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Cell-based Measurements of Mitochondrial Function in Human Subjects

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Abstract

There is growing evidence in the basic science field that aberrant metabolism plays an important role in tumorigenesis. Therefore, it is imperative to perform investigations in human subjects to determine: 1) whether the metabolic observations made in model systems are applicable to humans; and 2) if indeed applicable, whether the metabolic alterations are clinically significant for cancer development. As an initial step, here we describe methods for measuring the mitochondrial metabolism of blood lymphocytes and skeletal muscle myoblasts that can be obtained from human subjects.

Keywords

mitochondria; respiration; human; lymphocytes; myoblasts

1. INTRODUCTION

One proposed reason that cancer cells reprogram their metabolic activities is for survival and proliferation under adverse cellular environments. Although some types of cancer cells exhibit the “Warburg effect” (Warburg, 1956), growing evidence indicate that this increase in aerobic glycolysis is not intrinsically associated with cancer but, rather, that it may provide adaptive or growth advantages under specific conditions such as hypoxia (Zu and Guppy, 2004). Many cancer cells actually exhibit a concurrent increase in mitochondrial respiration and glycolysis as detailed in a comprehensive review of the Warburg effect (Koppenol et al., 2011). For example, increased oxygen consumption is associated with higher tumorigenic potential, and a subset of diffuse large B cell lymphoma has recently been shown to display a metabolic signature of increased oxidative phosphorylation and fatty acid oxidation (Ramanathan et al., 2005; Caro et al., 2012). In our own recent translational study, we have observed that both lymphocytes and myoblasts of patients with Li-Fraumeni syndrome, an autosomal dominant premature cancer syndrome caused by germline mutations of *TP53*, exhibit increased oxidative metabolic activity (Wang et al., 2013). Therefore, further studies measuring mitochondrial function in tissue samples obtained from human subjects with different conditions may provide insights into the

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contribution of mitochondrial function in cancer pathogenesis that may have clinical implications.

2. BLOOD LYMPHOCYTE PROTOCOL

Human lymphocytes are prepared using a protocol as previously described with some modifications (Jackson et al., 2004). Peripheral blood mononuclear cells (PBMCs) are collected by density centrifugation using citrate-containing Vacutainer CPT tubes. The PBMCs are then activated on immobilized anti-CD3 and anti-CD28 antibodies for 3 days. Anti-CD3 antibody specifically activates the T cells to proliferate via the T cell receptor complex. The stimulated T cells are subsequently cultured in RPMI media supplemented with IL-2 for 1 to 3 weeks and then attached to Seahorse XF-96 plates, which are pre-coated with Cell-TAK for metabolic analysis.

2.1. Materials

1. Citrate-containing vacutainer Cell Preparation Tubes (CPT) (Becton Dickinson, cat# 362761)
2. Human recombinant IL-2 (Boehringer Mannheim, cat# 799068)
3. Anti-human CD3e antibody (clone OKT3) (eBioscience, cat# 16-0037-85)
4. Anti-human CD28 antibody (clone CD28.2) (eBioscience, cat# 16-0289-85)
5. 10% BSA solution (MACS cat# 130-091-376)
6. Phosphate buffered saline (PBS), pH 7.4
7. 24-well tissue culture plate
8. 50 ml sterile conical tube
9. Trypan blue
10. Dimethylsulfoxide (DMSO)
11. RPMI 1640 with GlutaMAX (Invitrogen, cat# 61870-036)
12. Fetal bovine serum (FBS) (certified, US origin, Invitrogen, cat# 16000-044)
13. Penicillin-Streptomycin (P/S, 10,000 U/ml and 10,000 µg/ml, respectively)
14. β-Mercaptoethanol (BME, 55 mM) (Invitrogen cat # 21985)
15. Complete RPMI medium (supplemented with 10% FBS, 1% P/S and 20 µM BME)
16. XF96 4-port FluxPak (comprised of sensor cartridges, utility plates, cell culture microplates, and a calibrant solution) (Seahorse Bioscience cat# 102310)
17. XF assay medium (Seahorse Bioscience, cat# 102352)
18. BD Cell-Tak (BD Biosciences, cat# 354240)

2.2. Isolation of peripheral blood mononuclear cells

Note: Step 1–3 are performed at room temperature and the rest of the steps are at 4 °C or on ice with pre-chilled solutions.

1. If T cell culturing as described in section 2.3 is anticipated, 24 well plates need to be coated with anti-CD3e antibody the day before PBMC isolation. Prepare by pipeting 300 µl of an anti-CD3e antibody solution (5 µg/ml in sterile PBC solution) into each well of a 24 well tissue culture plate and incubate at 4 °C overnight (alternatively, 37 °C for 2 hrs may be an option).
2. Draw blood directly into citrate-containing Vacutainer CPT tubes (~8 ml per tube).
3. Mix by gentle inversion and then centrifuge the tubes at $1800 \times g$ at room temperature for 20 min.
4. In a tissue culture hood, transfer the mononuclear cell layer (2–3 ml volume per CPT tube) containing lymphocytes and monocytes into a 50 ml sterile conical tube (Figure 1).
5. Add at least three volumes of 0.5% BSA PBS to the mononuclear cell layer to bring up the total volume to ~15 ml and then centrifuge at $300 \times g$ at 4 °C for 10 min.
6. Discard the supernatant, wash the cell pellet by resuspending in 10 ml of 0.5% BSA in PBS, and centrifuge at $300 \times g$ at 4 °C for 10 min.
7. Resuspend the cells again in 10 ml of 0.5% BSA in PBS and count with Trypan blue. The expected yield is $\sim 1 \times 10^6$ mononuclear cells per ml of blood.

2.3. T cell culture

Note: The isolated peripheral blood mononuclear cells can be cultured at this point to expand T cells or they can be resuspended in FBS supplemented with 7% DMSO at a concentration of 2×10^6 cells/ml and stored in liquid nitrogen for future experiments. Human T cells can proliferate in culture for up to 3 to 4 weeks.

1. The PMBCs are centrifuged again at $300 \times g$ at 4 °C for 5 min and then resuspended at 2×10^6 cells/ml in complete RPMI media containing 1 µg/ml anti-CD28 antibody.
2. Remove the previously incubated anti-CD3e antibody solution from each well of the 24 well tissue culture plate (see section 2.2) and rinse once with PBS.
3. Dispense 1 ml of the PBMC suspension into each antibody-coated well to co-stimulate the T cells for 3 days.
4. Harvest the stimulated T cells by centrifuging at $300 \times g$, wash once with complete RPMI media, and resuspend at 1×10^6 cells/ml in the medium supplemented with IL-2 (10 U/ml). T cells can then be expanded for 1 to 3 weeks under this condition.

2.4. Measuring respiration in T cells using the Seahorse XF96 analyzer

Note: Unless otherwise specified, all 37 °C incubations should be performed in room air without CO₂ supplementation. Mitochondrial respiration can be measured using a Seahorse Analyzer that is designed for either 24- or 96-sample format, which requires appropriate cell number and volume adjustments. The Seahorse XF96 Extracellular Flux Analyzer uses a 96-well microplate assay format, and in our experience the optimal density for human T cells is $2-4 \times 10^5$ cells per well which may need to be adjusted for other cell types. Measuring uncoupled respiration requires the addition of an uncoupler such as FCCP which needs to be titrated depending on the cell type. Detailed guides for using the Seahorse Analyzer can be found at the following website (<http://www.seahorsebio.com/learning/protocols.php>).

1. The day before the metabolic assay, add 200 µl of Seahorse Bioscience XF Calibrant pH 7.4 to each well of the XF-96 utility plate, position the sensor cartridge on top of the utility plate, and incubate at 37 °C overnight.
2. Prepare Cell-Tak solution by adding 73 µl of 1.5 mg/ml stock concentration Cell-Tak to 1.1 ml sterile H₂O. This results in a final concentration of 0.1 mg/ml and is of sufficient volume for one XF96 microplate.
3. Add 10 µl of the Cell-Tak solution to each well of a XF96 cell culture microplate, and then add 40 µl of 0.1 M NaHCO₃ (pH 8.0) to each well to neutralize and promote Cell-Tak adsorption to the microplate.
4. Incubate the microplate at 37 °C for 1 hr to overnight.
5. Pre-warm the Seahorse XF assay media (supplemented with 25 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate) to 37 °C and adjust pH to 7.4 with 1 M KOH.
6. Count cell number, centrifuge the cells at $300 \times g$ for 5 min and resuspend in the supplemented XF assay media at 2.5×10^6 cells/ml.
7. Wash each well of the Cell-Tak coated XF96 cell culture microplate with 200 µl of sterile water.
8. Add 120 µl of cell suspension to each well.
9. Centrifuge the microplate containing the cell suspension at $700 \times g$ for 2 min to facilitate cell attachment.
10. Incubate at 37 °C for at least 1 h to allow cell attachment.
11. Up to four different compounds can be injected via ports A, B, C, and D at the appropriate concentrations to modulate mitochondrial respiration. It is important to make the appropriate concentrations of injected compounds to adjust for the sequential increase in total volume after each injection. In an example of a four-compound injection experiment, the cells are in a starting volume of 120 µl and the total volume increases to 200 µl with the addition of four sequential 20 µl injections of compounds. To adjust for the increase in volume, the stock concentrations of the injected compounds are appropriately diluted in warm XF medium to provide the indicated final concentration at each of the respective final volume. Hence, the

order in which the injections are made cannot be altered after the diluted compounds have been prepared.

Port	Compounds	Stock conc.	Final conc. (vol.)	Injection compound dilution
A	Oligomycin	50 mM	2.5 μ M (140 μ l)	1.5 μ l stock + 4.28 ml media
B	FCCP	5 mM	1 μ M (160 μ l)	6 μ l stock + 3.75 ml media
C	FCCP	5 mM	2 μ M (180 μ l)	6 μ l stock + 3.33 ml media
D	Antimycin A	5 mM	1 μ M (200 μ l)	6 μ l stock + 3 ml media

12. Aliquot 20 μ l of each compound into the corresponding ports of the sensor cartridge including the control wells for background correction.
13. Reposition the sensor cartridge over the utility plate and place them in the 37 °C incubator for at least 30 min.
14. Load the pre-warmed sensor cartridge and utility plate into the instrument and start calibration.
15. After the calibration is complete, replace the calibrant utility plate with the cell-attached XF96 cell culture microplate and run the XF software program. The data analysis is briefly summarized in section 4.

3. SKELETAL MUSCLE MYOBLAST PROTOCOL

The needle biopsy of the human skeletal muscle must be performed by a trained operator using sterile clinical grade equipments and anesthetics with appropriate approval as required by the investigator's institutional review board (IRB). The biopsy technique itself is not described here as it is beyond the scope of an experimental methods chapter. Needle biopsy specimens can be obtained from an accessible skeletal muscle group such as the *vastus lateralis* (lateral quadriceps of the leg). In our experience, a standard skeletal muscle biopsy needle (Ref# 8065, Cadence Science) can yield up to 50–100 mg of tissue from which about 25–50 mg is used for optimal myoblast preparation. The myoblast culture protocol is a modification of a previously described quantitative technique for growing adult skeletal muscle cells (Yasin et al., 1977; Taanman et al., 1997). Briefly, the muscle tissue is enzymatically dissociated using a combination of trypsin and collagenase, and the myoblasts are expanded in medium supplemented with uridine and high concentration of fetal bovine serum under reduced atmospheric oxygen (5% O₂).

3.1 Materials

1. DMEM (supplemented with GlutaMax-I, 4.5 g/l glucose, 1 mM pyruvate) (Invitrogen cat# 10569-010)
2. Fetal bovine serum (FBS) (certified, US origin, Invitrogen, cat# 16000-044)
3. Uridine (20 mM stock solution)
4. Penicillin-Streptomycin (P/S, 10,000 U/ml and 10,000 μ g/ml, respectively)
5. Trypsin (2.5%) (Invitrogen-Gibco, cat# 15090-046)
6. Versene (~0.5 mM EDTA in PBS) (Invitrogen-Gibco, cat# 15040-066)

7. Collagenase, type II (555 units/mg) (Sigma-Aldrich, cat# C6885)
8. BSA (essentially fatty acid free) (Sigma-Aldrich, cat# A8806)
9. 60 mm tissue culture dish (collagen type I coated) (BD cat# 354401)
10. 75 cm² vented flask (collagen type I coated) (BD cat# 354485)
11. Cell strainer (40 µm nylon) (BD, cat# 352340)
12. Sterile glass beaker (100 ml)
13. Sterile magnetic stir bar
14. Sterile hypodermic needle
15. Sterile aluminum foil
16. Water bath with shaker
17. Tissue culture incubator with 5% CO₂ and 5% O₂ capability
18. Diluted trypsin solution:

<u>Reagent</u>	<u>Stock conc.</u>	<u>Volume</u>	<u>Final conc.</u>
Trypsin	2.5%	4 ml	0.1 %
Versene (EDTA in PBS)	~0.5 mM	100 ml	~0.5 mM
Total		104 ml	

19. Enzymatic dissociation solution (prepare fresh before each experiment):

<u>Reagent</u>	<u>Stock conc.</u>	<u>Volume/mass</u>	<u>Final conc.</u>
Collagenase, type II	555 U/mg	21.6 mg	240 U/ml
BSA (fatty acid free)		40 mg	
DMEM medium		10 ml	
Filter the above mix through a 0.2 µm filter into a 50 ml sterile tube, then add:			
Diluted trypsin solution	0.1 %	3.2 ml	0.0064%
DMEM medium		40 ml	
Total		50 ml	

20. Complete myoblast medium:

<u>Reagent</u>	<u>Stock conc.</u>	<u>Volume</u>	<u>Final conc.</u>
DMEM		500 ml	
FBS	100%	100 ml	20%
P/S		3 ml	50 U/ml and 50 µg/ml
Uridine	20 mM	60 µl	2 µM
Total		603 ml	

3.2 Myoblast culture

Note: Care should be taken to split the primary human myoblast cultures before they reach confluency because they can fuse and form myotubes. The myoblasts should be maintained below 70% confluency and can be passaged about 10 times before they lose proliferative capacity. Unless indicated otherwise, it is important to note that human myoblasts are

maintained in 5% CO₂ under reduced oxygen tension (5% O₂) to maintain their proliferative potential.

1. Use 25–50 mg of skeletal muscle needle biopsy sample. A minimum of 25 mg is recommended for successfully culturing and expanding human myoblasts.
2. Place the muscle tissue in a sterile 100 ml beaker and add 10 ml of enzymatic dissociation solution. Using two sterile needles mounted on syringes to tease the muscle tissue apart for about 3 min.
3. Cover the beaker with a sterile aluminum foil and incubate it in a water bath at 37 °C with slow (40 rpm) shaking for 12 min.
4. Add 10 ml of complete myoblast medium which inactivates trypsin and triturate the muscle fragments by pipetting up and down 10 times through a 10 ml pipette.
5. Allow the muscle fragments to settle, then use a 10 ml pipette to transfer the supernatant and filter through a cell strainer (40 µm nylon) into a sterile 50 ml conical tube.
6. Add another 10 ml of enzymatic dissociation solution to the beaker with residual muscle fragments and incubate at 37 °C for 12 min.
7. During this time, centrifuge the supernatant from step 5 in the 50 ml tube at $300 \times g$ for 10 min to sediment the cells. Aspirate off the supernatant, resuspend the cell pellet in 0.5 ml of complete myoblast medium, and place the loosely capped 50 ml tube in a 37 °C humidified incubator (5% CO₂ and 5% O₂).
8. Perform 2 to 3 cycles of steps 2 to 7 until the muscle tissue fragments are essentially dissociated.
9. Pool the cells suspended in 0.5 ml of complete myoblast medium and transfer to a collagen-coated 60 mm culture dish. Add an additional ~4 to 6 ml of complete medium to the dish and mix by swirling.
10. Incubate the cells and change the media every 3 to 4 days until the cells reach ~70% confluency at which time they can be gently trypsinized and replated on larger collagen-coated 75 cm² vented flask.
11. Myoblasts can be resuspended in complete myoblast medium containing 7% DMSO and stored in liquid N₂ for future use. (Remember to thaw out and maintain human myoblasts under 5% O₂ condition for improved viability unless 21% O₂ is required for experimental purposes.)

3.3 Measuring respiration in myoblasts using the Seahorse XF24 analyzer

Note:

1. Unlike mononuclear cells that require a smaller surface area for attachment, myoblasts need more space provided by the 24-well format of the Seahorse XF24 Extracellular Flux Analyzer. A uniform distribution of cells over the bottom of the well is also critical for consistent results.

2. Optimal cell numbers should be used for the experiment. Depending on the cell type, the oxygen consumption rate (OCR) should fall within the linear range of 50 to 500 pmol/min and the extracellular acidification rate (ECAR) should be higher than 20 milli-pH/min.
 1. Gently dispense 100 μ l of cell suspension containing around 30,000 myoblasts into the bottom of a XF24 cell culture microplate well. Allow 1 to 5 hr for cell attachment (~80% confluent) then gently add an additional 150 μ l of the complete myoblast medium for overnight culture. Four wells (A1, B4, C3 and D6) containing only medium are used as the temperature correction controls.
 2. Pre-incubate the sensor cartridge and utility plate containing the calibrant solution overnight in a non-CO₂ room air 37 °C incubator.
 3. Leave the XF24 analyzer with the software running overnight to stabilize the analyzer.
 4. Next day, check that the analyzer temperature is at 37 °C.
 5. Pre-warm the Seahorse assay medium and reagents to 37 °C.
 6. Replace the myoblast medium with the Seahorse XF assay medium, which should be made fresh by adding 25 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate prior to adjusting the pH to 7.4 with 1 M KOH. The wells should not be allowed to dry during media changes. Rinse once with 1 ml of the XF assay medium then add 700 μ l of the medium to each well.
 7. Incubate the cell plate in a non-CO₂ incubator at 37 °C for 1 hr before starting the metabolic measurements.
 8. As described in Section 2.4, step 11, up to four different compounds placed in the sensor cartridge can be injected via ports A, B, C, and D at the appropriate concentrations to modulate mitochondrial respiration. The working stocks of the compounds are prepared as previously described except a larger volume of 75 μ l is loaded.
 9. Calibrate the analyzer by loading the sensor cartridge and the calibrant solution-containing utility plate into the instrument tray ensuring correct orientation.
 10. When complete, replace the calibration utility plate with the cell-containing microplate for the assay.

4. DATA ANALYSIS

In this chapter, we describe an *in vitro* approach for measuring aerobic metabolism in tissue samples obtained from human subjects. We have focused on mitochondrial function using the Seahorse XF Analyzer although it also measures extracellular acidification representing glycolysis. A basic whole cell Seahorse assay provides the rate of oxygen consumption in the following states using specific pharmacologic compounds: state 3 respiration that is

coupled to ATP synthesis; state 4 respiration in the absence of ATP synthesis; maximum mitochondrial respiration that can be achieved by uncoupling to ATP synthesis; and non-mitochondrial oxygen consumption (Figure 2). These measurements can be further analyzed with statistics software such as GraphPad Prism or GraphPad InStat (GraphPad Software, Inc.). Detailed guides to data analysis can be found at the following resource website (<http://www.seahorsebio.com/learning/protocols.php>).

Basal ADP-dependent respiration coupled to ATP synthesis (state 3) is affected by many factors such as substrate availability, cell proliferation rate and cellular energy requirements. In our study of human lymphocytes and myoblasts, we have observed that alterations in respiratory chain components can affect mitochondrial respiration (Wang et al., 2013). For example, the basal OCR values obtained using T cell samples of different human subject show a significant positive correlation with the levels of mitochondrial respiratory complex V ATP synthase subunit α protein (Figure 3).

Respiration in the absence of ATP synthesis (state 4) is largely determined by mitochondrial membrane proton leakage which can be estimated by the respiratory control ratio (RCR, ratio of state 3 versus state 4 OCR). A high RCR indicates tighter coupling of respiration to ATP synthesis and may serve as a functional assessment of mitochondrial integrity. With the addition of the mitochondrial uncoupler FCCP, the maximum respiratory capacity of the cell can be measured (Figure 2). It should be noted that about 10% of the basal OCR represents non-mitochondrial oxygen utilization such as by NADH/NADPH oxidases, various desaturases and detoxification enzymes (Herst et al., 2004). The addition of respiratory complex III inhibitor antimycin A at the end of each Seahorse assay provides an estimate of the oxygen consumption contributed by non-respiratory chain sources. Such *in vitro* measurements using samples obtained from patients may provide insights into the metabolic changes underlying disease conditions.

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References

- Caro P, Kishan AU, Norberg E, Stanley IA, Chapuy B, Ficarro SB, Polak K, Tondera D, Gounarides J, Yin H, Zhou F, Green MR, Chen L, Monti S, Marto JA, Shipp MA, Danial NN. Metabolic signatures uncover distinct targets in molecular subsets of diffuse large B cell lymphoma. *Cancer Cell*. 2012; 22:547–560. [PubMed: 23079663]
- Herst PM, Tan AS, Scarlett DJ, Berridge MV. Cell surface oxygen consumption by mitochondrial gene knockout cells. *Biochim Biophys Acta*. 2004; 1656:79–87. [PubMed: 15178469]
- Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol*. 2004; 5:818–827. [PubMed: 15258578]
- Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer*. 2011; 11:325–337. [PubMed: 21508971]
- Ramanathan A, Wang C, Schreiber SL. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. *Proc Natl Acad Sci U S A*. 2005; 102:5992–5997. [PubMed: 15840712]

- Taanman JW, Bodnar AG, Cooper JM, Morris AA, Clayton PT, Leonard JV, Schapira AH. Molecular mechanisms in mitochondrial DNA depletion syndrome. *Hum Mol Genet.* 1997; 6:935–942. [PubMed: 9175742]
- Wang PY, Ma W, Park JY, Celi FS, Arena R, Choi JW, Ali QA, Tripodi DJ, Zhuang J, Lago CU, Strong LC, Talagala SL, Balaban RS, Kang JG, Hwang PM. Increased oxidative metabolism in the Li-Fraumeni syndrome. *N Engl J Med.* 2013; 368:1027–1032. [PubMed: 23484829]
- Warburg O. On the origin of cancer cells. *Science.* 1956; 123:309–314. [PubMed: 13298683]
- Yasin R, Van Beers G, Nurse KC, Al-Ani S, Landon DN, Thompson EJ. A quantitative technique for growing human adult skeletal muscle in culture starting from mononucleated cells. *J Neurol Sci.* 1977; 32:347–360. [PubMed: 886361]
- Zu XL, Guppy M. Cancer metabolism: facts, fantasy, and fiction. *Biochem Biophys Res Commun.* 2004; 313:459–465. [PubMed: 14697210]

Figure 1. Separation of lymphocytes from whole blood

The appearance of a CPT tube filled with blood before and after centrifugation is shown. Density centrifugation allows the separation of peripheral mononuclear cells from other blood cell types and plasma. The mononuclear cell layer is gently transferred with a pipet for further processing and culturing.

Figure 2. Measuring mitochondrial respiration

Shown are representative tracings of oxygen consumption rate (OCR) of lymphocytes from two human subjects (red and blue) using the Seahorse XF Analyzer. State 3 respiration in the presence of ADP, state 4 respiration with inhibition of ATP synthesis using oligomycin, uncoupled respiration in the presence of increasing doses of FCCP (1 and 2), and non-mitochondrial oxygen consumption with inhibition of respiration using antimycin A are shown. Arrows indicate the approximate points at which the indicated compounds were added.

Figure 3. Correlation between mitochondrial function and biogenesis

A significant positive correlation between basal oxygen consumption rate (OCR) and protein level of mitochondrial respiratory complex V α subunit can be demonstrated in this set of human lymphocyte samples. The protein levels are normalized by a control such as β -actin after quantification using fluorescent western blot imaging (LI-COR Biosciences).