Running title: Adenine nucleotide exchange in mitochondria

Measurement of ADP-ATP exchange in relation to membrane potential and oxygen consumption in mitochondria with improved calibration

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We have previously described a fluorimetric method to measure ADP-ATP exchange rates in mitochondria of permeabilized cells where phosphorylases, kinases, ion pumps exhibiting substantial ATP hydrolase activity and other competing reactions interconverting adenine nucleotides are present. This was achieved by recording changes in free extramitochondrial [Mg^{2+}] reported by the Mg^{2+}-sensitive fluorescent indicator 'Magnesium Green', exploiting the differential affinity of ADP and ATP to Mg^{2+}. Cells are permeabilized with digitonin in the presence of BeF_3^- and Na_3VO_4 inhibiting all ATP-ADP utilizing reactions except for the adenine nucleotide translocase-mediated mitochondrial ATP-ADP exchange. The rate of ATP appearing in the medium after addition of ADP to energized mitochondria is calculated from the measured rate of change in free extramitochondrial [Mg^{2+}] using standard binding equations. Here, we have enhanced this method with an improved calibration step, which minimizes errors introduced during conversion of the dye-emission signal to free extramitochondrial [Mg^{2+}] and further to ATP. Furthermore, we can combine the methodologies addressing the kinetics of ADP-ATP exchange to mitochondrial membrane potential and to oxygen consumption in the same sample. This may prove to be particularly useful for studies of cancer cells which are known to thrive in hypoxic environments, harboring mitochondria with respiratory chain dysfunctions.

Abbreviations: ANT: adenine nucleotide translocase; AP_5A: P^1,P^5-di(adenosine-5′) pentaphosphate; cATR: carboxyatractyloside; DMEM: Dulbecco's Modified Eagle Medium; EDTA: Ethylenediaminetetraacetic acid; EGTA: Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; MgGr: magnesium green; SF 6847: Tyrphostin 9, RG-50872, Malonaben, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile, 2,6-di-t-butyl-4-(2',2'-dicyanovinyl)phenol.

INTRODUCTION

Adenine nucleotide translocase (ANT) exchanges ATP for ADP across the inner mitochondrial membrane (Klingenberg 1980;Pebay-Peyroula and Brandolin 2004). Because ANT transports adenine nucleotides only in the Mg^{2+}-free state (Klingenberg 1980;Kramer 1980), and Mg^{2+} has differential affinity for ADP and ATP, we are able to measure ANT activity using the membrane-impermeable Mg^{2+}-sensitive fluorescent indicator 'Magnesium Green'. In this assay, the rate of change in free extramitochondrial [Mg^{2+}] in the experimental medium is measured following addition of ADP to mitochondria. The changes in free extramitochondrial [Mg^{2+}] exhibit complete sensitivity to submicromolar amounts of the ANT inhibitor, carboxyatractyloside (cATR) (Chinopoulos *et al.* 2009;Metelkin *et al.* 2009). The rate of change in free [Mg^{2+}] is converted to rate of ATP exported from mitochondria using

standard binding equations (Chinopoulos *et al.* 2009).The ATP-ADP exchange rate mediated by the ANT from isolated mitochondria has been validated in (Chinopoulos *et al.* 2009), especially in view of the contribution of the ATP-Mg^{2+}/P_i carrier (Aprille 1993) and a homologue of the Mrs2 protein originally described in yeast that mediates an electrophoretic uptake of Mg^{2+} in mitochondria (Kolisek *et al.* 2003).
In isolated mitochondria, besides ANT, adenylate kinase and creatine kinase in the intermembrane space interconvert adenine nucleotides, but the former is effectively inhibited by P^1,P^5-di(adenosine-5′) pentaphosphate (AP_5A) (Lienhard and Secemski 1973), and the latter by excluding creatine or its phosphate derivatives in the medium (Chinopoulos *et al.* 2009). In permeabilized cells, however, numerous additional enzymes that interconvert adenine nucleotides exist. These include phosphorylases, phosphatases and kinases, as well as the Na^+/K^+ ATPase, the plasmalemmal and endoplasmic Ca^{2+} ATPase, and in contractile cells the myosin ATPase. These reactions hamper the use of the binding equations that convert free [Mg^{2+}] to ANT-dependent ADP-ATP exchange rate (Chinopoulos *et al.* 2009). Thus, in permeabilized cells, all competing adenine nucleotide interconverting reactions need to be inhibited. This is accomplished by the use of BeF_3^- and vanadium compounds, which effectively inhibit ADP and/or ATP utilizing reactions (Cantley, Jr. *et al.* 1977;Gordon 1991;Mukherjee *et al.* 2004;Davies and Hol 2004;Robinson *et al.* 1986;Werber *et al.* 1992;Baukrowitz *et al.* 1994). In (Kawamata *et al.* 2010), we used this approach and demonstrated ANT-dependent ADP-ATP exchange rate in permeabilized cells, using BeF_3^- and Na_3VO_4. This method was also applied in determining ANT dysfunction in relation to mitochondrial membrane potential in myotubes expressing ANT1 harboring mutations linked to autosomal dominant progressive external opthalmoplesia (Kawamata *et al.* 2011). In these studies the conversion of dye emission signal to [Mg^{2+}], and subsequently to ATP, was calibrated by obtaining the maximal fluorescence signal with excess [Mg^{2+}] and the minimal fluorescence by the addition of the cation chelator, EDTA. However, because Magnesium Green is a fluorimetric dye with single excitation and emission, it is subject to the potential pitfalls of non-ratiometric dyes related to variations in dye concentration and/or bleaching. In practice, this method introduced variability in the measurement of ADP-ATP exchange rate and a better calibration was required. Below we describe a step-by-step measurement of ANT-dependent adenine nucleotide exchange with a modified calibration method, allowing for an error-free conversion of Magnesium Green signal to free extramitochondrial [Mg^{2+}]. Furthermore, we present the methods to correlate ADP-ATP exchange rate to mitochondrial membrane potential and oxygen consumption. These could become particularly informative when studying bioenergetic parameters of cancer cell mitochondria that exhibit decreased or complete loss of electron flux associated with impaired respiration and ATP synthesis (Kwong *et al.* 2007).

METHODOLOGY

Reagents: Standard laboratory chemicals, P^1, P^5-Di(adenosine-5′) pentaphosphate (AP_5A), safranin O, and digitonin from Sigma (St. Louis, MO, USA). Magnesium Green $5K^+$ salt from Life Technologies (Carlsbad, CA, USA). Carboxyatractyloside from Calbiochem (San Diego, CA, USA). SF 6847 from Biomol (catalogue number EI-215, BIOMOL GmbH, Hamburg, Germany).

Stock reagents and buffers:

25 mM Na_3VO_4, pH 8.7 (see below how to prepare)
0.2 M $BeSO_4$ (see below how to prepare)
0.5 M NaF (see below how to prepare)
25 mM P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A), dissolved in distilled water
1 mM Carboxyatractyloside (cATR), dissolved in distilled water

5 mM Oligomycin (if a mixture of oligomycins A, B, and C are used, *i.e.* item # O4876 from Sigma-Aldrich, assume MW=800), dissolved in ethanol
0.2 M EDTA
1 mM Safranin O, dissolved in distilled water
1.1 mM Magnesium Green 5K^+ salt, dissolved in distilled water
1 M Glutamate, dissolved in distilled water, pH 7 with KOH
0.5 M Malate, dissolved in distilled water, pH 7 with KOH
0.2 M ADP, K^+ salt (see below how to prepare)
0.2 M ATP K^+ salt (see below how to prepare)
2.5 mM Digitonin, dissolved in DMSO (see below)
1 M $MgCl_2$
1 mM SF 6847, dissolved in ethanol. Do not substitute with FCCP, CCCP, or 2,4-dinitrophenol (see below).

Buffer A:
8 mM KCl
110 mM K-gluconate
10 mM NaCl
10 mM Hepes
10 mM KH_2PO_4
0.005 mM EGTA
10 mM Mannitol
1 mM $MgCl_2$
0.5 mg/ml Bovine serum albumin (fatty acid-free)
pH 7.2

Buffer B:
Buffer A without $MgCl_2$, but containing 25 µM AP_5A, 5 mM NaF, 0.2 mM $BeSO_4$, 30 µM Na_3VO_4, 5 µM EDTA (to chelate nominal amount of Mg^{2+} in the buffer) and 50 µM digitonin.

Buffer C:
Same as buffer A, but without $MgCl_2$, and containing 4 µM cATR, 2 µM oligomycin, and 5 µM EDTA.

Preparation of reagents:
ADP and ATP are purchased as a K^+ salt of the highest purity available and titrated to pH=6.9 with KOH to make stocks of 0.2 M. Concentration of ADP and ATP stock solutions is corrected by measuring absorbance at 260 nm using an extinction coefficient εM= 15,400 $M^{-1}*cm^{-1}$.
All other reagents should be dissolved in distilled water, except SF 6847 and oligomycin which should be dissolved in ethanol, and digitonin which should be dissolved in DMSO.

Preparation of sodium orthovanadate (Na_3VO_4) and beryllium trifluoride (BeF_3^-): A 25 mM Na_3VO_4 solution is prepared in distilled water (>17 megaOhm resistance). The pH of the solution is adjusted to 8.7 with HCl, upon which it turns yellow. This solution is boiled until it turns colorless and cooled to room temperature. The pH is reassessed, and readjusted to pH 8.7 with HCl, upon which the solution turns yellow again. This cycle of boiling until colorless and readjusting the pH is repeated until the solution remains colorless at pH 8.7. Finally, the solution is brought up to the initial volume with distilled water and stored in aliquots at -80 °C. This treatment removes all decavanadate ions present in the Na_3VO_4 solution, which induces mitochondrial membrane depolarization and inhibition of oxygen consumption (Aureliano and Crans 2009). Orthovanadate inhibits the oxidation of only disrupted mammalian mitochondria (Byczkowski *et al.* 1979). Likewise, fluoroberyllium nucleoside diphosphate complexes inhibit only the exposed F_1F_0-ATPase (Issartel *et al.* 1991). $BeSO_4$ and NaF are prepared as aqueous stock solutions of 0.2 M and 0.5 M, respectively, and kept at

+4 °C for several years. BeF_3^- is formed immediately in solution upon mixing of $BeSO_4$ and NaF, provided that NaF is in excess in the working solution.
Vanadate, beryllium and fluoride salts are highly toxic to tissues and to the environment, and thus require proper handling and disposal. The combination of orthovanadate and BeF_3^- will inhibit kinases, mutases, phosphatases, and ATPases (Ray *et al.* 1990;Climent *et al.* 1981). However, some kinases, such as pyruvate kinase, will remain uninhibited (Lord and Reed 1990). In this respect, upon permeabilization of the cells one has to totally separate pyruvate kinase from its substrate, phosphoenol pyruvate, i.e. there must be no glucose present in the medium prior to permeabilization, and a few minutes lag time must be allowed prior to ADP-ATP exchange rate measurements in order for the remaining reactions by kinases to 'die-out'.

Digitonin stock: Our digitonin powder stocks were purchased as "approximately 50% estimated by thin layer chromatography"; therefore we cannot be certain of the exact concentration of digitonin present in the measuring chamber. Optimum digitonin amount added to the chamber is determined by measuring oxygen consumption in non-permeabilized cells using succinate as respiratory substrate, as detailed in (Kawamata *et al.* 2010) and is the one conferring the highest respiratory control ratio. The entire study should be performed using the same digitonin stock solution.

Equipment: Oroboros Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) equipped with an LED exhibiting a wavelength maximum of 465 +/- 25 nm (current for light intensity adjusted to 2 mA, i.e., level '4') and either an <505 nm shortpass excitation filter (dye-based, filter set "Safranin" or an <495 nm shortpass excitation filter (dye-based, filter set "MgG / CaG"). Emitted light is detected by a photodiode (range of sensitivity: 350-700 nm), through either an >560 nm longpass emission filter (dye-based) for safranin O or an >525 nm longpass emission filter for MgGr. A cuvette-based fluorimeter equipped with the capability of stirring and thermoregulation would also be suitable (e.g., Hitachi F-7000 spectrofluorimeter, Hitachi High Technologies, Maidenhead, UK).

Culturing and harvesting of the cells: The amount of cells required varies from one type to another. In cell types that contain extremely high amounts of mitochondria, such as in differentiated myotubes from mouse C_2C_{12} myoblasts, plating 140,000 myocytes for differentiation (by exchanging horse for fetal bovine serum at the time when myocytes are over 90% confluent) is sufficient for the assay. For other cells, such as human fibroblasts, 1-2 million cells are required. Below, we present results obtained from HEK293 cells (~400,000 cells per assay). Regardless of the cell type: cells are washed once in phosphate-buffered saline and harvested with 0.1 ml of 0.25% trypsin-EDTA, inactivated by 0.9 ml calf serum, followed by centrifugation at 1,100 g for 2 minutes. Next, cells are washed once in buffer A without disturbing the pellet. After the wash, cells are resuspended in 0.2 ml of the buffer B. This buffer contains the same base composition as buffer A described above, but includes substrates (glutamate and malate) as well as inhibitors. See steps below for specific concentrations of substrates and inhibitors to include. The rationale for using this particular buffer composition is elaborated in (Chinopoulos *et al.* 2009). Glutamate and malate as mitochondrial substrates were chosen on the basis that they support mitochondrial substrate-level phosphorylation, and as such, they contribute to greater ATP efflux rates (Chinopoulos *et al.* 2010), (Kiss *et al.* 2013).

K_d determination of ATP and ADP for Mg^{2+}:
First, one should measure the apparent K_d values of ADP for Mg^{2+} and ATP for Mg^{2+} for the pertaining conditions (media, temperature, ionic strength, type and amount of cells, etc), steps 1-7.

1) Cells resuspended in 2 ml of buffer C are added to a chamber of an Oroboros Oxygraph-2k. The presence of cATR and oligomycin in the buffer is only required for the K_d

determination. Magnesium Green fluorescence is recorded by the O2k-Fluorescence LED2-Module at a 1 Hz acquisition rate. Experiments are performed at 37 °C. Digitonin (see above regarding remarks about its concentration) and Magnesium Green $5K^+$ salt (MgGr; 1 μM) are subsequently added to the chamber.

2) MgGr fluorescence signal is recorded upon stepwise additions of 0.1 mM $MgCl_2$ for a total of 10 additions (about 50 sec of recording time per addition). This is shown in figure 1A, on the *left* side of the dashed line.

3) Continuing in same cells in the chamber, add 0.25 mM ADP in subsequent steps for a total of 19 steps (about 50 sec of recording time per addition). This is shown in figure 1A, on the *right* side of the dashed line.

4) Likewise, in a new cell preparation, repeat Steps 1- 3, but with 11 additions of 0.2 mM ATP instead of ADP, also shown in figure 1A.

5) Convert the MgGr signal of both left and right part of figure 1A to free $[Mg^{2+}]$. To do this, plot the steady-states of MgGr after each addition of $MgCl_2$ (as seen in the left part of figure 1A) on the y-axis as a function of $[Mg^{2+}]$ (x-axis) and apply the following fit equation (you can use either trace): f=y0+a*(1-exp(-b*x)). This is an exponential rise to maximum equation with a 3 parameter function, where y0, a, and b are coefficients. The results are shown in figure 1B.

6) Calibrate the right part of figure 1A, using the coefficients y0, a, and b determined from step 5, shown in figure 1B, by solving the following exponential function for x: x=(-1/b)*ln(1-((f-y0)/a)). After calibration of MgGr signals obtained in figure 1A, it should look like figure 1C. If calibration was performed correctly, the left parts of both traces should be almost identical.

7) Next, from the calibrated right part of figure 1C, the K_d of ADP for Mg^{2+} and the K_d of ATP for Mg^{2+} is estimated by fitting the following equation with the least squares method to the data points:

$$[Mg^{2+}]_f = 0.5 \cdot \left([Mg^{2+}]_t - K_d - [L]_t + \sqrt{\left([Mg^{2+}]_t - K_d - [L]_t\right)^2 + 4K_d[Mg^{2+}]_t} \right),$$

where $[Mg^{2+}]_t$ is the total $[Mg^{2+}]$ (1 mM), $[L]_t$ is the total concentration of added ADP (or ATP). The least square fitting method can be performed by a customized Excel tool or other freely available software. The fitted curves are shown in figure 1D for both ADP and ATP. The K_d determined are K_d _ADP= 0.914 +/- 0.028 mM, and K_d _ATP=0.147 +/- 0.008 mM. Since K_d values are now known for the pertaining experimental conditions, ADP-ATP exchange rates can be calculated from the MgGr recordings.

$[Mg^{2+}]_{free}$ determination from Magnesium Green fluorescence in permeabilized cells and conversion to ADP-ATP exchange rate:

1) Add 1.1 μM of MgGr in 1.8 ml of buffer B. Record MgGr fluorescence for a few minutes and allow the signal to stabilize. In the meantime, harvest cells by trypsinization as elaborated above. Cells are washed once with buffer A and then resuspended in 0.2 ml of buffer B.

2) Upon addition of cells to the measuring chamber, wait 3 minutes and resume recording. After 2 minutes, add a known amount of ADP; we suggest 2 mM ADP. When ADP is added, the fluorescence signal will drop, as shown in figure 2A. Subsequently, the uncoupler SF 6847 is added to the chamber in 10 nM increments (~100 seconds between each interval); this will be used later on to correlate the ATP efflux rates with membrane

potential changes, see below. It is important to use SF 6847 and not the more widely used FCCP, CCCP, or 2,4-dinitrophenol, because SF 6847 is the only uncoupler that does not quench Magnesium Green fluorescence up to a concentration of 1 μM (SF 6847 is also appropriate to use with Calcium Green, a Ca^{2+}-sensitive dye; this is not unexpected as both Calcium Green and Magnesium Green are fluorescein derivatives).

3) Figure 2B shows the free [Mg^{2+}] calibrated from MgGr raw fluorescence. One may notice that initial total [Mg^{2+}] was measured to be 1.1486 mM, i.e. not exactly 1 mM, which was the actual amount added into the chamber. After the addition of 2 mM ADP, free [Mg^{2+}] was measured to attain the value of 0.5553 mM, i.e. not the exact value of 0.3958 mM, which is what would be expected. The value of 0.3958 mM was derived from the equation below:

$$[Mg^{2+}]_f = 0.5 \cdot \left([Mg^{2+}]_t - K_d - [L]_t + \sqrt{\left([Mg^{2+}]_t - K_d - [L]_t \right)^2 + 4 K_d [Mg^{2+}]_t} \right)$$

where $[Mg^{2+}]_t$ is the total [Mg^{2+}] (1 mM), [L]t is the total concentration of added ADP (or ATP), and K_d is the fitted value of K_{ADP} or K_{ATP}, respectively. These errors stem from the fact that MgGr is not a ratiometric dye, and thus it is subject to the pitfalls elaborated above. It is therefore necessary to adjust the whole trace shown in figure 2B so that the value just prior to addition of 2 mM ADP is 1 mM [Mg^{2+}] (the total), and the first value of [Mg^{2+}] after the addition of 2 mM ADP is 0.3958 mM. From figure 1B, it is apparent that the relation between free [Mg^{2+}] and MgGr fluorescence signal in the 0-0.5 mM range is fairly linear, see also (Chinopoulos et al. 2009). Thus, one may apply a series of simple arithmetic operations to correct the errors: the difference between the measured total [Mg^{2+}] (1.1486 mM) and the measured first value of [Mg^{2+}] after the addition of 2 mM ADP (0.5553 mM) is 0.5933 mM, while the difference between the actual total [Mg^{2+}] (1 mM) and the expected first value of [Mg^{2+}] after the addition of 2 mM ADP (0.3958 mM) is 0.6042 mM; therefore, the measured changes in free [Mg^{2+}] are underestimated by a factor of 0.6042/0.5553=1.01837. Thus, the whole trace of figure 2B is multiplied by 1.01837. This would generate a trace in which the value of [Mg^{2+}] just prior to addition of 2 mM ADP is 1.169 mM (the estimated total), and the first value of [Mg^{2+}] after the addition of 2 mM ADP is 0.5655 mM (not shown). By subtracting the difference of 1.169-1=0.169 mM from this whole trace, the final trace should look like the one depicted in figure 2C. Note that in the trace shown in figure 2C the value just prior to addition of 2 mM ADP is 1 mM [Mg^{2+}] (the total), and the first value of [Mg^{2+}] after the addition of 2 mM ADP is 0.3958 mM. The trace depicting the estimated [Mg^{2+}] has therefore been corrected, and the conversion to ATP appearing in the medium can now be applied, as shown in the next step.

4) We convert the corrected [Mg^{2+}] values to ATP appearing in the medium, using the equation below:

$$[ATP]_t = \left(\frac{[Mg^{2+}]_t}{[Mg^{2+}]_f} - 1 - \frac{[ADP]_t(t=0) + [ATP]_t(t=0)}{K_{ADP} + [Mg^{2+}]_f} \right) \Bigg/ \left(\frac{1}{K_{ATP} + [Mg^{2+}]_f} - \frac{1}{K_{ADP} + [Mg^{2+}]_f} \right)$$

The above equation is available for download as an executable file at http://www.tinyurl.com/ant-calculator. The rate of ATP appearing in the medium can be calculated by making a linear regression for the ATP values as a function of time, as shown in figure 2D.

Mitochondrial membrane potential (ΔΨm) determination in in situ mitochondria of permeabilized cells:

ΔΨm is estimated using fluorescence quenching of the cationic dye safranin O due to its accumulation inside energized mitochondria (Akerman and Wikstrom 1976), also taking into

account the considerations discussed in (Perevoshchikova *et al.* 2009) and (Figueira *et al.* 2012).

1) Cells are treated exactly as described for free [Mg^{2+}] determination, except that MgGr is replaced by 5 μM safranin O.

2) Fluorescence is recorded in an Oroboros Oxygraph-2k at a 1 Hz acquisition rate, using the 495 nm excitation and 585 nm emission wavelengths. Experiments are performed at 37 ^{o}C. After the baseline signal has stabilized, 2 mM ADP is added to the chamber and fluorescence is allowed to stabilize. The raw signal of safranin O fluorescence is shown in figure 3A.

3) After addition of ADP, ΔΨm is further depolarized stepwise by addition of 10 nM SF 6847 until the signal after depolarization becomes stable (~100 sec). Safranin O fluorescence is converted to mV (figure 3B) using the Nernst equation and assuming a matrix [K^+]=120 mM, which was determined by a voltage-fluorescence calibration curve of safranin O fluorescence in the presence of 2 nM valinomycin and increasing [K^+] (0.2-120 mM; (Akerman and Wikstrom 1976)). An important note is that cancer cells express very variable levels of IF1 (Sanchez-Arago *et al.* 2013), which inhibits the ATP-hydrolytic function of the ATPase, thus hindering reversal rates. Therefore, it is not recommended to study ATP *influx* rates in mitochondria from cancer cells, i.e. at very depolarized values. The method is suitable for studying cancer mitochondria during ATP synthesis.

Mitochondrial respiration:
Oxygen consumption is simultaneously measured with Safranin O reflecting ΔΨm or MgGr fluorescence reflecting ADP-ATP exchange rates in the same samples polarographically using the Oxygraph-2k. Oxygen concentration (black trace of figure 3C) and oxygen flux (grey trace of figure 3C) expressed as pmol·s^{-1}·mg^{-1}; negative time derivative of oxygen concentration, divided by tissue mass per volume and corrected for instrumental background oxygen flux arising from oxygen consumption of the oxygen sensor and back-diffusion into the chamber) were recorded using DatLab software (Oroboros Instruments).

From the above measured parameters, one is able to estimate the rate of ATP appearing in the medium as a function of ΔΨm or oxygen consumption rate.

CONCLUSIONS AND COMMENTS

Using this technique we have previously addressed ANT-dependent adenine nucleotide exchange in disease conditions (Kawamata *et al.* 2011). By generating ADP-ATP exchange rate/ΔΨm profile for myotubes expressing ANT1 with pathogenic mutations, we found that the mutations caused a reduced ADP-induced depolarization of membrane potential. This suggested that mutant ANT1 expressing cells require more ΔΨm to generate the same amount of ATP. In the same cells, ATP synthesis measured by a luciferase-based method did not show significant differences between mutant and wild type ANT1 expressing cells. Thus, this is a sensitive method to determine ATP production from mitochondria. Furthermore, with the presently described methodology, one is provided the capacity to measure ADP-ATP exchange rate in a kinetic mode together with oxygen consumption, in addition to recording changes in ΔΨm. This may be particularly informative when studying bioenergetic parameters of cancer cell mitochondria, as these are known to exhibit decreased or complete loss of electron flux, which leads to impaired respiration and ATP synthesis (Kwong *et al.* 2007).

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FIGURE LEGENDS

Figure 1: Magnesium green fluorescence calibration and estimation of K_d of ATP and ADP for Mg^{2+}. **A**: Reconstructed time-recordings of MgGr raw fluorescence traces in permeabilized HEK293 cells as a function of extramitochondrial [Mg^{2+}] (left part of the traces), and as a function of extramitochondrial ADP or ATP (right parts of the traces). **B**: MgGr fluorescence changes are dependent on extramitochondrial [Mg^{2+}]. **C**: Calibrated time-recordings of extramitochondrial [Mg^{2+}] (left part of the traces), and as a function of extramitochondrial ADP and ATP (right parts of the traces) are shown. **D**: Calibrated extramitochondrial Mg^{2+} plots as a function of ADP or ATP are shown, from which we estimated K_d of ADP and ATP for Mg^{2+} using the least squares method to fit the data.

Figure 2: Determination of extramitochondrial [Mg^{2+}] and conversion to ATP. **A**: Reconstructed time recording of MgGr raw fluorescence in permeabilized HEK293 cells upon addition of 2 mM ADP (where indicated), followed by incremental 10 nM additions of the uncoupler SF 6847. **B**: Calibrated time recording of extramitochondrial [Mg^{2+}] obtained from panel 2A. **C**: Corrected calibrated trace of panel 2B, as described in the text. **D**: Calculated amount of ATP appearing in the medium converted from panel 2C. The rate of ATP appearing in the medium is indicated in μmol/min/mg protein.

Figure 3: Mitochondrial membrane potential and oxygen consumption determination in permeabilized cells. A: Reconstructed time recording of Safranin O raw fluorescence in permeabilized HEK293 cells upon addition of 2 mM ADP (where indicated), followed by incremental 10 nM additions of the uncoupler SF 6847. **B**: Calibrated time recording of ΔΨm obtained from panel 3A. **C**: Reconstructed time recording of oxygen concentration in the medium (black trace) and oxygen flux (grey trace) recorded simultaneously with either MgGr signal (panel 2A) or Safranin O signal (panel 3A).