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RGB-color intensiometric indicators visualize spatiotemporal dynamics of ATP in single cells

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Conflict of interest

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Abstract

Adenosine triphosphate (ATP) provides energy for the regulation of multiple cellular processes in living organisms. Capturing the spatiotemporal dynamics of ATP in single cells is fundamental to our understanding of the mechanisms underlying cellular energy metabolism. However, it has remained challenging to visualize the dynamics of ATP in and between distinct intracellular organelles and its interplay with other signaling molecules. Using single fluorescent proteins, we developed multicolor ATP indicators, enabling the simultaneous visualization of subcellular ATP dynamics in the cytoplasm and mitochondria of cells derived from mammals, plants, and worms. Furthermore, in combination with additional fluorescent indicators, we were able to visualize the dynamic interplay of ATP, cAMP, and Ca^{2+} in activated brown adipocyte. This set of indicator tools will facilitate future research into energy metabolism.

Graphical Abstract

A multicolor palette of fluorescent indicators is of clear benefit to studies in biology and biochemistry. Here, we engineered red, green, and blue (RGB) color genetically encoded indicators, which are capable of reporting changes in ATP concentration as a change in single

fluorescence emission. The expanded palette enables simultaneous imaging of ATP dynamics in the cytoplasm versus mitochondria which has never been achieved with previous techniques.

Keywords

fluorescent probes; ATP; protein engineering; live cell imaging; brown adipocytes

Adenosine 5'-triphosphate (ATP), a ubiquitous energy currency for cellular activities, such as cell motility, exocytosis, and development, in living organisms, functions as an important signaling molecule [1]. Thus, the ability to visualize the spatiotemporal distribution of ATP at a subcellular resolution would be invaluable to cell metabolism research [2]. To achieve high resolution spatiotemporal imaging at a single cell level, fluorescent indicators capable of reporting intracellular ATP levels have emerged as promising tools compared with classical luciferin-luciferase ATP bioluminescence assays. Recent advances in technologies, including Förster resonance energy transfer (FRET) or ratiometric-based genetically-encoded indicators [3][4][5][6] and chemical probes [7][8], have helped to elucidate the roles of ATP in various cellular functions. However, simultaneously imaging the dynamics of ATP in different organelles within the same cell has not been possible as a result of the limited usability of indicators to accommodate existing excitation and emission wavelength windows. Here, we describe single fluorescent protein-based intensimetric indicators, which exhibit an increase in fluorescence emission at a single wavelength upon sensing ATP. These indicators are available in red, green, and blue (RGB-color) hues. A multicolor palette of single fluorescent protein-based indicators has been achieved previously through the evolution of a series of fluorescent Ca^{2+} indicators [9][10], but its application in other settings remains limited [11].

To generate the fluorescent ATP indicators, we inserted the ϵ subunit of the ATP-binding region on the bacterial F_0F_1 -ATP synthase into the green fluorescent protein (GFP) Citrine variant, the red fluorescent mApple protein, and a blue fluorescent protein variant (Scheme 1) [3]. The position of the insertion was designed to allow close proximity with the fluorescent protein chromophore upon folding into its tertiary structure. We anticipated that the ATP-dependent conformational change of the ϵ subunit, triggered by ATP binding, would alter the environment surrounding the fluorescent protein chromophore and lead to changes in its fluorescence [3]. We first assessed the effect of varying spacer peptides linking the ϵ subunit of the F_0F_1 -ATP synthase with the fluorescent protein on the indicator's dynamic range (F/F_0). Constructs exhibiting maximal fluorescent increases in the presence of ATP (10 mM), were designated MaLionG ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 505/522$), MaLionR ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 565/585$), and MaLionB ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 373/446$) (Monitoring aTP Level intensimetric turn-on Green/Red/Blue indicator), and exhibited increases of 390%, 350%, and 90%, respectively (Table S1–3 and Figure 1a–b). In the presence of ATP, the absorption peaks at 500 nm (MaLionG) and 564 nm (MaLionR) were increased, and those at 420 nm (MaLionG) and 450 nm (MaLionR) were decreased (Figure 1c). The former peaks correspond to anionic chromophores with a higher quantum yield and the latter peaks to protonated ones with a lower quantum yield [9]. The mechanism underlying MaLionG and R would be originated from the change in the balance between protonated and anionic forms.

In the case of MaLionB, a histidine-based chromophore showed less change in the absorption spectra. We assumed that it would undergo some kind of structural change in chromophore upon ATP binding. We also selected negative control indicators, designated negMaLionG, R, and B, with negligible changes in fluorescence intensity in the presence of ATP ($\pm 6\%$ range; Figure S1, S2). The dissociation constants (K_d) of MaLionG, R, and B for ATP were compatible with the expected intracellular physiological range of ATP (Figure 1d, Figure S3, and Table S4)^[12]. We further confirmed that MaLions work in the temperature range of room temperature to 37 °C, making MaLions applicable for all species, including mammals, worms and plants (Figure S4). Furthermore, MaLions exhibited negligible responses in the presence of other nucleotides, confirming the specificity of the ϵ subunit (Figure 1e). We then investigated the effect of pH on MaLion fluorescence intensity across a physiological range of pH 5 to 9. Fluorescence intensities of MaLionG and R decreased at a lower pH, whereas MaLionB showed relatively little pH sensitivity (Figure S5), most likely due to the low pK_a (= 4.9) of the histidine-derived chromophore of the blue fluorescent protein^[13].

We then evaluated the capability of MaLions in live cells. MaLion-expressing HeLa cells treated with an inhibitor of enolase (NaF) exhibited a decrease in normalized fluorescence of 30 to 40% in response to ATP depletion using all three indicators (Figure 2a,b and Figure S6). The same treatment applied to negMaLion-expressing HeLa cells resulted in minimal fluorescence changes of 5 to 8% (Figure 2b,c), confirming the ability of the indicators to monitor changes in intracellular ATP levels^[14]. Next, we fused a mitochondrial or a nuclear targeting signal sequence to the N-termini of the MaLions, named mitoMaLions and nlsMaLions, respectively (Figure S7). Co-expression in HeLa cells of nlsMaLionB, mitoMaLionG, and non-targeted MaLionR showed distinct targeting of the three indicators (Figure 2d and Figure S8).

Simultaneous monitoring of ATP dynamics in the cytoplasm and mitochondria of the same cell has not been possible using previously available fluorescent indicators. When HeLa cells co-expressing MaLionG and mitoMaLionR were stimulated with oligomycin, an inhibitor of oxidative phosphorylation (Oxphos), the fluorescence of cytoplasmic MaLionG exhibited an initial elevation and then reduction, while mitoMaLionR exhibited a sustained reduction (Figure 3a,b). This experiment was repeated in murine brown adipocytes differentiated from brown fat precursors^[15], in which Oxphos inhibition induced a rapid decrease in mitochondrial ATP followed by a subsequent drop in cytoplasmic ATP (Figure 3c,d). The differences in responses obtained with these two cell types are consistent with the Warburg effect, namely a high rate of glycolysis, in proliferating cells (e.g., HeLa cells)^[16]. It is assumed that excess metabolites due to Oxphos inhibition could be consumed by the strong glycolytic activity of the HeLa cells, resulting in cytoplasmic ATP elevation. This is the first direct observation of crosstalk between the cytoplasm and mitochondria consistent with the Warburg effect in individual proliferating cells. Conversely, as mitochondria are major contributors to ATP production in differentiated cells (e.g., brown adipocytes) in the absence of increased glycolytic activity, it is expected that cytoplasmic ATP would start to decrease shortly after mitochondrial ATP depletion^[17].

We next studied the ability of MaLions to monitor ATP levels in plant cells using the protoplast of *Arabidopsis thaliana* as a model (Figure 3e). Unlike animal cells, plant cells produce ATP through chloroplast photosynthesis as well as through glycolysis and Oxphos. A 1-min illumination of the protoplast expressing MaLionG and mitoMaLionR using blue light, before being placed back in the dark, resulted in transient ATP elevation in both the cytoplasm and mitochondria (Figure 3f). When treated with an inhibitor of photosynthesis, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, the light-induced elevation in ATP was suppressed (Figure S9). We speculate that photosynthesis triggered by exposure to light induces the production of carbohydrates via the Calvin cycle, leading to the detected transient ATP elevation in the cytoplasm and mitochondria.

The performance of MaLions in the whole animal body was assessed through generation of a transgenic model of *Caenorhabditis elegans* expressing MaLionG and mitoMaLionR in the pharyngeal muscle *in vivo*. When exposed to the gastropod anesthetizing compound, 1-phenoxy-2-propanol, which is known to interfere with the mitochondrial respiratory chain [18][19], MaLionG and mitoMaLionR revealed a decrease in cytoplasmic and mitochondrial ATP levels (Figure 3g,h and Figure S10). Differences in the rate of fluorescence reduction were observed between MaLionG (fast decrease) and mitoMaLionR (slow decrease), which may reflect the different affinities of MaLionG and MaLionR for ATP, discussed in more detail later on.

An additional merit of the expanded color palette is the ability to visualize sequential events. We tested this in brown adipocytes subjected to adrenergic stimulation, which results in heat production through the depolarization of the mitochondrial membrane via uncoupling protein-1, and causes a drop in ATP production through Oxphos. The potential benefit of targeting brown adipose tissue as a means to manage obesity and diabetes has generated new interest in the study of signal transduction events during thermogenesis [20]. We combined mitoMaLionR (red, ATP) with additional indicators, including B-GECO (turn-on type, blue, Ca^{2+}) and Flamingo2 (turn-off type, green, cAMP) [21]. Treatment with isoproterenol, a β -adrenergic agonist, resulted in an elevation of cAMP and a reduction in mitochondrial ATP corresponding to heat generation, which is consistent with the well-recognized adrenergic mechanism of non-shivering thermogenesis (Figure 4a–c and Movie S1)[22]. In contrast, treatment with phenylephrine, an α_1 -adrenergic agonist, resulted in a negligible decrease of ATP during the early stages of stimulation (c.a. 20 min.) (Figure 4c,f and Figure S11). In addition, peak B-GECO fluorescence occurred at an earlier time point following phenylephrine stimulation (Figure 4d–f and Movie S2). These differences in Ca^{2+} dynamics are expected because α_1 -adrenergic agonists induce Ca^{2+} release via inositol 1,4,5-trisphosphate (IP_3) receptors directly at the endoplasmic reticulum (ER), whereas β -adrenergic agonists induce the Ca^{2+} release from the mitochondria through the cascade [23]. Notably, Ca^{2+} elevation induced by α_1 -agonists was recently shown to sustain the proton gradient across the mitochondrial membrane, which would compete with the disruption of ATP production [23]. It was also reported that Ca^{2+} elevation activates adenylate cyclase and can trigger elevation of cAMP under certain conditions [24]. These findings support our results that mitochondrial ATP was not decreased despite the elevation in cAMP induced by α_1 -adrenergic stimulation.

Although single fluorescent protein-based indicators are powerful tools for multicolor imaging, it is important to consider the potential effects of changes in pH on these types of intensimetric indicators. In fact, previous studies reported that the metabolic stress caused the intracellular pH change, to varying extents [25]. We performed control experiments using negMaLions with comparable pH sensitivities to MaLions in order to evaluate changes in fluorescence due to fluctuations in pH. We established that these effects were minimal compared with the changes in fluorescence induced by alterations in intracellular ATP and as such would not interfere critically with the data produced using these indicators (Figure 2c and Figure S12–13). However, where harsh pH changes are anticipated, a correction of the pH effect using fluorescent pH indicators should be considered, as previously established by the Yellen group [6]. Using this methodology, we also showed how to obtain the ‘pH-corrected fluorescence’ by removing any effects of pH fluctuation from the fluorescence of MaLions using a pH calibration curve (Figure S14). This approach may be useful for end-users performing experiments under conditions involving harsh pH changes. Furthermore, when considering dual-imaging of ATP in both the cytoplasm and mitochondria of the same cell, the combination of mitoMaLionR and MaLionG is likely to be the most appropriate, given that ATP levels in mitochondria are relatively lower than in the cytoplasm [26]. In fact, MaLionG ($K_d = 1.1$ mM) was able to sense the elevation of cytoplasmic ATP levels during accelerated glycolysis in HeLa cells as shown in Figure 3b, whereas MaLionR ($K_d = 0.34$ mM) was unable to sense this (Figure S15), most likely due to saturation of fluorescence under high cytoplasmic ATP levels. However, relative cytoplasmic/mitochondrial ATP levels may differ across different cells/organisms, such that empirical testing of both combinations would be desirable. In the other cases, we observed similar behaviors of MaLionG and mitoMaLionG (or MaLionR and mitoMaLionR) regardless of their targeted organelles in brown adipocytes, the pharyngeal muscle of *C.elegans* and the protoplast of *Arabidopsis* (Figure S16–18), indicating that different K_d between MaLionG and MaLionR may affect relative fluorescence as a result of variations in their affinity for ATP.

In conclusion, we successfully demonstrate a novel method for the imaging of ATP spatiotemporal dynamics in single cells using a multicolor palette. If the current ATP binding domain in MaLions is replaced with one with nano-micromolar affinity, the usability of indicators will be expanded in future. This accessible technology may be applied by researchers across a range of disciplines, facilitating the study of underlying metabolic processes in health and disease across both animal and plant kingdoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of MaLions *in vitro*.

(a-c) Normalized excitation (a), emission (b), and absorption spectra (c) of MaLionB (blue), MaLionG (green), and MaLionR (red) in the absence (dashed line) or presence of 10 mM ATP (solid line). (d) Normalized fluorescence of MaLions at different ATP concentrations. Dissociation constants (K_d), and Hill coefficients were calculated as 1.1 mM and 2.0 (MaLionG), 0.34 mM and 1.6 (MaLionR), and 0.46 mM and 1.4 (MaLionB), respectively. The limits of the detection of MaLionG, R, and B were estimated to be 0.50, 0.02, and 0.11 mM. (e) Specificity of MaLions. The fluorescence change (F/F_0) in response to the other nucleotides (10 mM) was normalized to that in response to ATP (10 mM). Error bars indicate mean $\pm$ standard deviation.

Figure 2. Validation of MaLions in HeLa cells.

(a) Fluorescence images of HeLa cells expressing MaLions before (top) and after inhibition (NaF, 10 mM) (bottom). Scale bars, 20 μ m. (b) Timelapse experiments of MaLion and negMaLion fluorescence following NaF treatment. Average normalized fluorescence intensity (FI) in single cells was calculated per dish (gray lines). Thick color lines represent the average of three independent experiments. (c) Bar graph of (b). The average normalized FI at the time-point where the biggest change occurred was calculated from cells expressing each negMaLions or MaLions (9–11 cells). * $P < 0.05$, Student's t-test. (d) Triple-color imaging of MaLionB, G and R in HeLa cells. Scale bar, 10 μ m. Error bars indicate mean $\pm$ standard deviation.

Figure 3. Dual-color imaging of subcellular ATP.

(a,b) Monitoring of ATP dynamics in the cytoplasm and mitochondria of a single HeLa cell. Oligomycin (20 $\mu\text{g/ml}$) was added at 3 min. The light green and red lines represent the normalized FI change of MaLionG and mitoMaLionR in each cell, respectively. Thick color lines are representative of three independent experiments. Scale bar, 20 μm . (c,d) Experiments described in (a,b) were repeated in brown adipocytes. Scale bar, 20 μm . (e) Images of Arabidopsis protoplast expressing MaLionG and mitoMaLionR in three different channels. Scale bar, 5 μm . (f) Monitoring of ATP dynamics in Arabidopsis protoplast following light stimulation-induced photosynthesis. During light stimulation using a 458-nm laser, the recording was paused for 1 min. (g) Images of cytoplasmic and mitochondrial ATP in the pharyngeal muscle of *C. elegans*. Scale bar, 25 μm . (h) Anesthesia of *C. elegans* was applied at 3 min using 1-phenoxy-2-propanol (0.5 %). Light green and red lines represent the normalized FI change of MaLionG and mitoMaLionR, respectively, in each *C. elegans*. Thick color lines are representative of three independent experiments.

Figure 4. Triple-color imaging of a single brown adipocyte.

(a,b) Visualization of the dynamics of Ca^{2+} (turn-on type, B-GECO), cAMP (turn-off type, Flamindo2), and mitochondrial ATP (mitoMaLionR) in brown adipocytes. Timelapse images in three different channels and normalized FI changes in a representative cell are shown in (a) and (b). Isoproterenol (1 μM) was added at 5 min. (c) Light green (Flamindo2) and red (mitoMaLionR) lines represent normalized FI changes in individual cells. Thick lines represent the average of three independent experiments. (d,e,f) Experiments in (a,b,c) repeated using phenylephrine stimulation (10 μM). Scale bar, 10 μm .

Scheme 1.

Design of single fluorescence protein-based ATP indicators. MaLionG, MaLionR, and MaLionB (PDB ID: 3DPW, 2E5Y, and 2E5U).