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Pancreatic Acinar Cell Preparation for Oxygen Consumption and Lactate Production Analysis

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[Abstract] Mitochondrial dysfunction is a principal feature of acute pancreatitis (AP) although the underlying mechanisms are still unclear. AP precipitants induce Ca^{2+}-dependent formation of the mitochondrial permeability transition pore (MPTP) in pancreatic acinar cells (PACs), leading to ATP depletion and necrosis. Evaluations of mitochondrial bioenergetics have mainly been performed in isolated PACs using confocal microscopy, with assessment of mitochondrial membrane potential, NADH/FAD^+ and ATP levels, coupled with patch-clamp electrophysiology. These studies are technically demanding and time-consuming. Application of Seahorse flux analysis now allows detailed investigations of bioenergetics changes to be performed in cell populations using a multi-well plate-reader format; rates of oxygen consumption (OCR) and extracellular acidification (ECAR) provide important information about cellular respiration and glycolysis, respectively. Parameters such as maximal respiration, ATP-linked capacity and proton leak can be derived from application of a respiratory function “stress” test that involves pharmacological manipulation of the electron transport chain. The use of Seahorse Flux analysis therefore provides a quick, and convenient means to measure detailed cellular bioenergetics and allows results to be coupled with other plate-reader based assays, providing a fuller understanding of the pathophysiological consequences of mitochondrial bioenergetics alterations.

Keywords: Mitochondrial dysfunction, Bioenergetics, Seahorse, Respiration, Glycolysis, Pancreatic acinar cells, Acute pancreatitis

[Background] Mitochondrial dysfunction is a core feature of acute pancreatitis (AP), a debilitating and potentially fatal disease for which there is currently no specific therapy (Criddle 2016; Habtezion *et al.*, 2019). The elucidation of pivotal pathological mechanisms which underlie mitochondrial damage in pancreatic acinar cells (PACs) is paramount for the development of new therapies. Previously evaluation of mitochondrial bioenergetics in isolated PACs have been mostly performed using confocal microscopy, including assessments of mitochondrial membrane potential (tetramethyl rhodamine methyl ester: TMRM), NADH/FAD^+ autofluorescence and ATP (Magnesium Green), coupled with patch-clamp electrophysiology (Voronina *et al.*, 2002; Criddle, *et al.,* 2004; Criddle *et al.*, 2006). Such studies have pinpointed a reduction of ATP in response to precipitants of AP, via the opening of the mitochondrial permeability transition pore (MPTP), as a critical event that leads to necrotic cell death (Criddle *et al.*, 2006; Mukherjee *et al.*, 2016); supplementation with intracellular ATP ameliorated damage. Furthermore,

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luciferase measurements in PACs have provided details of changes of both mitochondrial and cytosolic ATP concentrations induced by pathophysiological stimulation (Voronina *et al.*, 2010). Such experimental approaches in single cells, however, are technically difficult and time-consuming. In contrast, population-based assays provide important information about mitochondrial dysfunction and cell death using a convenient plate-reader format (Armstrong *et al.*, 2019). The use of Seahorse Flux analysis allows a detailed evaluation of bioenergetics changes to be performed in PACs, measuring rates of oxygen consumption (OCR) and extracellular acidification (ECAR); these inform about cellular respiration and glycolysis, respectively. A respiratory function "stress" test can further be applied in which pharmacological manipulation of the electron transport chain (ETC) is used to derive parameters such as the maximal respiration, Spare Respiratory Capacity, ATP-linked turnover and non-mitochondrial respiration. Such detailed bioenergetics information can be coupled with parallel studies of apoptosis and necrosis to inform the influence of mitochondrial dysfunction on cell death patterns (Armstrong *et al.*, 2018).

Materials and Reagents

1. 70 µm filters (Fisherbrand, catalog number: 22363548)
2. Tissue paper
3. 30G needle
4. 1.5 ml microcentrifuge tube
5. 15 ml falcon tube
6. Pipette tips
7. Male CD1 or C57BL6/J mice, 8-12 week old (Charles River).
8. Collagenase (Worthington Biochemical Corporation, Lakewood, NJ)
9. PI-Cassette™ (Chemometec, Nucleocounter, catalog number: 941-0001)
10. Reagent A100, Lysis buffer (SKU: 910-0003, Chemometec, Nucleocounter)
11. Reagent B; Stabilizing buffer (SKU: 910-0002, Chemometec, Denmark)
12. XF24 Fluxpak, containing cell plates, cartridge consisting of sensor and utility plates and Seahorse XF calibrant (Agilent, Seahorse, catalog number: 100850-100)
13. Matrigel basement membrane matrix (Corning, catalog number: 354234)
14. Dimethyl sulfoxide (DMSO, Sigma, catalog number: D2650)
15. Ethyl alcohol, Pure (Sigma, catalog number: 459836)
16. Oligomycin A (Sigma, Merck, catalog number: 75351)
17. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, C2920, Sigma, Merck)
18. Antimycin A from *Streptomyces* sp. (Sigma, Merck, catalog number: A8674)
19. Rotenone (Sigma, Merck, catalog number: R8875)
20. DMEM (Sigma, Merck, catalog number: D5030)
21. L-Glutamine 100x (Gibco, Life Technologies, catalog number: 25030-081)
22. D-Glucose (Sigma, Merck, catalog number: G7528)

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23. Sodium pyruvate (Sigma, Merck, catalog number: P8574)
24. Sodium chloride (Sigma, Merck, catalog number: S9888)
25. Potassium chloride (Sigma, Merck, catalog number: P3911)
26. Magnesium chloride (Sigma, Merck, catalog number: M1028)
27. Calcium chloride (Sigma, Merck, catalog number: 21115)
28. Extracellular solution (see Recipes)
29. Seahorse media (see Recipes)

Equipment

1. Surgical scissors
2. Water bath
3. -20 °C freezer
4. Seahorse XF24 Extracellular Flux Analyser (Agilent)
5. Cell Counter NucleoCounter® NC-100™ (900-0004, ChemoMetec, Denmark)
6. Inverted light microscope

Software

1. Wave (Seahorse, Agilent. https://www.agilent.com)
2. Prism (GraphPad Software Inc., La Jolla, CA. https://www.graphpad.com)

Procedure

Day 1

A. *Pancreatic acinar cell preparation*

1. Terminate young (8-12 week old) adult CD1 or C57BL/6 (wild type) mouse using an approved Schedule 1 procedure.
2. Place mouse down on right hand side on fresh tissue paper.
3. Using forceps and scissors cut away fur in the side abdominal area. Once removed make a similar removal of lower skin epidermis to expose the abdominal cavity.
4. Locate the pancreas below the spleen gently with forceps and cut free with a small pair of surgical scissors.
5. Place the freshly excised pancreas in 7 ml extracellular solution and keep on ice until digestion.
6. Pre-warm 1 ml of collagenase (200 U ml^{-1}) to 37 °C in a water bath.
7. Carefully inject the pre-warmed collagenase using a 30G needle into the pancreas samples placed in a weighing boat until the pancreas inflates. Draw up any remaining collagenase not retained within the pancreas from the weighing boat and re-inject into pancreas.
8. Place the inflated pancreas into a 1.5 ml microcentrifuge tube and add any remaining

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collagenase.

9. Place in a 37 °C water bath for 17 min
   Note: This time is dependent on the brand and concentration of collagenase used. You may need to optimize so that you get a optimal amount of cell viability and cell number achieved.
10. After incubation for 17 min remove from water bath and decant contents of the 1.5 ml microcentrifuge tube into a 15 ml falcon tube labelled 'Panc', quickly add 5 ml of extracellular solution.
11. Using a 1 ml pipette tip, cut at an angle across the tip and the volume set at 700 µl vigorously pipette the whole pancreas tissue up and down in and out of the pipette tip to allow for tissue dissociation (trituration).
12. Repeat pipetting up and down until the mixture becomes cloudy with the release of cells.
13. Upon cessation of pipetting quickly remove the cellular solution into a fresh 15 ml tube labelled 'Cells', using a squeezy pipette being careful to leave behind any pieces of pancreas tissue.
14. Add 5 ml of extracellular solution to the 15 ml tube 'Panc'.
15. Using a fresh 1 ml pipette tip, cut at smaller angle than previously across the tip and the volume set at 700 µl vigorously pipette the whole pancreas tissue in and out of the pipette tip to allow further tissue dissociation.
16. Upon cessation of pipetting quickly remove the cellular solution into 15 ml tube 'Cells', using a squeezy pipette and being careful to leave behind any pieces of pancreas tissue.
17. Repeat Steps A14-A16 for a third and final time.
18. Discard any tissue remaining in a 15 ml tube labelled 'Panc'
19. Centrifuge 15 ml tube labelled 'Cells' for 2 min, 130 *x g*, room temp.
20. Carefully pour off the supernatant leaving behind the loose cell pellet.
21. Resuspend the cell pellet in 1 ml of extracellular solution.
22. Add an additional 9 ml of extracellular solution.
23. Set up a 70 micron filter in a 50 ml tube (labelled and dated) and pre-wet with a small amount of extracellular fluid.
24. Filter the 10 ml of cell suspension from the 15ml tube 'Cells' through the filter into the 50 ml tube.
25. Rinse the filter with plenty of extracellular fluid until the tube is filled to the 40 ml mark.
26. Centrifuge 50 ml tube labelled 'Cells' for 2 min, 130 *x g*, room temp.
27. Carefully pour off the supernatant leaving behind the loose cell pellet.
28. Resuspend the cell pellet in 1 ml of extracellular solution.
29. Add an additional 9 ml of extracellular solution.
30. Place the tube overnight in at 4 °C.

B. Oxygen consumption and lactate production analysis cell plate preparation and cartridge rehydration

1. Remove a Seahorse XF 24 well cell plate and remove from packaging
2. Take a pre-aliquotted 250 µl vial of Matrigel from storage in a -80 °C freezer and thaw on ice.

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3. Adapt Corning's 'Thin Coating Method' for use on a Seahorse XF24 cell plate as follows:
4. Using cooled pipettes, mix the BD Matrigel Basement Membrane Matrix to homogeneity.
5. Dilute Corning Matrigel Basement Membrane Matrix using serum-free medium Seahorse media.
6. Carefully add 36 µl diluted BD Matrigel Basement Membrane Matrix to Seahorse XF 24 well cell plate being sure not to create any bubbles.
7. Incubate at room temperature for 30 min.
8. Aspirate unbound material and gently rinse using serum-free Seahorse media.
9. Pipette 100 µl serum-free Seahorse media into each well and leave at 4 °C until ready for use.
10. Remove a Seahorse XF 24 cartridge from packaging.
11. Remove the sensor plate (green upper half of the cartridge) and place lid down on the bench.
12. Pipette 1 ml of XF callibrant into each of the 24 wells of the utility plate and return the sensor plate so the probes of each well are submerged in XF callibrant.
13. Place in a 37 °C CO_2 free incubator overnight until ready for use.

Day 2

C. Assess pancreatic cell viability

1. The next day the cell suspension is mixed to obtain a homogenous suspension by gently pipetting up and down using a 1 ml pipette.
2. Pipette a 1 ml cell sample from the cell suspension into a 1.5 ml microcentrifuge tube.
3. Label a PI-Cassette™ 'NV' (non-viable).
4. Draw the cell suspension into a PI-Cassette™ by inserting the tip of the cassette into the cell suspension in the 1.5 ml microcentrifuge tube and pressing the piston.
5. Set aside the 'NV' PI-Cassette™ to be analysed.
6. Pipette 100 µl of the 1ml cell sample from the 1.5 ml microcentrifuge tube into a second 1.5 ml microcentrifuge tube. Return any cell sample remaining to the original cell suspension 50 ml centrifuge tube 'Cells'.
7. Add 100 µl volume of Reagent A100 to the microcentrifuge tube with the 100 µl cell sample.
8. Mix by pipetting.
9. Add 100 µl volume of Reagent B to the mixture of cell suspension and Reagent A100.
10. Mix by pipetting.
11. Label a PI-Cassette™ 'T' (total)
12. Draw the diluted cell suspension into a PI-Cassette™ by inserting the tip of the cassette into the cell suspension and pressing the piston.
13. Immediately place the loaded PI-Cassette™ in the NucleoCounter® NC-100™sample tray, press RUN.
14. After approximately 45 s the total cell concentration (cells/ml) is presented in the bottom right.
15. The cell count produced will normally be in the order of x 10^5. Divide by 10 to achieve x 10^6 for the following calculations
16. Calculate total cell number and percent viability as follows:

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Cell viability = ['NV' / ('T' x 3)] x 100
Total viable cell count = [('T' x 3) – 'NV'] x volume of cell suspension

Examples of total cell count and percent viability previously achieved when isolating acinar cells from two separate pancreas (taken from two animals) at the same time and pooled for analysis are shown in Table 1.

Table 1. Examples of total cell count and percent viability previously achieved

Total read out	Total cell count	Non-viable cell count	Viability (%)	Volume (ml)	Total cell count (x10 ^{6})
0.324	0.972	0.137	85.9	10	8.35
0.211	0.633	0.091	85.6	10	5.42
0.462	1.386	0.161	88.4	10	12.25
0.351	1.053	0.018	98.3	10	10.35
0.302	0.906	0.165	81.8	10	7.41
0.326	0.978	0.145	85.2	10	8.33
0.238	0.714	0.152	78.7	10	5.62
0.224	0.672	0.101	85.0	10	5.71
0.372	1.116	0.207	81.5	10	9.09
0.364	1.092	0.219	79.9	10	8.73
		Mean ± S.D	85.0 ± 5.6		8.1 ± 2.2

17. Centrifuge 50 ml tube labelled 'Cells' for 2 min, 130 *x g*, room temp.
18. Carefully pour off the supernatant leaving behind the loose cell pellet.
19. Resuspend the cell pellet with 1 ml of extracellular solution.
20. The cell suspension needs to be adjusted to a concentration of 1 x 10^6/ml. This is achieved by adjusting the volume to the same value as the total number of cells. (Total viable cell count = 5 x 10^6, adjust cell volume to 5 ml). Centrifuge 50 ml tube labelled 'Cells' for 2 min, 130 *x g*, room temp.
21. The cells are now ready to be seeded onto the Matrigel coated Seahorse XF 24 well cell plate (Figure 1).
22. Remove the XF 24 well cell plate and remove the 100 µl serum-free Seahorse media from each well.
23. Continuously mixing the cell suspension during seeding by pipetting up and down, transfer 75 µl of cell suspension into each well leaving the following cells blank to give a total of 75,000 murine pancreatic cells per well.

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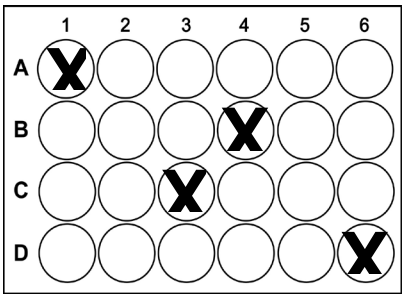

Figure 1. Well allocation for blank well for XF24 cell plate

24. Place the lid back onto the XF24 cell plate and place in a 37 °C CO_2 free incubator for 1 hour for the cells to adhere.
25. Whilst the cells are adhering the XF24 cartridge can be loaded with the compounds that comprise a mitochondrial “stress” test.
26. After one hour remove the 75 µl of extracellular solution from the cells. Quickly add 450 µl pre-warmed serum free Seahorse media to every well. Return to a 37 °C CO_2 free incubator until ready for use.

D. Preparing and loading compounds for the mitochondrial “stress” test

1. There are two types of assays that can be performed:
   Standard Assay – only involves the injection of modulators included in the kit.
   Modified Assay – includes an additional injection of a test compound prior to oligomycin injection, and Port A is used for the testing compound.
2. Refer to Table 2 for loading volume and port designation for compounds in different types of assays.

Table 2. Loading regime for mitochondrial stress test

Port	Standard Assay	Modified Assay	Port Concentration	Port Volume
A	Oligomycin	Test compound	10x	50
B	FCCP	Oligomycin	10x	55
C	Rotenone & Antimycin A	FCCP	10x	62
D	Empty	Rotenone & Antimycin A	10x	68

3. Make a working concentration of 10x the final required concentration required from stock solutions stored at -20 °C as follows:
   a. Oligomycin stock solution in DMSO
      10 µl in 1 ml of Seahorse media
      For a 10 x solution of 10 µg/ml for a final concentration of 1 µg/ml.
   b. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) stock solution in DMSO

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3 µl in 1 ml of Seahorse media
For a 10 x solution of 3 µM for a final concentration of 0.3 µM.

c. Rotenone stock solution in DMSO and Antimycin A stock solution in ethanol
10 µl of each in 1 ml of Seahorse media
For a 10 x solution of 20 µg/ml for a final concentration of 2 µg/ml.

4. Make a vehicle control solution for any well or port that is not receiving a compound or stress test with appropriate amounts of DMSO/Ethanol or other vehicle utilized for specific compounds.
5. Remove both the sensor plate and the corresponding utility plate together from the 37 °C CO_2 free incubator and remove the lid.
6. Load the corresponding volume of each compound appropriate for the assay type into the appropriate port on the Sensor plate.
7. Once all the compounds have been added to the ports, the lid can be replaced on to the Sensor plate and return to a 37 °C CO_2 free incubator until ready for use.

E. Oxygen consumption and lactate production analysis

1. Programme the Seahorse Extracellular Flux instrument as follows:
   a. Calibration
   b. Equilibration
   c. [Mix – 2 min, Wait – 2 min, Measure – 2 min] x 5 to ensure a stable baseline
   d. Inject Port A
   e. [Mix – 2 min, Wait – 2 min, Measure – 2 min] x 3 for standard assay oligomycin injection or x 5 typically for modified assay which gives 30 min for any test compound application.
   f. Inject Port B
   g. [Mix – 2 min, Wait – 2 min, Measure – 2 min] x 3 for standard assay FCCP injection and also x 3 for modified assay, oligomycin injection
   h. Inject Port C
   i. [Mix – 2 min, Wait – 2 min, Measure – 2 min] x 2 for standard assay Antimycin A/Rotenone injection and x 3 for modified assay, FCCP injection
   j. Inject Port D (modified assay only)
   k. [Mix – 2 min, Wait – 2 min, Measure – 2 min] x 2 for modified assay only, Antimycin A/Rotenone injection
2. After reviewing the group definitions, plate map layout, click Start Run.
3. After you enter the save location for your result file (following completion of the assay), the tray door on the XF24 Analyser will open.

Important! Before starting calibration, ensure:

4. The sensor cartridge fits properly on the Utility plate.
5. The lid is removed from the sensor cartridge.
6. Proper orientation (direction) of the sensor cartridge on the Utility plate.
7. Place the sensor cartridge (hydrated and loaded with compounds) and Utility plate onto the tray

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when prompted.

8. Press “Start” to initiate sensor cartridge calibration.
9. Time to complete calibration is approximately 10-20 min (for assays at 37°C). For XF assays performed at temperatures other 37°C an additional 30 min of pre-calibration time will be added to ensure accurate data acquisition.
10. Once sensor cartridge calibration is complete, the instrument controller will display the Load Cell Plate dialogue
11. Click Open Tray to eject the Utility plate and load the Cell Plate on the tray. The sensor cartridge remains inside the XF Analyser for this step.

Important! Before loading the Cell plate, ensure:

12. The lid is removed the Cell Plate.
13. Proper orientation (direction) of the Cell Plate on the tray.
14. After placing the Cell Plate on the tray, click Load Cell Plate to initiate equilibration.
15. After completing equilibration, the assay will automatically begin acquiring baseline measurements (as outlined in your instrument protocol).
16. Once the final measurement command in the instrument protocol is completed, Wave Controller software will display the Unload Sensor Cartridge dialog.
17. Click Eject when ready to eject the sensor cartridge and cell plate. Set aside for later analysis if necessary (example - cell count normalization).
18. After removing the sensor cartridge and cell plate, the Assay Complete dialogue will appear.
19. Click View Results to immediately open your assay result file. Download both files for the experiment
20. Excel file
21. Xfd. file

Data analysis

1. Open the Xfd file in Wave software.
2. You need to change the file format into a Prism file by selecting ‘save as’ and then choosing the .pzfx format.
3. The data can now be opened directly in prism.
4. Each experimental condition needs at least 3 wells per plate and for statistics needs at least n = 6 plates using fresh pancreas isolated from a separate mouse each time.
5. Exclusion criteria:
   a. Unstable baseline, usually when the baseline shows a steady rate of decline excessively low or high baseline <200 or > 800 pMol/min OCR.
   b. Lack of response by stress test control wells to stress test compounds.
   c. Outlier data points – entire series for a well and/or individual data points

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Notes

Important! – Before you start your XF Assay

1. Visually inspect the injection ports for even loading. The liquid should be in the port, make sure there are no residual drops on the top of the sensor cartridge.
2. View cells under a microscope to:
   a. Confirm cell health, morphology, seeding uniformity and purity (no contamination).
   b. For *adherent cells*, ensure cells are adhered with a consistent monolayer and were not washed away during washing step.

Recipes

1. Extracellular solution (mM)
   140 mM NaCl
   4.7 mM KCl
   1.13 mM $MgCl_2$
   1 mM $CaCl_2$
   10 mM D-glucose
   10 mM HEPES (adjust to pH 7.25 using NaOH)
2. Seahorse media
   a. Prepare DMEM by adding 800 ml dH_2O to powdered DMEM.
   b. Prepare assay medium by supplementing DMEM medium with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose as in the table below (Table 3):

Table 3. Recipe for serum free non-buffered Seahorse media

Reagent/Part Number	Final Concentration	Volume
DMEM Medium	-	1.0 L
D-Glucose (1.0 M solution)	10 mM	1.8 g
Pyruvate (100 mM solution)	1 mM	10 ml
L-Glutamine (200 mM solution)	2 mM	10 ml

   c. Bring XF medium with supplements to pH 7.4 and adjust the final volume to 1 litre, transfer into a Class 2 microbiological safety cabinet.
   d. Filter with a filter pipette into sterile 50 ml aliquot centrifuge tubes. Store in a fridge for up to 1 month.

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Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Ethics

The animals were humanely sacrificed by increasing CO_2 (schedule 1 procedure) in accordance with the Animals (Scientific Procedures) Act (1986) under Establishment License 40/2408 with approval by the University of Liverpool Animal Welfare Committee and Ethical Review Body (X70548BEB and PPL 70/8109).

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