Chapter 14

Application of FRET-Based Biosensor "ATeam" for Visualization of ATP Levels in the Mitochondrial Matrix of Living Mammalian Cells

Tomoki Yoshida, Soaad Alfaqaan, Norio Sasaoka, and Hiromi Imamura

Abstract

Genetically encoded biosensors utilizing the Förster resonance energy transfer (FRET) are powerful tools for live cell imaging of various cellular processes. Our group has previously developed a series of FRET-based biosensors, named "ATeam," for visualization of ATP levels inside a single living cell. ATeam not only provides a window of insight into a single cell but also allows for visualization of ATP levels in mitochondrial matrix of a single living cell. This novel tool is able to monitor alterations in cellular ATP in response to various treatments in real time. Here we present a method for the evaluation of ATP levels in mitochondria in living cells by using ATeam. At the end of this chapter, an example of experimental results is described for a better understanding of the presented procedure.

Key words Fluorescent biosensor, FRET, Live imaging, ATP, ATeam

1 Introduction

Genetically encoded biosensors utilizing the Förster resonance energy transfer (FRET) principle with fluorescence proteins have been extensively applied for live cell imaging of various cellular processes. FRET is an electrodynamic phenomenon between a donor fluorophore and an acceptor molecule. An overlap between the emission spectrum of a donor and the absorption spectrum of an acceptor, and a donor and an acceptor are in close proximity, then the energy of the excited donor can be transferred to the acceptor. The efficiency of FRET depends on factors including: the extent of the spectral overlap, and the relative distance and orientation between the donor and the acceptor. A thorough description of the principle can be found in "Principles of Fluorescence Spectroscopy" textbook [1]. In general, genetically encoded FRET-based biosensors are composed of donor and acceptor fluorescent proteins, separated by a sensory protein. A conformational change of the sensory protein, induced by, for example, ligand

Dejana Mokranjac and Fabiana Perocchi (eds.), *Mitochondria: Practical Protocols*, Methods in Molecular Biology, vol. 1567,
DOI 10.1007/978-1-4939-6824-4_14,

binding, alters the relative distance and orientation between the two fluorophores. Thus, a change in the cellular environment can be correlated to the changes in FRET efficiency between two fluorescent proteins. The detailed procedure for designing biosensors based on fluorescent fusion proteins can be found in "Live Cell Imaging; Methods and Protocols" [2] previously published by Method in Molecular Biology. One major advantage of FRET-based biosensors is their highly quantitative nature. The output signal is typically a ratio of the donor-to-acceptor emissions, or a fluorescence lifetime of the donor, which are both almost insensitive to the expression level of the biosensor itself.

Our group has previously developed a series of FRET-based biosensors for visualization of ATP levels inside a single living cell, named "ATeam" (ATP indicator based on Epsilon subunit for Analytical Measurement) [3]. The ATP imaging technique using ATeam provides an alternative method by which disadvantages in conventional ATP quantification methods can be overcome. For example, measurements of ATP by using luciferase [4] or chromatography [5] can only provide average ATP concentrations of the cell extract. In addition, they carry the risk of loss and decomposition of ATP during the extraction processes. Although valid attempts to monitor ATP levels in real-time using chemiluminescence with intracellular expression of firefly luciferase have been reported [6, 7], their applications are limited as they depend on the level of expression and activity of luciferase. Moreover, consumption of ATP by luciferase itself may disturb intracellular ATP levels.

A schematic of the mechanism of ATeam for measurement of ATP is illustrated in Fig. 1. An original ATeam (ATeam1.03) employs the ε subunit of the bacterial F_oF_1-ATP synthase as an ATP sensory domain sandwiched between two fluorescence proteins—a variant of cyan fluorescent protein (mseCFP) [8] as the donor fluorophore present N-terminally to the ε subunit and a circularly permuted monomeric Venus having the 173rd amino

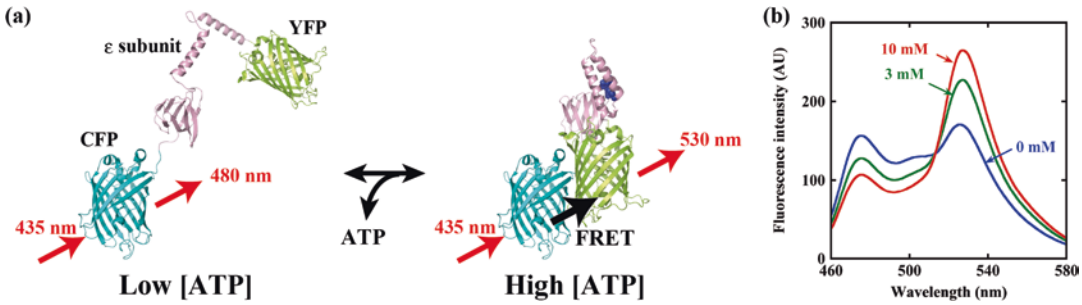

Fig. 1 Mechanism of ATeam for measurement of ATP. (**a**) Schematic of ATeam. (**b**) ATP-dependent fluorescence spectra of purified ATeam1.03 in 50 mM MOPS–KOH (pH 7.3), 50 mM KCl, 0.5 mM $MgCl_2$, 0.05% Triton X-100

acid as its N terminus (cp173-mVenus) [9] as the acceptor at the C-terminus of the ε subunit (Fig. 1a). The bacterial ε subunit is an ATP-binding protein (14 kDa) composed of an N-terminal β-barrel domain and two C-terminal α-helices [10]. The ε subunit binds to ATP by bundling α-helices without hydrolysis, and greatly changes its conformation. In the absence of ATP, the ε subunit extends and adopts a flexible conformation, resulting in the separation of the two fluorescence proteins. Conversely, in the presence of ATP, the ε subunit binds to ATP and contorts, drawing the two fluorescence proteins closer to each other. Thus, ATP alters the fluorescent spectra of ATeam by changing the FRET efficiency between CFP and YFP (Fig. 1b). In turn, this allows the ATP levels to be quantitatively measured by calculating the emission ratios of YFP-FRET/CFP from ATeam, although it is still challenging to determine absolute intracellular ATP concentrations of living cells.

There are several variants of ATeam with different binding affinities for ATP and the fluorescent spectra. The properties of these ATeams are summarized in Table 1 [3, 11, 12]. Properties of the spectra are altered by fluorescent proteins used as the donor and the acceptor fluorophores. It should be noted that temperature highly affects the affinity of the biosensors [3]. ATP measurements using ATeam can be properly achieved by applying a suitable ATeam selected from the series with the appropriate properties for the experimental conditions. For example, AT1.03, AT1.03YEMK, GO-ATeam1, or GO-ATeam2 biosensors are suitable for mammalian cells that are maintained at 37 °C, while AT1.03NL biosensor

Table 1
Properties of ATeam series

Name	Donor	Acceptor	K_d	References
ATeam1.03	mseCFP (CΔ11)	cp173-mVenus	3.3 mM	
ATeam1.03YEMK	mseCFP (CΔ11)	cp173-mVenus	1.2 mM	
ATeam1.03R122K/R126K	mseCFP (CΔ11)	cp173-mVenus	no detectable binding	Imamura et al. [3]
ATeam3.10	mseCFP (CΔ10)	mVenus	7.4 μM	
ATeam3.10MGK	mseCFP (CΔ10)	mVenus	14 μM	
GO-ATeam1	cp173-mEGFP	mKOκ	7.1 mM	
GO-ATeam2	cp173-mEGFP	mKOκ	2.3 mM	Nakano et al. [11]
GO-ATeam3	cp173-mEGFP	mKOκ	no detectable binding	
ATeam1.03NL	mseCFP (CΔ10)	cp173-mVenus	1.8 mM (24 °C)	Tsuyama et al. [12]

The value of K_d represents apparent dissociation constants for ATP of ATeam at 37 °C. CΔ10 and CΔ11 represent the deletion of 10 and 11 residues of C-terminal domain, respectively

works best for organisms with much lower body temperatures, such as *C. elegans* and *D. melanogaster*.

ATeam is a useful tool for investigating the relationship between cellular functions and ATP levels in each cell. Additionally, it enables for visualization of ATP levels in subcellular compartments of a single living cell [3]. Fluorescence imaging assay with ATeam revealed that ATP levels in the mitochondrial matrix are significantly lower than those in the cytosol and nucleus [3], and positively correlate with mitochondrial membrane potential [13] in HeLa cells. A recent report using ATeam has successfully visualized a decrease in mitochondrial ATP levels after a decrease in Ca^{2+} transporting ATPase activity during activation of cellular protective machinery in the inflammatory processes [14]. Employing ATeam and an ATP production assay, another study successfully illustrated an increase in mitochondrial ATP production in response to hypoxic stress as a means to protect cells from a critical energy crisis [15]. Furthermore, our group succeeded in elucidating the role of increased cytosolic ATP on Ca^{2+} oscillation trends in glucose-stimulated pancreatic islet by using a combination of ATeam and a fluorescent Ca^{2+} dye [16].

Herein, we describe the method for the evaluation of ATP levels in the mitochondrial matrix of living mammalian cells using ATeam1.03. The procedures described below consist of three steps: (1) preparation of cells that are stably or transiently expressing ATeam in mitochondria, (2) acquisition of fluorescent images of cells expressing ATeam in mitochondria and (3) processing of fluorescent images. At the end of this chapter, an example of experimental results is described for a better understanding of the presented procedure.

2 Materials

2.1 Cell Culture

1. Growth Medium: Dulbecco's modified Eagle's medium (DMEM, high glucose) containing 10% fetal bovine serum (FBS) supplemented with 100 μg/mL penicillin and streptomycin.
2. HEK293A cells are routinely cultured in growth medium in 90 mm dishes at 37 °C in 5% CO_2. At 80% confluence, cells are trypsinized and used to seed for transfection.

2.2 Transfection of Cells

1. D-PBS without Ca^{2+} and Mg^{2+}.
2. Trypsin–EDTA (0.25%) containing phenol red solution.
3. DMEM (high glucose).
4. 35 mm glass bottom dish: P35-1.5-14C (MatTek Corp., Ashland, MA).

5. Cellmatrix Type I-C (Nitta-Gelatin Co. Ltd, Osaka, Japan).
6. Lipofectamine® Transfection reagent (Invitrogen, San Diego, CA).
7. Lipofectamine® 2000 Transfection reagent (Invitrogen, San Diego, CA).
8. 1× Opti-MEM® medium (Gibco/BRL, Bethesda, MD).
9. PLUS™ reagent (Invitrogen, San Diego, CA).
10. pcDNA-mitAT1.03 [3]: Expression plasmid for AT1.03 with a tandem repeat of a mitochondrial targeting signal of cytochrome c oxidase subunit 8 at N-terminus. To obtain the plasmid, please contact corresponding author.
11. G418 (Geneticin®).
12. Cloning cylinders (8 mm × 8 mm).
13. Sterile silicon grease.
14. 24-well plate.
15. Fluorescence microscope.

2.3 Imaging

1. Microscope: Eclipse Ti-E inverted microscope with a perfect focus system (PFS), a motorized stage, a xenon light source, and filter wheels (Nikon Instrument Inc., Tokyo, Japan) (*see* **Note 1**).
2. Objective lens: CFI Plan Apo VC 60× oil (NA 1.40) (Nikon Instrument Inc., Tokyo, Japan).
3. Stage-top incubator: INUBG2-TIZB (Tokai Hit Co. Ltd, Fujinomiya, Japan).
4. Electric shutter: SmartShutter (Sutter Instruments, Novato, CA).
5. Camera: Zyla 4.2 sCMOS camera (Andor Technology Ltd, Belfast, UK) (*see* **Note 2**).
6. Optical filters for FRET imaging (Semrock, Rochester, NY): FF01-438/24 filter for excitation, FF458-Di01 dichroic mirror, FF01-483/32 filter for CFP emission and FF01-542/27 filter for YFP-FRET emission.
7. Optical filter set for YFP: YFP-A-Basic filter set (Semrock, Rochester, NY).
8. System controlling software: NIS-Elements 4.2 (Nikon Instrument Inc., Tokyo, Japan).
9. Immersion oil.

2.4 Processing Images

1. MetaMorph 7.6 (Molecular Devices, Sunnyvale, CA) (*see* **Note 3**).

3 Methods

3.1 Preparation of Cells Expressing ATeam in Mitochondria

3.1.1 Stable Transfection of Cells

1. Prepare HEK293A cells in 60 mm dish at 70% confluency in 3 mL growth medium.
2. Dilute 4 μL PLUS™ reagent and 2 μg pcDNA-mitAT1.03 in 250 μL DMEM. In another tube, dilute 6 μL Lipofectamine® in 250 μL DMEM and incubate both mixtures for 15 min at room temperature (25 °C).
3. After incubation, combine the two reagent mixtures together and ensure blending by pipetting about 10 times. Incubate the mixed solution at room temperature (25 °C) for 15 min.
4. Add the mixed solution to the 60 mm dish (in addition to the 3 mL medium in the dish).
5. Incubate at 37 °C in 5% CO_2 for 3 h.
6. Change the medium to fresh growth medium and incubate at 37 °C in 5% CO_2 for 24 h (*see* **Note 4**).
7. Wash the cells with 3 mL D-PBS.
8. Add 1 mL 0.25% trypsin–EDTA and swirl the solution around the plate gently for 30 s.
9. Remove ≈750 μL of trypsin–EDTA leaving ≈250 μL remaining in the dish.
10. Incubate at 37 °C in 5% CO_2 for 1 min.
11. Suspend the cells in 1 mL growth medium.
12. Transfer the cell suspension into 10 mL of growth medium containing 0.75 mg/mL G418 onto 90 mm dish (*see* **Note 5**).
13. Routinely culture the transfected cells in growth medium containing 0.75 mg/mL G418 in 90 mm dishes at 37 °C in 5% CO_2 for 2 weeks.
14. Re-passage the cells in 10 mL of growth medium in a 90 mm dish at a density of 1×10^2 cells/dish (Five dishes prepared as specified are adequate for making a stable transfection cell line).
15. Incubate at 37 °C in 5% CO_2 for 72 h.
16. Using fluorescence microscopy, individually select a single colony (about 10–20 cells/colony) expressing the fluorescent emission of ATeam in the mitochondria (*see* **Note 6**). To identify the location of the selected colonies, mark the bottom of the dish with a permanent marker at the selected areas.
17. Remove the medium from the dish and wash once with 3 mL D-PBS.

18. Isolate each individual colony using a cloning cylinder greased with sterile silicon grease on one end to allow the cylinder to seal to the plate.
19. Place 100 μL 0.25% trypsin–EDTA into the cloning cylinder.
20. Incubate the dish at 37 °C in 5% CO_2 for 3 min.
21. Gently pipette the solution within the cloning cylinder to allow the cells to detach. Individually remove the cells in suspension and transfer them to a 24-well plate with 2 mL growth medium (*see* **Note 7**).
22. At this stage, the stable transfection is complete and cells can be re-passaged onto larger plates after 3 days of incubation. Routinely culture the cells permanently expressing ATeam in mitochondria in growth medium and incubate at 37 °C in 5% CO_2.
23. For imagining purposes, cells must be re-passaged onto 35 mm collagen coated glass bottom dish (*see* **Note 8**) in 2 mL of growth medium at desired density (*see* **Note 9**).

3.1.2 Transient Transfection of Cells

1. Wash the cells at 80% confluence in a 90 mm dish with 3 mL D-PBS.
2. Add 1 mL 0.25% trypsin–EDTA and swirl the solution around the plate gently for 30 s.
3. Remove ≈750 μL of trypsin–EDTA leaving ≈250 μL remaining in the dish.
4. Incubate at 37 °C in 5% CO_2 for 1 min.
5. Suspend the cells in 3 mL growth medium.
6. Transfer aliquots of the cell suspension into 2 mL of growth medium onto a 35 mm collagen coated glass bottom dish at a density of 4–6 × 10^4 cells/dish.
7. Gently rock the dish in a back-to-forth and side-to-side manner for 20 s at room temperature (25 °C).
8. Incubate cells at 37 °C in 5% CO_2 for 24 h.
9. Individually dilute 3 μL Lipofectamine® 2000 and 1 μg pcDNA-mitAT1.03 in 150 μL Opti-MEM® medium, and incubate both mixtures for 5 min at room temperature (25 °C).
10. Mix the solutions well, and incubate at room temperature (25 °C) for 5 min.
11. Add 300 μL of the mixed solution directly into the medium and incubate cells at 37 °C in 5% CO_2 for 1 h (*see* **Note 10**).
12. Change the medium to fresh growth medium.
13. Incubate at 37 °C in 5% CO_2 for 1–3 days (*see* **Notes 9** and **11**).

3.2 Acquisition of Fluorescent Images of Cells Expressing ATeam in Mitochondria

For fluorescent live imaging in our experiments, images in CFP and YFP-FRET channels are captured from cells expressing ATeam in mitochondria by using a Nikon Ti-E-PFS inverted microscope equipped with a 60× oil-immersion objective (Nikon), a sCMOS camera (Andor), and stage-top incubator (Tokai Hit) as shown in Fig. 2. To illuminate the cells, a 75-W xenon lamp with 12.5 and 25% neutral density filters and an excitation filter (426–450 nm) was used. Excitation light was controlled by an electric shutter. To achieve dual-emission ratio imaging of ATeam, we used a motorized filter wheel to alternate between two (CFP and YFP-FRET) emission filters. The microscope system was controlled with NIS-Elements software. The detailed procedure for capturing fluorescent images is described below.

1. Stabilize the stage-top incubator at 5% CO_2 at 37 °C and warm the oil-immersion lens to 37 °C by wrapping it with the lens warmer (*see* **Note 12**).
2. Stabilize the xenon lamp for more than 30 min before starting the imaging assay.
3. Change the medium to 2 mL phenol red free DMEM (low glucose) pre-warmed at 37 °C (*see* **Note 13**).
4. Add a drop of immersion oil onto the warmed lens.
5. Place and fix the dish on the stage. For treatment that requires the addition of an external reagent such as oligomycin (*see* **Note 14**).
6. Turn on the perfect focus system.

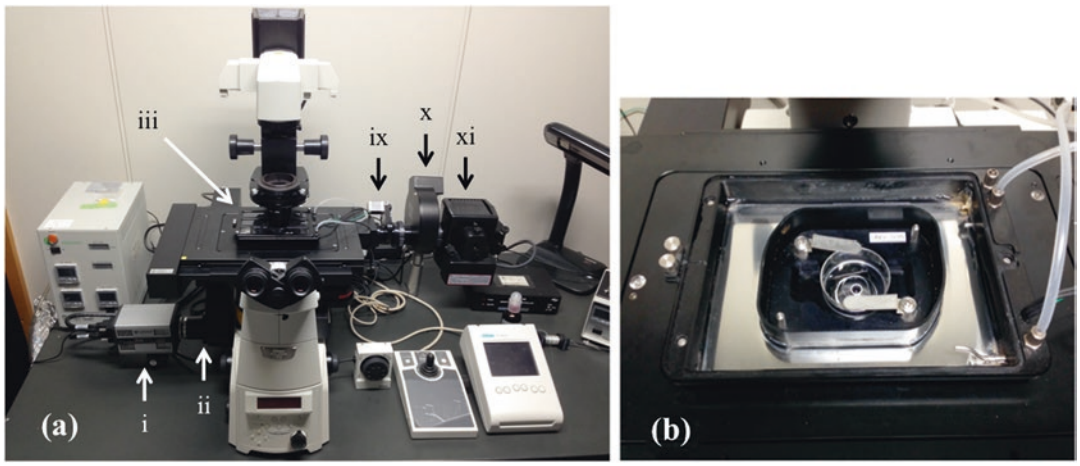

Fig. 2 Fluorescent microscope system for imaging of ATeam. (**a**) General view of the fluorescence microscope system used in our experiments. The numbers represent the parts as following; (*i*) Zyla 4.2 sCMOS camera, (*ii*) filter wheel (emission), (*iii*) stage-top incubator, (*ix*) electric shutter, (*x*) filter wheel (excitation), (*xi*) xenon lamp. (**b**) Stage-top incubator with a sample prepared for imaging of ATeam

7. Find ATeam-expressing cells by visual detection, using a YFP-filter set. Adjust xy position and focus (*see* **Note 15**).
8. Readjust the xy positions and refocus using a CFP or YFP-FRET fluorescent image obtained by sCMOS camera by using NIS-Elements (*see* **Note 12**).
9. Input the xy positions to NIS-Elements.
10. Repeat **steps 8–10** in order to select multiple view fields. Typically, 5 or more cells should be imaged to obtain reliable results.
11. Input the time interval between each shot (*see* **Note 16**).
12. Set the exposure times for both CFP and YFP-FRET channels (*see* **Note 17**).
13. Start image acquisition.

3.3 Processing of Fluorescent Images

According to the principle of ATeam described in the introduction, ATP levels in mitochondria can be evaluated by ratiometric changes of YFP-FRET per CFP emission signals from ATeam. In our experiments, MetaMorph (Molecular devices) has been used for calculation of the ratio value by processing fluorescent images in each channel as described below.

1. Open the image files of CFP and YFP-FRET channel by using processing software MetaMorph.
2. Select a region-of-interest (ROI) at the same position in CFP and YFP-FRET images within the area where no cells exist.
3. Subtract a constant background intensity value estimated from statistics of a selected ROI in CFP and YFP-FRET images, respectively, by using the "statistical model" function equipped in MetaMorph.
4. Select a ROI to surround a single mitochondria expressing ATeam in CFP and YFP-FRET images subtracted background signal, respectively.
5. Calculate integrated intensities of CFP and YFP-FRET emissions within a ROI.
6. Calculate the emission ratio of YFP-FRET/CFP by using the integrated intensity of YFP-FRET emission and CFP emission in ROI.
7. If a photographic display of the cellular ATP levels is desired, intensity modulated display (IMD) images can be obtained from background-subtracted images generated at **step 3**.

An example of the presented methods for evaluation of ATP levels in mitochondria is shown in Fig. 3. The experimental results display a mitochondrial ATP depletion as judged by a decrease in emission ratio of FRET-YFP/CFP in HEK293A cells permanently expressing ATeam1.03 in mitochondria after treatment with oligomycin A, inhibitor of oxidative phosphorylation.

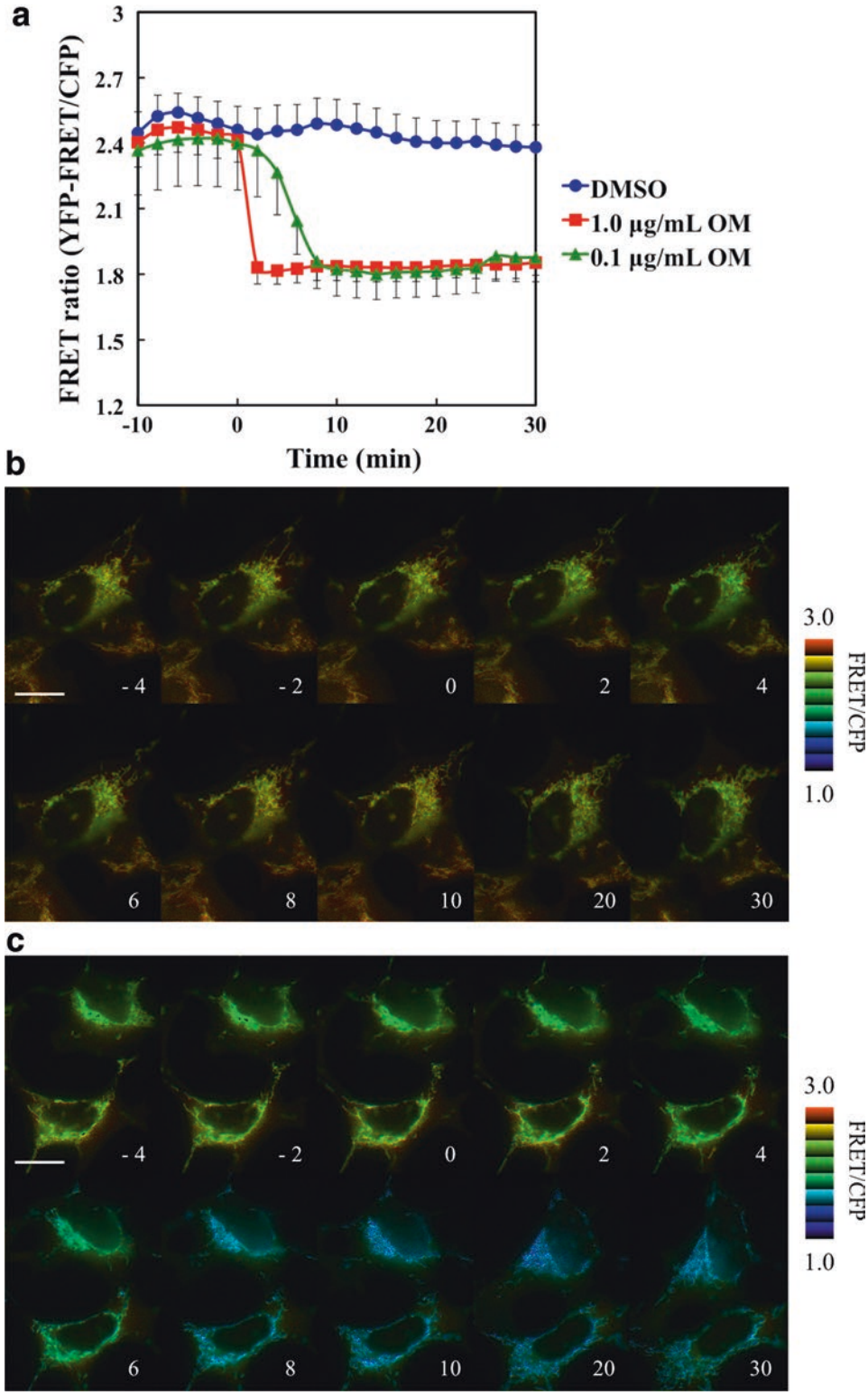

Fig. 3 Monitoring mitochondrial ATP levels in HEK293A cells. (**a**) Time course of averaged YFP-FRET/CFP emission ratio of ATeam1.03 expressed in the mitochondrial matrix of HEK293A cells. The cells treated with 0.1% DMSO (*blue*, $n = 6$) and 0.1 (*red*, $n = 6$) and 1.0 (green, $n = 5$) µg/mL oligomycin for 30 min at 5% CO_2 at 37 °C. Error bars indicate SD. (**b**, **c**) Sequential pseudocolored images of YFP-FRET/CFP emission ratio of the mitochondria of HEK293A cells. DMSO (**b**) or 0.1 µg/mL oligomycin A (**c**) was added at time = 0 (min). The numbers represent time in minutes. Scale bar represents 30 µm

4 Notes

1. A PFS is quite useful if long-term imaging is required, and a motorized stage enables to obtain data from many cells in one experiment. But they are not indispensable. A xenon light source may be replaced by a mercury, metal-halide or LED light source.
2. A cooled CCD or EMCCD camera may be used. It is important to use a camera with high sensitivity, high linearity, and low noise.
3. Other image processing software, such as ImageJ, may be used.
4. When changing the medium, make sure to pre-warm the medium to 37 °C.
5. For effective selection, cells should be passaged with an antibiotic (G418) to weed out cells not expressing ATeam.
6. On average, 5 colonies of ~10–20 cells expressing ATeam in the mitochondria per plate are selected.
7. Each colony is to be plated individually in 24-well plate. For instance, if you select 5 colonies, you will have 5 wells.
8. For regular re-passaging, cells can be plated on regular non-coated plates.
9. We usually perform imaging at 50–80% confluency when the desired condition is a single cell analysis. This is due to the fact that at this confluency, cells can be captured in a single state and not crowded by other cells. On the other hand, when investigating cell communication for instance, a confluency of 90% is desirable due to the overlapping of the cells. Re-passage of cells to new glass bottom dishes may be required to adjust confluency.
10. Transfection procedure using Lipofectamine® 2000 requires an incubation of 1–3 days. However, incubation for just 1 h has proven to yield successful transfection efficiency in our laboratory. Thus, by our recommendation, exposure of 1 h is optimal and should not exceed 3 h as it might decrease cell viability.
11. The cells can be used for imaging assay between 1 and 3 days post-transfection. Prior to this time, the fluorescent protein goes through several stages until it becomes functional, termed maturation. Although the protein is already synthesized, it is not fluorescent until completion of maturation. Three days post-transfection, expression decreases, thus not allowing for accurate measurements.
12. It is critical to maintain the temperature of the culture dish during imaging via a stage-top incubator. The temperature

significantly affects the affinity of ATeam to ATP and hence the values of YFP-FRET/CFP emissions ratio.

13. Other culture media can be used for the imaging. However, culture media containing components displaying higher background signal at the imaging, such as phenol red, should be avoided.
14. When adding an external reagent to the medium during time-lapse imaging, this can be done while the dish is on the stage followed by very gentle pipetting to homogenize the mixture. Calculation of final concentration of the reagent in the medium must take into account the volume already present in the dish. Reagent solutions should be pre-warmed to 37 °C.
15. Excess exposure of the cells to excitation light causes photobleaching of fluorescent proteins, especially YFP, resulting in ATP-independent decrease in YFP-FRET/CFP emission ratio. Thus, it is important to reduce excitation light using neutral density filters and to minimize opening the excitation shutter when adjusting xy positions and focuses.
16. We usually use the time interval of 1 min or longer to avoid photobleaching of ATeam. It should be noted that a shorter interval (e.g., 10 s) can be applied for GO-ATeam, since GO-ATeam is much less subject to photobleaching than ATeam [11].
17. We typically use the same exposure time for both channels, ranging from 200 to 700 ms. In the situation where protein expression levels are low, a longer exposure time of 700 ms is recommended. Alternatively, if the expression levels are high, a shorter exposure time of 200 ms is recommended.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (B) to H.I. (26291027) from the Ministry of Education, Culture, Sports, Science and Technology in Japan (MEXT), and by the Platform Project for Supporting in Drug Discovery and Life Science Research (Platform for Dynamic Approaches to Living System) from MEXT and Japan Agency for Medical Research and Development (AMED).

References

1. Lakowicz JR (ed) (2006) Principles of fluorescence spectroscopy, 3rd edn. Heidelberg, Springer
2. Pham E, Truong K (2010) Design of fluorescent fusion protein probes. In: Papkovsky DB (ed) Live cell imaging methods and protocols. Humana press, Herefordshire, pp 69–92
3. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H (2009) Visualization of ATP levels inside sin-

gle living cells with fluorescence resonance energy transfer-based genetically encoded indicators. Proc Natl Acad Sci U S A 106:15651–15656

4. Ford SR, Chenault KH, Bunton LS, Hampton GJ, McCarthy J, Hall MS, Pangburn SJ, Buck LM, Leach FR (1996) Use of firefly luciferase for ATP measurement: Other nucleotides enhance turnover. J Biolum Chemilum 11:149–167
5. Smolenski RT, Lachno DR, Ledingham SJ, Yacoub MH (1990) Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. J Chromatogr 527:414–420
6. Kennedy HJ, Pouli AE, Ainscow EK, Jouaville LS, Rizzuto R, Rutter GA (1999) Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells—Potential role for strategically located mitochondria. J Biol Chem 274:13281–13291
7. Saito K, Chang YF, Horikawa K, Hatsugai N, Higuchi Y, Hashida M, Yoshida Y, Matsuda T, Arai Y, Nagai T (2012) Luminescent proteins for high-speed single-cell and whole-body imaging. Nat Commun 3:1262
8. Matsuda T, Miyawaki A, Nagai T (2008) Direct measurement of protein dynamics inside cells using a rationally designed photoconvertible protein. Nat Methods 5:339–345
9. Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A (2004) Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. Proc Natl Acad Sci U S A 101:10554–10559
10. Yagi H, Kajiwara N, Tanaka H, Tsukihara T, Kato-Yamada Y, Yoshida M, Akutsu H (2007) Structures of the thermophilic F_1-ATPase ε subunit suggesting ATP-regulated arm motion of its C-terminal domain in F_1. Proc Natl Acad Sci U S A 104:11233–11238
11. Nakano M, Imamura H, Nagai T, Noji H (2011) Ca^{2+} regulation of mitochondrial ATP synthesis visualized at the single cell level. ACS Chem Biol 6:709–715
12. Tsuyama T, Kishikawa JI, Han YW, Harada Y, Tsubouchi A, Noji H, Kakizuka A, Yokoyama K, Uemura T, Imamura H (2013) In vivo fluorescent adenosine 5′-triphosphate (ATP) imaging of *Drosophila melanogaster* and *Caenorhabditis elegans* by using a genetically encoded fluorescent ATP biosensor optimized for low temperatures. Anal Chem 85:7889–7896
13. Kiyonaka S, Kajimoto T, Sakaguchi R, Shinmi D, Omatsu-Kanbe M, Matsuura H, Imamura H, Yoshizaki T, Hamachi I, Morii T, Mori Y (2013) Genetically encoded fluorescent thermosensors visualize subcellular thermoregulation in living cells. Nat Methods 10:1232–1240
14. Shintani Y, Kioka H, Takashima S, Suzuki K (2014) Toll-like receptor 9 protects cardiomyocytes from stress by modulating mitochondrial ATP synthesis through the inhibition of SERCA2. EMBO Rep 15:438–445
15. Kioka H, Kato H, Fujikawa M, Tsukamoto O, Suzuki T, Imamura H, Nakano A, Higo S, Yamazaki S, Matsuzaki T, Takafuji K, Asanuma H, Asakura M, Minamino T, Shintani Y, Yoshida M, Noji H, Kitakaze M, Komuro I, Asano Y, Takashima S (2014) Evaluation of intramitochondrial ATP levels identifies G0/G1 switch gene 2 as a positive regulator of oxidative phosphorylation. Proc Natl Acad Sci U S A 111:273–278
16. Tanaka T, Nagashima K, Inagaki N, Kioka H, Takashima S, Fukuoka H, Noji H, Kakizuka A, Imamura H (2014) Glucose-stimulated single pancreatic islets sustain increased cytosolic ATP levels during initial Ca^{2+} influx and subsequent Ca^{2+} oscillations. J Biol Chem 289:2205–2216