Video Article

Quantitative and Temporal Control of Oxygen Microenvironment at the Single Islet Level

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Abstract

Simultaneous oxygenation and monitoring of glucose stimulus-secretion coupling factors in a single technique is critical for modeling pathophysiological states of islet hypoxia, especially in transplant environments. Standard hypoxic chamber techniques cannot modulate both stimulations at the same time nor provide real-time monitoring of glucose stimulus-secretion coupling factors. To address these difficulties, we applied a multilayered microfluidic technique to integrate both aqueous and gas phase modulations via a diffusion membrane. This creates a stimulation sandwich around the microscaled islets within the transparent polydimethylsiloxane (PDMS) device, enabling monitoring of the aforementioned coupling factors via fluorescence microscopy. Additionally, the gas input is controlled by a pair of microdispensers, providing quantitative, sub-minute modulations of oxygen between 0-21%. This intermittent hypoxia is applied to investigate a new phenomenon of islet preconditioning. Moreover, armed with multimodal microscopy, we were able to look at detailed calcium and K_{ATP} channel dynamics during these hypoxic events. We envision microfluidic hypoxia, especially this simultaneous dual phase technique, as a valuable tool in studying islets as well as many *ex vivo* tissues.

Video Link

The video component of this article can be found at http://www.jove.com/video/50616/

Introduction

Dynamic hypoxia is important in biology, specifically for islet transplants

Dynamic hypoxia is an important physiological as well as pathophysiological parameter in many biological tissues. Change in oxygen, for example, is a potent developmental signal in angiogenesis. Moreover, spatial and temporal patterns in hypoxia modulate HIF1-alpha and play roles in diseases like pancreatic cancer. Hypoxia is also a confounding factor affecting islet transplant outcomes. Recently, temporally oscillations of hypoxia, or intermittent hypoxia (IH) have demonstrated benefits in "preconditioning" islets[1]. However, both static and transient hypoxia effects on islet physiology have yet to be well understood or studied, primarily due to the lack of appropriate tools to control islet's microenvironment.

Islets are well vascularized *in vivo*

Pancreatic islets are 50-400 µm spheroidal aggregates of endocrine cells, including beta-cells and alpha-cells that are responsible for glucose homeostasis. When islets are exposed to stimulatory glucose in the blood, uptake and glycolysis lead to ATP production, which opens up ATP-sensitive potassium (K_{ATP}) channels and results in calcium influx that triggers the exocytosis of insulin granules. Oxygen is important to drive this heavily metabolic process and insulin secretion is significantly influenced by the dynamics of blood flow and oxygen supply in addition to glucose gradients. Islets readily perform this glucose-insulin response *in vivo* as they are highly perfused in the pancreas, each within one cell length from a capillary vessel. However, the dense network of intraislet capillaries is removed by collagenase during islet isolation[2,3]. Consequently, both oxygen and nutrient supplies are constrained to a 100 µm perimeter due to diffusion limitations.

Current techniques have limited success in recreating islet microenvironment

Recreating islet's native oxygen and glucose dynamics, key to modeling physiological and pathophysiological conditions, is difficult to achieve with standard hypoxic chambers that require elaborate flow and lack continuous monitoring of islet functions. Moreover, transplant therapies for Type I diabetes expose isolated islets to hypoxia in the hepatic portal system[4] which has much lower pO_2 (<2%, 5-15 mmHg) compared to physiological pancreas (5.6%, 40 mmHg). Post-transplant, the islet grafts take two weeks or more to be revascularized. It has been demonstrated that hypoxic exposure impairs islet's glucose-insulin coupling mechanism. Among the stimulus-secretion coupling factors, calcium signaling, mitochondrial potentials, and insulin kinetics can be easily monitored using microfluidics. Our previous microfluidic technique

demonstrated this real-time monitoring with precise modulation of the aqueous microenvironment around single islet[5,6]. However, quantification of islet's hypoxic impairment is stymied by the lack of simultaneous stimulation and monitoring techniques. Therefore, combining microfluidic control of oxygen and islet monitoring can improve islet hypoxia studies.

Microfluidics can recreate and modulate the aqueous and oxygen microenvironment

The standard technique for tissue and culture hypoxia studies has been based on hypoxic chambers. In general, the hypoxic chambers provide single oxygen concentrations with equilibration times in ~10-30 min, incompatible with minute-scaled dynamic hypoxia. Two recent studies used small custom chambers for intermittent hypoxia exposure on whole mice, with conflicting results on glucose-induced insulin response[7,8]. Bear in mind that at the whole animal level, the respired oxygen is not directly translated to islet capillary pO_2, due to controls in the respiratory system. Furthermore, these studies do not have standardized oxygen levels, nor do they provide real-time measures at the tissue level of islets.

On the other hand, oxygen microfluidics can surpass these limitations by controlling oxygen via gas channel networks. Moreover, microfluidics is compatible with live imaging during oxygen modulation, a feat currently not possible with standard hypoxic chambers. A number of these novel microfluidics approaches utilize the gas permeability of polydimethylsiloxane to dissolve oxygen concentrations into microchannels that flow media over target cells[9-14]. These devices have also integrated multiple discrete oxygen concentrations, fluorescence based oxygen sensors, and even chemical oxygen generation on-chip.

Liquid solvation-based microfluidics have a hard time maintaining stable, continuous gradients as it depends on convective mixing which is sensitive to flow conditions. In comparison, the technique we use here focuses on decreasing the diffusion path of oxygen delivery. The gas solvation and shear flow are eliminated by directly diffusing oxygen across a membrane seeded with cells or islet tissues. This removes the extra microfluidics required to control solvation and prevents unnecessary shear stress to the islets, which itself can trigger insulin release. This platform has been used to demonstrate reactive oxygen species (ROS) up-regulation at both hyperoxic and hypoxic extremes (2-97% O_2) in cell culture[1,15]. Because of the direct delivery of oxygen and removal of shear flow, our diffusion-based platform provides the optimal microfluidic solution for studying islet hypoxia.

Multimodal stimulation and monitoring

Diffusion-based microfluidics also brings additional benefits when adapted for studying islet microphysiology. By using a membrane as a diffusion barrier, the liquid can be isolated from the oxygen modulations, enabling controls of aqueous glucose stimulations independently from hypoxic stimulations. This creates a sandwich-like simultaneous stimulation that spatially pin-points delivery to the islets. Furthermore, as the gas is temporally modulated via computerized microinjectors, we can modulate the oxygen concentration from 21-0% digitally with transient time less than 60 sec. The dynamic controls of the oxygen and glucose microenvironment at the microscope allow a real-time multimodal protocol that would not be possible or extraordinarily cumbersome using standard hypoxic chambers. Using this device, calcium signaling (Fura-AM), mitochondrial potentials (Rhodamine 123), and insulin kinetics (ELISA) were monitored to provide a complete picture of the dynamic glucose-insulin response under hypoxia.

Protocol

1. Preparing the Mouse Islets

1. Dissect C57BL/6 mice and isolate islets by collagenase digestion and Ficoll density gradient separation. (Refer to JOVE articles referenced in[2,3]).
2. Incubate islets in RPMI-1640 medium containing 10% FBS, 1% penicillin/streptomycin, and 20 mM HEPES in Petri dishes (37 °C, 5% CO_2). Post-isolation, culture islets for 24 hr prior to use in experiments. Use the islets within 1-2 days to ensure consistent results.

2. Making the Microfluidic Platform

1. Generate the microstructure geometries on transparency photomasks for each device layer: 1) inlet and outlet, 2) Glucose microfluidic layer with perifusion chamber (8 mm diameter x 3 mm, 150 µl), 3) 200 µm membrane with microwells (500 µm diameter, 100 µm deep), and 4) gas microfluidic layers.
2. To fabricate the Inlet and outlet layer, pour degassed, premixed PDMS to a height of 1.5 mm in a blank Petri dish and cure at 80 °C for 2 hr. Punch 2 mm diameter input/out ports.
3. To fabricate the glucose microfluidic layer, spin two 350 µm layers of SU8-2150 to form a single 700 µm layer on a 4 in silicon wafer.
    1. Expose UV light through the appropriate photomask to transfer microchannel pattern onto the SU8 after development, producing a 700 µm tall master.
    2. Pour degassed, premixed PDMS onto this master to a height of 3 mm and cure at 80 °C for 2 hr. Cut this PDMS to shape with a razor blade.
    3. Punch 2 mm diameter input/output ports as well as the 8 mm diameter chamber.
4. To fabricate the 200 µm membrane with microwells, spin SU8-2100 to 100 µm. Apply the same UV-lithography to transfer microwell patterns onto this 100 µm tall master.
    1. Spin degassed, premixed PDMS onto this master at 900 rpm for 30 sec and cure at 80 °C for 10 min. Spin a second layer on top of the first using the same conditions, resulting in a 200 µm PDMS membrane containing microwell patterns.
    2. Punch 2 mm diameter input/output ports through this membrane
5. To fabricate the gas microfluidic layer, spin SU8-2100 to 100 µm. Apply the same UV-lithography to transfer gas microfluidic patterns onto this 100 µm tall master.

1. Pour degassed, premixed PDMS onto this master to a height of 1.5 mm and cure at 80 °C for 2 hr. Cut the PDMS to shape with a razor blade.
2. Punch 2 mm diameter input/output ports through this layer

6. To bond the multiple layers, prepare them by cleaning with scotch tape, exposing to corona arc, and then aligning by hand in the following order.
   1. Bond the membrane to the bottom gas layer with microwells facing up.
   2. Bond the glucose microfluidic on top of the microwell membrane.
   3. Bond the inlet and outlet layer on the very top, encapsulating the whole assembly.
   4. Reinforce the bonding by adding 1 kg weight on top and baking at 100 °C for 3 hr.
7. Leak-test the completed device by loading water into the aqueous layer, then submerging the entire device under water. Then, flow air through the gas layer with an empty syringe.
8. Sterilize leak-free devices with 70% ethanol through the aqueous layer then flush with PBS buffer, in which the device is stored at 4 °C until use.

3. Microdispenser Setup

1. Obtain 2 microdispensers, 5 V and 20 V DC power supplies, and a digital I/O board to construct the oxygen mixing and delivery setup.
   1. Connect the microdispensers' control leads to the included driver units.
   2. Connect the drivers to the digital I/O board at ports corresponding to Labview controls.
   3. Connect the 5 V and 20 V power supplies to corresponding contacts on the drive units.
   4. Connect the digital I/O to a laptop to execute Labview codes for gas control (Appendix).
2. Connect one microdispenser to nitrogen while another to compressed air, both with 5% CO_2. Set both gases to 2 psi. Hook up the dispensers' outputs in a T-junction prior to entering the microfluidic device.
3. Using a leak-tested device, characterize the transient response of oxygen modulation by cycling the microdispensers between 5-21% oxygen, while measuring dissolved oxygen in water with a fiber optic oxygen sensor (see equipment list and representative results).

4. Setting Up at the Microscopy

1. Mount the device to the heated stage on the inverted microscope.
2. Connect 0% and 21% O_2 gases to the device and calibrate with the oxygen sensor.
3. Connect the output port of the glucose microfluidics to a fraction collector.
4. Prepare the Krebs Ringer bicarbonate buffer (KRB): 129 mM NaCl, 5 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES pH 7.35-7.40, 2 mM basal glucose, and 5% FBS.
5. Prepare two buffer solutions containing 2 mM and 14 mM glucose respectively in 50 ml conical tubes bathed in 37 °C water bath.
6. Connect a peristaltic pump to draw buffer at 250 µl/min into tubing. Flow the tubing over a 37 °C hotplate before entering the microfluidic device.
7. To stain the islets, add Fura-2 AM in DMSO and Rh123 in 100% ethanol to 2 ml of KRB to reach final dye concentrations of 5 µM and 2.5 µM, respectively.
8. Pick up islets with a 10 µl pipette and incubate in dyes for 30 min at 37 °C.
9. Load approximately 20 islets into the device via the glucose microchannel inlet. Direct the islets into the chamber by priming buffer from the outlet back into the inlet.
10. Perfuse the islets in buffer for 10 min to wash away excess dyes.

5. Running the Simultaneous Oxygen and Glucose Stimulation

1. Deliver a glucose stimulation pulse with 5 min of baseline established by KRB2, followed by 15 min of 14 mM stimulation, then wash for 15 min in KRB2.
2. Record 340/380 nm excitation wavelength for Fura-2 and 534 emission for Rh123.
3. Make measurements of a normal pulse (14 mM normoxic) before each experiment.
4. For oxygen modulation (hypoxia/intermittent hypoxia), only apply buffer flow during stimulation and washing steps and no flow in other steps, to minimize convective disturbances.
5. Collect effluents from glucose outlet at 1 min intervals for ELISA insulin assay.

Representative Results

Central to this islet hypoxia technique is the ability to modulate aqueous and gaseous phase stimulation in the same microfluidic chamber with minute-scale transients. **Figure 1** is a representative result of the a) dual stimulations and b) fast modulations measured within the islet chamber. Aqueous modulation, shown by introduction of fluorescein into the chamber, achieves equilibrium in three to four minutes of mixing. Furthermore, oxygen can be stepped from 5-21% with fast transients, enabling cycling of oxygen with periods as short as 2 min. Different cycling depths and frequencies can also be achieved as shown in **Figure 2**.

When this cycling is applied to create intermittent hypoxia at the islets, one can observe the benefits of preconditioning islets against hypoxia, as compared to a regular, normoxic pulse, **Figure 3a**. Because intracellular calcium flux—the signaling mechanism of insulin secretion—is monitored in real-time, effects of hypoxia and IH can be observed in the overshoot and oscillation damping of calcium transients, **Figure 3b**. These are important parameters associated with K_{ATP} channels suggested to control the preconditioning process. Furthermore, mitochondria's

link to metabolism and hypoxia can be visualized by monitoring mitochondrial potentials using Rh123. Finally, collection of microfluidic effluents allows ELISA assay of the total insulin quantity. Calcium, mitochondrial potential, and insulin are three parameters that begin to build a multimodal view of the glucose-insulin response under hypoxic transients, **Figure 3c**.

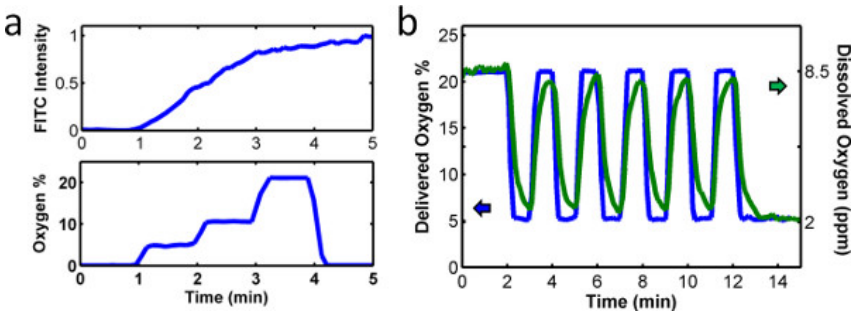

Figure 1. Simultaneous oxygen and glucose stimulations. (a) Static controls. Top: chamber introduction of FITC molecule at 250 µl/min stabilizes within 3 min of mixing time. Bottom: gas control provides stable delivery of 5, 10, and 21% oxygen at the membrane. **(b)** Temporal control. Cycling of oxygen between 5-21% is possible with 1 min period as measured both at the gas delivery (diffused) and surface of microwells (dissolved).

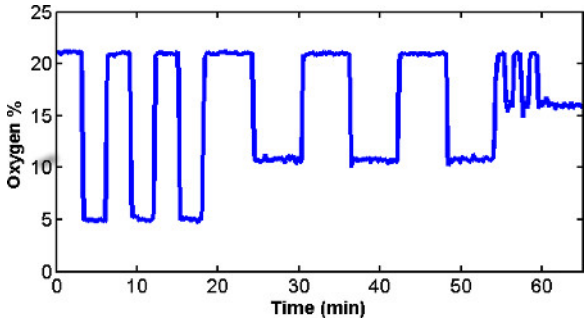

Figure 2. Microfluidic device can also generate other oxygen profiles. Different IH profiles can be obtained by varying the depth (5-10-15%) as well as the periods (3-6-1 min) of the cycling via gas mixing from the computerized microinjectors.

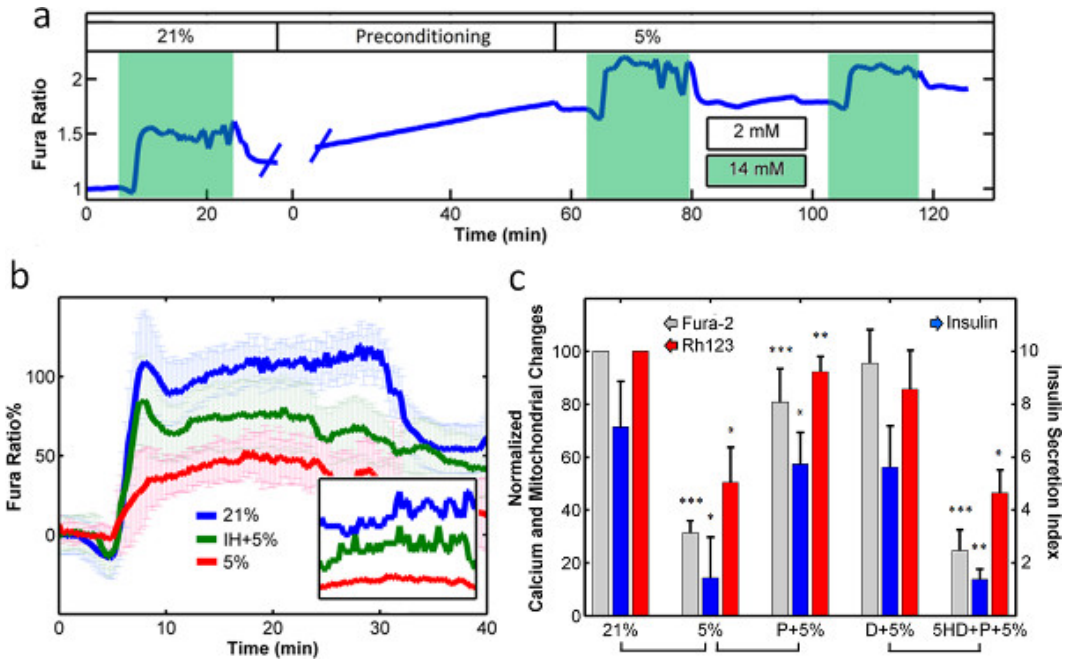

Figure 3. IH preconditioned islets have improved responses to hypoxia. (a) Representative overview of preconditioning using IH, totaling 30 min exposure at 5%, showing enhanced hypoxic response compared to normoxic pulse (same islet batch in a previous experiment). 10 more minutes of subsequent hypoxia still retains preconditioning. **(b)** Overlaid comparison of mean normoxic, hypoxic, and preconditioned hypoxic responses. Inset shows representative traces with recovery of oscillation behavior. **(c)** Multimodal Fura-2, insulin, and Rh123 responses for different oxygen and chemical conditions: 21% oxygen, 5% oxygen, preconditioning (P+5%), diazoxide (D+5%), and 5HD (5HD+P+5%). Diazoxide is consistent with preconditioning while 5HD negates the benefits of preconditioning by opening and blocking KATP channels, respectively. Two-tailed t-tests: 5% vs. 21%, 5% vs. P+5%, D+5% vs. 5HD+P+5%; *p<0.05, **p<0.01, ***p<0.001.

Discussion

The multiple modalities integrated in this islet hypoxia technique present several points noted here for troubleshooting. First the isolated islets continue to degrade and disintegrate in culture due to digestive enzymes from acinar cells. Standardizing experiments to 1-2 days after islet isolation is thus critical in obtaining consistent results. Second, the aqueous flow was stopped during hypoxia and intermittent hypoxia to prevent convective clearance at the boundary between laminar flow and diffusion. This seems to limit the duration of islet preconditioning. Future integration of a gas exchange in the aqueous channel can eliminate this minor clearance while still allowing rapid gas modulations at the membrane. Third, while loading the islets, the aqueous tubing should be reconnected carefully in reverse order (outlet then inlet) to avoid trapping air bubbles. Lastly, future device can be augmented with a fanned-out microfluidic distributor, to help distribute the cluster of islets over the entire chamber, at bottom of which can be patterned with an array of trapper pockets. This microfluidic distribution in addition to the pocket array will help create a positional array of islets for high-throughput experiments.

Prior to this microfluidic islet hypoxia technique, the fastest intermittent hypoxia modulations were achieved in one to three minute cycles by using small custom hypoxic chambers with high flow of pressurized gas. However, these can only be used on whole animals and not at the single islet level. Besides the uncertainty of the actual hypoxia level achieved in the whole animals' pancreas (after respiratory equilibrium) there is also the inability to probe glucose-insulin response in real-time, well-controlled microenvironments. In comparison, both aqueous and gas stimulations are controlled to minute time-scales in our microfluidics. These modulations are also mounted directly at the microscope for multiparametric monitoring. Prior to our technique, repeatable and well-characterized islet precondition has not been achievable. Oxygen-sensitive *ex vivo* tissues such as islets are optimally suited to this microfluidic platform as their microscaled dimensions (*i.e.* 100 µm radius) are trapped between smaller cell-culture platform and larger chamber apparatus. Beyond islets, a number of *ex vivo* tissues—including cardiac tissue, brain slices, and embryos—can be investigated using this technique.

Disclosures

The authors declare that they have no competing financial interests.

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