**Introduction**

The pre-adipocyte 3T3-L1 cell culture line, derived from mouse embryos, is a widely used model for the study of adipocyte differentiation. Robust and well-characterized, 3T3-L1 pre-adipocytes differentiate and form fat pads indistinguishable from normal adipose tissue when injected into mice.

In this protocol, differentiation is achieved in vitro through the sequential application of adipogenic factors to 3T3-L1 cells over a period of several days in culture. Following differentiation, metabolic functionality of adipocytes is characterized using the XF Analyzer from Seahorse Bioscience and the XF Cell Mito Stress Test Kit. The XF Cell Mito Stress Test Kit measures the four key parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, and maximal respiration, revealing critical information not evident in basal metabolism measurements alone.

**Protocol Flow Chart**

<table>
<thead>
<tr>
<th>10 Days Prior to Day of Assay</th>
<th>Day Prior to Day of Assay</th>
<th>Day of Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepare gelatin-coated microplates</td>
<td>Hydrate Sensor Cartridge</td>
<td>Load Cartridge &amp; Calibrate</td>
</tr>
<tr>
<td>Seed Cells</td>
<td>XF Cell Mito Stress Test Kit</td>
<td>Prepare Aliquots</td>
</tr>
<tr>
<td>Follow 8-day regime of induction media exchanges</td>
<td>Prepare Assay Medium Stock</td>
<td>Run Experiment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyze Data</td>
</tr>
</tbody>
</table>

**XF Cell Mito Stress Test Profile**

The fundamental parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, and maximal respiration, revealing critical information not evident in basal metabolism measurements alone.
Reagents and Materials

- XF96 FluxPak (Seahorse Bioscience #102310-001) or XF-96 FluxPak (Seahorse Bioscience #102416-100)
- XF Cell Mito Stress Test Kit (Seahorse Bioscience #101706-100)
- Gelatin (Sigma G9391)
- 3T3-L1 cells (ATCC CL-173)
- Media (except where otherwise indicated, media & chemical additives were obtained from Sigma Aldrich):
  - Growth Medium (DMEM + 10% FCS)
  - Induction Medium (DMEM + 10% FCS supplemented with IBMX (3-isobutyl-1-methylxanthine), DEXA (dexamethasone), TZD (thiazolidine) and insulin)
  - XF Assay Medium (Seahorse Bioscience #102365-100) supplemented with 25 mM glucose, 1 mM pyruvate

Cell Line Differentiation

(The following cell seeding & differentiation protocol was developed by researchers at the Hotamisligil laboratory in conjunction with Seahorse Bioscience field application scientists and is published with their permission.)

I. Plate Coating Procedure:

Prepare gelatin-coated 96-well XF microplates according to manufacturers’ instructions with the following adaptations:

1. Prepare a 0.2% w/v solution of gelatin in sterile tissue-culture grade H₂O at 37°C.
2. Gently rock plate to coat with 10 µl/cm² gelatin solution (XF microplates surface area = 0.13 cm²) and pour off any excess.
3. Prep the coated plates for cell seeding by exposure to two 60 minute doses of UV radiation generated by the ultraviolet lamps in the cell culture hood (class II laminar flow biological safety cabinet).
4. Allow coated plates to dry for two hours at room temperature (18-22°C) prior to cell seeding.

II. Seeding Cells

1. Dispense media only in columns 1 and 12.
2. Seed 5,000 3T3-L1 cells/well (DMEM + 10% FCS; 37°C @ 10% CO₂). Do not seed cells in two columns of the plate (include medium only) in these columns. These columns, typically column 1 and column 12 of the 96-well plate, will be used as the background correction wells during the XF Assay.
3. Allow cells to grow for two days until they reach confluence.
4. Once cells have reached confluence, maintain cells for two days in growth medium, then begin differentiation protocol.

III. Differentiation Protocol

Two days after cells become confluent, exchange growth medium for induction medium and culture according to the table below:

<table>
<thead>
<tr>
<th>Timeline</th>
<th>Induction Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 – 4</td>
<td>DMEM supplemented with 10% FCS + 500 µM IBMX + 1 µM DEXA + 1 µM TZD + 5 µg/ml insulin (change medium after day 2)</td>
</tr>
<tr>
<td>Day 4 – 6</td>
<td>DMEM supplemented with 10% FCS + 1 µM TZD + 5 µg/ml insulin</td>
</tr>
<tr>
<td>Day 6 – 8</td>
<td>DMEM supplemented with 10% FCS + 0.5µg/ml insulin</td>
</tr>
</tbody>
</table>

On day 8, the cells will be differentiated adipocytes.

IV. XF Cell Mito Stress Test (Refer to the XF Cell Mito Stress Test User Manual for detailed instructions)

NOTE: XF Cell Mito Stress Test compounds (Oligomycin and FCCP) must be optimized prior to running the stress test. Follow the instructions in the XF Cell Mito Stress Test Kit User Manual to optimize these compounds. Concentrations cited below are provided as an example. These concentrations correspond to the injections used to generate the example data included in the Results section of this protocol.

1. Perform a medium exchange on the cells in the plate by removing the differentiation medium from each well and replacing with XF Assay Medium supplemented with 25mM glucose + 1mM pyruvate:
   A. Remove all but 10 µl of differentiation medium
   B. Rinse cells twice with 215 µl XF Assay Medium supplemented with 25mM glucose + 1mM pyruvate
C. Add 215 μl XF Assay Medium supplemented with 25mM glucose + 1mM pyruvate
Final volume in each well is 225 μl

Note: The Seahorse XF Prep Station is recommended to ensure accurate final volumes during media exchanges.

2. Place the cells in a non-CO₂ incubator at 37 ºC for one hour prior to the start of the assay.

3. Create an assay template in the XF software using the Assay Wizard specifying 3 minute MIX and 3 minute MEASURE times. This example includes two groups:

   ![Assay Template Image]

   Blank  No Stress Test  Stress Test  Blank

A. Record three rate measurements to establish basal rate
   Note: In the example, basal OCR measurement average was 150 pmol/min

B. Inject Port A: Group 1: Medium only (red); Group 2: Oligomycin (blue)
   i. Oligomycin injection concentration 10μM (final concentration in the assay 1μM)
   ii. Injection volume 25μl
   Note: Concentration provided for example; must be optimized

C. Record six rate measurements following oligomycin injection

D. Inject Port B: Group 1: Medium only; Group 2: FCCP
   i. FCCP injection concentration 6μM (final concentration in the assay 600nM)
   ii. Injection volume 25μl
   Note: Concentration provided for example; must be optimized

E. Record three rate measurements following FCCP injection

F. Inject Port C: Group 1: Medium only; Group 2: Antimycin A and Rotenone
   i. Antimycin A and Rotenone injection concentration 150μM (final concentration in the assay 15μM)
   ii. Injection volume 25μl

G. Record three rate measurements following Antimycin A and Rotenone injection

Results

Cells in Group 1 (red) received medium only. Cells in Group 2 (blue) demonstrated a robust response to the XF Cell Mito Stress Test compounds.

![Graph Image]

Figure 1. Differentiated adipocytes demonstrate a robust response in the XF Cell Mito Stress Test.
References


Further Information

1. Seahorse Bioscience Cellular Bioenergetics Webinar Series: New dimensions in adipocyte metabolism: Using the XF to probe human and rodent adipocytes and adipose tissue
   Presented by: Sheila Collins, PhD, Diabetes and Obesity Research Center, Sanford-Burnham Medical Research Institute and Einav Yehuda-Shnaidman PhD, RD, The Hebrew University of Jerusalem
   Air date: February 9, 2011