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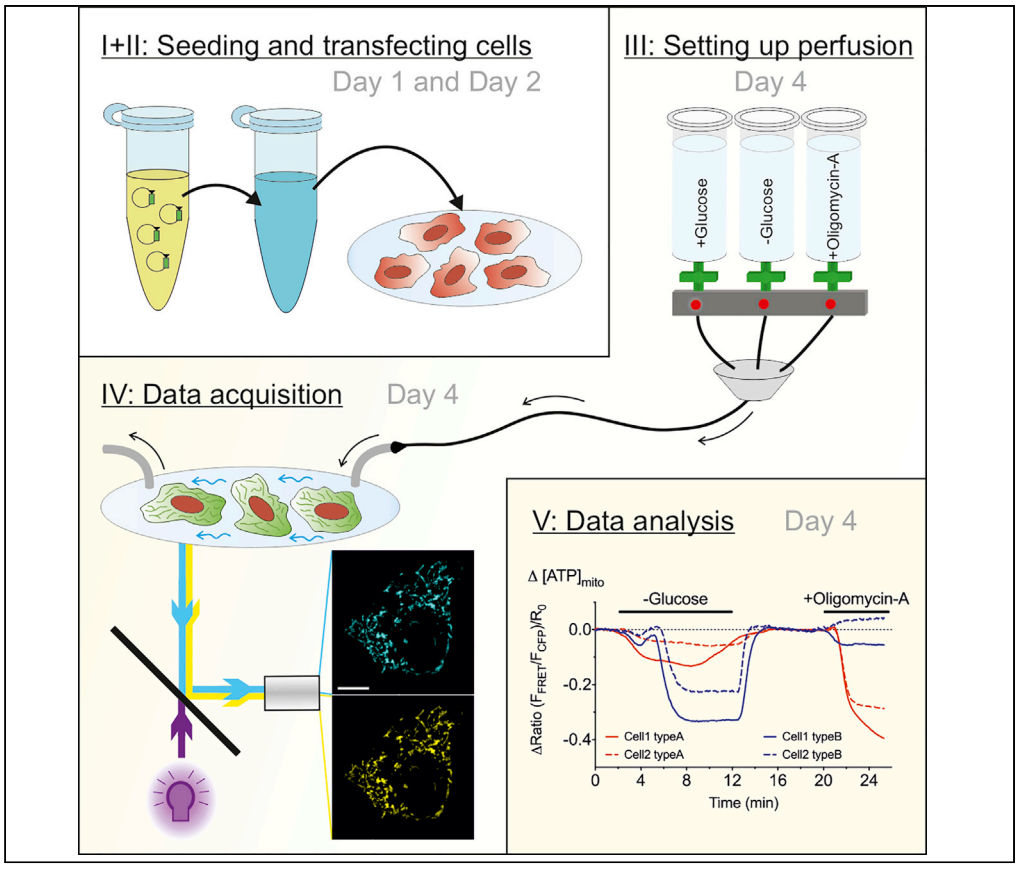
Metabolic Profiling of Single Cancer Cells Using Mitochondrial ATP Probes



The metabolic activity of cells is interrelated with cell signaling, functions, and fate. Uncontrolled cancer cell proliferation requires metabolic adaptations. Research focusing on understanding the characteristics of cell metabolism is crucial for the development of novel diagnostic and therapeutic strategies. Here, we describe protocols for the ATP profiling of single cancer cells by fluorescence live-cell imaging. In response to distinct metabolic inhibitions, we record individual mitochondrial ATP dynamics using established Förster resonance energy transfer-based genetically encoded fluorescent ATP probes.

Thomas Rauter, Maria R. Depaoli, Helmut Bischof, Wolfgang F. Graier, Roland Malli

thomas.rauter@medunigraz.at (T.R.)
mdepaoli@cemm.oeaw.ac.at (M.R.D.)
wolfgang.graier@medunigraz.at (W.F.G.)
roland.malli@medunigraz.at (R.M.)

HIGHLIGHTS

ATP profiling of single cancer cells by fluorescence live-cell imaging

Glucose removal or OXPHOS inhibition characteristically affects ATP in mitochondria

Mitochondrial ATP dynamics are a valuable readout of cell metabolism

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Metabolic Profiling of Single Cancer Cells Using Mitochondrial ATP Probes

Thomas Rauter,[1,3,5,*] Maria R. Depaoli,[1,3,4,5,*] Helmut Bischof,[1] Wolfgang F. Graier,[1,2,*] and Roland Malli[1,2,6,*]

[1]Molecular Biology and Biochemistry, Gottfried Schatz Research Center, Medical University of Graz, Neue Stiftingtalstraße 6/6, Graz 8010, Austria

[2]BioTechMed, Graz, Austria

[3]These authors contributed equally

[4]Present address: CeMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

[5]Technical Contact

[6]Lead Contact

*Correspondence: thomas.rauter@medunigraz.at (T.R.), mdepaoli@cemm.oeaw.ac.at (M.R.D.), wolfgang.graier@medunigraz.at (W.F.G.), roland.malli@medunigraz.at (R.M.)
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SUMMARY

The metabolic activity of cells is interrelated with cell signaling, functions, and fate. Uncontrolled cancer cell proliferation requires metabolic adaptations. Research focusing on understanding the characteristics of cell metabolism is crucial for the development of novel diagnostic and therapeutic strategies. Here, we describe protocols for the ATP profiling of single cancer cells by fluorescence live-cell imaging. In response to distinct metabolic inhibitions, we record individual mitochondrial ATP dynamics using established Förster resonance energy transfer-based genetically encoded fluorescent ATP probes.

For complete details on the use and execution of this protocol, please refer to Depaoli et al. (2018).

BEFORE YOU BEGIN

The workflow described in this protocol (Figure 1) takes several days and requires preparation and access to certain equipment (see Materials and Equipment). Before starting the experiment itself, required buffers and media must be prepared.

Depending on the cell type the composition of the cell culture medium can vary significantly. We recommend following the cultivation guidelines recommended by the cell line provider. In this protocol we used standard cell culture media and conditions for cultivation of respective cell lines as published in Depaoli et al., 2018. In the course of our studies we have not encountered any media additives that interfere with the protocol. Furthermore, times indicated for cell cultivation in this protocol might also be different depending on the cell type. The protocol serves as a reference for HeLa, HEK293a and INS-1 cells and represents a good starting point for a number of established cell lines. For some cell lines slight adjustments to the protocols might be necessary. In that case the Troubleshooting part contains detailed explanations and solutions for all possible challenges we have encountered with the protocol. All buffers required should be prepared according to the recipes provided in the Materials and Equipment section.

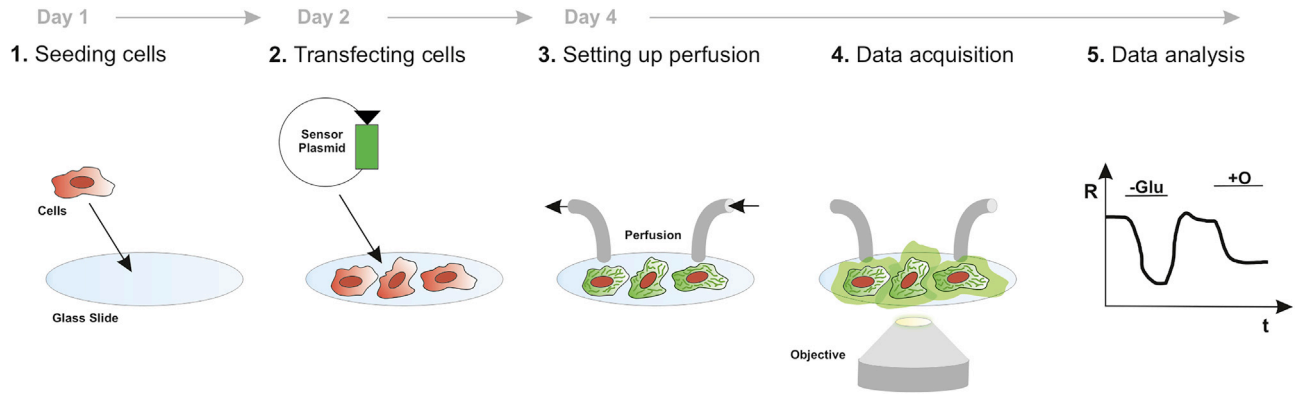

Figure 1. Step-By-Step Workflow for ATP Profiling of Live Cancer Cells Using Fluorescence Microscopy

1. Cells are seeded and grown on glass coverslips positioned in 6-well plates. 2. Cells are transfected with the plasmid encoding the mitochondria-targeted Förster Resonance Energy Transfer (FRET)-based ATP-sensor mtAT1.03. 3. The glass coverslip with adherent cells is transferred to a perfusion chamber and the perfusion system is set up and attached to the chamber allowing optimal buffer exchange during the measurements. 4. Measurement of mitochondrial ATP-dynamics of living cells via fluorescence microscopy is conducted. 5. The live-cell imaging data are analyzed, enabling a type of metabolic profiling of the tested cells by comparing mitochondrial ATP-profiles under certain conditions as a crucial metabolic parameter.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
NaCl	Carl Roth, Graz, Austria	Cat#3957
KCl	Carl Roth, Graz, Austria	Cat#6781
$MgCl_2$ x $6H_2O$	Carl Roth, Graz, Austria	Cat#2189
$CaCl_2$ x $2H_2O$	Carl Roth, Graz, Austria	Cat#T885
$NaHCO_3$	Carl Roth, Graz, Austria	Cat#8551
Na_2HPO_4 x $2H_2O$	Carl Roth, Graz, Austria	Cat#4984
KH_2PO_4	Merck, Darmstadt, Germany	Cat#A681173
NaOH	Carl Roth, Graz, Austria	Cat#6771
Dimethyl sulfoxide (DMSO)	Carl Roth, Graz, Austria	Cat#A994
L-Glutamine	Carl Roth, Graz, Austria	Cat#HN08
HEPES	Carl Roth, Graz, Austria	Cat#9105
D(+)-Glucose Monohydrate	Carl Roth, Graz, Austria	Cat#6780
Oligomycin-A	Tocris, Bristol, UK or Sigma Aldrich, Vienna, Austria	Cat#4110 or Cat#75351
D-Mannitol	Sigma Aldrich, Vienna, Austria	Cat#M4125
Gibco™ MEM Vitamin solution (100x)	Thermo Fisher Scientific, Vienna, Austria	Cat#11120052
Gibco™ MEM Amino Acid solution (50x)	Thermo Fisher Scientific, Vienna, Austria	Cat#11130051
Penicillin-Streptomycin (10,000U/ml)	Thermo Fisher Scientific, Vienna, Austria	Cat#15140122
Amphotericin B	Thermo Fisher Scientific, Vienna, Austria	Cat#15290018
TransFast™ Transfection Reagent	Promega, Walldorf, Germany	Cat#E2431
PolyJet™ In Vitro DNA Transfection Reagent	SignaGen® Laboratories, Rockville, MD, USA	Cat#SL100688

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
Human: HeLa S3	ATCC, Guernsey, UK	N/A
Human: HEK293a	ATCC, Guernsey, UK	N/A
Rat: INS-1 (832/13)	C.B. Newgard, Duke University School of Medicine, USA Hohmeier et al., 2000	N/A
Recombinant DNA		
pcDNA3.1 mtAT1.03	H. Imamura; Imamura et al., 2009	N/A
Software and Algorithms		
Visiview v4.2.0.1	https://www.visitron.de/products/visiviewr-software.html	N/A
GraphPad Prism v5.01	https://www.graphpad.com/scientific-software/prism/	N/A
Microsoft Excel 2013	office.com	N/A
Fiji/ImageJ	Schneider et al., 2012	https://fiji.sc/
Other		
Incubator HeraCell	Heraeus Holding GmbH, Hanau, Germany	N/A
Sterile Workbench HeraSafe	Heraeus Holding GmbH, Hanau, Germany	N/A
30mm Coverslip Glasses	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany	Cat#0111700
6-well Plates	Greiner Bio-One International GmbH, Kremsmünster, Austria	Cat#657160
Perfusion System	NGFI Next Generation Fluorescence Imaging GmbH, Graz, Austria	N/A
Perfusion Chamber PC30	NGFI Next Generation Fluorescence Imaging GmbH, Graz, Austria	N/A
Chemistry diaphragm pump ME 1c (vacuum pump)	Vacuubrand, Wertheim, Germany	N/A
Fluorescence microscopy setup	see Equipment	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Roland Malli (roland.malli@medunigraz.at).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

MATERIALS AND EQUIPMENT

We highly recommend preparing stock solutions of certain components in ddH_2O beforehand to minimize measuring inaccuracy. For the preparation of some stock solutions the hydrated salt was used (see Key Resources Table), however, it can also be exchanged by the anhydrous version of the same purity grade depending on availability. It is indicated in the respective tables, where a stock solution is preferred.

Preparation of Cell Storage Buffer

Timing: day 0

The cell storage buffer (CSB) enables a smooth transition for cells from the incubator to measuring conditions. It lacks fetal calf serum (FCS) and relies on a HEPES buffer system. For one well of a 6-well plate 2 mL of CSB are needed. The buffer can be prepared in advance and should be stored at 4°C after sterile filtration.

CSB is composed of the following ingredients (Table 1).

Preparation of Experimental Buffers for Perfusion

Timing: day 0

All experimental buffers (EBs) for cell perfusion during live-cell imaging have the same basic composition. The EBs contain physiological concentrations of important salts, are isotonic, and their pH is buffered via HEPES. The exact composition of the EBs is given in Table 2. The buffer should be prepared freshly, during storage the buffer capacity can decrease. Add D(+)-Glucose Monohydrate, D-Mannitol, or Oligomycin-A as well as other substrates and modulators of cell metabolism to the basic EB. Notably, Table 2 shows three EBs essential for the experiment (EB +-Glucose, EB -Glucose, and EB +Oligomycin-A). These are approved EBs for distinct and acute metabolic modulations of (cancer) cells of interest during live-cell imaging of mitochondrial ATP dynamics. If the reader extends the protocol by the use of additional substances that require preparation of a stock solution in an organic solvent, it is important to check if the solvent itself has any effect on cell metabolism or shows auto-fluorescence, thereby creating artifacts. In such cases, we recommend to perform control experiments with EBs that contain solvents in respective concentrations.

Table 1. Formulation of CSB for Cell Storage before Measurement

Component	Concentration	Amount for 1 L
NaCl	138 mM	138 mL of 1 M stock
KCl	5 mM	50 mL of 0.1 M stock
$CaCl_2$	2 mM	20 mL of 0.1 M stock
$MgCl_2$	1 mM	10 mL of 0.1 M stock
HEPES	10 mM	10 mL of 1 M stock
$NaHCO_3$	2.6 mM	26 mL of 0.1 M stock
Na_2HPO_4	0.34 mM	3.4 mL of 0.1 M stock
KH_2PO_4	0.44 mM	4.4 mL of 0.1 M stock
D(+)-Glucose Monohydrate	10 mM	1.98 g
Gibco MEM Vitamin solution (100x)	1x	10 mL
Gibco MEM Amino Acid solution (50x)	1x	20 mL
Penicillin-Streptomycin (10,000U/ml)	1 %	10 mL
L-Glutamine	2.5 mM	367 mg
Amphotericin B	0.5 %	5 mL
ddH_2O		to 1 L
NaOH		adjust pH to 7.4

Table 2. Formulation of Isotonic EBs for Cell Super-Fusion during Live-Cell Imaging of Cellular ATP Dynamics

Component	Concentration	Amount for 1 L
Basis for all EBs		
NaCl	138 mM	138 mL of 1 M stock
KCl	5 mM	50 mL of 0.1 M stock
$CaCl_2$	2 mM	20 mL of 0.1 M stock
$MgCl_2$	1 mM	10 mL of 0.1 M stock
HEPES	10 mM	10 mL of 1 M stock
ddH_2O		to 1 L
NaOH		adjust pH to 7.4
for EB with Glucose (+ Glu) add:		
D(+)-Glucose Monohydrate	10 mM	1.98 g
for EB without Glucose (– Glu) add:		
D-Mannitol	10 mM	1.82 g
for EB with Oligomycin-A (+ Oligo) add:		
D(+)-Glucose Monohydrate	10 mM	1.98 g
Oligomycin-A stock solution in DMSO (10 mM)[a]	to 2 μM final conc.	0.2 mL

[a]Oligomycin-A should be added freshly on the day of the measurement!

Equipment

- Cell culture

  A standard cell culture setup, a sterile workbench and a 37°C incubator at 5% CO_2 are sufficient for the experiment. The choice of culturing dishes depends on whether perfusion – as recommended – will be used during the experiment or not. If so, 30 mm coverslip glass slides (see Key Resources Table; alternatively Carl Karl Hecht GmbH & Co. KG Microscope Cover Glasses No.1) and 6-well plates can be used, if not, single imaging dishes are recommended (see Alternatives).
- Imaging setup

  There are certain requirements for the imaging setup used for this protocol. These can serve as a general technical guideline for all protocols involving the measurement of FRET-based probes composed of a CFP/YFP-FRET-pair in living cells including e.g. calcium ions (Palmer et al., 2006), potassium ions (Bischof et al., 2017) or pH (Burgstaller et al., 2019). Since the composition of imaging systems can vary significantly in different laboratories depending on their research focus, funding and availability of components in the respective country, the authors list the imaging setup with all its components used in our laboratory as a reference for a suitable system for this purpose (Table 3). It is important to mention that systems with equal quality and features from varying distributors can also be a viable option. Regarding data analysis, for example, the open source software ImageJ (Fiji) can be used.
- Perfusion system

  A gravity-based perfusion system (NGFI, Graz, Austria; https://www.ngfi.eu) that allowed quick exchange of buffer solution for optimal metabolic supply was used and is highly recommended for this type of experiment. This system can be successfully applied for the measurement of a number of different genetically encoded probes (as shown for measuring nitric oxide in our laboratory, Eroglu et al., 2017; link to video: https://www.jove.com/video/55486/application-genetically-encoded-fluorescent-nitric-oxide-no-probes?status=a57492k).

  The perfusion system was installed onto a perfusion chamber (PC30; Figure 2A) with a negative pressure at the exit side to allow constant super-fusion (Operating principle as displayed by manufacturer: https://www.youtube.com/watch?v=q2-scfjasAw&feature=emb_title). A round 30 mm glass coverslip with 0.13–0.16 mm thickness (carrying the cells in the experiment) was

Table 3. Components of the Imaging System Used in Our Laboratory

Component	Note	Company
Olympus IX73	Inverted setup is necessary due to the nature of the protocol	Olympus, Vienna, Austria
Omicron LEDHub	Light source equipped with 455 nm LED	Omicron, Klaus, Austria
Excitation filter	Semrock 427/10 Brightline HC	IDEX Health & Science, Rochester, NY, USA
Emission filter	Semrock LED-CFP/YFP/mCherry-3x	IDEX Health & Science, Rochester, NY, USA
Photometrics DV2	Optical beam splitter	Photometrics, Tucson, AZ, USA
Retiga R1 CCD camera	Camera	TELEDYNE QIMAGING, Surrey, Canada
PC	For control and monitoring of measurement via imaging software	N/A
Visiview v4.2.0.1	Imaging software	https://www.visitron.de/products/visiviewr-software.html

mounted in the chamber (Figure 2B). Our standard measurement procedure is carried out at room temperature (22–24°C) in ambient atmosphere.

It is also possible to apply simple protocols without the use of perfusion. However, one needs to consider that concentrations of substances decrease over longer incubation times, and results might differ. While changing buffer parameters by injection of concentrated solutions can be easier in such a setup, it is often not possible to remove added compounds again to observe the recovery of signals. Furthermore, multiphasic protocols with varying compounds or compound-concentrations are not possible at all.

Alternatives: Alternative to perfusion chamber: Despite the described advantages of using a perfusion system (gravity-based or pressured), it is also possible to alter the protocol slightly. If no perfusion system is available in the laboratory, we recommend using single imaging dishes with coverslip bottom, which can be used for cultivation and imaging (e.g. µ-Dish 35 mm, low; ibidi GmbH, Gräfelfing, Germany; Cat.#80136).

STEP-BY-STEP METHOD DETAILS

Seeding Cells

Timing: day 1

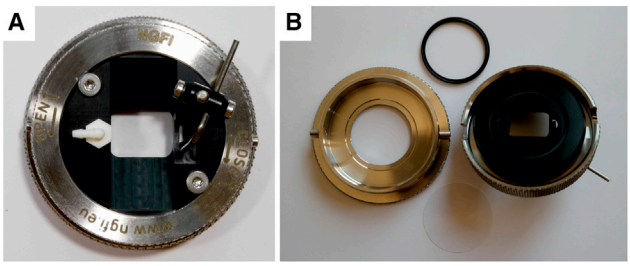

Figure 2. The Perfusion Chamber Applied in the Experimental Setup

The perfusion chamber (PC30, NGFI) used in the experiment is shown in the closed (A) and opened configuration (B).
(A) The positions of inlet (left, white) and outlet for the buffer (right) allow laminar flow over the imaged cells.
(B) In addition to the bottom (left) and top part of the chamber (right), the sealing ring (top) and a 30 mm glass slide (bottom) is shown. For the experiment the glass slide carrying the cells is placed in the slot of the lower chamber-part, the sealing ring is placed in the slot of the upper chamber-part and the chamber is closed via a quick-release fastener.

Handling the cells for this experiment does not require special treatment. However, in addition to the recommended cell confluencies for transfection, which might deviate from manufacturer protocols, and required cell culture dishes, there is a general point to consider: Any disturbances that may influence cell metabolism (e.g. fluctuations in atmosphere, temperature) should be strictly avoided!

Empirically optimized confluency values for model cell lines for this protocol are given in Table 4. For cell cultivation, 30 mm glass coverslips are placed in the 6-well culture plates. The cells are seeded in the culture dishes and attach to the glass slide, which in further consequence serves as the coverslip in the inverted microscopy setup. In case other cell lines are used a confluency of around 80% on the measuring day can be taken as a reference point.

Transfecting the Sensor Plasmid

Timing: day 2

1. Depending on the transfection method, there are different protocols to follow. In our laboratory, mostly lipid-based transfection using TransFast Transfection Reagent or PolyJet In Vitro DNA Transfection Reagent is applied for mitochondria-targeted constructs. (We have not tested primary cell lines with this exact protocol yet, however, from our experience, expressing the sensor in primary cells might be challenging)
2. Follow the manufacturer's protocol for the transfection of mammalian cells using TransFast (Technical Bulletin TB260; 1.5 μg/ml plasmid concentration) or PolyJet (General Protocol for Transfecting Mammalian Cells, SignaGen Laboratories website: signagen.com, Cat#SL100688; 1 μg/mL plasmid concentration).

Note: Empirical testing in our laboratory has shown that the incubation period with DNA and TransFast Reagent can be lengthened up to an overnight treatment (14–16 h) and the time after medium exchange can be as short one day in some cases. Times may vary depending on the cell line and sensitivity of the imaging system. Increasing incubation times may increase the expression levels of mtAT1.03 but may also lead to mislocalization of the ATP probe outside of mitochondria.

Note: Application of a different transfection reagent is possible depending on the cell type. While using other reagents might be beneficial in some cases, there might also be adverse effects – especially in case of excessive sensor production (see Troubleshooting). Transfection methods based on other physical phenomena like electroporation have not been tested within this experimental setup in our laboratory.

⚠ CRITICAL: The transfection is a critical step to return to later on, in case of problems with probe mislocalization or dysfunctionality of the sensor (see Troubleshooting).

Table 4. Recommended Confluencies at the Day of Transfection

	HeLa	HEK293a	INS-1
TransFast (2d)	40%	40%–50%	40%–50%
TransFast (1d)	40%–50%	40%–50%	40%–50%
PolyJet™ (2d)	25%–30%	25%–30%	25%–30%
PolyJet™ (1d)	40%	40%	40%

Values are given for the cell lines HeLa, HEK293a and INS-1 for either of the two mentioned lipid-based transfection reagents (TransFast, PolyJet). "2d": transfection performed two days before measurement; "1d": transfection performed on the day before the measurement day.

Preparation of Cells and Imaging Setup for Measurement

Timing: day 4

3. Get culture dish containing cells out of the incubator.
4. Completely remove full medium and add the same amount of CSB.
5. Leave cells on CSB for 60 min at room temperature (22–24°C) in ambient atmosphere to ensure that cells have adjusted and that the metabolic state is stabilized.

⚠ CRITICAL: The equilibration in CSB at room temperature (22–24°C) must not be shortened since ATP-levels can fluctuate significantly, when cells are in a state of adjustment. When keeping the cells on CSB, it is recommended not to exceed a maximum time of 8 h in order to ensure reproducibility of results. It might be favorable to stagger the transfer of cells to CSB.

Note: If buffers have not been prepared before the experiment or unstable additives have to be added this is the recommended time point to do that. Now, Oligomycin-A should be added to the standard EB containing 10 mM glucose to yield EB +Oligomycin-A.

6. Prepare perfusion system at the microscopy site

   a. Fill containers of the perfusion system with your buffer solutions
   b. Open every valve consecutively to ensure functionality and removal of air out of the tubings
   c. Ensure that the flow rate of all buffers is similar to avoid interruptions when switching channels
   d. Use two containers for washing solutions (ddH_2O and 70% ethanol)
   e. Wash the system with 70% ethanol to remove any substances not soluble in water from the tubes
   f. Wash the system with ddH_2O to remove any remaining buffer and ethanol residues
   g. Repeat steps e. and f. after every experiment!

7. After the 60 min CSB-incubation, transfer the coverslip into the perfusion chamber and close the chamber (Figure 2). Do not let dry out, immediately add basal buffer (usually basic EB + Glucose) when the chamber is closed (also see Eroglu et al., 2017; link to video: https://www.jove.com/video/55486/application-genetically-encoded-fluorescent-nitric-oxide-no-probes?status=a57492k).
8. Dry the bottom of the glass slide with a lint-free tissue
9. Fix chamber on the microscope table and add immersion oil if an oil objective is used. We recommend using 20x and/or 40x objectives to control the correct targeting of mtAT1.03 in single cells.

Note: In case of using single imaging dishes, the CSB is replaced by the starting buffer with an optional washing step with starting buffer; perform step 9., then skip to step 13.

10. Make sure that the outlet of the perfusion system contains basal buffer before starting the experiment.
11. Switch on the perfusion and connect the perfusion system to your chamber. Immediately attach the vacuum pump to avoid buffer overflow.
12. Establish a laminar flow of buffer through your chamber at a flow rate of approximately 1 mL/min. Conventional tube clamps might be used on both sides to control buffer inflow and vacuum.
13. Check the sample for a suitable imaging section with good signal intensity.

⚠ CRITICAL: Make sure that cells show a correct targeting of the ATP-sensor to the mitochondrial matrix and do not show cytosolic and/or nuclear mistargeting. We highly recommend

performing a preliminary experiment testing for correct targeting and expression of the sensor. The application of a mitochondrial tracking dye (e.g. MitoTracker Red CMXRos, Thermo Fisher Scientific, Vienna, Austria) for this purpose can be an option but has to be done with caution with regard to potential side effects on the (metabolic) activity of the organelle. Ensure that cells are not too dense, (i.e., changed in their morphology due to lack of space) within the area of interest.

Note: The signal-to-background ratio needed to yield good results depends on the imaging system. We recommend choosing cells with similar signal intensities corresponding to equal expression levels across all experiments.

14. The protocol for measuring ATP-dynamics can now be started.

Measurement of Mitochondrial ATP Dynamics

Timing: day 4 (4–6 h)

15. Setting up measurement parameters
    The mtAT1.03 sensor is excited at a wavelength of 430 nm; emission is collected at 535 nm and 480 nm, images are acquired every 2 s (see Note). Select regions of interest (ROIs) for each cell with correct targeting and expression of the sensor individually and at least one background region. The fluorescence intensities for data analysis will be calculated as averaged intensities of the ROIs for each channel (FRET and CFP).
    Take into account that a full measurement of all parameters in one sample can take up to 35–50 min. We recommend measuring at least three wells of each treatment condition and controls on three separate days to ensure reliable results. Usually this takes 4–6 h for one full set of experiments per day. However, there is no limit for the maximum number of individual measurements per day, if you have free access to an appropriate imaging system.
16. Record baseline
    Baseline mitochondrial ATP in the presence of glucose is measured for approximately 2 min (see Note).

    Note: In order to avoid photobleaching and phototoxicity, we recommend increasing the camera binning, if available on the system (e.g. binning 4), and minimizing the intensity of the excitation light as well as the exposure time. To improve the signal-to-noise ratio, increase exposure time rather than light intensity. For the setup described in the Material-part, we used 300 ms exposure time at a camera binning of 4 and recommend around 150 mW LED power with a 6% transmission filter as a starting point. These parameters can vary depending on microscope setup, cell type, and expression levels.

    Note: The interval between measurement points can vary depending on the desired temporal resolution. Increasing the intervals reduces light exposure of cells over time, which will also reduce photobleaching and phototoxicity.

    Note: It is essential to record a stable baseline, which is required to correct for photobleaching during data analysis if necessary.

    ⚠ CRITICAL: Do not change imaging parameters such as exposure time and light intensity from experiment to experiment, as this may cause artifacts.

17. Assessing glycolytic ATP production

a. Switch to buffer without glucose (preferentially via a perfusion system; exchanging the buffer manually might produce severe noise). Follow the dynamics of mitochondrial ATP changes for at least 10 min until they stabilize again at a certain value. In HeLa cells, ATP levels sometimes first drop slightly, then they usually increase, and finally, strongly decrease until they reach a stable minimum (see Note).

Note: The sensor is ratiometric, which means FRET intensity (535 nm) decreases when ATP levels decline, while CFP intensity (480 nm) increases under such conditions (Figure 3A). The exact timing depends on the cell type – test, which protocol is most useful for your purposes. We advise being patient to not miss potentially meaningful signals.

b. Switch back to glucose-containing buffer and follow the signal for at least 10 min until mitochondrial ATP levels increase back to baseline (or stabilize; see Note).

Note: Usually, mitochondrial ATP levels reach the baseline again. Sometimes the signal first overshoots above basal levels and then declines again. The kinetics may vary significantly between different cells. You may have to wait for up to 20 min until the signal stabilizes.

18. Measuring mitochondrial ATP changes in response to ATP synthase inhibition by Oligomycin-A
Switch to Oligomycin-A-containing buffer (2 μM). Mitochondrial ATP levels will either increase or decrease. Follow mitochondrial ATP changes for at least another 5 min. In this part of the protocol mitochondrial ATP production is assessed by inhibiting ATP synthase with Oligomycin-A (2 μM for all cell lines tested in our lab so far; may need to be adapted when using other cell lines).

Note: This protocol aims to assess both glycolytic and mitochondrial ATP production of individual cells in one single experiment. As it might be difficult to completely adverse Oligomycin-A induced inhibition of ATP-synthase, we add it at the end of the experiment. Moreover, residual Oligomycin-A might remain in the tubes and confound the following experiment. Hence, it is crucial to properly rinse the tubes of the perfusion system and the perfusion chamber with ethanol after each experiment.

⚠ CRITICAL: It is possible that the first part of the protocol (glucose depletion and re-addition) may affect ATP synthase activity in your cells. We recommend performing an additional set of experiments, in which only ATP synthase activity is measured (18.) without previous glucose depletion (17.), and comparing the results.

19. End experiment
Stop measurement and export data (average fluorescence intensities of ROIs over time), e.g. as a Microsoft Excel file. Dispose of the coverslip, clean perfusion system and chamber with ethanol and ddH_2O.

Data Analysis

Timing: day 4/any time after measurement

20. Background correction:
Subtract the background values for each channel (FRET and CFP), respectively.
21. Plot fluorescence intensities of FRET (F_{535}) and CFP (F_{480}) over time and ensure that signal changes are ratiometric to exclude any possible artifacts (Figures 3A and 3D).
22. Calculate the FRET ratio signal R=(F_{535}/F_{480}) by dividing FRET by CFP values (Figure 3B).
23. Select baseline values and calculate a function for the fluorescence bleaching effects (see Note) using the following equation: $R_0 = R_{inital} \bullet \exp(-K \bullet Time) + R_{plateau}$. R_{inital}: maximal fluorescence ratio signal once imaging is started; K: rate constant of fluorescence bleaching over time;

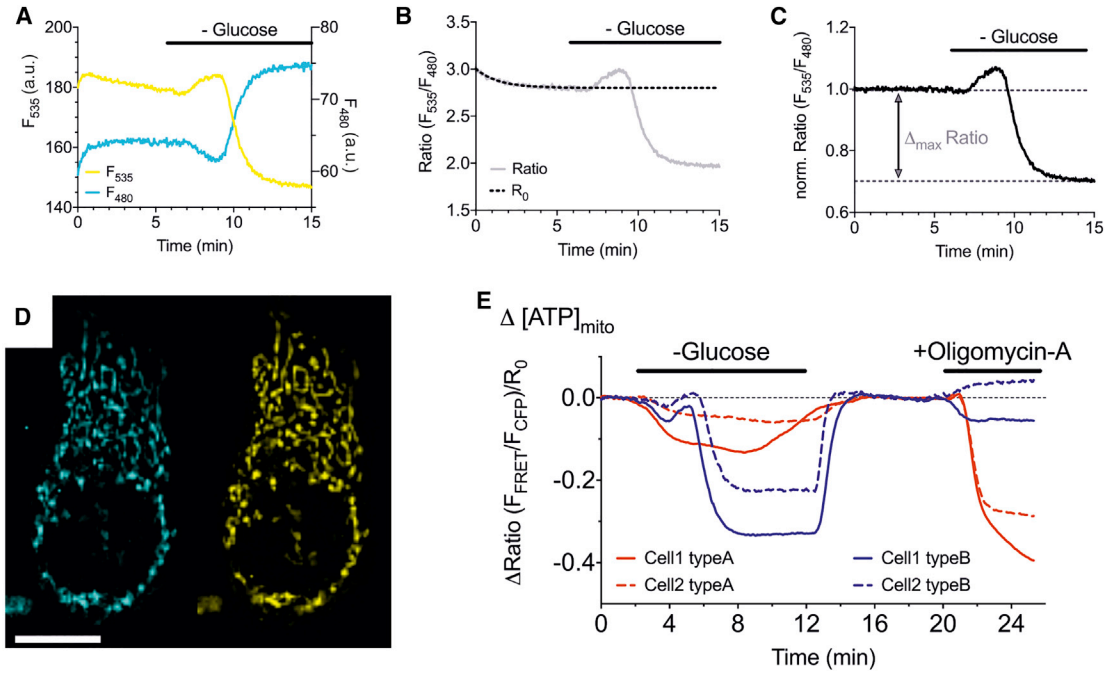

Figure 3. Exemplary Data of the ATP Profiling Protocol

(A–C) Show a single-cell response to glucose-removal measured with mtAT1.03. (A) Fluorescence intensity curves of CFP (480 nm, cyan) and FRET (535 nm, yellow) showing a mirror-inverted reaction upon glucose-removal underlining the ratiometric nature of the sensor. Values are given in arbitrary units (a.u.). (B) The single curves from A are used to calculate a ratio-value over time (grey) representing mitochondrial ATP-content. The function R_0 is calculated using GraphPad Prism (dotted curve, see Data Analysis). (C) A normalized and bleaching-corrected Ratio-curve enabling a readout of the maximum delta upon glucose-removal.

(D) Fluorescence microscope images of mitochondria-targeted ATP-sensor mtAT1.03 showing the CFP- (left) and FRET-channel (right), respectively, in HeLa cells using a 40x objective and binning 4. Scale bar, 20 μm.

(E) Comparison of normalized ratios when measuring with mtAT1.03 over the course of the protocol between two example cell lines ("typeA": red, "typeB": blue). The example nicely illustrates the power of the single-cell imaging protocol: resolving differences in general metabolic profiles between cell lines based on their mitochondrial ATP dynamics but also minor differences between single cells of the same cell line! For data used in this figure see Depaoli et al., 2018.

$R_{plateau}$: fluorescence ratio minimum reached by bleaching over time. (GraphPad Prism: one phase decay; Figure 3B).

24. From this function, calculate R_0 for each time point (Figures 3C and 3E).
For data normalization and bleaching correction to initial ratio, divide R/R_0 for each time point. Display the data in a scatter plot with smooth lines (x-axis: time, y-axis: $R = (F_{535}/F_{480})/R_0$). (For bleaching correction without normalization, calculate $R = (F_{535}/F_{480}) - R_0 + R_{initial}$.)
25. Use the normalized data to calculate the maximal ATP change during glucose depletion (Δ_{max} $[ATP]_{mito}$ by glucose depletion, Figure 3C), and the maximal ATP change upon Oligomycin-A treatment (Δ_{max} $[ATP]_{mito}$ by Oligomycin-A) for each cell (it may be a positive or negative value; Figure 3E). Blot single cell values on a scatter dot blot (x-axis: Δ_{max} $[ATP]_{mito}$ by Oligomycin-A, y-axis: Δ_{max} $[ATP]_{mito}$ by glucose depletion; see Depaoli et al., 2018).

Note: Photobleaching is an unwanted side effect and should be reduced to an absolute minimum by adjusting imaging parameters. Note, that numerous different approaches for bleaching correction are available – the given protocol describes one of them.

EXPECTED OUTCOMES

The starting ratio in the protocols enables a simple readout of basal values giving information about the basal concentration of ATP in mitochondria, which may already provide valuable information about the metabolic state of a cell and can differ significantly between cell types and/or treatment conditions.

By performing the whole protocol, one can acquire detailed information about the balance of glycolytic ATP-production and activity as well as the mode of action (forward or reversed) of the ATP-synthase, the main enzyme responsible for converting (forward, i.e., ATP production allowing protons to enter the matrix) or maintaining (reversed, i.e., ATP consumption and proton pumping to the intermembrane space) the electron/proton gradient across the inner mitochondrial membrane. These parameters represent crucial readouts for estimating the metabolic setting of a cell, as demonstrated in Depaoli et al., 2018. The metabolic state and its sensitivity to an infinite number of treatment conditions, including knockdown, knockout or overexpression of genes or compound treatment, can be assessed for single cells by using this method and expanding it, respectively. In contrast to cell population measurements, it can additionally provide valuable information about cell-to-cell heterogeneities.

LIMITATIONS

Due to the fact that perfusion is applied for buffer exchange during measurement a certain adherence of the cells used in the experiment is highly recommended. In order to allow adequate long-term measurements, cells should move as little as possible. Coating of glass coverslips prior to cell seeding has not been tested in this particular experimental setup. However, the authors recognize the possibility that it can work based on other experiments.

Furthermore, cells have to be resistant to light shear stress. Decreasing the perfusion flow rate can push the limits of this factor, but it has to be taken into account that a very low flow rate can lead to suboptimal buffer exchange and, hence, varying results.

TROUBLESHOOTING

Problem

The sensor construct does not target correctly to mitochondria. / There is a strong signal in the cytosol in addition to the mitochondrial signal.

Potential Solution

If the sensor does not target correctly, the measurements must not be conducted!

Depending on the cell type, transfection parameters might have to be changed to tackle this problem. Suggestions for that would be: shortening the time between transfection and measurement, reducing the amount of plasmid-DNA or switching to a different transfection reagent or virus infection. Cell clones stably expressing the mitochondria-targeted ATP probe might be generated and used instead. Notably, recently single FP-based ATP probes, QUEEN (Yaginuma et al., 2014) and MaLions (Arai et al., 2018), which might be better targetable to mitochondria, have been developed.

Problem

A fluorescence signal can be detected, but there is no proper reaction during the protocol.

Potential Solution

First, it has to be ensured that all buffers are made freshly and according to protocol. The perfusion should be checked for proper flow rate in all required channels.

Another possible reason for the insensitivity of ratiometric fluorescence signal to protocol changes can be excessive overexpression of the probe, followed by sensor aggregation or ATP-independent intermolecular FRET. Altering transfection parameters, as explained in the last point, can be a solution for that.

The included "starving" of cells due to a lack of fetal calf serum in the medium when using certain transfection reagents (like TransFast) can alter the result depending on cell type – no such effect has been observed during our measurements with the given cell lines.

Problem

The fluorescence intensities in both channels decrease quickly over time in a non-ratiometric manner.

Potential Solution

This would likely indicate photobleaching of your sample. To avoid this unwanted effect, it is necessary to adjust light source intensity, exposure time and frequency of time points measured. For that, usually, excitation light intensity and the number of images taken per minute are decreased while moderately increasing exposure time.

If adjusting these parameters does not yield a good signal-to-background ratio and desired temporal resolution, it is possible that the transfection efficiency was not good enough.

Problem

There is no fluorescence signal in any of the cells.

Potential Solution

Check if all imaging settings and parameters are correct, e.g., make sure that the correct excitation wavelength is used and that correct optical filters and mirrors are installed properly. Check if cosmetic covers in the optical pathways are removed. If the optical setting of your imaging system is, however, correct, but a major increase in excitation light intensity and/or exposure time does not succeed in producing fluorescence, cells might not express enough of the fluorescent ATP probe. In this case, the most likely solution is to adjust transfection parameters.

Problem

The cells do not adhere to the imaging glass slides and/or are detached by starting the perfusion via the perfusion system.

Potential Solution

A possible solution for that would be to coat the glass slides before seeding the cells. However, this has not been tested for the described experimental setup. Reducing the speed (flow rates) of cell perfusion is recommended if cells detach upon starting the perfusion system. It has to be kept in mind that too slow perfusion rates lead to suboptimal buffer exchange.

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AUTHOR CONTRIBUTIONS

T.R., M.R.D., H.B., W.F.G., and R.M. wrote the manuscript.

DECLARATION OF INTERESTS

W.F.G. and R.M. are founders of a company, referred to as NGFI GmbH, and are members of its scientific advisory board.

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