MethodsX 8 (2021) 101520

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method article

Dynamic calculation of ATP/O ratios measured using Magnesium Green (MgGr)™

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A B S T R A C T

Mitochondria generate aerobic cellular energy (i.e., ATP) through the reduction of oxygen to water via oxidative phosphorylation. The efficiency of this pathway can be measured by the phosphate/oxygen (ATP/O) ratio, which is the amount of ATP produced per oxygen atom reduced. This ratio thus provides a measure of the efficiency of mitochondrial respiration that can be readily compared between species. The magnesium green (MgGr) fluorometric method permits easy measurement of ATP/O ratios from isolated mitochondria but the standard analysis approach employs an endpoint method to calculate ATP/O ratios. Here, we present a modified method of ATP/O calculation that permits dynamic observation of fluorescent measurements of the consumption of O_2 (JO_2) and the production of ATP (JATP). Specifically, by substituting the slope of a straight line within a given period of time (seconds to minutes) with the slope of a tangent to each time point (per second), it is possible to evaluate JO_2, JATP and the ATP/O ratio in a dynamic manner.

- Provides second-by-second visualization of ATP/O ratios throughout experiments vs. a single measurement.
- Dynamic visualization allows for easy identification of outlying data and more accurate calculation of mean ATP/O ratios.

A R T I C L E I N F O
Method name: Dynamic calculation of ATP/O ratios measured using Magnesium Green (MgGr)™
Keywords: ATP production, Oxygen consumption, Fluorescence, Mitochondrial efficiency
Article history: Received 2 December 2020; Accepted 17 September 2021; Available online 20 September 2021

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https://doi.org/10.1016/j.mex.2021.101520

Specifications table

Subject Area	Agricultural and Biological Sciences
More specific subject area	mitochondrial physiology, *In vitro* physiology
Method name	Dynamic calculation of ATP/O ratios measured using Magnesium Green (MgGr)™
Name and reference of original method	Chinopoulos, G. Kiss, H. Kawamata, and A. A. Starkov, "Measurement of ADP-ATP exchange in relation to mitochondrial transmembrane potential and oxygen consumption," Methods Enzymol., vol. 542, no. 6, pp. 333–348, 2014, doi: 10.1016/B978-0-12-416618-9.00017-0. K. Salin et al., "Simultaneous measurement of mitochondrial respiration and ATP production in tissue homogenates and calculation of effective P/O ratios," Physiol. Rep., vol. 4, no. 20, Oct. 2016, doi: 10.14814/phy2.13007.
Resource availability	Software, OriginLab (https://www.originlab.com/)

Methodological protocols

1. All steps up until and including "ATP concentration calculation" in Fig. 1 are as described in [1]. Briefly, $K_{d\text{-}ATP}$ and $K_{d\text{-}ADP}$ were measured in the absence of mitochondria and ATPase inhibitors [2]. Skeletal muscle mitochondria (0.04 mg protein ml^{-1}) were added to 2 ml of respiration medium that had been air equilibrated at 32°C. Respiration was stimulated by glutamate (5mM), malate (1mM), and ADP (2mM). Oxygen concentration was monitored throughout all experiments at the range of 80-220 µM. Reoxygenation was achieved by opening the chambers to equilibrate the solution with room air [3]. An Excel file should be created for subsequent steps (Supplementary file 1).

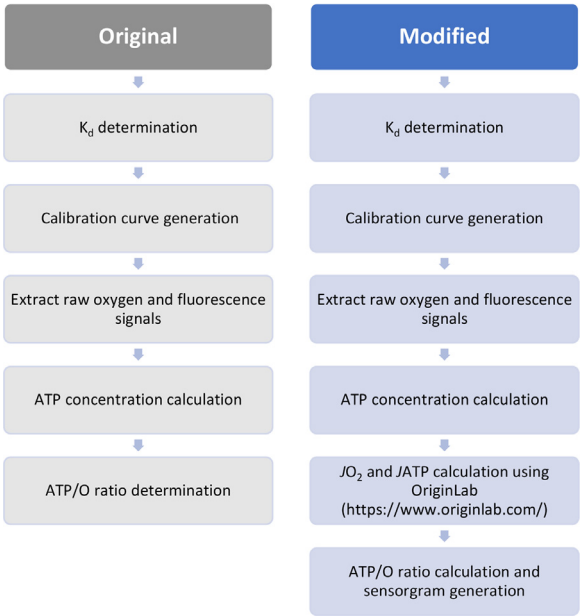

Fig. 1. Workflow schematic comparing the modified and original methods of ATP/O ratio calculations. Briefly, a modified method of JO_2 and JATP calculation was performed using OriginLab that permitted simple and dynamic visualization of the ATP/O ratio in a temporal view.

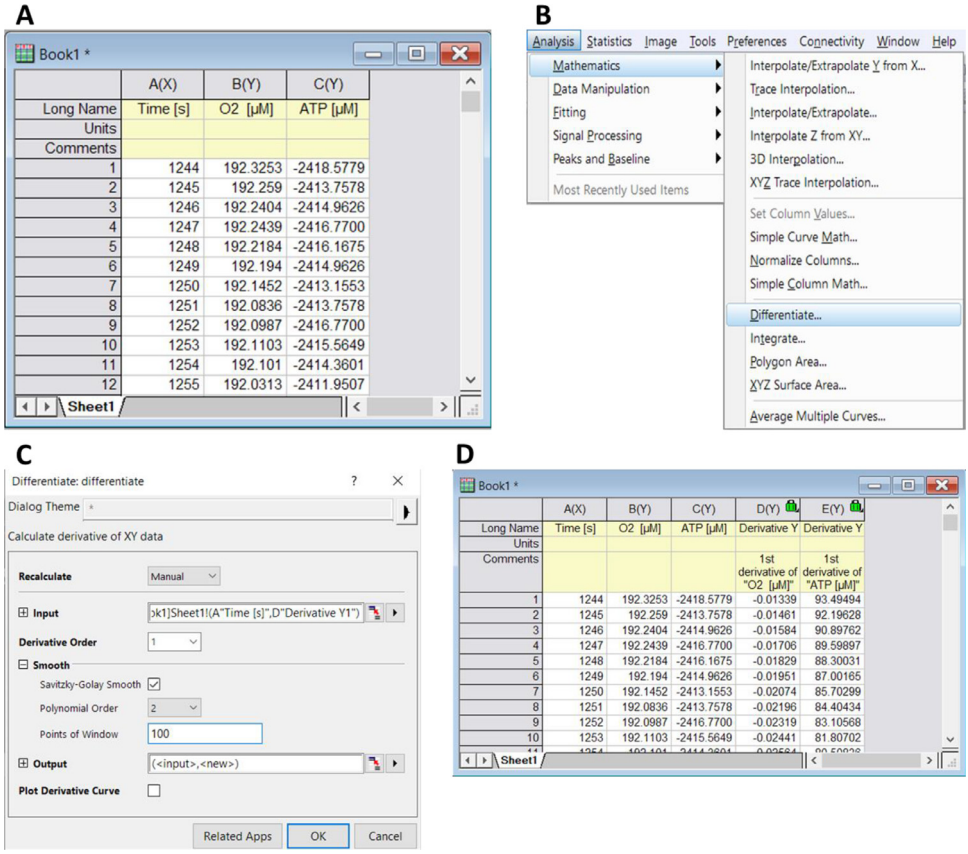

Fig. 2. Step-by-step guide to OriginLab operations. (A) The primary worksheet contains measurements of O_2 concentration (µM) and ATP concentration (µM) corelated to Time (S). (B) Apply "Differentiate" tool for slope calculation. (C) Parameters setup for the "Differentiate" step. (D) Processed result corresponding to raw value in (A).

2. Calculating JO_2 and JATP using OriginLab. Copy three columns: time (s), O_2 concentration (µM), and ATP concentration (µM) from the final step described in [1,2], as above, and paste these values into columns X, Y1 and Y2 in an OriginLab worksheet; Fig. 2A. **Note: O_2 and ATP must be in the same unit.**
3. Select the column labelled "O_2 concentration", and then click "Analysis" → "Mathematics" → "Differentiate"; Fig. 1B. Set paraments as per Fig. 1C, then select "Savitzky-Golay Smooth" and set "Points of Window" to 100 and click OK.
4. Select the column labelled "ATP concentration", and "Differentiate" using the same parameters in step 3 to get results shown in Fig. 2D.
5. Copy values from the columns labelled "Time", "Derivative O_2", and "Derivative ATP", and paste these into an Excel sheet (Supplementary file 2).
6. Select $JATP_{st4}$ and $JO_{2\text{-}ROX}$ as appropriate to each individual experiment; Fig. 3A. Corrected $JATP_{st3} = JATP +$ numerical value of $JATP_{st4}$, and corrected $JO_{2st3} = JO_2 +$ numerical value of $JO_{2\text{-}ROX}$. $ATP/O\ ratio = -JATPst3/(2 * JO2st3)$.
7. Overall, two graphs can be generated using the results obtained in step 6' Fig. 3. These graphs are JO_2 and JATP sensorgrams (Fig. 3A) and provide visualized dynamic ATP/O ratios on a second-by-second basis (Fig. 3B). An average mean of a steady state period can then be visually selected as the mean ATP/O ratio for a given sample.

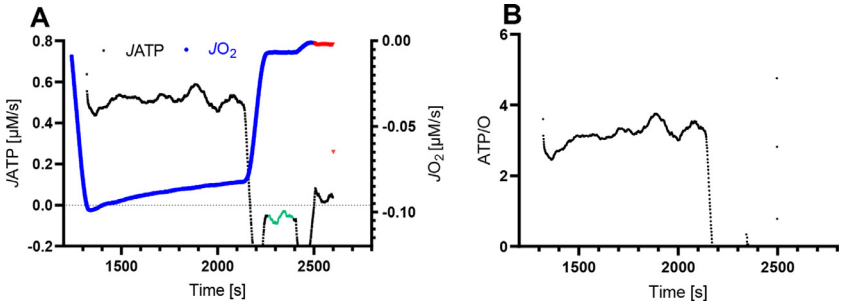

Fig. 3. Dynamic visualization of O_2 consumption, ATP production, and ATP/O ratio. (A) *J*ATP (black) and JO_2 (blue) sensorgrams correlated to time. $JATP_{st4}$ and $JO_{2\text{-ROX a}}$ are highlighted in green and red, respectively. (B) ATP/O ratios correlated to time.

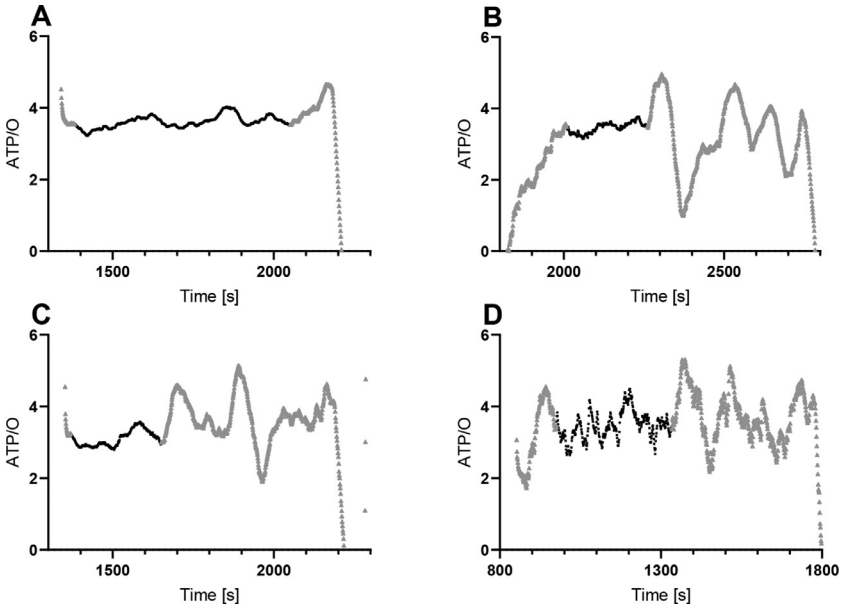

Fig. 4. Impact of fluorescence signal variability on ATP/O ratio calculations. (A-D) Sample experiments demonstrating the variability in ATP/O ratio calculation through time. Using the modified method to dynamically visualize the full experiment, relatively stable periods may be selected (black dots) among variable ATP/O values (grey dots) (B-D) to limit error due to variability in the fluorescence signal. (A) An experiment in which the ATP/O ratio value was stable during the full experiment.

Data validation

Our modified method dynamically generates ATP/O values for each time point of JO_2 and *J*ATP, Fig. 4. This permits real-time visualization of fluctuations in the ATP/O ratio when we introduce a "slope of a tangent" function for JO_2 and *J*ATP instead of taking a "slope of a straight line" as outlined in the original method. Such variability may be due to the high sensitivity of the fluorometric probe, which may detect unspecific signals produced by biological samples. Note that a higher "Savitzky-Golay Smooth" setting in OriginLab might smooth the curve for particularly variable data.

To validate this method, we processed data from 18 experiments previously performed on isolated skeletal muscle mitochondria samples from naked mole-rat gastrocnemius muscle using both the original (Supplementary file 1) and modified (Supplementary file 2) methods. Using our modified

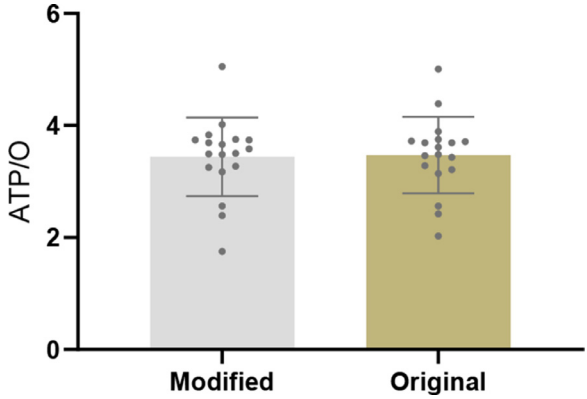

Fig. 5. Comparison of ATP/O values derived using both the modified and original methods. The ATP/O ratio was calculated from 18 individuals experiments using isolated naked mole-rat skeletal muscle mitochondria. Relatively stable periods were selected for calculating the average means of ATP/O ratio using the modified method. The original approach used the same periods as thatthose selected for the modified ATP/O ratio calculation. Data are presented as mean ± s.e.m.

method, we dynamically visualized the full experiment and chose relatively stable periods of recording to include in our calculations. To validate our calculations, we also analyzed the same subset of data using the original method as chosen using the modified method. The ATP/O ratios calculated using the two methods to analyze the same data were highly similar: 3.44 ± 0.07 (modified, mean ± s.e.m.) and 3.47 ± 0.07 (original, mean ± s.e.m.) (Fig. 5; $p = 0.89$, t-test with a Holm-Sidak *post-test*.)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by an NSERC Discovery grant and a Canada Research Chair awarded to MEP and a FRQNT fellowship to DM.

We would like to thank the uOttawa animal care and veterinary services team for their assistance in animal handling and husbandry, and Kenny Huynh for his help in data collection.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101520.

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[3] H. Cheng, D. Munro, K. Huynh, M.E. Pamenter, Naked mole-rat skeletal muscle mitochondria exhibit minimal functional plasticity in acute or chronic hypoxia, Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 255 (2021) 110596 no. December 2020Aug, doi:10.1016/j.cbpb.2021.110596.