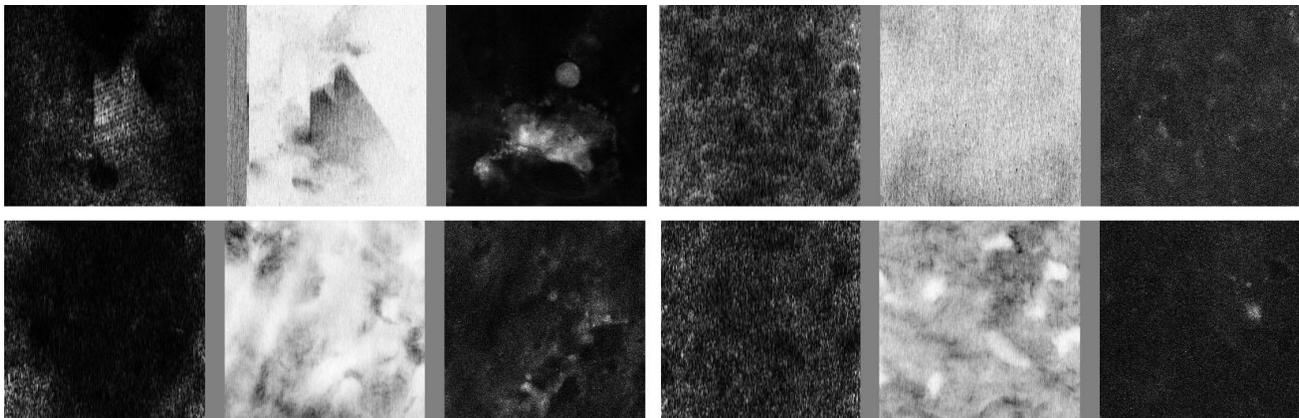

Fig. 3. OC, SHG, and TPEF trios from various depths in a non-stained, non-treated raft. Top left, surface of the raft showing keratin. Top right, inside the keratinocyte layer. Bottom left, at the interface between keratinocyte and collagen/fibroblast layer. Bottom right, inside the collagen/fibroblast layer. Grayscale intensity is in arbitrary units and scales are different for each image. OC and TPEF scales black (hypointense) to white (hyperintense). SHG is reverse scale.

3.3 Imaging of stained, untreated rafts

Rhodamine 123 and Hoechst 33342 staining was used to confirmed the origin of signal in OC and TPEF images. Figure 4 shows OC/TPEF image pairs taken in the keratinocyte layer of a non-treated, Hoechst-stained raft. "Holes" in the OC image correspond to brightly stained nuclei in the TPEF image. Signal in the nucleus in the OC image may be condensed chromatin in the apoptotic cells in the superficial layers (e.g. top left image pair in figure 3), or it may correspond to reflection from the nuclear membrane (bottom right and left image pairs in figure 3, which come from deeper, presumably healthy, cells). Imperfect correspondence may be due to the nature of Hoechst 33342 staining, which is brighter in apoptotic cells. For example, the top right image pair in figure 3 shows two large hypointense regions in the OC image, whereas the TPEF image at first appears to show only one large stained nucleus. Careful inspection reveals that the second structure is revealed by a faint ring in the TPEF image, perhaps a nucleus that did not stain well. SHG images are not shown in this series because the short-wavelength fluorescence emission of the Hoechst 33343 stain caused contamination of the SHG channel.
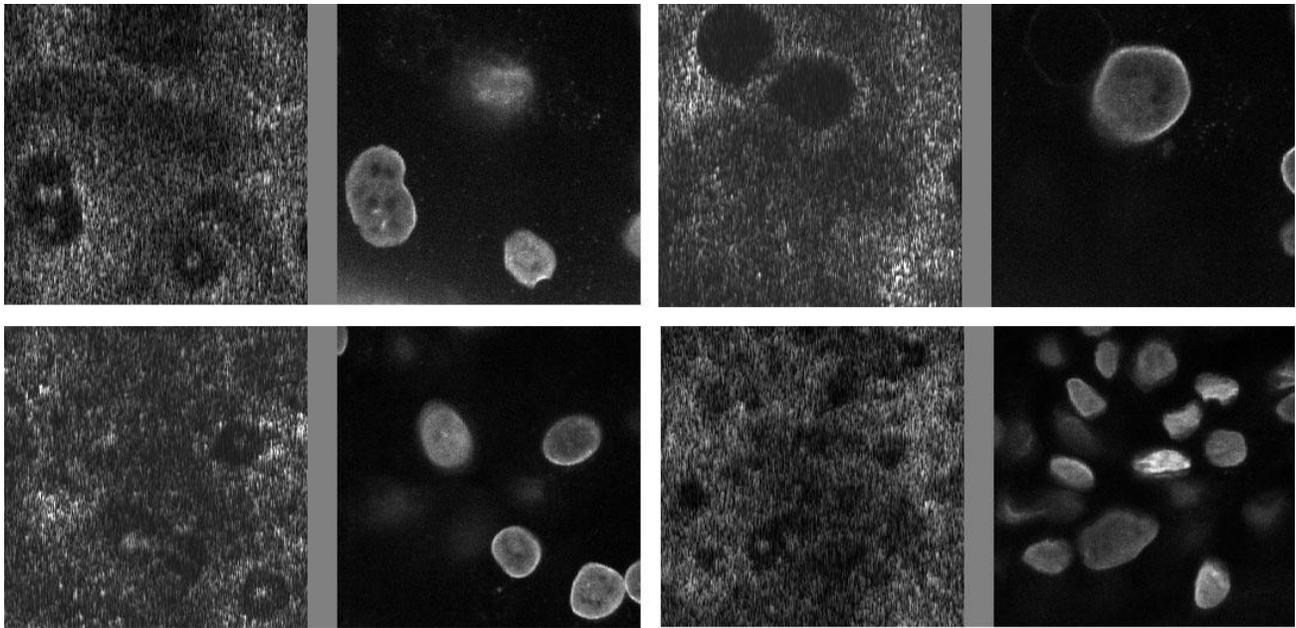
Fig. 4. OC and TPEF pairs from a Hoechst 33342-stained raft in the keratinocyte layer. Top left image pair: very superficial showing possibly apoptotic cells with condensed chromatin. Top right image pair: slightly deeper showing large nuclei, one of which did not stain well. Bottom left image pair: deeper showing smaller nuclei. Bottom right image pair: just above the interface with the collagen/fibroblast layer, showing many small nuclei.

Figure 5 shows images of untreated rafts stained with rhodamine 123. The top image shows a slice in the keratinocyte layer, but sectioning through to possibly include some of the basement membrane, as evidenced by SHG in the center of the image. The area shown as mitochondrial staining in the TPEF image is also backreflecting in the OC image. The bottom images show the collagen/fibroblast layer. The enlongated nature of the fibroblast cytoplasm is most evident in the TPEF image showing stained mitochondria. Hypointense regions are seen in the area of the fibroblasts in the SHG image. The OCT image shows a characteristic texture, but does not clearly show hypointense regions in the area of fibroblast nuclei.
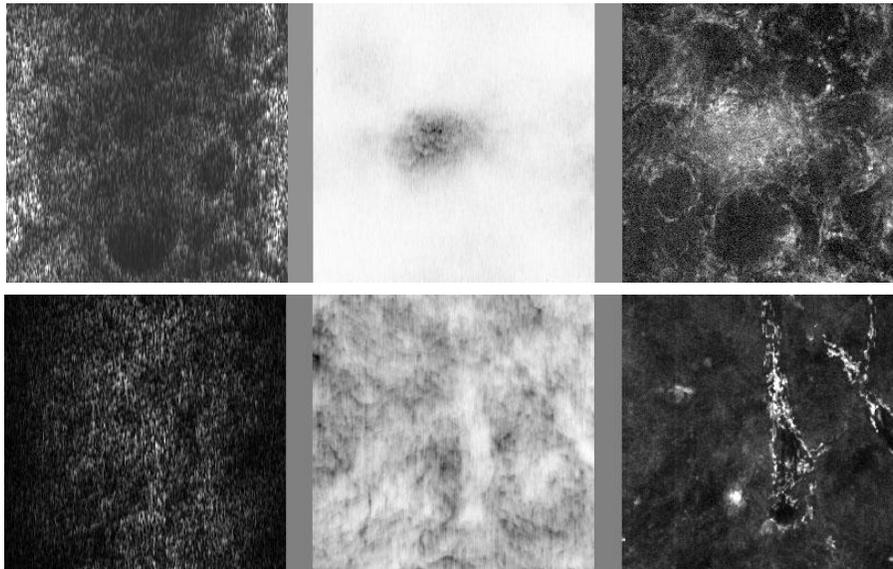

Fig. 5. OC, SHG, and TPEF trios from a Rhodamine 123-stained raft. Keratinocyte layer (top) shows mitochondrial staining and collagen in the center. Collagen/fibroblast layer (bottom) shows mitochondrial staining and fine collagen structure.

3.4 Imaging of treated rafts

The SHG signal was severely reduced in topically DMSO-exposed rafts, and nearly absent in rafts immersed in DMSO. This finding is in agreement with Yeh et al [8], who studied glycerol-immersed collagen-fibroblast constructs. They showed that collagen fibrils disassociate, leading to a decrease in light scattering (visible clearing of the construct) and about 90% loss of the SHG signal. In this study, the appearance of cells was altered, as seen in the below set of image trios taken from a DMSO-immersed (.5 hours), rhodamine 123- stained raft (figure 6). Little OC signal is seen in any layer, corresponding to visual increase in transparency of the raft. Weak SHG is seen, mainly near the top of the collagen/fibroblast layer. The rhodamine staining reveals that the structure of the keratinocyte layer appears slightly distorted. The collagen/fibroblast layer is strongly altered. Compare the bottom images in figure 6 with the bottom image in figure 5. The brightly stained spidery structures appear to be fibroblasts (further evidenced by lack of SHG signal in the stained regions), but they are unusually shaped. Deeper in this layer, the fibroblasts appear to be shrunken and no dark nucleus is seen.
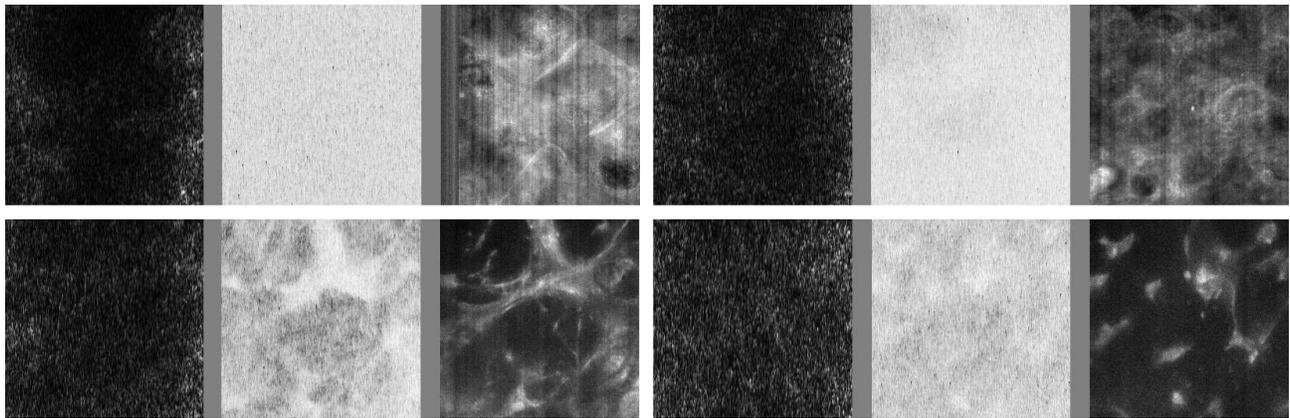

Fig. 6. OC, SHG, and TPEF trios from various depths in a rhodamine 123-stained, DMSO-treated raft. Top left, near the surface of the raft showing rhodamine fluorescence in keratin layer, but little OC or SHG signal. Top right, inside the keratinocyte layer. Bottom left, at the interface between keratinocyte and collagen/fibroblast layer, unusual spidery structure with bright rhodamine staining and absent in SHG. Bottom right, inside the collagen/fibroblast layer.

TPEF images of the keratinocyte layer of another raft, immersed in DMSO for one hour, then stained with Hoechst 33343, is shown in figure 7. The left image shows a section from near the top of the keratinocyte layer, and the right image shows an image from near the bottom of the keratinocyte layer. In the left image, the cell nuclei appear to be normally shaped, but nuclei appear overlapping. Given that the axial resolution of the system is approximately 1.5 µm, it would not be expected to section through two nuclei. The DMSO has apparently decreased the volume of the cells, such that these flattened nuclei are now packed together in the axial direction. The signal is also very weak. In the right image, the staining is unusual. The cell nuclei appear elongated. Signal seems to appear from throughout the tissue. This signal may be from small amounts of non-specific dye that was not washed from the raft, which appears in these images (but not figure 4) because the signal from the nuclei is relatively weak requiring high signal amplification.
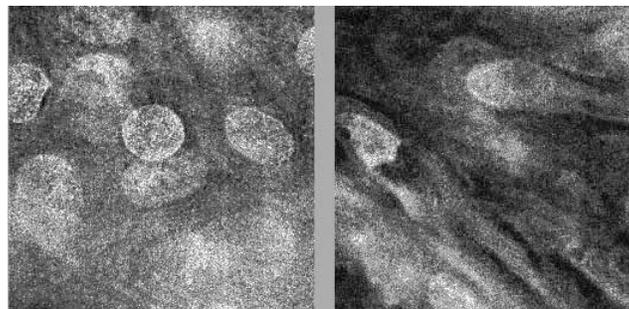

Fig. 7. TPEF image from the keratinocyte layer of a raft immersed in DMSO and stained with Hoechst 33343. The left image is from near the top of the keratinocyte layer and the right from near the bottom. The left image may show the effect of cell shrinkage from DMSO, with multiple overlapping nuclei now appearing in the image. The right appears to show distorted cell nuclei, and possibly non specific staining.

4. CONCLUSIONS

OC, SHG, and TPEF images show complimentary information. This multi-modality microscope appears to be useful for probing the structure of skin-equivalent rafts, and may allow non-destructive evaluation of investigative agents. In the future, it would be helpful to evaluate contrast agents for optical coherence microscopy, as these types images showed the least contrast, yet showed sufficient signal even from deep in the collagen/fibroblast layers.

5. ACKNOWLEDGEMENTS

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