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Essential Design Considerations for the Resazurin Reduction Assay to Noninvasively Quantify Cell Expansion within Perfused Extracellular Matrix Scaffolds

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Abstract

Precise measurement of cellularity within bioartificial tissues and extracellular matrix (ECM) scaffolds is necessary to augment rigorous characterization of cellular behavior, as accurate benchmarking of tissue function to cell number allows for comparison of data across experiments and between laboratories. Resazurin, a soluble dye that is reduced to highly fluorescent resorufin in proportion to the metabolic activity of a cell population, is a valuable, noninvasive tool to measure cell number. We investigated experimental conditions in which resazurin reduction is a reliable indicator of cellularity within three-dimensional (3D) ECM scaffolds. Using three renal cell populations, we demonstrate that correlation of viable cell numbers with the rate of resorufin generation may deviate from linearity at higher cell densities, lower resazurin working volumes, or longer incubation times that all contribute to depleting the pool of resazurin. In conclusion, while the resazurin reduction assay provides a powerful, noninvasive readout of metrics enumerating cellularity and growth within ECM scaffolds, assay conditions may strongly influence its applicability for accurate quantification of cell number. The approach and methodological

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recommendations presented herein may be used as a guide for application-specific optimization of this assay to obtain rigorous and accurate measurement of cellular content in bioengineered tissues.

Keywords

AlamarBlue; bioreactor; decellularization; extracellular matrix; liver; renal

INTRODUCTION

The increasing use of cellularized three-dimensional (3D) scaffolds in regenerative medicine [1, 2] has expanded biomanufacturing, originally centered on using cells to produce recombinant proteins and biological therapeutics [3], to now encompass the cells themselves—either alone or within a tissue scaffold—as a final product for pharmaceutical testing, disease modeling, or direct patient cellular therapy [4]. In response to a shift toward 3D cell culture, often carried out within bioreactors [5–8] or intricate cell-based microfluidic systems [9], there is a need to accurately measure viable cell numbers without disturbing or sacrificing the complex tissue under examination [10], which may be produced from scarce patient-specific cells [11–13] or patient-derived induced pluripotent stem cells [14]. In addition to providing information on cellular growth kinetics within these systems, noninvasive measurement of cell number is necessary to provide an accurate reference point for tissue function and phenotype. Rigorous, reproducible, and noninvasive cell counting measurements are fundamental tools needed to enable evaluation of cell-based products (matrices with cells [8]) and biofabricated tissues [7, 13, 15–23]. Measurement assurance of cell counting methods is an important element of process control to accurately reference cell and tissue behavior, measure tissue function on a per-cell basis, evaluate for batch-to-batch variability, and compare data across experiments and between laboratories [24, 25].

Resazurin is a blue dye that is internalized by cells and metabolically reduced to the highly fluorescent pink compound resorufin (Figure 1) that is freely released from cells [26]. The irreversible reduction of resazurin to resorufin is mediated by intracellular diaphorase enzymes [26, 27] and generates a strong fluorescent signal that may be measured using a spectrophotometer to noninvasively provide a comprehensive assessment of cellular metabolic activity within a population of cells. The resazurin reduction assay is inexpensive and non-toxic to cells at low concentrations and brief incubation periods (i.e., <4 hours [28]), and is therefore a useful method to indirectly measure cell proliferation kinetics. Resazurin has been used to gauge cell number within bioengineered muscle scaffolds [29], lung matrices [30, 31], and kidney scaffolds [19]. The calculated viable cell number within a scaffold is determined from a standard curve in which a linear relationship is derived between cell number (on a per-volume basis) and fluorescence intensity (FI), following treatment of the examined cell population with a known volume of resazurin over a specific time-period [26, 30, 32]. However, the reliability of the calculated cell number using this method is dependent upon (1) a constant average metabolic activity across the cell population that does not change under the desired experimental conditions (i.e., at different

evaluation time points) and (2) a constant resazurin reduction rate or, more specifically, a stable rate of FI increase.

Over the past few years, the resazurin reduction method has been used by the bioengineering and tissue engineering community for indirect measurement of total viable cell numbers present within 3D ECM scaffolds containing living cells (i.e., recellularized scaffolds) [6, 30, 33]. By design, perfusion bioreactor culture systems provide a technological means to deliver nutrient-rich culture medium to cells deep within 3D scaffolds that would normally be prohibited by the diffusible distance of oxygen and nutrients from the scaffold surface that interfaces with the culture medium in traditional stagnant culture. The penetration of media, and nutrients or metabolic indicators carried within it, into scaffolds within perfusion bioreactors is afforded by tiny embedded conduits within synthetic biomaterials or the retained vasculature within decellularized organ scaffolds [5, 6, 34]. By taking advantage of direct media delivery to growing cells within these scaffolds, via the infiltrating vasculature, resazurin may be directly delivered to cells and its reduced product, resorufin, then sampled within the recirculated media to measure metabolic activity and indicate cell number [5, 6, 19]. Our laboratory [6] and others [30, 31] have used this approach to estimate the number of growing cells within perfusable 3D ECM scaffolds. Importantly, this noninvasive method provides valuable data to compare scaffold seeding methods or gauge the efficiency of scaffold recellularization [6], evaluate the effects of cytotoxic drugs on cell viability within scaffolds [30, 31], and analyze cell proliferation during extended, continuous culture over multiple days [5, 19, 30].

While prior studies demonstrate that total viable cell number can be accurately correlated with FI generated over a specific time period [26, 32], the resazurin reduction assay has important limitations that are often overlooked. We found that large numbers of cells and extended assay times may cause the observed reduction rate to slow at longer incubation periods by depleting the resazurin pool. This is particularly important for applications involving engineered tissues or biomaterial scaffolds with high cell densities due to the layered geometry of 3D cell culture. Moreover, while the primary reduction of resazurin to resorufin is irreversible, an important, but lesser-known, byproduct may be generated from a secondary reaction that depletes resorufin and produces the colorless and non-fluorescent hydroresorufin (Figure 1) [26, 27]. These factors may cause the resazurin assay to greatly underestimate the actual number of cells present in cell-dense biomaterial scaffolds. Therefore, the purpose of this study was to provide a comprehensive evaluation of the resazurin reduction assay, specifically to noninvasively measure cell number during growth within thick 3D ECM scaffolds. With this investigation, we share valuable insights not given elsewhere, and provide guidelines for more accurately quantifying cell number during extended cell proliferation within perfusion bioreactor systems for tissue engineering.

MATERIALS AND METHODS

Cell culture

Immortalized Madin-Darby Canine Kidney (MDCK) epithelial cells were cultured in DMEM/F12 medium with L-glutamine (Life Technologies #11320033) supplemented with 10% fetal bovine serum (FBS; Corning #35-010-CV) and 1% Penicillin-Streptomycin

(Mediatech #30-002-CI). Immortalized human renal proximal tubule epithelial (RPTE; ATCC #CRL-4031) cells were cultured in Clonetics renal epithelial growth medium (Lonza #CC-3190) supplemented with an additional 10% FBS. Immortalized TK188 human fibroblasts derived from fibrotic human kidneys [35] were cultured in low-glucose DMEM with sodium pyruvate (Thermo Fisher Scientific #11885-084) supplemented with 10% FBS, 1% MEM non-essential amino acids (Corning #25-025-CI), 1% L-glutamine (Thermo Fisher Scientific #25030-081), and 1% Penicillin-Streptomycin. Human HepG2 hepatocarcinoma cells (ATCC #HB-8065) were cultured in MEM (Gibco #11095) supplemented with 10% FBS and 1% Penicillin-Streptomycin. Medium was replenished every 2–3 days, and cells were routinely passaged using 1× TrypLE Express (Life Technologies #12605-028).

Kidney recovery and decellularization

All procedures involving animals were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of Northwestern University. Kidneys were recovered from male Sprague Dawley rats (200–250 g) as previously described [19]. 24G catheters (BD Insyte Autoguard # 381412; BD Biosciences) were inserted into the renal artery and ureter of each kidney. Kidneys were frozen in Dulbecco's phosphate-buffered saline (PBS; Corning #21-030-CM) and stored at –80°C until used for decellularization. Thawed kidneys were decellularized by sequential perfusion of 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS) solutions through the renal artery as described [5, 19]. Decellularized kidney ECM scaffolds were stored in PBS at 4°C for a maximum of 2 weeks until use.

Bioreactor perfusion circuits and recellularization of kidney ECM scaffolds

Custom glass bioreactors built to house kidneys during cell seeding and perfusion culture (described in [6]) were treated with Sigmacote (Sigma #SL2) to prevent attachment of cells that exit the ECM scaffolds to the bottom reservoir surface. Bioreactors were assembled into perfusion circuits, and kidney ECM scaffolds were sterilized and prepared for seeding by sequential 1 hour perfusions of 50 mL volumes at 4 mL/min with: 0.1% peracetic acid/4% ethanol in reverse osmosis water (roH₂O), PBS (3 rinses), and culture media specific for each cell type infused (within the 37°C incubator), as described in-depth in [5]. Each ECM scaffold was then injected through the renal artery with a 2 mL cell suspension volume (5–10×10⁶ cells/mL), then immediately perfused antegrade at a high flow rate (25 mL/min, ~232 mmHg) for 15 minutes before restoring the flow rate to 4 mL/min [5, 6]. Culture medium within each perfusion circuit was replenished the day after seeding and every other day thereafter.

Resazurin reduction assay

The resazurin reduction assay was performed as described [5, 6]. A 440 µM (10×) stock solution of resazurin sodium salt (Sigma #R7017) diluted in Dulbecco's PBS was prepared and filter sterilized (0.2 µm pore diameter; VWR #28145-501). Stock solutions were stored at 4°C for no longer than 2 weeks. Resazurin working solutions (44 µM resazurin) were prepared on the same day of use by diluting resazurin stock solutions 1:10 (with respect to total volume) in standard culture media specific to each cell type.

After aspirating the residual media, working solutions containing 10% (v/v) resazurin stock solutions or 10% AlamarBlue (Thermo Fisher Scientific #DAL1025) diluted in culture media were added to each bioreactor reservoir as described [5]. The resazurin solution was perfused through each kidney ECM scaffold at a rate of 4 mL/min for 1 hour (unless otherwise specified) in a dry incubator maintained at 37°C and 5% CO₂, after which time partially reduced resazurin working solutions were aspirated and fresh medium was added. For experiments in which consecutive resazurin assays were run on the same day, 20 mL of fresh culture media was added to each reservoir and circulated for 10 minutes to rinse the organ and perfusion circuit of residual resazurin.

Conditioned medium as a resazurin diluent

Prior to evaluating the use of cell-conditioned (nutrient-depleted) medium as a resazurin diluent, the entire volume of culture medium was aspirated from each bioreactor reservoir and used to create a working solution containing 44 µM (10%) resazurin. To evaluate the influence of glucose levels on resazurin reduction, conditioned media samples were supplemented with enough D-(+)-glucose (Sigma #G7528) to increase the concentration to equivalent levels present in normal basal medium for each cell type (RPTE: 14.4 mg/mL; MDCK: 35.0 mg/mL; TK188: 11.1 mg/mL). Resazurin working solutions created using conditioned medium (50 mL) were first circulated for 1 hour before collecting samples for spectrophotometric analysis. After a 10 minute rinse using 20 mL of fresh culture medium, 50 mL volumes of resazurin working solutions created using either fresh medium or glucose-supplemented, conditioned media were added to the bioreactor reservoir and circulated for 1 hour. Media samples were then collected, and fresh medium was added to each bioreactor reservoir.

Spectrophotometer settings

Samples (100 µL) of resazurin-containing solutions were pipetted into black, opaque 96-well plates (Corning #3915) and measured fluorometrically using a monochromator-based spectrophotometer (Cytation 3, BioTek Instruments, Inc.) as described (excitation: 540 nm; emission: 590 nm) [5]. To determine an appropriate gain setting for the spectrophotometer, resorufin solutions (e.g. completely reduced, positive controls) were developed by autoclaving (121°C for 30 minutes) resazurin or AlamarBlue working solutions of each cell culture media formulation. The instrument was then set to scale the relative fluorescence units (RFU) according to these high-fluorescence wells. Non-reduced resazurin working solutions (negative controls) were stored in the same incubator as the test plate or bioreactor while the assay was performed to eliminate ambient temperature and carbon dioxide levels as confounding variables. FI units were normalized by subtracting RFU values of negative controls from RFU values of test samples; all FI units are presented in this fashion.

Calculation of cell number using resazurin reduction assay

For each scaffold recellularization experiment, unseeded cells obtained from the same passage as those injected into organ scaffolds were plated at defined, serially-diluted densities to generate a standard curve. After a 1-hour incubation with resazurin working solutions, a linear regression equation (1) was generated to relate measured FI (RFU) with the cells:volume ratio (10⁶ cells plated per mL resazurin working solution) [30]:

$$FI = \text{slope} \times (\text{cells:volume ratio}) + \text{intercept} \quad (1)$$

After performing the resazurin perfusion assay and measuring FI in perfusate samples, equation (2) was used to calculate the number of cells present (10^6 cells) within each scaffold:

$$\text{Millions of cells} = \text{Volume}_{\text{perfusate}} \times (\text{FI}_{\text{perfusate}} - \text{intercept}) / \text{slope} \quad (2)$$

where $\text{Volume}_{\text{perfusate}}$ is the volume of resazurin working solution perfused (mL), $\text{FI}_{\text{perfusate}}$ is the FI measured from the perfusate sample, and the slope and intercept are those obtained using the linear regression equation from the standard curve. Normalized FI is also presented as $(\text{FI} \times \text{volume perfused}) / \text{time perfused}$.

Media sampling during extended resazurin perfusion assays

For extended resazurin perfusion assays, 100 mL volumes of resazurin or AlamarBlue working solutions were added to each bioreactor reservoir and recirculated through recellularized kidney ECM scaffolds for up to 24 hours. 1 mL volumes of partially reduced resazurin or AlamarBlue solutions were collected after 5 minutes and thereafter every hour for the first 9 hours, as well as after 24 hours, and stored protected from light at 4°C until all samples were obtained. Alternatively, 35 mL working solution volumes were circulated, and 0.2 mL samples were collected every 15–30 minutes. Samples were analyzed simultaneously as described above.

Manual cell counting

Proximal RPTE cells (passage 18 (P18)), distal MDCK cells (P30), or TK188 fibroblasts (P7) were plated in separate 48-well plates at a density of 5,000 cells/cm². Three hours after plating and every day thereafter, the resazurin reduction assay was performed. Medium was aspirated from 6 wells and 100 µL resazurin was dispensed into each well and incubated for 1 hour. After collecting partially reduced resazurin samples, cells were lifted using TrypLE, pelleted, and resuspended in an appropriate volume of trypan blue for manual counting using a hemocytometer. The calculated number of viable cells (i.e., those that excluded trypan blue) per well was then compared to the estimate provided by the resazurin reduction assay in concert with a standard curve as described above.

Liver ECM scaffold recellularization experiments

Livers were recovered from Sprague Dawley rats and decellularized as described [6]. Custom glass bioreactors built to house liver ECM scaffolds during seeding and perfusion culture were assembled into perfusion circuits and liver right lobe ECM scaffolds were sterilized and seeded with 12.5 or 50×10^6 HepG2 cells (P8–18) via injection through the hepatic portal vein as described [6]. Culture medium (200 mL) was replenished the day after seeding and every other day thereafter. 100 mL resazurin working solutions were circulated for 1 hour.

Live/dead stain of recellularized scaffolds

MDCK-recellularized kidney ECM scaffolds were perfused for 1 hour with 2 μ M calcein AM and 2 μ M ethidium homodimer-1 diluted in DMEM/F12 medium, according to Live/Dead Cell Viability/Cytotoxicity Kit for mammalian cells instructions (Thermo Fisher Scientific #L3224). Scaffolds were then manually cut into thin (~1 mm) sections and imaged using a Nikon C2+ confocal microscope.

Statistical analysis

Statistical analyses were performed using SPSS (IBM). Mean perfusate FI values of samples obtained after the resazurin perfusion assay was performed under different test conditions were compared using paired Student's t-test. Mean numbers of cells calculated using resazurin assay or hemocytometer were compared using independent sample's t-test. Statistical significance was set at $p < 0.05$.

RESULTS

Characterization of temporal changes in perfusate fluorescence intensity as a result of resazurin reduction to resorufin within recellularized kidney ECM scaffolds

Rat kidneys were decellularized [19] and then repopulated using an optimized seeding protocol [5] with one of three cell types: proximal tubule-derived epithelial cells (RPTE), distal tubule-derived epithelial cells (MDCK), or renal fibroblasts (TK188) [35]. Cell proliferation within the recellularized kidney scaffolds was supported by antegrade arterial perfusion of media within customized perfusion bioreactor circuits [6]. Culture medium containing live/dead cell viability markers perfused through the retained vascular network reaches living cells and non-viable cells alike, indicating that resazurin within the perfused media has access to the cellular population within ECM scaffolds (Figure S1). This same bioreactor set-up was used to circulate defined volumes of resazurin-supplemented culture medium at specified intervals to measure cellular metabolic activity and calculate cell number.

We first characterized the temporal change in FI mediated by the metabolic reduction of resazurin to resorufin by each cell type. Recellularized kidney scaffolds were perfused with working solutions containing 44 μ M resazurin in culture media, and perfusates were sampled periodically to evaluate FI as a function of circulating time. For the first 1–2 hours, the slope of the FI vs. time plot, or rate of resazurin reduction to resorufin, was constant. After 2–4 hours, the slope gradually decreased until FI reached a maximum value, suggesting that all resazurin had been converted to resorufin at this time (Figure 2A). Generally, the slope of the FI vs. time curve over the first few hours was greater, and FI reached a peak value earlier, when higher numbers of cells were present within the ECM scaffolds (*data not shown*). We also compared FI trends in working solutions of culture media containing either the AlamarBlue reagent or resazurin alone. For all three cell types, the resazurin solution FI reached a slightly lower maximum than those containing AlamarBlue, but the maximal values were reached nearly simultaneously, suggesting a similar reduction rate and equivalent performance for both formulations (Figure 2A).

After the maximum perfusate FI was reached in RPTE-or TK188-recellularized scaffolds, the FI remained stable until the assay was terminated, even up to 24 hours (Figure 2A). In contrast, for MDCK-recellularized kidney scaffolds, the FI peaked and then gradually decreased for the remainder of the assay, decaying to zero by 24 hours (Figure 2A inset). The decrease in perfusate FI may be attributed to further reduction of resorufin to hydroresorufin, a non-fluorescent, colorless compound (Figure 1) [26, 27]. Between 3 and 9 hours, there was an obvious color change in the MDCK cell perfusate from pink to pale orange, and this occurred regardless of whether working solutions contained AlamarBlue or resazurin alone (Figure 2B). In contrast, the pink color and maximum measured FI remained stable in solutions perfused through RPTE-recellularized kidney ECM scaffolds (Figure 2). Thus, among the three cell types evaluated, only the distal tubule-derived MDCK cells converted resorufin to hydroresorufin.

These results demonstrate that incubation of resazurin with large numbers of cells growing within 3D tissues can result in rapid and complete reduction of resazurin to resorufin. Because the rate of resazurin reduction consistently increased with increasing numbers of cells (*data not shown*), we hypothesized that further modification of the resazurin volume and incubation time were essential for accurate quantification of cell number and to evaluate proliferation during long-term culture.

Optimization of the incubation time to characterize long-term proliferation

We showed that the rate of increase in FI will eventually slow as the concentration of resazurin decreases during its metabolic conversion to resorufin (Figure 2). This observation highlights the need to avoid conditions that decrease the rate of resazurin reduction during the course of an experiment in which extended cell proliferation is expected, such as during growth of cells in 3D scaffolds with dramatically greater cell capacities compared with traditional monolayer culture. We therefore investigated the optimal incubation period for a maximum range of cell densities that can be plated on two-dimensional (2D) tissue culture dishes. MDCK cells were plated at a saturating seeding density (~ 1000 cells/mm²), then serially diluted 1:2 to produce a range of cell densities per well. After 3 hours, the attached cells were incubated with a set volume of resazurin working solution for 1, 2, or 4 hours. Consistent with our findings in recellularized kidney scaffolds, FI increased in monolayer cultures as a function of cell number at all time points (Figure 3A), and also increased as a function of incubation time for each given cells:volume ratio (Figure 3B). However, incubating for 4 hours led to a decrease in the slope at higher cells:volume ratios (Figure 3A). If the data had not been plotted as such, the clear deviation from linearity at 4 hours could be overlooked due to the relatively high R^2 value ($R^2 = 0.947$; Figure 3A). For almost all cells:volume ratios evaluated, the rate of increase in FI slowed after 2 hours (Figure 3B). Thus, at higher cells:volume ratios, long incubation periods with resazurin (>2 hours) may produce a FI that is lower than anticipated and not truly representative of the actual number of cells present. To avoid this error, we standardized the resazurin incubation time at 1 hour and similar cells:volume ratios as used in monolayer standard curves to generate data for experiments employing recellularized organ scaffolds. For the perfused bioreactor cultures, $\sim 95\%$ of the 100-mL working volume of resazurin solution is present at any given time within the bioreactor reservoir and completely recirculates 2.4 times through the

recellularized scaffold at the applied flow rate of 4 mL/min. The one-hour incubation (perfusion) period allows the 100-mL working volume of resazurin solution (50 times the scaffold resident volume of ~2 mL) to recirculate and mix, yet is short enough to minimize cell exposure to resazurin and prevent decay of the resazurin reduction rate.

Standard curve generation and modifications

Standard curves were generated in monolayer culture using cells:volume ratios at regular intervals between 0 and 1 million cells/mL resazurin to improve the accuracy of the linear correlation between cell number and FI. We evaluated three kidney-derived cell lines (RPTE, MDCK, and TK188) with different intrinsic metabolic activity profiles. Standard curves were generated by incubating different numbers of cells with a constant volume of resazurin working solution (Figure 4A). TK188 fibroblasts exhibited the highest average activity per cell – or greatest conversion of resazurin to resorufin per unit time – and distal MDCK cells showed the lowest activity. The slope of the plots, or increase in FI per cells:volume ratio, tended to decrease at the highest ratios evaluated (Figure 4A). This effect was most pronounced in TK188 fibroblasts, which occupy the largest surface area per cell of the three cell lines analyzed, and least pronounced in distal MDCK cells with the smallest cell size. After generating extended standard curves in which the number of cells plated (up to 5 million cells/mL) was increased to well beyond a saturating surface density, the measured FI no longer increased linearly in proportion to cells:volume ratio (Figure S2). This effect is due to a saturating number of cells (i.e., above a cells:volume ratio of 1×10^6 cells/mL), resulting in limited cell attachment to the plate surface and a lower actual cells:volume ratio than what was calculated. In order to obtain a linear standard curve between the number of cells present and FI generated by resorufin, the cell seeding density must not limit cell attachment to the plate surface. The maximum plating density should be empirically determined for each cell type.

To determine whether the FI generated was inversely proportional to resazurin working volume, we produced additional standard curves by plating a constant, saturating number of cells (~ 1000 cells/mm²) in each well and adjusted the volume of resazurin working solution to produce the same series of cells:volume ratios as those shown in Figure 4A. Linear regression equations generated by varying resazurin volume were very similar to those produced by varying cell number, but with greater slopes, especially for MDCK cells (Figure 4B). The higher slopes of the varied-volume regression plots (Figure 4B) compared to the varied-cell regression plots (Figure 4A) are likely because lower attachment efficiencies noted above at high plating densities were avoided, as the same number of cells were plated in each well in the latter experiment. However, because the main objective of the present study is to quantify total cell number based on resazurin reduction within a given working volume, we elected to use linear regression equations generated using variable numbers of cells and avoided saturating levels at the high end of these curves where linearity decays (Figure 4A).

Comparison between calculation of the cell number using the resazurin reduction assay or direct manual cell counting during extended culture of cells within a constrained 2D environment

Cell viability and overall metabolic activity can decrease at higher cell densities due to contact inhibition [36], and we postulated that, after cells reached a state of confluence, the average metabolic activity per cell would decrease. RPTE, MDCK, or TK188 cells were plated at low densities (~ 50 cells/mm²), and allowed to proliferate for 7 days. Average cell number per well was calculated daily using both the resazurin reduction assay and manual counting with a hemocytometer. Figure 5A compares the two methods used to calculate the number of cells present in each well. At sub-confluence (up to 5 days after plating), the estimates obtained using the resazurin reduction assay with linear regression analysis were slightly higher than those obtained by manual counting. There are several possible causes for the discrepancy between the two methods. During manual counting, some cell loss occurs during enzymatic detachment, centrifugation, and resuspension of pelleted cells in the small volumes necessary for accurate direct counting – all contributing to underestimation. Conversely, the resazurin assay may slightly overestimate cell number as the standard curve used to correlate the increase in FI with cell number is based on the assumption of 100% cell attachment (i.e., no cell loss) during plating. Despite this, the cell number estimates provided by the resazurin reduction assay correlated very well with those obtained using manual counting for RPTE cells (Figure 5A), consistent with results reported by Ren et al. [30]. In contrast, the resazurin estimate obtained for MDCK cells correlated closely with manual counting up to day 5, but fell short at days 6 ($p < 0.001$) and 7 ($p = 0.021$). A similar trend was noted for TK188 cells at days 6 ($p = 0.031$) and 7 ($p = 0.005$). Increasing the resazurin incubation volume five-fold in a repeat experiment to decrease the effective cells:volume ratio did not improve this result, indicating that excessive reduction of resazurin to resorufin was not the reason for this discrepancy (*data not shown*). As shown in the representative images in Figure 5B, proliferation of MDCK or TK188 cells continued even after confluence was reached on day 5. Because the cell cultures remained in monolayer, the average cell size (i.e., surface area occupied) decreased. In contrast, overgrown RPTE cell cultures were characterized by vertical growth of cells over the surface of one another (compare day 5 images to day 7 images). These results highlight the possibility that dimensional constraints imposed by the culture environment may result in morphological alterations, such as a reduction in average cell size and a resulting decrease in metabolic activity per cell. Thus, morphological changes in cells may limit the correlation of resazurin reduction with total cell number when standard curves are generated from cells lifted after growth at lower cell densities.

Accurate calculation of cell number during growth within 3D ECM scaffolds using perfusion bioreactors depends upon resazurin perfusate volume

Due to the much greater capacity and surface area for cell growth within 3D ECM scaffolds compared to 2D culture plates, additional optimization of the resazurin reduction assay is warranted with respect to the cells:volume ratio. The volume of resazurin working solution circulated is influenced by the bioreactor design, as it is critical that a sufficient volume be used to prevent air from being drawn into the recirculation tubing from the reservoir outlet and subsequently perfused into the recellularized scaffold. In general, it is preferable to

minimize the volume circulated to conserve reagents and increase the sensitivity of metabolic assays. However, cell proliferation will increase the ratio of cells to resazurin volume, and this will lead to faster reduction of resazurin to resorufin, which may result in a gradual decrease in the reaction rate during the incubation period. To investigate the influence of resazurin volume, kidney ECM scaffolds were repopulated with proximal RPTE cells, distal MDCK cells, or TK188 fibroblasts, and the resazurin perfusion assay was performed using the minimum (10 mL) and maximum (100 mL) volumes allowed by our current bioreactor design. Linear regression equations obtained from standard curves in 2D cultures (Figure 4A) were used to calculate cell number based on FI values obtained from each resazurin perfusate volume. As shown in Figure 6A, the 100 mL resazurin volume yielded at least a two-fold greater estimate for cell number compared to the 10 mL volume for all three cell types analyzed (RPTE: 13.4 ± 6.0 vs. $6.0 \pm 1.9 \times 10^6$ cells, $p=0.041$; MDCK: 38.2 ± 14.5 vs. $7.3 \pm 1.9 \times 10^6$ cells, $p=0.017$; TK188: $30.5 \pm 9.1 \times 10^6$ vs. 7.0 ± 2.1 cells, $p=0.003$). This was initially unexpected, because it had previously been determined that FI inversely correlated with resazurin working volume. Based on the average cell number determined the previous day for each cell type (calculated using a 50-mL resazurin volume), and the fact that we have observed consistent proliferation of RPTE, MDCK, and TK188 cells during the first several days of perfusion culture within kidney ECM scaffolds ([5] and unpublished data), it is clear that the 100 mL volume provided a more accurate estimate. We performed a follow-up experiment using MDCK cell-recellularized kidney ECM scaffolds (20×10^6 cells per scaffold) 1 day after seeding – perfusing 10, 25, 50, 75, or 100 mL resazurin working volumes for 1 hour. As shown in Figure 6B (left and center panels), the cells:volume ratios calculated from the FI values obtained using each volume ranged from ~ 0.2 to ~ 0.8 . Using these values, the number of cells calculated using 50, 75, or 100 mL volumes (at cells:volume ratios of 0.42, 0.27, and 0.20, respectively) was consistent at $20.6 \pm 0.3 \times 10^6$ cells per scaffold (Figure 6B, right panel). However, when the resazurin volume applied was 25 or 10 mL, the number of cells calculated decreased to 16.7 or 8.1×10^6 cells, respectively. Based on the 20.6×10^6 average cell estimate, the actual cells:volume ratio would have been 0.83 for 25 mL and 2.06 for 10 mL circulating volumes. We therefore concluded that for accurate estimation of cell number in similar perfusion bioreactors, a resazurin volume should be perfused (for 1 hour) that will provide a cells:volume ratio below 0.5×10^6 cells/mL resazurin. However, this specific value will depend on the cell type and scaffold configuration tested, so application-specific optimization is required.

Rate of metabolic reduction of resazurin to resorufin is lower in fresh media relative to conditioned media

A practical consideration, and potential limitation, for the eventual scale-up of 3D ECM scaffold recellularization technologies from small animal model organs to human-sized organs is the large volume of medium that will be necessary to support growth of billions of cells. To conserve costly culture media, we explored whether nutrient-depleted (i.e., cell-conditioned) culture media could be used as a diluent for resazurin within perfusion bioreactors. Resazurin stock solutions were diluted in either media that had been recirculating through the recellularized kidney scaffolds for 24–48 hours (nutrient-depleted media) or fresh culture media, and perfused for 1 hour. We observed a 50–100% greater

increase in the FI and calculated number of cells present using depleted medium vs. fresh medium for all 3 cell types (Figure 7A - RPTE: 11.3 ± 2.0 vs. $5.6 \pm 2.0 \times 10^6$ cells, $p=0.010$; MDCK: 33.7 ± 8.2 vs. $22.0 \pm 6.8 \times 10^6$ cells, $p=0.048$; TK188: 15.2 ± 4.3 vs. $7.8 \pm 2.6 \times 10^6$ cells, $p=0.007$). We first hypothesized that this effect was due to a decrease in oxidative metabolic activity in the presence of fresh, glucose-rich culture medium. However, after supplementing nutrient-depleted medium with an equivalent amount of glucose, we observed little if any inhibitory effect on resazurin reduction or the number of cells calculated using depleted vs. glucose-supplemented culture medium (Figure 7B - RTPE: 18.3 ± 4.6 vs. $17.7 \pm 4.4 \times 10^6$ cells, $p=0.228$; MDCK: 50.5 ± 19.3 vs. $60.7 \pm 33.0 \times 10^6$ cells, $p=0.482$; TK188: 35.9 ± 7.7 vs. $36.1 \pm 9.9 \times 10^6$ cells; $p=0.909$). A similar decrease in resazurin reduction was observed when fresh medium was used relative to conditioned medium perfused through HepG2-recellularized liver ECM scaffolds grown within an alternative bioreactor configuration [6] (Figure S3D). Consistent with these findings, greater FI was observed at each cell density evaluated, resulting in an increased slope when standard curves were generated in 2D cultures using conditioned medium (obtained from the same culture flask from which cells were lifted for plating), compared to fresh medium (Figure 7, C vs. D). Therefore, we suggest that the lower resazurin reduction rate in the presence of fresh culture medium is not due to a change in cellular metabolic activity, but rather to pre-reduction of other compounds present in fresh culture medium that may act as electron acceptors (i.e., oxidizing agents) that compete with the enzymatic reduction of resazurin to resorufin. Nutrient-depleted medium may therefore be used as a diluent for the resazurin reduction assay as long as the standard curve used to produce the linear regression equation is similarly produced using conditioned medium.

Using the optimized resazurin perfusion assay to characterize cell proliferation within perfused organ scaffolds and other organ systems

The results described above provide a set of experimental parameters (e.g., incubation time, resazurin volume, plating density range for standard curve generation, and medium conditioning) that, when appropriately optimized, will ensure that the resazurin perfusion assay yields an accurate estimate of cell number in 3D tissue engineered biomaterials (see Table 1 for a list of common issues and proposed solutions related to the assay). We used these optimal conditions to characterize proliferation trends of proximal RPTE cells, distal MDCK cells, and TK188 fibroblasts during 3D growth within kidney ECM scaffolds. All three cell types demonstrated the consistent ability to proliferate over one week of 3D perfusion culture (Figure 8), and these results are consistent with previous histological and metabolic findings showing the capability of MDCK cell proliferation and tubule formation during extended culture in decellularized rodent kidney scaffolds [5, 6, 19]. To demonstrate versatility of these optimization guidelines, we similarly adapted the resazurin perfusion assay to characterize HepG2 hepatocellular carcinoma cell growth within decellularized rat liver ECM scaffolds using a distinct bioreactor configuration, and noted rapid early growth followed by stabilization of cell numbers after one week of culture (Figure S3) [6]. The resazurin perfusion assay can be adapted for use within any perfusion bioreactor system to provide noninvasive metrics of cell proliferation during growth in 3D scaffolds composed of similar decellularized tissues, synthetic biomaterials containing a perfusable vasculature, or other perfusable substrates.

DISCUSSION

Resazurin is the primary active compound in a variety of commercially available viability/cytotoxicity assays, including AlamarBlue, PrestoBlue, and others. Using cell-supporting 3D kidney ECM scaffolds with substantially greater surface areas for cell growth compared to traditional 2D culture systems, we highlight limitations of the resazurin assay and point out recommendations to minimize inaccuracy in perfusable 3D scaffolds. In particular, we showed how the rate of resazurin reduction to resorufin slows as the concentration of resazurin within the perfused working solution decreases. The standard curve method previously used by others to relate FI to viable cell number present [26, 30, 32] implicitly assumes that the resazurin reduction rate does not change over the incubation period. Importantly, we demonstrate in the present study that, when the ratio of cells exposed to the volume of resazurin working solution incubated increases, as occurs when cells proliferate, resazurin is more rapidly reduced to resorufin and the reaction rate will progressively slow. This can potentially result in a significant underestimation of cell number. Our principal suggestion based on this finding is that users increase the volume of resazurin working solution, when greater proliferation is expected, to ensure that the reaction rate is steady over the course of the assay.

Metabolic demand varies among organs and between tissue types and, as such, the inherent metabolic rates observed among specific cell populations can be expected to vary [37]. In the present study, we profiled three distinct kidney cell populations derived from various regions of the nephron and renal interstitium. RPTE cells, an established human epithelial cell line derived from the proximal tubule, displayed greater average metabolic activity per cell than MDCK epithelial cells isolated from the distal tubule, which consistently displayed the lowest activity per cell. This distinction reflects the larger size of RPTE cells and the greater physiological demands for proximal tubules, which are responsible for active reabsorption of glucose, solutes, and amino acids from the urinary filtrate, relative to distal tubules [38]. Investigators have previously noted that the secondary reaction (reduction of resorufin to dihydroresorufin) is favored after all resazurin has been reduced to resorufin [26]. Why the MDCK cell line was uniquely, and reproducibly, capable of secondary reduction of resorufin to dihydroresorufin, despite displaying the slowest reduction of resazurin to resorufin, is unclear.

We also highlight differences in morphological adaptation among the three cell lines within a constrained 2D environment. Unlike the proximal tubule epithelial cell cultures, in which cells were observed to grow across the surface of one another after reaching confluence, distal tubule-derived MDCK cells and TK188 fibroblasts remained in a monolayer while decreasing in size during continued proliferation. The reduction in average cell size resulted in a corresponding decrease in the average metabolic activity per cell, such that the initial linear regression equation could no longer accurately reflect cell number. A reduction in cell size due to environmental constraints is less likely to occur in kidney ECM scaffolds with dramatically larger surface areas due to the presence of an extensive, integrated network of tubular basement membranes. Nonetheless, these results highlight that morphological or phenotypic changes in growing cells, which often occur during stem cell differentiation or

maturation, are significant transformative events that must be taken into account when using metabolism-based assays.

Finally, culture medium and its contents may be greatly conserved by using conditioned medium obtained from bioreactor perfusion circuits, rather than fresh medium, as a diluent for resazurin in perfusion assays during evaluation of 3D bioengineered tissues. The greater resazurin reduction rate in conditioned medium will increase assay sensitivity, but a lower cells:volume ratio must be used than for fresh medium.

CONCLUSIONS

Collectively, these results underscore the importance of appropriately optimizing the resazurin reduction assay for accurate cell quantification during proliferation within donor organ-derived, recellularized ECM scaffolds. Importantly, we show it is critical that a sufficient concentration of the substrate, resazurin, be present for the entire duration of the assay so that the rate at which resazurin is reduced to resorufin remains constant to provide an accurate estimate. As cells proliferate during extended culture, the higher numbers of cells present will convert a greater amount of resazurin to resorufin within a set time period. To avoid excessive reduction of resazurin to resorufin without increasing the working concentration of resazurin (which could potentially be cytotoxic [26]) the ratio of cells exposed to the volume of resazurin can be lowered by increasing the resazurin perfusate volume. The bioreactor and perfusion circuit design should appropriately accommodate the maximum number of cells present in a cell-laden tissue or 3D scaffold, both for sufficient nutrient delivery within the perfused culture medium, and to allow an increase in the perfusate volume to enable accurate quantification of cell number using the resazurin perfusion assay. In conclusion, the resazurin perfusion assay is an inexpensive, simple, and non-invasive test that, when used correctly, is tremendously useful for the characterization of growing cell populations within 3D culture. This assay will benefit the biomanufacturing and regenerative medicine research community by allowing investigators to more closely monitor temporal proliferation trends of cells grown within 3D biomaterials and decellularized tissue matrices without sacrificing developing tissue for frequent direct cell measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Stepwise conversion of resazurin to resorufin and hydroresorufin

Resazurin (blue) diffuses into cells where it is irreversibly reduced by diaphorase enzymes to a highly fluorescent (pink) compound, resorufin. In a subsequent, reversible reaction, resorufin may be further reduced to colorless, non-fluorescent hydroresorufin [26, 27].

Figure 2. Changes in perfusate fluorescence intensity (FI) over time mediated by metabolic reduction of resazurin to resorufin within recellularized 3D kidney ECM scaffolds

Kidney ECM scaffolds ($n=2$ scaffolds per cell type) were repopulated with proximal RPTE cells (12.5×10^6), distal MDCK cells (20×10^6), or TK188 fibroblasts (10×10^6), and cells were allowed to proliferate under perfusion culture for up to 7 days. Kidney scaffolds were perfused with working solutions containing 10% AlamarBlue or 10% stock resazurin, and samples were withdrawn periodically. A: Representative graphs show the resulting change in FI as a function of circulating time. Inset plots with dashed outlines show the same data set with an additional sample obtained at 24 hours for RPTE and MDCK-recellularized kidney ECM scaffolds. B: Representative images of RPTE- or MDCK-recellularized kidney ECM scaffolds being perfused with AlamarBlue (top row) or resazurin solutions (bottom row) are shown at the beginning (0 hours), near-maximum FI (6 hours for RPTE cells; 3 hours for MDCK cells), and end (9 hours) of each experiment.

Figure 3. Effect of incubation time and cells: volume ratio on the increase in fluorescence intensity measured during resazurin reduction

Distal MDCK cells were plated at different cell densities to produce a range of cells:volume ratios from 8×10^3 to 10^6 cells per mL of resazurin working solution. After allowing cells to attach for 3 hours, medium was aspirated and 200 μ L resazurin working solution was incubated in each well for 1, 2, or 4 hours. A: Standard curves generated at different incubation times are shown. As expected, longer incubation periods led to an increase in FI generated by conversion of resazurin to resorufin. However, longer incubation times (i.e., 4 hours) also resulted in a loss of linearity (decrease in slope of FI vs. cells:volume ratio curve) at higher cells:volume ratios, as demonstrated by the decreased R^2 value associated with the linear trendline. B: FI values are plotted on a logarithmic scale as a function of incubation time for each cells:volume ratio. The rate of increase in measured FI per hour decreased between 2 and 4 hours. Data are presented as mean $\pm$ standard deviation (n=3 wells per data point).

Figure 4. Generation of standard curves by varying cell number or resazurin working volume Proximal RPTE cells, distal MDCK cells, or TK188 fibroblasts were plated in separate 48-well plates. After allowing cells to attach to the plate for 3 hours, medium was aspirated and resazurin working solution was added to each well and incubated for 1 hour. A: Standard curves were generated by using a constant resazurin working volume (100 μ L), and varying the number of cells plated in each well to produce a range of cells:volume ratios ranging from 0.05 to 1 (10^6 cells per mL resazurin). B: Standard curves were generated by plating a constant number of cells (10^5 cells per well), and varying the volume of resazurin working solution added to each well to produce a range of cells:volume ratios ranging from 0.1 to 1 (10^6 cells per mL resazurin). Results are presented as mean $\pm$ standard deviation (n=3 wells per data point). Linear regression trendline equations and associated R^2 values are shown in each graph.

Figure 5. Comparison of cell number calculated from resazurin reduction or by direct hemocytometer counting during cell growth to and beyond confluence

Proximal RPTE cells, distal MDCK cells, or TK188 fibroblasts were plated in separate 48-well plates at a density of 5×10^3 cells/cm². Three hours after plating and subsequently every day, the resazurin reduction assay was performed and cells were lifted from replicate wells for manual counting. A: Results show the average number of cells per well calculated using either a hemocytometer (n=3) or the resazurin reduction assay (n=6). Data are presented as mean $\pm$ standard deviation. The vertical arrow shows the approximate time point (day 5) at which the cells have reached confluence. Asterisks (*) indicate a significant difference in means between calculation method at each time point as determined by independent samples Student's t-test ($p < 0.05$). B: Representative phase contrast images at various time points show that cells continued to proliferate even after reaching confluence. By day 7, RPTE cells had begun to grow vertically atop one another, whereas MDCK and TK188 cells remained as a monolayer, despite continued proliferation. Scale bars: 100 μ m.

Figure 6. Accurate estimation of cell number in kidney perfusion bioreactors depends on resazurin working volume

Kidney ECM scaffolds (n=4 scaffolds per cell type) were repopulated with proximal RPTE cells (12.5×10^6), distal MDCK cells (20×10^6), or TK188 fibroblasts (10×10^6), and cells were allowed to proliferate under perfusion culture. Two days after seeding, 10 mL or 100 mL of resazurin working solution was circulated through each reservoir for 1 hour, and medium samples were withdrawn. A: The calculated number of cells varied greatly between the two volumes of resazurin circulated, with the lower volume providing a significantly lower estimate. The dashed line shows the number of cells calculated via the resazurin perfusion assay, using 50 mL of working solution, on the previous day. Data are presented as mean $\pm$ standard deviation (n=4 ECM scaffolds per data point). Asterisks (*) indicate a significant difference in means as determined by paired samples Student's t-test ($p < 0.05$). B: In a separate experiment, a standard curve generated by plating varying numbers of distal MDCK cells in 2D cultures (left panel) was used to derive a linear regression equation to relate FI to cells:volume ratio. One day after injecting 20 million MDCK cells into a kidney ECM, The resazurin assay was run for 1 hour each, circulating volumes of 10, 25, 50, 75, or 100 mL (middle panel), and the number of cells calculated using each volume is shown in the right panel. The 50, 75, or 100 mL volumes provided a consistent estimation at approximately 20 million cells (the number of cells injected the previous day, indicated by the dashed line), whereas the 25 mL and, especially, the 10 mL volumes provided much lower estimations of cell number.

Figure 7. Comparison of fresh vs. nutrient-depleted culture medium as a diluent for resazurin Kidney ECM scaffolds (n=4 scaffolds per cell type) were repopulated with proximal RPTE cells (12.5×10^6), distal MDCK cells (20×10^6), or TK188 fibroblasts (10×10^6), and cells were allowed to proliferate under perfusion culture. A: One day after seeding, 5 mL of resazurin stock solution was diluted in 45 mL of nutrient-depleted media obtained from the bioreactor reservoir, and the solution was recirculated through each scaffold for 1 hour. After a 10 minute wash using 20 mL of fresh medium, a new 50 mL volume of resazurin working solution prepared using fresh medium as a diluent was circulated for 1 hour. As shown, the calculated number of cells present was lower when fresh medium was used as a diluent, due to a decrease in metabolic reduction of resazurin to resorufin. Results are presented as mean $\pm$ standard deviation (n=4 scaffolds per data point). Asterisks (*) indicate a significant difference in means as determined by independent samples Student's t-test ($p < 0.05$). B: Four days after seeding, resazurin was diluted in nutrient-depleted medium with or without supplementation to basal concentrations of D-glucose. As shown, glucose supplementation had no inhibitory effect on resazurin reduction. Results are presented as mean $\pm$ standard deviation (n=2 scaffolds per data point). C-D: Resazurin working solutions were prepared by diluting resazurin in nutrient-depleted (C) or fresh (D) culture media, and varying numbers of plated cells were incubated with these solutions to prepare standard curves as previously described. As shown, FI generated by resazurin reduction was greater in nutrient-depleted medium (C) compared with fresh medium (D). Results are presented as mean $\pm$ standard deviation (n=3 wells per data point). Linear regression trendline equations and associated R² values are shown within each graph.

Figure 8. Proliferation trends measured within recellularized kidney scaffolds using the standardized resazurin perfusion assay

Kidney ECM scaffolds (n=2 scaffolds per cell type) were repopulated with proximal RPTE cells (12.5×10^6), distal MDCK cells (20×10^6), or TK188 fibroblasts (10×10^6), and cells were allowed to proliferate for up to 7 days in perfusion bioreactors. The optimized resazurin reduction assay (100 mL of working solution created using cell-conditioned medium circulated for 1 hour) was performed in concert with a standard curve from 2D culture to quantify the number of cells present at each time point. Data are presented with normalized FI on the primary vertical axes, and the calculated number of cells on the secondary vertical axes.

Table 1
Potential issues and proposed solutions for the resazurin reduction assay

This table lists common issues that may affect the accuracy of the resazurin reduction assay for estimating cell number. Each issue and its potential cause(s) are described briefly, and solutions are proposed to overcome each problem. Figures that demonstrate several of the issues are also listed.

Potential Issue	Description	Cause	Proposed Solution	Figure References?
Inaccurate estimates of cell number using standard curve	At low cells:volume ratios, cell number estimated by the standard curve indicates a negative number of cells	Few cells or high background fluorescence intensity of resazurin negative control	<ul style="list-style-type: none"> Force linear regression line through zero (i.e. set y-intercept as zero) Increase incubation time to increase sensitivity 	N/A
Non-linearity of standard curve	Signal appears to “plateau” at increasing cell:volume ratios	Incubation time is too long	Decrease incubation time	Figure 3 (4 hours)
	FI signal appears to plateau only at highest cell:volume ratios (0.8 and above)	Cell seeding density is too high for efficient attachment at higher cell:volume ratios	Remove outlying points at highest cells:volume ratios for linear regression analysis	Figure 4A, Figure S2
Lower cell numbers are calculated than the number of cells actually present	The number of cells present, as determined by alternative means (e.g., manual counting), is higher than that estimated by the resazurin assay	Majority of resazurin is converted to resorufin and dihydroresorufin; Reaction rate slows during incubation OR Average metabolic activity per cell decreases (e.g., due to contact inhibition – Figure 5A) [No solution]	<ul style="list-style-type: none"> Reduce incubation time Decrease cells:volume ratio by increasing resazurin working volume. (Cells:volume ratio should be kept below 0.5 million cells/mL resazurin) 	Figure 2A, Figure 3, Figure 5A, Figure 6
Low assay sensitivity	Low measured fluorescence intensity/no visual color change due to little conversion of resazurin to resorufin	Cell-hours:volume ratio is too low	<ul style="list-style-type: none"> Extend incubation time Increase cells:volume ratio by decreasing resazurin working volume Increase gain setting on spectrophotometer 	Figure 3 (1 vs. 2 vs. 4 hours)
Limited cell numbers available for standard curve	Primary and other sensitive cell lines may be too limited in number to produce a full standard curve	Cells with limited expansion potential may not proliferate enough for both experiments and standard curve generation	Use smaller-well plate and/or decrease number of points used for cells:volume ratios	N/A
Increased fluorescence measurement in perfusion systems during repeat assays	Fluorescence intensity measurements seem higher than normal at the time each assay is run.	Perfusion circuits often retain a small volume of culture medium in the tubing and within the scaffold itself, despite medium in the reservoir being exchanged with fresh medium. Residual amounts of resazurin that remain in the medium will be reduced to resorufin during normal perfusion culture, and at the time of the next assay, this	<ul style="list-style-type: none"> Dilute residual resazurin to trace amounts. Briefly perfuse the scaffold with a volume of fresh medium after the resazurin assay. Aspirate, and replace with fresh medium Use nutrient-depleted culture medium as the resazurin diluent during assays. This will provide an accurate “background” sample to 	N/A

Potential Issue	Description	Cause	Proposed Solution	Figure References?
		will lead to artificially high fluorescence readings.	normalize the data against.	