METHODS ARTICLE

Optical Metric Assessed Engineered Tissues Over a Range of Viability States

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Many conventional methods to assess engineered tissue morphology and viability are destructive techniques with limited utility for tissue constructs intended for implantation in patients. Sterile label-free optical molecular imaging methods analyzed tissue endogenous fluorophores without staining, noninvasively and quantitatively assessing engineered tissue, in lieu of destructive assessment methods. The objective of this study is to further investigate label-free optical metrics and their correlation with destructive methods. Tissue-engineered constructs ($n=33$ constructs) fabricated with primary human oral keratinocytes ($n=10$ patients) under control, thermal stress, and rapamycin treatment manufacturing conditions exhibited a range of tissue viability states, as evaluated by quantitative histology scoring, WST-1 assay, Ki-67 immunostaining imaging, and label-free optical molecular imaging methods. Both histology sections of fixed tissues and cross-sectioned label-free optical images of living tissues provided quantitative spatially selective information on local tissue morphology, but optical methods noninvasively characterized both local tissue morphology and cellular viability at the same living tissue site. Furthermore, optical metrics noninvasively assessed living tissue viability with a statistical significance consistent with the destructive tissue assays WST-1 and histology. Over the range of cell viability states created experimentally, optical metrics noninvasively and quantitatively characterized living tissue viability and correlated with the destructive WST-1 tissue assay. By providing, under sterile conditions, noninvasive metrics that were comparable with conventional destructive tissue assays, label-free optical molecular imaging has the potential to monitor and assess engineered tissue construct viability before surgical implantation.

Keywords: tissue engineering, optical metric, noninvasive, label-free, viability, imaging

Impact Statement

The U.S. Food and Drug Administration has identified the lack of noninvasive evaluation method as an essential missing experimental release criterion strongly desired for clinical translation of tissue-engineered constructs. This study addresses the need by investigating label-free optical metrics for monitoring engineered tissues with a range of viability states. The noninvasive optical method was compared with multiple traditional assays and was found to correlate with a destructive tissue assay. The study would draw interest from many fields, including academics and professionals working in biomedical optics, tissue engineering, regenerative medicine, and the broader medical audience due to its potential applications to any cell-based construct.

Introduction

TISSUE ENGINEERING HAS advanced to a state that diseased tissues can be replaced or repaired by patient's own healthy tissues.^{1,2} However, tissue-engineered construct manufacturing is strictly regulated by the U.S. Food and Drug

Administration, which requires assessment of product effectiveness and safety before release for use in patient treatment. Inhomogeneous engineered tissues require techniques to reliably assess their local viability. Conventional methods to assess local tissue viability include histology, WST-1 assays, and Ki-67 immunostaining imaging, which require tissue

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sections or biopsies followed by labor-intensive analysis protocols. Naturally, the tissue specimens analyzed in these ways cannot be implanted due to the destructive nature of the methods and, because of tissue inhomogeneity, the remaining engineered tissue available for implantation has uncertain viability. Therefore, nonlinear optical microscopic imaging methods have been developed to assess inhomogeneous engineered tissues noninvasively.³⁻⁶

Previously, we demonstrated that label-free nonlinear optical molecular imaging with quantitative metrics noninvasively and reliably assessed the viability of living tissue-engineered human oral mucosa equivalents.³ The nonlinear optical technique provides microscopic images of thin optical sections (a few micrometers thick, comparable with a cellular layer) of tissue constructs,^{7,8} thereby reducing the presence of background optical signals from other tissue layers.^{9,10} In addition, the near-infrared wavelengths employed for nonlinear optical imaging make it optimal for deeper, sub-surface tissue imaging (millimeter scale, depending on the wavelength and tissue type), as compared with conventional linear optical microscopy at visible wavelengths.^{11,12}

Importantly, the high sensitivity of nonlinear optical molecular microscopy makes it ideal for measuring endogenous tissue fluorophores, enabling label-free noninvasive optical monitoring of tissue-engineered constructs without stains or fluorescent dyes. By minimizing chemical perturbations to the living biological system, the interrogated tissues retain their inherent functionality and can then be studied with other assays or employed clinically. Endogenous tissue fluorophores include keratin, melanin, nicotinamide adenine dinucleotide (phosphate) [NAD(P)H], flavin adenine dinucleotide (FAD), lipofuscin, tryptophan, collagen, and elastin. The optical properties of these tissue fluorophores have been reported¹³ and widely investigated for biological and biomedical applications.¹⁴⁻¹⁶ In particular, intracellular NAD(P)H and FAD molecules are involved in mitochondrial metabolic pathways, serving as natural biomarkers for cellular metabolic function.¹⁷⁻¹⁹ Therefore, a quantitative ratiometric method was developed that employs measures of intracellular NAD(P)H and FAD fluorescence intensity^{3,20} to form a quantity known as the redox ratio (RR), a metric used to evaluate cellular metabolism that can be mapped across optical sections of living tissues.

The objective of this study is to further investigate label-free optical metrics and their correlation with destructive methods. We compare optical metrics obtained noninvasively from living tissues to standard, but destructive, tissue viability measures over a range of viability states in engineered tissue

constructs manufactured from primary human oral keratinocytes. We introduced rapamycin treatment to selected tissue constructs during fabrication. Rapamycin is a drug regulating cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription.²¹ Rapamycin, a specific inhibitor of the protein kinase mTOR (mechanistic or mammalian target of rapamycin), maintains primary human oral keratinocytes as an undifferentiated cell population capable of retaining their proliferative capacity.^{22,23} Rapamycin treatment controls cellular proliferation in culture and in engineered tissues, which can create a range of states of tissue viability.²³ Data were obtained from control, rapamycin-treated, and thermally stressed tissue constructs, with results supporting the ability of noninvasive label-free optical molecular imaging to safely and effectively assess a range of viability states in living engineered tissues.

Materials and Methods

Study design

Tissue-engineered constructs were manufactured with primary human oral keratinocytes freshly harvested from 10 individuals (Table 1). With one subject's cells, one batch of tissue-engineered constructs was cultured. Five batches were studied for the rapamycin experiment and five for the thermal-stress experiment. Constructs were fabricated under both control and experimental procedures for each batch. Tissue-engineered constructs were assessed by standard assays and by optical methods, as illustrated in Figure 1. The equipment and settings for all the images shown are in Supplementary Table S1.

All 10 batches of engineered tissue constructs were sectioned for histological assessment and characterized with label-free optical imaging at three randomly selected sites (Table 1 and Fig. 1). Two batches of engineered tissue constructs from the rapamycin experiment were randomly selected for Ki-67 immunostaining imaging and five batches (three from the rapamycin experiment and two from the thermal experiment) were randomly selected for punch biopsies for the WST-1 assay. As shown in Figure 1, a tissue section was sliced across the construct, near the edge, for hematoxylin and eosin (H&E) histology and Ki-67 immunostaining imaging.

A tissue-engineered construct consisted of three layers: a keratin layer, the living cellular layer, and a dermal equivalent layer, as shown in a representative histology section (Fig. 1). At each tissue site imaged optically, one optically sectioned image was recorded in the proliferating basal layer

TABLE 1. STUDY DESIGN

Experimental condition (cell culture+tissue culture)	<i>control+control</i>	rapa+control	rapa+rapa ^a	control+rapa ^a	thermally stressed
Number of batches (patients)	<i>10</i>	5	5	5	5
Number of constructs/histology	<i>12</i>	6	4	3	5
Number of WST-1 measurements	<i>7</i>	4	2	1	2
Number of optical measurements	<i>64</i>	36	21	15	30

The color indicates the expected range of viability states for the fabricated tissues constructs, from most viable (italics, left) to least viable (bold, right).

^aOf the 33 tissue constructs fabricated for the study, 3 constructs (1 from rapa+rapa and 2 from control+rapa) were found through both histology measures and optical imaging assessment to have no cells present atop the dermal equivalent. Therefore, rapa+rapa and control+rapa had less number of constructs and measurements for analysis than the other experimental conditions.

FIG. 1. Tissue-engineered constructs were developed with varying viability and evaluated by histology, WST-1 assay, immunohistochemistry imaging, and label-free optical molecular imaging. A tissue construct ~ 1 cm in diameter was sectioned for histology and/or immunohistochemistry imaging, and/or punch biopsied for WST-1 assay (blue). (The numbers of measurements are detailed in Table 1). The remainder of the construct was assessed noninvasively with optical imaging (orange) at three randomly selected locations (red arrows). z represents the optical axis of the microscope. DE, dermal equivalent (AlloDerm® scaffold); K, keratin layer; LC, living cellular layer. Color images are available online.

(lower depth) and another was recorded in the differentiating cellular layer (upper depth). At tissue sites without an analyzable upper cellular layer, only the basal layer was measured.

Ex vivo produced oral mucosa equivalent culture protocol

Discarded keratinized oral mucosa was collected from patients undergoing minor oral surgical procedures at the University of Michigan (UM) Hospital. The UM Medical School's 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.

The standard culture protocol can be split into two stages: cell culture and tissue fabrication. In the cell culture stage, primary human oral keratinocytes were harvested from procured discarded keratinized oral mucosa and cultured according to previously described protocols.¹ In brief, 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) containing 0.06 mM calcium.

In the tissue fabrication stage, *ex vivo* produced oral mucosa equivalent (EVPOME) constructs were manufactured by first seeding oral keratinocytes on 1 cm² acellular cadaver dermis (AlloDerm®; LifeCell Corporation, Branchburg, NJ). Resulting keratinocytes and AlloDerm (dermal equivalent) were submerged in a 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. Cells and constructs were cultured in incubators at 37°C with 5% CO₂ for all culture days.

Rapamycin treatment and thermal stressing protocols

For the rapamycin experiment, cells were cultured with or without the presence of rapamycin during the last 5 days of the cell culture stage. Then, five batches of tissue-engineered constructs were fabricated with or without the presence of rapamycin for 11 days during the tissue fabrication stage.

Thus, four experimental conditions were created (Supplementary Fig. S1):

1. control+control: No rapamycin was in the culture medium during either the cell culture stage or the tissue fabrication stage.
2. control+rapa: No rapamycin was in the culture medium during the cell culture stage; culture medium containing 2 nM rapamycin was employed during the tissue fabrication stage.
3. rapa+control: Culture medium containing 2 nM rapamycin was employed during the last 5 days of the cell culture stage; no rapamycin was in the culture medium during the tissue fabrication stage.
4. rapa+rapa: Culture medium containing 2 nM rapamycin was employed during both the last 5 days of the cell culture stage and the tissue fabrication stage.

For the five batches of constructs employed for the thermal-stress study, stressed constructs were cultured at 43°C for 24 h beginning on day 9 of the tissue-fabrication stage (postseeding) and were returned to normal culture conditions starting on day 10. Thermal stressing was expected to yield tissue constructs with the lowest viability.³ Table 1 summarizes the overall study design with the five experimental conditions, the numbers of batches and constructs studied, and the number of measurements obtained from tissue constructs through histology, WST-1 assays, and optical imaging.

WST-1 cellular viability assay protocol

Punch biopsies obtained from engineered tissues (Fig. 1) were incubated for 4 h at 37°C and 5% CO₂ with 10 μ L/well cell proliferation reagent WST-1 (Roche) in 100 μ L of culture medium without phenol red (Life Technologies). The samples were shaken thoroughly for 1 min on a shaker before measurement. Viability was characterized by comparing the samples' optical absorbance at 440 nm using 630 nm as the reference wavelength to that of the background control sample containing only the solution using a microplate reader. The WST-1 assay monitored intracellular mitochondrial activity, as assessed by WST-1 readings of

the biopsied tissues. High WST-1 readings indicate high mitochondrial activity. Negative WST-1 readings were assigned a value of 0.

Ki-67 immunostaining imaging protocol

Histology procedures were performed by the Histology Core Facility at UM. Tissue sections were fixed in 4% phosphate buffered formalin, paraffin-embedded, and sectioned on a microtome. Tissue section slides were deparaffinized in xylene, rehydrated through graduated alcohols, and washed in distilled water. Before staining, antigen retrieval was performed in the Biocare Decloaking Chamber. Immunoperoxidase staining was performed on a DAKO AutoStainer at room temperature with primary antibody Ki-67 (dilution 1:200; Abcam), secondary antibody rabbit HRP polymer (Biocare), DAB chromogen, and hematoxylin counterstain (dilution 1:10; Biocare). Ki-67 images were analyzed with ImageJ to count the number of dark stained nuclei in an image.

Quantitative histology scoring protocol

Tissue sections were fixed in 4% phosphate buffered formalin, paraffin-embedded, and sectioned for H&E staining. All H&E histology slides were assessed by a panel of three blinded expert readers (Y.K., C.L.M., and S.E.F.). Classification criteria (previously developed and detailed³) yielded tissue viability scores of 1 (least viable), 2, 3, 4, or 5 (most viable). Classification criteria included basal cell layer health, construct cellular organization, and keratin layer structural quality. The readers individually assigned a score to each EVPOME histology slide, although blinded to the construct's experimental condition and to the other readers' scores. Each cultured cell batch was categorized based on its control+control (no rapamycin treatment) construct histology score into either a well-growing or a poorly growing culture: a control+control score >3 defined a well-growing culture, whereas a control+control score ≤3 defined a poorly growing culture.

Label-free optical molecular imaging protocol

Detailed imaging protocols for living engineered tissues were reported previously.¹ In brief, tissue autofluorescence (attributed to cellular metabolic cofactors NAD(P)H and FAD, as well as extracellular structural proteins collagen and keratin) and tissue second harmonic generation (SHG) signals (attributed to collagen) were acquired in independent channels for both cross-sectional (*x-z* plane) and *en-face* (*x-y* plane) imaging (Fig. 1) with a Leica TCS SP5 microscope equipped with an ultrafast Ti:Sapphire laser (Mai Tai, Spectra-Physics). The laser light delivered to living tissue specimens and the light emitted from the tissues were coupled through an inverted microscope with a 25× water immersion objective lens (0.95 NA, 2.5 mm working distance) to image optical sections of EVPOME constructs. NAD(P)H fluorescence was excited at 705 nm and detected from 435 to 485 nm. FAD fluorescence and collagen SHG were excited at 900 nm and detected from 500 to 550 nm (Leica Dapi/FITC filter cube) and 440 to 460 nm, respectively. Detector gain and offset were consistent for each measurement to avoid detector saturation. Images (1024×1024 pixels, 0.391×0.391 μm² pixel size, 8-bit image

depth) were acquired in ~40 s with a 200 Hz line scanning speed. To reduce background noise, a line average of eight was employed for all images. Constructs were inverted onto measurement dishes. All measurements were collected in a sterile controlled environment (37°C with 5% CO₂) to mimic EVPOME culture.

For display, the optical images were overlaid with two signal channels: endogenous fluorescence from NAD(P)H and collagen (cyan) and SHG solely from collagen (dark blue). SHG images were employed to remove collagen signals from fluorescence images. NAD(P)H and FAD fluorescence images devoid of collagen signals were then employed to derive a RR map, calculated as $[FAD]/([NAD(P)H]+[FAD])$ at each image pixel. For each image containing cells, a mean optical RR-metric was calculated for viability assessment, with lower RR-metrics indicating higher tissue viability. RR maps of the differentiating cellular layer would contain pixels with abnormally high RRs (attributed to background keratin signals), so these pixels were excluded from the mean RR-metric calculation.

Statistical analysis

To account for the hierarchical nature of the data, statistical analyses used mixed-effects models.²⁴ Specifically, assessments from each construct generated under an experimental condition had assessments from a control construct generated from the same batch. Furthermore, for optical imaging data, measures were taken from multiple sites per tissue construct, and, for histology data, each histological section from a construct was read by three expert readers. Hence, for the analysis of optical imaging data, mixed-effects models included batch (patient) and construct as random intercepts to account for the two levels of nesting where multiple sites were sampled from each construct, and multiple constructs were obtained from each batch. Using these models, we tested for differences in various optical measures between experimental and control constructs by including an indicator for experimental condition as the primary explanatory variable.

Correlation between the optical RR-metric values and the WST-1 readings was assessed after averaging all metrics over each construct by experimental condition. The mixed-effects model was then employed with the optical RR-metric as the dependent variable and the WST-1 reading as a predictor, with batch as random intercepts to account for within-patient correlation. The Pearson correlation coefficient *r* and its associated *p*-value from the model were used to test for the significance of a linear relationship between the optical RR-metric and WST-1.

For data presented in box plots, the central mark on each box is the median and the edges of the box are the 25th and 75th percentiles. The whiskers above and below the box indicate the 99.7th and 0.03th percentiles, ~2.7 standard deviations from the mean.

Results

A range of tissue viability states were demonstrated as detailed in Supplementary Figures S2 and S3. The well-growing and the poorly growing batches demonstrated different levels of viability change with rapamycin treatment, measured by quantitative histology and Ki-67 images.

Label-free optical molecular imaging achieved rapid noninvasive assessment of local tissue morphology that was consistent with destructive tissue histology

In contrast to destructive tissue analysis methods described earlier, label-free optical molecular imaging assessed living engineered tissue morphology rapidly and noninvasively. The optical technique was performed repeatedly, in real time, at multiple tissue sites under sterile conditions to more fully and rapidly characterize engineered tissue constructs than labor-intensive, destructive techniques such as tissue histology.

Figure 2 compares histology sections with cross-sectioned label-free optical images of living tissues for the control+control and the rapa+control constructs of a poorly growing batch. Nonuniform tissue growth was observed in the control+control construct: Figure 2a (top) shows a histology section with a well-growing region (right) and a poorly growing region (left). This control+control engineered tissue received a histology score of 1.2, and thus was defined as a poorly growing batch. Label-free cross-sectioned optical molecular images of the living construct also revealed highly variable tissue growth in different spatial regions. In Figure 2a, the left optical image shows a thin layer of cells atop the dermal equivalent layer (AlloDerm scaffold), whereas the right optical image shows the full three tissue layers, thereby noninvasively confirming non-uniform tissue growth in this living construct. It is noted that the optical images were acquired by randomly selecting three

sites, not exactly the same spots as the histology image. Alternatively, the rapa+control construct in this batch had morphological characteristics consistent with a viable tissue construct (Fig. 2b): both the histology section of fixed tissue and the noninvasive optical images of the living tissue construct revealed full, relatively uniform, and thick cellular and keratin layers.

As noted in Table 1, 3 of the 33 tissue constructs fabricated were found through both destructive histology measures and noninvasive optical imaging assessment to have no cells present atop the dermal equivalent. Thus, the presence or absence of subsurface cells, their size, density, and uniformity, as well as the presence, absence, and uniformity of the surface keratin layer, could all be assessed rapidly and noninvasively in the living tissue construct through label-free optical molecular imaging, which provided results consistent with destructive tissue histology.

Label-free optical molecular imaging noninvasively and simultaneously assessed local tissue morphology and viability

While cross-sectioned label-free optical molecular images assessed local tissue morphology, *en-face* optical molecular images with the RR-metric assessed local tissue cellular metabolic function. Figure 3a and b shows cross-sectioned and *en-face* optical molecular images of optical sections of engineered tissues from all the experimental conditions of a

FIG. 2. Both histology sections of fixed tissues and noninvasive cross-sectioned label-free optical molecular imaging of living tissues provided spatially selective information representative of local tissue morphology. **(a)** In a poorly growing batch, a control+control construct had inhomogeneous tissue growth characterized by a fully growing region (*right*) and a poorly growing region (*left*). These features were visible in both histology and noninvasive optical images. **(b)** For the same poorly growing batch, the rapa+control tissue construct exhibited an intact layered structure visible in both the histology section and in the optical images of the living tissue. In the optical images, endogenous tissue fluorescence (*cyan* channel, as pointed by the *arrow*) was overlaid with dermal equivalent second harmonic generation from collagen (*blue* channel as pointed by the *triangles*) to show optical demarcation of cell and dermal equivalent layers. Scale bars: 100 μ m. Color images are available online.

FIG. 3. Label-free optical molecular imaging non-invasively characterized local tissue morphology and viability at the same site. For a poorly growing batch, (a) cross-sectioned optical images provided initial assessments of tissue morphology, whereas (b) *en-face* images of optical sections of tissue at the basal cellular layer revealed cellular spatial organization (*left*) and their corresponding optical RR-metric maps of cellular viability (*right*). The rapa+control construct had the lowest mean RRs, reflecting the highest cellular viability of the four tissue constructs, consistent with the results in Figures 3–5. In the *left* and *middle columns*, endogenous tissue fluorescence (cyan channel) was overlaid with dermal equivalent second harmonic generation from collagen (*blue* channel). Scale bar: 100 μ m. RR, redox ratio. Color images are available online.

poorly growing batch. For a tissue site on a control+control construct, the cross-sectioned image showed an irregular keratin surface, consistent with the observation from the Figure 2a histology image. In addition, the control+control *en-face* image revealed that the cells in the basal cellular layer were disorganized. In contrast, the cross-sectioned images of the control+rapa and the rapa+control constructs demonstrated well-layered tissue structures. In particular, the rapa+control construct had a thick living cellular layer ($\sim 21 \mu\text{m}$ thick). Its *en-face* image demonstrated that basal cellular layers were composed of small closely packed cells (well organized). The rapa+rapa construct had a relatively poor growth in this batch. Optical RR-metric maps assessed local tissue viability, with green indicating high viability and yellow indicating relatively low viability. Thus, rapa+control constructs had the highest viability, control+rapa and poorly growing control+control constructs had medium viability, and rapa+rapa constructs had the lowest viability. Thus, for the same living tissue site, optical molecular imaging noninvasively and simul-

taneously assessed local tissue morphology and composition, as well as cellular organization and viability.

Label-free optical molecular imaging noninvasively assessed living tissue viability with statistical significance equal to or better than destructive tissue assays

Figure 4 shows box plots comparing control+control and rapa+control constructs for poorly growing control+control batches. As compared with control+control constructs, rapa+control constructs show high WST-1 readings ($*p < 0.001$ for $n = 2$ batches with 4 measurements from the control+control and 3 measurements from the rapa+control constructs), high histology scores ($p = 0.002$ for $n = 3$ batches with 5 measurements from the control+control and 4 measurements from the rapa+control constructs), and low RRs ($*p < 0.001$ for $n = 3$ batches with 23 measurements from the control+control and 24 measurements from the rapa+control constructs). The results indicate that rapa+control constructs

FIG. 4. The optical RR metric (*right panel*) noninvasively assessed living tissue viability with a statistical significance equal to or better than the destructive tissue assays WST-1 (*left panel*) and histology (*center panel*), respectively. (**WST-1, $p < 0.001$ for $n = 2$ batches with 4 measurements from control+control and 3 measurements from rapa+control; *Histology, $p = 0.002$ for $n = 3$ batches with 5 measurements from control+control and 4 measurements from rapa+control; **Optical RR metric, $p < 0.001$ for $n = 3$ batches with 23 measurements from control+control and 24 measurements from rapa+control.) Color images are available online.

had high mitochondrial activity, high cellular metabolism, and high tissue growth, measured by WST-1 assay, optical metrics, and histology scores, respectively. All three metrics detected that the rapa+control constructs had better tissue viability than the control+control constructs in the poorly growing batches.

Optical RR metrics from living tissues correlated with WST-1 assay readings from tissue biopsies over a range of cell viability states

Optical RR metrics were compared with WST-1 assay readings for individual tissue constructs (Fig. 5). A total of 16 constructs were shown on the plot with each data point representing 1 tissue-engineered constructs and is color coded per 6 categories: 2 control+control conditions separated by their histology scores (score > 3 for well-growing control+control; score ≤ 3 for poorly growing control+control), 3 rapamycin treatment experimental conditions, and 1 thermal stress experimental condition. Over the wide range of viability states characterized by the WST-1 assay, the optical RR metric showed a correlation with WST-1 readings. Based on the optical measurement data averaged at the construct level where the metrics measured viability in tissue independent of experimental conditions, a moderate linear correlation was found with the correlation coefficient $r = -0.47$ ($p = 0.07$, $n = 16$), although it was only marginally significant. For the data at the optical measurement level, the correlation was significant, $r = -0.50$ ($p < 0.001$, $n = 88$). In addition, the slope estimate, β , was -0.30 ($p < 0.001$, $n = 16$) based on the linear mixed-effects model accounting for patient variability. The slope estimate indicated that 1 U of WST-1 reading increase was associated with 0.3 U decrease in RR.

The data points (tissue constructs) plotted in Figure 5 tended to cluster by experimental classification. The thermally stressed constructs (red open circles) had the highest optical RR metrics and low WST-1 readings, indicating low overall tissue viability. All the control+control constructs (blue) had comparable optical RR metrics but lower than the thermally stressed constructs. The poorly growing control+control constructs (blue open circles) usually had lower WST-1 readings than well-growing control+control constructs (blue filled circles). In general, the highest tissue

viability was found in the rapa+control (green filled circles) and rapa+rapa (black filled circle) constructs, with low optical RR metrics and high WST-1 readings.

Discussion

We previously showed¹ that quantitative optical imaging reliably assessed the viability of engineered tissues after extreme stressing (thermal-stressing and metabolic stressing). In

FIG. 5. Label-free optical molecular imaging noninvasively and quantitatively characterized living tissue viability and correlated with the destructive WST-1 tissue assay over a range of cell viability states, as determined from measurements on 19 tissue constructs fabricated with primary cells from 5 batches (patients). For all overall batches, the optical RR-metric showed statistically significant negative correlation with the WST-1 readings ($r = -0.47$ and $p = 0.07$ for $n = 16$ constructs; $r = -0.50$ and $p < 0.001$ for $n = 88$ optical measurements). Constructs with lower values for the optical RR-metric had higher WST-1 readings and better tissue viability. The *red line* is the linear fit to data. (Error bars show standard deviations for the optical RR metric. As only one WST-1 reading was obtained from each construct, there is no standard deviation for WST-1 readings.) Color images are available online.

this study, a range of tissue viability states was created with rapamycin treatments to test the optical technique. The viability difference between engineered tissues with rapamycin treatments versus control tissues was smaller than the extreme cases (e.g., thermal stress), observed by standard tissue assessment assays and optical metrics.

Tissue-engineered constructs were assessed by histology scoring, optical RR metrics, and WST-1 assay. The three methods monitored different aspects of tissue constructs: histological assessments monitored tissue morphology; optical RR metrics and WST-1 assay monitored cellular metabolic function. Over the range of tissue viability, all three metrics identified the viable tissue constructs with statistical significance. In addition, we analyzed the correlation between the three methods. Both WST-1 assay readings and the optical RR metrics revealed weak correlations to histology scores, as histology score is generally a better measure of tissue morphology than cellular metabolic function. Alternatively, a moderate correlation was found between WST-1 readings and the optical RR metrics, both of which measure cellular metabolic function.

Although a correlation was found between WST-1 assay readings and optical RR metrics, statistical analysis of the optical data showed significance at the measurement level but marginal significance at the construct level. This may be a result of the WST-1 assay readings being taken randomly from one location per construct due to its destructive nature, thus biasing the reading to specific locations. Alternatively, optical measurements were taken at multiple sites in a construct, characterizing the construct's overall viability. In general, high tissue inhomogeneity was observed. Low RR metrics were found with high WST-1 assay readings as well as high histology scores, indicating high tissue viability in our study.

Although the three metrics reliably assessed tissue viability, WST-1 and histology are destructive. In contrast, label-free optical molecular imaging is noninvasive and can be performed numerous times at multiple sites in tissues. Therefore, the method is useful to assess engineered tissues manufactured at a large scale (10×10 cm). When manufacturing such engineered tissues, nonuniformity is expected and a method for noninvasive assessments at multiple sites over the entire tissue is necessary. With an automated system programmed to monitor multiple local areas of interest with the optical RR metric, surgeons can noninvasively and quantitatively assess a large-scale tissue-engineered construct in real time, selecting the most uniform and, thus, healthiest construct to minimize less than optimal clinical outcomes.

It was found that rapamycin treatment during the cell culture stage improved the viability of tissue-engineered constructs in poorly growing culture.²³ This could be because rapamycin inhibited apoptosis, cell senescence, or differentiation, thus preserving cellular proliferative capability during the cell culture stage.²⁵ Therefore, during the tissue fabrication stage when rapamycin was not present in the culture medium, the cells with high proliferation capability actively grew. Enhanced tissue viability in the rapa+control constructs as compared with control+control constructs was found from poorly growing batches. The well-growing batches had comparable tissue growth among the experimental conditions (control+control, rapa+control, control+rapa, and rapa+rapa) as measured by histology, Ki-67

immunostaining imaging, WST-1 reading, and the optical RR metric data.

Conclusions

In this study, we demonstrated that label-free optical metrics successfully assessed tissue-engineered constructs with a range of states of viability. Label-free optical molecular imaging noninvasively, quantitatively, and reliably assessed tissue-engineered construct cellular metabolic function and spatial morphological information. Using this approach, reliable adjunctive tools could be developed to provide clinicians with quantitative feedback on engineered construct viability, thus enabling clinicians to select the most viable cellular engineered constructs for implantation.

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Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Table S1

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