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Full Length Research Paper

Ultrasonic analyses algorithm on an *EX VIVO* produced oral mucosal equivalent

Frank Winterroth[1]*, Junho Lee[2], Shiuhyang Kuo[3], J. Brian Fowlkes[1,4], Stephen E. Feinberg[1,3], Scott J. Hollister[1] and Kyle W. Hollman[1,5]

[1]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA.
[2]School of Dentistry, University of Michigan, Ann Arbor, MI, USA.
[3]Department of Oral and Maxillofacial Surgery, University of Michigan, Ann Arbor, MI, USA. [4]Department of Radiology, University of Michigan, Ann Arbor, MI, USA.
[5]Sound Sight Research, Livonia, MI, USA.

Abstract

This study examines the use of high-resolution ultrasound with an analyses algorithm to accurately monitor development of an *EX VIVO* produced oral mucosal equivalent (EVPOME). We used ultrasonic profilometry to examine EVPOME development as seeded cells on its surface proliferate. As these engineered structures develop, seeded cells stratify from their differentiation and produce a keratinized protective upper layer. Some of these transformations could alter backscatter of ultrasonic signals and produce scattering of the signal similar to an unseeded scaffold. Developing non-invasive *IN VITRO* ultrasonic monitoring allows adjusting tissue cultivation in-process, accounting for biological variations in the development of the EVPOME.

Key words: Acoustic microscopy, oral mucosa, non-invasive assessment, tissue engineering, ultrasound.

INTRODUCTION

The need for soft tissue replacements of oral mucosa in cases of disease, injury, or defect is enormous. Hence, developing a practical and cost - effective engineered tissue device which is compatible with the patients is essential toward proper treatment of soft tissue conditions. We have found high-frequency acoustic microscopy to be an advantageous method to study the physical and anatomical characteristics of tissues, both engineered and natural.

This study is a complement to our previous examination: using scanning acoustic microscopy (SAM) to study changes in radiofrequency (RF) data between tissues which have undergone periods of elevated thermal incubation (stressed) to those which were incubated continuously at physiological temperature (unstressed) (Winterroth et al., 2011). Here, we examine these same tissues using SAM, coupled with filtering and an analyses algorithm to effectively study their surface characteristics. The advantages of using SAM over conventional optical microscopy include being able to image the cells and tissues without doing any preparations which could potentially kill or alter the tissues; this provides a more accurate representation of the tissues' natural properties (Cohn et al., 1997a). It will also provide evidence as to the degree of differentiation which the cells undergo without chemically affecting their properties (Kolios et al., 2002; Saijo et al., 2004).

Previously, we used SAM to examine and compare surface characteristics between the commercially available cellular cadaveric dermis (AlloDerm®) and an in-house developed *ex vivo* produced oral mucosal equivalent (EVPOME) (Winterroth et al., 2009). We further copared changes in the RF data in EVPOME specimens undergoing differentiation, apoptosis, and keratinization by studying the reflectivity off their surfaces and analyzing the degree of surface irregularities (Winterroth

*Corresponding author. **E-mail**: fwinterr@umich.edu. **Tel**: (734) 936-2781. **Fax:** (734) 647-4834

et al., 2009). We then compared these results to EVPOME specimens which underwent standard histological preparation and examination under optical microscopy: a strong linear correlation was found between the optical and SAM images when quantifying these surface characteristics. By correlating changes in the RF data to the EVPOME (and natural tissue cells in general) undergoing differentiation, apoptosis, and keratinization, we can better understand the physiological processes of these cells as they evolve.

For this study, we examined and compared the surface acoustic profile characteristics (ultrasonic profilometry) between two EVPOME specimens: one underwent a thermal elevation for 24 h (stressed) while the other was an unstressed control. Ultrasonic profilometry uses an analysis algorithm to remove any major debris from the scanned specimens – which could potentially alter the image of the scanned surface; it is a promising method to analyze tissue types and their respective characteristics on the basis of acoustic transmission and scatter properties (Matsuyama et al., 1989; Zuber et al., 1999).

MATERIALS AND METHODS

The protocol for harvesting human oral mucosal tissue was approved by a University of Michigan Internal Review Board. Details of the EVPOME development are similar to those described elsewhere (Winterroth et al., 2009; Izumi et al., 2004). Briefly, oral mucosa keratinocytes were enzymatically dissociated from the tissue sample, and a primary cell culture was established and propagated in a chemically-defined, serum- and xenogeneic products-free culture medium, with calcium concentration of 1.2 mM. The AlloDerm® was soaked in 5 µg/cm^2 human type IV collagen at 4°C overnight prior to seeding cells to assist the adherence of cells, then approximately 1.5 x 10^5 cells/cm^2 of oral keratinocytes were seeded onto the type IV collagen pre-soaked AlloDerm®. The com-posites of the keratinocytes and the AlloDerm® were then cultured, in the submerged condition at 37°C for 4 days to form a continuous epithelial monolayer. After 4 days, the equivalents were raised to an air-liquid interface to encourage cellular stratification and cultured for another 7 days, resulting in fully-differentiated, well-stratified epithelial layers on the AlloDerm®. At Day 11 post-seeding, EVPOME samples were collected for SAM imaging. On Day 9 day post-seeding, one set of EVPOME specimens were incubated at 43°C for 24 h, then switched back to 37°C for another 24 h (thermally stressed). Another set of EVPOMES were kept at 37°C up through Day 11 post-seeding (non-stressed). The culture times for both conditions – stressed and non-stressed - were equal. The experiments were conducted as single-blind studies: we received two specimens, labeled as "1" and "2" for each study set. We were not told which specimens were stressed or unstressed until after presenting and announcing the results from the SAM scans.

Details of the scanning and ultrasound system are similar to those described elsewhere (Cohn et al., 1997 b). The transducer's parameters are: 15 µm scanning step size in both the transverse and horizontal directions, a lateral resolution of 37 µm, an axial resolution of 24 µm, and a depth of field of 223 µm. Axial resolution is the resolution in the direction of propagation and is determined by the length of the ultrasound pulse propagating in the tissue; lateral resolution is the resolution orthogonal to the propagation direction of the ultrasound wave. Sampling along the Z-axis was performed at a rate of 300 mega samples/second. B-scan images were obtained by stepping the transducer element laterally across the desired region. At each position, the transducer fired and an RF A-line was recorded. After repeated firings at one position, the transducer moved to the next, where the image was constructed from A-lines acquired at all lateral positions. Because of the transducer's short depth of field, a composite B-scan image (the 2D cross-sectional display based on the time required for return of the echo to the transducer) was generated from multiple scans at different heights.

Surface profilometry was determined by first finding the instance of threshold value, fitting and subtracting the planar surface, then calculating root-mean-squared (RMS) height. RMS was computed in time domain:

$$RMS = \sqrt{\frac{1}{n}\sum_{n} x^2(t)}$$

where n is number of $x(t)$ samples. An analysis algorithm was applied to all the scans performed for each of the specimens used in this study and to 6 scans of AlloDerm® specimens. After detecting the front surface of each specimen and subtracting any tilt, a filter was applied to determine the broad underlying surface of the specimen; this filter was then subtracted from the tilt removed surface. A second filter was then applied to the resulting surface to remove any possible noise.

RESULTS AND DISCUSSION

The 2D B-scans from the SAM and their comparative histology images show a clear partitioning of the keratin layer from the apical surface of the specimens under stressed conditions. Comparing the B-scans taken of EVPOME at 11 days post-seeding, there is a distinct reflection off of the surface (Figure 1a) – a result of the seeded keratinocytes differentiation - which is not found in the stressed EVPOME specimen (Figure 1b). The histology images of these same specimens- AlloDerm® and EVPOME at 11-days post seeding (Figures 1c and 1d, respectively) - verify these findings as the keratinized layer in the latter image shows a smooth surface, with less surface irregularities compared to the former image. SAM C-Scans performed on the unstressed and stressed EVPOMEs after removing any tilt and applying the aforementioned filters show greater surface homogeneity in the unstressed control; evident of the keratinized layer (Figure 1e). In contrast, the stressed EVPOME show greater variations on the surface (Figure 1f).

Removing any tilt (which potentially occurs when mounting and positioning the specimens) lowers the measured RMS heights for all specimens but it maintains the mean difference between the stressed and non-stressed specimens in SAM C-scans of their surface (Figures 1e and 1f); the same is true when adding the weak filter. However, adding the weak filter seems to slightly increase standard deviation; the filter parameters may require further adjustments. B-scans taken along the transverse axis of the stressed specimen where the surface variations are the highest show significant changes in the surface at the point where there is a significant increase in the RMS height (Figure 2), accounting

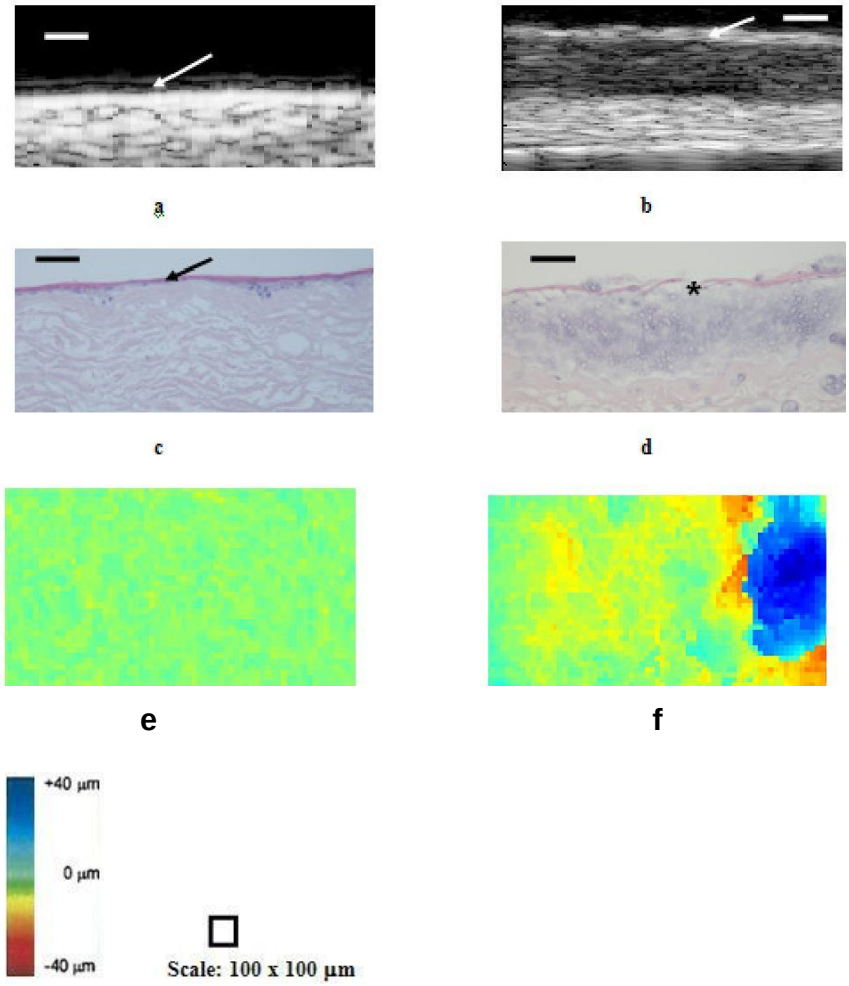

Figure 1. SAM 2D B-scans of EVPOMEs under unstressed (a) and stressed (b) conditions. Note the differences in the reflectivity on the surfaces (arrows). The histology images of the unstressed (c) and stressed (d) devices verify these findings: the keratinized layer (arrow) and the debris in the partitioned area (asterisk). The scale bars represent 100 ∝m. SAM C-scans of an unstressed (e) and stressed (f) EVPOME specimen. The images result from taking the image and removing any tilt, followed by fitting the planar surface and subtracting the result.

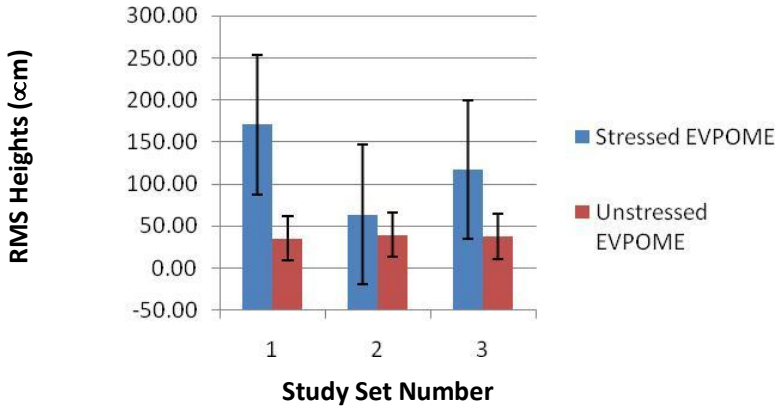

Figure 2. RMS variations in height for the stressed and unstressed EVPOME specimens for each set of studies conducted. The standard deviation of the scaffold as a percentage of the mean is 70.71.

for the higher variation in the RMS value for the stressed specimen.

This is verified by calculating the mean values for the height variations between the stressed and non-stressed specimens and determining their respective standard errors in all the study sets performed. The error bars represent the percent standard deviation of the mean. Because there are more RMS variations in the stressed specimens, there would be the likelihood for higher error (Figure 2). There is also the likelihood of the tissue samples tilting during their mounting process which would contribute to the increased RMS profiles, for both stressed and non-stressed specimens. Further EVPOME analyses will involve use of the filters and tilt corrections to validate these profilomtery findings.

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REFERENCES

Cohn NA, Emelianov SY, Lubinski MA, O'Donnell M (1997a). An Elasticity Microscope. Part I: Methods. IEEE Trans. Ultrason. Ferr. Freq. Ctrl., 44(6): 1304-1319.

Cohn NA, Emelianov SY, O'Donnell M (1997b). An Elasticity Microscope. Part II: Experimental Results. IEEE Trans. Ultrason. Ferr. Freq. Ctrl., 44(6): 1320-1331.

Izumi K, Song J, Feinberg SE (2004). Development of a tissue-engineered human oral mucosa: from the bench to the bed side. Cells Tiss. Org., 176:134–152.

Kolios MC, Czarnota GJ, Lee M, Hunt JW, Sherar MD (2002). Ultrasonic Spectral Parameter Characterization of Apoptosis. Ultrasound in Med. Biol., 28(5): 589–597.

Matsuyama T, St Goar FG, Tye TL, Oppenheim G, Schnittger I, Popp RL (1989). Ultrasonic Tissue Characterization of Human Hypertrophied Hearts *In Vivo* with Cardiac Cycle-Dependent Variation in Integrated Backscatter. Circulation, 80: 925-934.

Saijo Y, Miyakawa T, Sasaki H, Tanaka M, Nitta S (2004). Acoustic Properties of Aortic Aneurysm Obtained with Scanning Acoustic Microscopy. Ultrasonics, 42: 695–698.

Winterroth F, Fowlkes JB, Kuo S, Izumi K, Feinberg SE, Hollister SJ, Hollman KW (2009). High-Resolution Ultrasonic Monitoring of Cellular Differentiation in an *Ex Vivo* Produced Oral Mucosal Equivalent (EVPOME). Proc. IEEE Bioultrasonics Conference.

Winterroth F, Lee J, Kuo SJ, Fowlkes JB, Feinberg SE, Hollister SJ, Hollman KW (2011). Acoustic Microscopy Analyses to Determine Good vs. Failed Tissue Engineered Oral Mucosa Under Normal or Thermally Stressed Culture Conditions. Ann. Biomed. Eng., 39(1): 44-52.

Zuber M, Gerber K, Erne P (1999). Myocardial Tissue Characterization in Heart Failure by Real-Time Integrated Backscatter. Eur. J. Ultrasound, 9: 135–143.