CHAPTER SIXTEEN

Methods to Monitor and Compare Mitochondrial and Glycolytic ATP Production

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Abstract

ATP is commonly considered as the main energy unit of the cell and participates in a variety of cellular processes. Thus, intracellular ATP concentrations rapidly vary in response to a wide variety of stimuli, including nutrients, hormones, cytotoxic agents, and hypoxia. Such alterations not necessarily affect cytosolic and mitochondrial ATP to similar extents. From an oncological perspective, this is particularly relevant in the course of tumor progression as well as in the response of cancer cells to therapy. In normal cells, mitochondrial oxidative phosphorylation (OXPHOS) is the predominant source of ATP. Conversely, many cancer cells exhibit an increased flux through glycolysis irrespective of oxygen tension. Assessing the relative contribution of glycolysis and OXPHOS to intracellular ATP production is fundamental not only for obtaining further insights into the peculiarities and complexities of oncometabolism but also for developing therapeutic and diagnostic tools. Several techniques have been developed to measure intracellular ATP levels including enzymatic methods based on hexokinase, glucose-6-phosphate dehydrogenase, and firefly luciferase. Here, we summarize conventional methods for measuring intracellular ATP levels and we provide a detailed protocol based on cytosol- and mitochondrion-targeted variants of firefly luciferase to determine the relative contribution of glycolysis and OXPHOS to ATP synthesis.

1. INTRODUCTION

The mitochondria are part of a complex cellular signaling network that play a central role in several physiological processes (Bonora, Bononi, et al., 2013; Marchi, Patergnani, & Pinton, 2014; Patergnani et al., 2013), in cellular processes (Houtkooper, Pirinen, & Auwerx, 2012), during the stress response (Manoli et al., 2007), and in regulating the homeostasis of second messengers (Giorgi et al., 2012; Marchi & Pinton, 2014). These highly specialized organelles are also the primary source of ATP: during the mitochondrial tricarboxylic acid cycle, the NADH (nicotinamide adenine dinucleotide reduced) produced by the oxidation of glycolytic pyruvate is transferred along the electron transport chain across the inner mitochondrial membrane to boost ATP production. ATP acts as an instant source of energy within the cells of all living organisms, and it participates in a variety of cellular processes. Most importantly, ATP is highly involved in tumorigenesis (Rodriguez-Enriquez et al., 2010; Shackelford & Shaw, 2009).

For example, tumor cells are able to sustain high rates of glycolysis for ATP/energy generation, regardless of oxygen availability (Scatena, 2012). Studies have clearly demonstrated that the metabolic shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis is a common hallmark

of cancer and that the signaling pathways controlling energy production in cancer cells are greatly altered during tumor progression (Gatenby & Gillies, 2004; Pollak, 2013).

A key molecular player that is highly interconnected to ATP synthesis and storage, the 5′ AMP-activated protein kinase (AMPK), has been linked to cancer metabolism and progression. AMPK is a Ser/Thr protein kinase that is sensitive to the cellular AMP/ATP ratio. AMPK acts to phosphorylate several downstream substrates in response to metabolic and energy crises caused by significant alterations in ATP synthesis. The overall effect of AMPK activation promotes ATP synthesis and consequential inhibition of anabolic (ATP-utilizing) processes such as protein and lipid synthesis.

Another critical regulator of ATP homeostasis involved in tumorigenesis is the mitochondrial ADP/ATP carrier ANT, which is responsible for the exchange of ADP for ATP in a 1:1 stoichiometry. Because of its critical role in energy metabolism, defects in the ADP/ATP transport system result in severe disorders. Studies have shown that neoplastic transformations are often associated with mutated expression of members of the ANT family and that ADP/ATP exchange is compromised of different pathological conditions.

Thus, understanding the importance of intracellular ATP levels, the rate of ADP/ATP exchange, and the intracellular ADP levels will undoubtedly be of great importance in elucidating the metabolic behavior of tumor cells.

The most commonly used methods to determine ADP levels are based on the activity of pyruvate kinase (PK). The production of ADP is evaluated by measuring the decrease in absorbance or fluorescence concomitant with the oxidation of NADH to NAD^+ by PK in the presence of phosphoenolpyruvate (Shapiro, Hajec, & Gao, 2011). One limitation of this approach is that the use of UV absorbance or excitation renders the assay prone to interference by the absorbance or fluorescence of the samples being tested. On the other hand, these methods for determining ADP/ATP exchange have been well developed (Duee & Vignais, 1969). Recently, a novel technique to measure this mitochondrial exchange based on the Mg^{2+}-sensitive fluorescent probe Magnesium Green has been described. Using this method, it is now possible to determine the ATP–ADP exchange rate mediated by the ANT and to estimate the molecular turnover of this mitochondrial carrier (Chinopoulos et al., 2009; Kawamata, Starkov, Manfredi, & Chinopoulos, 2010).

Similarly, the assessment of cellular ATP content is a critical measure in many types of experiments, especially in those involving hypoxic, metabolic, or cytotoxic cell injury. Several techniques have been developed. Some approaches are based on reactions catalyzed by different kinases, such

as adenylate and PK, and permit an excellent measurement of the total ATP levels. Nevertheless, these methods require a permeabilization step that allows the hydrophilic substrates to penetrate biological membranes. Using an FRET-based sensor, it is possible to measure the level of ATP in intact cells and to determine its cellular distribution and dynamics. Unfortunately, this approach possess critical pitfalls (such as ATP buffering or a substantial modification of ATP homeostasis; Liemburg-Apers et al., 2011). The best method for measuring intracellular ATP employs firefly luciferase, an enzyme that causes the oxidation of luciferin (an oxidizable substrate). This reaction can be quantified because the energy produced releases a photon of light (bioluminescence).

In the following sections, we review the luciferase–luciferin systems currently used to measure intracellular ATP concentrations. We describe accurate methods for the detection of ATP in different cellular compartments using recombinant-targeted chimeras of luciferase. Finally, we describe an enzymatic-based method for accurately measuring ATP levels in cultured cells.

2. MONITORING INTRACELLULAR ATP USING LUCIFERASE-BASED TECHNIQUES

The luciferase isolated from the firefly *Photinus pyralis* is a monomeric enzyme that catalyzes light production in bioluminescent organisms, requiring luciferin, ATP, and O_2 as substrates (DeLuca & McElroy, 1974; de Wet, Wood, DeLuca, Helinski, & Subramani, 1987). The reactions catalyzed by firefly luciferase are:

$$\text{Luciferase} + \text{luciferin} + \text{ATP} \overset{Mg^{2+}}{\longleftrightarrow} \text{Luciferase} - \text{luciferyl} - \text{AMP} + PP_i$$

$$\text{Luciferase} - \text{luciferyl} - \text{AMP} + O_2 \rightarrow \text{Luciferase} + \text{oxyluciferin} + \text{AMP} + CO_2 + h\nu$$

Because of its great sensitivity, the firefly luciferase assay has been widely employed to measure intracellular ATP content, and different luciferin–luciferase kit assays have been engineered.

Again, a simple microinjection of purified luciferase lacking a targeting sequence into single isolated cells permits effective assessment of cytosolic ATP levels (Bowers, Allshire, & Cobbold, 1992).

Luciferase can be expressed in the cytosol by introducing an appropriate cDNA into the cell. An example of a luciferase vector is pcDNA3LuciferaseLL/V, a mutagenized luciferase with the C-terminal

amino acid substitution L550V, which eliminates the peroxisomal targeting sequence (Bell, Manfredi, Griffiths, & Rutter, 2007).

Similarly, the cytosolic ATP luminescence of luciferase-expressing HeLa cells (HeLa-LUC cells) was continuously monitored by integrating the photon counts over 10-s intervals at 37 °C. The sample was rotated at a frequency of approximately 100 rpm, and the direction was changed every 3–5 s to ensure proper mixing of the solution (Zamaraeva, Sabirov, Manabe, & Okada, 2007). Using the adenoviral-driven expression of recombinant firefly luciferase (AdCMVcLUC), Ainscow and collaborators monitored cytosolic ATP concentrations in real time in isolated human and mouse pancreatic β-cells (Ainscow & Rutter, 2002).

A recent study used a protein transduction domain (PTD)-conjugated luciferase (LUC) to assay intracellular ATP levels in live cells. PTD-LUC can penetrate the cell membrane without affecting its integrity or interfering with cellular metabolism, and its luciferase activity is proportional to the ATP concentration (Lee et al., 2012).

Specifically targeted luciferase chimeras have been engineered based on recombinant DNA technology. A cDNA encoding luciferase can be introduced and expressed in cells and, with the addition of signal peptides, luciferase constructs have been targeted to subcellular compartments (Jouaville, Pinton, Bastianutto, Rutter, & Rizzuto, 1999; Kennedy et al., 1999). Furthermore, recombinant luciferase has been used to study variations in ATP concentration after cell stimulation by agonists that mobilize intracellular calcium (Bonora, Bononi, et al., 2013; Bonora et al., 2012; Brini et al., 1999; Giorgi et al., 2010; Giorgi, Romagnoli, Pinton, & Rizzuto, 2008; Rimessi, Giorgi, Pinton, & Rizzuto, 2008; Rimessi et al., 2009; Voronina et al., 2010). Jouaville and collaborators demonstrated that mitochondrial Ca^{2+} accumulation triggers increased ATP synthesis in the mitochondria and enhanced ATP levels in the cytosol. In contrast, a decrease in mitochondrial calcium accumulation leads to diminished mitochondrial ATP concentrations (Jouaville et al., 1999).

We present a detailed protocol below that uses recombinant luciferase chimeras targeted to discrete intracellular domains and a luminescent reader (luminometer) to monitor basal and induced levels of intracellular ATP.

2.1. Measuring the intracellular ATP concentration with a luminescence reader

In this method, the luminescence is dependent on the presence of luciferin, and it is proportional to the perfused luciferin concentration, between

20 and 200 μ*M*. The light emission is proportional to the ATP concentration in the sample.

The protocol allows the measurement of ATP levels with a recombinant-targeted luciferase in a wide variety of cell types (e.g., HeLa, HEK 293T, CHO, COS-7, SH-SY5Y, A7r5, PC12, mouse embryonic fibroblasts (MEFs), as well as primary cultures of neurons and skeletal muscle myotubes).

2.2. Equipment setup

Luminescence reader (luminometer) for cell population analyses. The photons emitted during the experimental procedure can be recorded and analyzed using relatively inexpensive equipment. The instrument is constructed as follows:

- Photon-counting head H7360-01 (Hamamatsu Photonics)
- Perfusion chamber (Elettrofor)
- Elettrofor Termositc group GT-80/P (Elettrofor)
- Photon counter C8855-01 (Hamamatsu Photonics)
- Peristaltic pump

The luminometer is composed of an upper part containing a low-noise photomultiplier inserted into a dark box housing a manual shutter (to protect it from light exposure) and a removable temperature-controlled chamber to hold the sample. During ATP measurements, the shutter is open and the perfusion chamber (13 mm in diameter, 2 mm in height, with a volume capacity of 265 μl) is placed in close proximity (<3 mm) to the photomultiplier. The cells are continuously perfused with temperature-controlled medium (water bath, GT-80/P) at 37 °C. A peristaltic pump allows liquid perfusion. The signals generated by the phototube are collected and converted by a digital counting unit connected to a computer (Bonora, Giorgi, et al., 2013; Chiesa et al., 2001; Cobbold, Cuthbertson, Goyns, & Rice, 1983; Webb, Rogers, Karplus, & Miller, 2010).

2.3. Reagent preparation

1. Modified Krebs–Ringer solution (KRB): 125 m*M* NaCl, 5 m*M* KCl, 1 m*M* KH_2PO_4, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 5.5 m*M* glucose, and 20 m*M* HEPES.
2. Luciferin solution (Luciferin): 20 μ*M* luciferin (Promega, E1605) in KRB solution.

3. Ca^{2+}-mobilizing solution (CMS): Dissolve the Ca^{2+}-perturbing agent in the luciferin solution; different cell types require different agents (e.g., histamine for HeLa cells, bradykinin for human fibroblasts, ATP for MEFs and HEK293T cells, and carbachol for SH-SY5Y cells).

2.4. Sample preparation and transfection

The cells are seeded on glass coverslips (13 mm in diameter) for single-sample luminescence measurements, and they are allowed to grow until they reach 50% confluence. After seeding the cells, wait at least 24 h. The cells are then transfected with a cytosolic (untargeted) firefly luciferase construct (cytLUC) and a mitochondrial-targeted HA1-tagged luciferase construct (mtLUC) using an appropriate transfection method. Cancer and immortalized cell lines are easily transfected using Ca^{2+} phosphate or lipoamine transfection methods. Primary cultures that are notably "hard-to-transfect" require electroporation or infection with adenoviral vectors. After transfection, wait for 36–48 h.

An 876-nucleotide *Bgl*II*Hin*dIII fragment of the cytosolic (untargeted) firefly luciferase was transferred from the pGL3 basic plasmid (Promega) to the pcDNA3 plasmid (Invitrogen), and it was then transferred to VR1012 to generate the cytLUC plasmid.

To obtain the mitochondrial luciferase construct, the photoprotein was engineered with DNA sequences encoding the mitochondrial protein subunit VIII of cytochrome *c* oxidase (COX8) and the hemagglutinin HA1 tag. The entire final construct (mtLUC) was cloned into the expression vectors pcDNAI and VR1012 (Jouaville et al., 1999; Kennedy et al., 1999; Porcelli et al., 2001).

2.5. Measurements

1. Remove the cells from the incubator and place the 13-mm coverslip inside the temperature-controlled (37 °C) perfusion chamber.
2. Begin perfusing the cell monolayer with KRB using a peristaltic pump (standard flow rate: 2.5 ml/min) and place the chamber in close proximity (2–3 mm) to the surface of the low-noise photomultiplier tube.
3. Measure the background luminescence. Begin by recording the basal cps (counts per second) luminescence value for at least 30 s to obtain a stable signal, and then record the lower value as the background (see Fig. 16.1A).

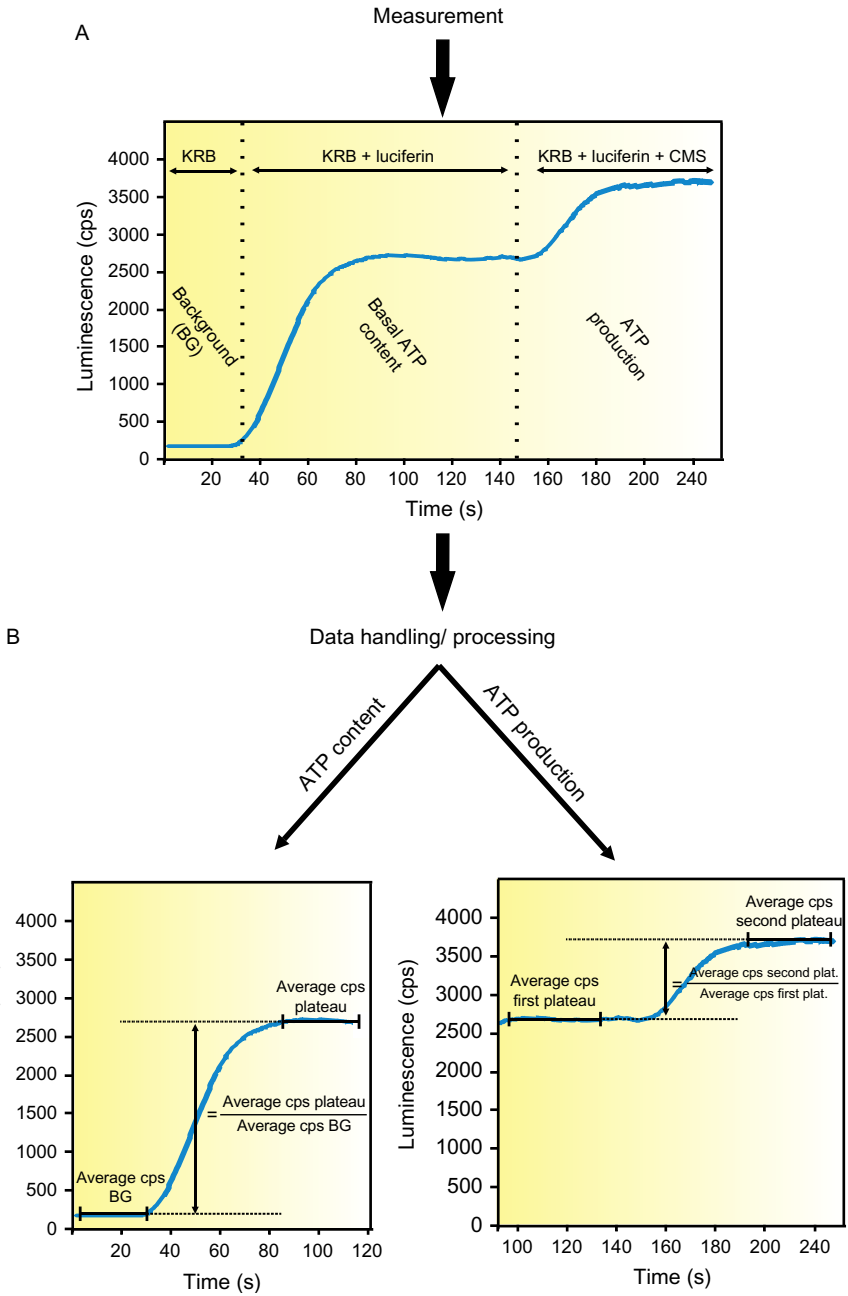

Figure 16.1 *Calibration and measurement of intracellular ATP levels using a luminometer.* (A) A representative luminescence trace employed to measure free [ATP] in intracellular compartments using a specifically targeted chimera of the ATP-sensitive photoprotein luciferase. The resting luminescence (background, BG) was recorded before the addition of luciferin to assess the basal ATP levels. Next, the cells were perfused with a Ca^{2+}-mobilizing solution (CMS) to determine the amount of ATP production. (B) The data were processed to estimate the basal ATP content and the levels of ATP produced. KRB, modified Krebs–Ringer solution; cps, counts per second.

4. To record the basal ATP content, pause the perfusion, change from the modified KRB to the luciferin solution, and resume the perfusion. Following the addition of luciferin, light emission (and thus cps values) rapidly increases and reaches a plateau within approximately 2 min (see Fig. 16.1A).
5. Record the ATP production. Wait until the cps values remain unchanged for approximately 15–30 s. Pause the perfusion, change the medium to the CMS solution, and resume the perfusion. Under these conditions, the light emission (and therefore, the cps values) rapidly increases and reaches a second plateau within approximately 1–1.5 minutes. Wait until the cps values remain unchanged for approximately 20–30 s (see Fig. 16.1A).
6. Stop the analysis system, switch off the power supply and the perfusion, and remove the 13-mm coverslip from the chamber notch. Reseal the chamber and wash the entire system extensively by perfusion with distilled water, thus avoiding the occurrence of spurious signals in later experiments

2.6. Data handling/processing

To calculate the basal ATP content, estimate the luminescence values of the plateau generated after the addition of the luciferin solution (see Fig. 16.1B). As the basal ATP content is dependent on the abundance of luciferase transfected, an immunoblot assay with an antibody against luciferase is recommended. To calculate the amount of ATP produced, divide the luminescence values of the plateau generated after the addition of the luciferin solution by the luminescence values of the second plateau generated after the addition of the CMS solution (see Fig. 16.1B).

2.7. Discrimination of glycolytic versus OXPHOS ATP generation

By using both luciferase constructs, it is possible to dynamically monitor the effect of OXPHOS and glycolysis inhibitors to capture an accurate measurement of the contribution of glycolysis and oxidative phosphorylation to the intracellular ATP production.

To mimic OXPHOS suppression, use uncouplers of mitochondrial OXPHOS, such as pentachlorophenol or carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone. Alternatively, the same results may be obtained using inhibitors of the respiratory chain, such as cyanide

(NaCN, an inhibitor of complex IV). However, the most commonly used inhibitor is oligomycin, a potent inhibitor of the mitochondrial ATP synthase.

Different methods may be used to inhibit ATP production by the glycolytic pathway. If it is available, we recommended utilizing the non-metabolizable analog of glucose, 2-deoxyglucose (2DG); if 2DG is not available, it is possible to block glycolytic ATP synthesis with iodoacetamide (a glyceraldehyde 3-phosphate blocker) or fluoride (NaF, an enolase inhibitor).

Considering the fact that different cell lines display different respiration levels and glycolysis rates, the specific pharmacological effects of the compound used should be evaluated initially to determine the ideal experimental conditions. In the next sections (Sections 2.8–2.11), we describe how to discriminate between glycolytic and OXPHOS ATP generation and discuss the optimal inhibitor dosages for an immortalized and cancer cell line (HeLa cells) and for primary cells (MEFs).

2.8. Reagent setup

1. Modified Krebs–Ringer solution (KRB): 125 m*M* NaCl, 5 m*M* KCl, 1 m*M* KH_2PO_4, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 5.5 m*M* glucose and 20 m*M* HEPES.
2. Luciferin solution (luciferin): 20 μ*M* luciferin in KRB solution.
3. 2DG solution: 5 m*M* (for immortalized or cancer cell lines) or 20 m*M* (primary cell cultures) deoxyglucose (Sigma-Aldrich, D8375) in luciferin solution.
4. Oligomycin solution (oligomycin): 1 μ*M* (for immortalized or cancer cell lines) or 5 μ*M* (primary cell cultures) oligomycin (Sigma-Aldrich, 75351) in 2DG solution.

2.9. Measurement

1. After seeding the cells as described in Section 2.4, transfect the cells with cytLUC or mtLUC. HeLa cells are easily transfected using Ca^{2+}-phosphate or lipoamines. Primary cells are less sensitive to standard methods and require electroporation or adenoviral vector infection.
2. After transfection, wait for 36–48 h.
3. Begin the measurement by following the instructions provided in Section 2.5 until step 4 (see Fig. 16.2A).

4. Wait until the cps values remain unchanged for approximately 15–30 s. Pause the perfusion, change the medium to the 2DG solution, and resume the perfusion. Under these conditions, the nonmetabolizable analog of glucose triggers a large decrease in the luciferase signal. The light emission (and therefore cps values) decreases until reaching a plateau after approximately 1 min. At this point, wait until the light emission does not change further (approximately 30 s; see Fig. 16.2A).
5. Stop the perfusion system and change the medium to the oligomycin solution. Restart the perfusion. As a consequence of adding the mitochondrial ATP synthase inhibitor, OXPHOS is suppressed and the cps values decrease further until they are comparable to resting luminescence. Wait until the values reach a plateau (approximately 30–60 s; see Fig. 16.2A).
6. Repeat step 6 from Section 2.5.

2.10. Data handling/processing

To calculate the total ATP levels, divide the luminescence values of the plateau generated after the addition of the luciferin solution by the luminescence values of the plateau generated after the addition of the oligomycin solution. To estimate the contribution of oxidative phosphorylation, divide the total ATP level by the ratio calculated between the plateau generated after the addition of oligomycin and the plateau generated after the addition of 2DG. To compute the amount of ATP produced by glycolysis, divide the total ATP level by the rate of oxidative phosphorylation (see Fig. 16.2B).

2.11. Notes

Unlike fluorescent reporters, luciferase has the advantage that no external light source is required to reveal its presence; thus, there is no background light originating from the sample, and it is possible to avoid photobleaching, phototoxicity, and autofluorescence (Welsh & Kay, 2005). However, this enzyme has its own disadvantages. For instance, luciferase becomes extensively inactivated during the coupling reaction. Importantly, the ATP concentration and light emission are not proportional. Furthermore, this assay is highly dependent on ATP and oxygen; thus, it is necessary to define the endogenous ATP levels to accurately determine the kinetics of ATP production. Several strategies have been developed to overcome these problems (Wibom, Lundin, & Hultman, 1990). Adding aliquots of an ATP standard during a continuous luminescence recording ensures a quantification of the

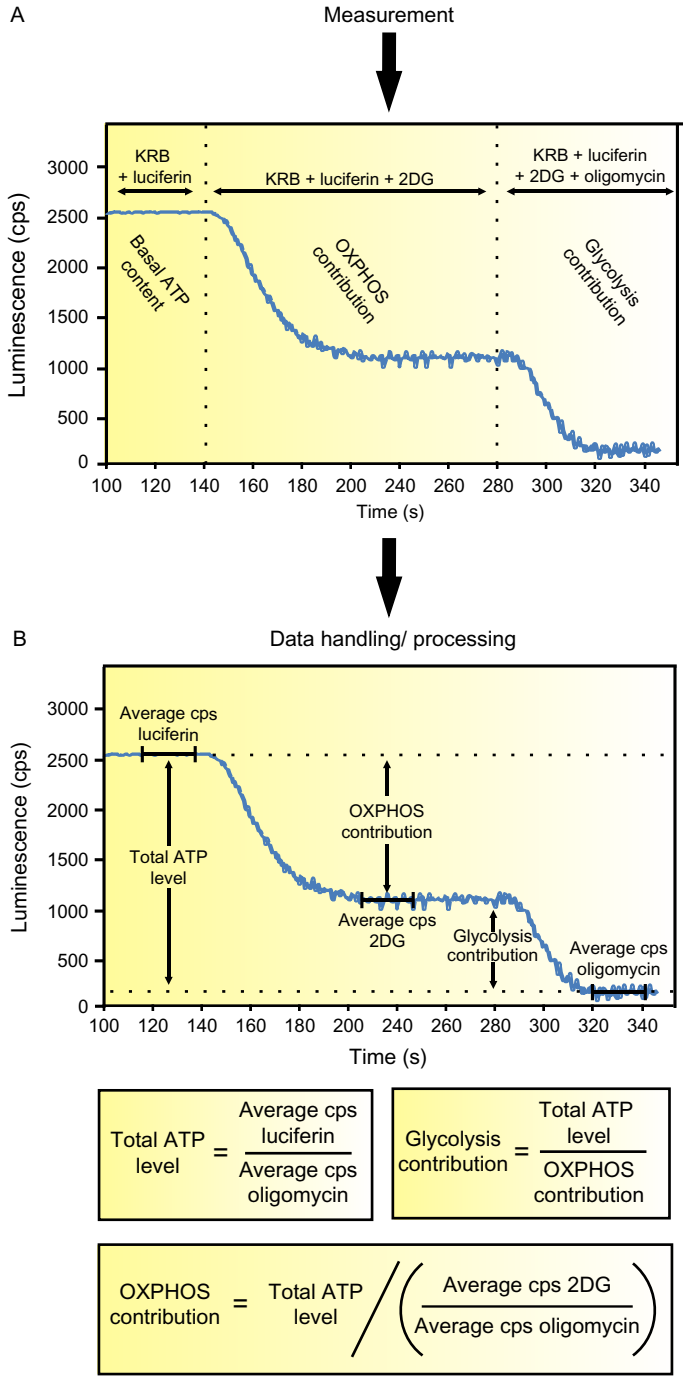

Figure 16.2 *Assessment of the OXPHOS and glycolytic contribution to ATP content*. (A) A luminescence trace for determining glycolytic and OXPHOS ATP in the presence of 2DG and oligomycin. (B) The data were processed to determine the contribution of glycolysis and OXPHOS to ATP production. The luminescence is expressed in relative light units. cps, counts per seconds; 2DG, 2-deoxyglucose.

ATP concentration (Lemasters & Hackenbrock, 1976). Alternatively, it is possible to perform measurements with mutant luciferases that improve the enzyme's stability or increase its catalytic efficiency (Fujii et al., 2007; Hirokawa, Kajiyama, & Murakami, 2002).

Nevertheless, the major drawback of this method is that luciferin is an amphipathic molecule that, due to its carboxyl group, is charged at physiological pH, thus preventing easy passage across cell membranes. It is possible to increase the rate of luciferin entry into cells by manipulating the buffer conditions. For example, improved luciferin uptake into cells has been demonstrated using a buffer with a low pH (Craig, Simmonds, Watmore, McCapra, & White, 1991). Other ways to enhance luciferin entry in mammalian cells include the employment of uncharged luciferin derivatives (Gould & Subramani, 1988) or membrane-permeable luciferin esters (Harwood, Mofford, Reddy, & Miller, 2011).

3. MONITORING INTRACELLULAR ATP USING ALTERNATIVE ENZYMATIC METHODS

The main advantage of the luciferin–luciferase assay is the very rapid detection of ATP levels, but this technique requires a luminometer. Should a luminometer not be available, it is possible to use alternative procedures that take advantage of standard enzymatic methods. Two alternative enzymatic methods for ATP measurement are proposed below.

The first method is based on the glycerokinase/glycerolphosphate oxidase/horseradish peroxidase-coupled assay. Briefly, the assay is composed of a three-step enzymatic reaction:

1. ATP (from a measured sample) + glycerol + glycerokinase → glycerol-3-phosphate + ADP
2. Glycerol-3-phosphate + O_2 + glycerolphosphate oxidase → phosphodioxyacetone + H_2O_2
3. H_2O_2 + TMPD (TetraMethylPhenyleneDiamine) + horseradish peroxidase → H_2O + TMPD*

   *The absorbance of oxidized TMPD is measured spectrophotometrically at 610 nm.

This method was originally proposed for the detection of triglycerides rather than ATP.

The second method is based on the hexokinase/glucose-6-phosphate dehydrogenase-coupled assay. This method is the most common and was

originally used for glucose detection. After some modification, it was also used for hexokinase detection in subfractionated mitochondrial protein complexes, as described by Beutner, Ruck, Riede, Welte, and Brdiczka (1996) or by Wieckowski, Brdiczka, and Wojtczak (2000) and Wieckowski et al. (2001). Briefly, the assay is composed of a two-step enzymatic reaction:

1. ATP + glucose + hexokinase → glucose-6-phosphate + ADP
2. Glucose-6-phosphate + NADP + glucose-6-phosphate dehydrogenase → 6-phosphogluconate + NADPH

   The absorbance of reduced NADPH can be measured spectrophotometrically at 340 nm or fluorimetrically at an excitation/emission of 340/450 nm, respectively.

Below, we include an accurate description of this tool and provide a precise standardization of the method for quantifying the amount of ATP.

3.1. Reagent setup

1. Reaction buffer: 100 m*M* TEA (triethanolamine), 16 m*M* $MgSO_4$ (magnesium sulfate) and 10 m*M* EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate); pH 7.6
2. Stock solutions: glucose (1 M stock), NAD (1 M stock), ATP (0.75 M stock), hexokinase (0.5 U/ml stock), and glucose-6-phosphate dehydrogenase (200 U/ml stock)

3.2. Sample preparation

1. Resuspend the cells in a small amount of PBS (depending on the pellet size).
2. Add TCA (trichloroacetic acid) to a final concentration of 2%. The TCA should contain 0.001% of xylenol blue dye as a pH indicator. A red color indicates an acidic pH.
3. After the TCA extraction, the sample should be diluted to 0.1% (concentration of TCA).
4. Neutralize the sample using the Tris–acetate buffer, pH 7.7. The color of the neutralized sample should change from red to yellow. If necessary, add more of the Tris–acetate buffer, pH 7.7.
5. Keep the sample on ice and use it for ATP measurements or store it at -80°C.

An alternative method has been described by Manfredi, Yang, Gajewski, and Mattiazzi (2002).

1. Resuspend the cells in a small amount of PBS (depending on the pellet size).
2. Add 10 µl of ice-cold 0.4 *M* perchloric acid per milligram of protein.
3. Keep the suspension on ice for 30 min, then centrifuge it at 14,000 rpm for 10 min at 4 °C.
4. Collect the supernatant and neutralize it with 10 µl of 4 *M* K_2CO_3 per 100 µl of supernatant.
5. Keep the suspension on ice for 10 min and then at −80 °C for 1–2 h to precipitate the perchlorate.
6. Centrifuge the sample again at 14,000 rpm for 10 min at 4 °C.
7. Keep the collected supernatant on ice and use it for ATP measurements. Alternatively, store it at −80 °C.

3.3. Measurements

1. Dispense 2.5 ml of the reaction buffer into the cuvette and add the following:
   - 10 µl of 1 *M* glucose
   - 10 µl of 1 *M* NAD^+
   - 20 µl of glucose-6-phosphate dehydrogenase (200 U/ml stock)
   - 25–50 µl of the sample
2. Record the fluorescence trace. Begin the reaction with the addition of 20 µl of hexokinase (0.5 U/ml stock) and wait for the plateau. If the change in fluorescence is small, add more sample. To ensure that the amount of hexokinase in the reaction was not limiting, it is possible to add an additional hexokinase to the cuvette. No significant change in the fluorescence should be observed (see Fig. 16.3).

3.4. Standardization of the method

After reaching the plateau, add small volumes of the ATP stock solution (diluted to the proper concentration, if necessary). Measure the increase in fluorescence (see Fig. 16.3). It is important to perform this standardization on the same day of the experiment (e.g., for each ATP stock and sample buffer).

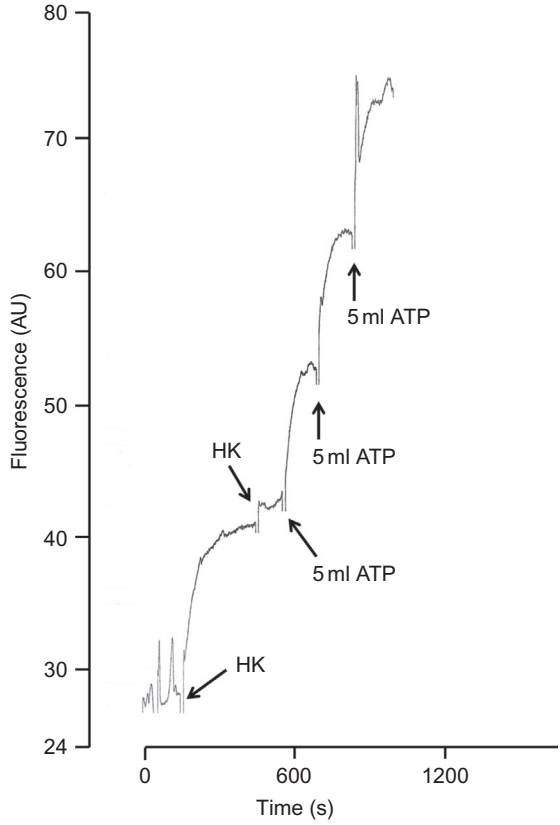

Figure 16.3 *Representative trace of an ATP measurement in Zajdela tumor cell lysates.* The data were obtained from 50 μl of Zajdela tumor cell lysates. HK, hexokinase; ml, milliliter; AU, arbitrary unit.

3.5. Notes

The protocol presented above (in Section 3) is useful for measuring the total cellular ATP level. It is very difficult to distinguish between the cytosolic and mitochondrial ATP levels using this method. These difficulties are based on the practical impossibility of separating the cytosolic fraction without damaging the mitochondria (during mild homogenization or plasma membrane permeabilization using digitonin). Mitochondrial damage will cause the release of ATP from broken mitochondria and contaminate the cytosolic ATP pool. Recently, Soccio et al. described a fast method for isolating cytosol-enriched fractions in which mitochondrial integrity is preserved by using an isotonic grinding medium (Soccio, Laus, Trono, & Pastore,

2013). This fluorimetric method to assay ATP content is practically identical to the one presented in the section above (Section 3).

4. SUMMARY

Because ATP regulates many aspects of cellular life, detecting the levels of this molecule is fundamentally important in many biological fields. Importantly, the alteration of ATP storage is found in several human disorders, including cancer. Currently, the measurement of ATP levels is also used to evaluate tumor growth and to determine tumor chemosensitivity (Bradbury, Simmons, Slater, & Crouch, 2000; Sevin et al., 1988). Thus, an efficient measurement of intracellular ATP levels will allow the assessment of proliferation and the effective energetic state of a tumor cell. Furthermore, it will be possible to determine whether a specific pharmacological treatment is able to block tumor growth and to reverse the malignant phenotype.

In this chapter, we have described luciferase/luciferin-based techniques that guarantee a rapid and highly accurate determination of ATP levels in different compartments, particularly in the cytosol (the site of glycolysis) and the mitochondria (the site of OXPHOS). However, in certain cases, the use of firefly luciferase may not always be possible, and alternative methods are necessary. We have presented two efficient techniques to measure intracellular ATP without luciferase: one based on the glycerokinase/glycerolphosphate oxidase/horseradish peroxidase-coupled assay and the other based on the hexokinase/glucose-6-phosphate dehydrogenase-coupled assay (for the latter, we also provided a detailed description).

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REFERENCES

Ainscow, E. K., & Rutter, G. A. (2002). Glucose-stimulated oscillations in free cytosolic ATP concentration imaged in single islet beta-cells: Evidence for a Ca^{2+}-dependent mechanism. *Diabetes*, *51*(Suppl. 1), S162–S170.

Bell, C. J., Manfredi, G., Griffiths, E. J., & Rutter, G. A. (2007). Luciferase expression for ATP imaging: Application to cardiac myocytes. *Methods in Cell Biology*, *80*, 341–352.

Beutner, G., Ruck, A., Riede, B., Welte, W., & Brdiczka, D. (1996). Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. *FEBS Letters*, *396*(2–3), 189–195.

Bonora, M., Bononi, A., De Marchi, E., Giorgi, C., Lebiedzinska, M., Marchi, S., et al. (2013). Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. *Cell Cycle*, *12*(4), 674–683.

Bonora, M., Giorgi, C., Bononi, A., Marchi, S., Patergnani, S., Rimessi, A., et al. (2013). Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. *Nature Protocols*, *8*(11), 2105–2118.

Bonora, M., Patergnani, S., Rimessi, A., De Marchi, E., Suski, J. M., Bononi, A., et al. (2012). ATP synthesis and storage. *Purinergic Signal*, *8*(3), 343–357.

Bowers, K. C., Allshire, A. P., & Cobbold, P. H. (1992). Bioluminescent measurement in single cardiomyocytes of sudden cytosolic ATP depletion coincident with rigor. *Journal of Molecular and Cellular Cardiology*, *24*(3), 213–218.

Bradbury, D. A., Simmons, T. D., Slater, K. J., & Crouch, S. P. (2000). Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. *Journal of Immunological Methods*, *240*(1–2), 79–92.

Brini, M., Pinton, P., King, M. P., Davidson, M., Schon, E. A., & Rizzuto, R. (1999). A calcium signaling defect in the pathogenesis of a mitochondrial DNA inherited oxidative phosphorylation deficiency. *Nature Medicine*, *5*(8), 951–954.

Chiesa, A., Rapizzi, E., Tosello, V., Pinton, P., de Virgilio, M., Fogarty, K. E., et al. (2001). Recombinant aequorin and green fluorescent protein as valuable tools in the study of cell signalling. *The Biochemical Journal*, *355*(Pt. 1), 1–12.

Chinopoulos, C., Vajda, S., Csanady, L., Mandi, M., Mathe, K., & Adam-Vizi, V. (2009). A novel kinetic assay of mitochondrial ATP-ADP exchange rate mediated by the ANT. *Biophysical Journal*, *96*(6), 2490–2504.

Cobbold, P. H., Cuthbertson, K. S., Goyns, M. H., & Rice, V. (1983). Aequorin measurements of free calcium in single mammalian cells. *Journal of Cell Science*, *61*, 123–136.

Craig, F. F., Simmonds, A. C., Watmore, D., McCapra, F., & White, M. R. (1991). Membrane-permeable luciferin esters for assay of firefly luciferase in live intact cells. *The Biochemical Journal*, *276*(Pt. 3), 637–641.

DeLuca, M., & McElroy, W. D. (1974). Kinetics of the firefly luciferase catalyzed reactions. *Biochemistry*, *13*(5), 921–925.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., & Subramani, S. (1987). Firefly luciferase gene: Structure and expression in mammalian cells. *Molecular and Cellular Biology*, *7*(2), 725–737.

Duee, E. D., & Vignais, P. V. (1969). Kinetics and specificity of the adenine nucleotide translocation in rat liver mitochondria. *The Journal of Biological Chemistry*, *244*(14), 3920–3931.

Fujii, H., Noda, K., Asami, Y., Kuroda, A., Sakata, M., & Tokida, A. (2007). Increase in bioluminescence intensity of firefly luciferase using genetic modification. *Analytical Biochemistry*, *366*(2), 131–136.

Gatenby, R. A., & Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis? *Nature Reviews Cancer*, *4*(11), 891–899.

Giorgi, C., Baldassari, F., Bononi, A., Bonora, M., De Marchi, E., Marchi, S., et al. (2012). Mitochondrial Ca(2+) and apoptosis. *Cell Calcium*, *52*(1), 36–43.

Giorgi, C., Ito, K., Lin, H. K., Santangelo, C., Wieckowski, M. R., Lebiedzinska, M., et al. (2010). PML regulates apoptosis at endoplasmic reticulum by modulating calcium release. *Science*, *330*(6008), 1247–1251.

Giorgi, C., Romagnoli, A., Pinton, P., & Rizzuto, R. (2008). Ca2 + signaling, mitochondria and cell death. *Current Molecular Medicine*, *8*(2), 119–130.

Gould, S. J., & Subramani, S. (1988). Firefly luciferase as a tool in molecular and cell biology. *Analytical Biochemistry*, *175*(1), 5–13.

Harwood, K. R., Mofford, D. M., Reddy, G. R., & Miller, S. C. (2011). Identification of mutant firefly luciferases that efficiently utilize aminoluciferins. *Chemistry & Biology*, *18*(12), 1649–1657.

Hirokawa, K., Kajiyama, N., & Murakami, S. (2002). Improved practical usefulness of firefly luciferase by gene chimerization and random mutagenesis. *Biochimica et Biophysica Acta*, *1597*(2), 271–279.

Houtkooper, R. H., Pirinen, E., & Auwerx, J. (2012). Sirtuins as regulators of metabolism and healthspan. *Nature Reviews Molecular Cell Biology*, *13*(4), 225–238.

Jouaville, L. S., Pinton, P., Bastianutto, C., Rutter, G. A., & Rizzuto, R. (1999). Regulation of mitochondrial ATP synthesis by calcium: Evidence for a long-term metabolic priming. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(24), 13807–13812.

Kawamata, H., Starkov, A. A., Manfredi, G., & Chinopoulos, C. (2010). A kinetic assay of mitochondrial ADP-ATP exchange rate in permeabilized cells. *Analytical Biochemistry*, *407*(1), 52–57.

Kennedy, H. J., Pouli, A. E., Ainscow, E. K., Jouaville, L. S., Rizzuto, R., & Rutter, G. A. (1999). Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells. Potential role for strategically located mitochondria. *The Journal of Biological Chemistry*, *274*(19), 13281–13291.

Lee, M. S., Park, W. S., Kim, Y. H., Ahn, W. G., Kwon, S. H., & Her, S. (2012). Intracellular ATP assay of live cells using PTD-conjugated luciferase. *Sensors (Basel, Switzerland)*, *12*(11), 15628–15637.

Lemasters, J. J., & Hackenbrock, C. R. (1976). Continuous measurement and rapid kinetics of ATP synthesis in rat liver mitochondria, mitoplasts and inner membrane vesicles determined by firefly-luciferase luminescence. *European Journal of Biochemistry*, *67*(1), 1–10.

Liemburg-Apers, D. C., Imamura, H., Forkink, M., Nooteboom, M., Swarts, H. G., Brock, R., et al. (2011). Quantitative glucose and ATP sensing in mammalian cells. *Pharmaceutical Research*, *28*(11), 2745–2757.

Manfredi, G., Yang, L., Gajewski, C. D., & Mattiazzi, M. (2002). Measurements of ATP in mammalian cells. *Methods*, *26*(4), 317–326.

Manoli, I., Alesci, S., Blackman, M. R., Su, Y. A., Rennert, O. M., & Chrousos, G. P. (2007). Mitochondria as key components of the stress response. *Trends in Endocrinology and Metabolism*, *18*(5), 190–198.

Marchi, S., Patergnani, S., & Pinton, P. (2014). The endoplasmic reticulum-mitochondria connection: One touch, multiple functions. *Biochimica et Biophysica Acta*, *1837*(4), 461–469.

Marchi, S., & Pinton, P. (2014). The mitochondrial calcium uniporter complex: Molecular components, structure and physiopathological implications. *The Journal of Physiology*, *592*(Pt 5), 829–839.

Patergnani, S., Marchi, S., Rimessi, A., Bonora, M., Giorgi, C., Mehta, K. D., et al. (2013). PRKCB/protein kinase C, beta and the mitochondrial axis as key regulators of autophagy. *Autophagy*, *9*(9), 1367–1385.

Pollak, M. (2013). Targeting oxidative phosphorylation: Why, when, and how. *Cancer Cell*, *23*(3), 263–264.

Porcelli, A. M., Pinton, P., Ainscow, E. K., Chiesa, A., Rugolo, M., Rutter, G. A., et al. (2001). Targeting of reporter molecules to mitochondria to measure calcium, ATP, and pH. *Methods in Cell Biology*, *65*, 353–380.

Rimessi, A., Giorgi, C., Pinton, P., & Rizzuto, R. (2008). The versatility of mitochondrial calcium signals: From stimulation of cell metabolism to induction of cell death. *Biochimica et Biophysica Acta*, *1777*(7–8), 808–816.

Rimessi, A., Marchi, S., Fotino, C., Romagnoli, A., Huebner, K., Croce, C. M., et al. (2009). Intramitochondrial calcium regulation by the FHIT gene product sensitizes to apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 12753–12758.

Rodriguez-Enriquez, S., Carreno-Fuentes, L., Gallardo-Perez, J. C., Saavedra, E., Quezada, H., Vega, A., et al. (2010). Oxidative phosphorylation is impaired by prolonged hypoxia in breast and possibly in cervix carcinoma. *The International Journal of Biochemistry & Cell Biology*, *42*(10), 1744–1751.

Scatena, R. (2012). Mitochondria and cancer: A growing role in apoptosis, cancer cell metabolism and dedifferentiation. *Advances in Experimental Medicine and Biology*, *942*, 287–308.

Sevin, B. U., Peng, Z. L., Perras, J. P., Ganjei, P., Penalver, M., & Averette, H. E. (1988). Application of an ATP-bioluminescence assay in human tumor chemosensitivity testing. *Gynecologic Oncology*, *31*(1), 191–204.

Shackelford, D. B., & Shaw, R. J. (2009). The LKB1-AMPK pathway: Metabolism and growth control in tumour suppression. *Nature Reviews Cancer*, *9*(8), 563–575.

Shapiro, A. B., Hajec, L., & Gao, N. (2011). High-throughput, homogeneous, fluorescence intensity-based measurement of adenosine diphosphate and other ribonucleoside diphosphates with nanomolar sensitivity. *Analytical Biochemistry*, *415*(2), 190–196.

Soccio, M., Laus, M., Trono, D., & Pastore, D. (2013). A new simple fluorimetric method to assay cytosolic ATP content: Application to durum wheat seedlings to assess modulation of mitochondrial potassium channel and uncoupling protein activity under hyperosmotic stress. *Biologia*, *68*(3), 421–432.

Voronina, S. G., Barrow, S. L., Simpson, A. W., Gerasimenko, O. V., da Silva Xavier, G., Rutter, G. A., et al. (2010). Dynamic changes in cytosolic and mitochondrial ATP levels in pancreatic acinar cells. *Gastroenterology*, *138*(5), 1976–1987.

Webb, S. E., Rogers, K. L., Karplus, E., & Miller, A. L. (2010). The use of aequorins to record and visualize Ca(2+) dynamics: From subcellular microdomains to whole organisms. *Methods in Cell Biology*, *99*, 263–300.

Welsh, D. K., & Kay, S. A. (2005). Bioluminescence imaging in living organisms. *Current Opinion in Biotechnology*, *16*(1), 73–78.

Wibom, R., Lundin, A., & Hultman, E. (1990). A sensitive method for measuring ATP-formation in rat muscle mitochondria. *Scandinavian Journal of Clinical and Laboratory Investigation*, *50*(2), 143–152.

Wieckowski, M. R., Brdiczka, D., & Wojtczak, L. (2000). Long-chain fatty acids promote opening of the reconstituted mitochondrial permeability transition pore. *FEBS Letters*, *484*(2), 61–64.

Wieckowski, M. R., Vyssokikh, M., Dymkowska, D., Antonsson, B., Brdiczka, D., & Wojtczak, L. (2001). Oligomeric C-terminal truncated Bax preferentially releases cytochrome c but not adenylate kinase from mitochondria, outer membrane vesicles and proteoliposomes. *FEBS Letters*, *505*(3), 453–459.

Zamaraeva, M. V., Sabirov, R. Z., Manabe, K., & Okada, Y. (2007). Ca(2+)-dependent glycolysis activation mediates apoptotic ATP elevation in HeLa cells. *Biochemical and Biophysical Research Communications*, *363*(3), 687–693.