Time-dynamics of mitochondrial membrane potential reveal an inhibition of ATP synthesis in mitosis

Joon Ho Kang¹,²,³,#, Georgios Katsikis¹,#, Max A. Stockslager¹,⁴, Daniel Lim¹,⁵, Michael B. Yaffe¹,⁵, Scott R. Manalis¹,⁴,⁶, Teemu P. Miettinen¹,⁷,*

Affiliations:
¹ Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA.
² Department of Physics, Massachusetts Institute of Technology, Cambridge, MA, USA.
³ Center for BioMicroSystems, Korea Institute of Science and Technology, Seoul, Korea.
⁴ Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA.
⁵ Center for Precision Cancer Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA.
⁶ Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA.
⁷ MRC Laboratory for Molecular Cell Biology, University College London, London, UK.
# These authors contributed equally to the work
* Corresponding author, email: teemu@mit.edu

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Abstract

The energetic demands of a cell are believed to increase during mitosis 1-7. As cells transit from G2 into mitosis, mitochondrial electron transport chain (ETC) activity increases 4,8,9, and cellular ATP levels progressively decrease until the metaphase-anaphase transition 3,7,10, consistent with elevated consumption. The rates of ATP synthesis during mitosis, however, have not been quantified. Here, we monitor mitochondrial membrane potential of single lymphocytes and demonstrate that cyclin-dependent kinase 1 (CDK1) activity causes mitochondrial hyperpolarization from G2/M until the metaphase-anaphase transition. By using an electrical circuit model of mitochondria, we quantify the time-dynamics of mitochondrial membrane potential under normal and perturbed conditions to extract mitochondrial ATP synthesis rates in mitosis. We found that mitochondrial ATP synthesis decreases by approximately 50% during early mitosis, when CDK1 is active, and increases back to G2 levels during cytokinesis. Consistently, acute inhibition of mitochondrial ATP synthesis failed to delay cell division. Our results provide a quantitative understanding of mitochondrial bioenergetics in mitosis and challenge the traditional dogma that cell division is a highly energy demanding process.

Main text

In animal cells, including most cancer cells, energy in the form of ATP is produced mainly through oxidative phosphorylation in mitochondria (reviewed in 11-13). However, largely due to a lack of quantitative single-cell approaches, little is known about ATP synthesis during mitosis. To study mitochondrial bioenergetics at the single-cell level, we combined suspended microchannel resonators (SMR), a non-invasive single-cell buoyant mass sensor, with a fluorescence detection system. This allowed us to monitor cell mass-normalized fluorescence signals with a temporal resolution of 2 min and a fluorescence measurement error of 2% without perturbing normal growth 14,15 (Supplementary Fig. 1, Supplementary note 1). Using this setup, we examined the murine lymphocytic leukemia cell line L1210 grown in the presence of non-quenching concentrations (10 nM) of TMRE, a fluorescent probe for mitochondrial membrane potential (∆Ψm) 16 (Supplementary Fig. 2A, Supplementary note 2). Monitoring the mass-normalized TMRE signal over multiple cell generations revealed oscillatory TMRE behavior with a robust, transient and extensive spike-like increase in TMRE signal preceding the end of each cell cycle (Fig. 1A). The rate of TMRE increase and decrease were not limited by TMRE diffusion speed (Supplementary Figs. 2C-E, Supplementary note 2). Similar TMRE behavior persisted in both glucose and galactose-based culture media and in other cell types, including mouse BaF3 pro-B lymphocytes, chicken DT40 lymphoblasts, suspension HeLa cells and, importantly, in primary CD8+ and CD3+ human T-cells (Supplementary Fig. 3). However, the mouse Fl5.12 pro-B
lymphocytes did not display any change in TMRE at the end of cell cycle (Supplementary Fig. 3A).

L1210 cells were utilized as model system in all further studies.

To validate that the spike-like TMRE increase reflects an increase in $\Delta \Psi_m$, we used quenching concentrations (10 µM) of an alternative $\Delta \Psi_m$ probe, Rhod123 (Supplementary Fig. 2B, Supplementary note 2). Rhod123 signal suddenly decreased approximately 1h prior to cell division (Fig. 1B), consistent with a spike-like increase in $\Delta \Psi_m$. In contrast, the $\Delta \Psi_m$-insensitive MitoTracker Green probe did not display any changes at the end of cell cycle (Fig. 1C). We next examined changes in plasma membrane potential ($\Delta \Psi_p$) using a 1 µM DiBAC$_4$(3) probe. The DiBAC$_4$(3) signal was reduced before and during the TMRE increase, indicative of increased $\Delta \Psi_p$ (Supplementary Fig. 4A). We speculated that this $\Delta \Psi_p$ change may be a feature of mitotic cell swelling 17,18, which can be inhibited with 5-(N-ethyl-N-isopropyl)amiloride (EIPA) 17. Indeed, treatment of L1210 cells with 5 µM EIPA partially inhibited the observed decrease in the DiBAC$_4$(3) signal but did not affect the TMRE signal increase (Supplementary Fig. 4). This indicates that mitotic cell swelling associates with changes in plasma membrane potential, but the extent to which plasma membrane hyperpolarizes is not affecting the reliability of TMRE as a reporter for $\Delta \Psi_m$ (Supplementary note 2).

Next, we studied the exact timing of the $\Delta \Psi_m$ increase. Inhibition of mitotic entry using 2.5 µM CDK1 inhibitor RO-3306 completely eliminated the observed increase in TMRE signal, despite the fact that cells continued to increase in size beyond the typical G2/M transition (Fig. 1D). We then monitored single-cell density to compare TMRE signal increase to the timing of mitotic cell swelling, an event that is known to start in prophase 17,18. The increase in the TMRE signal was observed immediately following the onset of density reduction, indicating that mitochondrial hyperpolarization begins shortly after mitotic entry (Fig. 1E). This timing of TMRE increase was further validated using biophysical markers of G2/M transition (Supplementary Fig. 5) 14,15. We next compared the timing of TMRE signal increase to the degradation of the protein Geminin using L1210 FUCCI cells, which express fluorescently labelled Geminin (Geminin-mAG) 15,19. The Geminin-mAG signal was fully degraded in approximately 8.6 minutes at the metaphase-anaphase transition (Fig. 1F, Supplementary Fig. 6), and the loss of Geminin aligned exactly with the maximal TMRE signal (Fig. 1G). In most cells, the TMRE signal declined back to typically G2 levels before the final abscission of the daughter cells (Figs. 1E, 1G). Together, these results indicate that the mitochondrial hyperpolarization begins shortly after the G2/M transition, reaches a maximum at the metaphase-anaphase transition and returns to G2 levels during cytokinesis.

Previous work has shown that the CDK1/Cyclin B complex localizes to mitochondria during mitosis and directly phosphorylates components of the mitochondrial ETC 4. CDK1 also associates with other metabolic proteins, including the $\alpha$ subunit of ATP synthase 20. CDK1 activity
is minimal before mitotic entry, after which the switch-like activation of CDK1/cyclin B complex results in high CDK1 activity until the onset of anaphase \(^{21-23}\). Since the timing of mitochondrial hyperpolarization coincided exactly with the reported CDK1 activity, we hypothesized that the switch-like CDK1 activity was causally responsible for mitochondrial hyperpolarization. To test this, we first arrested cells in a CDK1 active-state (prometaphase and metaphase) using three different chemicals: the kinesin motor inhibitor S-trityl-l-cysteine (STLC), the microtubule polymerization inhibitor nocodazole, and the anaphase-promoting complex inhibitor proTAME. In response to any of these three chemicals we observed that TMRE signal increased following mitotic entry and plateaued to a high level during the mitotic arrest, indicative of mitochondria reaching a steady, hyperpolarized state. Next, we partially inhibited CDK1 with RO-3306 (1 µM) or with an alternative CDK1 inhibitor BMS-265246 (400 nM) and examined the level of TMRE during STLC mediated prometaphase arrest. Note that complete inhibition of CDK1 blocks mitotic entry, but it is possible to partially inhibit CDK1 while allowing mitotic entry and progression \(^{15,17}\). We observed that CDK1 inhibition reduces the mitotic mitochondrial hyperpolarization (Figs. 2B, 2C). Finally, we arrested cells in prometaphase with STLC and after the TMRE signal had reached a new equilibrium in mitosis we treated the cells with 100 nM okadaic acid (O.A.) to inhibit the protein phosphatase PP2A and block the dephosphorylation of CDK1 targets \(^{23}\). The O.A. treatment increased TMRE signal (Fig. 2D). In contrast, when the CDK1 activity of prometaphase-arrested cells was inhibited with 5 µM RO-3306 the TMRE signal returned to G2 levels (Fig. 2E). Together, these results indicate that CDK1 activity drives the mitochondrial hyperpolarization in early mitosis.

CDK1 has been suggested to promote mitochondrial ATP synthesis \(^4\). Considering the prevailing dogma that mitosis is energy intensive \(^1-7\), we studied whether acute inhibitions of mitochondrial ATP synthesis affected cell division. Direct measurements of oxygen consumption validated that L1210 cells maintain active mitochondrial ATP synthesis, which could be completely inhibited by 1 µM oligomycin, a specific inhibitor of Fo-ATP synthase (Supplementary Figs. 7A-C). Unexpectedly, when we treated L1210 cells in the G2 cell cycle phase with 1 µM oligomycin and monitored their growth using the SMR, the cells still proceeded through mitosis and divided symmetrically (Fig. 3A), although the magnitude of the TMRE signal increase in mitosis was reduced (Fig. 3B). To further quantitatively analyze the role of mitochondrial ATP synthesis in mitotic entry and progression, we synchronized cells to G2 using RO-3306, treated the cells with 1 µM oligomycin for 15 min, released the cells to enter mitosis in the presence of oligomycin, and collected samples for cell cycle analysis at different timepoints. Surprisingly, mitochondrial ATP synthesis inhibition had little effect on mitotic entry and the subsequent appearance of G1 cells (Figs. 3C, 3D). Similar results were observed in BaF3 and DT40 lymphocytes (Supplementary Fig. 8). To further examine
the extent to which ATP synthesis inhibition influences L1210 cell behaviour, we monitored single-cell mass accumulation (growth) rates using a serial SMR \(^{15,24}\). We observed that oligomycin treatment caused a major reduction in cell growth rates that persisted for several hours (Fig. 3E). Thus, mitochondrial ATP synthesis is required to support cell growth, but not cell division. This finding is consistent with prior observations that mitochondrial ATP synthesis inhibition influences L1210 cell behaviour, we monitored single-cell mass accumulation (growth) rates using a serial SMR \(^{15,24}\). We observed that oligomycin treatment caused a major reduction in cell growth rates that persisted for several hours (Fig. 3E).

Next, to more directly examine bioenergetics and oxidative stress in mitosis, various fluorescence based metabolic reporters were expressed in both L1210 and BaF3 cells. However, the expression of these exogenous proteins resulted in the loss, or even the reversal of the normal mitotic mitochondrial hyperpolarization (Supplementary Fig. 9, Supplementary note 3), indicating that these genetic tools can bias quantitative analyses of mitotic mitochondrial bioenergetics in our model system.

As an alternative approach to understand mitotic bioenergetics, we developed a model to derive ATP synthesis rates from the TMRE signal dynamics (Supplementary Fig. 10, Supplementary note 4). First, we converted the TMRE signal to approximate ∆Ψm using existing measurements of tetramethylrhodamine ester dye accumulation and membrane potentials \(^{26}\). Second, we assumed that the voltage across the inner mitochondrial membrane (ΔΨm) is determined by the currents through ATP synthesis (I_{ATP}), voltage-dependent leakage \(^{27}\) (I_{Leak}) and the ETC (I_{ETC}). Third, we modelled the inner mitochondrial membrane as an electrical circuit with voltage (ΔΨm), capacitance (C) and resistances (R) for each one of the currents (Fig. 4A, Supplementary note 4), constituting a simple model that is consistent with the biochemical view of mitochondria \(^{16,28}\). We assumed that the circuit behaves in a switch-like manner between two distinct states whether CDK1 is activated (CDK1 on) or inactivated (CDK1 off). By fitting our model’s analytical solution to the ΔΨm data we derive RC values, which reflect the time constant of the ΔΨm change (Figs. 4B, 4C, Supplementary notes 5 and 6). We derived these RC values separately for each control and oligomycin-treated cell during CDK1 on and CDK1 off states (Fig. 4D, Supplementary Fig. 11).

Comparing the RC values between control and oligomycin-treated cells, we extracted the resistance of ATP synthase (R_{ATP}) during the CDK1 on and CDK1 off states (Fig. 4B). We found that R_{ATP} is higher during the CDK1 on state than during CDK1 off state (Supplementary Fig. 11C, Supplementary note 4). In addition, comparing RC values during the CDK1 on and off states in each control cell revealed that in order for R_{ATP} to increase, R_{Leak} and R_{ETC} are required to cumulatively decrease during the CDK1 on state (Supplementary note 7). We then derived the current through ATP synthase (I_{ATP}),
i.e. the ATP synthesis rate, throughout mitosis using Ohm’s law (I_{ATP}=V/R_{ATP}) (Supplementary notes 7-9). Surprisingly, our modelling revealed that the mitochondrial ATP synthesis rate is inhibited by 54 % ± 11 % (mean±s.e.m.) during prometaphase and metaphase, when compared to G2 ATP synthesis rates (Figs. 4E, 4F). During anaphase, only a minor increase (<10%) in comparison to G2 levels was observed (Fig. 4E). Overall, this temporal control of ATP synthesis results in 40 % decrease in total mitochondrial ATP production during early mitosis (between G2/M transition and metaphase-anaphase transition) when compared to a situation where mitochondrial ATP synthesis would remain at G2 levels (Fig. 4G).

It is important to recognize the limitations of our approach. Especially, i) the TMRE signal is a proxy for ΔΨm, subject to systematic errors, ii) we assumed that oligomycin only perturbs mitochondrial ATP synthase, and iii) our electric circuit model, while including all major elements of mitochondrial bioenergetics, may oversimplify the dynamics of ATP synthesis. To support our conclusions, we independently quantified the average ATP synthesis rate of prometaphase and G2 arrested cell populations by measuring oligomycin sensitive oxygen consumption (Supplementary Figs. 7D, 7E). Consistently with our modeling approach, this indicated that mitochondrial ATP synthesis rate is significantly reduced in mitotic cells when compared to G2 cells (Fig. 4F). Furthermore, oxygen consumption measurements revealed that mitotically arrested cells have higher leakage than G2 cells (Supplementary Figs. 7D, 7E), consistent with our modeling (Supplementary note 7). In addition, our results on ATP synthesis have little sensitivity to the model-specific parameters, including those used for TMRE-to-ΔΨm conversion (Supplementary Figs. 12-14, Supplementary note 10). Since our modeling relies on comparisons between control and oligomycin-treated cells, any systematic bias that affects TMRE in both samples will not affect our ATP synthesis results.

Our findings are compatible with existing literature. Our observation that ΔΨm can increase in mitosis in the presence of oligomycin (Fig. 3B) is supported by the reported mitotic activation of the ETC 4,8,9. Both ETC activation and ATP synthesis inhibition cause ΔΨm to radically increase, and high ΔΨm is known to promote mitochondrial protein import 9, reactive oxygen species (ROS) generation 29, proton leakage 27 and heat production 30. Consistently, mitochondrial protein import, ROS levels and cellular heat output have been reported to increase during mitosis in a CDK1-dependent manner 9,31-33. Furthermore, the ATP synthesis dynamics we discover can explain the reported ATP level dynamics in mitosis 3,7,10.

Overall, our work reveals the previously unknown dynamics of ΔΨm and mitochondrial ATP synthesis during mitosis. Considering that mitochondria are responsible for the majority of cellular ATP synthesis 11-13 and that ATP consumption in interphase consumes cellular ATP pools
within minutes\textsuperscript{32}, our discovery that mitochondrial ATP synthesis is inhibited during mitosis suggests a much lower rate of ATP consumption during mitosis than previously assumed. Notably, cells maintain ATP at concentrations near 4 millimolar\textsuperscript{7,34}, but most enzymes have Michaelis constants ($K_m$) for ATP in the micromolar range. Thus, even a major decrease in cellular ATP levels will not affect enzymatic reaction rates, and the intracellular ATP pools may be able to fulfill the energetic needs of mitosis even in the absence of additional ATP synthesis, as suggested by early work on antephase\textsuperscript{35,36}. The decreased ATP levels may even promote cell division by facilitating cellular reorganization and chromatin condensation\textsuperscript{7,37}.

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Competing interests

Scott R Manalis is a co-founder of Travera and Affinity Biosensors, which develops techniques relevant to the research presented.

Author contributions

J.H.K., M.A.S. and T.P.M. planned and carried out the experiments. G.K. carried out the modeling. D.L. and T.P.M. generated the reporter cell lines. J.H.K., G.K. and T.P.M. wrote the manuscript with input from all authors. M.B.Y., S.R.M. and T.P.M. supervised the work. T.P.M. conceived the study.

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**Figure 1.**

(A) Buoyant mass (black) and mass-normalized TMRE (red) trace for a single L1210 cell and its progeny over 10 full generations with a measurement interval of 1.9 min. At each cell division (orange arrows), one of the daughter cells is randomly kept and monitored, while the other is discarded. TMRE was used in a non-quenching concentration (10 nM).

(B) Mass-normalized Rhod132 trace for a L1210 cell around cell division. Cells were loaded with a quenching concentration of Rhod123 (10 μM) and immediately analyzed with no Rhod123 in the culture media. In the quenching mode, the fluorescence signal behavior is reversed.

(C) Mass-normalized MitoTracker Green (50 nM) trace for a L1210 cell around cell division.

(D) Buoyant mass (black) and mass-normalized TMRE (red) trace for a L1210 cell treated with 2.5 μM RO-3306 to inhibit mitotic entry. Typical size for mitotic entry is illustrated with light yellow area.

**Fig. 1: Mitochondria transiently hyperpolarize during prophase and metaphase**

(A) Buoyant mass (black) and mass-normalized TMRE (red) trace for a single L1210 cell and its progeny over 10 full generations with a measurement interval of 1.9 min. At each cell division (orange arrows), one of the daughter cells is randomly kept and monitored, while the other is discarded. TMRE was used in a non-quenching concentration (10 nM).

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(D) Buoyant mass (black) and mass-normalized TMRE (red) trace for a L1210 cell treated with 2.5 μM RO-3306 to inhibit mitotic entry. Typical size for mitotic entry is illustrated with light yellow area.
(E) Cell density (black) and mass-normalized TMRE (red) trace for a L1210 cell around cell division. Mitotic entry (G2/M transition) is illustrated with light yellow area.

(F) Representative phase contrast (grey) and mAG-hGeminin cell cycle reporter (green) images of a L1210 cell in mitosis. Scale bars denote 10 µm.

(G) Mass-normalized mAG-hGeminin (black) and mass-normalized TMRE (red) trace for a L1210 cell around cell division. Metaphase-to-anaphase transition (M/A transition) is illustrated with light yellow area.
Fig. 2: CDK1 drives a switch-like mitochondrial hyperpolarization

(A) Mass-normalized TMRE traces for L1210 cells treated with 5 µM STLC (dark blue), 1 µg/ml Nocodazole (light blue) or 15 µM proTAME (red) to induce mitotic arrest. TMRE signal reaches a stable but highly elevated level when mitosis is prolonged, indicative of a metabolic switch in mitosis. Boxplots on the right indicate the level to which TMRE increases during mitotic arrest. p-value was obtained using ANOVA.

(B) Mass-normalized TMRE traces for L1210 cells treated with 5 µM STLC alone (dark blue), in combination with 1 µM RO-3306 (light blue) or in combination with 400 nM BMS-265246 (red) to partly inhibit CDK1. Partial inhibition of CDK1 reduces the extent of the metabolic switch in mitosis. Boxplots on the right indicate the level to which TMRE increases during mitotic arrest. p-values were obtained using ANOVA followed by Sidakholm test.

(C) Schematic indicating how the chemical inhibitors (red) affect CDK1 activity.

(D) Mass-normalized TMRE trace for a L1210 cell treated with 5 µM STLC. Once the cell was arrested in mitosis, 100 nM O.A. was added (black arrow) to the culture media. Boxplots on the right indicate the TMRE level before and after O.A. addition. p-value was obtained using two-tailed Student’s t-test.
(E) Mass-normalized TMRE trace for a L1210 cell treated with 5 µM STLC. Once the cell was arrested in mitosis, 5 µM RO-3306 was added (black arrow) to the culture media to inhibit CDK1. Boxplots on the right indicate the TMRE level before and after RO-3306 addition.
Fig. 3: Mitochondrial ATP synthesis is required for cell growth, but not for cell division

(A) Buoyant mass (black) and mass-normalized TMRE (red) trace for a L1210 cell around cell division in the presence of 1 µM oligomycin. Despite reduced growth rate, the cell proceeds through mitosis.

(B) Quantifications of the TMRE increase in mitosis relative to G2 levels in control and 1 µM oligomycin-treated L1210 cells. p-value was obtained using two-tailed Student’s t-test.
(C) Quantifications of mitotic entry in control and 1 µM oligomycin-treated L1210 cells. Cells were synchronized to G2, released and collected for cell cycle analysis at indicated timepoints. 1 µM oligomycin treatment was started 15 min before release from G2 arrest. Each dot represents a separate culture (n=3).

(D) Quantifications of mitotic exit (appearance of G1 cells) for samples shown in (C).

(E) Quantification of mass accumulation (growth) rate in individual control and 1 µM oligomycin-treated cells. Oligomycin blocks cell growth within 1h. Each dot represents the growth rate of a single-cell. Quantifications of the growth rates after 1h drug exposure are shown on the right. p-value was obtained using two-tailed Student’s t-test.
Fig. 4: Mitochondrial ATP synthesis is inhibited in mitosis

(A, B) Electrical circuit model of mitochondria, where $\Delta \Psi m$ is the voltage across the circuit (panel A). The key components controlling $\Delta \Psi m$ (ETC, ATP synthase and leakage), all have their respective resistances ($R_{ETC}, R_{ATP}$ and $R_{Leak}$) which together form the total resistance ($R$) in the circuit (panel B). In the presence of oligomycin, the $R_{ATP}$ value increases to infinity. The difference in $R$ values between control and oligomycin-treated cells reflects the $R_{ATP}$ value.

(C) Model fit to a typical L1210 single-cell TMRE data. TMRE signals were converted to approximate voltages and the period where CDK1 is on (light blue) and off (dark blue) were fitted separately to derive the time constant (RC) values for each period.

(D) Derived time constant (RC) values for CDK1 on (light blue) and off (dark blue) periods in control and 1 µM oligomycin-treated L1210 cells. p-values were obtained using ANOVA followed by Sidakholm test.

(E) ATP synthesis rate ($I_{ATP}$) for 85 separate L1210 cells around cell division. Traces of individual cells are drawn with thin opaque lines, whereas the population average is drawn as thick solid line. The colored areas reflect total amounts of ATP synthesized during early mitosis (red) and anaphase (light yellow).

(F) Quantifications of relative ATP synthesis rates ($I_{ATP}$) in G2 (dark blue) and mitosis (light blue) as modelled on a single-cell level in non-arrested cells (left), and as estimated based on oxygen consumption rates (OCR$_{ATP}$) in cell populations arrested to G2 and mitosis (right). Note that both
approaches rely on comparing control and oligomycin treatment conditions to derive ATP synthesis rates, but the model accounts for changes in $\Delta \Psi_{m}$ and does not require cell cycle synchronizations. Data depicts mean ± s.e.m. (n=85-32 for model, n=7-8 for OCR).

(G) Quantifications of the total amount of mitochondrial ATP synthesis during early mitosis (from G2/M to M/A transition, red) and during anaphase (from M/A transition to end of anaphase where cell elongation is complete, light yellow), when compared to a null-hypothesis where ATP synthesis rate remains at G2 levels throughout mitosis (dashed horizontal line). Data depicts mean ± s.e.m. of the $R_{ATP}$ values (N=40-32, n=85-32).