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Analysis of Non-Human Primate Pancreatic Islet Oxygen Consumption

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cover letter

VANDERBILT UNIVERSITY School of Medicine
Division of Diabetes, Endocrinology, and Metabolism

September 24, 2019

Dear Dr. Jewhurst:

Attached you will find the revised version of our manuscript JoVE60696. We have addressed to all of the reviewers' and editor's comments. A detailed response is attached. Based on your comments, we have changed the title to "Analysis of non-human primate pancreatic islet oxygen consumption". We hope that our manuscript is now acceptable for consideration for publication in *Journal of Visualized Experiments.* In this manuscript, we describe measurement of oxygen consumption (a proxy for mitochondrial function and cellular metabolism) in whole pancreatic islets. The methods described in our manuscript should be applicable to any spherical structure, including tissue organoids. Given the critical role that mitochondrial function plays in the insulin-producing beta cell and diabetes etiology, we feel that this methodology will be of great importance to the field.

Yours sincerely,

Maureen Gannon, Ph.D.
Professor of Medicine,
Molecular Physiology and Biophysics, and
Cell and Developmental Biology

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TITLE:

Analysis of Non-Human Primate Pancreatic Islet Oxygen Consumption

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KEYWORDS:

islet, oxygen consumption, spheroid, mitochondria, β cell, pancreas, non-human primate

SUMMARY:

This protocol demonstrates the accurate and reproducible measurement of oxygen consumption in non-human primate pancreatic islets. The islet loading techniques and coating of the microplate provide a framework for efficient measurement of respiration in other types of cultured spheroids.

ABSTRACT:

The measurement of oxygen consumption in spheroid clusters of cells, such as *ex vivo* pancreatic islets, has historically been challenging. We demonstrate the measurement of islet oxygen consumption using a 96 well plate microplate designed for the measurement of oxygen consumption in spheroids. In this assay, spheroid microplates are coated with a cell and tissue adhesive on the day prior to the assay. We utilize a small volume of adhesive solution to encourage islet adherence to only the bottom of the well. On the day of the assay, 15 islets are loaded directly into the base of each well using a technique that ensures optimal positioning of islets and accurate measurement of oxygen consumption. Various aspects of mitochondrial respiration are probed pharmacologically in non-human primate islets, including ATP-dependent respiration, maximal respiration, and proton leak. This method allows for consistent, reproducible results using only a small number of islets per well. It can theoretically be applied to any cultured spheroids of similar size.

INTRODUCTION:

In order to maintain normal blood glucose levels, the pancreatic β cell must sense elevations in glucose and secrete insulin accordingly. The coupling of insulin secretion with glucose levels is directly linked to glucose metabolism and the production of ATP through mitochondrial oxidative phosphorylation. Thus, mitochondria play a critical role in stimulus-secretion coupling[1]. Assessing β-cell mitochondrial function can reveal defects that lead to impaired insulin secretion. The secretion of glucagon by pancreatic α cells is also closely tied to mitochondrial function[2]. Although immortalized islet cell lines have proven useful for some types of assays, the physiology of these cells does not accurately recapitulate whole islet function, as illustrated by the potentiation of insulin secretion by glucagon[3,4] and the inhibition of glucagon secretion by insulin/somatostatin[5,6] in intact islets. This demonstrates the need for measuring oxygen consumption on whole, intact islets.

Techniques for the measurement of islet cell respirometry have evolved over time, from the use of oxygen-sensitive fluorescent dyes[7] to solid-state sensors that directly measure oxygen consumption[8]. Initially designed for monolayer, adherent cells, commonly used cell culture plate systems have proven to be ineffective for pancreatic islets. As islets do not naturally adhere to the wells, they are prone to being pushed to the periphery of the culture well resulting in inaccurate measurement of oxygen consumption[9]. To combat this problem, specialized 24-well plates with a central depression that could contain islets were developed[9]. However, the 24-well plate system was limited by the large number of islets required (50-80 per well) and the number of conditions that could be tested simultaneously[10]. The recent development of 96 well microplates designed specifically for extracellular flux analysis in spheroids has overcome these barriers, enabling the measurement of islet respirometry with 20 or fewer islets per well[10].

Here, we demonstrate the use of this system to measure oxygen consumption in islets from the Japanese macaque (*Macaca fuscata*), an animal model with similar islet biology to humans[11,12]. In this protocol, 15 macaque islets are analyzed per well. In our hands, 15 islets per well produced higher baseline oxygen consumption than fewer islets, with robust activation and repression of respiration in response to pharmacologic manipulation. We highlight the steps to prepare for the assay, an effective method for consistent loading of islets at the center of each well, and common challenges when performing this assay.

PROTOCOL:

Islet retrieval was conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Oregon National Primate Research Center (ONPRC) and Oregon Health and Science University and were approved by the ONPRC IACUC. The ONPRC abides by the Animal Welfare Act and Regulations enforced by the United States Department of Agriculture (USDA) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

1. Preparation of microplate and sensor cartridge on the day prior to running the assay

NOTE: Islets were isolated from three-year-old Japanese macaques as previously described[13]. This method is very similar to that used to isolated human islets from cadaver donors, but differs from mice, in which pancreata are often inflated with collagenase solution while the animal is under sedation and prior to organ removal.

1.1. Preparation of the spheroid microplate

1.1.1. Prepare 3 mL of a 0.1 M solution of sodium bicarbonate. Filter sterilize the solution using a 0.45 µm filter (**Table of Materials**).

1.1.2. In a cell culture hood, add 200 µL of cell and tissue adhesive (**Table of Materials**) to 2.8 mL of 0.1 M sodium bicarbonate. Then, add 20 µL of this solution to the bottom of each well of the 96 well spheroid microplate (**Table of Materials**). Ensure that air bubbles are removed and only the bottom of the plate is covered.

NOTE: Coating of the microplate prevents islets from moving up the sides of the wells when drugs are added and mixed into the well during the assay. It is only necessary to coat as many wells as will be used for islets when the assay is run. If only a few wells will be used, the cell adhesive solution can be scaled down appropriately. The cell adhesive used in the assay is a formulated protein solution extracted from the marine mussel. Alternatively, microplate wells can be coated with 20 µL of 100 µg/mL poly-D-lysine.

1.1.3. Incubate the plate in a 37 °C, non-CO_2 incubator for 1 h.

1.1.4. In a sterile environment, aspirate cell adhesive solution from the plate. Wash each well 2x with 400 µL of 37 °C sterile water using a multichannel pipet. Allow to air dry.

1.1.5. After 30–40 min, cover the plate and store overnight at 4 °C.

1.2. Hydration of the sensor cartridge

1.2.1. In a sterile environment, open the sensor cartridge package (**Table of Materials**) and remove the contents. Place the sensor cartridge upside down next to the utility plate.

1.2.2. Fill each well of the utility plate with 200 µL of sterile water and lower the sensor cartridge back into the utility plate. Place assembled sensor cartridge and utility plate in a 37 °C, non-CO_2 incubator overnight.

1.3. Preparation of calibrant

1.3.1. Aliquot 25 mL of calibrant (**Table of Materials**) into a 50 mL conical tube. Place tube in a 37 °C, non-CO_2 incubator overnight.

2. Media preparation, loading of islets, and loading of sensor cartridge on the day of assay

2.1. Preparation of media

2.1.1. To 48.5 mL of base media (minimal Dulbecco's modified Eagle medium [DMEM], **Table of Materials**), add 500 µL each of 1 mM sodium pyruvate and 2 mM glutamine. Additionally, add 496 µL of 200 mg/mL glucose (final concentration of glucose in the media = 5.5 mM).

NOTE: Baseline glucose concentration was kept constant for these experiments. However, glucose can also be used to stimulate respiration in addition to the pharmacologic manipulations described blow[10].

2.1.2. Confirm that the pH is 7.4 ± 0.1, adjusting if necessary.

2.2. Preparation of sensor cartridge

2.2.1. Take sensor cartridge out of the incubator, remove sensor cartridge lid, and dump out water from wells. Place 200 mL of pre-warmed calibrant into each well and replace sensor cartridge lid.

2.2.2. Place sensor cartridge back in incubator for ~1 h (until needed).

2.3. Preparation of drugs for mitochondrial stress assay

2.3.1. Open the stress test kit (**Table of Materials**) and remove the contents. Make up stock and working solutions for oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone/antimycin A (AA) as follows.

2.3.1.1. For oligomycin, add 630 µL of assembled media to tube to make 100 µM stock. Then, dilute to 45 µM with media to obtain port concentration (final well concentration after injection = 4.5 µM).

2.3.1.2. For FCCP, add 720 µL of assembled media to tube to make 100 µM stock. Dilute to 10 µM with media to obtain port concentration (final well concentration = 1 µM).

NOTE: Bovine serum albumin (BSA) and/or fetal bovine serum (FBS) should not be added to media, as this can affect the action of mitochondrial uncouplers[14].

2.3.1.3. For rotenone/AA, add 540 µL of assembled media to tube to make 50 µM stock. Dilute to 25 µM with media to obtain port concentration (final well concentration = 2.5 µM).

NOTE: The above concentrations have produced consistent results in authors' hands. Additional FCCP led to no further increase in respiration. However, drug concentrations may need to be adjusted depending on islet size, and especially with islets from different species or non-islet

spheroids. For reference, the average diameter of a non-human primate (NHP) islet is about 150 µm[15].

2.4. Preparation of spheroid plate and transfer of islets

2.4.1. Hand-pick pancreatic islets into a 60 mm x 15 mm cell culture dish containing assembled media to obtain near 100% purity.

NOTE: Islets should appear rounded, with a clearly defined periphery, and appear denser than exocrine tissue contaminants. Avoid islets that appear damaged or frayed. In this experiment, islet size was not directly measured, islets of approximately equivalent size were picked for each well and sample.

2.4.2. Load each well of the spheroid microplate with 175 µL of assembled media using a multichannel pipette.

2.4.3. Using a P20 pipette set to 15 µL, aspirate 15 islets from cell culture dish.

NOTE: Islets should be visible in the pipette tip to the naked eye.

2.4.4. To transfer islets to the spheroid microplate, lower pipette tip to the bottom of the well, barely lift up, and slowly pipette out a very small volume (~5 µL). Confirm that all islets have left the pipette tip. Occasionally check the spheroid microplate under the microscope to verify that islets are at the bottom of each well rather than stuck to the sides.

NOTE: Each well should receive 15 islets. Avoid loading the corner wells with islets. Oxygen and pH flux out of the plastic can occur during the assay, and this is exacerbated in the four corner wells.

2.4.5. Once all islets have been transferred to the spheroid microplate, incubate the microplate in a 37 °C, non-CO_2 incubator while completing the steps below.

2.5. Loading the sensor cartridge and running the assay

2.5.1. Remove sensor cartridge from incubator. Load port A (top left of each sensor) with 20 µL of oligomycin or media. Load port B (top right) with 22 µL of FCCP or media. Load port C (bottom left) with 25 µL of rotenone/AA.

NOTE: Ports A–C for each well must be loaded with either drug or media. Wells containing islets and the four corner wells should be loaded with drug. Non-corner wells without islets should be loaded with media. Port D can be left empty.

2.5.2. Program an extracellular flux analyzer assay for a 30 min baseline respiration period (5 cycles of 3 min mix, 3 min measure), 42 min oligomycin measurement period (7 cycles of 3 min

mix, 3 min measure), 30 min FCCP (5 cycles of 3 min mix, 3 min measure), and 30 min rotenone/AA (5 cycles of 3 min mix, 3 min measure).

2.5.3. Run the assay and follow instructions on screen for calibrating the sensor and inserting the microplate.

REPRESENTATIVE RESULTS:

To load islets into microplate, 15 islets should be aspirated in 15 µL of media, as shown in **Figure 1A**. Islets will naturally settle toward the bottom of the pipet tip within a few seconds. Then, the pipet tip is lowered to the bottom of the well. The tip is very slightly lifted, and a small volume (about 5 µL) is pipetted out along with the islets. This technique results in consistent placement of islet at the bottom of the microplate well (**Figure 1B**) allowing for accurate oxygen consumption measurements.

Figure 2 shows representative results by individual well for oxygen consumption throughout the assay. This particular experiment demonstrates what can happen when wells are loaded poorly, with many islets stuck up on the sides of the well rather than at the bottom of the well. This can be caused by pipetting too much media into the well after the islets have already been pipetted out of the tip. The flow of additional media pushes the islets out of the bottom of the well. When this happens, the baseline level of oxygen consumption will be very low, and this well will show little to no response to FCCP. The bolded line in **Figure 2** demonstrates this phenomenon.

Figure 3 shows a different experiment in which most wells did show significant baseline respiration and response to drugs. However, two wells (with bolded lines) showed no response to rotenone/AA. This suggests that the drug was not properly released into the well. In this case, as with cases of no basal oxygen consumption, these wells can be excluded from further analysis.

Figure 4 shows the results of a successful assay. Here we show an example of summary data from a separate experiment in which wells were properly loaded with islets and correctly injected with drugs. ATP-dependent mitochondrial oxygen consumption was effectively inhibited by oligomycin, maximal respiration—well above basal levels—was induced by FCCP, and mitochondrial respiration was completely shut down—below oligomycin levels—by inhibition of the electron transport chain with rotenone/AA.

FIGURE LEGENDS:

Figure 1: Pipetting technique for loading islets into microplate. (**A**) Approximately 15 islets (red arrow) pipetted with 15 µL of media. (**B**) Islets centered at the bottom of the spheroid microplate.

Figure 2: Well-to-well variability due to difference in islet loading. Wells loaded improperly (bold blue line) show little to no basal oxygen consumption and minimal response to cell stress test drugs.

Figure 3: Failure of drug injection. Two wells (bold blue lines) were not injected with rotenone/AA and show no significant decrease in oxygen consumption.

Figure 4: Averaged data across all wells in an experiment after exclusion of poorly loaded or injected wells. Average oxygen consumption throughout the cell stress test assay in a separate experiment including 16 technical replicates. Error bars show standard deviation for n = 16 wells.

DISCUSSION:

The study of islet oxygen consumption has previously been hampered by the spherical shape of islets, their lack of adherence to culture surfaces, and the number of islets required per well. In this protocol, we highlight the efficacy of the 96 well spheroid microplate for measuring islet oxygen consumption on a small number of islets and demonstrate a technique for handling and loading islets which is technically feasible and produces consistent results.

In order for islets to adhere to the bottom of the microplate and be unperturbed during mixing steps throughout the assay, the 96 well microplate is coated with a cell adhesive solution the day before the assay. While this is generally beneficial, if islets are not loaded properly they are liable to stick to the side of the well rather than the bottom, thus affecting or precluding accurate measurement. To combat this, we recommend coating wells with a very small volume of adhesive solution—only 20 µL. Additionally, the pipetting technique we demonstrate ensures that islets are loaded to the bottom of the well and not pushed up the sides by the flow of additional media.

The 96 well spheroid microplate has overcome previous limitations of measuring islet oxygen consumption, including the high number of islets required and the number of conditions that can be tested simultaneously. Indeed, a recent study[10] demonstrated the use of this system with a single human islet per well. However, measurement of oxygen consumption using single islets resulted in very low basal measurements of oxygen consumption, which were significantly above background in only the largest islets (diameter above 290 µm). NHP islets tend to be smaller than human islets, with an average diameter of only 150 µm[15]. In our hands, 15 NHP islets per well showed higher baseline and a more responsive profile during the cell stress test than fewer islets. We also tested five and ten islets per well, but found that basal oxygen consumption and signal to noise were significantly reduced.

In addition to the ability to use a low number of islets per well, the 96 well microplate allows for the testing of multiple different conditions at once. This is beneficial in cases where islets are treated with different compounds or genetically manipulated. However, when comparing groups of human or non-human primate islets that come from different sources (e.g., diabetic versus non-diabetic islets), samples are often received on different days. Thus, in these cases it is not possible to take full advantage of this system.

In order to quantify various aspects of mitochondrial oxygen consumption, we manipulated different aspects of respiration pharmacologically. Drug concentrations used were based on a pervious study in human islets[10]. The dose of FCCP was optimized to induce maximal mitochondrial respiration in our hands. Specifically, the concentration of oligomycin was 4.5 µM, FCCP was 1 µM, and rotenone/AA was 2.5 µM. However, these concentrations likely need to be calibrated for different applications of this protocol.

The data produced can be either analyzed directly or normalized using a number of methods. In this experiment, equal numbers of islets of similar sizes were used for each sample, and data was not normalized by cell number or islet size, which can be difficult to directly quantify. An alternative method of normalization is by total cellular protein, which involves lysing cells after the assay is performed and quantifying protein levels. Data can also be normalized to basal respiration levels. This may be informative in certain cases, such as the quantification of reserve capacity as a percentage of basal levels. However, with these normalization methods and others, information can be lost during normalization as previously described[16].

The system described here provides a unique tool to better understand islet physiology. Indeed, the importance of oxygen consumption in islet health is illustrated by the observation that islet oxygen consumption rates are closely tied with islet health and the probability of successful islet transplantation[17]. This system provides an excellent platform for consistent quantification of islet oxygen consumption which can, in theory, also be applied to any similarly-sized cultured spheroids.

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DISCLOSURES:
The authors have nothing to disclose.

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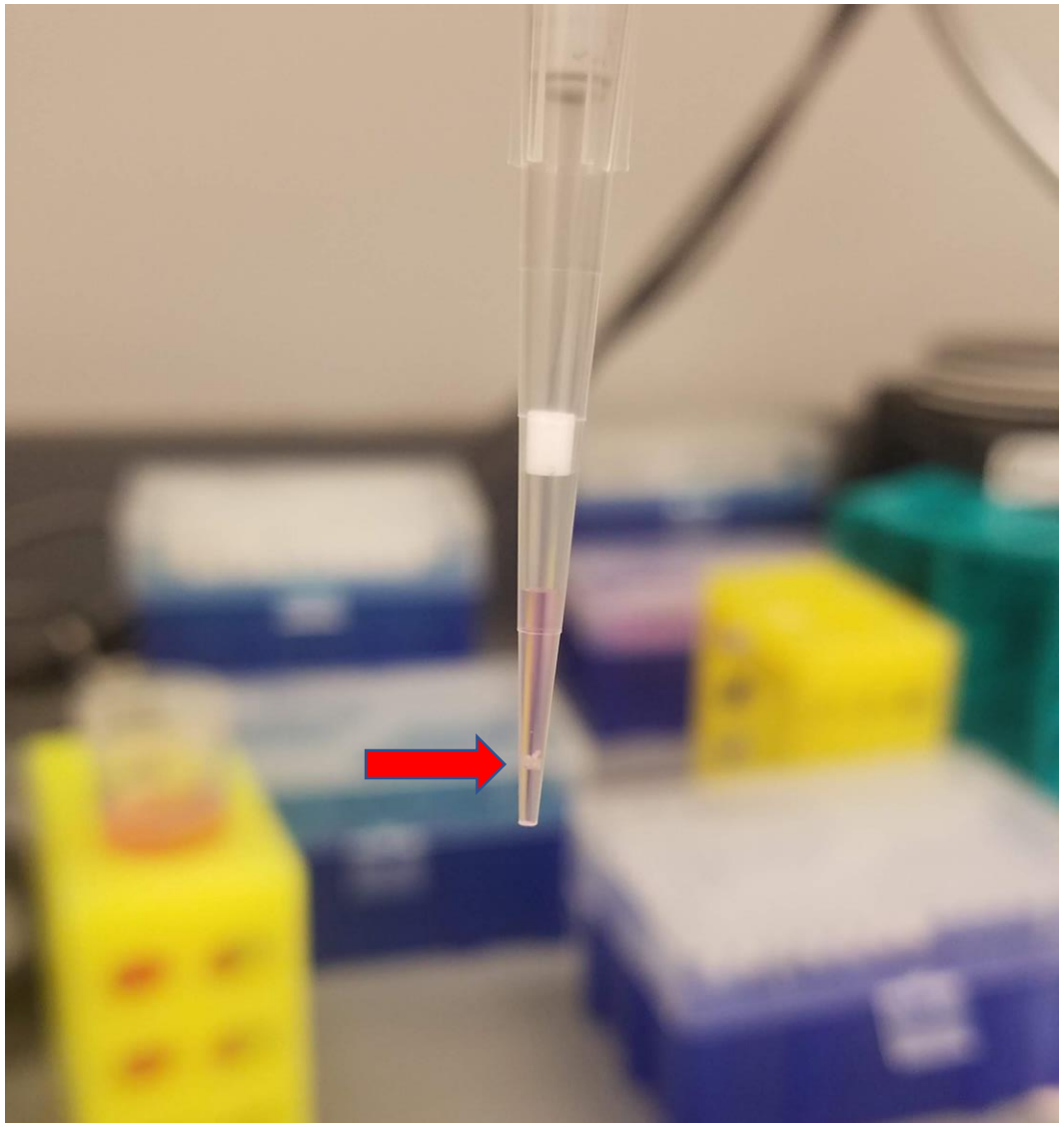
Figure 1

A



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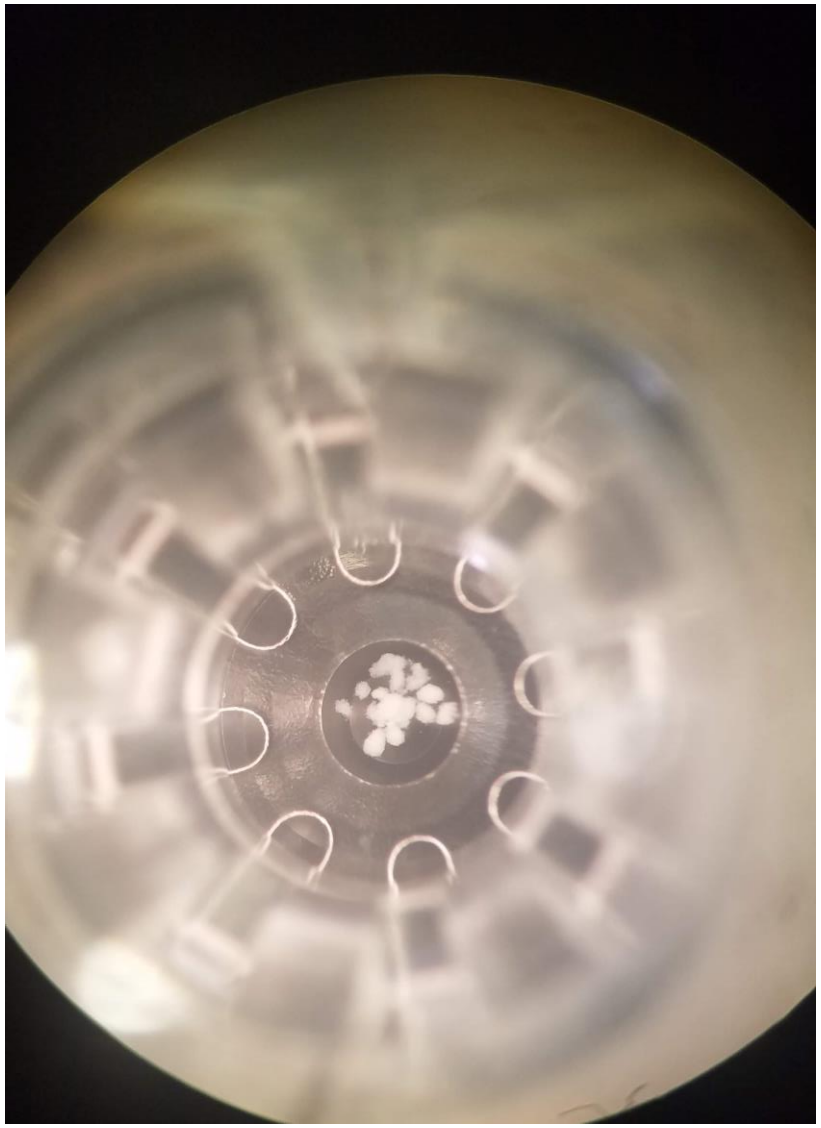

Figure 2

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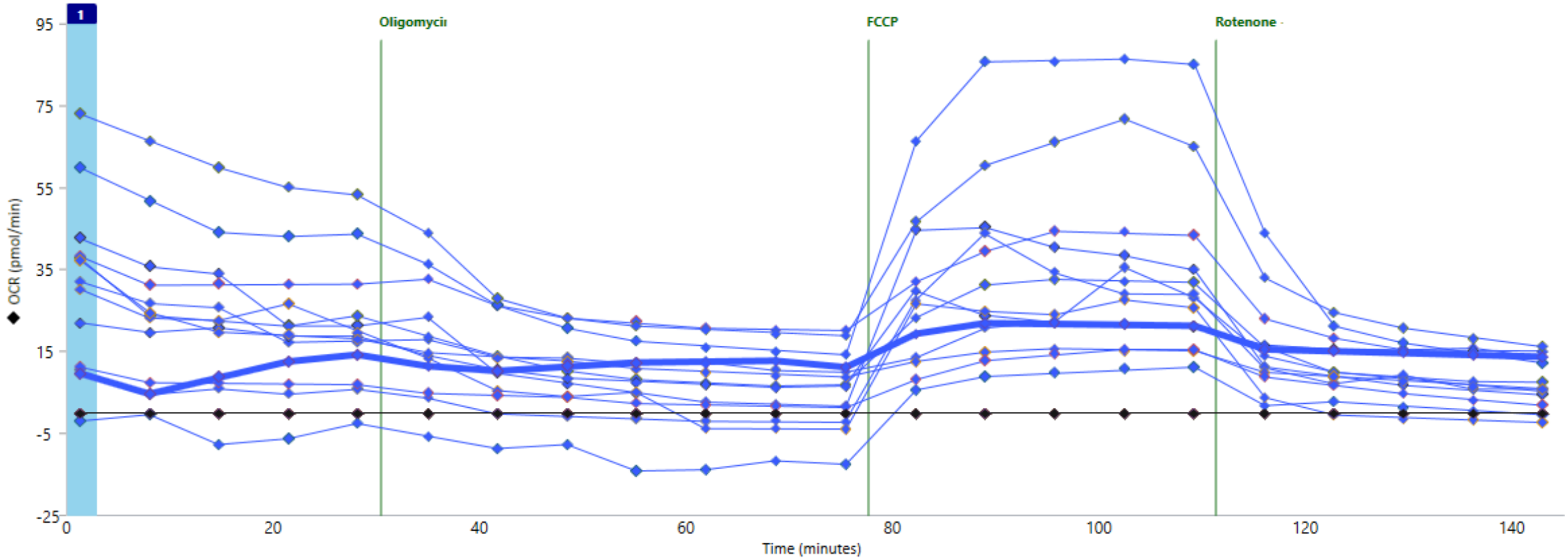

Figure 3

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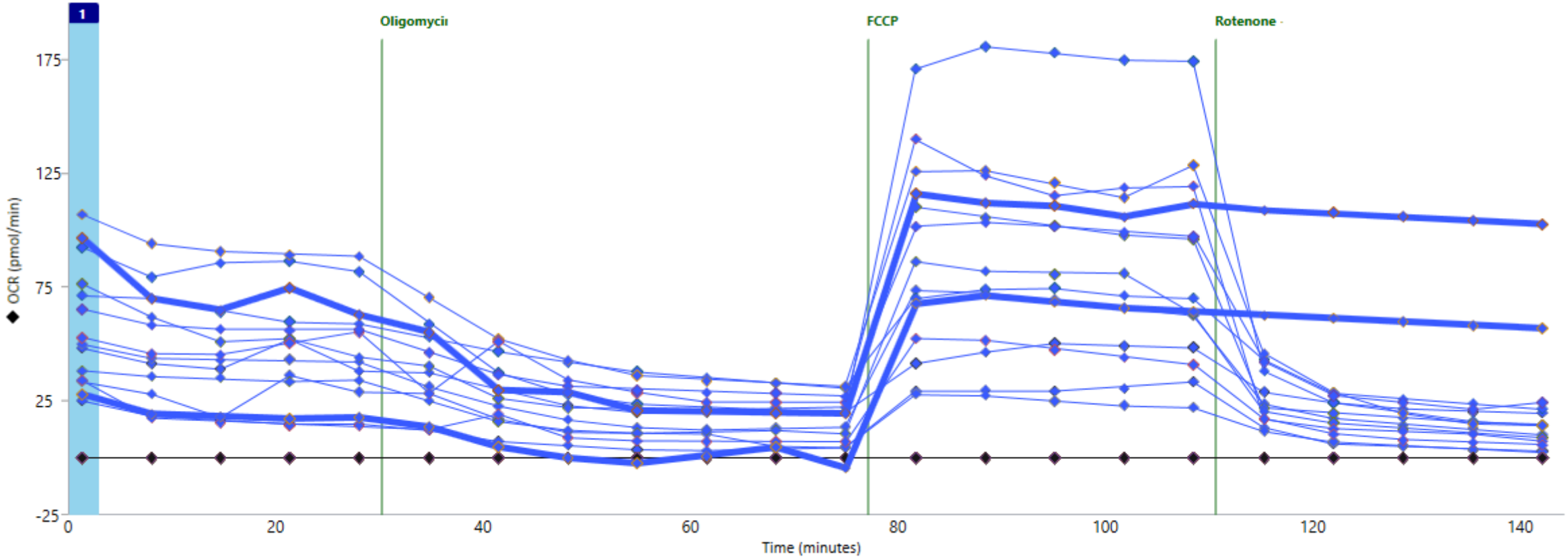

Figure 4

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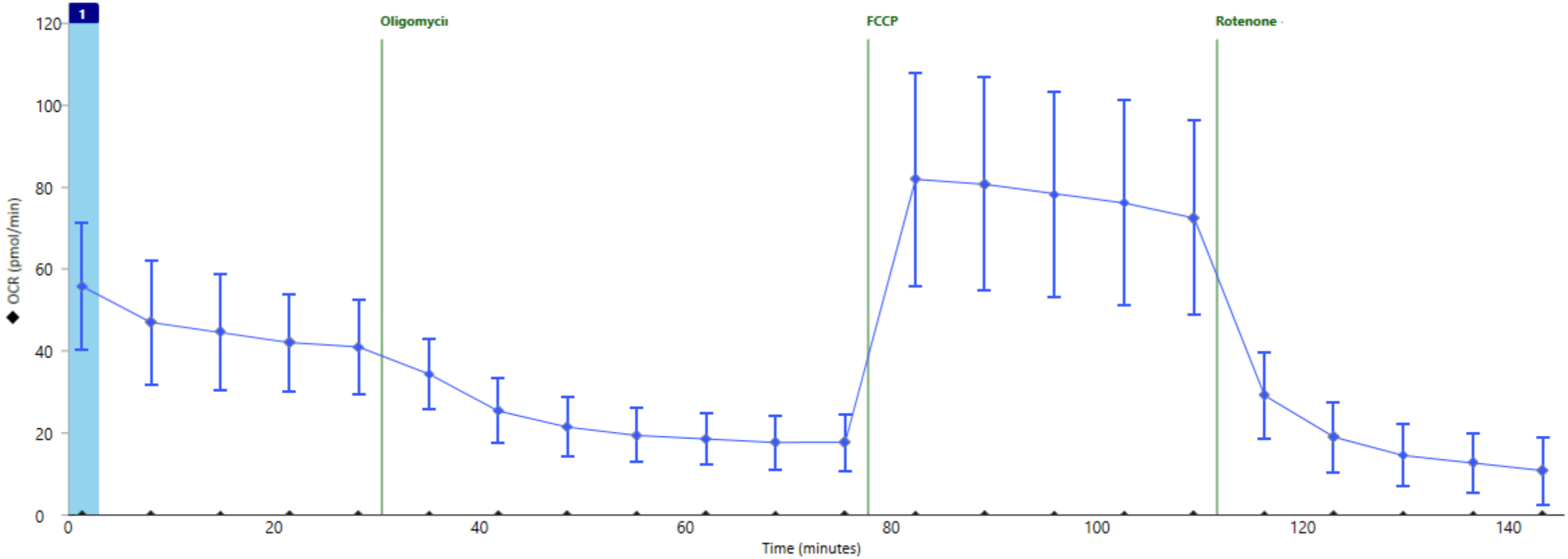

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table of Materials Elsakr et al final.xlsx

Name of Material/ Equipment	Company	Catalog Number
Cell culture dish, 60 mm X 15 mm style	Corning	430166
Cell-Tak Cell and Tissue Adhesive	Corning	354240
Conical tube, 50 mL	Falcon	352070
Dextrose anhydrous	Fisher Scientific	BP350-1
Disposable reservoirs (sterile), 25 ML	Vistalab	3054-1033
EZFlow Sterile 0.45 µm PES Syringe Filter, 13 mm	Foxx Life Sciences	371-3115-OEM
L-glutamine	Gibco	25030-081
Multichannel pipette tips	ThermoFisher Scientific	94410810
Multichannel pipette, 15-1250 µL	ThermoFisher Scientific	4672100BT
P20, P200, and P1000 pipettes	Eppendorf	2231000602
pH Probe	Hanna Instruments	HI2210-01
Pipette tips, 20 µL, 200 µL, 1000 µL	Olympus	24-404, 24-412, 24-430
Seahorse XF Base Media	Agilent	103334-100
Seahorse XF Cell Mito Stress Test Kit	Agilent	103015-100
Seahorse XFe96 Analyzer	Agilent	S7800B
Seahorse XFe96 Spheroid Fluxpak Mini	Agilent	102905-100
Sodium bicarbonate	Fisher Scientific	BP328-500
Sodium pyruvate	Gibco	11360-070
Stereo Microscope	Olympus	SZX9
Syringe (sterile), 5 mL	BD	309603
Water (sterile)	Sigma	W3500-500mL

Comments/Description

For glucose solution, 200 mg/ml, sterile filetered
for loading multichannel pipet

200 mM (100x)

Recommended

Includes Oligomycin, FCCP, and Rotenone/Antimycin A
Including prep station with 37℃ non-CO2 incubator
Includes sensor cartridge, spheroid microplate, and calibrant

100 mM (100x)

For sterile filtration

AAAAAH384Q8=

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been reviewed for spelling and grammar.

2. Title/Keywords: Please remove “Seahorse” as it is a commercial term.

The title and keywords have been changes as requested.

3. Please define acronyms/abbreviations upon first use in the main text.

All abbreviations have been defined, with the exception of very common abbreviations such as “ATP”

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Cell-Tak, Seahorse, Agilent, etc.

All commercial language has been removed from the manuscript, and the Table of Materials is referenced where applicable.

5. 1.1.1: Please list an approximate volume of solution to prepare.

The volume has been specified.

6. 2.4.1: Please specify the size of pancreatic islets and the culture dish used.

Islet size was not directly measured, but islets of approximately similar size and morphology were selected by eye. A note explaining this has been added. The average islet size for Japanese macaques was noted. Culture dish size has been specified as requested

7. 2.4.4: Are 15 islets transferred to the same well in the microplate?

Yes. This is now stated explicitly.

8. Figure 1 legend: Do you mean red arrow instead of blue arrow?

Yes, this has now been modified.

9. Figures 2-4: Please define error bars in the figure legend.

Figures 2 and 3 do not have error bars—each line is a single replicate. Error bars for Figure 4 are standard deviation, and this is now stated in the representative results and figure legend sections.

10. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

The Table of Materials has been expanded to include all supplies, reagents, and equipment, and is now sorted alphabetically as requested.

11. References: Please do not abbreviate journal titles; use full journal name.

All journal names have been expanded.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript the protocol to measure oxygen consumption in intact islets isolated from Japanese Macaques using the XF96e Spheroid plates. It will be of great use to the islet research community to have videos of the execution of this protocol, as islet seeding on the XF96e plate can be challenging. I would recommend acceptance after addressing these concerns:

We thank the reviewer for these positive comments.

Major Concerns:

-An in-depth discussion comparing the islet isolation procedure, islet size and number of cells per islet between the Japanese Macaque, humans and mice would help readers understanding why 15 islets per well are needed, when compared to what was reported in mouse and human islets. This discussion would allow readers to evaluate whether the requirement for 15 islets is something specific for Macaques' islets or whether it reveals differences in the islet isolation procedure/picking criteria used in this protocol versus other publications.

We now reference the publication from our group describing the methodology for isolating islets from this species. This method is similar to that used for isolating islets from human cadaver pancreata, but differs from mice, in which the pancreas is often inflated with collagenase solution in situ under sedation prior to removal from the animal. We also now discuss how we tested lower numbers of islets with less reliable results, and how only very large human islets work with 1 islet/well. We also mention the average size of an NHP islet.

Minor Concerns:

-Filter pore size for the filtering of the Cell Tak solution is missing.

This has now been added to 1.1.1

-Add a comment on the presence of FBS and/or BSA changing the efficacy of FCCP action.

A note and reference about this has been added to 2.3.1.2.

-Define the criteria of islet picking and exclusion of biological material (i.e. damaged islets? size of islets?).

A note about islet picking has been added to 2.4.1, which describes the visual criteria used to pick islets that are intact while excluding damaged islets and non-endocrine tissue. Islet size was not directly measured.

-Explain why corner loading should be avoided. Is it just the corner or all wells in the rows located at the edge of the plate?

It is just the four corner wells, because oxygen and pH can flux out of the plastic, and this is exacerbated in the corner wells. A note about this has been added to 2.4.4

Reviewer #2:
Manuscript Summary:
The manuscript describes a protocol of using the Seahorse instrumentation in order to measure oxygen consumption of non-human primate pancreatic islets. The focus is on how the isolated islets can be placed into special wells for this specific device and how the wells should be prepared with special buffers from the company.

Major Concerns:
My major concern is that the actual clue of the procedure remains quite unclear. Moreover, the usability of this protocol to quantify oxygen consumption of other biological preparations is not well elaborated. Specialized multi-wells and a product referred to as "Cell-Tak" seems essential to correctly place and keep the islets and measure oxygen consumption of them within the device. What is the composition of the Cell-Tak? What is the outcome without using Cell-Tak? Without such controls the meaning of the results are rather poor.

Cell-Tak is composed of a formulated protein solution extracted from the marine mussel. The actual ingredients are proprietary. Cell-Tak per se is not required (poly-D-lysine can be substituted), but an adhesive reagent must be added in order to keep islets from moving up the sides of the well during the assay. This can happen as drugs are injected into the well or during mixing steps. All the above information has been added to section 1.1.2.

Minor Concerns:
In the legend to Figure 1 it should be rather "red arrow" than "blue arrow". The arrow has a blue line but is filled red.

Thank you for noticing this. It has been fixed.

Reviewer #3:
Manuscript Summary:
This manuscript provides a detailed description of how to measure oxygen consumption in islets

from non-human primates using the Seahorse XFe96 Spheroid Microplate. The methodology outlined in this manuscript will be especially useful to researchers in the field of islet biology.

We thank the reviewer for these positive comments.

Major Concerns:
How were the islet respiration data normalized? Did the authors extract DNA/protein from the islets after the experiment for normalization?

In these particular experiments, the data was not normalized. Equal numbers of qualitatively similarly-sized islets were added to each well for each sample. A paragraph explaining this, methods of normalization, and how information can be lost during normalization has now been added to the discussion.

Minor Concerns:
(1) 2.1.1. It will be helpful if the authors state the final glucose concentration in the Seahorse Base Media that was used for these experiments. Did the authors perform experiments with different baseline glucose concentrations?

We did not vary the glucose concentration in these experiments, although high glucose can be used as an additional stimulus of respiration if desired. Glucose was kept at 5.5 mM. This information has been added to 2.1.1.

(2) Pg 3, line 173 Can the authors please clarify what they mean by this sentence?

This step has been modified to be more clear.

(3) 2.5.2 I think it will be very helpful if, here, the authors provide details of their measurement cycles i.e. the number of cycles and the time for mix, wait and measure.

Information on the number of cycles and the times for mix and measure steps has been added to 2.5.2.

(4) Figure and table legends: It is not clear whether Figures 2 - 4 are from the same experiment. Can the authors please clarify? The legend from Figure 4 implies that this is the case but description of the representative results for figure 4 implies that they were separate experiments. Also, can the authors please state how many technical replicates were performed relating to the data in Figure 4?

Figures 2 -4 were from three different experiments. This is now made clear in the representative results section and figure legends. There were 16 technical replicates in Figure 4—this is now stated explicitly.

(5) Is there a reason why Cell Tak was used instead of Poly-D-Lysine? The latter was used in Taddeo EP (2018) Molecular Metabolism, Volume 16, Pages 150-159. Is it essential to use Cell Tak or can the plates be coated with other agents that facilitate cell adhesion?

Plates can be coated with other adhesive reagents such as Poly-D-Lysine. A note stating this has been added.