Video Article

Measurement and Analysis of Extracellular Acid Production to Determine Glycolytic Rate

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Abstract

Extracellular measurement of oxygen consumption and acid production is a simple and powerful way to monitor rates of respiration and glycolysis[1]. Both mitochondrial (respiration) and non-mitochondrial (other redox) reactions consume oxygen, but these reactions can be easily distinguished by chemical inhibition of mitochondrial respiration. However, while mitochondrial oxygen consumption is an unambiguous and direct measurement of respiration rate[2], the same is not true for extracellular acid production and its relationship to glycolytic rate [3-6]. Extracellular acid produced by cells is derived from both lactate, produced by anaerobic glycolysis, and CO_2, produced in the citric acid cycle during respiration. For glycolysis, the conversion of glucose to $lactate^- + H^+$ and the export of products into the assay medium is the source of glycolytic acidification. For respiration, the export of CO_2, hydration to H_2CO_3 and dissociation to $HCO_3^- + H^+$ is the source of respiratory acidification. The proportions of glycolytic and respiratory acidification depend on the experimental conditions, including cell type and substrate(s) provided, and can range from nearly 100% glycolytic acidification to nearly 100% respiratory acidification [6]. Here, we demonstrate the data collection and calculation methods needed to determine respiratory and glycolytic contributions to total extracellular acidification by whole cells in culture using C2C12 myoblast cells as a model.

Video Link

The video component of this article can be found at http://www.jove.com/video/53464/

Introduction

The overall goal of this method is to accurately measure the glycolytic rate of cells using extracellular flux analysis. Quantitative measurement of glycolytic rate using extracellular acidification is the desired endpoint of many experiments. However, the total rate of extracellular acidification is the sum of two components: respiratory acidification, in the form of CO_2 (which hydrates to H_2CO_3 then dissociates to $HCO_3^- + H^+$), and glycolytic acidification, in the form of $lactate^- + H^+$.

The contributions of CO_2 to total extracellular acidification have until recently been considered negligible in the measurement platform used here, the XF24 analyzer [7]. However, it is clear in multiple other systems that CO_2 can be a major contributor to extracellular acidification[4-5]. Multiple papers acknowledge this contribution, but do not attempt direct quantitation of CO_2-derived acid [3,8,9]. We recently demonstrated quantitatively that CO_2 production is a significant source of extracellular acidification in this system [6]. Moreover, though there are multiple metabolic pathways that generate CO_2 from glucose catabolism, those carried out by matrix dehydrogenases in the citric acid cycle are the overwhelming contributors and all other sources generate amounts of CO_2 that are within experimental error [6].

Without correcting for CO_2 production, extracellular acidification is therefore an ambiguous indicator of glycolytic rate and cannot be used quantitatively. Our previous publication highlights several instances where respiratory CO_2 comprises the bulk of the total acidification signal, even in cells generally believed to primarily use glycolysis[6]. Additionally, the respiratory CO_2 contribution to total acidification varies widely during the course of common metabolic profiling experiments, demonstrating that correct comparison of the glycolytic rate during different parts of an experiment requires correction for CO_2.

To measure the glycolytic rate of cells using the rate of extracellular acidification, it is necessary to convert pH changes to changes in total H^+ generated, and to subtract the extracellular acidification caused by CO_2 released during operation of the citric acid cycle. Here, we describe a straightforward method for measuring extracellular proton production rate (from extracellular changes in pH and the calibrated buffering power of the assay medium) and CO_2 production (from extracellular changes in O_2 concentration), and demonstrate how to calculate glycolytic rate using these measurements.

This method strengthens the utility of extracellular acidification measurement by using it to properly calculate glycolytic rate as defined by lactate production. Without correction for respiratory CO_2 (or direct measurement of lactate), it is impossible to determine if and to what extent the

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total acidification rate reflects glycolytic rate, confounding the interpretation of experiments that use total extracellular acidification as a direct measurement of lactate production.

CALCULATIONS

CO_2 and lactate are, within experimental error, the only two contributors to extracellular acid production, based on experiments with myoblast cells[6]. Therefore, the rate of total extracellular acidification (PPR, proton production rate) can be defined as:

$PPR_{tot} = PPR_{resp} + PPR_{glyc}$ ***Equation 1***

where tot = total; resp = respiratory; glyc = glycolytic. Glycolytic PPR is thus:

$PPR_{glyc} = PPR_{tot} - PPR_{resp}$ ***Equation 2***

Here,

$PPR_{tot} = ECAR_{tot}/BP$ ***Equation 3***

where ECAR = extracellular acidification rate (mpH/min), and BP = buffering power (mpH/pmol H^+ in 7 µl), while

$PPR_{resp} = (10^{pH-pK_1}/(1+10^{pH-pK_1}))(\max H^+/O_2)(OCR_{tot} - OCR_{rot/myx})$ ***Equation 4***

where K_1 = combined equilibrium constant of CO_2 hydration and dissociation to HCO_3^- + H^+; max H^+/O_2 = the CO_2-derived acidification for a particular metabolic transformation such as complete oxidation of glucose[6]; OCR = oxygen consumption rate (pmol O_2/min), and $OCR_{rot/myx}$ = non-mitochondrial OCR.

Equation 4 isolates mitochondrial OCR by subtracting any non-mitochondrial OCR (defined as OCR that is resistant to the mitochondrial respiratory poisons rotenone and myxothiazol) and accounts for the maximum H^+ generated per O_2 consumed for each substrate (max H^+/O_2) (see [6]), as well as the proportion of CO_2 giving rise to H^+ at the experimental temperature and pH ($10^{pH-pK_1}/(1+10^{pH-pK_1})$. For full oxidation of glucose, mitochondrial Oxygen Consumption Rate (OCR) is exactly equal to the rate of CO_2 production. In the confined assay volume of extracellular flux measurement, CO_2 produced by respiration remains trapped in the assay medium. Most of the trapped CO_2 is hydrated to H_2CO_3, which then dissociates to HCO_3^- + H^+. A small fraction remains dissolved but not hydrated, and another small fraction is hydrated but not dissociated, as dictated thermodynamically by the combined equilibrium constant of CO_2 hydration and dissociation to HCO_3^- + H^+ at experimental temperature (37 °C) and pH (~7.4).

Thus, the complete equation for calculating PPR_g by subtracting PPR_{resp} from PPR_{tot} is:

$PPR_{glyc} = ECAR_{tot}/BP - (10^{pH-pK_1}/(1+10^{pH-pK_1}))(\max H^+/O_2)(OCR_{tot} - OCR_{rot/myx})$ ***Equation 5***

In this way, rates of respiration and glycolysis, as well as their associated ATP production rates, can be quantitatively determined from straightforward measurements (oxygen consumption, extracellular acidification, buffering capacity) and import or calculation of other required values (H^+/O_2, P/O, and the equilibrium constant K_1) [6]. The experiment described here expands on standard techniques for using the Extracellular Flux Analyzer such as Seahorse XF24 [10,11]; for other extracellular flux measurement formats (*e.g.*, XF^e96, or XFp), all volumes below should be scaled appropriately.

The buffering power of the assay medium can be measured by construction of a standard curve either directly in the extracellular flux platform or separately using a calibrated pH probe. Here, three options for measuring buffering by the extracellular flux assay medium are given, including using all injection ports of the extracellular flux analyzer with cell-free sample wells, or using only the last injection port in cell-containing wells (section 1) or by using an external pH measurement (section 2). See the attached spreadsheet for the full calculations of example data.

To measure buffering power using the pH-detecting capability of the extracellular flux instrument, it is safest to use cell-free wells to minimize signal variation. However, within the error, no statistical difference exists between cell-free and cell-containing wells when performing this measurement (data not shown). NOTE: The variation described in step 1.7 carries the advantage of accounting for any potential changes to buffering conferred by added compounds or by the presence of cells, with the disadvantage of noisier signal. However, as stated above, no significant differences were found in the calculated buffering power between the cell-free design shown in **Table 1** and the post-experiment design in **Table 2** under the experimental conditions described here.

Additionally, over small ΔpH ranges (<0.4 units; experimentally best restricted to 0.2 units), the linear slope obtained by plotting Δ mpH/pmol H^+ adequately approximates the logarithmic relationship between ΔpH and [H^+]. The slope of this standard curve therefore represents the buffering power of the assay medium under test in pH/nmol H^+ in 7 µl, or mpH/pmol H^+ in 7 µl. We recommend increasing medium buffering power or decreasing cell density for samples that exceed a 0.2 pH unit change during the measurement time. The measurement time may also be decreased, but this may shorten the steady state acidification rate and introduce error into the rate calculation.

Protocol

1. Measuring Buffering Power in an Extracellular Flux Instrument: Two Variations

NOTE: the calculations and methods described here were developed using an Extracellular Flux Analyzer. For other instruments, the measurement volume must be scaled appropriately.

1. Prepare 0.1 M standard HCl in water using HCl concentrate (see Materials and Equipment) according to manufacturer instructions.

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Note: An example calculation for preparing HCl injections for use in all four injection ports is shown in **Table 1**:

Table 1: Consecutive HCl injections into a cell-free assay well

Port (µL injected)	Stock (mM)	µl of stock	µl of medium	Resulting change in HCl (mM)	cumulative HCl (mM)	H^+ in 7 µL (nmol)	pH (n = 1)	Δ pH (mean of n = 10)	SEM
well	100	0	0	0	0	0	7.37	0.000	0.000
A (50)	100	1.1	48.9	0.2	0.2	1.4	7.30	0.067	0.006
B (50)	100	1.2	48.8	0.2	0.4	2.8	7.25	0.126	0.004
C (50)	100	1.3	48.7	0.2	0.6	4.2	7.18	0.195	0.005
D (50)	100	1.4	48.6	0.2	0.8	5.6	7.13	0.252	0.005

Table 1. Consecutive HCl injections into an extracellular flux assay well.

2. Prepare dilutions of the HCl standard in the medium to be assayed as in **Table 1** for the number of technical replicates being carried out in one plate, plus one to allow for pipetting error; *e.g.*, for four technical replicates of the port A injection, prepare a stock of (1.1 µl x 5) 0.1 M HCl in (48.9 µl x 5) assay medium.
3. Distribute 50 µl into each Port A of the measurement probe cartridge. Repeat this procedure for remaining ports B, C, and D.
4. Run an extracellular flux assay[10] with a standard calibration cycle, followed by two cycles of [2 min mix, 1 min wait, and 5 min measurement] for each of the four port additions (see **Figure 2**).
    1. Program the experiment above in the instrument software according to software instructions. Load the prepared cartridge into the machine and perform calibration according to software instructions.
    2. When prompted by the program, remove the calibrant-containing plate and insert the plate containing assay medium in each well into the instrument; continue the program.

5. Using the average of 8-10 data points obtained at steady state (typically the last 8-10 points) from before and after each port addition, calculate the (cumulative) difference in pH (ΔpH) caused by each injection of standard acid.
6. Plot ΔpH against nmol H^+ contained in the 7 µl volume trapped by the measurement probe. The linear slope is the buffering power (BP) in mpH/pmol H^+.
7. Alternatively to steps 1.2-1.3, carry out the ΔpH measurements following an assay in which Ports A, B, and C are used for conducting an experiment, followed by an HCl injection in Port D. As in **Table 2**, four technical replicates are used to generate each point of a 5-point standard curve in the 20 experimental wells (excluding the four background temperature correction wells) of the extracellular flux assay plate.

Table 2: Single HCl injection into a cell-containing assay well, injecting HCl in a 50 µL aliquot into Port D, varying concentration between wells

Port (µL injected)	Stock (mM)	µl of stock	µl of medium	Final in well (mM)	H^+ in 7 µL (nmol)	pH (n = 1)	Δ pH (mean of n = 9)	SEM
D (50)	100	0	50	0	0	7.35	-0.010	0.003
D (50)	100	0.7	49.3	0.1	0.7	7.33	0.025	0.003
D (50)	100	1.4	48.6	0.2	1.4	7.28	0.069	0.013
D (50)	100	3.5	46.5	0.5	3.5	7.16	0.186	0.011
D (50)	100	7	43	1	7	7.03	0.332	0.034

Table 2. Single HCl injection into an extracellular flux assay well.

2. Measuring Buffering Power Using an External pH Meter

NOTE: To measure the buffering power of a medium using an external pH probe, calibrate the probe at 37 °C and maintain this temperature for all reagents during the experiment.

1. Prepare 0.1 M standard HCl in water using HCl concentrate according to manufacturer instructions.
2. Warm pH probe, pH standards, the assay medium whose buffering power is to be measured, and 0.1 M HCl to 37 °C in a water bath.
3. Calibrate pH probe at 37 °C according to manufacturer instructions. Maintain all reagents at 37 °C throughout the assay by using a heat plate or water bath.
4. Aliquot 10 ml of the assay medium into a small beaker or conical tube. Monitor pH continuously using an immersed pH probe.
5. Add 0.1 M HCl to the assay medium in 10-20 µl aliquots.
    1. Ensure mixing by using a stir bar or by manually swirling the container after each acid addition.
    2. Allow a few seconds for the pH measurement to stabilize, then record the pH after each addition.
    3. As demonstrated in **Table 3**, make a sufficient number of additions to ensure accurate slope calculation and to cover the pH range expected during the experiment.

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Table 3: Measuring buffering power using a pH meter. Adding HCl in 0.2 mM increments to 10 mL assay medium. Plotting ΔpH vs nmol H^+ added per 7 µL volume yields buffering power (BP) as the slope of the graph.

Assay Medium (µL)	HCl stock (mM)	HCl added (µL)	resulting change in HCl (mM)	cumulative HCl (mM)	H^+ in 7 µL (nmol)	pH (example)	Δ pH (mean of n = 4)	SEM
10000.00	0.00	0.00	0.00	0.00	0.00	7.29	0.00	0.01
10000.00	100.00	20.00	0.20	0.20	1.40	7.24	0.06	0.02
10020.00	100.00	20.04	0.20	0.40	2.80	7.17	0.12	0.02
10040.04	100.00	20.08	0.20	0.60	4.20	7.11	0.18	0.01
10060.12	100.00	20.12	0.20	0.80	5.60	7.04	0.26	0.03
10080.24	100.00	20.16	0.20	1.00	7.00	6.94	0.34	0.03

Table 3. Measuring buffering power using a pH meter. Data represent a typical experiment with six 20 µl additions of 0.1 M HCl.

6. Plot ΔpH against nmol H^+ added per 7 µl, giving a linear slope that represents the buffering power (**Figure 1**).

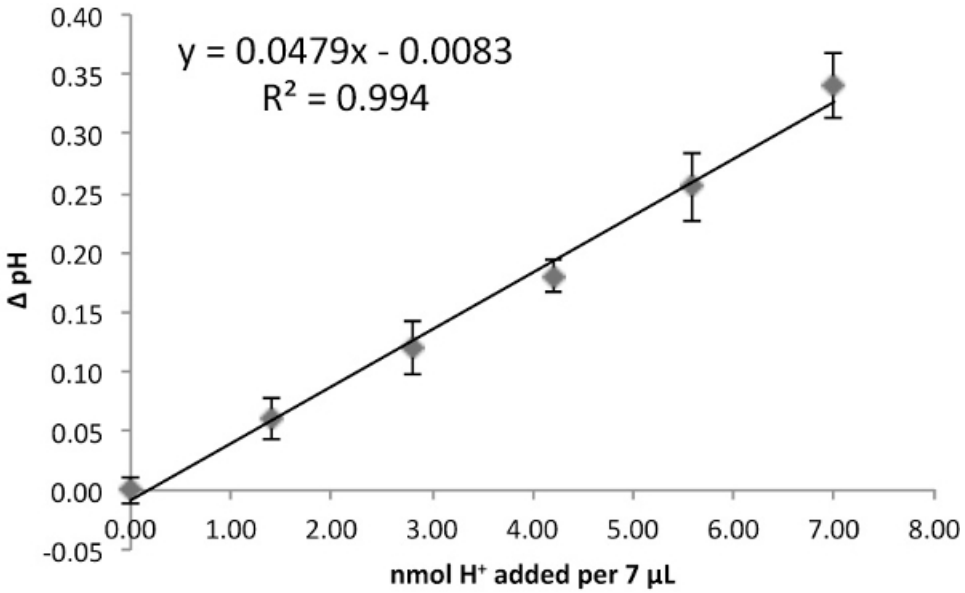

Figure 1. Determining buffering power. HCl standard curve measured as in **Table 1**, **Table 2** or (here) as in **Table 3**. The slope of the linear curve fit gives the buffering power (pH/nmol H^+ in 7 µl). Each point represents mean ± SEM of n = 9 technical replicates.

7. Once the buffering power is known through Method 1 or 2 above, convert the ECAR signal (mpH/min) to PPR (pmol H^+/min/µg protein) by dividing ECAR by buffering power (BP) (mpH/pmol H^+) and scaling to the protein content of each well:
   PPR_{tot} (pmol H^+/min/µg protein) = ECAR (mpH/min)/BP (mpH/pmol H^+ in 7 µl)/protein per well (µg) ***Equation 6***
8. Alternatively, use the same experiments in Methods 1 or 2 to calculate the Buffering Capacity (BC) value used by the instrument software to automatically calculate PPR during data collection.
   NOTE: The instrument user manual[12] (page 107) provides detailed information about calculating and using buffering capacity, where BC is described as
   BC (mol/L) = moles H^+/(ΔpH x buffer volume (L)) ***Equation 7***
   NOTE: The buffering capacity as defined in Equation 7 can be calculated in the instrument or external pH probe assays described above. Conversion between buffering power and buffering capacity is easily done (see attached spreadsheet):
   BC = 1 x 10^{-9}/BP ((mpH/pmol H^+ in 7 µl) / 7 µl) ***Equation 8***
   NOTE: If known prior to performing the assay, the buffering capacity can be entered directly into the instrument software during experimental setup.
9. Apply this procedure and the calculations used above to most conventional buffer systems, as described in previous publication [6].
   NOTE: **Table 4** lists the buffering power and buffering capacity of several conventional media.

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Table 4: Buffering power and buffering capacity of selected media				
Assay medium	Volume of medium (L)	BP (mpH/pmol H^+/7 µL)	BC (mol H^+/L*ΔpH)	Composition
KRPH 0.1% BSA	7.00E-06	0.045	0.0032	2 mM HEPES, 136 mM NaCl, 2 mM NaH_2PO_4, 3.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1% w/v fatty-acid-free bovine serum albumin, pH 7.4 at 37 °C
KRPH 1% BSA	7.00E-06	0.035	0.0041	2 mM HEPES, 136 mM NaCl, 2 mM NaH_2PO_4, 3.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1% w/v fatty-acid-free bovine serum albumin, pH 7.4 at 37 °C
minimal TES buffer	7.00E-06	0.010	0.0143	3.5 mM KCl, 120 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 1.2 mM Na_2SO_4, 2 mM $MgCl_2$, 15 mM glucose, 20 mM TES, and 0.3% w/v fatty-acid-free bovine serum albumin, pH 7.4 at 37 °C
XF DMEM	7.00E-06	0.100	0.0014	XF DMEM containing 5 mM glucose, 2 mM glutamine, 0.4 mM sodium pyruvate, pH 7.4 at 37 °C
XF RPMI	7.00E-06	0.041	0.0035	XF RPMI (bicarbonate-free) containing 11 mM glucose, 2 mM glutamine, 1 mM NaH_2PO_4, pH 7.4 at 37 °C

Table 4. Buffering power and buffering capacity of selected media.

3. Performing an Extracellular Flux Assay Using C2C12 Myoblast Cells

NOTE: In step 3.4.3, there were no observed differences in CO_2-derived acid production dependent on the presence of carbonic anhydrase in C2C12 culture, suggesting that its presence is not required for full conversion of CO_2 to HCO_3^- + H^+. However, empirically testing this in different experimental systems is recommended before omitting carbonic anhydrase.

1. Culture mouse C2C12 myoblasts [13] at 37 °C under 95% air/5% CO_2 in Dulbecco's modified Eagle medium (DMEM) with 11.1 mM glucose, 2 mM glutamine, 10% v/v fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.
2. 24 hr prior to assay, plate/seed cells in 100 µl of the same culture medium at 20,000 cells/well in a 24-well polystyrene extracellular flux assay plate (see Materials and Methods) with no additional coating.
3. Dilute oligomycin, FCCP, and rotenone plus myxothiazol, and HCl (optional) to 10x final concentration in Krebs Ringer Phosphate HEPES (KRPH) assay medium (2 mM HEPES, 136 mM NaCl, 2 mM NaH_2PO_4, 3.7 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1% w/v fatty-acid-free bovine serum albumin, pH 7.4 at 37 °C).
4. Cell preparation
    1. 30 min prior to the assay, wash adherent cells three times by aspirating to gently remove the medium from the well and then slowly adding 500 µl KRPH.
    2. Incubate cells after the third wash step at 37 °C under air (not under 5% CO_2, which will alter the pH of this bicarbonate-free medium).
    3. At assay start, replace KRPH in wells with 500 µl fresh KRPH containing 500 U/ml carbonic anhydrase and either glucose (10 mM) or medium only, with no additional substrate.
5. Loading the sensor cartridge
    1. Pipet 50 µl aliquots of each 10x compound prepared in Step 3.3 into cartridge ports of an extracellular flux sensor cartridge as follows (final concentrations in assay well given): Port A: 2 µg/ml oligomycin, Port B: 0.5 µM FCCP, Port C: 1 µM rotenone, 1 µM myxothiazol, Port D: HCl (if performing an in-assay acid calibration as described above and in **Table 2**).
    NOTE: for the purpose of complete respiratory chain inhibition described here, 1 µM myxothiazol may be used interchangeably with 1 µM antimycin A.
6. Extracellular flux assay:
    1. Perform a standard extracellular flux assay for determining respiratory control as described in [10].

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NOTE: For each segment of the experiment, determine the mix, wait, and measurement times desired, as well as the number of cycles per segment.

NOTE: The data in **Table 5** were collected over two assay cycles of 2 min mix, 1 min wait, and 5 min measure for each segment, with three assay cycles occurring after the Port D addition of different amounts of HCl (for calibration of buffering power as in **Table 2**).

Table 5: Seahorse assay configuration		
	Command	Time (min)
	Calibrate	0.00
	Equilibrate	
2 Cycles	Mix	2.00
	Wait	1.00
	Measure	5.00
	Inject	Port A (oligomycin)
2 Cycles	Mix	2.00
	Wait	1.00
	Measure	5.00
	Inject	Port B (FCCP)
2 Cycles	Mix	2.00
	Wait	1.00
	Measure	5.00
	Inject	Port C (rot/myx)
2 Cycles	Mix	2.00
	Wait	1.00
	Measure	5.00
	Inject	Port D (HCl)
3 Cycles	Mix	1.00
	Wait	0.50
	Measure	5.00

Table 5. Extracellular flux assay configuration.

4. Measuring End-point Lactate Concentration

NOTE: To validate the indirect assay described here in some different system, end point lactate concentration at the end of an extracellular flux experiment can be determined directly in a conventional 96-well plate by measuring the initial velocity (over 2 min) of reduction of $NAD^+ \rightarrow$ NADH catalyzed by lactate dehydrogenase, described in detail in our prior publication[6]. For the data presented in Representative Results, the end point lactate concentration in glucose-containing assay wells was ~40 µM.

1. Prepare 2x hydrazine medium: 1 M Tris, 20 mM EDTA, 400 mM hydrazine, pH 9.8 at 22 °C). Immediately before assay start, add NAD^+ to 4 mM and lactate dehydrogenase (LDH) to 40 U/ml. Final assay medium composition (1x): 500 mM Tris, 10 mM EDTA, 200 mM hydrazine, 2 mM NAD^+, 20 U/ml LDH.
2. Immediately following the extracellular flux assay, remove 100 µl of assay medium from each well of the extracellular flux assay plate and transfer to a well of an opaque (black) 96-well plate.
3. To each sample well, add 100 µl 2x hydrazine medium.
4. Immediately load the plate into a microplate reader and begin monitoring NADH fluorescence at 340 nm excitation/460 nm emission.
5. Record the initial velocity for approximately 2 min.
6. Run a similar experiment to construct a standard curve by plotting initial velocity against lactate concentration for added lactate concentrations from 0 to 50 µM.
7. Calculate lactate concentration in each experimental well using the standard curve.

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5. Measuring Protein Content

1. Remove remaining assay medium from each well of the assay plate.
2. Wash wells three times with 250 µl BSA-free KRPH, being careful to minimize the dislodging of cells from the bottom well surface.
3. Add 25 µl RIPA lysis medium (150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% v/v SDS, pH 7.4 at 22 °C) to each well of the assay plate.
4. Incubate plate on ice for 30 min.
5. Agitate plate on a plate shaker at 1,200 rpm for 5 min.
6. Measure protein concentration by standard methods, *e.g.,* by BCA assay, ensuring that the lysis buffer composition is compatible with the measurement method. The protein content in the experiment in **Figure 2** was ~4 µg/well.

Representative Results

Figure 2 shows the raw data for a typical experiment. The last 10 measurement points from the point-to-point recording of both OCR and pH (shaded vertical bars) were used for the calculations. Initial concerns that the average value (middle point measurement) of each assay cycle would not provide sufficient resolution of rate for an accurate calculation, particularly as there appeared to be a slight lag between port addition and steady state acidification rate, were not borne out, as this does not appear to contribute significantly to calculation error (not shown). Alternatively, if the correct buffering capacity is entered during experimental setup, PPR can be read directly from the instrument data collection readout by displaying the PPR output in the instrument software or in the PC-compatible format available as one of the data output settings.

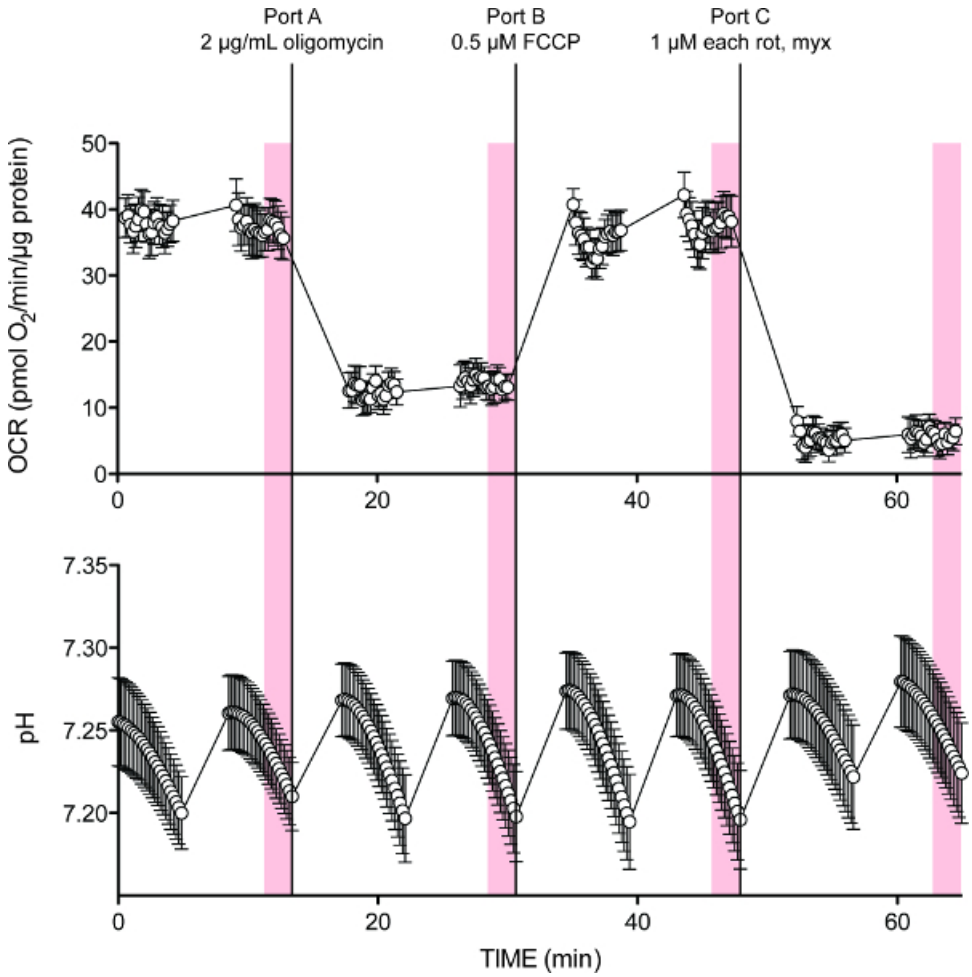

Figure 2. Representative extracellular flux traces of O_2 and H^+. OCR and pH traces for the example experiment in **Table 5**, containing 10 mM glucose at assay start. Port D had different HCl concentrations for calibration of buffering power (not shown in these averaged traces). Data from previous publication[6]. Each point represents mean ± SEM of n = 8 biological replicates. Please click here to view a larger version of this figure.

Data analysis of representative results

Using the spreadsheet shown in **Table 6** and provided as an attachment, data values from individual wells may be entered in the columns shown with yellow headers. All six columns to the right are calculated from these entries. The example in **Table 6** shows the calculations of PPR_{resp} and PPR_{glyc} using ECAR and OCR data from individual wells for the native conditions with or without added glucose, prior to Port A addition of oligomycin. Technical replicates on each biological preparation are normally averaged to give single values of the outputs in the last four

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columns, then data from different biological preparations are averaged with appropriate propagation of error statistics in BP and these four values.

Table 6: Calculation of respiratory and glycolytic acidification (green). Columns headed in yellow indicate values to be entered from calculation (e.g., BP, max H^+/O_2), or from data collection (e.g., $ECAR_{tot}$, OCR). The yellow cells are filled down and the yellow cells repopulated to calculate glycolytic rate for new datasets. We normally average technical replicates on each biological preparation to give single values to average data from different biological preparations with appropriate propagation of error statistics in BP and these four values.

$$PPR_{glyc} = ECAR_{tot}/BP - (10^{(pH-pK1)}/(1+10^{(pH-pK1)}))(\max H^+/O_2)(OCR_{tot} - OCR_{rot/mya})$$

identifier	medium	pH	BP (mpH/pmol H^+ in 7 µL)	pK, at 37°C CO_2 → HCO_3	cell type	substrate	max H^+/O_2	protein in well (µg)	OCR_{tot} (pmol O_2/min)	$OCR_{rot/mya}$ (pmol O_2/min)	$ECAR_{tot}$ (mpH/min)	mito OCR (pmol O_2/min/µg protein)	PPR_{glyc} (pmol H^+/min/µg protein)
example expt 1	KRPH 0.1%BSA	7.40	0.045	6.093	C2C12	glucose 10 mM	1.00	4.10	184.20	20.40	20.25	40.0	109.8
example expt 2	KRPH 0.1%BSA	7.40	0.045	6.093	C2C12	none	1.00	4.05	161.40	21.30	5.94	34.6	32.6
identifier	medium	1.00	1.000	6.093	cell type	substrate	1.00	1.00	1.00	1.00	1.00	0.0	1.0

Table 6. Calculation of respiratory and glycolytic acidification. Columns headed in yellow indicate values to be entered from calculation (*e.g.*, BP, max H^+/O_2), or from data collection (*e.g.*, $ECAR_{tot}$, OCR). Please click here to view a larger version of this table. | Please click here to download this table as an Excel spreadsheet.

Contributions of glycolysis and respiration to PPR after correction

Figure 3 shows the graphical output of data calculated as in **Table 6** for native rates of glycolytic and respiratory acidification, rates following oligomycin addition (Port A), and rates following FCCP addition (Port B). These data clearly demonstrate how the proportions of respiratory and glycolytic acidification change with choice of substrate (glucose vs. control (ctl) with none added) and with mitochondrial status (native function vs. pharmacologically altered function).

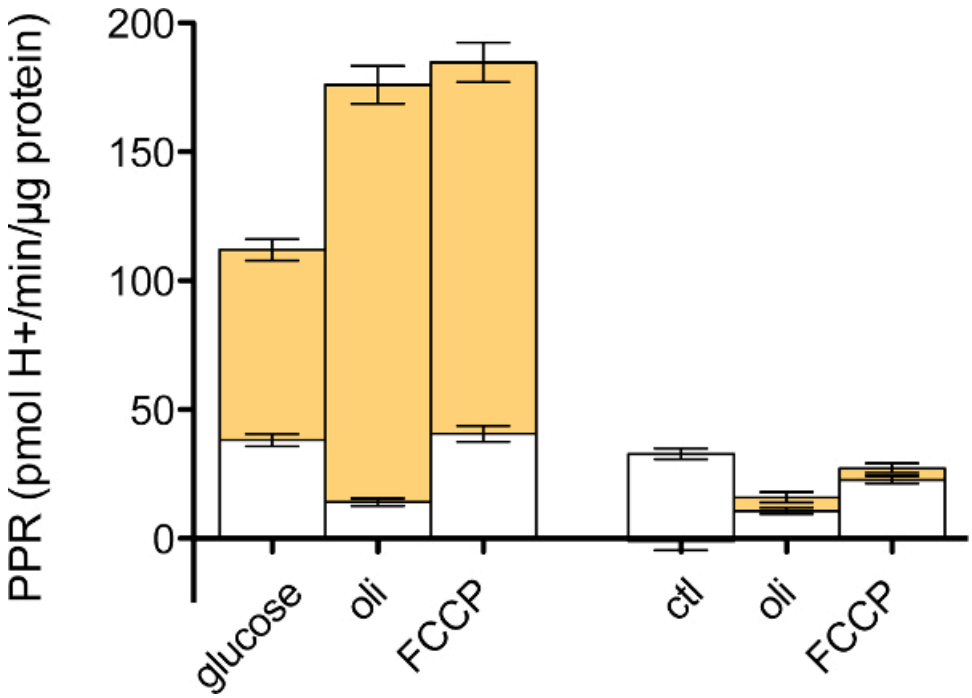

Figure 3. Proton production rate (PPR) from glycolytic and respiratory sources. PPR from respiration (open portions) and glycolysis (filled portions) of C2C12 cells calculated using Equation 5 with added glucose (three left bars) or without added glucose (three right bars). Data from[6]. All data represent mean ± SEM of n = 8 biological replicates.

Discussion

Extracellular acidification is an easily measured indication of cellular metabolic rate. To properly determine the rate of cellular glycolysis (as defined by lactate production) it is critical to know the buffering power of the assay medium, and to convert the extracellular flux measurements of oxygen consumption and acidification to proton production rates. By performing this calculation, the acidification resulting from CO_2 released in the citric acid cycle can be subtracted, leaving the acidification that results from lactate production.

The multiple different ways given here to measure buffering power for this correction carry different advantages and disadvantages. External measurement using a pH probe is highly accurate and reproducible, but may not reflect small differences in pH detection introduced by the

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fluorophores contained within the assay plate, the addition of compounds during the assay, or the presence of the cells themselves. The in-plate pH measurements address these issues, but also introduce varying degrees of experimental noise.

The CO_2 correction to ECAR allows for the first time the unambiguous and quantitative calculation of glycolytic rate, and reveals variation in respiratory and glycolytic contribution to total ECAR during the course of an experiment. Using Equation 5 and the measurements of OCR, ECAR, and buffering power, glycolytic rate can be calculated using the simple spreadsheet provided (**Table 6**). This rate can be verified by post-hoc lactate measurement if desired [6]. In cells where the pentose phosphate pathway is highly active, the use of pathway inhibitors such as 6-aminonicotinamide may be useful to isolate glycolytic rate. Calculation of the contributions of both CO_2^- and lactate-derived H^+ from the total measured Extra Cellular Acidification Rate and Oxygen Consumption Rate is an invaluable tool for using extracellular flux data to make powerful and quantitative statements about metabolic activity.

Using the procedures described here, including various modifications for measuring buffering power, and optimizing the extracellular flux experiment for the cells under investigation and data desired, the rate of glycolysis in intact cells can be quantified under a wide range of experimental conditions. This method is limited to cells that can grow in adherent culture on (or cells or organelles that can be adhered to) a polystyrene surface. It is most reliable when cultured cells are homogenous and confluent, though useful data may still be obtained over a range of these conditions. The calculations require some knowledge of the metabolism of the cells, as max H^+/O_2 ranges from 0.65 to 1.0 for full oxidation of different substrates and more for partial oxidation [6], however, if the cells are known to oxidize glucose, a value of 1.0 can be assumed.

Though relevant to all metabolic characterization, this method may be particularly helpful when used in systems in which a shift between respiratory and glycolytic metabolism to maintain cellular ATP supply is a critical phenotype, including the characterization of stem cells and tumor-derived cancer cells. Understanding metabolic control alterations in these and other contexts will allow a greater degree of sophistication and accuracy in the experimental design and analysis of these cell types.

Disclosures

Dr. Shona Mookerjee declares that she has no competing financial interests. Dr. Martin Brand has consulted for Seahorse Biosciences, which produces instruments and reagents used in this Article. Open Access fees for this article were paid for by Seahorse Biosciences.

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