Video Article

Calorespirometry: A Powerful, Noninvasive Approach to Investigate Cellular Energy Metabolism

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Abstract

Many cell lines used in basic biological and biomedical research maintain energy homeostasis through a combination of both aerobic and anaerobic respiration. However, the extent to which both pathways contribute to the landscape of cellular energy production is consistently overlooked. Transformed cells cultured in saturating levels of glucose often show a decreased dependency on oxidative phosphorylation for ATP production, which is compensated by an increase in substrate-level phosphorylation. This shift in metabolic poise allows cells to proliferate despite the presence of mitochondrial toxins. In neglecting the altered metabolic poise of transformed cells, results from a pharmaceutical screening may be misinterpreted since the potentially mitotoxic effects may not be detected using model cell lines cultured in the presence of high glucose concentrations. This protocol describes the pairing of two powerful techniques, respirometry and calorimetry, which allows for the quantitative and noninvasive assessment of both aerobic and anaerobic contributions to cellular ATP production. Both aerobic and anaerobic respirations generate heat, which can be monitored *via* calorimetry. Meanwhile, measuring the rate of oxygen consumption can assess the extent of aerobic respiration. When both heat dissipation and oxygen consumption are measured simultaneously, the calorespirometric ratio can be determined. The experimentally obtained value can then be compared to the theoretical oxycaloric equivalent and the extent of the anaerobic respiration can be judged. Thus, calorespirometry provides a unique method to analyze a wide range of biological questions, including drug development, microbial growth, and fundamental bioenergetics under both normoxic and hypoxic conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57724/

Introduction

In biological systems, the heat-release or enthalpy change during metabolism is typically monitored either directly (*via* direct calorimetry) or indirectly via O_2 consumption and/or CO_2 production (via respirometry). Unfortunately, when these techniques are used in isolation, critical information is lost, such as the contribution of anaerobic pathways to cellular metabolism. Calorespirometry is a powerful technique that relies on the concurrent measurement of both heat dissipation and respiration. Pioneering calorespirometric work investigated the anaerobic metabolism in fully oxygenated mammalian cells and demonstrated simultaneous contributions of both aerobic and anaerobic pathways to energy homeostasis despite the transformed cells being in a fully oxygenated environment[1]. Calorespirometry has since been applied to a wide variety of biological questions. Some examples include the study of animal energetics at low oxygen levels, the effects of both herbicide and estrogen on the gills of bivalves, the metabolism of terrestrial organisms, and the microbial decomposition of organic soil matter[2,3,4,5,6]. Furthermore, calorespirometry has revealed how metabolic preconditioning prior to freezing improves the cryopreservation of mammalian cells[7]. Each approach, both calorimetry and respirometry, has independently increased our knowledge of cellular and organismal bioenergetics. However, fundamental biological questions that can be answered through the use of calorespirometry remain relatively unexplored.

Hess's law states that the total enthalpy change of a reaction is independent of the pathway between the initial and final states. For example, the total enthalpy change for a biochemical pathway is the summation of the change in enthalpies of all reactions within the pathway. Calorimetry offers a real-time approach for measuring cellular heat production, which indiscriminately detects both aerobic and anaerobic pathways. This is based on the foundation that no energy is exchanged in the system except through the walls of the experimental ampule[8]. A change in heat dissipation is equal to the change in enthalpy released from all metabolic reactions in the ampule. Thus, a negative enthalpy correlates to a loss of heat from the system. Exhaustive research over the last four decades has characterized the thermodynamic landscape of both catabolism and anabolism. This is represented by a steady rise in research articles found under the search terms "biological" and "calorimetry" as indexed by the United States National Library of Medicine (NLM) at the National Institutes of Health (PubMed). The search reveals that prior to 1970, a total of 27 publications reference biological calorimetry; meanwhile, in 2016 alone, 546 publications utilized the technique.

Calorimetric methods are well established to determine heat production. However, more flexibility is granted for resolving the respirometric value. The respirometric measurement can consist of O_2, CO_2, or both O_2 and CO_2. Further, the measurement of O_2 or CO_2 can be accomplished by various techniques, including optrodes, Clark-type electrodes, and tunable diode laser absorption spectroscopy[7,9,10,11]. While CO_2 production

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is a valuable metric in many respirometric studies, the medium for cultured cells often utilizes a bicarbonic buffer system for pH control[12,13]. To avoid complications of CO_2 measurement in the bicarbonate system, the following protocol for the calorespirometry of cells in culture utilizes O_2 as the sole respirometric parameter.

Concurrent with measuring oxygen flux, certain respirometers (see **Table of Materials**) are designed for detailed assessments of mitochondrial function. Substrate-uncoupler-inhibitor-titrations (SUIT) protocols are well established and are compatible with experiments designed to measure membrane potential or reactive oxygen species (ROS) formation[14]. The presented protocol for calorespirometry of intact cells is compatible with the introduction of chemical uncouplers such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and the F_0F_1-ATP synthase inhibitor oligomycin. Through the addition of FCCP, oxygen consumption can be uncoupled from ATP production, which is useful to assess the impact of potential therapeutics on mitochondrial peformance[15]. Furthermore, the addition of oligomycin illuminates the extent of leak respiration. Thus, the respirometric measurements performed during calorespirometry are compatible with extensive protocols designed to further elucidate mitochondrial physiology.

The simultaneous measurement of both heat dissipation and oxygen flux allows for the calculation of the calorespirometric (CR) ratio. This ratio is then compared to the Thornton's constant or the theoretical oxycaloric equivalent, which ranges between -430 to -480 kJ mol^{-1} depending on the cell line or tissue of interest and the supplemented carbon substrates[1,16]. Thus, a more negative CR ratio reveals increased contributions from anaerobic pathways to overall metabolic activity. For example, the CR ratio for routine muscle tissue respiration without the active performance of work ranges from -448 to -468 kJ mol-1 which is within the range of the theoretical oxycaloric equivalent[17,18]. Meanwhile, mammalian cancer cells cultured in medium that is high in glucose display enhanced lactic acid fermentation following glycolysis and relatively low mitochondrial engagement[19]. This phenotype results in CR ratios in the range of -490 to -800 kJ mol^{-1}, demonstrating a heightened involvement of the anaerobic pathways in the cellular metabolism as indicated by more negative CR ratios[1,7,16,20].

Both commercial and non-profit cell and tissue distributors currently offer cell lines from over 150 species, with nearly 4,000 cell lines derived from humans. Immortalized cell lines are convenient tools for quickly evaluating the toxicity of potential therapeutics, many of which may directly or indirectly interfere with mitochondrial functions. Using transformed cells during drug screening may be of limited predictive value in part because of the Warburg effect, a hallmark of many cancers. Often, cancers generate ATP from substrate-level phosphorylation and maintain redox balance through the production of lactate without fully engaging the mitochondrion under aerobic conditions[19]. Pharmaceutical development is notoriously costly and inefficient, with approximately 8 out of 9 compounds tested in human clinical trials failing to achieve market approval[21]. While potential therapeutics may pass initial screening due to low cytotoxicity in cell lines, it is possible that some of these compounds are mitotoxic. Without a suitable method to detect how these toxins can impair the energy balance in primary cells that do not display the Warburg effect, critical information is often over-looked, bottlenecking therapeutic development in early stages.

Calorespirometry is a practical, noninvasive approach to analyze metabolic activity in a variety of biological samples, including cells and tissues. The core of the presented protocol is compatible with a range of applications. One complication, however, has been identified. Immortalized cells are often cultured in a glucose-free medium supplemented with galactose to increase the contribution of oxidative phosphorylation (OXPHOS) for energy production, in order to sensitize the cells to potential mitotoxins[22,23]. This metabolic reprogramming appears to obscure analysis when samples are placed in the stainless steel ampules used by the calorimeter[15]. Cells cultured in a glucose medium continue to engage in high metabolic activity for several hours. Meanwhile, cells cultured in galactose medium decrease the heat production within 30 min of their placement in the ampule, making measurements restricted to early experimental time points. This behavior, unfortunately, hinders the opportunity to assess their cellular proliferation. Despite this specific limitation, most applications are compatible with calorespirometric analysis and detailed metabolic information can be obtained through this approach.

Protocol

1. Cell Culture

1. Maintain human hepatocellular carcinoma (HepG2) cells in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and additional substrates (10 mM glucose, 2 mM glutamine, and 1 mM pyruvate) at 37 °C in a cell culture incubator (5% CO_2, 95% air, 100% humidity).
   NOTE: Use the above DMEM formulation when DMEM is referenced in later steps for respirometry and calorimetry.
2. Plate the cells at 1 x 10^6 cells per 100 mm culture plate and subculture them every 7 days or before reaching 90% confluency and renew the medium every 3 - 4 days.
3. Perform the experiments when cells are 60 - 80% confluent.

2. Preparation of Respirometer and Calorimeter

1. Pipet 2.2 mL of DMEM into the respirometer chamber, insert the stopper fully, and adjust it with the stopper spacer tool to allow for optimal oxygen diffusion from air to medium.
2. Stir continuously at 37 °C until oxygen concentration (blue trace) and oxygen flux (red trace) are stable. Ensure the respirometer's software is programmed to account for the oxygen solubility of the medium used in the experiment.
   NOTE: Different media have different oxygen solubility coefficients (*e.g.*, DMEM = 0.92).
3. After stabilization of the oxygen concentration in DMEM with the chamber open, select a stable portion of the oxygen concentration trace and calibrate it for 100% air saturation.
4. Calibrate for 0% oxygen selecting a stable portion of the oxygen concentration trace when no oxygen is present in the chamber. Perform this step after 20 µL titration of 230 mM sodium dithionite or after all oxygen is consumed by the biological sample at the end of an experiment.
5. After the 100% air calibration of the respirometer, close the stopper and ensure that no bubbles are present in the chamber.
   NOTE: a slight increase in oxygen flux will be detected in the closed chamber as the polarographic oxygen sensor (POS) consumes oxygen in order to measure the oxygen partial pressure.

6. After ~10 min of the stopper being closed, confirm that the respirometer is ready for HepG2 cells by observing a stable oxygen flux.
   NOTE: The calorimeter used here utilizes stainless steel ampules and requires one ampule to be used as a reference.
7. Add 2.5 mL of H_2O (dH_2O) [equal volume as the samples (step 4)] to the reference ampule of the calorimeter and tighten the cap, using pliers if necessary. Clean the sealed ampule with airflow and slowly lower the ampule to position 1 in the reference channel of the calorimeter. Remain at position 1 until biological sample is prepared.

3. Preparation of HepG2 Cells for Respirometry and Calorimetry

1. Confirm that the respirometer and calorimeter are prepared and calibrated. Warm DMEM and trypsin to 37 °C in a water bath.
2. Add DMEM to a fresh 100-mm plate and place in the incubator to allow for the equilibration of the medium for both CO_2 and temperature.
   NOTE: This medium will be used for the calorimetry experiment, so the appropriate volume depends on the number of ampules that will be used.
3. Confirm with an inverted light microscope that the cells are at 60 - 80% confluent, then wash the 100-mm plate 2x with 10 mL of room temperature phosphate-buffered saline (PBS).
4. Add 3 mL of the 37-°C trypsin to the 100-mm plate of cells and incubate them for 7 - 10 min at 37 °C in the cell culture incubator. Neutralize the trypsin with 3 mL of 37-°C DMEM and suspend by gently pipetting it ~20 times with a 5 mL pipet.
5. Transfer the 6 mL of resuspended cells in DMEM and trypsin into a sterile 15-mL conical tube and centrifuge it for 5 min at 175 x g. Remove the liquid without disturbing the cell pellet.
6. Resuspend the cell pellet in ~5 mL of DMEM and pipet it thoroughly to ensure the cells are sufficiently dissociated from one another.
7. Remove ~100 µL of the well-mixed sample and perform a thorough cell count utilizing all 8 fields on a hemocytometer to determine the total number of cells. Then calculate the volume for resuspension of the cells in order to generate ~2 x 10^6 cells per 50 µL.
   NOTE: This will ensure that 2 x 10^6 cells are added to each chamber of the respirometer with minimal medium displacement.
8. Centrifuge the cells for 5 min at 175 x g. Resuspend the pellet in the calculated volume of DMEM from step 3.7.
9. Perform a cell count to determine the volume required to inject 2 x 10^6 cells per chamber in the respirometer (this should be ~50 µL). Store the cells for the respirometric measurement on ice until ready.
10. While the concentrated cell sample is on ice, determine the dilution required to produce a concentration of 1 x 10^5 cells/mL for the calorimetry.
11. Mix the sample well and prepare ~3 mL of the cells at 1 x 10^5 cells/mL in DMEM for each ampule.
    NOTE: The DMEM used for the sample dilution should be the equilibrated medium prepared in step 3.3.

4. Calorimetry

1. Ensure that calorimeter is connected to the computer and the calorimetric signal in µW is observable and stable.
2. Add 2.5 mL of the cells at 1 x 10^5 cells/mL (~2.5 x 10^5 cells) to each ampule, ensuring sufficient air is between the lid of the ampule and the medium to allow for gas diffusion.
3. Immediately tighten the cap, using pliers if necessary. Clean the sealed ampule, using airflow to remove any external debris, and slowly lower the ampule to position 1 of the calorimeter. Record the time the ampule is lowered to position 1.
4. Allow the ampule to sit at position 1 for 15 min.
   NOTE: During this 15 min period, cells are to be injected into the respirometer (step 5). This ensures that the same interval of time is used for both heat production and oxygen consumption when the CR ratio is determined.
5. After 15 min at position 1, slowly lower both the reference and sample ampules to position 2. Record the time the ampules were lowered and measure for at least 30 min.

5. Respirometry

1. During the 15-min interval in which the ampule is at position 1 in the calorimeter, begin the respirometric studies.
2. Thoroughly mix the cell sample by pipetting to ensure a homogenous solution and inject <50 µL of the cell sample into each chamber of the respirometer for a final concentration of ~2 x 10^6 cells/chamber. Record the time the cells are injected into the respirometer.
3. After the injection, the HepG2 cells are continuously stirred at 37 °C. Wait at least 20 - 30 min for both a stabilization of the oxygen flux and a steady decrease in the oxygen concentration.
4. Select the **O_2 flux per V** tab on the right of the screen and highlight a stable portion of oxygen flux. Select marks, then statistics and record data for O_2 flux per V. This recording will be used in calculation of the CR ratio.
   NOTE: Steps 5.5 and 5.6 are optional. Leak respiration and maximal uncoupled respiration will be revealed by titrations of oligomycin and FCCP.
5. Inject 1 µL of 4mg/mL oligomycin into each chamber and allow ~ 10 min for oxygen flux stabilization. Record stable leak respiration rate by following steps performed in 5.4 by highlighting a stable portion of the oxygen flux trace after oligomycin addition.
6. Titrate 2-µL injections of 0.2 mM FCCP into each chamber, allowing for oxygen flux stabilization after each injection. Continue the titrations until the maximal flux is reached, as indicated by a slight decline in the subsequent FCCP titration. Record the stable respiration rate during the maximal flux.

6. Calculation of Calorespirometric Ratio

1. Perform a final cell count of the samples prepared at ~ 1 x 10^5 cells/mL utilizing all 8 fields of a hemocytometer to determine the total number of cells added to the ampule (2.5 mL).
2. Determine a time to record measurements from both calorimeter and respirometer at which stable signals are present at identical times. Reference the time recorded when the ampule was lowered to position 2 and the time of injection of cells into the respirometer.
3. Record the heat production by placing the cursor over the trace displaying heat output for the respected ampule at the time determined in step and express as µW/million cells. Collect oxygen consumption data and ensure that it is expressed as pmol O_2 x s^{-1} x million cells.

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4. To calculate the CR ratio, first convert µW to kJ/s, and then divide kJ/s over mol of O_2/s to obtain the CR ratio expressed as kJ/mol O_2. NOTE: The CR ratio is presented in kJ/mol O_2.
(see Supplementary FIle 1)

Representative Results

The reproducibility of calorespirometric measurements depends on a proper and consistent sample preparation. Samples prepared from cell culture should not be used if the plates are overgrown, as cell counts can become inaccurate due to clumping. Further, reduced heat flows due to limited substrate diffusion in the clumped cells may occur. Therefore, when using adherent cells, it is critical to select a plate with the confluency between 60 - 80% and to change the medium 24 h prior to the experiment.

Proper instrumental calibration and maintenance are also critical for informative and reproducible calorespirometric measurements. By following the manufacturer's established cleaning protocol for the respirometer, extensive washes with ethanol are implemented to ensure that residual hydrophobic uncouplers and inhibitors are removed and do not compromise the measurements by partially inhibiting or uncoupling mitochondria. When the respirometer chamber is operating prior to the sample measurement, both the oxygen concentration and the oxygen flux traces should be stable as shown in **Figure 1**. If the POS is in need of sensor service, the oxygen flux will not stabilize as seen in **Figure 2**. Measurements should not be performed when the POS is not properly serviced. Additionally, the ampules utilized in the calorimeter should be cleaned, sterilized, and dried prior to use, as biological contamination can interfere with the heat production of the sample.

Cell samples are prepared after the proper calibration and preparation of both the respirometer and the calorimeter. First, the stopper in the respirometer is closed and the chamber is inspected to ensure no air bubbles are present. Then, HepG2 cells are concentrated to ~2 x 10^6/50 µL for respirometry and immediately placed on ice. For calorimetry, HepG2 cells from the concentrated sample are diluted in equilibrated DMEM at ~1 x 10^5 cells/mL. After cells are thoroughly mixed by pipetting, 2.5 mL of the suspension is added to a sterile ampule. The ampule is tightly sealed, and any external debris is removed by blowing the ampule with a stream of air from an air pump. The ampule is then slowly lowered to position 1, where it remains for 15 min. After 15 min has passed, both the reference and sample ampules are then slowly lowered to position 2, and measurements can begin to be recorded once the heat output is stable (**Figure 3**).

A final cell count is performed after the cells are added to both the calorimeter and the respirometer to determine the precise number of cells introduced into the calorimeter. The heat production is then expressed per million cells. If the HepG2 cells are cultured in the absence of glucose and galactose is supplemented to increase mitochondrial involvement, the cells do not proliferate inside the calorimeter, but instead decrease their metabolic activity after about 30 min in the ampule. This restricts the CR calculation to the earliest time point at which the heat dissipation was measured (**Figure 4**).

It is critical to record the time the cells are added to the ampule so that the measurements of heat production and oxygen consumption are collected at identical time points. If this precaution is not taken, important experimental errors can be introduced in the determination of the CR ratio. During the 15-min period, while the ampule is at position 1 in the calorimeter, the cells are sufficiently mixed by pipetting them, and 2 x 10^6 cells are injected into the respirometer *via* a Hamilton syringe. Oxygen flux stabilizes after approximately 15 min and oxygen concentration steadily declines (**Figure 5**). Again, it is critical that the time of the sample injection is recorded for data analysis. The stabilized oxygen flux of the cells serves as the surrogate for oxygen consumption when calculating the CR ratio. Additional titrations are performed to assess the leak respiration (1 µL oligomycin) indicated by a drop to a lower oxygen flux and the maximal uncoupled respiration (3 - 5 titrations of 2 µL FCCP) indicated by the failure to increase the respiration after successive FCCP titrations. An incomplete uncoupling or an inhibition of respiration may not be observed if the FCCP stock has expired (**Figure 6**).

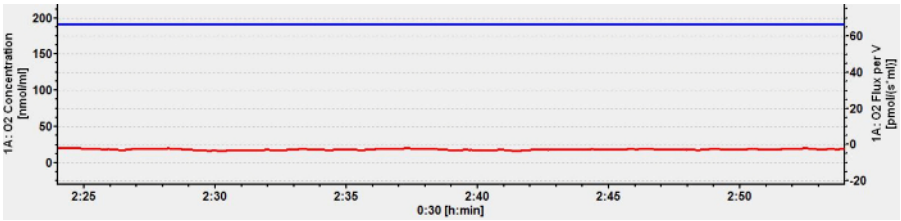

Figure 1: 100% air calibration of respirometer and status of polarographic oxygen sensor (POS). The signals for the oxygen concentration (blue line) and the oxygen flux (red line) both stabilize prior to the 100% air calibration. The stable lines indicate that the respirometer is ready for calibration. The calibration should be performed in DMEM, and not in H_2O. Please click here to view a larger version of this figure.

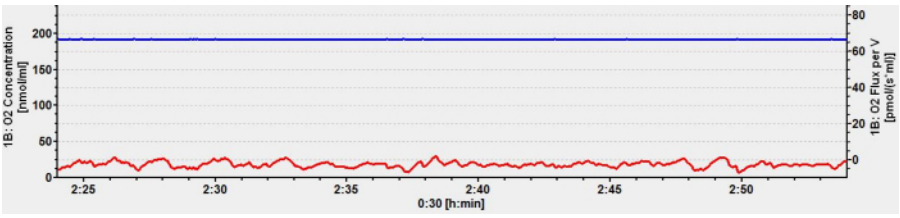

Figure 2: POS in need of service. After the stabilization of the oxygen concentration (blue trace), the oxygen flux (red trace) fails to stabilize. The POS should not be used for experimentation and service should be applied as described by the manufacturer. An increased instability of the oxygen flux correlates with an increased demand for POS service. Please click here to view a larger version of this figure.

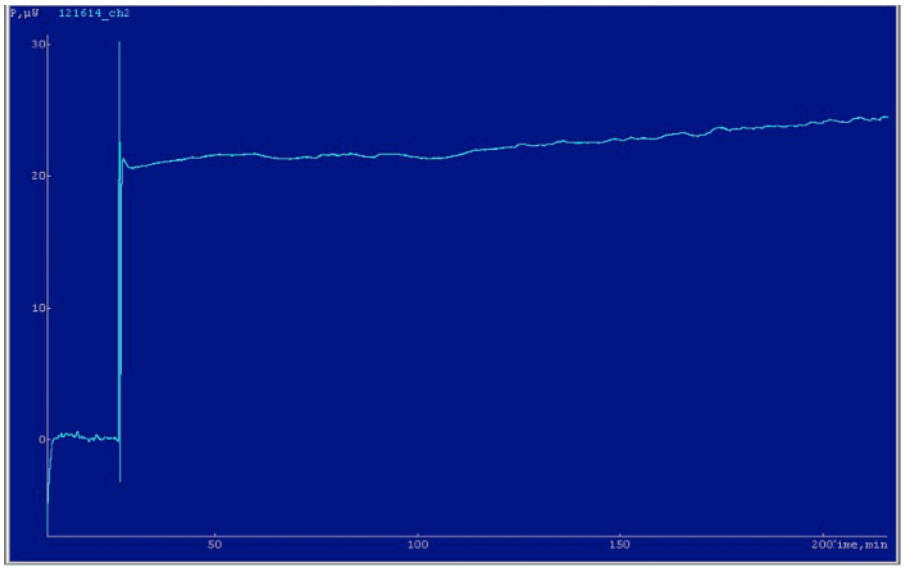

Figure 3: Approximately 2.5 x 10^5 HepG2 cells cultured in glucose medium show signs of proliferation in the calorimeter. Loading ~2.5 x 10^5 HepG2 cells cultured in the presence of glucose per ampule generates a constant heat production of -20 to -28 µW using the 30-µW setting on the instrument and an increased heat production over time correlates with cellular proliferation inside the ampule. Please click here to view a larger version of this figure.

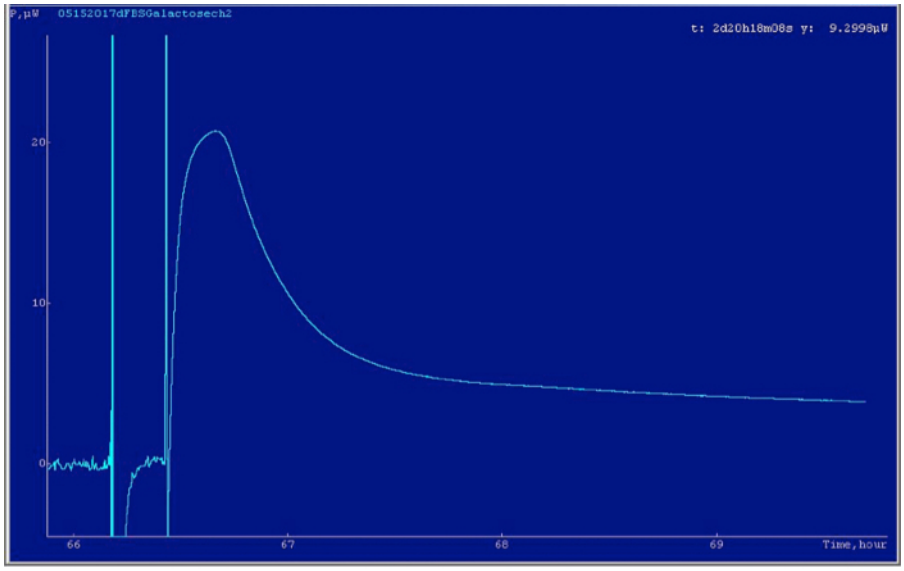

Figure 4: Approximately 2.5 x 10^5 HepG2 cells cultured in galactose medium do not proliferate and heat production decreases over time. Loading ~2.5 x 10^5 HepG2 cells cultured in galactose per ampule results in an immediate heat production of ~-20 µW. However, the heat production decreases over time and restricts CR-ratio measurements to the early time points of the experiment. Please click here to view a larger version of this figure.

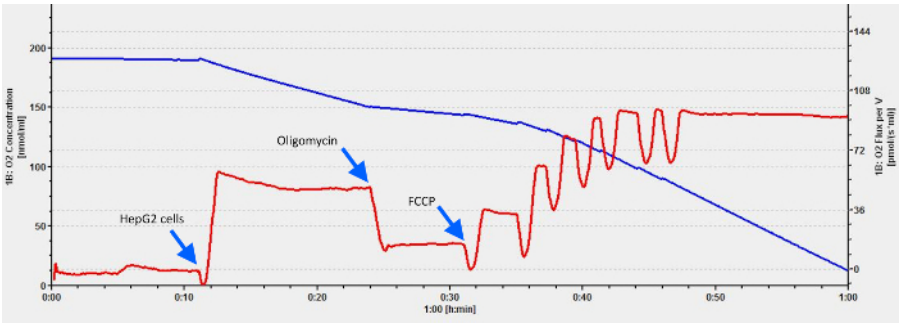

Figure 5: Routine, uncoupled, and leak respiration of HepG2 cells. Once cells were injected into the respirometer, oxygen concentration steadily decreased (blue trace). After the stabilization of the oxygen flux (red trace) during the routine respiration of intact cells, oligomycin was added to inhibit the F_0F_1-ATP synthase. This is indicated by the stabilization of oxygen flux after oligomycin addition, which reveals leak respiration. Approximately 3 - 5 titrations of FCCP should be performed to uncouple respiration from ATP synthesis and to reveal electron transport system (ETS) capacity. Please click here to view a larger version of this figure.

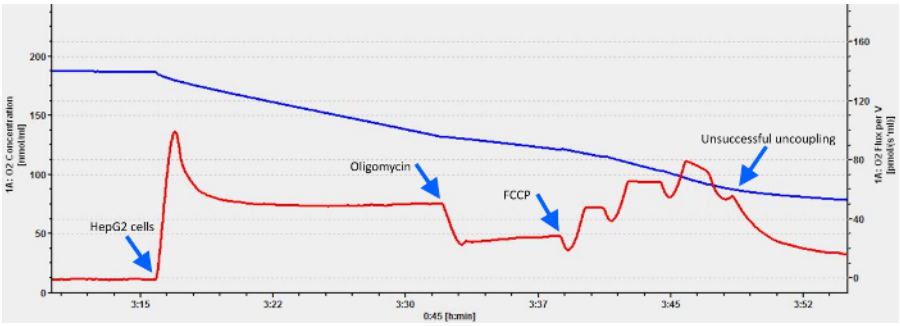

Figure 6: Unsuccessful uncoupling of HepG2 cell respiration. After addition of cells, oxygen flux stabilized (red trace) during routine respiration and oxygen concentration steadily decreased (blue line). Titration of FCCP resulted in an incomplete uncoupling and eventual inhibition, preventing the maximal ETS capacity from being reached. FCCP was likely contaminated or degraded from long-term storage. If observed, a fresh FCCP stock must be prepared. Also, confirm that oligomycin does not inhibit ETS capacity during uncoupling. Please click here to view a larger version of this figure.

Discussion

The objective of calorespirometry is to quantitatively evaluate the contributions of aerobic and anaerobic pathways to metabolic activity and obtain a composite view of cellular energy flux. This is accomplished by a simultaneous measurement of heat dissipation and oxygen flux followed by a comparison of the calculated CR ratio with the theoretical oxycaloric equivalent. Several critical steps must be considered for reproducible and reliable data. The maintenance of a sterile, healthy cell culture is critical. Contamination by bacteria or fungi may result in misappropriated heat production or oxygen consumption, rendering the CR ratio meaningless. Further, improper cell counts, poor sample mixing, or clumped cells can also result in an inaccurate CR ratio. A critical step in regard to the respirometer is proper sample preparation so that the injection is below 50 µL in volume. This volume ensures a minimal amount of medium is displaced from the chamber and the oxygen consumption is correctly attributed to 2 x 10^6 cells. Once routine respiration is observed, the proposed protocol is compatible with two additional titrations (oligomycin and FCCP), which illuminate leak respiration and ETS capacity of the mitochondria. For this application, excessive addition of FCCP can inhibit respiration and prevent maximal flux; therefore, at least 3 - 5 titrations should be performed prior to reaching the maximal uncoupled respiration.

Maintenance and proper calibration of both the respirometer and calorimeter is critical for calorespirometry. The titration protocol for background oxygen consumption is highly recommended and is performed with step-wise titrations of dithionite to reach decreased oxygen levels within the chamber. This is an essential measure to confirm tightly sealed chambers and correct for oxygen back diffusion into the chambers, especially if measurements are performed at a low oxygen tension. Also, performing a stirrer test before addition of cells can provide information about the responsiveness of the POS. If responsiveness is poor or the oxygen flux is not stable (**Figure 2**), service to the POS is required. Improper cleaning of the respirometer chambers can leave behind residual amounts of inhibitors or uncouplers, which may affect subsequent experiments. Air bubbles in the sealed chamber are also capable of interfering with the measurements and can be introduced during the closing of the chamber or during sample injection if air bubbles are present within the syringe. The calorimeter's protocol is more direct, but it is also susceptible to experimental errors. For example, the improper sealing of the ampule will allow evaporation to occur which massively changes heat flow.

One of the limitations of this method is that not all cells can be cultured in suspension and many cell lines are adherent to the cell culture plate or substrate. In order to perform experiments, attached cells have to be enzymatically dislodged from the plate through the use of dissociation agents such as trypsin. Cells in this state may not be in the logarithmic growth phase and their physiology may be altered. Thus, subsequent analysis *via* the calorimeter and respirometer of recently trypsinized cells may assess disrupted cellular activities. Further, a limitation in the presented calorimetric protocol is the sedimentation of cells in the enclosed ampules, which may lead to local hypoxia around the cells due to slow oxygen diffusion in aqueous solutions. We have determined that ~3 x 10^5 cells is the upper limit compatible with the ampules used in this protocol. Increasing the number of cells can generate a hypoxic environment from the sedimentation of the cells, compromising the results. Thus,

optimization is critical if a different cell line is investigated and alternative approaches should be considered such as increasing the medium density to reduce the sedimentation of cells[24]. Ampules are available that permit stirring; however, it is critical to ensure a minimal signal-to-noise ratio is present during the stirring. Additionally, calorimeters besides the one employed in this protocol are compatible with calorespirometry and their capabilities may be beyond what we have presented. Another limitation of calorespirometric analysis *via* a two-chamber respirometer is the inability for a high-throughput analysis, which would be most relevant for pharmaceutical development. This limitation, however, is offset by the capacity for a high-resolution characterization of the compound's influence on the respiratory chain and mitochondrial physiology.

The capacity for both high-resolution and simultaneous measurement of heat output and oxygen flux from the same sample is a significant strength of this method. A potential gold standard when using adherent cells would be to both culture and analyze cells grown on microcarrier beads in suspension. This method would avoid the negative impact of the dissociation from the cell culture surface and allow for the analysis of cellular functions in different phases of the growth curve (logarithmic phase *vs.* contact-inhibited phase[7]). Unfortunately, using microcarrier beads can be problematic due to the high stirring speed required in the respirometer and the potential for grinding of the beads along with cells underneath the stir bar. Despite these limitations, a broad range of applications remains compatible with calorespirometry. In particular, this method is poised to better facilitate pharmaceutical development by quickly identifying mitotoxic compounds in cultured cells.

Disclosures

The authors have nothing to disclose.

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