METHODS ARTICLE

Noninvasive Optical Assessment of Implanted Engineered Tissues Correlates with Cytokine Secretion

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Fluorescence lifetime sensing has been shown to noninvasively characterize the preimplantation health and viability of engineered tissue constructs. However, current practices to monitor postimplantation construct integration are either qualitative (visual assessment) or destructive (tissue histology). We employed label-free fluorescence lifetime spectroscopy for quantitative, noninvasive optical assessment of engineered tissue constructs that were implanted into a murine model. The portable system was designed to be suitable for intravital measurements and included a handheld probe to precisely and rapidly acquire data at multiple sites per construct. Our model tissue constructs were manufactured from primary human cells to simulate patient variability based on a standard protocol, and half of the manufactured constructs were stressed to create a range of health states. Secreted amounts of three cytokines that relate to cellular viability were measured *in vitro* to assess preimplantation construct health: interleukin-8 (IL-8), human β -defensin 1 (hBD-1), and vascular endothelial growth factor (VEGF). Pre-implantation cytokine secretion ranged from 1.5 to 33.5 pg/mL for IL-8, from 3.4 to 195.0 pg/mL for hBD-1, and from 0.1 to 154.3 pg/mL for VEGF. *In vivo* optical sensing assessed constructs at 1 and 3 weeks postimplantation. We found that at 1 week postimplantation, *in vivo* optical parameters correlated with *in vitro* preimplantation secretion levels of all three cytokines ($p < 0.05$). This correlation was not observed in optical measurements at 3 weeks postimplantation when histology showed that the constructs had re-epithelialized, independent of preimplantation health state, supporting the lack of a correlation. These results suggest that clinical optical diagnostic tools based on label-free fluorescence lifetime sensing of endogenous tissue fluorophores could noninvasively monitor postimplantation integration of engineered tissues.

Keywords: *in vivo* fluorescence lifetime spectroscopy, tissue viability, oral mucosa, EVPOME, intravital spectroscopy, endogenous fluorescence

Introduction

COMBINATION CELL–SCAFFOLD CONSTRUCTS, commonly used for skin¹ and oral mucosa² applications, are rapidly transitioning to clinical use.³ A major obstacle to the clinical translation of combination constructs is the lack of reliable evaluative tools to ensure manufacturing consistency (before implantation) and to validate clinical efficacy (after implantation).⁴ Such evaluative tools must assess construct viability quantitatively and noninvasively.

For preimplantation assessment, biochemical assay of constitutively produced cytokines in the spent culture medium correlates with the number of viable functional

cells in the living cell layer,⁵ and, therefore, assists in predicting *in vivo* tissue integration.⁶ However, current practices for postimplantation assessment are either qualitative (visual assessment) or destructive (histology). Thus, there is an unmet clinical need for a quantitative and noninvasive methodology for evaluating engineered tissues postimplantation.

Optical techniques including fluorescence spectroscopy,⁷ optical coherence tomography,⁸ laser speckle imaging,⁹ and Raman spectroscopy¹⁰ offer the potential to quantitatively and noninvasively assess engineered tissues in real time during the *in vivo* tissue integration process.¹¹ Of these, fluorescence spectroscopy can sense endogenous reporters

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of cellular metabolism, enabling label-free assessment of construct viability, as has been demonstrated in engineered cartilage,¹² bone constructs,¹³ and skeletal muscle units.¹⁴

Our model system, *Ex Vivo* Produced Oral Mucosa Equivalent (EVPOME), comprises an acellular dermal extracellular matrix and a living epithelial cell layer manufactured from primary human oral keratinocytes.¹⁵ Using noninvasive and label-free nonlinear optical microscopy, our group demonstrated that fluorescence lifetime¹⁶ parameters can indicate local cellular viability of EVPOMEs *in vitro* by sensing endogenous reporters of cellular metabolism.¹⁷ That study motivated us to implement handheld, fiber-optic-based fluorescence lifetime spectroscopy (FLS) instrumentation^{18–20} to interrogate EVPOMEs implanted into a murine model.²¹ This observational study demonstrates promise that clinical optical diagnostic tools based on fluorescence lifetime sensing could monitor the health of tissue-engineered constructs, an unmet clinical need.

Materials and Methods

Manufacturing of EVPOME human keratinocyte-based tissue-engineered constructs

The clinical study protocol was approved by the University of Michigan (UM) Medical School Institutional Review Board. Discarded, surgically resected oral mucosa samples were obtained from patients undergoing minor oral surgical procedures. All study practices were in accordance with the Declaration of Helsinki guidelines.

The detailed protocols for culturing human oral keratinocytes and manufacturing EVPOME have been previously published.^{22,23} In brief, primary human oral keratinocytes were enzymatically dissociated from the tissue samples, isolated, established in culture, and cryopreserved. Frozen keratinocytes were thawed and passaged up to two times when needed for EVPOME manufacturing. A total of 2×10^5 cells were seeded onto a 1 cm^2 acellular cadaver dermis (AlloDerm®; LifeCell), hereafter referred to as the dermal equivalent, precoated with type IV collagen (Sigma) to promote cell attachment. The seeded dermal equivalent was immersed for 4 days in culture medium containing 1.2 mM calcium to promote cell proliferation, then raised to an air–liquid interface for an additional 7 days to enhance stratification of the epithelial layer.

To create a range of preimplantation health states, half of the EVPOMEs were manufactured per standard protocol (control) and the other half were thermally stressed to reduce cell viability and proliferation capabilities (stressed). Control EVPOMEs were incubated at 37°C with 5% CO₂ for all culture days. The incubation temperature of the stressed EVPOMEs was increased to 43°C on day 9 for 24 h and then returned to 37°C.¹⁷

Preimplantation assessment of construct health by biochemical assay and histology

To quantitatively assess the preimplantation construct health, cytokine concentrations in the culture medium were measured by biochemical assay on the day of implantation (day 11). Concentrations were measured by the microfluidic enzyme-linked immunosorbent assay (ELISA) kit Optimiser™ (Siloam Biosciences) according to the manufacturer's

instructions, as previously described.⁶ The three constitutively secreted cytokines measured were interleukin-8 (IL-8), human β -defensin 1 (hBD-1), and vascular endothelial growth factor (VEGF). IL-8 promotes re-epithelialization by inducing host keratinocyte migration and proliferation.²⁴ The antimicrobial peptide hBD-1 promotes differentiation by protecting keratinocytes from apoptosis.²⁵ VEGF induces angiogenesis *in vivo*, a critical factor in tissue integration.^{5,26}

Histological sections were also collected on day 11 to directly observe the preimplantation health of the constructs. Approximately one-fifth of the construct was cut, fixed in 10% formalin, washed with phosphate-buffered saline, and stored in a 70% ethanol solution. Specimens were cut into 5 μm thick sections and stained with hematoxylin and eosin.

Study design

Primary cells from four distinct patients were used in this study. Figure 1 depicts the experimental study design per patient. Cells from each patient were used to manufacture EVPOMEs in 1- or 3-week implantation cohorts. Cell seeding was staggered by 2 weeks so that *in vivo* optical measurements could be performed on the same day for both cohorts. Each cohort was split into stressed and control groups at day 9 of the manufacturing protocol, with each group containing duplicates. Therefore, eight EVPOMEs were manufactured from each patient. One exception was the 1-week cohort of patient 1, which contained triplicates instead of duplicates.

Biochemical and histological assessments performed on day 11 characterized preimplantation construct viability. Each construct was then implanted into a mouse. The implanted constructs were noninvasively assessed by FLS either 1 or 3 weeks after implantation. Mice were euthanized after optical assessment, and constructs were sectioned and processed for postimplantation histological assessment.

Construct implantation protocol

All animal experiments were approved by the UM Committee on Use and Care of Animals. After preimplantation assessments were completed on day 11, constructs were implanted into 7- to 8-week-old female severe combined immunodeficiency mice, as previously described.²¹ In brief, constructs were implanted into subcutaneous pouches ($\sim 2 \text{ cm}^2$) created by a curvilinear incision in the dorsal skin of anesthetized mice. The constructs were oriented such that the dermal equivalent was in contact with the muscular fascia. A thin silastic sheet ($\sim 130 \mu\text{m}$ thick, $\sim 2 \text{ cm}^2$) was placed over the construct to prevent adhesion with the skin. The pouch was secured using removable surgical staples (Autoclip™; Becton Dickinson).

FLS instrumentation

Time-resolved fluorescence decays were acquired *in situ* from the implanted constructs by employing clinically compatible FLS instrumentation,²⁰ shown in Figure 2. A pulsed 355 nm laser (1 kHz repetition rate; 500 ps pulse width) excited endogenous fluorophores in the constructs, including nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), an indicator of cellular metabolism.^{17,27–30} Laser excitation light was directed to the constructs by a central “source”

FIG. 1. Study design for each patient. Cells from one patient were seeded on AlloDerm® (day 0) in two cohorts: one to be implanted into mice for 1 week and the other for 3 weeks. The incubation temperature of stressed constructs (red) was increased to 43°C, whereas control constructs (blue) were maintained at 37°C (days 9–10). At day 11 for each construct, three aliquots of spent culture medium were collected for ELISA, one-fifth of the construct was cut for histology, and the remainder was implanted into an SCID mouse. Constructs were optically assessed by FLS for both 1- and 3-week postimplantation cohorts (days 18 and 32). After optical assessment, the mice were sacrificed, and the construct was processed for histology. Note that “day 0” for 1- and 3-week cohorts was staggered so that FLS measurements could be acquired on the same day. ELISA, enzyme-linked immunosorbent assay; EVPOMEs, *Ex Vivo* Produced Oral Mucosa Equivalents; FLS, fluorescence lifetime spectroscopy; hBD-1, human β -defensin 1; SCID, severe combined immunodeficiency; VEGF, vascular endothelial growth factor. Color images available online at www.liebertpub.com/tec

optical fiber that was surrounded by six adjacent “detector” fibers employed to collect fluorescence emission (all fibers were of 200 μ m core diameter, R200-ANGLE-UV; Ocean Optics). The fiber-optic probe tip had a 30° angled window. Minimizing “source–detector” separation and angling the probe increased the optical interrogation volume of the superficial tissue layers relative to the underlying native mouse tissue.^{31,32} The angle also reduced collection of reflected excitation light from the tissue surface.

The collected fluorescence emission was detected by an avalanche photodiode (APD, C5658; Hamamatsu) and sampled at 4 MHz by a transient digitizer (TDS 784A; Tektronix). A 387 nm long-pass filter blocked reflected excitation light. A 540 ± 20 nm band-pass filter increased

sensitivity in the spectral regime dominated by NAD(P)H fluorescence relative to collagen.²⁰ A photodiode detected the laser pulse to synchronize the digitizer data acquisition to the excitation pulse. Instrument response functions (IRFs) of the APD were characterized by removing the filters from the light path, illuminating 0.54 μ m diameter silica microspheres suspended in deionized water (1.7%), and recording the diffusely reflected signal passed to the APD.^{18,20}

FLS measurement protocol

Mice were placed prone and anesthetized using isoflurane administered through nose cone. A curvilinear incision was made in the area where constructs were implanted, and the

FIG. 2. Schematic of FLS system components. The distal end of the fiber-optic probe is positioned at varying stand-off distances from the *top* surface of the tissue sample. The portable FLS system acquires data in <1 s per measurement. The handheld probe is sufficiently small to be positioned at multiple measurement sites on each construct. The angled probe design aids in localizing optical interrogation to the top living cell layer of the construct and reduces collection of reflected excitation light. ND, neutral density filter; PD, photodiode; L1, L2, and L3, lenses; LP, long-pass filter; BP, band-pass filter; APD, avalanche photodiode. Color images available online at www.liebertpub.com/tec

TABLE 1. TABLE OF MEASUREMENTS

<i>Condition</i>	<i>Patients</i>	<i>Constructs</i>	<i>Sites</i>	<i>Measurements acquired</i>	<i>Measurements included</i>
One week postimplantation					
Control	4	7	14	70	48
Stressed	4	9	18	90	80
Total		16	32	160	128
Three weeks postimplantation					
Control	4	7	14	70	52
Stressed	4	7	14	70	40
Total		14	28	140	92

Optical measurements were acquired *in vivo* from two sites per construct, and from five stand-off positions per site. Included measurements met criteria for raw signal strength and quality of model fit.

silastic sheet was removed to expose the implanted constructs for measurement. Mice were placed on a stage equipped with a heating pad set to 38°C to maintain body temperature. The stage could be tilted to align the construct surface with the angled window of the FLS probe. Measurements were acquired from two randomly selected sites on the implanted constructs at five stand-off distances from the tissue surface (0 to 2 mm in increments of 0.5 mm). A computer-controlled, micrometer-guided, three-axis linear translation stage precisely ($\sim 10 \mu\text{m}$ resolution) located the FLS probe to select measurement sites and set stand-off distances. Control measurements were acquired from exposed muscle tissue adjacent to the construct and from neighboring bare skin. The duration of each FLS measurement was ~ 500 msec.

FLS data analysis

The protocol for analyzing fluorescence decays acquired by the FLS was previously reported.^{18,20} In brief, time-resolved fluorescence measurements acquired from tissues were interpreted as a convolution of the intrinsic fluorescence decay from the sample and the IRF. After an iterative deconvolution process, the intrinsic fluorescence decay was fitted to a simulated multiexponential model. The data were best fitted to a double-exponential decay model:

$$f(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}. \quad (1)$$

Here, τ_1 and τ_2 represent electronic excited-state lifetimes of long-lived and short-lived component fluorophores within the sample, respectively, and A_1 and A_2 represent percentage contributions of those components to the measured fluorescence.

Only measurements that met certain criteria were included for data analysis. First, the peak fluorescence intensity of the measurement had to be greater than the threshold, defined as 1 standard deviation below the average peak signal intensity for all measurements within the cohort. Decays with low signal intensity do not have sufficient photon count to accurately parameterize the curves. Second, the double-exponential model had to produce an excellent fit to the data, defined as a Pearson's cumulative chi-squared test statistic, χ^2 , < 0.1 . Poor fits may arise from measurements with low signal-to-noise ratios. Finally, one measurement was excluded for exhibiting saturation. The number of measurements that met these three inclusion

criteria for each experimental group within each cohort is listed in Table 1.

Fluorescence decays were background corrected for ambient light before analysis.²⁰ IRF measurements were iteratively shifted temporally with respect to the fluorescence decay to align the decays. We performed the Levenberg-Marquardt least-squares fitting algorithm (MATLAB R2013b) for all measurements on each mouse for each IRF temporal shift. The temporal shift that produced the smallest χ^2 value was used as the best fit for that mouse. We calculated the optical parameter A_1/A_2 for each measurement after fitting the fluorescence decay profiles according to Equation 1.

Statistical analysis

Given the hierarchical grouping of the measurements (stand-off distances within sites, sites within constructs, constructs within patients), we fit the data using a linear mixed-effects model.³³ The response variable was the postimplantation optically measured parameter A_1/A_2 , and the model was fit for each implantation cohort (1 and 3 weeks) separately. The primary independent variable was the preimplantation construct health, quantified as the concentration of secreted cytokines, that is, IL-8, hBD-1, or VEGF, each considered as a predictor in separate models. These fixed-effects terms contain the linear regression coefficients that indicate correlations between A_1/A_2 values and cytokine concentrations. Patients and constructs were each included as random intercepts, accounting for correlations from sites sampled within constructs and constructs sampled within patients. Five levels of stand-off distances were also included as dummy variables to adjust for potential differences due to stand-off distance. Statistical significance was set at an alpha of 0.05. Analysis was performed using the Statistics Toolbox (v. 8.3) in MATLAB (R2013b).

Results

Histological assessment of preimplantation and postimplantation construct health

Representative preimplantation and postimplantation histologies are shown in Figure 3. Control constructs developed three characteristic layers: a top keratin layer, a middle living cell layer, and a bottom dermal equivalent layer. Stressed constructs typically did not develop healthy

FIG. 3. Representative tissue-engineered construct histology. Control preimplantation constructs developed three distinct layers: a top keratin layer (K), a middle living cell layer (LC), and a bottom dermal equivalent layer (DE). Stressed constructs did not develop a healthy cell layer preimplantation. One week after implantation, cell layers in control constructs were thicker and more organized than those of stressed constructs. However, cell layers of both control and stressed groups were comparably healthy 3 weeks after implantation, consisting of re-epithelialized cell layers and neovasculation (arrowheads).²¹ All scale bars are 50 μ m. Color images available online at www.liebertpub.com/tec

cell layers. One week after implantation, cell layers in control constructs were thicker and more organized than those of stressed constructs. After 3 weeks, both control and stressed constructs consisted of cells and neovasculation originating from the mouse.²¹

Characterization of in vivo FLS measurements

Figure 4A shows representative fluorescence decays, all acquired from one mouse at a stand-off distance of 0.5 mm, to illustrate characterization of the FLS system. Measurements are shown from the construct (on site), adjacent exposed muscle tissue (off site), and neighboring bare skin. Fluorescence intensities shown in Figure 4A are normalized to the maximum intensity of the depicted measurements. Raw signal intensity of control measurements acquired from off-site muscle tissue was <30% of that from on-site of the construct. Raw signal intensity acquired from neighboring bare skin was <6% of the intensity from the construct.

The solid curve shown in Figure 4A represents the simulation fit resulting from the least-squares iterative deconvolution algorithm. The goodness of fit can be visualized from the residual gray curve, which has been multiplied fivefold to be visible on the plot. For this representative decay, the residual difference between the measured data and the simulation fit is <2%. The average reduced chi-squared for all included measurements in the study was 0.05, ensuring confidence in the accuracy of the extracted fit parameters A_1 , A_2 , τ_1 , and τ_2 .

Figure 4B shows a box plot that categorizes fluorescence intensities measured from the implanted constructs by stand-off distance of the probe from the tissue sam-

ple. The data shown here are for the control group of the 1-week implantation cohort. Each measurement is normalized to the intensity at the stand-off distance that has the strongest raw signal intensity within that site. The fluorescence intensity was always strongest at a stand-off distance of 0.0 mm, when the probe is in contact with the tissue surface, and consistently decreased as the stand-off distance increased. In contrast, as shown in Figure 4C, the variability and range of the corresponding fluorescence lifetime parameters A_1/A_2 extracted from simulation fits were largely independent of stand-off distance.

Correlation between in vivo optical parameters and in vitro cytokine secretion

The scatter plots in Figure 5 show the relationship between the fluorescence lifetime parameter A_1/A_2 and each of the three preimplantation cytokine secretion levels, for the 1-week implantation cohort. Each data point represents the average of all measurements per construct (averaged across sites and stand-off distances). *In vivo* fluorescence sensing revealed that A_1/A_2 values correlated negatively with preimplantation cytokine secretion levels. Constructs that exhibited high cytokine secretion levels before implantation yielded smaller A_1/A_2 values after 1 week of tissue integration. The linear regression coefficients were -0.30 ($p=0.012$), -0.09 ($p<0.001$), and -0.06 ($p=0.001$) for 10 pg/mL increase in IL-8, hBD-1, and VEGF, respectively, indicating a significant negative correlation between A_1/A_2 values and cytokine concentration for all three proteins.

FIG. 4. FLS data analysis. (A) Representative time-resolved fluorescence decays acquired from one mouse, at one stand-off distance. Fluorescence data acquired from the construct *in situ* (on site, diamonds) were fit to a biexponential decay (simulation fit, solid line), for extraction of fluorescence decay parameters: τ_1 , τ_2 , A_1 , and A_2 . The residual difference between the data and the simulation fit is no more than 2% (gray line). Signals acquired from muscle tissue adjacent to the construct (off site, dashed line) and neighboring skin (dotted line) are significantly lower than signal from the construct. (B) Box plots of peak fluorescence intensity, categorized by stand-off distance of probe from tissue surface. Mean peak fluorescence intensity from the constructs decreased consistently as the stand-off distance increased. (C) However, the extracted fluorescence lifetime parameter A_1/A_2 was stable for all stand-off distances. Therefore, the fluorescence lifetime parameters from all stand-off distances per construct may be combined for data analysis. Data for box plots are for 1-week implantation control measurements ($n=9, 12, 12, 8$, and 7 for stand-off distances 0.0 – 2.0 mm, respectively). Center horizontal line, median; Box, middle 50% of data; Whiskers, upper and lower 25% of data.

Although A_1/A_2 values also varied across the 3-week implantation population with similar range and variability as the 1-week population, the data did not indicate any significant correlation with respect to preimplantation cytokine concentrations (data not shown). The linear regression co-

efficients were -0.18 ($p=0.122$), 0.01 ($p=0.335$), and 0.00 ($p=0.819$) for 10 pg/mL increase in IL-8, hBD-1, and VEGF, respectively, indicating no significant correlation between A_1/A_2 values and cytokine concentration for any of the three proteins for the 3-week implantation cohort.

FIG. 5. Noninvasive optical assessment of implanted engineered tissues correlates with preimplantation cytokine secretion. The fluorescence lifetime parameter A_1/A_2 varied across the 1-week implantation study cohort, correlating negatively with preimplantation protein concentration levels for all three constitutively secreted proteins (linear mixed effects model, $p=0.012$ for IL-8, $p<0.001$ for hBD-1, and $p=0.001$ for VEGF). Each data point represents the average value per construct, where vertical error bars represent standard errors and horizontal error bars represent 5% relative standard deviations. IL-8, interleukin-8.

Discussion

Tissue fluorescence spectroscopy was employed for this study for its ability to noninvasively and quantitatively assess local tissue biochemistry and morphology *in vivo* through endogenous sources of contrast.³⁴ The use of FLS was motivated by our finding that label-free fluorescence lifetime-based sensing differentiated control from stressed engineered tissue constructs *in vitro*.¹⁷ In that study, we found that the percentage contribution of long-lived fluorescence decay cellular components (A_1) was significantly lower for control constructs than for stressed constructs. Our *in vivo* results for the 1-week implantation cohort are, therefore, consistent with our *in vitro* results.

These results suggest that optical parameters measured using FLS can noninvasively assess the health of implanted tissue-engineered constructs. Healthy constructs that secreted high levels of the cytokines IL-8, hBD-1, and VEGF before implantation tended to have lower optically measured A_1/A_2 values 1 week after implantation into mice. Post-implantation histology confirmed that control constructs (which had high cytokine secretion levels) had thicker and better organized living cell layers than stressed constructs at this time point, as expected.⁶

In excised rabbit oral mucosa, depth-resolved FLS revealed that the percentage contribution of the short-lived fluorophore was greater in the epithelial cell layer than in the dermal layer.³⁴ The difference was attributed to the presence of nicotinamide adenine dinucleotide in the superficial cell layer, which has a shorter fluorescence lifetime than the collagen in the underlying dermal layer.³⁵ Our *in vitro* studies on engineered tissue constructs found notably higher levels of cellular NAD(P)H fluorescence emission from control constructs than from stressed constructs.¹⁷ Similarly, we found that constructs with high pre implantation cytokine secretion levels, and, therefore, more viable cells, had lower A_1/A_2 values postimplantation, indicating greater contribution from short-lived fluorophores (A_2).

Unlike the 1-week implantation cohort, A_1/A_2 did not correlate with preimplantation cytokine secretion after the constructs had been implanted into mice for 3 weeks. For the 3-week implantation cohort, histology showed that native mouse cells had infiltrated most of the constructs, regardless of their preimplantation health state, consistent with our previously published results.²¹ This is expected, as even the implantation of bare dermal equivalent aids re-epithelialization and revascularization by providing a substrate.³⁶ The infiltration of mouse cells may explain the lack of correlation of 3-week postimplantation A_1/A_2 values with preimplantation cytokine secretion. Thus, the utility of this approach could be best suited to monitor the viability of the living, manufactured epithelial cell layer of a construct at early stages postimplantation.

FLS has certain advantages for tissue assessment in clinical environments. Local tissue absorption and scattering *in vivo* can unpredictably affect spectral line shape, but fluorescence lifetime is less sensitive to these artifacts.³⁷ The acquisition time per measurement is <500 msec, which allows for rapid assessment and overcomes of patient-related motion artifacts.³⁸ Our *in vitro* study¹⁷ employed nonlinear optical microscopy with multiphoton excitation, which has inherent optical sectioning capability to enhance

spatial targeting of the epithelial cell layer. However, the large size and expense of multiphoton laser sources create a barrier to clinical translation. Here, we have employed single-photon excitation for fluorescence sensing, an approach that has successfully evaluated the composition of explanted porcine vascular grafts³⁹ and has been demonstrated for a handheld fluorescence lifetime imaging system for oral cancer imaging *in vivo*.⁴⁰ The extended spatial volume is beneficial when the clinician cannot precisely handle the probe while accessing small confines, as the instrument is insensitive to stand-off distance. Together, these considerations demonstrate promise that our FLS system could be used in future human applications.

In summary, we demonstrated a methodology to rapidly and noninvasively assess engineered tissue constructs *in vivo* by measuring endogenous optical signals through FLS. This is the first demonstration of postimplantation assessment of the cellular metabolic activity of engineered epithelial tissues *in vivo*, to our knowledge. Since optical parameters measured *in vivo* correlated with preimplantation cytokine concentrations, which indicate cell viability and functionality and predict healthy construct integration,⁶ this clinically compatible approach can be a promising tool for assessing tissue integration and graft success.

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Disclosure Statement

No competing financial interests exist.

References

- MacNeil, S. Progress and opportunities for tissue-engineered skin. *Nature* **445**, 874, 2007.
- Moharamzadeh, K., Colley, H., Murdoch, C., *et al.* Tissue-engineered oral mucosa. *J Dent Res* **91**, 642, 2012.
- Fisher, M.B., and Mauck, R.L. Tissue engineering and regenerative medicine: recent innovations and the transition to translation. *Tissue Eng Part B Rev* **19**, 1, 2013.
- Lee, M.H., Arcidiacono, J.A., Bilek, A.M., *et al.* Considerations for tissue-engineered and regenerative medicine product development prior to clinical trials in the United States. *Tissue Eng Part B Rev* **16**, 41, 2010.
- Xu, Q., Izumi, K., Tobita, T., Nakanishi, Y., and Feinberg, S.E. Constitutive release of cytokines by human oral keratinocytes in an organotypic culture. *J Oral Maxillofac Surg* **67**, 1256, 2009.
- Kuo, S., Zhou, Y., Kim, H.M., *et al.* Biochemical indicators of implantation success of tissue-engineered oral mucosa. *J Dent Res* **94**, 78, 2015.
- Lloyd, W.R., Chen, L., and Mycek, M. Fluorescence spectroscopy. In: Morgan, S., Rose, F., and Matchar, S., eds. *Optical Techniques in Regenerative Medicine*. Boca Raton, FL: CRC Press, 2013, p. 171.
- Tan, W., Oldenburg, A.L., Norman, J.J., Desai, T.A., and Boppart, S.A. Optical coherence tomography of cell dynamics in three-dimensional tissue models. *Opt Express* **14**, 7159, 2006.

9. White, S.M., Hingorani, R., Arora, R.P., Hughes, C.C., George, S.C., and Choi, B., Longitudinal in vivo imaging to assess blood flow and oxygenation in implantable engineered tissues. *Tissue Eng Part C Methods* **18**, 697, 2012.
10. Khmaladze, A., Ganguly, A., Kuo, S., *et al.* Tissue-engineered constructs of human oral mucosa examined by Raman spectroscopy. *Tissue Eng Part C Methods* **19**, 299, 2013.
11. Georgakoudi, I., Rice, W.L., Hronik-Tupaj, M., and Kaplan, D.L. Optical spectroscopy and imaging for the non-invasive evaluation of engineered tissues. *Tissue Eng Part B Reviews* **14**, 321, 2008.
12. Sun, Y., Responde, D., Xie, H., *et al.* Noninvasive evaluation of tissue engineered cartilage using time-resolved fluorescence spectroscopy and ultrasound backscatter microscopy. *Tissue Eng Part C Methods* **18**, 215, 2012.
13. Ashijian, P., Elbarbary, A., Zuk, P., *et al.* Noninvasive *in situ* evaluation of osteogenic differentiation by time-resolved laser-induced fluorescence spectroscopy. *Tissue Eng* **10**, 411, 2004.
14. Syverud, B.C., Mycek, M.A., and Larkin, L.M. Quantitative, label-free evaluation of tissue-engineered skeletal muscle through multiphoton microscopy. *Tissue Eng Part C Methods* **23**, 616, 2017.
15. Izumi, K., Song, J., and Feinberg, S.E. Development of a tissue-engineered human oral mucosa: from the bench to the bed side. *Cells Tissues Organs* **176**, 134, 2004.
16. Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*. New York: Springer Science & Business Media, 2013.
17. Chen, L., Lloyd, W.R., Kuo, S., *et al.* The potential of label-free nonlinear optical molecular microscopy to non-invasively characterize the viability of engineered human tissue constructs. *Biomaterials* **35**, 6667, 2014.
18. Pitts, J.D., and Mycek, M.A. Design and development of a rapid acquisition laser-based fluorometer with simultaneous spectral and temporal resolution. *Rev Sci Instruments* **72**, 3061, 2001.
19. Vishwanath, K., Pogue, B., and Mycek, M.A. Quantitative fluorescence lifetime spectroscopy in turbid media: comparison of theoretical, experimental and computational methods. *Phys Med Biol* **47**, 3387, 2002.
20. Chandra, M., Vishwanath, K., Fichter, G.D., Liao, E., Hollister, S.J., and Mycek, M.A. Quantitative molecular sensing in biological tissues: an approach to non-invasive optical characterization. *Opt Express* **14**, 6157, 2006.
21. Izumi, K., Feinberg, S.E., Terashi, H., and Marcelo, C.L. Evaluation of transplanted tissue-engineered oral mucosa equivalents in severe combined immunodeficient mice. *Tissue Eng* **9**, 163, 2003.
22. Izumi, K., Takacs, G., Terashi, H., and Feinberg, S.E. Ex vivo development of a composite human oral mucosal equivalent. *J Oral Maxillofac Surg* **57**, 571, 1999.
23. Izumi, K., Terashi, H., Marcelo, C.L., and Feinberg, S.E. Development and characterization of a tissue-engineered human oral mucosa equivalent produced in a serum-free culture system. *J Dent Res* **79**, 798, 2000.
24. Rennekampff, H.O., Hansbrough, J.F., Kiessig, V., Dore, C., Sticherling, M., and Schroder, J.M. Bioactive interleukin-8 is expressed in wounds and enhances wound healing. *J Surg Res* **93**, 41, 2000.
25. Frye, M., Bargon, J., and Gropp, R. Expression of human beta-defensin-1 promotes differentiation of keratinocytes. *J Mol Med (Berl)* **79**, 275, 2001.
26. Nakanishi, Y., Izumi, K., Yoshizawa, M., Saito, C., Kawano, Y., and Maeda, T. The expression and production of vascular endothelial growth factor in oral mucosa equivalents. *Int J Oral Maxillofac Surg* **36**, 928, 2007.
27. Wu, Y., and Qu, J.Y. Autofluorescence spectroscopy of epithelial tissues. *J Biomed Opt* **11**, 054023, 2006.
28. Pogue, B.W., Pitts, J.D., Mycek, M., *et al.* In vivo NADH fluorescence Monitoring as an assay for cellular damage in photodynamic therapy. *Photochem Photobiol* **74**, 817, 2001.
29. Pitts, J.D., Sloboda, R.D., Dragnev, K.H., Dmitrovsky, E., and Mycek, M.A. Autofluorescence characteristics of immortalized and carcinogen-transformed human bronchial epithelial cells. *J Biomed Opt* **6**, 31, 2001.
30. Sud, D., Zhong, W., Beer, D.G., and Mycek, M.A. Time-resolved optical imaging provides a molecular snapshot of altered metabolic function in living human cancer cell models. *Opt Express* **14**, 4412, 2006.
31. Vishwanath, K., and Mycek, M. Time-resolved photon migration in bi-layered tissue models. *Opt Express* **13**, 7466, 2005.
32. Zhu, C., Liu, Q., and Ramanujam, N. Effect of fiber optic probe geometry on depth-resolved fluorescence measurements from epithelial tissues: a Monte Carlo simulation. *J Biomed Opt* **8**, 237, 2003.
33. Fitzmaurice, G.M., Laird, N.M., and Ware, J.H. *Applied Longitudinal Analysis*. Hoboken, NJ: John Wiley & Sons, 2012.
34. Wu, Y., and Qu, J.Y. Combined depth- and time-resolved autofluorescence spectroscopy of epithelial tissue. *Opt Lett* **31**, 1833, 2006.
35. Vishwanath, K., and Mycek, M.A. Do fluorescence decays remitted from tissues accurately reflect intrinsic fluorophore lifetimes? *Opt Lett* **29**, 1512, 2004.
36. Berthiaume, F., Maguire, T.J., and Yarmush, M.L. Tissue engineering and regenerative medicine: history, progress, and challenges. *Annu Rev Chem Biomol Eng* **2**, 403, 2011.
37. Lloyd, W.R., Wilson, R.H., Chang, C.W., Gillispie, G.D., and Mycek, M.A. Instrumentation to rapidly acquire fluorescence wavelength-time matrices of biological tissues. *Biomed Opt Express* **1**, 574, 2010.
38. Mycek, M.A., Schomacker, K.T., and Nishioka, N.S. Colonic polyp differentiation using time-resolved autofluorescence spectroscopy. *Gastrointest Endosc* **48**, 390, 1998.
39. Fatakda, H., Griffiths, L.G., Humphrey, S., and Marcu, L. Time-resolved fluorescence spectroscopy and ultrasound backscatter microscopy for nondestructive evaluation of vascular grafts. *J Biomed Opt* **19**, 080503, 2014.
40. Cheng, S., Cuenca, R.M., Liu, B., *et al.* Handheld multi-spectral fluorescence lifetime imaging system for in vivo applications. *Biomed Opt Express* **5**, 921, 2014.

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