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Hardware Article

A three-zone hypoxia chamber capable of regulating unique oxygen and carbon dioxide partial pressures simultaneously

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ABSTRACT

Oxygen is a vital but often overlooked variable in tissue culture experiments. Physiologically relevant oxygen tensions range from partial pressures of 100 mmHg at the alveolar-capillary interface in the lung to less than 7.6 mmHg in the hypoxic regions of solid tumors. These values are markedly lower than the partial oxygen pressure of ambient air, which is standard experimental practice. Physiologically relevant culture environments are needed to better predict cellular and tissue-level responses to drugs or potential toxins. Three commonly used methods to regulate in vitro oxygen tension involve placing cells in 1) a hypoxia chamber, 2) setups that rely on mass transport-limited microenvironments, and 3) microfabricated devices. Hypoxia chambers have the lowest barrier to entry, as they do not require laboratories to change their tissue culture setups. Here, we present a gas-regulation system for a three-zone hypoxia chamber. Each zone can maintain independent environments, with partial pressure compositions of 1–21 % O_2 and 1–10 % CO_2. The design incorporates small-scale fabrication techniques (e.g., laser cutting and 3D printing) and off-the-shelf electronic components for simple assembly. The hypoxia chambers are significantly lower in cost than the commercial counterparts: $1,400 for the control system or $4,100 for a complete three-zone chamber system.

Specifications table

Hardware name	3-zone hypoxia chamber with independent control of oxygen and carbon dioxide partial pressure
Subject area	• Chemistry and biochemistry • Medical (e.g., pharmaceutical science) • Biological sciences (e.g., microbiology and biochemistry) • Educational tools and open-source alternatives to existing infrastructure • General
Hardware type	• Measuring physical properties and in-lab sensors • Biological sample handling and preparation
Closest commercial analog	Hypoxia Chamber

(*continued on next page*)

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(*continued*)

Hardware name	3-zone hypoxia chamber with independent control of oxygen and carbon dioxide partial pressure
Open source license	Creative Commons Attribution-ShareAlike license
Cost of hardware	$4,100 USD
Source file repository	Mendeley Data: http://dx.https://doi.org/10.17632/xm3njtmpmd.1

1. Hardware in context

Under standard tissue culture practices, cells are maintained in a mixture of 95 % ambient air and 5 % CO_2. This partial carbon dioxide pressure ($P_{CO2} = 38$ mmHg) maintains the buffer capacity of the culture medium, while the partial oxygen pressure ($P_{O2} = 150$ mmHg at sea level) maintains oxidative phosphorylation processes.

Oxygen is a vital but often overlooked variable in tissue culture experiments, as the P_{O2} values found in vivo are tissue-dependent and significantly lower than ambient air. The average oxygenation at the alveolar-capillary interface ($P_{O2} \approx 100$ mmHg) [1] drops substantially in blood reaching the capillaries of healthy breast tissue ($P_{O2} \approx 65$ mmHg) [2]. Spatially defined oxygen gradients maintain cellular function along each liver sinusoid. Blood entering the sinusoid through the portal triad is $PO_2 = 45 – 50$ mmHg, and blood exiting the sinusoid through the hepatic vein is $P_{O2} = 15 – 20$ mmHg [3]. In humans, the median value under the liver capsule of 12 donors pre-resection was $P_{O2} = 30.7$ mmHg [4]. This gradient regulates xenobiotic metabolism through hepatocyte post-differentiation and liver zonation.

Oxygen gradients result in poorly vascularized solid tumors and spheroid models because oxygen consumption outpaces its diffusion [5–7]. Hypoxia, or the inadequate supply of oxygen needed to maintain ATP production through the electron transport chain, occurs in 90 % of solid tumors [8]. The extent of hypoxia depends on tumor diameter and vascularization and is evaluated with molecular markers such as hypoxia-inducible factors [9,10]. These transcription factors reprogram the cell to survive in these oxygen-poor environments. Another consequence of hypoxia is the increased concentration of reactive oxygen species [11]. In cancer, the extent of hypoxia is linked with aggressiveness and phenotypes such as increased invasiveness, drug resistance, and mortality [12–14].

There is an increasing focus on conducting in vitro studies at physiologically representative environments, including evaluating potential chemotherapeutics in the hypoxic conditions found in tumors (e.g., $P_{O2} = 7.6$ mmHg or 1 % O_2). Three commonly employed strategies for incorporating physiologically relevant oxygen partial pressures include 1) hypoxia chambers, 2) culture setups that rely on mass transport-limited microenvironments, and 3) microfabricated devices. Hypoxia chambers maintain a defined P_{O2} value through external gas tanks and electronically controlled feedback mechanisms. Much like the tissue culture incubators, cells in these

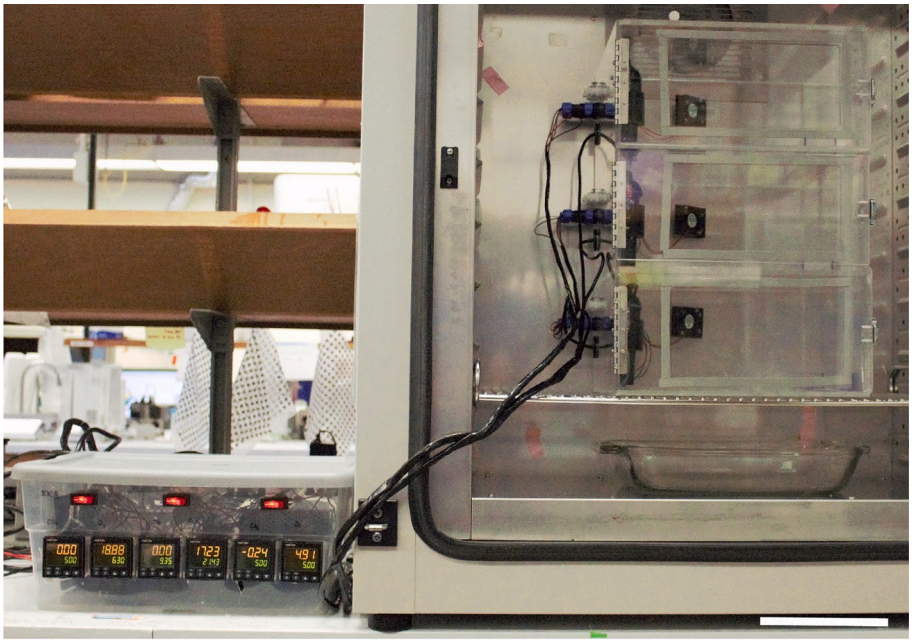

Fig. 1. Fully assembled three-zone hypoxia chamber in a standard culture incubator and the associated control box. The scale bar represents 150 mm.

chambers are maintained at 5 % CO_2 and a defined temperature (e.g., 37 °C). Glovebox-like chambers are also available, manipulating cells under reduced oxygen partial pressures without returning them to the ambient. We have used hypoxia chambers to demonstrate the importance of oxygen in estrogen signaling in breast cancer cell lines and the regulation of cytochrome P450 enzyme activity in hepatocytes [15–17].

Mass transport-limited setups that confine the exchange of fresh medium to a fraction of the cells in culture result in hypoxic microenvironments. Reproducibility generating and maintaining these oxygen gradients can prove experimentally challenging and are best for evaluating the dynamics of cellular responses when sub-populations are exposed to different partial pressures of oxygen. Spheroids are a well-characterized example of such a setup [5]. Whitesides first described an alternative to generating spheroid-like structures with cell-laden paper scaffolds [18]. An oxygen gradient is formed when these scaffolds are stacked and placed in a holder that restricts medium exchange. We confirmed the oxygen gradients in these stacks with biochemical assays, cell-based readouts, and a culture-compatible optode [19–21]. Microfabricated devices place cells in micron-sized channels. The large surface area-to-volume ratios of these channels allow for the rapid exchange of gases. These devices can impose spatially and temporally defined oxygen gradients on monolayer or 3D cell culture formats. The gradients rely on oxygen-permeable materials, chemical reactions that produce or remove oxygen species from the culture medium, or a supply of pre-sparged medium [22–25]. We showed that imparting an oxygen gradient on small numbers of cells suspended in an extracellular matrix is straightforward with gas-permeable materials and laser-cut cast acrylic components [26].

While each of the methods described above can maintain cells at a defined P_{O2}, hypoxia chambers have the lowest barrier to entry for tissue culture laboratories. A hypoxia chamber can accommodate monolayer or 3D cultures but costs approximately $15,000 for a 0.16 m^3 space [27]. Here, we describe the fabrication and validation of a three-zone system capable of generating and maintaining three distinct P_{CO2} and P_{O2} combinations simultaneously: 1 – 10 % CO_2 and 1 – 20 % O_2. A CO_2 gas tank was used to maintain a preset P_{CO2} value of 5 %, and a N_2 gas tank was used to displace oxygen from the chambers to maintain a preset P_{O2} value. Fig. 1 is a photograph of the assembled hypoxia chamber, which costs approximately $4,100 for three 0.013 m^3 spaces. The three-zone system chamber fits directly into an incubator, allowing for temperature control. We chose the three-zone setup because it allows for more than one culture condition to be evaluated in parallel, alleviating biological variation from comparing different cell passage numbers.

2. Hardware description

The three-zone hypoxia chamber consisted of an acrylic container divided into three compartments of equivalent volume ($1.25x10^{-2}$ m^3), each independently controlled and capable of maintaining defined P_{O2} and P_{CO2} setpoints. Acrylic was used because it can be processed with a laser cut and solvent bonded with non-whitening acrylic cement. The chamber(s) can be prepared with any material. However, the assembly process should not result in an air-tight unit, as the chambers rely on gas displacement to regulate the gas composition.

Each chamber was connected to an electronic control unit and external gas tanks with quick-connect ports. The chamber was placed in a standard laboratory incubator to minimize the electronics needed to maintain the unit's temperature. The humidity was maintained with large-surface area water pans. The same method of humidification is used in commercial tissue culture incubators.

The gas-regulation system relied on a non-dispersive infrared CO_2 sensor and an electrochemical O_2 sensor. Both sensors were attached to a Red Lion Control Unit. These units are standard PID controllers capable of real-time monitoring and adjusting partial gas pressures based on error minimization algorithms. The control units allowed gas partial pressures to be set and monitored without disturbing the contents of the box. They also eliminated the need for an external computer or control interface. The system was constructed to provide the experimental flexibility needed to maintain cells from the same passage or patient sample at different oxygen tensions in parallel while reducing the cost needed for a commercial system. A single chamber with a volume of 0.013 m^3 costs approximately $1,300. A three-zone chamber with the same overall volume divided amongst three equally sized compartments costs roughly $4,100.

Hardware Overview:

- A hypoxia chamber capable of maintaining cells at desired partial pressures of O_2 and CO_2 for prolonged culture periods of 72 + h.
- Three separate chambers enable three experimental variables to be run in parallel.
- Easy-to-operate user interface.
- Significantly less expensive than commercial chambers, which maintain cells at a single partial pressure of O_2 and CO_2.

3. Design files summary

The components of the three-zone hypoxia chamber were constructed with parts prepared by laser cutting or 3D printing. The 3D-

printed parts were prepared using fused deposition modeling (FDM) or stereolithographic polymerization (SLA) methods.

Design file name	File type	Open-source license	Location of the file
Water Pan [a]	STL	CC BY 4.0	All files in this table are available at:http://dx.https://doi.org/10.17632/xm3njtmpmd.1.
CO_2 Sensor Mount [b]	STL	CC BY 4.0	
O_2 Sensor Mount [b]	STL	CC BY 4.0	
O_2 Sensor Wire Nut [b]	STL	CC BY 4.0	
Fan Mount [b]	STL	CC BY 4.0	
Latch Spacer [b]	STL	CC BY 4.0	
Vernier Protoboard Adapter Mount [b]	STL	CC BY 4.0	
Acrylic Chambers [c]	Adobe Illustrator	CC BY 4.0	

[a] Components were 3D printed on a Form Labs 3B + printer with Form Labs BioMed Clear resin. Each component was cleaned with the Form Wash twice (fresh IPA, 20 min) and cured in the Form Cure (1 h, 60 °C).

[b] Components were 3D printed from ColorFabb_XT Copolyester filament on a Qidi Plus FDM 3D printer with a build plate temperature of 80 °C, a nozzle temperature of 265 °C, and a speed of 10 mm/sec without cooling.

[c] Components were cut from ¼"-thick clear cast acrylic on a Universal Laser Systems laser cutter at 2 % speed and 100 % power.

4. Bill of materials summary

Designator	Component	Number	Cost per unit (USD)	Total cost (USD)	Source of materials	Catalog number
Control Unit	Red Lion Controls PXU21A20 [a]	6	214.21	1285.26	Allied Electronics	70,425,302
Control Unit	MEAN WELL RD-65A	1	28.15	28.15	Allied Electronics	70,406,962
Control Unit	Replacement Power Cord (6-foot, black)	1	8.79	8.79	Amazon	B072BYGKZZ
Control Unit	15A 250 V Rocker Switch Power Socket Inlet Module Plug	1	7.99	7.99	Amazon	B07RRY5MYZ
Control Unit	Rocker Switch Lighted On/Off 120 V	3	9.99	29.97	Amazon	B0821NF28Y
Control Unit	KEYFANCLUB 2-Pack 120 mm Fan DC 12 V Computer Fan	1	13.99	13.99	Amazon	B08BLTW8RX
Control Unit	Lever Wire Connectors, 3 Port Lever Wire Nut Suitable for Multiple Types of Wires	1	11.99	11.99	Amazon	B09PRMVNGY
Control Unit	10 Pieces Tie Rod Nut Wire Connector 8-Port PCT-218 Classified Conductor Compact Terminal Cable Connect	1	8.99	8.99	Amazon	B08ZSFXWN7
Control Unit	K30 10 % CO_2 Sensor	3	149.00	447.00	CO2 Meter	SE-0118
Control Unit	ASCO™ 8262H020DC12/DCD	6	87.00	522.00	Emerson	8262H020DC12/DCD
Control Unit	Firm Polyurethane Tubing for Air and Water 1/16″ ID, 1/8″ OD	1	14.00	14.00	McMaster-Carr	5648 K22
Control Unit	Yor-Lok Fitting for Steel Tubing Straight Adapter for 1/8″ Tube OD x 1/4 NPT Male	6	11.98	71.88	McMaster-Carr	5929 K34
Control Unit	316 Stainless Steel Pan Head Phillips Screw, 4–40 Thread Size, 1/4″ Long (Pack of 50)	3	3.73	11.11	McMaster-Carr	91735A102
Control Unit	High-Pressure Brass Pipe Fitting, Straight Adapter with Hex Body, 1/4 NPT Female x Male	6	2.66	15.96	McMaster-Carr	50785 K27
Control Unit	Wire Assortment, 22 Gauge, Five 100-feet Spools	1	92.17	92.17	McMaster-Carr	9948 T31
Control Unit	Stranded Wire: 300 V AC, 18 Gauge	1	5.66	5.66	McMaster-Carr	8054 T15
Control Unit	Waterproof 3 Contact Cable Plug & Panel Jack	18	4.95	89.10	MPJA.com	35,802 PL
Control Unit	Sensata – Crydom DC60S3	6	29.70	29.70	Newark	37 M2360
Control Unit	Vernier Analog Protoboard Adapter	3	12.00	36.00	Vernier	BTA-ELV
Control Unit	Vernier Analog Extension Cable	3	12.00	36.00	Vernier	EXT-BTA
Control Unit	O_2 Gas Sensor	3	210.00	630.00	Vernier	O2-BTA
Control Unit	16Q storage box	1	7.48	7.48	Home Depot	073,149,644,688
Control Unit	316 Stainless Steel Pan Head Phillips 4–40 Thread Size, 7/8″ Long	1	9.19	9.19	McMaster-Carr	91735A115
Chamber	2-Pack 40 mm x 10 mm DC 12 V Brushless Cooling Fan	2	13.98	27.96	Amazon	B0711FVD48
Chamber	10-ft x 3/4-in Gray Window Seal Foam	1	4.48	4.48	Lowes	66,682
Chamber	Gorilla Max Strength Clear Construction Adhesive	1	7.96	7.96	Amazon	B0916KZ598
Chamber	Plastic Quick-Disconnect Tube Coupling for Air and Water Plug, 1/8 Coupling Size, for 1/16″ Barbed Tube ID	6	3.92	23.52	McMaster-Carr	5012 K24

(continued on next page)

(*continued*)

Designator	Component	Number	Cost per unit (USD)	Total cost (USD)	Source of materials	Catalog number
Chamber	Plastic Quick-Disconnect Tube Coupling for Air and Water Thru-Wall Socket, 1/8 Size, Shut-Off, 1/16″ Barbed Tube	6	21.86	130.08	McMaster-Carr	5012 K33
Chamber	Tight-Hold Draw Latch Screw on, Steel, 2–3/4″ Long x 1–1/2″ Wide	3	5.80	17.40	McMaster-Carr	1734A45
Chamber	Clear Scratch- and UV-Resistant Cast Acrylic Sheet, 12″ x 24″ x 1/4″	5	31.55	157.75	McMaster-Carr	8560 K355
Chamber	Clear Scratch- and UV-Resistant Cast Acrylic Sheet, 12″ x 12″ x 1/4″	4	18.38	73.52	McMaster-Carr	8560 K354
Chamber	Surface-Mount Hinge with Holes	3	27.88	83.64	McMaster-Carr	1586A31
Chamber	Non-whitening Cement for Acrylic	1	11.82	11.82	McMaster-Carr	7517A1

4.1. Sensors and electronic control system design

Commercially available sensors regulated the P_{O2} and P_{CO2} levels in each zone of the chamber. The oxygen sensor relied on a two-electrode electrochemical cell, which reduced oxygen molecules entering the electrolyte at a gold cathode. The carbon dioxide sensor relied on transmission measurements of a CO_2 laser passing through a region with a defined path length where ambient air can freely exchange. The composition of the chamber was monitored in real time by the sensors and adjusted to the user-defined gas composition with a proportional-integral-derivative (PID) controller. The PID system calculated the gas composition in the chamber from the voltage output of the two gas sensors. If the tension was below the setpoint, a defined volume of gas entered the chamber via a PID-activated solenoid valve connected to external gas tanks. This closed-loop system used a combination of three coefficients to determine the appropriate volume of gas to introduce to the chamber. The iterative process of the defined partial pressure is 1) adding an excess of gas, 2) waiting for the chamber to recover, 3) adding a smaller volume of gas, and 4) waiting for the chamber to recover. This method of reaching the desired gas composition is why the hypoxia chambers require an equilibration period before use.

4.2. Hypoxia chamber components

Acrylic was ideal for constructing the chamber because it is gas-impermeable, lightweight, and optically transparent. The transparency allowed us to visually inspect experiments, ensuring the water pan was filled (needed to maintain an appropriate humidity and CO_2 concentration) and the cells had an adequate volume of culture medium. Quick-connect ports for the electronics and gas lines ensured the chamber could be easily assembled and disassembled. These ports also prevented the unwanted diffusion of gas from the chamber. The weather-stripping seals that lined the door were gas permeable, allowing gas to escape from the chamber if it over-pressurized. The seals are not inherently sterile, but we did not observe any bacterial or fungal contamination over months of use. The spring latches on the door ensured a constant pressure was applied to weather stripping.

The 3D-printed components used cell culture-compatible resins. The ColorFabb_XT filament was extrusion printed and sterilized with ethanol. We determined that the extrusion-printed parts were not toxic to monolayers of cells maintained in standard culture medium and culture conditions. The Biomed Clear resin was lithographically printed. This material is commercially formulated and certified as USP class VI biocompatible. These components are water-impermeable.

5. Build instructions

5.1. Three-zone chamber

1. Laser-cut the chamber panels from ¼“ acrylic sheets using the files provided. Fig. 2 shows the schematics of each panel.

Note. If a laser cutter is unavailable, the panels can be cut from the acrylic sheets using other fabrication methods (e.g., a CNC router or saw).

2. Using a router with a 6.35 mm straight cut bit, remove half of the thickness (3.18 mm) in a 6.35 mm wide channel from the acrylic panels (Fig. 2). The channels follow the outside edge of each side wall. Two channels are also cut across the center of the board 140 mm from the edge channels.

Note. This step can be skipped if a router is not available. However, it will make the assembly process more difficult. The raster function of most laser cutters can be used but will significantly increase the cut time for each panel.

3. Using a 4–40 tap, thread the 2 mm laser-cut holes in the left, back, and door panels.

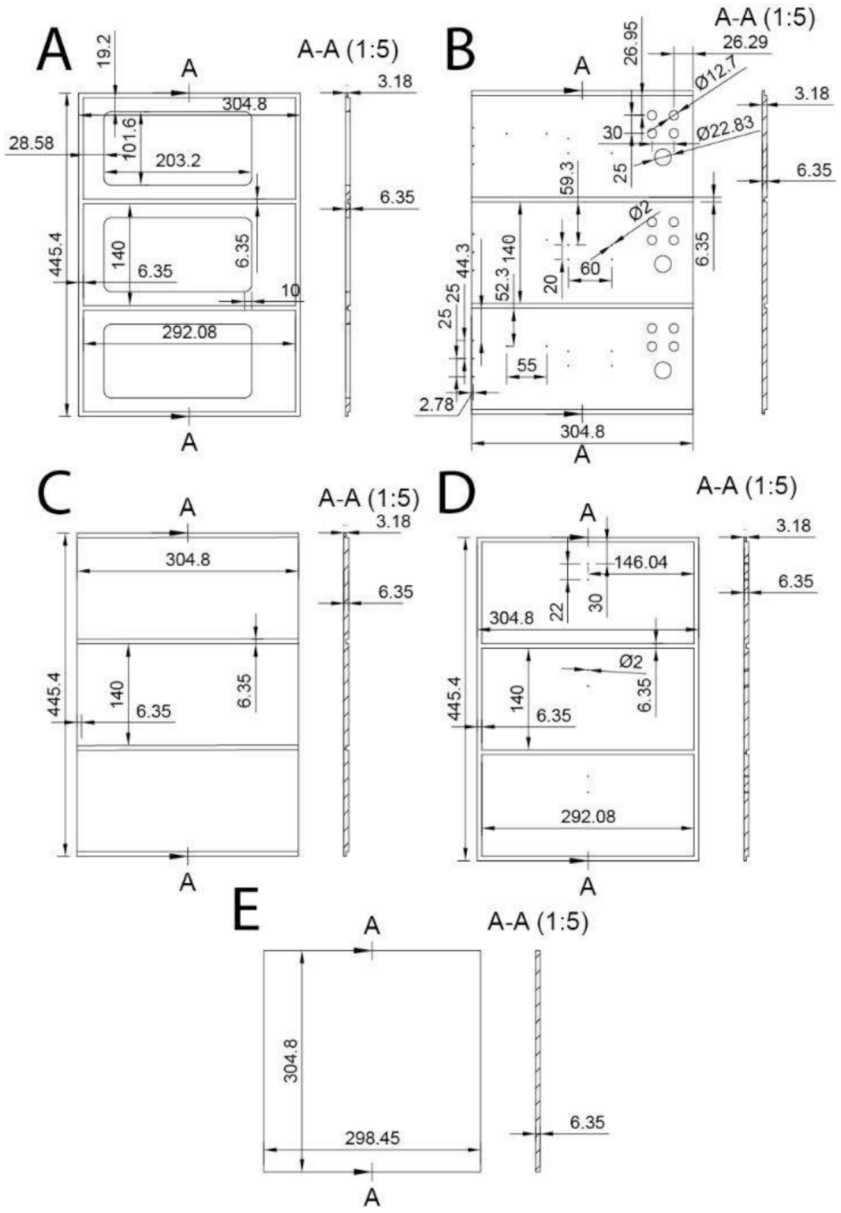

Fig. 2. Schematics for the A) front panel, B) left-side panel, C) right-side panel, D) back panel, and E) shelves for the three-zone hypoxia chamber. All values are millimeters. Laser cutter files are available in.AI and.EPS file formats at Mendeley data.

4. Remove the protective paper layer from the routed side of each acrylic panel and shelf panel.
5. Using non-whitening acrylic cement, glue each of the panels together, minimizing any gaps that may form between the panels.

Note. We used the shelf panels and clamps to ensure the side panels met at a right angle.

6. Allow the glue to harden overnight before continuing. The glue will set within 30 min and strengthen with time.
7. Using the 6 mm 4–40 screws, attach the hinge to the front panel of the chamber and the door panels.
8. Remove the remaining protective paper from the acrylic components.
9. Place weather stripping around each door frame, approximately 3 mm from the opening (Fig. 3**A**).
10. Once the door panels are attached, mark the screw hole locations of the Latch Spacer on each door panel (Fig. 3**B**).
11. Using a 2 mm drill bit, drill the holes into each door (approximately 10 mm) before threading the holes with the 4–40 tap.

Note. If the tap goes too far into the door, the threads can become too large for the screw. If this occurs, a small amount of glue can

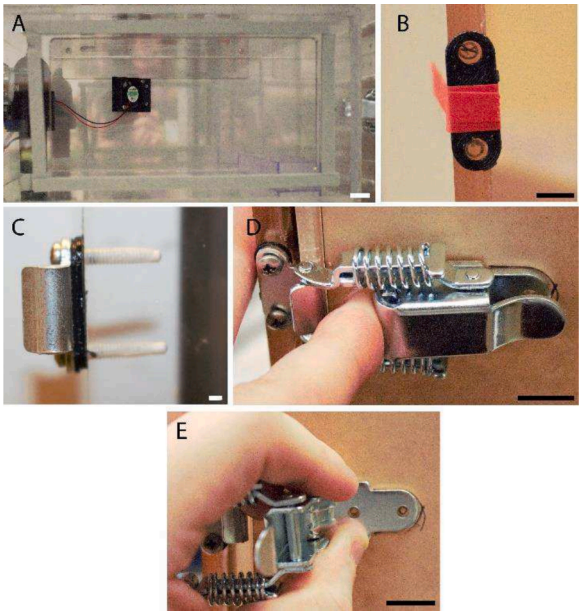

Fig. 3. Photograph of the steps needed to mount the doors on each hypoxia chamber. A) Once the chamber is assembled, place the weather stripping approximately 3 mm from the opening. B) Using the Latch Spacer to mark the screw holes on the edge of the door. C) Attach the Latch hook with a Latch Spacer to the door. D) Trace the location of the Latch on the hypoxia chamber. E) Mark the screw holes for the Latch using the trace. The scale bars represent A) 30 mm, B) 6.35 mm, C) 2 mm, D) 16 mm, and E) 14 mm.

be applied to the screw to hold it in place.

12. Attach a latch hook to each door, with the Latch Spacer between the door and the hook (Fig. 3**C**).
13. While placing a small amount of pressure on the door against the weather stripping, hold the Latch in place and trace the bottom part of the Latch for each chamber (Fig. 3**D**).
14. Open the door, hold the Latch in place according to the trace, and mark the screw hole locations for each chamber (Fig. 3**E**).
15. Using a 2 mm drill bit, drill out the holes before threading the holes with the 4–40 tap.
16. Place the oxygen sensor in the 3D-printed O_2 Sensor Mount and screw the holder into the four holes in the center of the left chamber wall inside each chamber (Fig. 4**A**).

Note. The cord for the sensor will need to be slightly bent to fit in the chamber. Ensure there is a gap (at least 1.5 mm) between the bottom of the sensor and the chamber floor. The sensor will not function properly if it is flush against the acrylic.

17. Feed the wire for the O_2 Sensor through the large hole on the left panel. Glue two of the O_2 Sensor Wire Nuts around the wire and glue the lip to the outside of the chamber (Fig. 4**B**).
18. Cut approximately 150 mm of nine 22-gauge wires and solder three to each CO_2 Sensor according to Fig. 4C.
19. Solder the free end of the three wires to the 3-Contact Cable Jack (Cable Jack) through one of the remaining holes directly above the CO_2 sensor wire in each chamber, ensuring the nut is threaded through the wires before soldering.

Note. Record the location of each of the three wires on the Cable Jack.

20. Feed the Cable Jack through the hole and secure the nut.
21. Mount the CO_2 Sensor Mount to the remaining holes on the left panel and attach the CO_2 Sensor (Fig. 4**B**).
22. Strip the wires on a 40 mm Fan and solder the wires to the Cable Jack through the remaining hole directly above the O_2 sensor wire, ensuring the nut is threaded through the wires before soldering.

Note. Record the location of each of the two wires on the Cable Jack.

23. Feed the Cable Jack through the hole and secure the nut.
24. Attach the fan to the Fan Mount using the 22 mm screws before mounting the Fan Mount to the holes on the back panel.

Note. The Fan Mount does not need to be threaded; the screws can be screwed into the plastic if a small amount of pressure is applied.

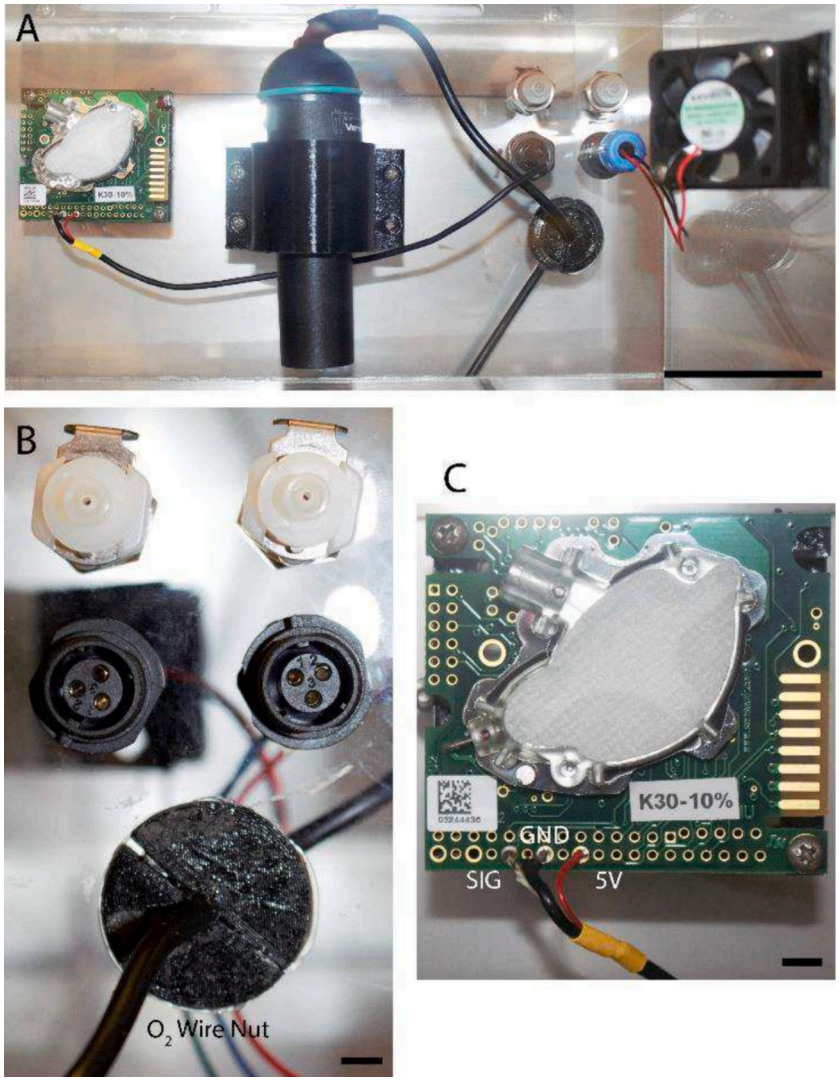

Fig. 4. Photographs of the assembled sensors, fan, and corresponding quick-connect ports in the chamber. A) The mounted CO_2 sensor (left) and O_2 sensor in its 3D-printed holder (right). B) The quick connect gas ports (top, white), 3-contact cable jacks for the CO_2 sensor and fan (middle), and the O_2 sensor wire sealed to the chamber with 3D-printed adaptors. C) The locations of the three wires connected to the CO_2 sensor, including the 5 V source (5 V, red), ground (GND, black), and signal (SIG, white) wires. The scale bars represent A) 57 mm, B) 5.7 mm, and C) 6 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

25. Attach the Quick-Connect Through Wall Sockets to the two remaining holes on the left panel (Fig. 4**B**).

5.2. Control Unit

1. Using the pin locations from Steps 19 and 22 in the Chamber Assembly Instructions section, prepare wire extenders to attach the control box to the hypoxia chambers. Repeat this step 3 times. Fig. 5**A** is a photo of our completed extension wiring harness for a three-chamber system.
   a. Cut 5 wires per chamber, approximately 3 m long or the estimated distance from the hypoxia chamber to the control box.
   b. Solder one wire to each of the complementary parts to the 3-Contact Cable Plug (Cable Plug) following the pin locations recorded in Step 19 in the Chamber Assembly Instructions section.
   c. Using the remaining two wires, repeat the process from step b using the recorded pin locations from step 22 in the Chamber Assembly Instructions section.

Note. After completing the wire extensions, we wrapped the wire bundles in electrical tape for easier wire management. Bundling the wires is not required.

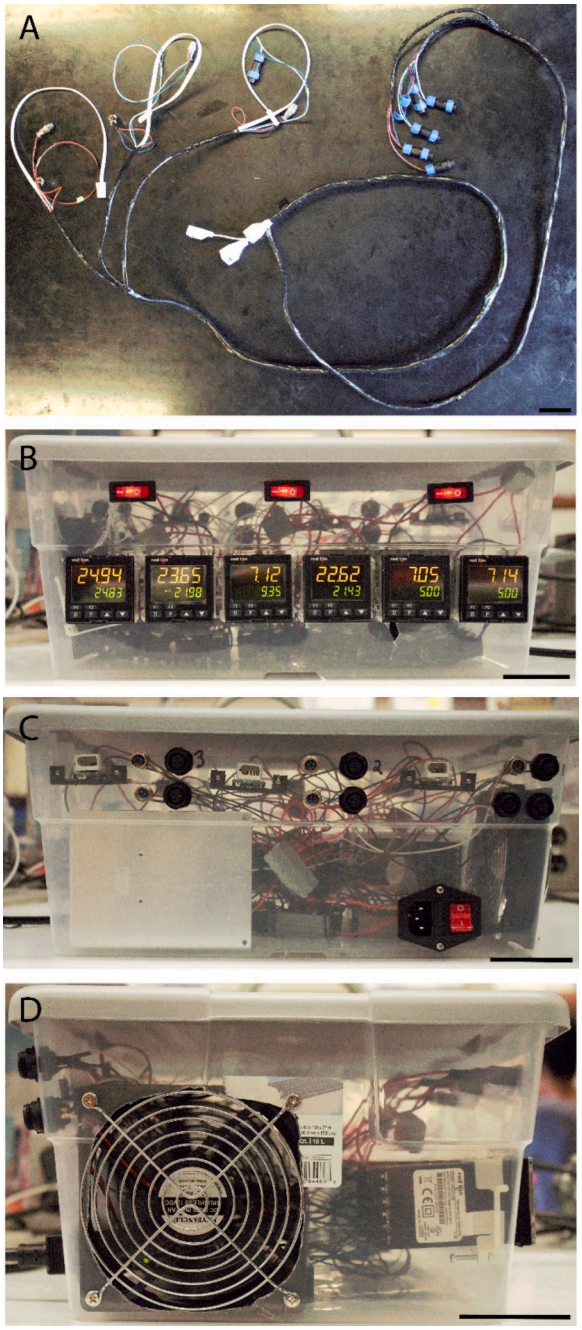

Fig. 5. Photographs of the control box. A) A wire extension harness for one zone of the three-zone hypoxia chamber. B) The front of the assembled control box contains the Red Lion Control Units and the rocker switches. C) The back of the assembled control box, which contains the Vernier protoboard adapter (white), the main power rocker switch, and the through-wall 3-Contact Cable Jacks (black and chrome). D) The side of the assembled control box contains a mounted fan to cool the interior electronics. The scale bars represent A) 40 mm, B) 48 mm, C) 60 mm, and D) 58 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. On the front of the Control Box, trace the location of the Red Lion Control Units (6 units) and the Rocker Switches (3 total). The placement of these components is shown in Fig. 5**B**. On the back of the control box, trace the location of the Power Socket and the Vernier Protoboard Adapters (3 in total). The placement of these components is shown in Fig. 5**C**. On the left-hand side of the control box, trace the location of the 120 mm x 120 mm Fan. The placement of the fan is shown in Fig. 5**D**.

   Note. We used a plastic tote as the housing for our control unit. If a metal box is used, ensure it is grounded appropriately (not

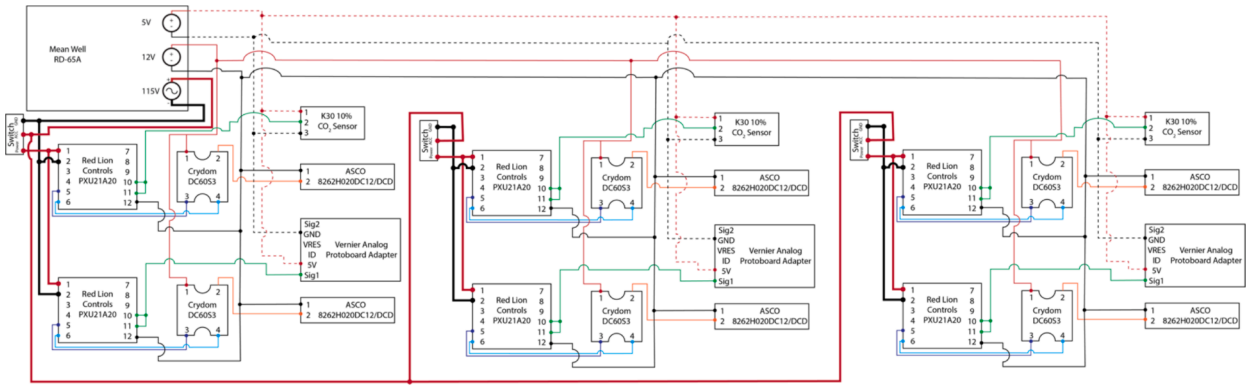

Fig. 6. Wiring diagram for the three-zone hypoxia chamber. The control system uses a two-voltage output DC power supply, 12 V (solenoid valves and fans), and 5 V (CO_2 and O_2 sensors). The Red Lion Control Units are powered by the 115 V AC from the outlet. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Photographs of gas-delivery mechanism. A) A solenoid valve and the associated wiring scheme, including the signal (SIG, 12 V from the relay) and the ground (GND). B) The solenoid valve is attached to the gas tank regulator using a straight brass adaptor. C) The assembled polyurethane tubing is connected to the solenoid valve with the Yor-lok fitting and is connected to the chamber with the quick-disconnect coupling. The scale bars represent A) 17.5 mm, B) 21 mm, and C) 6.35 mm.

described in this work).

3. Cut out the traced outlines from the previous step for each component from the control box.
4. Dry fit each component in the control box and mark the locations of the additional screw holes for each component in the control box.
5. Using a 19 mm drill bit, drill holes for the through-wall 3-Contact Cable Jacks (12 in total) in the back of the control box (Fig. 5**C**).

Note. We used two different Cable Jacks in this Step due to supplies in our lab. A single mount type can be used.

6. Using the pin locations from Step 1b and 1c, solder three or two wires (approximately 0.2 m each), respectively, to the through-wall cable jacks (e.g., three wires for the CO_2 Sensors and two wires for the fans).
7. Solder two wires at approximately 0.2 m to two more Cable Jacks. These wires will be connected to the Solenoid Valves (Step 11).
8. Using a 2 mm drill bit, drill out the marked locations for each of the screw holes and thread the holes using a 4–40 tap.
9. Place each component in the control box and fix their locations using the 6 mm 4–40 screws.
10. Attach the components using the wiring diagram in Fig. 6.

Note. We used Multi-Port Wire Nuts to simplify the system wiring and decrease the number of wires connected to the power supply.

11. Using the pin locations from Step 6, solder the wires from the Solenoid Valves to the corresponding pins (Fig. 7**A**).

Note. The wires on the solenoid valves may need to be extended based on the distance between the gas tanks and the control box. We used an 18-gauge wire to extend these wires when required.

12. Connect the Solenoid Valve to the gas tank using a Straight Adaptor Brass Pipe Fitting (Fig. 7**B**).
13. Connect the Yor-Lok Fitting to the Solenoid and cut the polyurethane tubing (approximately 1.5 m or the approximate distance from the gas tanks to hypoxia chambers).
14. Connect one side of the polyurethane tubing to the Yor-Lok Fitting and the other to the Quick-Disconnect Tube Fitting (Fig. 7**C**).
15. Place the control box in a designated location. It will remain fixed throughout the culture experiment. Connect the control box to the hypoxia chamber with the connecting wires from Step 1 (the fan and CO_2 sensor) or the Vernier O_2 sensor.

6. Operation instructions

6.1. Calibration instructions

1. Place the assembled three-zone hypoxia chamber inside an incubator, set it to the desired temperature (37 °C), and maintain it for at least 12 h before calibration.

Note. Each sensor must be calibrated individually due to slight differences in voltage output.

2. If the Red Lion Control Units were used previously, perform a factory reset using the following instructions. If the control units are new, then skip this step.
   a. Press and hold the P button until it displays TUNE.
   b. Press the P button three times. Press the up arrow until you reach 9-F5.
   c. Press the P button one time. Press the up arrow to set the value on the screen to 66.
   d. Press the P button one time. Power cycle to complete the reset.
3. Press and hold the P button until it displays TUNE.
4. Press the P button until it displays CNFP. Press the arrow buttons until you reach I-IN.
5. Press the P button one time. Press the arrow buttons until you reach 5u.
6. Press the P button until it displays DCPT. Press the down arrow until you reach 0.00 (two decimal places, the initial value will be 0.0).
7. Press the P button until you see DSP1. Use arrow buttons to set the value to 0.00 for the CO_2 and O_2 sensors.
8. Press the P button once until it reads DSP2. Use arrow buttons to set the values to 5.00 for the CO_2 sensor and 4.80 for the O_2 sensor.
9. Press the P button until it reads END.
10. Record O_2 voltage. Plug the sensor into the Vernier LabQuest3 Module and read the true oxygen tension (approximately 18–20 %, depending on the geographic location of the lab). Plug the O_2 sensor back into the control box.
11. Purge the chamber with N_2 gas (4.8 grade, Airgas NI UHP300) for approximately 5 min or until the voltage output on the Red Lion Control Units stabilizes. Record the voltage reading for the CO_2 and O_2 sensors once the values stabilize. Quickly plug the oxygen sensor into the Vernier LabQuest 3 Module and read the oxygen tension.

Note. We use a secondary CO_2 sensor from Vernier to get a more precise CO_2 tension in the chamber. This step is unnecessary if the assumed minimum voltage is 0 % CO_2.

12. Purge the chamber with CO_2 (USP Grade Medical Carbon Dioxide, Airgas CD USP50) for approximately 5 min and record CO_2 voltage. Record the CO_2 voltage.

Note. 10 % CO_2 is the maximum reading of this sensor.

13. Graph the voltage and tension values for each sensor. Determine the linear equation for each. Sample data is shown in Fig. 8.

Note. The response of the sensors is linear over the ranges we evaluate, allowing us to generate two-point curves.

14. Using the equations determined in the previous step, calculate the associated tensions for a voltage of 0 and 5 V for both sensors. These values will be the new inputs for DSP1 and DSP2, respectively. Replace the DSP1 and DSP2 values following steps 3 – 9.
15. Press and hold the P button until it displays CNCF. Use the arrow buttons until you reach 2-OP. Press the P button one time.
16. Input the settings from Table 1 into the control units for CO_2 and O_2 sensors. Press the P button between each value. Use the arrow buttons to reach the desired values.
17. Press the P button and use the arrows to set the following: PROP. 97, INTER. 150, DER. 25 for the CO_2 and O_2 sensors.

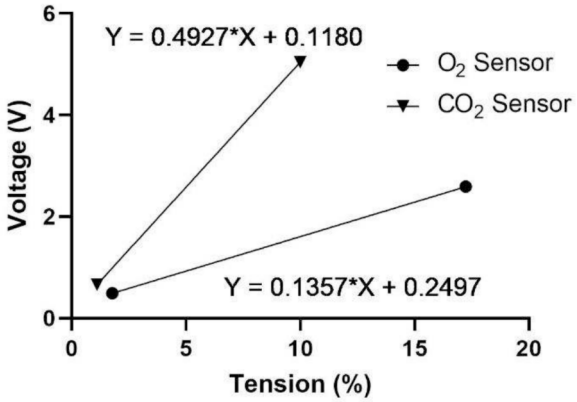

Fig. 8. Representative two-point voltage-to-tension relationships for the CO_2 and O_2 sensors used to calibrate the Red Lion Control Units.

Table 1
Red Lion Control Unit Settings for the CO_2 and O_2 sensors.

	CO_2	O_2
OPAC	RLR2	DID2
CTRL	PID	PID
STPL	SP	SP
CYC1	3.0	3.0
OPL1	0	0
OPIH	100	100
IFO1	0	N/A
CHYS	0.2	2
OP2L	N/A	0
OP2H	N/A	100

Note. For larger chambers such as tissue culture incubators, the settings for the CO_2 sensor are PROP. 7, INTER. 120, DER. 30.

18. Power cycle to save the changes to the Red Lion Control Unit.

6.2. Operation instructions

1. One day before the experiment, wipe down the inside of each chamber with 70 % ethanol and place the chamber in an incubator.
2. Connect the following:
   a. Gas lines to the quick connectors on each chamber.
   b. The CO_2 and O_2 sensor wires to the control box.
   c. The fan wires to the control box.
   d. The solenoid valve wires to the control box.
3. Ensure that there is sterile water (approximately 200 mL) in the water pans of each chamber.
4. Close the incubator door and allow the chambers to equilibrate overnight.
5. Turn on the main power of the control box using the toggle switch on the back of the control box.
6. Turn on the toggle switches for each chamber needed for the experiment.
7. Use the arrow buttons on the front of each Red Lion Control Unit to set the desired O_2 and CO_2 tensions.
8. Open the gas tanks.
9. Power cycle the Red Lion Control Units.
10. Allow the chambers to equilibrate to the desired gas mixture (i.e., partial pressures) for at least 1.5 h before use.

6.3. Maintenance instructions

1. Check the calibration of the O_2 and CO_2 sensors each month. Determine the slope of each two-point calibration curve and ensure the sensitivity of each is maintained.
2. Confirm the accuracy of each sensor with a known gas mixture, which can be prepared with a flow controller unit or specialty ordered from a gas tank supplier.
   a. If unable to accurately measure the gas composition inside the chamber, ensure the PID controller is programmed correctly.

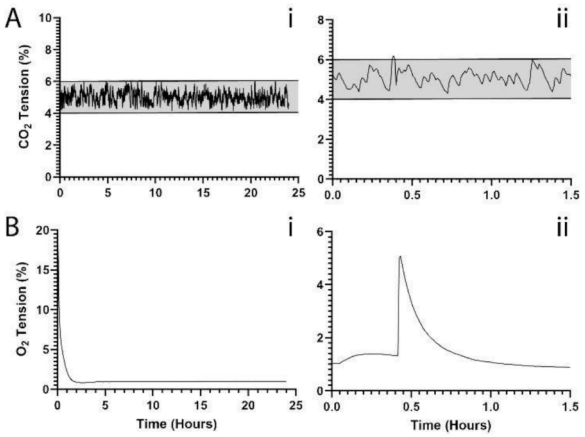

Fig. 9. Measured equilibration and recovery times required for the hypoxia chamber to reach a setpoint of A) $P_{CO2} = 5$ % and B) $P_{O2} = 1$ %. These data were collected with pre-calibrated Vernier CO_2 and O_2 Sensors, and the values were recorded on a Vernier LabQuest 3 Module. Partial pressure values were measured over i) a 24-hour period to determine equilibration times required to reach the setpoints from ambient tensions or ii) a 1.5-h period to determine recovery times when the chamber door was opened. The data graphed is a representative trial of the equilibration and recovery in the hypoxia chambers. These experiments were performed in a single chamber at room temperature (P_{CO2}) or 37 °C (P_{O2}).

3. Confirm the chamber can maintain a set O_2 and CO_2 partial pressure for 48 h.
   a. If unable to maintain the chamber environment, replace the weatherproofing seal on the door.
4. Place a beaker containing 25 mL of standard culture in the chamber, set the PID controller to maintain a 5 % CO_2 partial pressure environment, and measure the pH of the medium after 24 h.

7. Validation and characterization

Heat and Humidity. The hypoxia chambers rely on an incubator or lab-grade oven to maintain a temperature of 37 °C. Humidity in the chambers relies on passive evaporation of water from a 3D-printed water pan, which we fill with sterile water (i.e., deionized and autoclaved) the day before the experiment. The same mechanism of maintaining humidity is used in commercial tissue culture incubators.

P_{CO2} and P_{O2} Equilibration and Recovery. We used a Vernier LabQuest3 Module to record each chamber's equilibration and recovery times. This module records the P_{CO2} and P_{O2} values measured by a Vernier CO_2 Sensor (IR-based CO_2 sensor, approx. 30 sec) and a Vernier O_2 Sensor (electrochemical sensor, in the three-zone hypoxia chamber, approx. 1 sec). The P_{O2} measurements were performed at 37 °C. The P_{CO2} measurements were performed at room temperature because of the operating limitations of the CO_2 sensor. The noise associated with the P_{CO2} measurements is from the ± 20 % (or ± 1 % CO_2) of the Vernier CO_2 Sensor and is indicated by the shaded box on the graphs. The CO2Meter K30 CO_2 Sensor used in the chamber has an error of ± 3 % (or 0.2 % CO_2).

The equilibrium experiment measured the P_{CO2} *and* P_{O2} values for a 24-hour period after initiating the gas-regulation system with the following setpoints: $P_{CO2} = 5$ % to match standard culture conditions and $P_{O2} = 1$ % to match a commonly used hypoxia condition. The chamber reached the P_{CO2} setpoint of 5 % within 5 min of initiation (Fig. 9**Ai**). The chamber reached the P_{O2} setpoint (1 %) within ± 0.2 % error after 90 min and ± 0.01 % after 240 min (Fig. 9**Bi**). These data indicate that the chambers maintain the desired gas composition 1.5 h after initialization but require 4 h to reach a P_{O2} value within 0.01 % error. We have used this hypoxia system for 72-h experiments and observed similar trends in the ability to maintain the P_{CO2} and P_{O2} setpoint.

The recovery experiment measured the P_{CO2} *and* P_{O2} values for a 1.5-h period after the door to the chamber was opened. The chamber door was opened at T = 0.4 h and remained open for the time required to insert and remove another well plate. There was no measurable decrease in the P_{CO2} value when the door was opened (Fig. 9**Aii**). The P_{O2} value increased by 5.1 % and required 60 min to recover to the 1 % setpoint (Fig. 9**Bii**). These data indicate that the incubator door should remain closed throughout the experiment.

Support of cells at hypoxic conditions for prolonged periods. While the datasets for cell-based experiments are not included in this work, we have successfully maintained primary human hepatocytes [28], the bipotent progenitor human-derived HepaRG cell line that differentiates into biliary and hepatocyte phenotypes [17,28], and the human hepatoma HepG2 cell lines in these chambers [16,29]. In each of these studies, monolayers and scaffolds supporting 3D cell-laden gels were maintained in the three chambers simultaneously to compare the same passage or source of cells to normoxic (20 % O_2), periportal (11 % O_2), and perivenous (8 % O_2) conditions. The accuracy of the sensors in the chamber to different oxygen partial pressures was evaluated at the start and end of each experiment to ensure the cells were maintained at the correct set point. We observed no drift in the sensors when determining the calibration relationship (Fig. 8) or when measuring the partial pressure of a known gas mixture.

CRediT authorship contribution statement

Zachary R. Sitte: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Abel A. Miranda:** Writing – review & editing, Investigation. **Thomas J. DiProspero:** Conceptualization. **Matthew R. Lockett:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] M.C. Brahimi-Horn, J. Pouyssegur, Oxygen, a source of life and stress, FEBS J. 581 (2007) 3582–3591, https://doi.org/10.1016/j.febslet.2007.06.018.
[2] P. Vaupel, K. Schlenger, C. Knoop, M. Hockel, Oxygenation of human tumors: Evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements, Cancer Res. 51 (1991) 3316–3322.
[3] T. Kietzmann, Metabolic zonation of the liver: The oxygen gradient revisited, Redox Biol. 11 (2017) 622–630, https://doi.org/10.1016/j.redox.2017.01.012.
[4] A.J. Brooks, J. Eastwood, I.J. Beckingham, K.J. Girling, Liver tissue partial pressure of oxygen and carbon dioxide during partial hepatectomy, Br. J. Anaesth. 92 (2004) 735–737, https://doi.org/10.1093/bja/aeh112.
[5] F. Hirschhaeuser, H. Menne, C. Dittfeld, J. West, W. Mueller-Klieser, L.A. Kunz-Schughart, Multicellular tumor spheroids: An underestimated tool is catching up again, J. Biotechnol. 148 (2010) 3–15, https://doi.org/10.1016/j.jbiotec.2010.01.012.
[6] A. Gomes, L. Guillaume, D.R. Grimes, J. Fehrenbach, V. Lobjois, B. Ducommun, Oxygen partial pressure is a rate-limiting parameter for cell proliferation in 3D spheroids grown in physioxic culture condition, PLoS One. 11 (2016) e0161239.
[7] M. Hockel, P. Vaupel, Tumor hypoxia: Definitions and current clinical, biologic, and molecular aspects, J. Natl. Cancer Inst. 93 (2001) 266–276, https://doi.org/10.1093/jnci/93.4.266.
[8] I. Godet, Y.J. Shin, J.A. Ju, I.C. Ye, G. Wang, D.M. Gilkes, Fate-mapping post-hypoxic tumor cells reveals a ROS-resistant phenotype that promotes metastasis, Nat. Commun. 10 (2019) 4862, https://doi.org/10.1038/s41467-019-12412-1.
[9] N.R. Prabhakar, G.L. Semenza, Oxygen sensing and homeostasis, Physiol. 30 (2015) 340–348, https://doi.org/10.1152/physiol.00022.2015.
[10] L. Schito, G.L. Semenza, Hypoxia-inducible factors: Master regulators of cancer progression, *Trends*, Cancer. 2 (2016) 758–770, https://doi.org/10.1016/j.trecan.2016.10.016.
[11] L. Jagannathan, S. Cuddapah, M. Costa, Oxidative stress under ambient and physiological oxygen tension in tissue culture, Curr. Pharmacol. Rep. 2 (2016) 64–72, https://doi.org/10.1007/s40495-016-0050-5.
[12] D.C. Singleton, A. Macann, W.R. Wilson, Therapeutic targeting of the hypoxic tumour microenvironment, Nat. Rev. Clin. Oncol. 18 (2021) 751–772, https://doi.org/10.1038/s41571-021-00539-4.
[13] T. Shibue, R.A. Weinberg, EMT, CSCs, and drug resistance: The mechanistic link and clinical implications, Nat. Rev. Clin. Oncol. 14 (2017) 611–629, https://doi.org/10.1038/nrclinonc.2017.44.
[14] E.B. Rankin, A.J. Giaccia, Hypoxic control of metastasis, Science. 352 (2016) 175–180, https://doi.org/10.1126/science.aaf4405.
[15] N. Whitman, Z. Lin, R. Kenney, L. Albertini, M. Lockett, Hypoxia differentially regulates estrogen receptor alpha in 2D and 3D culture formats, Arch. Biochem. Biophys. 671 (2019) 8–17, https://doi.org/10.1016/j.abb.2019.05.025.
[16] T.J. DiProspero, E. Dalrymple, M.R. Lockett, Physiologically relevant oxygen tensions differentially regulate hepatotoxic responses in HepG2 cells, Toxicol. in Vitro. 74 (2021) 105156, https://doi.org/10.1016/j.tiv.2021.105156.
[17] T.J. Diprospero, L.G. Brown, T.D. Fachko, M.R. Lockett, Integration of physiological oxygen tensions and Wnt/B-catenin signaling into HepaRG cultures results in zonal patterning of drug metabolizing enzymes, Drug. Metab. Dispos. 50 (2022) 1098–1105, https://doi.org/10.1124/dmd.122.000870.
[18] R. Derda, A. Laromaine, A. Mammoto, S.K. Tang, T. Mammoto, D.E. Ingber, G.M. Whitesides, Paper-supported 3D cell culture for tissue-based bioassays, Proc. Natl. Acad. Sci. USA 106 (2009) 18457–18462, https://doi.org/10.1073/pnas.0910666106.
[19] A.S. Truong, M.R. Lockett, Oxygen as a chemoattractant: confirming cellular hypoxia in paper-based invasion assays, Analyst. 141 (2016) 3874–3882, https://doi.org/10.1039/C6AN00630B.
[20] M. Boyce, R. Kenney, A. Truong, M. Lockett, M., Quantifying oxygen in paper-based cell cultures with luminescent thin film sensors, Anal. Bioanal. Chem. 408 (2016) 2985–2992, https://doi.org/10.1007/s00216-015-9189-x.
[21] M. Boyce, G. LaBonia, A. Hummon, M. Lockett, M., Assessing chemotherapeutic effectiveness using a paper-based tumor model, Analyst. 142 (2017) 2819–2827, https://doi.org/10.1039/c7an00806f.
[22] K.R. Rivera, M.A. Yokus, P.D. Erb, V.A. Pozdin, M. Daniele, Measuring and regulating oxygen levels in microphysiological systems: design, material, and sensor considerations, Analyst. 144 (2019) 3190–3215, https://doi.org/10.1039/c8an02201a.
[23] P. Oomen, M. Skolimowski, E. Verpoorte, Implementing oxygen control in chip-based cell and tissue culture systems, Lab Chip. 16 (2016) 3394–3414, https://doi.org/10.1039/c6lc00772d.
[24] V. Palacio-Castaneda, N. Velthuijs, S. Le Gac, W.P.R. Verdurmen, Oxygen control: The often overlooked but essential piece to create better in vitro systems, Lab Chip. 22 (2022) 1068–1092, https://doi.org/10.1039/d1lc00603g.
[25] M.D. Brennan, M.L. Rexius-Hall, L.J. Elgass, D.T. Eddington, Oxygen control with microfluidics, Lab. Chip. 14 (2014) 4305–4318, https://doi.org/10.1039/c4lc00853g.
[26] M. Boyce, W. Simke, R. Kenney, M. Lockett, Generating linear oxygen gradients across 3D cell cultures with block-layered oxygen controlled chips (BLOCCs), Anal. Methods. 12 (2020) 18–24, https://doi.org/10.1039/C9AY01690B.
[27] Thermo Scientific HERA VIOS 160I CO2 SS TC 120V (catalog no. 51033719) cost estimate, website accessed in June 2023.
[28] T.J. DiProspero, L.G. Brown, T.D. Fachko, M.R. Lockett, HepaRG cells undergo increased levels of post-differentiation patterning in physiologic conditions when maintained as 3D cultures in paper-based scaffolds, BioRxiv (2023), https://doi.org/10.1101/2023.01.16.524330.
[29] Z.R. Sitte, T.J. DiProspero, M.R. Lockett, Evaluating the impact of physiologically relevant oxygen tensions on drug metabolism in 3D hepatocyte cultures in paper scaffolds, Curr. Protoc. 3 (2023) e662.

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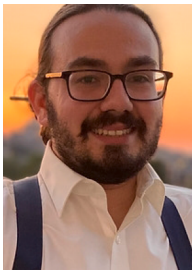

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