Oxygen gradients correlate with decrease in cell density and viability in engineered cardiac tissue

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Introduction:

For clinical utility, cardiac grafts should be thick and compact, and contain physiologic density of metabolically active and differentiated cells. This involves the need to control the levels of nutrients, and most critically oxygen, throughout the construct volume. Most culture systems involve diffusional transport within the constructs, a situation associated with gradients of oxygen concentration, cell density, cell viability, and function. For example, cultivation in Petri dishes resulted in cardiac constructs with viable cells confined at the top surface of the construct [1, 2] Medium flow at the surfaces of tissue constructs cultured in stirred flasks [3, 4] and rotating vessels [5]enhanced cell density, metabolism, differentiation and electromechanical coupling in the outer peripheral tissue layer [3-5], but not in the construct interior that remained mostly acellular. Relatively uniform distribution of oxygen (established by convective-diffusive transport through the construct that was perfused by culture medium) was associated with a relatively uniform cell distribution at cell densities that were in a physiologic range [6]. Moreover, constructs containing uniformly distributed cells at high spatial density had enhanced expression of cardiac markers, more aerobic cell metabolism, and markedly improved contractile properties [6]. Consistently, high density of viable differentiated cells in native heart tissue is supported by convective-diffusive transport of oxygen through dense vasculature, with capillaries spaced only about 20 μm apart [7], whereas the disruption of oxygen supply can lead to cell death (e.g., during myocardial infarction).

The main goal of our study was to measure oxygen gradients in the dish-grown cardiac tissue constructs, prepared by culturing neonatal rat cardiac myocytes on porous collagen scaffolds, and correlate them to the spatial distributions of cells and cell viability. To rationalize experimental data, a mathematical model of oxygen distribution was derived as a function of cell density, viability, and spatial position within the construct. It was confirmed that medium flow reduces oxygen gradients within engineered cardiac tissue, a finding consistent with the observed improvement in tissue properties.

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Methods:

Disc shaped cardiac tissue constructs (3.6 mm diameter, 1.8 mm thickness) based on neonatal rat cardiomyocytes ($3 \cdot 10^6$ cells/cm^3) were cultured on collagen scaffolds for 16 days under static conditions. The oxygen profile measurements were performed using microelectrodes in unconfined constructs placed on a layer of agarose in a stagnant culture medium (to reflect static culture conditions) or suspended on a needle with superficial medium flow (to reflect the culture in mixed flasks or rotating vessels). To asses cross-sectional cell viability the live constructs were stained using Reduced Biohazard Cell Viability Kit (Molecular Probes), cross-sectioned and imaged using fluorescent microscope. The viability was determined using Image Analysis. Cell distribution was determined from DAPI counterstained histological cross-sections using image analysis. To rationalize experimental data,

a mathematical model of oxygen distribution was derived as a function of cell density, viability, and spatial position within the construct.

Mathematical model:

A mathematical model was developed that correlates the distribution of oxygen within a construct cultured in static fluid to the distribution of cell density and cell viability. The construct was considered to be radially symmetrical, and the distributions of oxygen, total and viable cells were analyzed across the full cross section through the construct center. The model was solved numerically using a finite element method and a commercial software Femlab.

Governing Equations Assuming constant density and constant diffusivity of oxygen, the governing equation for oxygen in the disc-shaped cardiac tissue construct placed in the stagnant culture medium was written in the form:

(1)

$$0 = D_t\left[\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial C_t}{\partial r}\right)+\frac{\partial^2 C_t}{\partial z^2}\right]-\frac{V_{\max}\delta(z)C_t}{K_m + C_t}$$

where D_t is diffusivity of oxygen, and C_t is oxygen concentration. It was considered that oxygen was consumed by the cells according to the Michaelis-Menten kinetics where V_{max} is the maximum respiration rate per cell, K_m is the concentration of oxygen at which the respiration rate decreases to one half, and $\delta(z)$ is the position dependent live cell density. The live cell density was assumed to decrease exponentially with depth:

$$\delta(z) = ae^{-bz} \quad (2)$$

and the coefficients *a* and *b* in this equation were determined experimentally.

Boundary conditions Oxygen concentrations at the bottom and top construct surfaces were measured and used as boundary conditions for solving model equations:

$$C_t(r,0) = C_b \quad (3)$$

$$C_t(r,L) = C_t \quad (4)$$

Assuming the construct is axisymmetric (a reasonable assumption, based on histological data and previous studies, it was sufficient to model only one half of the construct and impose the symmetry boundary condition at the construct center-line:

$$\partial C_t / \partial r(0,z) = 0 \quad (5)$$

The concentrations at the lateral and top surfaces of the construct that are in contact with culture medium were assumed to be equal to each other:

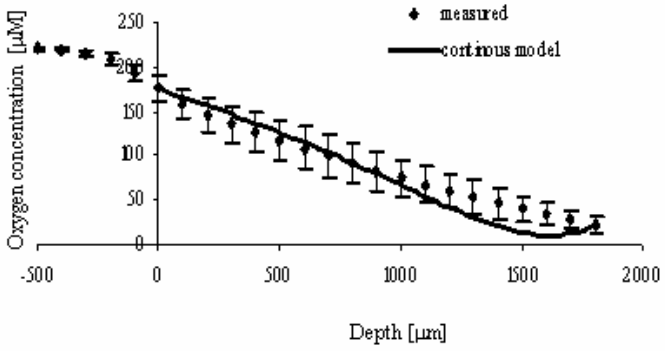
$$C_t(R_c,z) = C_t \quad (6).$$

The model for the measured parameters (Table I) using a finite element methods and a commercially available software, Femlab.

Table I Model parameters

PARAMETER	*VALUE*	*SOURCE*
R_c (construct radius)	1.8mm	Measured
L (construct thicnkess)	1.8 mm	Measured
V_{max} (maximum oxygen consumption per cell)	1.5 nmol/min/10^6cells	[8]
K_m	6.875μM	[8]
a (pre-exponential factor for cell distribution)	1.7053 [10^8 cell/cm^3],	Fitted to data
b (live cell density exponential decay constant)	0.0042 μm^{-1}	Fitted to data
D_t (oxygen diffusion coefficient within the tissue)	2.0 10^{-5} cm^2/s	[3]
C_t (oxygen concentration at the top surface)	175.6 μM	Measured
C_b (oxygen concentration at the bottom surface)	22.4 μM	Measured

A



B

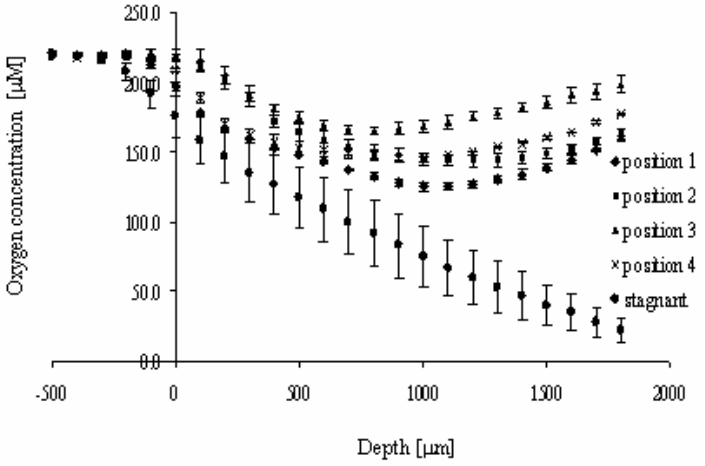


Figure 4. **Oxygen concentration along the cross sections of statically cultured cardiac constructs.** Constructs were harvested after 16 days of static culture and oxygen profiles were measured (A) in stagnant culture medium, across the construct centerline (n = 4 constructs) and (B) with the flow of culture medium around constructs, at four symmetrical positions along the construct diameter (n = 3 constructs). The average construct thickness is 1.84 ± 0.08 mm. Data are Average ± SE.

Results and Discussion:

At the end of cultivation, live cells expressing cardiac markers (sarcomeric α–actin, cardiac troponin I and connexin-43) were confined to the 250m thick surface layer. Oxygen distribution (175.6μM to 22.4μM, **Figure 1A**) and viability (60%-5%) decreased linearly and live cell density decreased exponentially with construct thickness with the decay constant of 236 μm (**Figure 2**). Live/dead staining indicated that the live cells were present only in the outer layer of the construct, while the interior was made up of dead cells (**Figure 2A**).

Cell viability along the construct center-line (**Figure 2B**) was approximately 60 % within the first 500 μm of the construct thickness, 40 – 30 % at 500 - 1000 μm beneath the upper construct surface, and 15 – 5 % at depths that were greater than 1000μm beneath the upper construct surface. Only one construct had enough cells at the bottom surface (in the segment from 1,532 – 1,787 μm) to allow the determination of cell viability. Interestingly, the viability decreased linearly with depth (r^2 = 0.9473) (**Figure 2B**), a trend consistent with the linear decrease in the concentration of oxygen (**Figure 1A**).

By summing the total numbers of live and dead cells along the entire construct cross section, (using data in **Figures 2B** and **D**) the overall cell viability was calculated to be 57 %, which is comparable to the viability of 47.4 ± 7.8 % measured previously by FACS analysis for similar cardiac constructs cultured statically [6].

Center-line cell density (**Figure 2C**), calculated from the total cell numbers measured in histological cross sections, decreased by two orders of magnitude over a distance of 1000 μm, from the values characteristic for the native rat heart (~10^8cells/cm^3) at the construct surface to (0.074 - 0.008)·10^8 cells/cm^3 for the remainder of the construct depth. By combining the data for cell density and cell viability (**Figure 2D**), it was determined that the density of live cells decreased from 1.6 to 1.0·10^8 cells/cm^3 within the outer 128 μm layer of the construct, in parallel with the decrease in oxygen concentration from 176 to 158 μM (**Figure 1A**). Within the 400 μm layer underneath this outer layer, the density of live cells was an order of magnitude lower than in the native heart (0.6 - 0.2·10^8 cells/cm^3), and the corresponding oxygen concentrations ranged from 158 to 117 μM. In the layer positioned 500 – 1000 μm beneath the top surface, the live cell density was in the range 0.08 - 0.02·10^8 cells/cm^3 and the oxygen concentration decreased from 117 to 74 μM. At thicknesses beyond 1000 μm from the outer surface, oxygen concentration was less than 74 μM and the live cell density was 3 to 4 orders of magnitude lower than in the native heart. The live cell density exhibited an exponential decrease with depth (Eq. 2) (r^2 = 0.9828). Consequently, the cell density decay constant (*1/b*) was 236 μm, indicating that the live cell density decreased by 95% at a depth of 708 μm beneath the construct surface.

The measured live cell density decreased exponentially with the decrease in measured oxygen concentration (r^2 = 0.9575):

$$\delta = \alpha e^{-\beta C_{O2}} \qquad (7)$$

where δ is live cell density in 10^8 cell/cm^3, α= 0.0004 [10^8cell/cm^3], β = 0.0518μM^{-1} and C_{O2} is local oxygen concentration in μM.

The modeled oxygen profile indicated that the areas of high cell viability co-localized with the areas of high oxygen concentration. For disc-shaped constructs with a low aspect ratio the edge effects are significant, and oxygen concentration within the construct can be increased due to diffusion from the sides. To account for this effect, we developed a two-dimensional model and solved it using a finite

element method. Overall, high cell viability (≥ 60 %) was maintained at concentrations of oxygen above 120 μM. The decrease in oxygen concentration below 70 μM (at 1,000 μm beneath the construct surface) led to the decrease in viability to below 20%. Interestingly, it was reported that cardiomyocytes begin to change their metabolism in response to hypoxia by down-regulating energy using processes at oxygen concentration of 70μM [8].

The predicted oxygen profile along the construct centerline was within the standard error of the measured oxygen concentration (**Figure 1A**). The Michaelis-Menten parameters used for oxygen consumption were taken from published work for suspensions of neonatal rat cardiac myocytes [8]. The glucose consumption rate measured in this work (~0.17 μmol/hr/10^6cells,) agreed well with the reported glucose consumption rates [8]. However, the published values of Vmax and Km used for modeling most likely varied slightly from the actual respiration values, as indicated by the minimum in predicted oxygen concentration at 1,600 μm. The diffusion coefficient for oxygen was assumed to be constant throughout the construct volume although local variations may exist, especially in the regions of high cell density.

We used a continuous model of a disc shaped construct, where live cell density data were fitted to an exponential model and the resulting profile was used to calculate oxygen distribution. The model resulted in an almost linear decrease along the centerline, a prediction consistent with experimental data (**Figure 1A**). However, oxygen concentration was underestimated at low cell densities (at depths beneath 1,000 μm from the outer surface). Further numerical simulations indicated that an accurate fit could be achieved by increasing Km and decreasing Vmax, suggesting that oxygen gradients within tissue construct affect the cell distribution in a manner dependent on the cell respiration rate.

Application of superficial medium flow during measurement significantly increased oxygen concentration in the construct cross-section, and reduced the thickness of the mass transport boundary layer (**Figure 1B**). This correlated with the improved tissue properties in mixed flasks and rotating vessels.

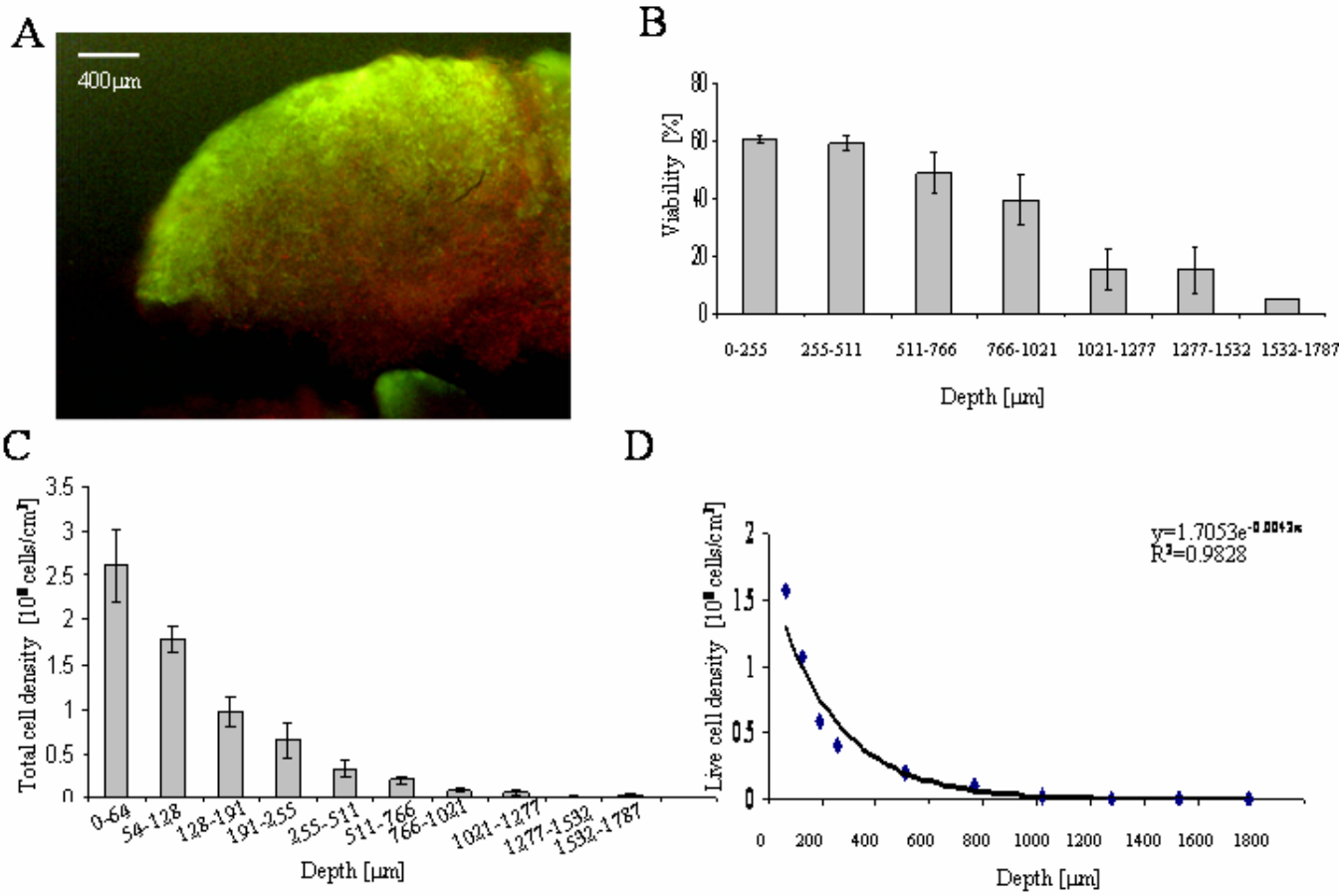


Figure 5. **Cell density and viability along the cross sections of statically cultured cardiac constructs:** (A) Live/dead stain of a representative cross-section (B) Measured cell viability along the construct cross–section (n = 3) (C) Measured total cell density along the construct cross-section (n = 4). Data expressed as Average ± SE. (D) Calculated live cell density and fitted exponential decay

Implications to cardiac tissue engineering The obtained correlation between live cell density and depth within the construct can be utilized to determine the construct dimensions necessary to achieve a desired density of live cells having a specific respiration rate for static culture conditions. For neonatal rat cardiomyocytes, the thickness of the construct that would have a physiologic cell density would be only 190 µm (**Figure 5C**). Therefore, for cells with high respiration rates (such as cardiomyocytes), alternative approaches, such as the interstitial flow of medium (perfusion through the construct) need to be taken to create constructs with high cell density and clinically relevant thicknesses (1 - 5mm). We recently described the cultivation of channeled cardiac tissue constructs perfused with culture medium that was supplemented with oxygen carriers [9]. We developed a mathematical model to describe oxygen distribution within the construct, which treated the construct as an array of cylindrical domains with a channel in the center and the surrounding tissue space. Notably, oxygen transport within the tissue space remains to be governed by diffusion. Therefore, the correlations and modeling results we describe here will enable us to predict the live cell density around each channel for different scaffold configurations and flow rates. This way, the present study enables a refinement of this recently published model and can thereby aid to the creation and analysis of thick and compact constructs.

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