Measurement of ATP concentrations in mitochondria of living cells using luminescence and fluorescence approaches

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Chapter outline

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Abstract

Adenosine 5′-triphosphate (ATP) is the central metabolite in the energy metabolism of cells and is hydrolyzed to ADP and inorganic phosphate to provide free energy in various cellular processes. ATP also functions as an intracellular signaling molecule. Thus, it is important to know the ATP concentration within cells to understand cellular activities. Here, we describe two methods to detect ATP concentrations in the cytoplasm and mitochondrial matrix using genetically encoded luminescent or fluorescent biosensors. These methods enable quantitative investigation of ATP concentration dynamics in living cells, single cells and cell populations.

1 The luciferase assay to measure ATP ATP levels

1.1 Rationale

ATP is the main source of energy for essential intracellular processes, as well as the intracellular modulator of ATP-sensitive potassium channels (Ashcroft, 2005), AMP-dependent protein kinase (AMPK) (Hardie, Ross, & Hawley, 2012) and various other metabolic enzymes. In addition, it is an important extracellular signaling mediator (Di Virgilio, Pinton, & Falzoni, 2016) of processes including muscle metabolism, cardiac contraction, and neurotransmission.

Glycolysis and oxidative phosphorylation (OXPHOS) are the major ATP-producing systems in animal cells (Bonora et al., 2012). Therefore, ATP is mainly compartmentalized in the cytosol and in mitochondria (Abrahams, Leslie, Lutter, & Walker, 1994; Miller & Horowitz, 1986). More recently, ATP has been detected in the nucleus where it is essential for hormone-induced chromatin remodeling,

transcriptional regulation, and cell proliferation (Wright et al., 2016). Because the rate of ATP synthesis should be balanced with that of ATP consumption to maintain ATP homeostasis in cells, malfunction and/or dysregulation of either glycolysis or OXPHOS, or both, will result in catastrophic changes in intracellular ATP levels (Koopman, Distelmaier, Smeitink, & Willems, 2013). Thus, intracellular ATP levels are closely related to the functions, viability, and fate of cells.

In biological research, intracellular ATP levels are generally quantified by a firefly luciferase assay (Morciano et al., 2017). The luciferase enzyme was isolated from the common North American firefly *Photinus pyralis* and then characterized for its intracellular kinetics and stability. In this assay, the amount of ATP from cells is measured as the number of photons that are emitted when firefly luciferase degrades ATP and luciferin. Importantly, the relative light units measured in living cells are proportional to those found in cell lysates (Ignowski & Schaffer, 2004).

Luciferase uses luciferin, ATP and O_2 as substrates to catalyze light production in a reaction described below (DeLuca & McElroy, 1974; Di Virgilio et al., 2016):

1. Formation of the intermediate Luc-D-luciferyl adenylate (LH_2-AMP), with release of inorganic phosphate (PPi):

$$\mathrm{Luc} + \mathrm{LH_2} + \mathrm{ATP} \rightarrow \mathrm{Luc \cdot LH_2 - AMP} + \mathrm{PPi}$$

2. The intermediate complex Luc-D-luciferyl adenylate is oxidized by molecular oxygen with the formation of an excited enzyme-oxyluciferin-AMP complex and the release of carbon dioxide (CO_2):

$$\mathrm{Luc \cdot LH_2 - AMP} + \mathrm{O_2} \rightarrow \mathrm{Luc \cdot AMP \cdot Oxyluciferin} + \mathrm{CO_2}$$

3. In the final step, energy loss from the excited complex produces photon emission and dissociation of the complex

$$\mathrm{Luc \cdot AMP \cdot Oxyluciferin} \rightarrow \mathrm{Luc} + \mathrm{Oxyluciferin} + \mathrm{AMP} + \mathrm{h}\nu$$

This reaction occurs only in the presence of Mg^{2+} and the product is a flash of yellow–green light with a peak emission at 560 nm that is dependent upon ATP levels and measured using a luminometer. The dynamic range of this assay is sufficient to monitor basal and agonist-induced elevated levels of ATP.

Given its versatility and sensitivity, firefly luciferase can be modified by the addition of specific signal peptides to allow it to localize to, and measure ATP concentrations in, specific intracellular compartments. Two regions in luciferase that mediate transport of the protein into peroxisomes were identified in 1987. One of those

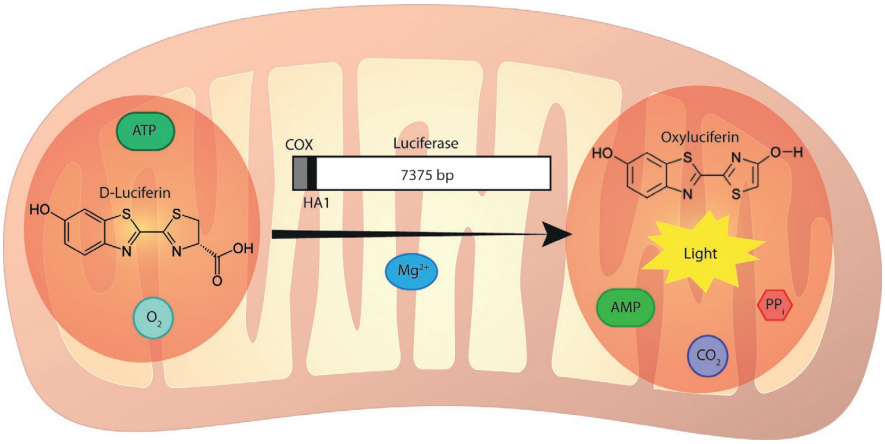

FIG. 1

Luciferin-luciferase reaction: the energy produced by luciferase as a result of the oxidation of its small-molecule substrate luciferin, can release a photon of light which can be captured by sensitive detector and it is proportional to the amount of intracellular ATP.

regions (region II) is located at the C-terminus of the protein (Gould, Keller, & Subramani, 1987) and contains the three amino acids: serine–lysine–leucine (Gould, Keller, Hosken, Wilkinson, & Subramani, 1989). Substitution of the terminal leucines with valine (luciferase LL/V) results in localization of the protein to the cytosol and allows for measurement of free ATP at that site (Manfredi, Yang, Gajewski, & Mattiazzi, 2002). The mutant luciferase LL/V has been fused to the simian virus (SV40) large T antigen nuclear localization signal (NLS), creating a nuclear luciferase (nls-luciferaseLL/V) (Michels, Nguyen, Konings, Kampinga, & Bensaude, 1995). Another chimeric protein engineered, pmeLUC, contains the N-terminal leader sequence and C-terminal glycosyl phosphatidylinositol (GPI) anchor of the folate receptor. pmeLUC is anchored in the plasma membrane with the luciferase moiety on the cell surface and is used to measure ATP concentrations in pericellular spaces (Pellegatti, Falzoni, Pinton, Rizzuto, & Di Virgilio, 2005). In 1999, Jouaville and colleagues constructed a chimeric cDNA, mitochondrial luciferase (mtLuc), which consists of the mitochondrial matrix targeting sequence of COX8 fused to LL/V luciferase (Fig. 1). Using this tool, these investigators found that mitochondrial calcium (Ca^{2+}) accumulation leads to increased mitochondrial ATP synthesis and enhanced ATP levels in the cytosol (Jouaville, Pinton, Bastianutto, Rutter, & Rizzuto, 1999).

1.2 Materials, equipment and solutions

1.2.1 Materials

- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- Potassium chloride (KCl; Fluka, cat. no. 60128)
- Potassium phosphate, monobasic (KH_2PO_4; Sigma-Aldrich, cat. no. P0662)

- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$; Sigma-Aldrich, cat. no. M5921)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Glucose (Sigma-Aldrich, cat. no. G7528)
- Calcium chloride ($CaCl_2$; Fluka, cat. no. 10043-52-4)
- Sodium hydroxide (NaOH; Fluka, cat. no. 71691)
- Sodium phosphate, dibasic (Na_2HPO_4; Sigma-Aldrich, cat. no. 255793)
- Milli-Q Direct System water (Millipore, http://www.merckmillipore.com/IT/it/product/Milli-Q-Direct-Water-Purification-System,MM_NF-C85358#ordering-information)
- Ethanol (Sigma-Aldrich, cat. no. 02860)
- Beetle luciferin, potassium salt (Promega, cat. no. E1605)
- Mitochondrial firefly luciferase DNA (mtLuc, available from our lab)
- Ca^{2+}-perturbing agents
- N,N′-dicyclohexylcarbodiimide (DCC; Sigma-Aldrich, cat. no. 36650)
- Transfection reagents (i.e., lipofectamine 2000, JetPEI, HiPerFect)
- L-glutamine 100× (Euroclone, cat. no. COD. ECB3000D)
- Fetal Bovine Serum (FBS, Thermo Fisher Scientific, cat. no. 11573397)
- Penicillin-Streptomycin 100× (Euroclone, cat. no. COD. ECB3001D)
- Adherent cultured cell lines

1.2.2 Equipment

- Parafilm (Sigma-Aldrich, cat. no. P7793)
- 1.5-mL Microcentrifuge tubes
- 50-mL Tubes
- Aluminum foil
- Glass coverslip, 13-mm diameter, thickness between 0.16 and 0.19 mm (Thermo Fisher Scientific)
- 24-multiwell plate (Corning, cat. no. CLS3527-100EA)
- Peristaltic pump (Elettrofor Scientific Instruments)
- Temperature-controlled bath (Elettrofor Scientific Instruments)
- Personal computer
- Custom-made luminescence reader (an aequorinometer) equipped with a single-photomultiplier tube (PMT), photon-counting head (Hamamatsu, model no. C8855-01), digitizer counting unit (Hamamatsu, model no. C9744) and high-voltage power supply (Hamamatsu, model no. C9525). Alternatively, luminometers are commercially available including the GloMax Discover System (Promega), Lucetta Luminometer (Lonza), LUMIstar Omega Plate Reader (BMG Labtech) or Luminoskan Ascent Microplate (Thermo Fisher Scientific)

1.2.3 Solutions

Culture medium: to be chosen on the basis of the cell type and supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, and 1% penicillin–streptomycin solution.

TRIS–EDTA: TRIS–EDTA (TE) buffer is 10 mM Tris–HCl and 1 mM EDTA at pH 7.4 in filter-sterilized dH_2O. Dissolve 121.14 mg of Tris–HCl and 37.24 mg of EDTA in 100 mL of Milli-Q water. Adjust the pH to 7.4 with HCl, and then bring the final volume to 100 mL. Sterilize by filtration and store the solution at room temperature (RT).

PBS 1 ×: PBS is 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in <1000 mL of Milli-Q water. Adjust the pH to 7.4 with NaOH; then, bring the final volume to 1000 mL. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 p.s.i. (1.05 kg/cm^2).

15 mg/mL D-Luciferin stock solution: Reconstitute 1 g of D-luciferin with 66 mL of sterile PBS 1 ×. Allow the luciferin solution to sit for a minimum of 15 min with gentle agitation before making 1 mL aliquots. Aliquots should be stored at −80 °C.

Krebs–Ringer buffer: Krebs–Ringer buffer (KRB) is 135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4{\cdot}7H_2O$, 20 mM HEPES and 5.5 mM glucose, pH 7.4. Dissolve 7.89 g of NaCl, 0.373 g of KCl, 0.054 g of KH_2PO_4, 0.247 g of $MgSO_4{\cdot}7H_2O$, 4.76 g of HEPES and 1 g of glucose in <1000 mL of Milli-Q water. Adjust the pH to 7.4 with NaOH; then, bring the final volume to 1000 mL.

Modified KRB: Add 0.5 mL of 1 M $CaCl_2$ to 500 mL of KRB.

10 mM Luciferin stock solution: Luciferin is delivered in powder form and it is usually dissolved at 10 mM in PBS, pH 7.4, then stored at −80 °C until use. Dissolve 0.064 g of luciferin in 20 mL of PBS and divide the stock solution into 1-mL aliquots.

25 μM Luciferin solution: Add 125 μL of the luciferin stock solution to 50 mL of modified KRB. The solution volume to be prepared depends on the cell type, the number of samples to be analyzed and the instrument settings.

CAUTION: Avoid multiple freeze–thaw cycles for luciferin and protect the luciferin solution from light.

Ca^{2+}-mobilizing solution (CMS): Dissolve the Ca^{2+}-perturbing agent in a 25-μM luciferin solution. This stimulus should be optimized for each the cell type, see below.

75 mM DCC stock solution: Dissolve 0.077 g of DCC in 5 mL of pure ethanol. Store the solution at −20 °C until use.

75 μM DCC solution: Add 50 μL of 75 mM DCC stock solution to 50 mL of 25 μM luciferin solution.

CAUTION: Avoid multiple freeze–thaw cycles for DCC solutions.

70% Ethanol solution: Prepare 500 mL of 70% ethanol by combining 150 mL of Milli-Q water with 350 mL of pure ethanol.

1.3 Protocols

1.3.1 Cell preparation and transfection

The firefly luciferase assay can be used with multiple mammalian cell lines. The culture and transfection protocol and CMS buffer must be optimized for different cell types. Here, we describe methods and times of a standardized procedure.

Cell growth

1. If needed, coat circular glass coverslips (13-mm diameter) with an optimal substrate in a 24-multiwell plate according to manufacturer's instructions;
2. Wash the coverslips twice with PBS and let them dry for several minutes;
3. Seed cells onto the circular glass coverslips for each luminescence measurement;
4. Allow the cells to grow until they reach 80% confluence.

Transfection

5. Transfect cells with 2 μg of plasmid encoding mitochondrial firefly luciferase per coverslip using the appropriate transfection reagent and protocol;
6. Incubate the cells for 24–36 h.

1.3.2 Setting up the luminometer

Most luminometers are equipped with a temperature controlled perfusion chamber that houses the sample, a peristatic pump to perfuse the sample, and a dark hollow cylinder for detection of light emitted from the sample using a high-performance photomultiplier (PMT) (Fig. 2).

7. Before and after each experiment, wash the peristaltic pump tubing with Milli-Q water and 70% ethanol;
8. Carefully check the tubing and chamber to insure that that there are no air bubbles or leaks;
9. Ensure that the perfusion chamber is at 37 °C.

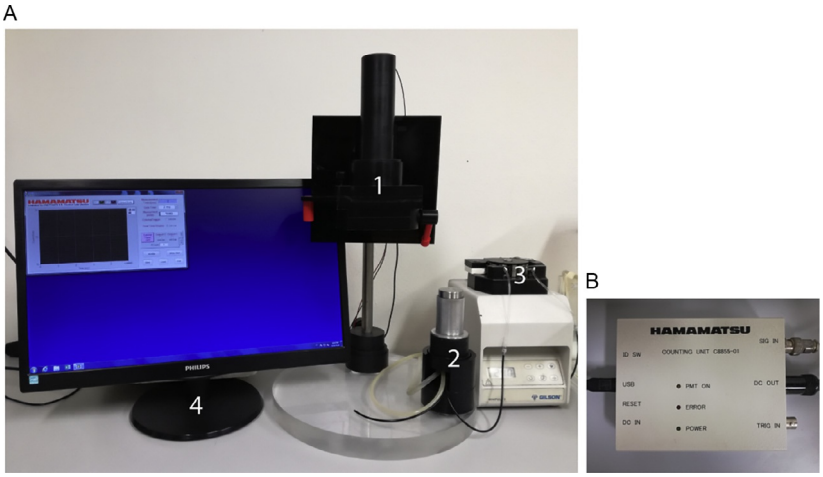

FIG. 2

The luminometer (A). The system consists of a perfusion chamber mounted on top of a hollow metal cylinder (2), which is temperature-controlled and continuously perfused with solutions using a peristaltic pump (3). Samples within the perfusion chamber are placed 2 mm from the surface of a high-performance photomultiplier tube (1). The photon counts are transferred from the photon-counting head and digitizer unit (B) to a computer (4).

1.3.3 Single-sample measurement of mitochondrial ATP basal content

10. Remove a 13-mm coverslip containing transfected cells from the incubator, rinse it twice with 1 mL of modified KRB to remove residual medium, transfer it to the perfusion chamber and seal the chamber;
11. Start the peristaltic pump to perfuse of cells with modified KRB at a flow rate of 2.5 mL/min;
12. Maintain constant perfusion of the cells with modified KRB (2.5 mL/min), place the chamber in the dark hollow cylinder so that cells are in close proximity to the PMT, and start recording;
13. Measure light output upon perfusion with modified KRB perfusion for at least 30 s to detect background signal;
14. To monitor the mitochondrial basal ATP content, temporarily stop the perfusion, change the perfusing solution from modified KRB to the luciferin solution and resume perfusion. Light emission rapidly increases and reaches a plateau within ~2–3 min after introduction of luciferin, depending on cell type.

1.3.4 Single-sample measurement of mitochondrial ATP production

At this point, the researcher can choose the method of interest. If mitochondrial ATP production is being measured, follow letter "a" of the bulleted list. If the contribution of oxidative phosphorylation (OXPHOS) to ATP production is being measured, follow the letter "b."

15a. Wait (~15–30 s) until the luminescence output stabilizes and remains unchanged. Temporarily stop the perfusion, initiate perfusion with CMS buffer, and restart the perfusion. Under these conditions, the light emission rapidly increases and reaches a second plateau within ~1–1.5 min, depending on the cell type. Wait (~20–30 s) until the luminescence stabilizes and remains unchanged;
16a. Stop perfusion. Remove the perfusion chamber from the photon-counting head and remove the sample from the chamber;
17a. Reseal the chamber and carefully wash the entire system with Milli-Q water followed by 70% ethanol and modified KRB solution.

1.3.5 Evaluation of OXPHOS contribution to intracellular ATP production

15b. Wait until the luminescence values remain unchanged for ~15–30 s. Temporarily stop the perfusion, change from the luciferin solution to the DCC buffer, and restart the perfusion. Under these conditions, the light emission rapidly decreases until a second plateau is reached within ~1–1.5 min, depending on the cell type. Wait until the luminescence values remain unchanged for ~20–30 s;
16b. Stop perfusion, remove the perfusion chamber from the photon-counting head and remove the sample from the chamber;
17b. Reseal the chamber and carefully wash the entire system with Milli-Q water, 70% ethanol and modified KRB solution.

1.3.6 Data saving

18. Save each experimental data set and export results to a spreadsheet-compatible format for data analysis;
19. Open exported files using a spreadsheet software (e.g., Microsoft Office Excel) and organize each luminescence value series in a single column.

1.4 Analysis and statistics

For all measurements, the recorded luminescence should be proportional to the concentration of the luciferin from 20 to 200 μM and luciferase should be expressed at similar levels in cells under all experimental conditions. If these criteria are met, the signal detected reflects the actual ATP concentration in mitochondria and represents a reliable and sensitive technique to use in living cells.

1.4.1 Measurements and representations

Once the photon counts are transferred to a computer from both the photon-counting head and the digitizer unit (see Equipment), the output signals are converted to kinetic data using the Hamamatsu Photonics software and are then ready for analysis. Fig. 3A shows an example of data obtained for single-sample measurement of mitochondrial ATP production (steps 1–17a). The kinetic data can be divided into three parts. The first part is the background measured upon perfusion of modified KRB for up to 30 s and represents the signal noise of the instrument recording in the absence of luciferin. The second part is the data obtained upon perfusion with

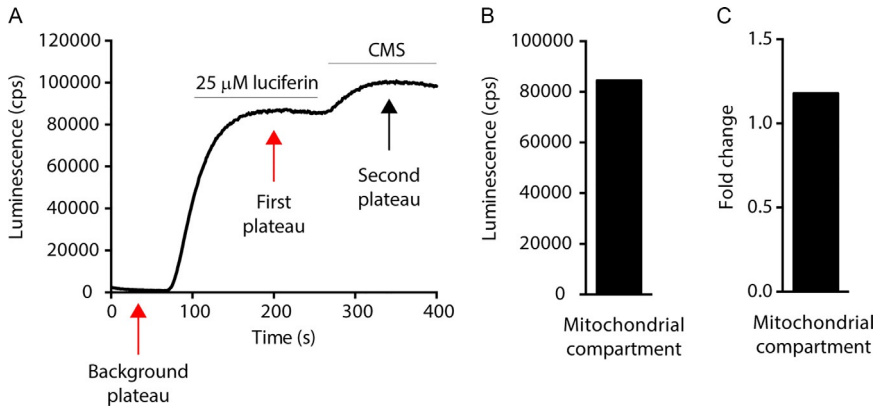

FIG. 3

Calculation of basal ATP content in and stimulated ATP production by mitochondria. (A) Representative data of background levels (background plateau), as well as basal (first plateau) and calcium-induced (second plateau) levels of ATP in mitochondria detected in living cells using 25 μM luciferin. Representative of basal ATP levels (B) and CMS-stimulated increases in ATP production (C) in mitochondria.

the luciferin, and reaction of that substrate with mitochondria-targeted luciferase in the presence of ATP, Mg^{2+} and O_2. This reaction generates a sigmoidal-shaped curve which reaches a plateau that is proportional to ATP concentration in the organelle. The third is ATP production above basal levels, which in this case is stimulated by perfusion of cells with Ca^{2+} containing solutions (CMS).

To calculate the ATP basal content of the mitochondrial compartment, the average signal upon perfusion with the modified KRB solution (Fig. 3A, background plateau) should be subtracted from the average signal obtained upon perfusion with luciferin (Fig. 3A, first plateau). To calculate the ATP produced by mitochondria in response to a stimulant (e.g., Ca^{2+}), the average of luminescence produced by the stimulant (Fig. 3A, second plateau) is divided by the average of luminescence values under basal conditions (Fig. 3A, first plateau). Basal ATP levels are expressed as counts of luminescence per second (cps) (Fig. 3B) and the level of ATP produced in response to a stimulant is expressed as fold change above basal levels (Fig. 3C).

Treatment with ATP synthase inhibitors such as DCC or oligomycin is used to determine the contribution of OXPHOS to total ATP production. In that case, a decrease in luminescence values reflects ATP produced by the respiratory chain (Fig. 4).

1.5 Statistical methods

The most common method to evaluate if the basal or stimulate-induced ATP content varies among experimental conditions is to apply either a Students *t*-test or a one-way Anova statistical test for two or more experimental groups, respectively.

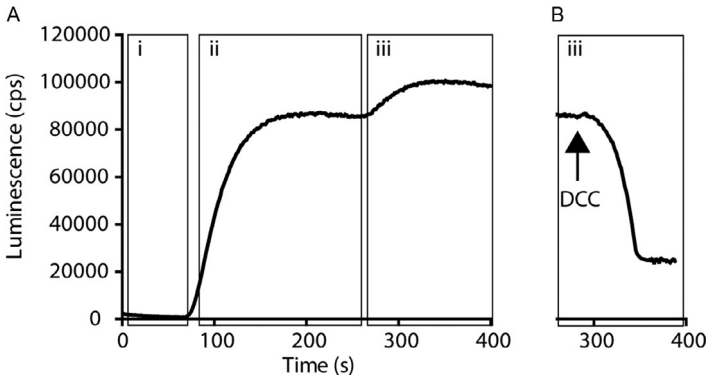

FIG. 4

(A) Three parts (i, ii, iii) of a standard experiment as explained in Fig. 3. (B) Representative data obtained for assessment of the OXPHOS contribution in ATP synthesis. The cell population in luciferin solution was perfused with high concentrations of DCC. Inhibition of ATP synthase promotes a decrease in ATP detection, and hence cps, revealing the OXPHOS contribution in that cell line.

1.6 Pros and cons

Pros	Cons
Mammalian cells lack chemiluminescent proteins and thus do not produce background signal	Cells must express plasmids containing luciferase. It is important to verify that the enzyme is expressed in the absence of an autocalibration method
Light is emitted by oxyluciferin very efficiently and does not result in photobleaching, phototoxicity or background chemiluminescence	The reaction depends not only on ATP but also on the amount of O_2 and the exogenous delivery of luciferin
	ATP amounts are generally expressed as bioluminescence signals and not as concentrations
	The activity of other ATP-consuming enzymes may affect the ATP measurement performed by this assay (Morciano et al., 2017)

1.7 Troubleshooting and optimization

Step	Problem	Reason	Optimization
13	High noise during background recording	Photon-counting head wrongly exposed to light	Wait for signal to decrease and restart recording
		Buffer perfusion by peristaltic pump and tubes is not optimal	Stop recording and perfusion. Check chamber perfusion for air bubbles
14	Luminescence signal does not increase upon perfusion with luciferin	Luciferase transfection is not efficient	Improve the transfection method
		Low number of cells	Seed more cells
		Luciferin perfusion is not optimal	Increase luciferin concentration or check its perfusion
15a	Luminescence signal does not increase with treatment with stimulant	Perfusion of luciferin and CMS is not optimal or CMS is prepared improperly	The solution of Luciferin and CMS for all trials/replicates within an experiment should be prepared in one batch
		Cells under investigation are producing ATP by glycolysis and not OXPHOS	Measure only the ATP basal content or change cell line

2 Visualization of ATP with subcellular resolution using the fluorescent biosensor, ATeam

Another method to measure intracellular ATP levels is to image the ATP concentration in cells using a fluorescence microscope and genetically encoded fluorescent biosensors for ATP. The ATP-imaging method has large advantages over other

major methods. First, this method does not require disruption of cells, but measures ATP concentrations inside living cells. Second, this method has high spatial resolution and can be used to quantify the ATP concentration of a single cell or individual organelles within a cell. Third, the method has high temporal resolution and can be used to monitor changes in intracellular ATP concentrations of living cells in real time.

"ATeam" is the first and most frequently used genetically-encoded fluorescent biosensor for ATP. Measurements with this biosensor utilize the principle of Förster resonance energy transfer (FRET) (Imamura et al., 2009). FRET is a physical phenomenon in which the excitation energy of a donor fluorescent chromophore is transferred to an acceptor chromophore. The efficiency of FRET is highly dependent upon the distance and relative angle between the donor and acceptor chromophores (Miyawaki, 2003). ATeam is a tandem fusion of a cyan fluorescent protein (CFP; mseCFP), an ATP binding protein (F_oF_1-ATP synthase ε subunit [F_oF_1-ε]), and a yellow fluorescent protein (YFP; cp173-mVenus) (Fig. 5). CFP and YFP serve as the donor and acceptor, respectively. Large conformational changes in F_oF_1-ε, produced by ATP binding, lead to rearrangement of the positions and orientations of the two fluorescent proteins, resulting in increased efficiency of FRET from a donor to an acceptor.

ATeam should be expressed in mammalian cells either transiently or permanently. ATeam without any targeting signal is localized mainly in the cytoplasm (Fig. 5B). As with Luciferase, ATeam can be localized to any cellular location by fusion to an appropriate targeting sequence or to a protein known to have a specific cellular localization pattern, in order to measure the ATP concentrations at specific cellular locations. As an example, ATeam with a tandem repeat of a mitochondrial targeting signal from subunit VIII of cytochrome *c* oxidase localizes to and reports on ATP concentrations in the mitochondrial matrix (Fig. 5B).

2.1 Materials, equipment and solutions

2.1.1 Materials

- PEI Max (Polysciences, cat. no. 24765-1)
- OPTI-MEM (Thermo Fisher Scientific, cat. no. 31985062)
- Glass-bottom dish (MatTek, cat. no. P35G-1.5-10-C)
- G418 stock solution (50 mg/mL; Nacalai Tesque, cat. no. 09380-86)
- Cellmatrix type I-C (3 mg/mL; Nitta Gelatin, cat. no. 637-00773)
- PBS powder (Nissui, cat. no. 08190)
- DMEM low glucose (Nacalai Tesque, cat. no. 08490-05)
- L-glutamine (Nacalai Tesque, cat. no. 16948-04)
- 2-Deoxy-D-glucose (Nacalai Tesque, cat. no. 10722-11)
- Oligomycin A (Sigma-Aldrich, cat. no. 75351)
- pcDNA3.1(−)-AT1.03: A plasmid harboring cDNA for cytoplasmic ATeam (AT1.03). The vector backbone is pcDNA3.1(−) (Thermo Fisher Scientific).

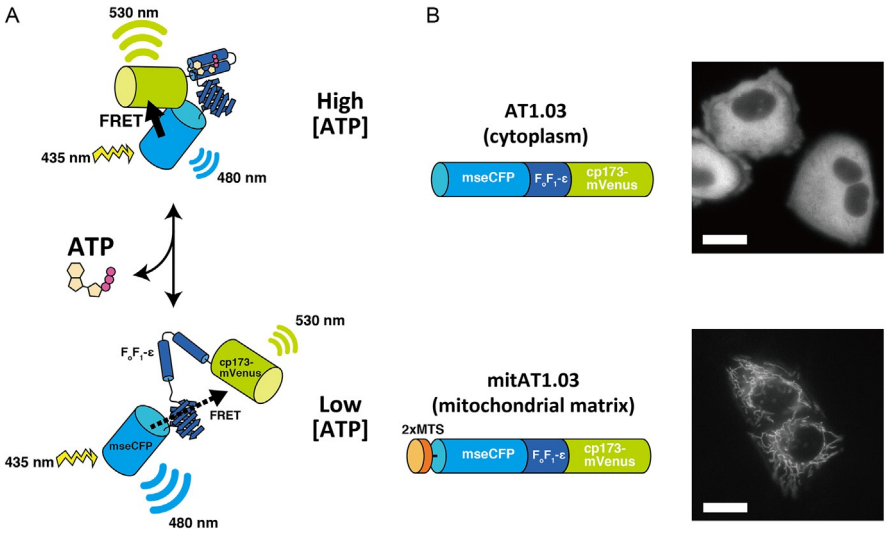

FIG. 5

Genetically encoded ATP biosensor ATeam. (A) Schematic drawing of how the ATeam biosensor works. At low ATP concentrations, most ATeam molecules are in an ATP-free state, in which FRET efficiency from a donor to an acceptor is low. In contrast, most ATeam molecules are in an ATP-bound state with high FRET efficiency. (B) Architecture and localization of ATeam in mammalian cells. ATeam (AT1.03) is a tandem fusion of mseCFP, F_oF_1-ε, and cp173-mVenus. AT1.03 expressed in mammalian cells localizes to the cytoplasm. A duplex of mitochondrial targeting signal (2xMTS) is fused at the N-terminus in mitAT1.03, which is localized to the mitochondrial matrix when expressed in mammalian cells. Bars = 20 μm.

It should be prepared with a transfection-grade plasmid purification system such as PureLink (Thermo Fisher Scientific) (Imamura et al., 2009).

– pcDNA3.1(−)-mitAT1.03: A plasmid harboring cDNA for mitochondrial ATeam (mitAT1.03) (Imamura et al., 2009). It should be prepared with a transfection-grade plasmid purification system such as PureLink (Thermo Fisher Scientific).

2.1.2 Equipment

– Standard laboratory tools (as previously mentioned for the first method)
– Plan Apo 40 ×, 0.95 numerical aperture, dry objective lens (Nikon), or a Plan Apo 60 ×, 1.40 numerical aperture, oil objective lens (Nikon) is usually used
– A fluorescence microscope for live cell ATP imaging. The system that we use is a Nikon Ti-E inverted microscope equipped with a 75-W xenon lamp housing (Hamamatsu photonics), a motorized excitation filter wheel (Nikon), an electric shutter (Sutter Instrument), a stage-top CO_2 incubator (Tokai Hit), a motorized stage (Nikon), a motorized emission filter wheel (Nikon), and a sCMOS camera

(Zyla4.2, Andor Technologies). An excitation filter (FF01-438/24, Semrock), a dichroic mirror (FF458-Di02, Semrock), and emission filters (FF02-483/32 for CFP and FF01-542/27 for YFP, Semrock) are installed in the excitation filter wheel, the filter cube turret, and the emission filter wheel, respectively.

2.1.3 Solutions

PEI Max solution (0.1% (*w/v*)): Dissolve 50 mg PEI Max in 40–45 mL of ultrapure water, then adjust pH between 6.9 and 7.1 with NaOH. Adjust the volume to 50 mL with ultrapure water. Filter sterilize the solution with a 0.2 μm filter unit. Store aliquots at −20 °C. Warm a frozen stock at 37 °C for 30 min to dissolve. Once dissolved, a stock can be stored at 4 °C for at least 6 months.

D-PBS(−): Dissolve Dulbecco's PBS(−) powder in ultrapure water to a final volume of 1 L and autoclave.

Phenol red-free DMEM: Mix 25 mL of phenol red-free DMEM low glucose with 0.5 mL of 200 mM L-glutamine. The mixture can be stored for 3 months at 4 °C.

2-Deoxyglucose stock solution (1 M): Dissolve 2-deoxy-D-glucose in ultrapure water to a final concentration of 1 M. The stock solution can be stored at −20 °C.

Oligomycin A stock solution (10 mg/mL): Dissolve oligomycin A in DMSO to a final concentration of 10 mg/mL. The stock solution can be stored at −20 °C.

2.2 Protocols

2.2.1 Transient expression of ATeam in cultured HeLa cells

1. Trypsinize HeLa cells and seed $\sim 2 \times 10^5$ cells into a 35-mm plastic dish with 2 mL DMEM containing 10% FBS;
2. Culture cells in a CO_2 incubator for 1 day.
3. Mix 2 μg of pcDNA3.1(−)-AT1.03 or pcDNA3.1(−)-mitAT1.03 plasmid with 200 μL OPTI-MEM in a 1.5 mL tube;
4. Add 8.5 μL of PEI-Max solution to a mixture of plasmid and OPTI-MEM and vortex for 10–15 s;
5. Incubate the above mixture for 30 min at room temperature;
6. Add 1 mL of pre-warmed DMEM containing 10% FBS to OPTI-MEM/plasmid/PEI-Max mixture (step 5), and mix using a pipette;
7. Replace the medium of a culture dish (step 2) with the medium containing plasmid (step 6);
8. Culture cells in a CO_2-incubator for 1 day;
9. Just before step 10, spread ~50 μL of collagen (Cellmatrix type I-C) on a glass surface of a 35-mm glass-bottom dish, followed by rinsing the surface twice with 500 μL D-PBS(−);
10. Trypsinize and seed the transfected cells into the collagen-coated glass-bottom dish at an appropriate density.

Imaging may be performed 24–72 h after transfection.

2.2.2 Establishment of HeLa cells stably expressing ATeam

11. After step 8, trypsinize the cells and passage them to a 100-mm dish, and culture in 10 mL medium containing 0.7 mg/mL G418;
12. Culture the cells for 1 week, replacing the G418-containing medium every 2 days;
13. Trypsinize the cells and suspend cells in 2 mL of phenol red-free DMEM;
14. Apply the cell suspension to a fluorescence-activated cell sorter, and sort each single YFP-positive cell to a well of a 96-well plate containing culture medium with penicillin-streptomycin;
15. After about 1 week of culture, investigate the wells of the 96-well plates to check whether the cells inside a well are actually derived from a single or multiple cells. Mark the wells containing only a single colony. If possible, confirm ATeam expression by visualization using a fluorescence microscope;
16. Culture the cells from a single colony until cells cover about 20% of the well surface;
17. Release cells by trypsinization, transfer the cells to 48-well plates, and culture them until confluent;
18. Release cells by trypsinization, transfer the cells to 12-well plates, and culture them until confluent;
19. Transfer half of the cells to 6-well plates, and the remaining to a collagen-coated glass-bottom 35-mm dish;
20. One day after step 19, visualize cells cultured in the glass-bottom dish under a fluorescence microscope. If the expressed ATeam is functional, FRET signals (YFP-FRET/CFP ratio) will decrease after treatment with the glycolysis inhibitor 2-deoxyglucose (10 mM) and an OXPHOS inhibitor oligomycin A (1 μg/mL) (see below for details);
21. Expand the culture of ATeam-positive clones from the 6-well plates in step 19, and prepare frozen stocks.

2.2.3 Live cell ATP imaging and image processing

The most commonly used technique to image FRET signals is intensity-based ratiometric imaging, which is described below. In this method, donor images (donor fluorescence images under excitation of the donor) and acceptor images (acceptor fluorescence images under excitation of the donor) are obtained, and used to calculate the ratio of fluorescence intensities (YFP-FRET/CFP ratio) in the cells. Because an acceptor channel generally contains a bleed-through fraction from a donor fluorophore due to spectral overlap, estimation of the actual contribution of FRET is required if the amounts of donor and acceptor fluorophores are different. However, in the case of imaging with a single polypeptide FRET biosensor like ATeam, this estimation is not necessary because single polypeptide FRET biosensors contain equimolar donor and acceptor fluorophores. The ratio of fluorescence intensities of the acceptor and donor are basically not affected by the biosensor expression level or by morphological changes in cells. Therefore, simple calculation of the

fluorescence intensity ratio of a donor image and an acceptor image is usually sufficient to evaluate FRET signals from cultured mammalian cells expressing ATeam biosensor.

For live imaging of mammalian cells expressing ATeam, images in CFP and YFP-FRET channels can be captured using an inverted wide-field fluorescence microscope. A confocal microscope may also be used to obtain images with higher spatial resolution. Because the affinity of ATeam for ATP is quite sensitive to temperature, changes in temperature will result in altered FRET signals independently of ATP concentration. Therefore, temperature control of cell culture dishes on the microscope is critical for precise assessment of intracellular ATP levels. An objective lens warmer is necessary, in addition to the stage mounted environmental control chamber, when an oil-immersion objective lens is used for imaging. Without the lens warmer, heat will dissipate from a cell culture dish through the lens. To avoid unnecessary exposure of cells to excitation light, the microscope should be equipped with a motorized shutter for excitation light. It is also good to use neutral density filter(s) to reduce the intensity of excitation light. Because chromatic aberration of an objective lens will cause mismatched focal planes of CFP and YFP-FRET images, the use of an apochromatic objective lens is highly recommended.

22. Stabilize the microscope, the xenon lamp and the stage-top incubator at 5% CO_2 at 37 °C for >30 min before starting image acquisition. (*Optional*) If an oil-immersion objective lens is used, warm the lens to 37 °C by wrapping it with the lens warmer;
23. Replace culture medium with 2 mL of phenol red-free DMEM pre-warmed at 37 °C;
24. Place and fix the dish onto the microscope stage;
25. (*Optional*) Turn on the autofocus system of the microscope;
26. Find cell(s) expressing ATeam through the eyepieces using a YFP fluorescence filter set;
27. Adjust the *xy* position and focus (*z* position) of cells using a CFP or YFP-FRET image obtained with the sCMOS camera using an imaging software NIS-Elements (Nikon). Input the *xyz* positions to NIS-Elements;
28. Repeat steps 26 and 27 to select multiple view fields;
29. Input the exposure times for both CFP and YFP-FRET channels, and the frequency of image acquisition to NIS-Elements;
30. Wait for >5 min to allow the recovery of YFP fluorescence, which may be partially decreased by photochromism during steps 26 and 27 (Miyawaki, Nagai, & Mizuno, 2013).
31. Start image acquisition;
32. Export individual CFP and YFP-FRET images as grayscale in tiff format using the export option of NIS-Elements software;
33. Open files for CFP and YFP-FRET images using the image processing software MetaMorph (Molecular Devices);

34. Mark a region of interest (ROI) within an area of CFP images where no cells exist. Copy the ROI in CFP images to the corresponding YFP-FRET images using the "Transfer regions" function;
35. Subtract intensity values within a ROI from an entire image using the "Background and shading correction" function with "Statistical correction" operation of MetaMorph. Repeat this procedure for all time-series images. It is highly recommended to make a "journal" (a kind of macro) that (semi-) automatically processes these steps, especially for long time-series data;
36. Mark ROIs that surround a whole cell or part of a cell in the background-subtracted CFP images. Transfer the ROIs to background-subtracted YFP-FRET images using the "Transfer regions" function;
37. Measure intensities within ROIs of both the CFP and the YFP-FRET images using the "Region measurements" function. Export the data to Microsoft Excel;
38. Calculate YFP-FRET/CFP ratios within ROIs on Microsoft Excel.

If photographic images with pseudo-colors that reflect YFP-FRET/CFP ratio values are desired, intensity modulated display images can be generated from background-subtracted CFP and YFP-FRET images (step 35) using the "Ratio images" function of MetaMorph.

An example of ATP imaging is shown in Fig. 6. HeLa cells expressing either AT1.03 or mitAT1.03 were treated sequentially with oligomycin A and 2-deoxyglucose. Mitochondrial ATP concentrations were partially decreased by oligomycin A, whereas cytoplasmic ATP concentrations were almost insensitive. Further treatment with 2-deoxyglucose significantly decreased both the mitochondrial and cytoplasmic ATP concentrations to a similar level. Notably, basal ATP concentrations are lower in the mitochondria than in cytoplasm.

2.3 Safety considerations and standards

Since these methods were designed to be applied in mammalian living cells and not human cells or whole in vivo studies. Safety considerations refer only to the use of dangerous reagents and sensitive tools as follows:

- The adherent cell lines used should be checked for mycoplasma contamination. The presence of this microorganism may compromise the final result.
- Avoid multiple freeze–thaw cycles for luciferin and DCC stock solutions and protect the luciferin solution from light.
- DCC is a mutagenic compound and is thus harmful to health; use adequate protection.
- Ethanol is dangerous to human health if inhaled.
- Avoid exposing the photon-counting head to a direct light source and keep the light source turned off or the shutter closed when not in use.

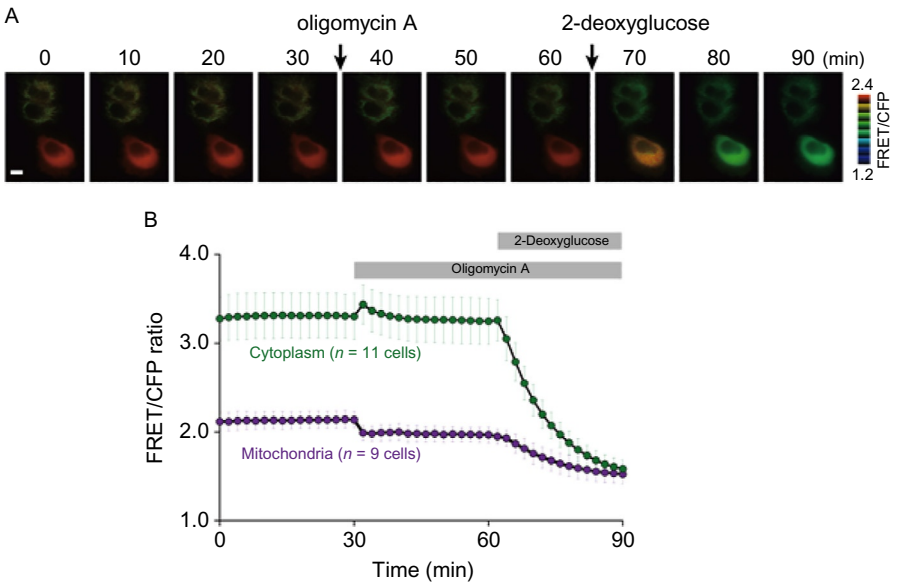

FIG. 6

Imaging ATP concentrations in individual living HeLa cells. (A) Time-lapse pseudocolored YFP-FRET/CFP images of HeLa cells expressing AT1.03 in the cytoplasm (lower) and in the mitochondria (upper). Oligomycin A (1 μg/mL) and 2-deoxyglucose (10 mM) were added at 30 and 60 min, respectively. Bar = 10 μm. (B) Quantified YFP-FRET/CFP ratios. Averaged ratios are shown. Error bars represent standard deviations.

2.4 Related techniques and alternative fluorescence methods

Perceval and its newer version, PercevalHR, is a genetically-encoded protein that permit a quick measure of the ATP/ADP ratio. These probes are based on circularly permutated fluorescent (Berg, Hung, & Yellen, 2009; Tantama, Martinez-Francois, Mongeon, & Yellen, 2013). There are also several descendants of ATeam, including GO-ATeam (Nakano, Imamura, Nagai, & Noji, 2011), which employs green fluorescent protein (GFP; cp173-mEGFP) as a donor and orange fluorescent protein (OFP; mKOκ) as an acceptor, and ATeamNL (Tsuyama et al., 2013), which is optimized for use at low temperature (around 25 °C). When using these probes, some modifications are needed in the experimental protocols, which are described above.

There are several techniques to image FRET signals from cells expressing the ATeam biosensor. Fluorescence lifetime imaging (FLIM) is one of the most quantitative imaging techniques to measure FRET efficiency. FRET efficiency (E) can be expressed as follows:

$$E = 1 - \tau_{DA}/\tau_{D}$$

where τ_D and τ_{DA} are the fluorescence lifetimes of a donor fluorophore in the absence and presence of an acceptor molecule, respectively. FRET efficiency can

be calculated directly from the fluorescence lifetime of a donor fluorophore, which is obtained with FLIM. Although FLIM is a very powerful tool for measuring FRET, it is not used frequently because it requires specialized and costly equipment, and often takes a long time to acquire each image.

For measuring the amount of ATP in cellular extract, one may use other alternative procedures that take advantage of standard enzymatic reactions. In this case, the detection of ATP is composed by a two-step reaction based on the hexokinase/glucose-6-phosphate dehydrogenase-coupled assay (Zhu, Romero, & Petty, 2011). Here, the amount of reduced NADPH is measured.

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

The second standard enzymatic convincing method to detect ATP levels is composed by three-step enzymatic reactions and is based on the glycerokinase/glycerolphosphate oxidase/horseradish peroxidase-coupled assay. The final product of the reaction is TMPD (TetraMethylPhenyleneDiamine) oxidized, which can be measured spectrophotometrically. To determine the ATP concentrations, unknown and control samples from a standard curve are run simultaneously with the samples (Patergnani et al., 2014).

1. ATP (from a measured sample) + glycerol + glycerokinase → glycerol-3-phosphate + ADP
2. Glycerol-3-phosphate + O_2 + glycerolphosphateoxidase → phosphodioxyacetone + H_2O_2
3. H_2O_2 + TMPD (TetraMethylPhenyleneDiamine) + horseradish peroxidase → H_2O + $TMPD^{oxidized}$

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