Measuring mitochondrial defects in human skin fibroblasts

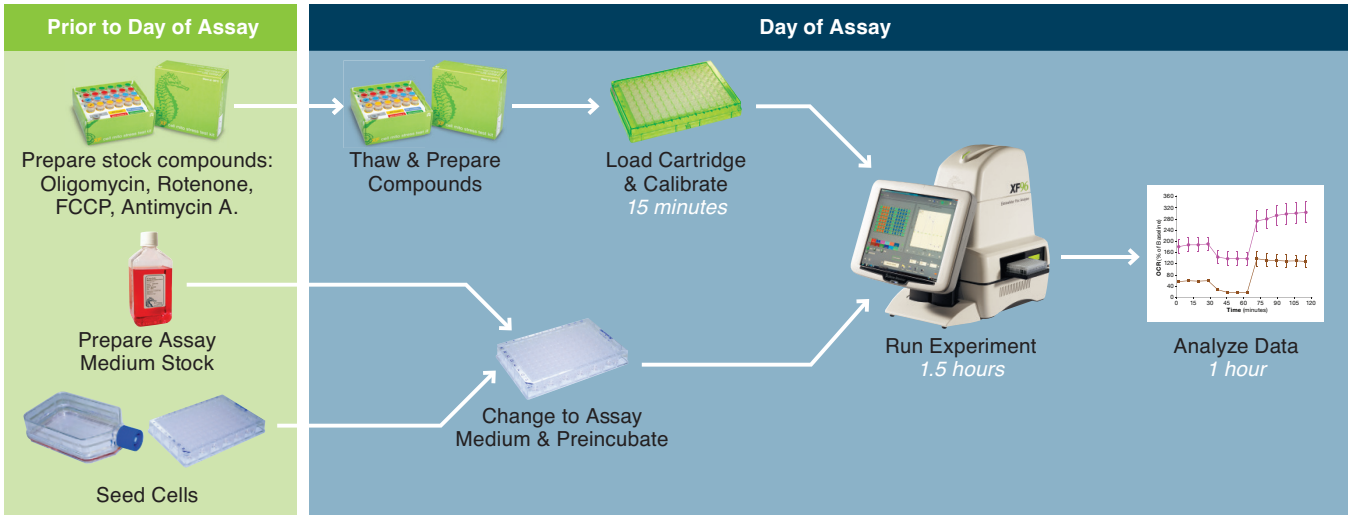
XF bioenergetic analysis of human skin fibroblasts identify mitochondrial disorders

Federica Invernizzi, PhD, IRCCS Foundation Neurological Institute "C.Besta", Milano, Italy
Massimo Zeviani, MD PhD, IRCCS Foundation Neurological Institute "C.Besta", Milano, Italy
Valeria Tiranti, PhD, IRCCS Foundation Neurological Institute "C.Besta", Milano, Italy
Per Bo Jensen, PhD, Seahorse Bioscience, Copenhagen, Denmark

Mitochondrial disorders are characterized by defective mitochondrial function in metabolically active tissues such as skeletal muscle and brain. Diagnosis is challenging. While spectrophotometric analysis is utilized to detect biochemical defects in muscle biopsies or skin fibroblasts, the samples are difficult to obtain and/or require a large number of cells. A more inclusive approach is needed to detect defects in the interplay between complexes and subunits of the oxidative phosphorylation system. In addition, mitochondrial disorders are heteroplastic, with only a fraction of mitochondria affected, and require a sensitive method for analysis.

This Technical Brief describes how to detect defects in the OXPHOS system in human skin fibroblasts with the Seahorse XF96 Extracellular Flux Analyzer.[1-4] In contrast to traditional methods, in which results obtained with skin fibroblasts often do not coincide with results from muscle biopsy-derived material, this method has the advantage of significantly increased sensitivity and requires 100 times fewer cells.

Figure 1 | **Flow Chart of XF Assay**



Materials and Methods

Reagents:

1. Culture Medium: Dulbecco's modified Eagle's medium (DMEM), bicarbonate buffered, containing 4.5 g/L glucose, 10 % (V/V) fetal calf serum, 1 mM sodium pyruvate, 200 U/ml Penicillin G, 200 mg/ml streptomycin, and 4mM glutamine.
2. Assay Medium: Unbuffered DMEM (no bicarbonate), containing 4.5 g/L glucose, 1 mM sodium pyruvate, 200 U/ml Penicillin G, 200 mg/ml streptomycin, and 4mM glutamine.
3. Injection Reagents: [10X in assay medium] 10 um Oligomycin, 7 uM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). XF Cell Mito Stress Test Kit. (Seahorse Bioscience #101706-100)
4. CyQUANT Cell Proliferation Kit (Invitrogen, cat # C7026)

Cell Culture:

Human skin fibroblasts: Cells were grown at 37°C in a humidified 5% CO_2 atmosphere, trypsinized once or twice a week, and medium replaced twice a week as previously described.

Seeding for XF analysis: Control and patient fibroblasts were seeded at 20×10^3 cells/well in 80 µL of DMEM in Seahorse XF96 Cell Culture Microplates using 8 replicates and incubated for 24 hours at 37 °C in 5% CO_2 atmosphere.

Due to the intrinsic variability of human primary fibroblast cultures, including variable cell growth and early senescence, it is necessary to include up to 12 replicates of each clinical sample to insure statistical significance. This is particularly important when comparing results among different experiments.

Normalization: Data were normalized by cell number using the CyQUANT Cell Proliferation Kit and expressed as pmol of O_2 per minute per cell.

XF Bioenergetic Analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in adherent fibroblasts with a Seahorse XF96 Extracellular Flux Analyzer, 24 hours after seeding in the Seahorse XF96 Cell Culture Microplate [Figure 1].

Load Seahorse XF96 Assay Cartridge:

The drug injections ports of the Seahorse XF Assay Cartridge were loaded with the assay reagents at 10X in assay medium. 20 µl of oligomycin (10 µM) and 22 µl of FCCP (7 µM) were added to ports A and B respectively.

Medium Exchange & Calibration:

Culture medium was exchanged with assay medium prior to measurements. Culture medium was aspirated and 180 µl prewarmed PBS added, aspirated and 180 µl prewarmed assay medium added. 180 µl of assay medium was added to the temperature control wells (without cells) and the microplate equilibrated in a CO_2 free incubator at 37ºC for 30 minutes. During this equilibration period, the Seahores XF96 Analyzer was calibrated with a calibration plate using the standard XF calibration protocol.

Skin Fibroblast Assay:

Following calibration, the calibration plate was replaced with the Seahorse XF96 Cell Culture icroplate containing fibroblasts and the experimental run started. The Instrument Run Protocol was as described in Table 1.

Result files:

At the end of the XF experiment, the software generates two files (".xfd" and ".xls"). The xfd file is analyzed using the XF reader software and the xls file analyzed using Microsoft Excel®. Both file types requires the installation of the XF software on the PC being used for analysis.

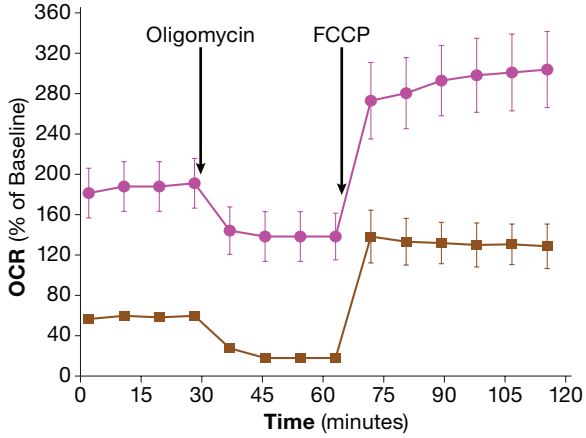
Table 1 | Instrument run protocol

Start Protocol		
Command	**Time (min.)**	**Port**
Calibrate		
Equilibrate		
Mix	4	*(repeat 3-5 times)*
Measure	5	
Inject		A
Mix	4	*(repeat 3-5 times)*
Measure	5	
Inject		B
Mix	4	*(repeat 2+ times)*
Measure	5	
End Protocol		

Interpretation of Results

The assay described is a version of the Seahorse XF Cell Mito Stress Test, which reveals key parameters of mitochondrial function: basal respiration; ATP production, and respiratory capacity[2, 3] [Figure 2].

Figure 2 | Mitochondrial stress profiles of healthy (pink) and patient (brown) fibroblasts



Oxygen consumption rate (OCR) measured in human skin fibroblasts derived from either a healthy control or a patient with a defined DNA mutation in an OXPHOS gene. Four baseline measurements were followed by sequential injections of Oligomycin and FCCP. Data are normalized to cell number.

The Seahorse XF Cell Mito Stress Test measures the basal oxygen consumption rate (OCR, reported as pmol per minute per cell) to obtain the basal respiration -- or resting state of the cells. A decreased basal rate in patient vs. healthy control samples may indicate a defect in the respiratory complexes.

Oligomycin blocks ATPase activity and the reduction in OCR is a measure of the respiration needed to sustain ATP consumption in the cells. The remaining respiration reflects the proton leak of the mitochondria (i.e. the flow of protons across the inner mitochondrial membrane (IMM) which generates heat but not ATP). The composition of the IMM (lipids, proteins) and the oxidative stress level of the system influence proton leak.

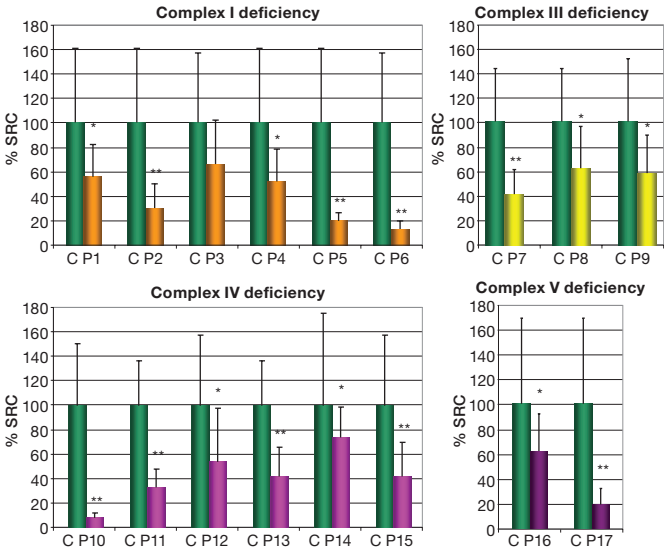
FCCP uncouples respiration by carrying protons across the IMM, FCCP dissipates the electrochemical gradient (membrane potential) that drives ATP synthesis. To maintain the membrane potential, the mitochondria increase the flow of electrons and oxygen consumption. In an XF Assay, the optimized concentration of FCCP reveals the maximal respiration for the sample. This measure reflects how a system reacts to an increased ATP demand. Reduced maximal respiratory may lead to an energetic crisis for the cells.

The fold increase in oxygen consumption rate using the XF software's "baseline" feature option might not reflect the deficiency. Mitochondrial diseases are often heteroplasmic [i.e. only a fraction of mitochondria are affected]. The 'healthy' fraction of mitochondria will be able to increase their respiration to the same fold as in the healthy control samples upon FCCP stimulation. Thus, looking at fold changes may give the impression of both populations being healthy, whereas viewing the data normalized to the number of cells per well would reveal the deficient maximal respiration.

Seventeen patient samples were tested in this study [Table 2]. After genetic analysis to classify the mitochondrial gene defect, the patient material was further tested. With traditional spectrophotometric methods, 9 of the 17 of the patient fibroblast samples were found to have genetic defect, while nearly half falsely tested normal. Analysis of mitochondrial-enriched homogenate from muscle yielded two false negatives [normal]. Upon analysis of the skin fibroblasts using the Seahorse instrument, all 17 yielded results indicative of a defective OXPHOS system, demonstrating the value of analyzing the intact oxphos system rather than individual complexes, and the value of the increased sensitivity of the Seahorse XF96 Extracellular Flux Analyzer.

Figure 3, below, shows the diminished spare respiratory capacity in the patient samples relative to the corresponding control samples. Complex V deficiencies could not be detected using the traditional methods using either skin fibroblasts or

Figure 3 | Diminished spare respiratory capacity in patient skin fibroblasts



OCR measured after FCCP addition indicates the spare respiratory capacity of the cells. Green is the corresponding control. The patients defective OXPHOS complex were determined by gene analysis

muscle homogenates, whereas these defects were identified by the Seahorse XF Analyzer using far less material.

Summary

In summary, the advantages of using the XF bioenergetic assay for measuring bioenergetic dysfunction in human skin fibroblasts are twofold. First, the intact oxidative phosphorylation machinery is being measured, allowing detection of mutations affecting the interplay between complexes and subunits of the respiratory chain. Second, only a very small amount of clinical material is required and sensitivity is greatly enhanced. Thus, defects that could not previously have been detected in a reliable way using skin fibroblasts are now detectable through bioenergetic analysis, suggesting that skin fibroblasts may be used as a first line of screening for suspected mitochondrial disease. The greatly enhanced reliability of detection allows the results to be used as justification for the more invasive sampling of muscle biopsies for further analysis. In the future, it is expected that methods will be developed describing the analysis of permeabilized cells, enabling researchers to determine which complex is affected.

Table 2 | Comparison of the spectrophotometric analysis of individual complexes and XF96 analysis of 17 patient samples with known mitochondrial gene defects

			Muscle	Fibroblasts	
Patient	**Affected Gene**	**Affected Complex**	**% Residual Activity (% Hetero-plasmy)**	**% Residual Activity (% Hetero-plasmy)**	**% MRR**
P1	MTND3	Complex I	43% (H >95%)	Normal (H 90%)	55%
P2	MTND3	Complex I	43% (H 90%)	42% (H 50%)	30%
P3	MTND5	Complex I	Normal (H 50%)	Normal (H 30%)	65%
P4	NDUFV1	Complex I	36%	Normal	55%
P5	NDUFA10	Complex I	10%	19%	30%
P6	NDUFS1	Complex I	14.5%	50%	20%
P7	BCS1L	Complex III	n.d.	30%	50%
P8	BCS1L	Complex III	30%	Normal	65%
P9	BCS1L	Complex III	20%	Normal	55%
P10	SURF1	Complex IV	n.d.	10%	10%
P11	SURF1	Complex IV	n.d.	15%	45%
P12	SURF1	Complex IV	10%	11%	45%
P13	SCO2	Complex IV	30%	Normal	40%
P14	COX15	Complex IV	42%	22%	70%
P15	COX6B1	Complex IV	15.3%	49.2%	45%
P16	MTATP6	Complex V	n.d.	Normal (H 80%)	65%
P17	MTATP6	Complex V	Normal	Normal (H >90%)	25%

Biochemical and molecular features of the patients tested: the colors indicate the respiratory chain complex affected (orange=complex (C) I, yellow=CIII, pink=CIV, purple=CV).

* the percentages are referred to the mean values obtained in the control cell line(s) used in each experiment

Assay Optimization Hints

1. The optimal cell seeding density yields basal rates OCR between 100-200 pmoles/min. The cell seeding required to achieve these rates can vary from lab to lab. Start with the cell numbers and conditions in this Technical Brief and titrate to determine optimal seeding density for your samples.
2. Optimize concentration of reagents as described in the Seahorse XF Cell Mito Stress Test Kit Manual available on the Seahorse Bioscience website. http://www.seahorsebio.com/resources/pdfs/mito-kit-manual-xf24.pdf
3. It is important to have the same cell number across the entire microplate after overnight incubation. Patient fibroblasts should be seeded at a density that accounts for their unique doubling time to ensure the same cell count in each well when the assay is run.
4. It is necessary to include 8-12 replicates of each clinical sample to account for the intrinsic variability of human primary fibroblast cultures.
5. Initially, measure 3-5 baseline rates to determine the basal OCR and the time required to observe a full response to modulators. Adjust accordingly; measure at least two rates per condition.

References

1) Garavaglia B and T Valeria: Cultured fibroblasts in the diagnosis of genetic mitochondrial diseases. Biochimica clinica 33(2) (2009) : 122-128

2) Brand, M. D. and D. G. Nicholls: Assessing mitochondrial dysfunction in cells. Biochemical Journal 435(2) (2011): 297-312.

3) Wu, M., *et al:* Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am J Physiol Cell Physiol (2007); 292:C125-C136.

4) Invernizzi, F., *et al:* Microscale oxygraphy reveals OXPHOS impairment in MRC mutant cells. Mitochondrion (2012); 12(2): 328-35.

Related Webinar

For more details about the work discussed in this brief, view the "Bioenergetic Evaluation in Mitochondrial Disorders" webinar presented by Dr. Massimo Zeviani, MD, PhD and Valeria Tiranti, PhD (two of the investigators involved in this research). This webinar was presented as part of the Seahorse Bioscience Cellular Bioenergetics Webinar Series, and is available On-Demand at www.seahorsebio.com.

Corporate Headquarters

Seahorse Bioscience Inc.
16 Esquire Road
North Billerica, MA 01862 US
Phone: 1.978.671.1600

European Headquarters

Seahorse Bioscience Europe
Fruebjergvej 3
2100 Copenhagen DK
Phone: +45 31 36 98 78

Asia-Pacific Headquarters

Seahorse Bioscience Asia
199 Guo Shou Jing Rd, Suite 207
Pudong, Shanghai 201203 CN
Phone: 0086 21 33901768

www.seahorsebio.com

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