Biomaterials 35 (2014) 6667–6676

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Leading opinion

The potential of label-free nonlinear optical molecular microscopy to non-invasively characterize the viability of engineered human tissue constructs

Leng-Chun Chen [a,1], William R. Lloyd [a,1], Shiuhyang Kuo [b,2], Hyungjin Myra Kim [c,3], Cynthia L. Marcelo [d,4], Stephen E. Feinberg [b,d,5], Mary-Ann Mycek [a,*]

[a] Department of Biomedical Engineering, University of Michigan College of Engineering & Medical School, 1101 Beal Avenue, Ann Arbor, MI 48109-2110, USA
[b] Department of Oral and Maxillofacial Surgery, University of Michigan School of Dentistry, 1150 W. Medical Center Drive, Ann Arbor, MI 48109, USA
[c] Center for Statistical Consultation and Research, University of Michigan School of Public Health, 915 E. Washington, Ann Arbor, MI 48109-1070, USA
[d] Department of Surgery, University of Michigan Medical School, 1150 W. Medical Center Drive, Ann Arbor, MI 48109, USA

ARTICLE INFO

Article history:
Received 28 March 2014
Accepted 22 April 2014
Available online 20 May 2014

Keywords:
Tissue engineering
Label-free optical molecular imaging
Tissue viability
Multiphoton excitation microscopy
Second-harmonic generation (SHG) imaging
Fluorescence lifetime imaging microscopy (FLIM)

ABSTRACT

Nonlinear optical molecular imaging and quantitative analytic methods were developed to non-invasively assess the viability of tissue-engineered constructs manufactured from primary human cells. Label-free optical measures of local tissue structure and biochemistry characterized morphologic and functional differences between controls and stressed constructs. Rigorous statistical analysis accounted for variability between human patients. Fluorescence intensity-based spatial assessment and metabolic sensing differentiated controls from thermally-stressed and from metabolically-stressed constructs. Fluorescence lifetime-based sensing differentiated controls from thermally-stressed constructs. Unlike traditional histological (found to be generally reliable, but destructive) and biochemical (non-invasive, but found to be unreliable) tissue analyses, label-free optical assessments had the advantages of being both non-invasive and reliable. Thus, such optical measures could serve as reliable manufacturing release criteria for cell-based tissue-engineered constructs prior to human implantation, thereby addressing a critical regulatory need in regenerative medicine.

1. Introduction

Recent advances in tissue engineering and regenerative medicine (TERM) have become an integral part of reconstructive surgery and tissue/organ regeneration. One such advance is the development of tissue-engineered constructs, which are classified by the United States Food and Drug Administration (FDA) as combination products comprised of an engineered extracellular matrix and a biological component. Biological device manufacturing is strictly regulated by the FDA prior to product release for patient treatment to assure effectiveness and safety. It is essential that the device is objectively (*i.e.* quantitatively) and non-invasively (*i.e.* without sectioning or staining) evaluated in real-time to assess cellular viability [1–3], which is the significant technical challenge we address here by developing quantitative methods for tissue-based, label-free nonlinear optical molecular microscopy.

Rather than employing exogenous fluorescent dyes that are bright but invasive to the manufactured tissue, the method described here targets biologically- and metabolically-relevant endogenous tissue fluorophores, primarily nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and flavin adenine dinucleotide (FAD). NAD(P)H denotes the simultaneous analysis of NADH and NADPH as one fluorophore, grouped because of their similar fluorescence spectrum and their important roles in cellular metabolic function. NADH and FAD are mitochondrial coenzymes that report on cellular metabolism and oxygen consumption (Supplementary Fig. 1), and NADPH is an anabolic coenzyme largely produced by fast growing cells for biosynthesis [4].

* Corresponding author. Tel.: +1 734 647 1361.
E-mail addresses: lengleng@umich.edu (L.-C. Chen), billlloy@umich.edu (W. R. Lloyd), skuo@med.umich.edu (S. Kuo), myrakim@umich.edu (H.M. Kim), cmarcelo@umich.edu (C.L. Marcelo), sefein@med.umich.edu (S.E. Feinberg), mycek@umich.edu (M.-A. Mycek).
[1] Tel.: +1 734 763 8113.
[2] Tel.: +1 734 615 8298.
[3] Tel.: +1 734 647 8158.
[4] Tel.: +1 734 763 6721.
[5] Tel.: +1 734 763 5963.

http://dx.doi.org/10.1016/j.biomaterials.2014.04.080

NAD(P)H and FAD fluoresce in their reduced and oxidized states, respectively [5,6]. Therefore, their concentrations typically trend oppositely to one another and their relative concentrations can be assessed with fluorescence intensity measurements characterized by a redox ratio (RR) metric, defined as [FAD]/([NAD(P)H] + [FAD]) [7]. Intensity ratios like RR enhance the metabolic variations measured while reducing intensity-based artifacts, including signal variations due to optical loss, which may be difficult to quantify or control in tissues. A complementary approach is fluorescence lifetime imaging microscopy (FLIM). In addition to two-dimensional spatial information, FLIM images contain a third dimension – time [6–8]. FLIM is advantageous for tissue-based sensing because fluorescence lifetime reveals molecular microenvironment (*e.g.*, temperature, pH, and binding), while being insensitive to intensity-based measurement artifacts.

Sensitive, spatially-resolved measures of NAD(P)H and FAD in unstained tissue constructs were achieved by developing quantitative nonlinear optical molecular microscopy methods to optically (non-invasively) section tissue samples into layers as thin as 1 μm [9–12]. Such image resolution enabled the detection of optical signals from a single cellular layer of a three-dimensional engineered tissue. In addition, nonlinear optical techniques enable specific measurements of collagen molecules through second harmonic generation (SHG). Because extracellular collagen and intracellular NAD(P)H and FAD have similar fluorescence characteristics, measuring collagen via SHG enables the spatial separation of intracellular (NAD(P)H and FAD) and extracellular (collagen) fluorescence via image post-processing. Therefore, by combining optical sectioning with fluorescence and SHG measurements, tissue fluorescence arising from cells can be analyzed to quantitatively characterize cellular metabolism and cellular organization for tissue viability assessment from a single cellular layer [7,13].

As a model system for our studies, we employed the engineered cell-based device EVPOME (*Ex Vivo* Produced Oral Mucosa Equivalent) [1]. EVPOMEs are manufactured by culturing primary human oral keratinocytes atop a dermal equivalent scaffold for tissue formation. EVPOMEs, developed for intraoral grafting procedures for reconstructive surgery of oral and dental soft tissues, were demonstrated to reduce patients' wound healing time by half [4]. In addition, EVPOMEs were implanted successfully in humans during an FDA approved Phase I clinical trial [14].

2. Materials and methods

2.1. Procurement of human oral mucosal tissues

Discarded keratinized oral mucosa was collected from patients undergoing minor oral surgical procedures at the University of Michigan (UM) hospital. The UM Medical School Institutional Review Board approved use of the mucosa and patients provided informed consent for research use. The study adhered to the Declaration of Helsinki Guidelines.

2.2. Standard protocols for culturing human oral keratinocytes and manufacturing EVPOMEs

Primary human oral mucosal tissues were harvested from procured discarded keratinized oral mucosa and cultured according to previously described protocols [1,2]. Briefly, primary human oral keratinocytes were enzymatically dissociated from the tissue samples. Oral keratinocyte cultures were established in a chemically-defined, serum-free culture medium (EpiLife and EDGS, Invitrogen/Life Sciences, Carlsbad, CA). The medium contained 0.06 mM calcium, 25 μg/ml gentamicin, and 0.375 μg/ml fungizone (both from Sigma, St. Louis).

For cell culture studies, the oral keratinocytes were seeded onto a 3.5 cm glass bottom dish (MatTek Corp., Ashland, MA) coated with collagen. Calcium concentration in the growth medium was controlled at 0.06 mM for cell proliferation and 1.2 mM to induce cell differentiation.

For EVPOME studies, EVPOME constructs were manufactured by first seeding 200,000 cells/cm^2 on 1 cm^2 acellular cadaver skin (AlloDerm®, LifeCell, KCl, Branchburg, NJ) that was pre-soaked in 0.05 μg/μL human type IV collagen at 4 °C overnight (Sigma–Aldrich, St. Louis, MO). Resulting keratinocytes and AlloDerm® were submerged in medium containing 1.2 mM calcium for 4 days and then raised to an air–liquid phase for an additional 7 days to induce cell stratification and differentiation. Control constructs were cultured in 100 mm dishes (for thermally-stressed) or in 6-well plates (for metabolically-stressed) with inserts in incubators at 37 °C with 5% CO_2 for all culture days. The day 11 glucose concentration of the culture medium was read by a glucose meter (ACCU-CHEK®, Aviva, Roche, Indianapolis, IN).

2.3. EVPOME stressing protocols

Thermally-stressed constructs were cultured at 43 °C for 24 h beginning on day 9 post-seeding and were returned to normal culture conditions starting day 10. To create metabolically-stressed constructs, constructs received no fresh culture medium for 6 days beginning on day 4 post-seeding and were returned to normal culture conditions starting day 10. We note that for the first batch of the metabolic-stress experiment, the construct was starved from day 4 to day 11. As a result, there is no glucose consumption measurement for this patient. In this study, we have grouped optical results from both metabolic-stress protocols because there were no observed differences from construct histology results. For two batches measured in the FLIM thermal stressing study, we note that two of the five batches had no histology or glucose samples measured. Their reported glucose and histology metrics were measured from constructs cultured in parallel with the same primary human cells.

2.4. Nonlinear optical microscopic imaging

Images were acquired with a Leica TCS SP5 microscope equipped with a Ti:Sapphire laser (Mai Tai, Spectra-Physics). The excitation laser source and the emission light were coupled through an inverted microscope with 40× (1.25 NA)/63× (1.4 NA) oil immersion objective lenses to image oral keratinocytes in culture and a 25× water immersion objective lens (0.95 NA, 2.5 mm working distance) to image EVPOME constructs. EVPOME constructs were imaged from the top surface down (*i.e.* through the topmost keratin layer) to mimic post-implantation conditions *in vivo*. Prior to measurement on an inverted microscope, constructs were flipped over onto measurement dishes. This process was not expected to alter cellular viability or layer thickness.

All measurements were collected in a controlled environment (37 °C with 5% CO_2) to mimic EVPOME culture. NAD(P)H was excited with excitation at 705 nm; FAD and collagen (to detect SHG) were excited with excitation at 900 nm. Before each measurement, the excitation power at the specimen surface plane at both excitation wavelengths with a 10× objective was calibrated at 20 mW for standard cell cultures and 30 mW for EVPOME constructs to limit photobleaching and non-reversible changes in the sample. The laser power was selected after consulting literature that concluded no short-term cellular damage occurred after 760 or 800 nm exposure with powers of 50 mW (~200 fs pulse width) [15] and 60 mW (~150 fs pulse width) [16], respectively. Currently, there are no FDA approved nonlinear optical microscopy systems for human use. However, a CE-certified device is commercially available in Europe and a preliminary clinical study in the US of this device [17] indicates that such imaging technologies will have medical risk profiles similar to FDA approved clinical imaging modalities, including X-ray and computed tomography instruments [18].

For NAD(P)H intensity detection, the backscattered fluorescence was collected through a band pass filter from 435 to 485 nm; for FAD intensity detection, the backscattered fluorescence was collected through a band pass filter from 500 to 550 nm (Leica Dapi/FITC filter cube). Emission light was collected with short-coupling non-descanned photomultiplier tubes to increase collection efficiency. An internal tunable photomultiplier collected SHG emission from 440 to 460 nm. Detector gain and offset were consistent for each measurement to avoid detector saturation. Images (1024 × 1024 pixels, 0.391 × 0.391 μm^2 pixel size, 8-bit image depth) were acquired in ~40 s with a 200 Hz line scanning speed. The two fluorescence and SHG signals were acquired sequentially, with all three measurements occurring at each site in less than 3 min. To reduce background noise, a line average of eight was employed for all images. For NAD(P)H FLIM detection, emission light was collected through a tunable monochromator from 410 to 490 nm coupled to a photomultiplier tube and photon counting add-on (HydraHarp TCSPC, Leica). FLIM measurements of cells in monolayer were collected (1024 × 1024 or 512 × 512 pixels, ~265 × 265 μm^2) until 100 counts were reached in the peak channel, lasting an average of ~2 min per FLIM image. FLIM measurements of EVPOMEs were collected (512 × 512 or 256 × 256 microns, ~400 × 400 μm^2) for 1.5 min.

The image acquisition settings above were applied for both cross-sectional and *en-face* imaging, except that the cross-sectional images were collected in *x–z* (vertical) direction and the *en-face* images were collected in *x–y* (horizontal) direction. Representative cross-sectional fluorescence images were overlaid with their corresponding SHG images, processed via Photoshop Screen function (Figs. 1 and 2). No post-processing was performed on representative *en-face* images in Figs. 1 and 3–6, Supplementary Figs. 1–3 and 6 unless indicated on the figures.

2.5. Chemical treatments

MitoTracker®, an exogenous fluorescence dye that only fluoresces within mitochondria, stained the cell culture samples for colocalized fluorescence from