Cell Culture

1. Two different formulations of cell culture medium are required. The primary growth medium should be nutrient rich medium such as DMEM or RPMI 1640.
2. A second growth medium for isotope labeling, which does not contain the components which will be isotopically labeled. For example, in experiments where [U-$^{13}C_6$] glucose will be used, glucose-free DMEM/RPMI-1640 should be used at the point.
3. Assess concentration of labeled substrate that will be used for the experiment. Physiological glucose levels are in the range of 5.5 to 6 mM. Assuming that the tracer compound is purchased at a purity of 99%, this concentration is more than sufficient to produce strong labeling of TCA cycle intermediates.
4. Dissolve isotopically enriched substrate using a small volume of second medium, sterile filter the solution and mix the solution with rest of the medium.
5. Prepare sterile media supplements (e.g. additional glutamine for several cancer cell lines) and antibiotics as appropriate.

To prepare cells for NMR based metabolomics and flux analysis, standard cell culture protocols can be followed with minimal modifications. Protocol for growing cells would need to be optimized based on each cell line. A generic protocol is presented below.

1. For several cancer cell lines, plating about 5 million cells per plate yields ~80% confluence in about 48 hours. These cells are grown in standard growth medium such as DMEM or RPMI.
2. In order to introduce ^{13}C label in the cells, the medium should be changed. Confluent cells should be washed with pre-warmed phosphate buffered saline (PBS) to remove unlabeled medium.
3. Repeat Step 2 several times to ensure near complete removal of standard growth medium.
4. Add medium containing ^{13}C substrates to the plate.
5. Incubate cells for 6 hours to 24 hours depending on the experimental needs (Also see *Notes: Duration of incubation*).
6. Repeat the steps above with as many dishes as needed to collect 30 million cells.
7. At the end of incubation period, detach the cells (using trypsin or cell scrapper as appropriate) and wash the cells with ice cold PBS 3 – 5 times.
8. Centrifuge the cells at 4°C and store the cell pellet at -80°C for further processing.

Some cell lines do not tolerate prolonged exposure to PBS. It is important to change media rapidly to avoid cells detaching from the dishes. An alternative would be to avoid PBS entirely and wash the cells with media without ^{13}C label prior to adding the media with ^{13}C label.

Temperature of cell culture media must be properly regulated. It is essential that medium containing ^{13}C label be pre-warmed prior to adding to the cells.

Duration of Incubation

In general, exposing the cells to ^{13}C labeled substrates for 6 hours is sufficient. However, there may be scenarios where longer or shorter incubation periods are necessary. It is therefore prudent to optimize the duration of incubation carefully prior to setting up duplicates or triplicates.

Concentration of isotopes

In order to avoid adverse effects of change in media composition, care should be taken to ensure that the concentration of ^{13}C substrates is the same as natural abundance substrates.

Oxygen Consumption

Assuming strong coupling between O_2 consumption ATP production, O_2 consumption can be used to normalize the relative fluxes obtained from isotopomer analysis, rendering quantitative estimates of metabolic turnover ***(1)***. Several different systems exist for measuring oxygen concentration in cell cultures. The authors prefer using Oxygraph+ (Hansatech Instruments, United Kingdom) since it enables measuring oxygen concentration in the medium the cells are present. The steps described in this protocol pertains to Oxygraph+ for all O_2 measurements.

1. Prepare solution containing 50 mM sodium sulphite in water. Using freshly prepared sodium sulphite solution yields best results in our experience.
2. Assemble the oxygen electrode according to manufacturer's instructions.

It is critical to measure oxygen consumption, a direct measure of energy metabolism, using the same cell culture as the one used for flux analysis. Cell density required for O_2 measurements varies between different cell types. In our experience, cell density of 1 - 2 million cells per mL are optimal for measuring oxygen consumption when using Oxygraph.

1. Calibrate Oxygraph+ according to instructions provided by the manufacturer. It is prudent to calibrate the electrodes at the start of each day of use.
2. Obtain a "blank" measurement by adding cell culture medium to the electrode that was incubated along with the cells.
3. To measure oxygen consumption, detach the cells from the dishes/T-flasks.
4. Resuspend the cells in warm medium that was kept in the incubator along with the cells. Add the resuspended cells to the sample chamber.
5. Start the magnetic stirrer and begin recording data. Each measurement should be carried out over several (typically, 2 – 5) minutes.
6. The slope of the curve yields the rate of oxygen consumption in the cell culture.

Refs

1. Malloy CR, Jones JG, Jeffrey FM, et al (1996) Contribution of various substrates to total citric acid cycle flux and anaplerosis as determined by ^{13}C isotopomer analysis and O_2 consumption in the heart. Magn Reson Mater Phys Biol Med 4:35–46