Chapter 7

Measuring Rates of ATP Synthesis

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Abstract

Here, we offer you a high-throughput assay to measure the ATP synthesis capacity in cells or isolated mitochondria. More specifically, the assay is linked to the mitochondrial' electron transport chain components of your interest being either through complex I (with or without a linkage to pyruvate dehydrogenase activity), through complex II, or through the electron transport flavoprotein and complex I (β-oxidation of fatty acids).

Key words ATP synthesis, Oxidative phosphorylation, β-oxidation, Mitochondria, Bioenergetics, Oxidative phosphorylation (OXPHOS)

1 Introduction

ATP, produced from ADP and inorganic phosphate by the magic machine oxidative phosphorylation (OXPHOS) complex V (CV) [1], is the energy currency of cells, driving a myriad of energetically unfavorable reactions. ATP levels in cells and tissues are highly dynamic, and are affected by numerous underlying factors such as levels of the intracellular adenylate pool (AMP, ADP and AMP) [2], and the mitochondrial membrane potential. The traditional static measurement of ATP levels in cells, which provides a snapshot of ATP levels, is undoubtedly a useful tool for understanding the bioenergetics of your system. To this end, there are numerous methods already available for measuring homeostatic ATP levels using kits such as the commercially available luminescence kits, or the more sensitive LC-MS approaches [3]. Here though, we describe a complementary method that measures the ATP synthesis capacity of cells linked to specific OXPHOS pathways, a method adapted from Wanders et al. [4]. That is, measuring ATP production linked to coupled respiration, or state

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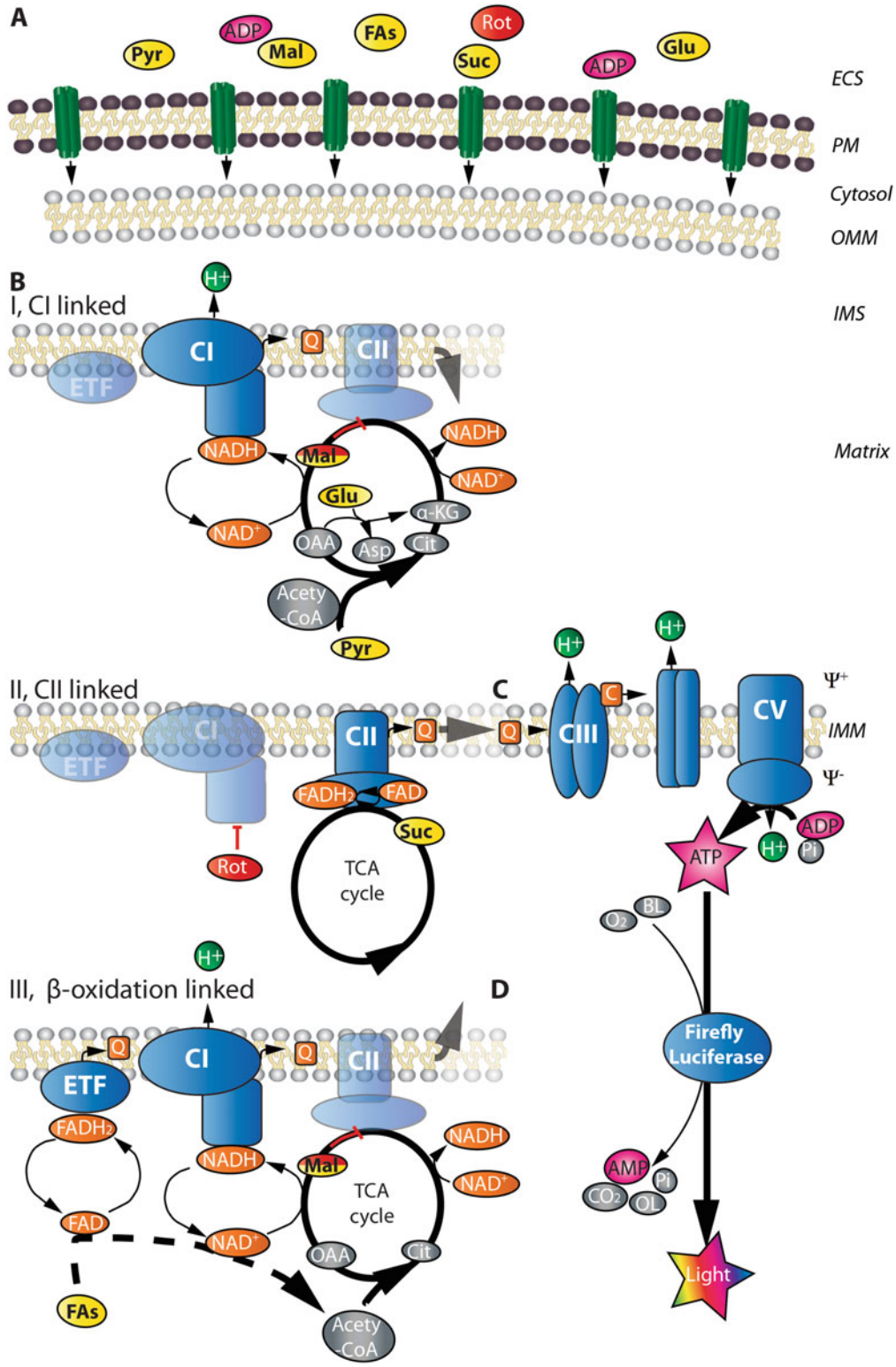

Fig. 1 Rates of ATP synthesis in cells or isolated mitochondria are measured linked to OXPHOS activity. (**a**) Cells or isolated mitochondria are incubated with a reaction buffer containing substrates (yellow), and

3 respiration. State 3 is where there is excess ADP and OXPHOS substrates, and an intact mitochondrial membrane potential ($\Delta\Psi_m$). Under such conditions, the OXPHOS system only works to maintain the $\Delta\Psi_m$ as it is dissipated by CV, hence it is "coupled". The utility of this assay lies in its ability to discern the "maximal" (under the in vitro conditions used) contribution of specific components of the OXPHOS system to ATP synthesis. Such findings can guide researchers in revealing if mitochondria are dysfunctional, and provide specific clues as to where any perturbations in the organelle might lie.

Here, we describe coupled ATP synthesis (Fig. 1) with either complex I (CI: making use of glutamate and malate, or pyruvate and malate as substrates), CII (succinate as substrate), or CI and the electron transferring flavoprotein (ETF: making use of fatty acids as substrate).

The role of malate in this assay is a little complex, warranting clarification about its use. Malate is unable to sustain CI linked ATP synthesis alone, as it rapidly reaches equilibrium with its forward reaction product in the tricarboxylic acid cycle (TCA) cycle, oxaloacetic acid (OAA) [5]. It also reaches equilibrium with its reverse product in the TCA cycle, fumarate, which in turn likely blocks CII activity by energetically discouraging the conversion of succinate to fumarate. It is possible that malate exerts an additional inhibitory effect on CII activity via OAA, where OAA forms a molecular merkin with CII, thus inhibiting CII [5, 6]. Malate is used then in conjunction with other substrates as follows. Malate is converted to OAA (deriving the CI substrate NADH). OAA and glutamate are then transaminated to

Fig. 1 (continued) inhibitors (red) linked to the pathway of interest, ADP (pink) and an optimal concentration of digitonin (green, concentration as determined in a digitonin titration assay, not required for isolated mitochondria) that selectively permeabilizes the plasma membrane but leaves both the OMM and the IMM intact. (**b**) Common substrate and inhibitor combinations include **I**, substrates linked to CI activity (pyruvate and malate, or glutamate and malate). Note that malate not only provides OAA linked to glutamate metabolism (via glutamate oxaloacetate transaminase to α-KG), but also energetically prevents the oxidation of succinate to fumarate by CII, essentially blocking its activity. **II**, CII linked (succinate) and **III**, CI and ETF linked (fatty acids via β-oxidation). (**c**) Q (orange, as for other electron carriers) generated from these pathways converge at CIII, where it transfers its electron load to C, which finally dumps the electrons via CIV on O_2 to form H_2O. The net result is to pump protons at CI, III and IV for the purpose of maintaining the $\Delta\Psi_m$. CV then harnesses this $\Delta\Psi_m$ to produce ATP from ADP and Pi. (**d**), ATP is finally detected in the assay by luminescence. This is achieved using a luciferase (blue, here firefly luciferase by Promega, E1501) that uses ATP and O_2 to oxidize a luciferin molecule, deriving light as product of the reaction. *α-KG* alpha ketoglutarate, *Acy-Coa* acetyl-CoA, *Asp* aspartic acid, *BL* beetle luciferin, *C* cytochrome C, *CI-V* oxidative phosphorylation complexes I-V, *Cit* citrate, *ECS* extra cellular space, *FAs* fatty acids, *Glu* glutamate, H^+ hydrogen proton, *IMM* inner mitochondrial membrane, *IMS* inter membrane space, *OAA* oxaloacetic acid, *OL* oxyluciferin, *OMM* outer mitochondrial membrane, *Mal* malate, *Pi* inorganic phosphate, *PM* plasma membrane, *Pyr* pyruvate, *Rot* rotenone, *Suc* succinate, *TCA* tricarboxylic acid cycle, *Q* coenzyme Q_{10}

oxaloacetic acid by the glutamate oxaloacetic acid transaminase (GOT) (Fig. 1). The α-ketoglutarate is then further converted to succinyl-CoA, deriving more NADH to drive CI in the process, and drive maximal activity of the GOT enzyme. Pyruvate is sufficient to drive CI linked respiration on its own, but addition of malate has the dual effect of indirectly blocking CII activity and providing a pool of OAA that can drive the conversion of acetyl-CoA (from pyruvate) to citrate at a maximal rate. Malate serves a similar function with β-oxidation, where it both indirectly blocks CII activity, and draws away acetyl-CoA produced through sequential rounds of β-oxidation, and again, regenerating NADH from NAD^+ in the process.

The distinction between the complex I substrate pairs of pyruvate and malate, or glutamate and malate is offered to provide additional insight into the activity of pyruvate dehydrogenase complex, which is a commonly mutated complex in the case of mitochondrial disease [7].

2 Materials

1. ATP buffer: 25 mM Tris base, 150 mM KCl, 2 mM EDTA, 10 mM K_2HPO_4, pH to 7.4, stored at 4 °C.
2. ADP (*see* **Note 1**): 10 mM stock solution prepared in ATP buffer, stored at −80 °C (avoid repeated freeze thawing).
3. ATP: 100 mM in distilled water, stored at −20 °C.
4. BSA, fatty acid free (*see* **Note 2**): 10 mg/mL stock solution prepared in ATP buffer, stored at −20 °C.
5. Digitonin: 0.2% stock solution prepared in ATP buffer by boiling in distilled water (1 min) and vortexing, stored at −20 °C.
6. L-glutamic acid (glutamate): 250 mM stock solution prepared in ATP buffer, pH to 7 with neutralization solution, stored at −20 °C. Requires vigorous vortexing to dissolve.
7. Luciferase: ATP detection luciferase solution (*see* **Note 3**).
8. Malic acid (malate): 250 mM stock solution prepared in ATP buffer, pH to 7 with neutralization solution, stored at −20 °C.
9. Malonic acid (malonate): 50 mM stock solution prepared in ATP buffer, pH to 7 with neutralization solution, fresh only.
10. Neutralization solution: 2 M KOH and 0.6 M MOPS prepared in distilled water, stored at room temperature.
11. Octanoylcarnitine: 100 mM stock solution prepared in ATP buffer, stored at −20 °C.

12. Pyruvic acid (pyruvate): 250 mM stock solution prepared in ATP buffer, fresh only.
13. Rotenone: 2.5 mM stock solution prepared in ethanol, stored at −20 °C.
14. Sodium succinate: 500 mM stock solution prepared in ATP buffer, stored at −20 °C.
15. Stop solution: 3 M $HClO_4$ (perchloric acid) prepared in distilled water, stored at 4 °C.
16. TE buffer: 1× prepared in distilled water, stored at 4 °C.

3 Methods

3.1 Sample Preparation

50 μL of sample is required for each condition to be tested. For a typical assay, 5–10 conditions are tested, necessitating a minimum volume of 250–500 μL of sample as described below. All samples must be prepared fresh.

- Cells are harvested by trypsinization and neutralized with fetal calf serum (for adherent cells only), pelleted (800 g, 5 min, 4 °C) and washed twice in phosphate-buffered saline (PBS). Cells are finally diluted in ATP buffer to 100 μg/mL.
- Mitochondria are isolated from cells or tissues as previously described [8]. Isolated mitochondria are finally diluted in ATP buffer to 10 μg/mL.

3.2 Digitonin Titration

Digitonin is used to selectively permeabilize the plasma membrane, allowing substrate to enter cells [9]. Add too little digitonin then, and substrates won't get into the cell to drive OXPHOS. Add too much though, and you will disrupt your mitochondria and any chance of obtaining reliable data. The first part of this assay then is to optimize the concentration of digitonin you will use with your cells (not required for isolated mitochondria). This is both necessary in light of cells' varying sensitivity to digitonin, as well to account for different sources of digitonin. An overview of this method is described in Fig. 2.

1. Prepare 100 μL of each digitonin titration master mix per cell line in ATP buffer containing: 2 mM ADP, 2 mg/mL BSA 20 mM succinate (complex II substrate), 5 μM rotenone (CI inhibitor rotenone, to block electron backflow from CII to CI), and a gradient of digitonin (suggested range of 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, and 0.05% digitonin).

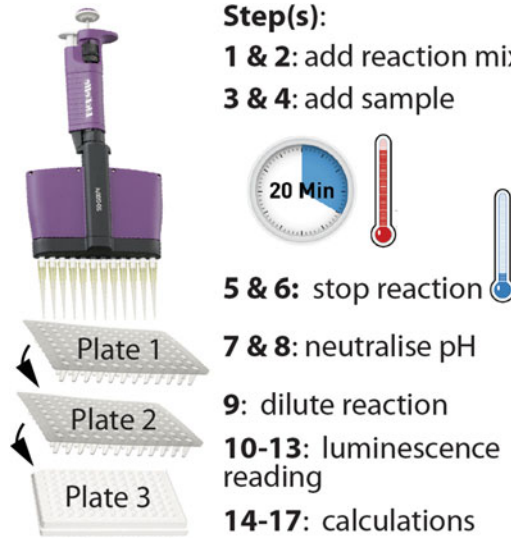

Fig. 2 Experimental workflow. Overview of method containing **steps 1–17** as described in Subheading 3.2

2. 25 μL of each master mix is aliquoted in duplicate into a round-bottomed 96-well plate (circa 100 μL well volume), or into 1.5 mL microfuge tubes (*see* **Note 4**).
3. The plate is then loaded into a thermal block, or a PCR machine, set at the optimal temperature for your organism (37 °C for human samples). If using tubes, they are either loaded into a pre-warmed water bath or a heat block (heat exchange is slower in a block, which will delay the reaction and potentially introduce bias).
4. 25 μL of sample is added in a staggered fashion (recommended every 20 s) to each well or tube.
5. 20 min after the first addition of cells, reaction tubes are stopped periodically (e.g., every 20 s) with the addition of 10 μL of stop solution and moved to ice (a 96-well plate can be moved to ice at the end of the stop solution additions), such that every sample has a reaction time of precisely 20 min. An adhesive plastic plate sealer should be used to seal the 96-well plate between subsequent steps.
6. Plates or tubes are incubated on ice for ≥10 min. Samples remain chilled for the rest of the procedure.
7. Samples are neutralized to pH 7 with neutralization solution, and the volume adjusted per well or tube to 120 μL with TE buffer. Samples must be mixed thoroughly. In a sealed 96-welll plate, this is easily achieved by repeated inversions of the plate. 1.5 mL microfuge tubes should be briefly vortexed.

The volume of neutralization solution and TE can be determined in one well, and then applied as a master mix to all remaining wells. It is not necessary to test every well for pH neutrality. Further, samples can be stored at this stage at −80 °C prior to completing the analysis.

8. Cellular debris is pelleted (4400 g, 10 min, 4 °C). This spin step has also been tested at room temperature, and the ATP levels were found to be stable.
9. 1 in 50 dilutions of samples are prepared in water, and mixed by 3 successive up and down aspirations with your pipette. Be careful to avoid transferring the pelleted debris at the bottom of the well/tube.
10. ATP standards are prepared by diluting ATP in water to 0, 3^{-6}, 1^{-6}, 3^{-7}, 1^{-7}, 3^{-8}, 1^{-8}, and 3^{-9} M.
11. 25 μL of each standard or sample is loaded in duplicate into a flat-bottomed black 96-well plate.
12. 25 μL of luciferase reagent is added to each well, and mixed by 3 successive up and down aspirations with your pipette, and the plate is incubated at room temperature for 10 min.
13. Luminescence levels are measured using a luminescence reader.
14. A linear regression is fitted on the x and y axes from Log10 (standards concentration in M) and Log10(standards luminescence units) respectively.
15. The concentration of ATP in each sample is determined by converting the luminescence values of each sample into Log10, and determining their ATP concentration from the standard curve above.
16. Rates of ATP production per sample are calculated as the concentration of ATP in each sample relative to the length of the assay (20 min) and the amount of sample loaded into each well (calculated as the amount of protein in the luminescence reaction). The amount of sample in each luminescence reaction is calculated as:

$$\text{Amount of protein per luminescence reaction } (\mu g) = \frac{\text{Original concentration of sample } (\mu g/\mu L) \times \text{volume of sample in first reaction } (\mu L) \times \text{volume of diluted sample in luminescence reaction } (\mu L)}{\text{End reaction volume pre-dilution } (\mu L) \times \text{dilution factor}}$$

$$= \frac{0.1(\mu g/\mu L) \times 25(\mu L) \times 25(\mu L)}{120(\mu L) \times 100}$$

$$= 0.0052\,\mu g$$

17. Rates of ATP synthesis are then plotted against the concentration of digitonin used for each sample to determine the

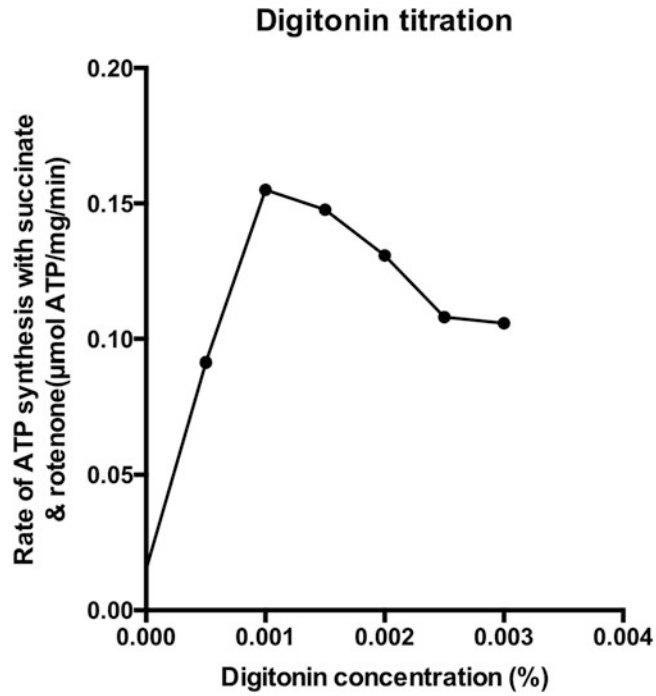

Fig. 3 Digitonin titration in isolated monocytes. The maximal rate of ATP synthesis was determined in isolated human monocytes with the complex II linked substrate succinate, and the complex I inhibitor rotenone (to prevent electron backflow from complex II to complex I) with an increasing percentage of digitonin in the reaction mix. Based on such data, an optimal concentration of digitonin in the reaction is 0.001%

maximal rate of ATP synthesis that can be measured from the minimum concentration of digitonin. See the example in Fig. 3 where it was determined that for isolated monocytes, 0.001% digitonin was optimal. This is the concentration that you should then use for testing the rate of ATP synthesis in your samples linked to other OXPHOS substrate and inhibitor combinations as now described.

3.3 Determining Rates of ATP Production in Cells or Isolated Mitochondria

The rate of ATP synthesis linked to specific OXPHOS substrate inhibitor combinations is determined using the same method as for the digitonin titration described above, except that the digitonin concentration is fixed (at a concentration determined using the above method, as before, digitonin is not added to the reaction mix if you are working with isolated mitochondria), and instead, specific substrate and inhibitor combinations are used to determine positional information about the activity of the OXPHOS system. To this end, many different substrate and inhibitor combinations can be tested. Table 1 describes 9 standard reaction master mixes

Table 1
Suggested reaction master mixes containing substrate and inhibitor combinations to test the maximal rate of ATP synthesis linked to the activity of specific OXPHOS pathways

Master mix	Substrates	Inhibitors	Description
1	No substrate		Internal substrate driven ATP synthesis only
2	20 mM succinate	5 μM rotenone	CII linked rate (rotenone to prevent electron backflow to CI)
3	20 mM succinate	5 μM rotenone + 2 mM malonate	CII linked rate negative control
4	20 mM glutamate + 4 mM malate		CI linked rate
5	20 mM glutamate + 4 mM malate	5 μM rotenone	CI linked rate negative control
6	20 mM pyruvate + 4 mM malate		CI and PDHC linked rate
7	20 mM pyruvate + 4 mM malate	5 μM rotenone	CI and PDHC linked rate negative control
8	1 mM octanoylcarnitine + 4 mM malate		ETF and CI linked rate
9	1 mM octanoylcarnitine + 4 mM malate	5 μM rotenone	ETF and CI linked rate negative control

All master mixes are prepared in ATP buffer containing 2 mM ADP, 2 mg/mL BSA and digitonin for intact cells at a concentration determined in Subheading 3.2
Abbreviations: *CI and II* complexes I and II, *ETF* electron transferring flavoprotein, *PDHC* pyruvate dehydrogenase complex

(*see* **step 1** above in sect. 3.2) which tests the maximal rate of ATP synthesis linked to either CI (± linked to pyruvate dehydrogenase activity), CII and CI with the ETF (with fatty acids). Figure 4 shows representative data obtained in isolated monocytes.

4 Comments

4.1 Notes

1. Commercial ADP powders can contain significant amounts of contaminating ATP. This generates a very high background signal in the assay. For this reason, we highly recommend ADP sourced from Calbiochem, 117105.
2. BSA must be fatty acid free, as any contamination with fatty acids can potentially be used by the mitochondria as a substrate, thus also returning a higher background rate of ATP synthesis than expected under some conditions.
3. The luminescence reagent is the major cost in this assay. For sensitivity and stability, we highly recommend firefly luciferase from Promega, E1501. We freeze this solution for re-use,

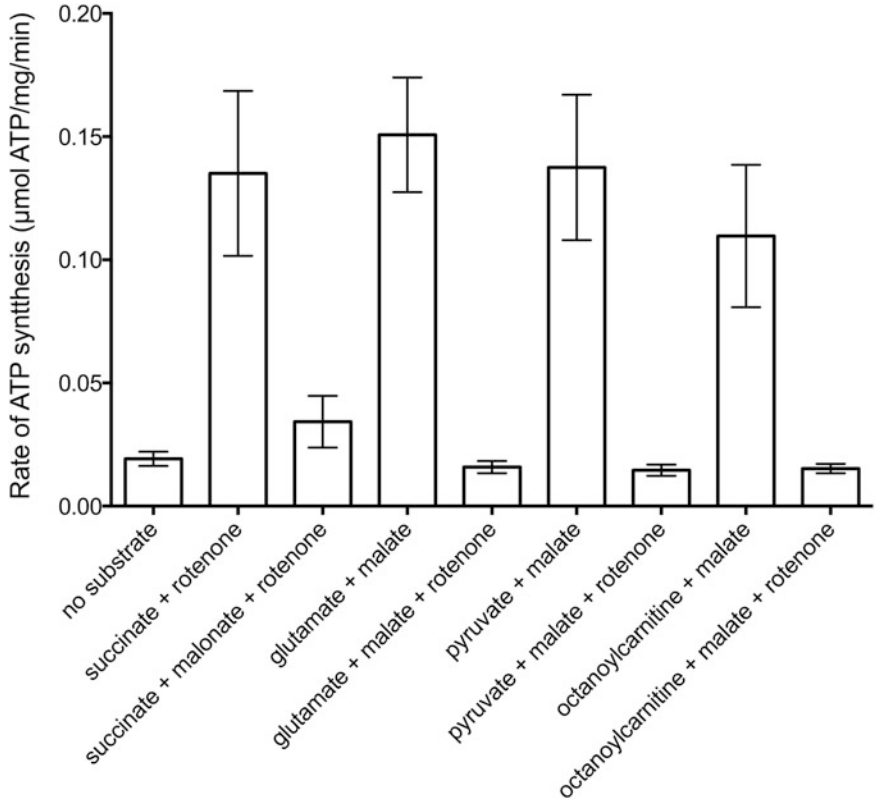

Fig. 4 Rates of ATP synthesis as determined in healthy monocytes. Rates of ATP synthesis were determined in isolated monocytes using 0.001% digitonin. Rates of ATP synthesis were determined linked to no substrate, complex II (succinate + rotenone ± the control inhibitor malonate), complex I (glutamate + malate ± the control inhibitor rotenone), complex I and the pyruvate dehydrogenase complex (pyruvate + malate ± the control inhibitor rotenone) and complex I with the electron transferring flavoprotein (octanoylcarnitine + malate ± the control inhibitor rotenone). $N = 6$, error bars are 95% confidence interval

which has stable activity for at least 3 months. Other ATP detecting luciferase solutions have been found to be less stable, and should be tested for stability when frozen.

4. This method has been optimized for a plate-based format with a multichannel pipette. Instructions have however also been provided for a tube format, which generates equivalent data, but requires significantly more pipetting and sample handling.

4.2 Common Problems

Technical duplicate values are highly inconsistent: this may be due to bubbles in the luminescence plate. To avoid bubble formation, be careful to mix solutions without introducing any air and avoid using the second stop on your pipette. You can also increase the number of technical replicates to more easily identify outliers.

Luminescence values are very low: The luminescence solution can be stored frozen, and we report that the firefly luciferase from Promega used here is stable for at least 3 months when frozen.

Other luciferase solutions may not be stable for such extended periods. If you are using a frozen solution of luciferase, and your signal is low, prepare a fresh solution of luciferase and repeat the luminescence readout (from **step 10**). If the values are still very low, you can prepare a less diluted sample for the luminescence readout (**step 9**), or repeat the assay with more concentrated starting material (*see* Subheading 3.1 "Sample preparation"). It may also be that what you consider as being "very low" luminescence values in your samples is actually a "very high" background signal that is masking your signal. This may be caused by impurities in your reagents, such as ADP, which is contaminated with ATP (common from other suppliers than Calbiochem as used here), or BSA with fatty acid (BSA must be fatty acid free).

Luminescence values are very high and do not fall within the linear range of the standards: Prepare a more diluted sample for the luminescence readout (**step 9**).

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Competing Interests
The authors declare no competing interests.

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