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Controlled gas exchange in whole lung bioreactors

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Abstract

In cellular, tissue-level or whole organ bioreactors, the level of dissolved oxygen is one of the most important factors requiring control. Hypoxic environments may lead to cellular apoptosis, while hyperoxic environments may lead to cellular damage or dedifferentiation, both resulting in loss of overall tissue function. This manuscript describes the creation, characterization and validation of a bioreactor system that can control oxygen delivery based on real-time metabolic demand of cultured whole lung tissue. A mathematical model describing and predicting gas exchange within the tunable bioreactor system is developed. In addition, the inherent gas exchange properties of the bioreactor and the inherent oxygen consumption rates of native rat lungs are determined, thereby providing a quantitative relationship between system parameters and levels of dissolved oxygen. Finally, the mathematical model is validated during whole lung culture under a range of system parameters. The system presented here provides a quantitative relationship between the concentration of dissolved oxygen, tissue oxygen consumption rates, and controllable system parameters that introduce gasses into the bioreactor. This relationship not only enables the maintenance of constant levels of dissolved oxygen throughout a culture period during which cells are replicating, but also provides noninvasive and real-time estimation of the metabolic and proliferative states of native or engineered lung tissue simply through dissolved oxygen measurements.

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Conflict of interest

The authors have declared that there is no conflict of interest.

Author contributions

A.J.E. and L.E.N. designed all experiments. A.J.E., A.V.L. and P.B. performed all experiments. A.J.E. carried out all analyses and mathematical modelling, and wrote the manuscript.

Disclosure

L.E.N. is a founder and shareholder in Humacyte, Inc, which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. L.E.N.'s spouse has equity in Humacyte, and L.E.N. serves on Humacyte's Board of Directors. L.E.N. is an inventor on patents that are licensed to Humacyte and that produce royalties for L.E. N. L.E.N. has received an unrestricted research gift to support research in her laboratory at Yale. Humacyte did not fund these studies, and Humacyte did not influence the conduct, description or interpretation of the findings in this report.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

S1. Mathematical model derivation – whole lung bioreactor

S2. Mathematical model derivation – sealed bioreactor

S3. Supplemental References

Keywords

bioreactor; oxygen; gas exchange; mathematical model; lung

1. Introduction

Bioreactors serve as vessels for *in vitro* or *ex vivo* study and growth of cellular and tissue systems. The pharmaceutical industry has spearheaded development of cellular bioreactors, enabling large-scale production of proteins and antibodies from individual cells (Ozturk and Hu, 2005). These bioreactors are highly controlled with regard to temperature, fluid flow, pH, nutrients and dissolved oxygen, and computational models have been developed to understand, monitor and control these factors (Almquist *et al.*, 2014; Cvijovic *et al.*, 2011; Koutinas *et al.*, 2012; Mata-Alvarez and Mitchell, 2008). In contrast, systems for culturing intact, functional organs, developed in both academic (Hogan *et al.*, 2014; Niklason *et al.*, 2010; Petersen *et al.*, 2011, 2010; Song *et al.*, 2011) and commercial settings (Asnaghi *et al.*, 2009; Clause *et al.*, 2010; Macchiarini *et al.*, 2008; Ott *et al.*, 2010; Price *et al.*, 2010, 2015), are crude by comparison. Whole organ culture systems provide little to no control over gas exchange and nutrients. Additionally, there is very little understanding about how mass transfer affects the growth of complex tissues, and computational models that contemplate *in vitro* or *ex vivo* organ maintenance are either rudimentary or nonexistent (Linder *et al.*, 1996; Sanchez *et al.*, 2012; Sundaresan *et al.*, 2009). In order to create bioreactor systems that are able to support the long-term culture of whole organs, a fundamental understanding of gas and nutrient exchange is required.

The level of dissolved oxygen within a bioreactor system is one of the most crucial factors to monitor and control. If the environment becomes too hypoxic, cells will no longer have sufficient oxygen to undergo aerobic metabolism, which can lead to cellular hypoxia, apoptosis and loss of overall organ function (Liu *et al.*, 1994; You *et al.*, 2002). Conversely, multiple studies have shown that exposing alveolar epithelial cells to super-physiologic levels of dissolved oxygen can lead to cell damage or dedifferentiation via epithelial-mesenchymal cell transition (Calle *et al.*, 2015; Hou *et al.*, 2015; Johnston *et al.*, 2010; Mach *et al.*, 2011; Pagano and Barazzzone-Argiroffo, 2003; Vyas-Read *et al.*, 2014).

In the culture of engineered organs, where a subconfluent number of cells are seeded onto a supporting matrix, cellular proliferation leads to a greater oxygen demand over time, and thus decreased levels of dissolved oxygen in the bioreactor during the culture period (Crapo *et al.*, 2011; Petersen *et al.*, 2010). As such, one of the largest obstacles to long-term whole organ culture is providing enough oxygen to meet the increasing – and difficult to measure – metabolic demand of the growing tissue. Hence, there exists an unmet need for bioreactor systems that can tune the levels of dissolved oxygen in the bioreactor, thereby providing physiological levels of dissolved oxygen throughout the entirety of the culture period, regardless of metabolic demands and cell number.

Herein, we demonstrate the creation of a bioreactor system for whole rat lungs that can control dissolved gas levels by virtue of four discrete design factors. The first factor is careful and standardized system design, enabling repeatable system setup and determination

of all components that contribute to system gas exchange. The second factor is the creation of a mathematical model of oxygen transfer within the system, which provides relationships between bioreactor settings/parameters and the resultant levels of oxygen in the system. The third factor is rigorous and separate characterization of both the inherent gas exchange properties of the bioreactor and the inherent oxygen consumption rates of native rat lungs, replacing unknowns within the mathematical model with experimental constants to obtain a quantitative relationship between system parameters and levels of dissolved oxygen. The last factor is the validation of this mathematical model by culturing native rat lungs under a range of system parameters. Taken together, not only is this system able to maintain desired levels of dissolved oxygen throughout a culture period regardless of metabolic demand, but this model also enables noninvasive and real-time estimation of cell number, simply through dissolved oxygen measurements.

2. Materials and methods

2.1. Bioreactor design

The bioreactor used for these studies is illustrated in Figure 1A, B. All bioreactor components were obtained from Cole-Parmer (Vernon Hills, IL, USA) unless otherwise noted. The main body of the bioreactor was a 500 ml glass jar that was fitted with a silicone stopper, filled with 225 ml of culture medium, and stirred at 60 revolutions/min. PharMed BPT tubing (Westlake, OH, USA), size L/S 16, was inserted through the silicone stopper to enable the necessary connections to the lung, including perfusion and oxygenation loops, and to a pair of 0.22 μm nylon air filters (Whatman, GE Healthcare Life Sciences, Pittsburgh, PA, USA) for passive gas exchange. An optical dissolved oxygen probe (Vernier, Beaverton, OR, USA) extended into the bioreactor, with data from the probe collected with a LabQuest 2 interface every 5–10 s.

The perfusion loop consisted of 1.50 m of PharMed silicone tubing and a Masterflex L/S roller pump (Masterflex, Vernon Hills, IL, USA) to draw culture medium from the jar and perfuse it into the pulmonary arterial cannula. Culture medium was allowed to flow passively out of the pulmonary vein via the left ventricle and into the bioreactor jar. The airways were fluid-filled and the trachea cannula was left uncapped in the culture medium. Parallel to this perfusion loop was an oxygenation loop, consisting of 1.50 m in total of PharMed silicone tubing fed through a second Masterflex L/S roller pump such that the speed of the two fluid loops could be independently controlled. Culture medium was drawn from the jar and pumped into a PDMS Hollow Fibre Cartridge (HFC; PermSelect, Ann Arbor, MI, USA), which then drained back into the bioreactor jar. This HFC consisted of 3200 thin-walled, hydrophobic fibres with an aggregate surface area of 2500 cm^2 , roughly equivalent to the alveolar surface area of a 200-g Sprague–Dawley rat (Tschumperlin and Marguiles, 1999). Incubator air (37°C, 5% CO_2 , 75% humidity) was pumped through the fibres at 1.0 SLPM using an aquarium pump (JW Aquatic, Arlington, TX, USA). The HFC was kept at a 30° orientation during culture using a 3D-printed stand to ensure it remained completely filled with fluid.

2.2. Modelling gas exchange

To quantify and predict gas exchange within the bioreactor, a lumped parameter model was constructed as shown in Figure 1C. The model contains three elements: (1) a *bioreactor* element, assumed to be a well-mixed fluid compartment with a concentration of dissolved oxygen C_B ; (2) a *lung* element which consumes oxygen from the system; and (3) an *oxygenator* element, which adds oxygen back into the system, consisting of all elements that introduce gasses into the system: the PharMed tubing, air filters and hollow fibre cartridge. Fluid flows out of the bioreactor at oxygen concentration C_B , through the perfusion loop at flow rate F_P , and into the lung element. The oxygen concentration drops through the lung to a concentration C_L , and flows back into the bioreactor. Simultaneously and independently, fluid flows out of the bioreactor and through the oxygenation loop at flow rate F_O . The oxygen concentration rises through the oxygenator element to concentration C_O , and then flows back into the bioreactor. Since this is a mixed-tank lumped parameter model, a governing differential equation for the bioreactor oxygen concentration can be found by summing each product of flow rate and oxygen concentration in or out of the bioreactor (Equation 1, Figure 1C).

$$\dot{C}_B = F_O(C_O - C_B) - F_P(C_B - C_L) \quad (1)$$

2.3. Bioreactor characterization

A schematic of the experiments performed for bioreactor characterization is shown in Figure 2A. The bioreactor jar was filled with 300 ml of phosphate-buffered saline (PBS) and was equilibrated to a hypoxic gas mixture of 5% O₂, 5% CO₂, 37°C, and 75% humidity. The cap, perfusion loop and oxygenation loop with hollow fibre cartridge were assembled in a Caron incubator (Caron, Marietta, OH, USA), at ambient O₂, 5% CO₂, 37°C and 75% humidity. The air pump was placed inside the incubator and attached to the hollow fibre cartridge. The speed of the peristaltic pump for perfusion was fixed at 4 ml/min, and the speed of the peristaltic pump for oxygenation was set at 0, 3, 9, 15, 21 or 27 ml/min. The data acquisition interface was set to record dissolved oxygen concentration every 5 s for 100 min.

To initiate testing, the bioreactor jar was capped and quickly brought to the incubator. The hollow fibre cartridge, oxygenation line and perfusion line were all primed with the hypoxic fluid (5% O₂). Data collection was begun concurrently with starting both peristaltic pumps. Data between $t = 5$ min and $t = 100$ min were fitted to a saturating exponential curve using the LabQuest interface, and the time constant and saturation point of the best fit curve were determined. For each nonzero HFC flow rate, six experiments were performed. Three experiments were performed for the oxygenation flow rate of 0 ml/min.

2.4. Lung harvest and preparation

All animal work was performed in accordance with AAALAC guidelines and was approved by the Yale Institutional Animal Care and Use Committee. Lungs were harvested from adult Sprague–Dawley male rats weighing 308.8 ± 11.1 g [mean $\pm$ standard deviation (SD)] as previously described (Calle *et al.*, 2011; Petersen *et al.*, 2010). Briefly, rats were

anesthetized with a mixture of 75 mg/kg of ketamine and 5 mg/kg xylazine, and the chest cavity was exposed following full deflation of the lungs. The heart was perfused with PBS containing heparin (100 U/ml; Sigma–Aldrich, St Louis, MO, USA) and sodium nitroprusside (10 µg/ml; Sigma–Aldrich) via the right ventricle. After 10 ml had been perfused, the heart, lungs, and trachea were removed *en bloc*.

Cannulae were inserted into the trachea and into the pulmonary artery (PA) via the right ventricle and attached with 4-0 polypropylene suture. Lungs were inflated with 10 ml of PBS with 1000 U/ml penicillin, 1000 µg/ml streptomycin (both from Gibco), 10 µg/ml amphotericin B, and 200 µg/ml gentamicin (both from Gemini Bioproducts). This solution was held in the airways while 120 ml of PBS/heparin/sodium nitroprusside were perfused into the pulmonary artery via gravity-driven flow at a pressure head of 30 cmH₂O. Fluid was allowed to flow passively out of the pulmonary vein via the left ventricle. After repeating this treatment with the antibiotics/antimycotics solution and final rinses with PBS and complete Dulbecco's modified Eagle's medium (high glucose), lungs were submerged first in 70% ethanol and next in PBS, then mounted in a bioreactor prepared with 225 ml of complete Dulbecco's modified Eagle's medium high glucose with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 3 µg/ml amphotericin B and 50 µg/ml gentamicin. The pulmonary artery cannula was attached to the perfusion loop, while the trachea cannula and pulmonary vein were left detached and free floating in the culture medium. The total preparation time was noted as the interval between the initiation of heart perfusion and the initiation of data collection inside the bioreactor.

2.5. Lung oxygen consumption characterization

To characterize the inherent oxygen consumption characteristics of native lungs, lungs prepared as described above were placed into a sealed bioreactor with all routes of oxygen entry minimized or eliminated, as shown in Figure 3A. The oxygenation loop with HFC was removed for these experiments, air filters were capped, and all silicone tubing replaced with low-oxygen-permeable PharMed tubing. A mathematical model for the sealed bioreactor is outlined in Figure 3B, with Equation 2 as the governing differential equation. The oxygen probe data acquisition interface was set to record the dissolved oxygen concentration every 10 s for 24 h of culture. The pulmonary artery perfusion speed was set to 4 ml/min for each culture period. Three experiments were performed in total.

$$\dot{C}_B = -F_P(C_B - C_L) = F_P(C_O - C_B) - \dot{Q}_N \quad (2)$$

The initial whole-lung oxygen consumption rate was calculated as the slope of a linear regression of the dissolved oxygen vs. time curve, between $pO_2 = 120$ and 80 mmHg. The equilibration point was calculated as the mean of the data between $t = 16$ and 20 h. The final oxygen consumption rate for the N cells in the lung, $\dot{Q}_N$, was calculated from the equilibration point by solving Equation 2 for the steady-state condition (see Supplement for derivation).

2.6. Solving the mathematical model

Results from the bioreactor characterization and the lung oxygen consumption characterization were incorporated into the mathematical model's differential equation (Equation 1) to determine a solution dependent upon only experimental parameters and experimentally-determined constants. A step-by-step derivation of this solution is included in the Supplemental material, and is summarized here.

The governing differential equation for the model outlined in Figure 1C (Equation 1). There are two unknowns: C_O , the concentration of oxygen leaving the oxygenator element; and C_L , the concentration of oxygen leaving the lung. C_B , the concentration of oxygen in the bioreactor, is directly measured, while the two flow rates are user-defined parameters for the HFC oxygenation loop (F_O) and for the pulmonary artery perfusion loop (F_P). To incorporate the results of bioreactor characterization testing, we may replace the expression $F_O(C_O - C_B)$ with the expression $(S(F_O) - C_B) \cdot V/\tau(F_O)$, where $S(F_O)$ is the experimentally-determined system saturation as a function of the oxygenation flow rate, $\tau(F_O)$ is the experimentally-determined system time constant as a function of the oxygenation flow rate, and V is the total amount of fluid volume in the system. Fick's principle –that $(C_B - C_L) = \dot{Q}/F_P$, where $\dot{Q}$ is the experimentally-determined oxygen consumption rate for a native rat lung (Boron and Boulpaep, 2008) – may be used, simply replacing the expression $F_P(C_B - C_L)$ with $\dot{Q}$. By setting $\dot{C}_B$ to zero (e.g. by assuming the bioreactor oxygen concentration is not changing), this may be solved for the steady-state concentration of oxygen within the bioreactor by rearranging the equation to isolate C_B :

$$C_B = S(F_O) - \dot{Q} \cdot \tau(F_O)/V \quad (3)$$

This equation is now dependent only upon the oxygenation flow rate, which is a known experimental parameter, as well as experimentally determined mathematical relationships. Equation 3 therefore represents a quantitation of the inherent, user-controlled gas transfer characteristics of the oxygenator-containing bioreactor system.

2.7. Model validation

To validate the model, lungs were cultured in the full bioreactor system over a range of HFC oxygenation flow rates: 4, 8, 16 or 32 ml/min. The perfusion flow rate was held constant at 4 ml/min. The data acquisition interface was set to record dissolved oxygen concentration every 10 s for 24 h of culture. Three independent experiments were performed at each of the four oxygenation flow rates, for a total of 12 native lung cultures. The equilibration point was determined as the mean of the flattest 4-h region after $t = 8$ h. By plugging the raw dissolved oxygen data (C_B) and the HFC flow rate (F_O) into the mathematical model, the dissolved oxygen data were then transformed into an expression for the whole-lung oxygen consumption rate ($\dot{Q}$) vs. time.

2.8. Lung takedown, histology, and immunofluorescence

After 24 h of culture, glucose and lactate measurements of the culture medium were taken with a GlucCell glucose meter (CESCO BioProducts, Atlanta, GA, USA) and an iSTAT

cartridge (CG4+, Abaxis, Union City, CA, USA) respectively. Glucose consumption and lactate production were calculated by subtracting baseline values of the culture medium from the postculture values. The accessory, right caudal and right medial lobes of the lung were tied off, removed, and weighed before snap-freezing. The left lobe and right cranial lobes were inflation-fixed for 5 min in 10% neutral buffered formalin (NBF) under 15 cmH₂O of intratracheal pressure, rocked for an additional 3-h in NBF, weighed, and then paraffin-embedded and sectioned. Three control lungs were also prepared by fixation immediately after explant. Routine histology (haematoxylin and eosin) was performed for each lung, as well as immunofluorescence for proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) to investigate proliferation and apoptosis, respectively.

For PCNA staining, 5 µm sections were rehydrated with a decreasing ethanol gradient, taken through antigen retrieval with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), permeabilized in PBS with 0.2% Triton X-100 for 15 min, and blocked in PBS with 0.75% glycine and 5% bovine serum albumin for 60 min at room temperature. Blocked sections were incubated overnight at 4°C with a primary PCNA antibody diluted in blocking buffer (Mouse, Abcam, 1:1000 dilution), and a secondary antibody was applied at a 1:500 dilution (Goat anti-Mouse, IgG Alexa Fluor 555, Invitrogen). Sections were rinsed with PBS, costained with DAPI (Biotium, 1:1000 in Millipore ddH₂O), mounted with Fluoromount (Sigma–Aldrich) and imaged.

For TUNEL staining, samples were permeabilized following rehydration using a solution of 0.1% Triton X-100 and 0.1% sodium citrate, and incubated at room temperature for 8 min. A terminal deoxynucleotidyl transferase (TdT) enzyme solution (100 µl) was added to 900 µl of a fluorescein-dUTP label solution from a TUNEL kit (Roche Applied Science, Indianapolis, IN, USA) and kept on ice. A 50-µl aliquot of the TUNEL reaction mixture was added to each slide, after which sections were covered with a coverslip and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Samples were then rinsed with PBS, costained with DAPI, mounted and imaged.

For histological quantification, three 40× images of distal alveolar regions with greater than 100 nuclei were taken for each PCNA and TUNEL slide, and the percentage of positive cells per high-powered field was calculated. Three separate images were taken for each three distinct lungs per experimental group.

2.9. Total deoxyribose nucleic acid analysis

Each accessory lobe was lyophilized overnight then digested in 1 ml of papain solution [1200 U papain (Sigma–Aldrich), 40 ml PBS, 400 µl ethylenediamine tetra-acetic acid 0.5 M pH 8.0, 35.2 mg cysteine HCl, and 320 mg sodium acetate] for every 10 mg of dry weight. Samples were digested at 65°C for 3 days and vortexed every 12 h. Digested samples were diluted 1:100 in 1× TE buffer (1 ml 1 M Tris-HCl pH 7.4, 0.2 ml ethylenediamine tetra-acetic acid, 99 ml Millipore ddH₂O) to a total volume of 400 µl. A 100-µl aliquot of each sample was placed in three wells in a 96-well plate along with 100 µL of PicoGreen fluorophore (Life Technologies, Waltham, MA, USA). The 96 well plate was placed in a fluorometer plate reader (Synergy HT Multidetector, Biotek, Winooski, VT,

USA) with excitation at 485 nm and emission at 535 nm. The estimated cell count in each sample was found by assuming 7 pg of deoxyribose nucleic acid (DNA) per cell (Stone *et al.*, 1992). The estimated whole lung cell count was found by dividing the estimated sample cell count by the wet weight of each accessory lobe and multiplying by the combined weight of all five lobes.

2.10. Variable correlations

To investigate correlations between experimental variables, data from the 12 model validation lungs were analysed in GraphPad Prism. Independent variables tested were the HFC flow rate, rat weight, total lung weight and preparation time. Dependent variables tested were the equilibrium pO_2 ; the whole-lung oxygen consumption rate at the equilibrium pO_2 ; glucose and lactate during culture; the ratio between lactate production and glucose consumption, Y_{lg} ; the estimated whole lung cell count; the single cell oxygen consumption rate, glucose consumption and lactate production; and the percentage of cells positive for either PCNA or TUNEL.

2.11. Statistics

In section 2.3, to determine if either the time constant or the saturation point were a function of oxygenation flow rate, a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test was used to test significance. Where significance was found, nonlinear curve fitting in MATLAB was used to determine the best fit relationship and quantify the correlation to oxygenation flow rate. For sections 2.8 and 2.10, a one-way ANOVA with Dunnett's multiple comparison test was used to test significance between experimental groups. For section 2.10, linear regressions were performed against rat weight, total lung weight or preparation time, and the correlation r^2 between the data was found. An F test was performed to test if the slope of the regression was significantly different than zero. All regressions and calculations for sections 2.3, 2.5 and 2.7 were performed in MATLAB, while those for sections 2.8 and 2.10 used the GraphPad Prism statistical analysis software. For all regressions and comparisons, significance was characterized by $p < 0.05$.

3. Results and discussion

3.1. Bioreactor characterization

A simplified diagram of the experimental setup for bioreactor characterization is shown in Figure 2A. Representative curves for each oxygenation flow rate are shown in Figure 2B. Oxygenation curves were fitted to the equation for a saturating exponential to determine the time constant and the saturation point. The time constant for the system showed an exponential dependence upon the oxygenation flow rate (Figure 2C), obeying the relationship $\tau(F_O) = A \cdot \exp(-F_O/T) + B$, where A , B and T are the constants determined through the nonlinear curve fitting in MATLAB. The time constant data fit the above equation with an r^2 of 0.9903, suggesting a first-order relationship between the oxygenation flow rate and the time constant, as expected ($p < 0.0001$). The time constant for equilibration without a hollow fibre cartridge (analogous to an oxygenation flow rate of 0 ml/min) was 84.5 ± 10.6 min.

Figure 2D shows the dependence of the saturation point on the oxygenation flow rate, demonstrating that the saturation point of the system has no statistically significant dependence on oxygenation flow rate ($p = 0.9971$). The mean of all values of the saturation point of oxygen in the bioreactor system was $S = 5.924 \text{ mg/l}$ (131.3 mmHg). In the experiments performed without a hollow fibre cartridge, the saturation value was $5.877 \pm 0.02 \text{ mg/l}$ ($130.3 \pm 0.5 \text{ mmHg}$). This result suggests that the addition of the hollow fibre cartridge does not significantly affect the total amount of dissolved oxygen that the system can hold.

3.2. Lung oxygen consumption and DNA analysis for cell number

A diagram of the experimental setup for lung oxygen consumption characterization is shown in Figure 3A, with a lumped parameter model of the simplified system shown in Figure 3B. The dissolved oxygen curves for the three lungs that were deprived of oxygen are shown in Figure 3C, with oxygen consumption rates and equilibration values listed in Table 1. The preparation time (time the tissue spent at room temperature between start of cardiac perfusion and start of data collection inside the bioreactor) for these three lungs was $33 \pm 5 \text{ min}$.

For lungs cultured without exogenous sources of oxygen, three different phases of oxygen consumption behaviour can be seen. The first phase is a linear decrease in the levels of dissolved oxygen, indicating that the overall rate of oxygen consumption is fairly constant. The initial oxygen consumption rate was defined as the slope centred around 100 mmHg , and was equal to $1.432 \pm 0.223 \text{ mmHg/min}$ (0.0676 mg/l/min , $0.475 \mu\text{mol/min}$). The second phase consists of a shift towards exponential-like behaviour at an inflection point occurring around $40\text{--}60 \text{ mmHg}$. This could mark a shift in the metabolic state of the lung, with possible upregulation of anaerobic metabolism over aerobic metabolism (Ozturk and Hu, 2005) or downregulation of ATP-intensive processes. Finally, there is an equilibration phase where the slope of the curve is approximately zero. This equilibration at $24.15 \pm 1.82 \text{ mmHg}$ could indicate that the lungs are beginning to utilize oxygen at the same rate that oxygen is entering passively through the PharMed tubing in the bioreactor system. In equation 2 (Figure 3B), this is equivalent to setting the left side of the equation to zero and rearranging to obtain: $\dot{Q}_N = F_P(C_O - C_B)$. Given the PharMed tubing's oxygen permeability coefficient, surface area, and thickness, the oxygen consumption rate at the equilibration point was $0.0751 \pm 0.0013 \text{ mmHg/min}$ ($0.025 \mu\text{mol/min}$), or approximately 5% of the initial oxygen consumption rate.

Taken together, some conclusions may be drawn about the oxygen utilization behaviour of native rat lungs. First, given sufficient levels of dissolved oxygen, lungs will consume oxygen at a fixed rate, proportional to the percentage of cells that are able to participate in aerobic metabolism. Second, when levels of dissolved oxygen drop too low and are maintained low, the lung will slow its consumption rate of oxygen, possibly due to a shift from largely aerobic to largely anaerobic metabolism, or possibly due to the ischaemia/necrosis of a percentage of cells in the lung. For the lungs tested here, this threshold value appears to range between 40 and 60 mmHg . Third, the lung is able to partially recover from

periods of minimal or zero oxygen consumption, as evidenced by the equilibration of dissolved oxygen levels following a local minimum and subsequent rise.

The estimated whole lung cell count was determined through the DNA assay as described above, with the lungs containing $390 \pm 103 \times 10^6$ cells. The mean single cell oxygen consumption rate was calculated as 1.301×10^{-15} mol/min/cell, which is in good agreement with previously reported values for lung epithelium at 1.12×10^{-15} mol/min/cell (Strovas *et al.*, 2010). This implies that the preparation time for the lungs is short enough to enable a majority of cells to participate in aerobic metabolism.

3.3. Model validation

The bioreactor and lung characterization data were incorporated into Equation 3 in order to obtain a quantitative relationship between the steady state concentration of oxygen in the bioreactor and the oxygenation flow rate. Experimentally-determined constants for $\tau(F_O)$ (Figure 2C) and $S(F_O)$ (Figure 2D) were combined with the value for $\dot{Q}_N$, the mean whole-lung oxygen consumption rate from Table 1 and Figure 3C. This results in:

$$C_B = S - (\dot{Q}_N/V) \cdot (A \cdot \exp(-F_O/T) + B)$$

$$C_B = (5.924 \text{ mg/l}) - (0.0676 \text{ mg/l/min}) \cdot ((27.16 \text{ min}) \cdot \exp(-F_O/(8.772 \text{ ml/min})) + (5.765 \text{ min}))$$

(3)

To assess the predictive value of this mathematical relationship, we cultured native lungs at four different rates of oxygenation flow for 24 h. Representative dissolved oxygen traces are shown in Figure 4A. The preparation time for these 12 lungs was 33 ± 3 min, and the estimated whole lung cell count was $442 \pm 101 \times 10^6$ cells. All four of the dissolved oxygen traces follow a similar trend, starting with an initial decrease followed by an equilibration within 1–4 h, with slower HFC flow rates resulting in a lower pO₂ at equilibrium.

Figure 4B shows the equilibration values as a function of the HFC flow rate, with $n = 3$ for each point. The model prediction from above is overlaid as a solid trace, and a best-fit curve calculated in MATLAB through nonlinear curve fitting is overlaid as a dashed line. The model prediction aligns well with the HFC flow rates of 8, 16 and 32 ml/min, but the actual equilibration values at 4 ml/min are lower than predicted. This may be caused by technical factors: small bubbles become trapped in the hollow fibre cartridge at slow flow rates, thereby lowering the surface area for gas exchange.

Given these findings, the constants in the mathematical model were updated to best reflect the actual behaviour of the oxygenator. B was assumed to be the least flexible variable, as Figure 2C demonstrates tight clustering at high HFC flow rates. As such, the value of B was maintained, and the values of S , A and T were updated to yield an updated Equation 3:

$$C_B = (5.997 \text{ mg/l}) - (\dot{Q}_N/0.225 \text{ L}) \cdot ((51.85 \text{ min}) \cdot \exp(-F_O/(5.829 \text{ ml/min})) + (5.765 \text{ min}))$$

The equation above provides a quantitative relationship between the dissolved oxygen, the oxygenation flow rate, and the oxygen consumption rate of the whole lung. With this relationship, the dissolved oxygen data in Figure 4A were transformed into real-time whole lung oxygen consumption rate data (Figure 4C). This demonstrates the initial acclimation of the organ to its new environment after explantation from the donor, and the subsequent increase in oxygen consumption after approximately 1 h of bioreactor culture. The oxygen consumption rates for all 12 lungs are shown in Figure 4D as a function of the HFC flow rate, compared to the final oxygen consumption rate for the lungs cultured without oxygen ($n = 3$ for all bars). There is no statistically significant difference between the four HFC flow rates ($p = 0.9429$), and increases in oxygen consumption rate as compared to the No O₂ culture ($p = 0.0552, 0.0439, 0.0918$ and 0.0331 for No O₂ vs. 4, 8, 16 and 32, respectively). This suggests that our culture system is able to support similar levels of whole organ oxygen consumption regardless of the pO₂ during culture, within this tested experimental range.

3.4. Immunofluorescence

Histological images were taken for control lungs (freshly excised from rats), for lungs maintained at each of the four HFC flow rates, and for lungs cultured without oxygen (No O₂; Figure 5A–F). Histology showed that all HFC flow rates maintain gross cell phenotype in both distal and proximal regions, while the No O₂ culture displays rounding of cell nuclei and detachment of cells from the extracellular matrix, demonstrating the adverse effects of extreme hypoxia.

Representative PCNA and TUNEL images are also shown (Figure 5G–R), along with quantification of positive nuclei for each stain (Figure 5S–T). Images contained 167 ± 33 nuclei (mean $\pm$ SD). Lungs cultured at HFC flow rates of 4 ml/min demonstrated significantly higher rates of proliferation as compared to the other experimental groups, suggesting that mild hypoxia may trigger increased proliferation. While not significant, these data also show slight increases in the percentage of cells staining for PCNA for all lungs cultured under oxygenation as compared to the controls, and decreases in PCNA staining for the lungs cultured without oxygen as compared to the controls. TUNEL staining shows a highly statistically significant increase in apoptosis in the group lacking oxygen as compared to every other group, with no statistically significant differences between any of the HFC lungs and the controls. When the No O₂ group is excluded from analysis and statistics repeated, the only statistically significant differences are between the HFC 16 group and the control, HFC 4 and HFC 8 groups ($p = 0.0076, 0.0123, 0.0388$, respectively). This difference is potentially caused by the HFC 16 group containing the lung with the longest preparation time, as discussed in the next section. Taken together, these proliferative and apoptotic marker data support the idea that the bioreactor with the hollow fibre oxygenator is able to support whole lung viability.

3.5. Variable correlations

The relationship between experimental variables was investigated through a series of one-way ANOVAs and linear regressions to determine significant correlations, with a complete listing of r^2 and p values reported in Table 2. The HFC flow rate only had statistically significant correlations with the equilibrium pO_2 , highlighting that the pO_2 values explored in these experiments had no significant effect on nutrient utilization. The preparation time for the lungs used in these experiments only had statistically significant correlations with TUNEL staining. This correlation emphasizes the exquisite sensitivity of lung tissues to periods of ischaemia, as the addition of a few minutes of prep time yields a significant increase in the percentage of cells staining positively for apoptotic markers.

Rat weight and lung weight have statistically significant correlations with whole-organ nutrient consumption. This relationship intuitively makes sense, as it suggests that heavier rats and larger lungs will consume more oxygen and glucose and will produce more lactate. However, neither rat nor lung weight show statistical significance at the single cell level for either oxygen, glucose or lactate. This absence of significance could suggest that this bioreactor system is an effective tool for investigating metabolic activity of an average single lung cell *in situ*, regardless of organ size or cell number. The mean single cell oxygen consumption rate for these lungs was $1.20 \pm 0.59 \times 10^{-15}$ mol/min/cell, compared to previously reported values for lung epithelium at 1.12×10^{-15} mol/min/cell (Strovas *et al.*, 2010). This would suggest that lung cells consume oxygen at roughly the same rate in their native environment as compared to isolated cells. However, the average values for single cell glucose consumption (0.402 ± 0.244 ng/cell/day) and lactate production (0.335 ± 0.163 ng/cell/day) were significantly lower than literature values for freshly isolated cells (glucose 0.912 ± 0.061 ng/cell/day, lactate 1.129 ± 0.108 ng/cell/day) (Pérez-Díaz *et al.*, 1977), suggesting that lung cells are less metabolically active *in situ* as compared to isolated, individual cells. These data also demonstrate no correlation between any variable to $Y_{l/g}$, the ratio of lactate consumption to glucose production and an indicator of overall metabolic state. Furthermore, the mean ratio of 0.78 ± 0.13 is lower than literature values of 1.24 ± 0.20 for freshly isolated lung cells (Pérez-Díaz *et al.*, 1977). Not only do these findings suggest that heavier lungs behave metabolically similarly to lighter lungs, but also that lung cells *in situ* participate in proportionally more aerobic metabolism (where $Y_{l/g} = 0$) than anaerobic metabolism (where $Y_{l/g} = 2$) as compared to isolated cells.

3.6. Proposed applications, limitations and conclusions

This bioreactor system allows for the real-time tracking of whole organ oxygen consumption rate, in addition to maintenance of desired levels of dissolved oxygen throughout the entirety of culture. In practice, this enables noninvasive and real-time estimation of cell number and the proliferative state of lung tissue simply through dissolved oxygen measurements. During the culture of engineered tissue where the initial cell number is known, a single cell oxygen consumption rate specific to the seeded cell type(s) may be calculated from initial whole organ oxygen consumption rates. As culture continues and as the cells proliferate, increases in whole organ oxygen consumption can subsequently be related back to cell number. These measurements could also be fine-tuned with periodic glucose and lactate measurements, using the expression $(2 - Y_{l/g})/2$ to estimate the percentage of cells participating in aerobic

metabolism. This addition could enable more accurate predictions of cell number, by compensating for metabolic shifts in nutrient utilization. Furthermore, the system described here enables controllable culture over a range of dissolved oxygen concentrations, with the mathematical model reporting a necessary oxygenation flow rate for a given cell number and desired culture pO_2 . This whole-organ culture system could therefore enable exploration of the effects of various oxygen tensions on cell phenotype, tissue metabolism and whole organ function.

While this bioreactor system offers greater quantitative control over dissolved oxygen than previous culture systems, there are improvements that could be made to address a few limitations of the current study. First, the solution to the system's differential equation assumes a steady state condition between oxygen consumption and oxygen delivery has been reached. In order to study the dynamics of tissue response to sudden environmental changes, the time derivative of oxygen concentration would have to be tracked in addition to raw dissolved oxygen measurements. Second, the use of native organs and the necessity of the rinsing and preparation steps to enable sterile bioreactor culture introduces periods of ischaemia to the organ that affect cellular apoptosis. While this should not affect the preparation of engineered tissue, the preparation protocol could be further optimized for native tissue culture. Third, further phenotypic and functional analysis will be required when culturing tissue for longer than 24 h. While this time period is sufficient to demonstrate functionality of the system and maintenance of tissue viability, longer culture periods for lung tissue would be likely to require an air-liquid interface and breathing motions. The bioreactor characterizations could be repeated for the more complicated system, and further phenotypic and functional analyses could be performed on the tissue after bioreactor culture.

In summary, the system presented here represents significant progress in controllable bioreactor development for whole lung culture. The bioreactor created and characterized enables predictable tuning of dissolved oxygen levels, regardless of the number of cells within the cultured tissue. It is because of this standardization and characterization that dissolved oxygen measurements can be used to calculate the real-time oxygen consumption rates of cultured tissue, and this feedback can be used to maintain constant levels of dissolved oxygen throughout culture. Practically, this system enables real-time, noninvasive estimation of oxygen consumption rates and cell number in engineered tissue simply through dissolved oxygen measurements. The data presented here offer further insight into *in vitro* oxygen consumption behaviour of lungs, and may enable advancement of controllable bioreactor systems for other whole organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

A	Constant relating the time constant of the system to the oxygenation flow rate [min]
B	Constant relating the time constant of the system to the oxygenation flow rate [min]
C_B	Dissolved oxygen concentration in the bioreactor [mg/l]
$\dot{C}_B$	Time derivative of dissolved oxygen concentration in the bioreactor [mg/l/min]
C_L	Concentration of dissolved oxygen exiting the lung [mg/l]
C_O	Concentration of dissolved oxygen exiting the <i>oxygenator</i> element [mg/l]
F_O	Flow rate of the oxygenation loop [ml/min]
F_P	Flow rate of the perfusion loop [ml/min]
N	Number of cells in the lung [unitless]
$\dot{Q}$	Oxygen consumption rate for the lung [mg/l/min]
S	Saturation point of dissolved oxygen in the bioreactor [mg/l]
τ	Time constant of gas exchange within the bioreactor [min]
T	Constant relating the time constant of the system to the oxygenation flow rate [ml/min]
V	Amount of circulating fluid in the bioreactor [l]
$Y_{l/g}$	Ratio of lactate produced to glucose consumed over a time interval, ranging between 0 (fully aerobic metabolism) and 2 (fully anaerobic metabolism) [unitless]

References

- Almquist J, Cvijovic M, Hatzimanikatis V, et al. Kinetic models in industrial biotechnology – improving cell factory performance. *Metab Eng.* 2014; 24:38–60. [PubMed: 24747045]
- Asnaghi A, Jungebluth P, Raimondi MT, et al. A double-chamber rotating bioreactor for the development of tissue-engineered hollow organs: from concept to clinical trial. *Biomaterials.* 2009; 30(29):5260–5269. [PubMed: 19647867]
- Boron, WF., Boulpaep, EL. *Medical Physiology.* 2. Saunders; Philadelphia, PA: 2008.
- Calle EA, Mendez JJ, Ghaedi M, et al. Fate of distal lung epithelium cultured in a decellularized lung extracellular matrix. *Tissue Eng Part A.* 2015; 21(11–12):1916–1928. [PubMed: 25789725]
- Calle EA, Petersen TH, Niklason LE. Procedure for lung engineering. *J Vis Exp.* 2011; 49:2651.
- Clause KC, Tinney JP, Liu LJ, et al. A three-dimensional gel bioreactor for assessment of cardiomyocyte induction in skeletal muscle-derived stem cells. *Tissue Eng Part C Methods.* 2010; 16(3):375–385. [PubMed: 19601695]
- Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. *Biomaterials.* 2011; 32(12):3233–3243. [PubMed: 21296410]

- Cvijovic M, Bordel S, Nielsen J. Mathematical models of cell factories: moving towards the core of industrial biotechnology. *Microb Biotechnol*. 2011; 4(5):572–584. [PubMed: 21375719]
- Hogan BL, Barkauskas CE, Chapman HA, et al. Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. *Cell Stem Cell*. 2014; 15(2):123–138. [PubMed: 25105578]
- Hou A, Fu J, Yang H, et al. Hyperoxia stimulates the transdifferentiation of type II alveolar epithelial cells in newborn rats. *Am J Physiol Lung Cell Mol Physiol*. 2015; 308(9):L861–872. [PubMed: 25681436]
- Johnston LC, Gonzales LW, Lightfoot RT, et al. Opposing regulation of human alveolar type II cell differentiation by nitric oxide and hyperoxia. *Pediat Res*. 2010; 67(5):521–525. [PubMed: 20098340]
- Koutinas M, Kiparissides A, Pistikopoulos EN, et al. Bioprocess systems engineering: transferring traditional process engineering principles to industrial biotechnology. *Comp Struct Biotechnol J*. 2012; 3:10022.
- Linder A, Friedel G, Fritz P, et al. The *ex-vivo* isolated, perfused human lung model: description and potential applications. *Thorac Cardiovasc Surg*. 1996; 44(3):140–146. [PubMed: 8858797]
- Liu JY, Nettesheim P, Randell SH. Growth and differentiation of tracheal epithelial progenitor cells. *Am J Phys*. 1994; 266(3 Pt 1):L296–307.
- Macchiaroni P, Jungebluth P, Go T, et al. Clinical transplantation of a tissue-engineered airway. *Lancet*. 2008; 372(9655):2023–2030. [PubMed: 19022496]
- Mach WJ, Thimmesch AR, Pierce JT, et al. Consequences of hyperoxia and the toxicity of oxygen in the lung. *Nurs Res Pract*. 2011; 2011:260482. [PubMed: 21994818]
- Mata-Alvarez, J., Mitchell, DA. *Encyclopedia of Life Support Systems*. 2008. Mathematical Modeling in Biotechnology.
- Niklason LE, Yeh AT, Calle EA, et al. Enabling tools for engineering collagenous tissues integrating bioreactors, intravital imaging, and biomechanical modeling. *Proc Natl Acad Sci U S A*. 2010; 107(8):3335–3339. [PubMed: 19955446]
- Ott HC, Clippinger B, Conrad C, et al. Regeneration and orthotopic transplantation of a bioartificial lung. *Nat Med*. 2010; 16(8):927–933. [PubMed: 20628374]
- Ozturk, S., Hu, WS. *Cell Culture Technology for Pharmaceutical and Cell-Based Therapies*. 1. CRC Press; 2005.
- Pagano A, Barazzzone-Argiroffo C. Alveolar cell death in hyperoxia-induced lung injury. *Ann N Y Acad Sci*. 2003; 1010:405–416. [PubMed: 15033761]
- Pérez-Díaz J, Martín-Requero A, Parrilla R, et al. Relationship between cellular energy production and rates of glucose utilization by lung cells. *Pflügers Arch*. 1977; 371(1–2):19–24. [PubMed: 563572]
- Petersen TH, Calle EA, Colehour MB, et al. Bioreactor for the long-term culture of lung tissue. *Cell Transplant*. 2011; 20(7):1117–1126. [PubMed: 21092411]
- Petersen TH, Calle EA, Zhao L, et al. Tissue-engineered lungs for *in vivo* implantation. *Science*. 2010; 329(5991):538–541. [PubMed: 20576850]
- Price AP, England KA, Matson AM, et al. Development of a decellularized lung bioreactor system for bioengineering the lung: the matrix reloaded. *Tissue Eng Part A*. 2010; 16(8):2581–2591. [PubMed: 20297903]
- Price AP, Godin L, Domek A, et al. Automated decellularization of intact, human-sized lungs for tissue engineering. *Tissue Eng Part C Methods*. 2015; 21(1):94–103. [PubMed: 24826875]
- Sanchez PG, Bittle GJ, Burdorf L, et al. State of art: clinical *ex vivo* lung perfusion: rationale, current status, and future directions. *J Heart Lung Transplant*. 2012; 31(4):339–348. [PubMed: 22423980]
- Song JJ, Kim SS, Liu Z, et al. Enhanced *in vivo* function of bioartificial lungs in rats. *Ann Thorac Surg*. 2011; 92(3):998–1006. [PubMed: 21871290]
- Stone KC, Mercer RR, Freeman BA, et al. Distribution of lung cell numbers and volumes between alveolar and nonalveolar tissue. *Am Rev Respir Dis*. 1992; 146(2):454–456. [PubMed: 1489139]
- Strovas TJ, McQuaide SC, Anderson JB, et al. Direct measurement of oxygen consumption rates from attached and unattached cells in a reversibly sealed, diffusionally isolated sample chamber. *Adv Biosci Biotech*. 2010; 5(5):398–408.

- Sundaresan A, Yuta T, Hann C, et al. A minimal model of lung mechanics and model-based markers for optimizing ventilator treatment in ARDS patients. *Comput Methods Prog Biomed.* 2009; 95(2): 166–180.
- Tschumperlin DJ, Marguiles SS. Alveolar epithelial surface area–volume relationship in isolated rat lungs. *J Appl Physiol.* 1999; 86(6):2026–2033. [PubMed: 10368370]
- Vyas-Read S, Wang W, Kato S, et al. Hyperoxia induces alveolar epithelial-to-mesenchymal cell transition. *Am J Physiol Lung Cell Mol Physiol.* 2014; 306(4):L326–340. [PubMed: 24375795]
- You Y, Richer EJ, Huang T, et al. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol Lung Cell Mol Physiol.* 2002; 283(6):L1315–1321. [PubMed: 12388377]

Figure 1. Design of the whole lung bioreactor for controlling gas exchange

(A) Schematic and (B) image of the system are shown. A hollow fibre cartridge (HFC) serves as a synthetic capillary network uncoupled from perfusion, enabling modulation of gas transfer properties at fixed perfusion rates. (C) A lumped parameter model for gas exchange in the bioreactor, with Equation 1 being the system's overall governing differential equation

Figure 2. Characterization of the bioreactor system

(A) A diagram of the *No lung* experimental setup for characterizing inherent gas exchange properties of the bioreactor as a function of the oxygenation flow rate F_O . (B) The hypoxic-to-ambient equilibration was characterized by its time constant (τ , how quickly the system can respond) and saturation (S , how much oxygen the system can hold). (C) The time constant τ has a first-order response to the oxygenation flow rate F_O . (D) The saturation S has no response to F_O . $n = 6$ for each flow rate

Figure 3. Characterization of native rat lung oxygen consumption

(A) A diagram of the *No O₂* experimental setup for characterizing the inherent oxygen consumption rate of native rat lungs. (B) A lumped parameter model for gas exchange in the sealed bioreactor, with Equation 2 being the system's overall governing differential equation. (C) Raw dissolved oxygen versus time curves for the three lungs tested. Lung 2 shows a local maximum at $t = 4$ h, caused by a stir plate malfunction starting at $t = 3$ and fixed at $t = 4$, demonstrating the contribution of stirring during culture

Figure 4. Model validation

(A) Sample dissolved oxygen vs. time curves for each of four HFC flow rates. (B) The model's predictions plotted against the actual equilibration values, calculated as the mean of the flattest 4 hour region after $t = 8$ h. $n = 3$ for each flow rate. (C) Real-time oxygen consumption rate versus time curves, obtained by transforming the data from (A) using the mathematical model. (D) Oxygen consumption rates were calculated from the equilibrium values used in 4B

Figure 5. Representative images and quantification

(A–F) Representative haematoxylin and eosin images, 40×, showing both distal and proximal airways from control lungs fixed immediately post explant, each of the four HFC speeds, and no oxygen culture. Scale bars are 50 μm. (G–L) Representative PCNA staining of distal alveolar regions for all six experimental groups. Scale bars are 20 μm. (M–R) Representative TUNEL staining of distal alveolar regions for all six experimental groups. Scale bars are 20 μm. (S–T) Quantification of PCNA and TUNEL, respectively, for $n = 3$ separate images, each of a distal alveolar region with greater than 100 nuclei, for each of $n = 3$ distinct lungs per experimental group. Percentage indicates the proportion of positive cells per high-powered field (HPF). Images contained 167 ± 33 nuclei (mean $\pm$ SD).

Table 1**Characterization of lung oxygen consumption**

The initial consumption rate was calculated as the slope in Figure 2C between 120 and 80 mmHg. The equilibration point was calculated as the mean of data between 16 and 20 h. The final consumption rate was calculated from the equilibration point by solving Equation 2. The estimated whole lung cell count was determined from the PicoGreen Total DNA Assay. The estimated single cell consumption rate was found by normalizing the initial consumption rate by cell number

Lung #	Initial O ₂ consumption rate (mmHg/min [mg/l/min])	Equilibration point (mmHg [mg/l])	Final O ₂ consumption rate (mmHg/min [mg/l/min])	Estimated whole lung cell count ($\times 10^6$)	Estimated single cell O ₂ consumption rate (mol/min/cell)
1	1.688 (0.0797)	22.29 (1.053)	0.0764 (0.00344)	299	1.877×10^{-15}
2	1.332 (0.0629)	24.23 (1.145)	0.0750 (0.00338)	502	0.882×10^{-15}
3	1.276 (0.0603)	25.93 (1.225)	0.0738 (0.00333)	370	1.144×10^{-15}
Mean $\pm$ SD	1.432 ± 0.223 (0.0676 $\pm$ 0.0105)	24.15 ± 1.82 (1.141 $\pm$ 0.086)	0.0751 ± 0.0013 (0.00338 $\pm$ 0.00005)	390 ± 103	$1.301 \pm 0.515 \times 10^{-15}$

Table 2

Variable correlations for model validation cultures

The p values for HFC speed represent the results of a one-way ANOVA between the four HFC flow rates, while p values for all other independent variables represent the slope of a linear regression, with correlation r^2 , being significantly non-zero. Bolded and italicized values represent $p < 0.05$ and $r^2 > 0.33$, while italicized values represent $p < 0.10$ and $r^2 > 0.24$

	p				r^2		
	HFC speed	Rat weight	Lung weight	Prep time	Rat weight	Lung weight	Prep time
Equilibrium pO ₂	<i>0.0005</i>	0.4778	0.5811	0.5181	0.0516	0.0315	0.0429
O ₂ consumption rate	0.9429	<i>0.0511</i>	<i>0.0263</i>	0.4258	<i>0.3293</i>	<i>0.4041</i>	0.0645
Glucose	0.8064	<i>0.0227</i>	0.1657	0.4645	<i>0.4200</i>	0.1827	0.0547
Lactate	0.7543	0.1177	<i>0.0477</i>	0.3571	0.2267	<i>0.3374</i>	0.0853
Y _{1/g}	0.3162	0.1522	0.7632	0.8725	0.1937	0.0095	0.0027
Total cell count	0.5723	0.5472	0.2124	0.2483	0.0374	0.1507	0.1307
Single cell O ₂ consumption rate	0.9878	<i>0.0991</i>	0.1091	0.2440	<i>0.2484</i>	0.2362	0.1329
Single cell glucose	0.9519	<i>0.0566</i>	0.2230	0.2719	<i>0.3172</i>	0.1444	0.1191
Single cell lactate	0.9893	0.1511	0.1011	0.1662	0.1947	<i>0.2458</i>	0.1823
% PCNA	0.2292	0.7572	0.7522	0.1646	0.0100	0.0104	0.1836
% TUNEL	0.1006	0.1538	0.8739	<i>0.0153</i>	0.1923	0.0026	<i>0.4605</i>