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ORIGINAL RESEARCH PAPER

Enhanced-acceptor fluorescence-based single cell ATP biosensor monitors ATP in heterogeneous cancer populations in real time

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Abstract Current methods to monitor cellular ATP do not provide spatial or temporal localization of ATP in single cells in real time or they display imperfect specificity to ATP. Here, we have developed a single cell, Enhanced Acceptor Fluorescence (EAF)-based ATP biosensor to visualize ATP in real time. This biosensor utilizes a modified mimic of the ε-subunits of the *Bacillus subtilis* F_0F_1 synthase and is coupled to the EAF fluorophores pairs, GFP and YFP. The sensor was then used to monitor ATP in a heterogeneous glioblastoma multiform cancer cell population. We anticipate that this innovative technology and our better understanding of the ATP machinery will have substantial influence on future translational studies.

Keywords ATP · Biosensor · Cancer · Enhanced acceptor fluorescence · Metabolism

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Introduction

ATP is the ubiquitous energy currency of living cells. It regulates the cell's capacity to do work and coordinates cellular signaling machinery in response to cell energy availability and rate of energy flux (Israelsen and Vander Heiden 2010; DeAngelis 2001). Abnormal ATP regulation and altered ATP machinery have been implicated in several pathologies, including cancer (Parsons et al. 2008; Das 2003). Cellular ATP levels have been used as a determinant for cell state, cell death by necrosis and cell metabolic efficiency (Tsujimoto 1997; Hide et al. 2000). In a study of Hispanic patients that had undergone renal transplantation, ATP release was utilized to determine risk of developing infection or rejection and response to therapy (Pérez-Flores et al. 2009). Studies in non-small-cell lung cancer (NSCLC) have correlated ATP flux to chemotherapy response (Glaysher and Cree 2011). Current available assays to monitor intracellular ATP are limited or display imperfect specificity to ATP.

ATP can be quantified via a bioluminescence assay, in which a luciferase and luciferin reagent reaction is monitored in a luminometer. This ATP-bioluminescence assay has been utilized to monitor tumor cell viability and has even been adapted to determine tumor chemo-sensitivity in vitro (Glaysher and Cree 2011). This assay, however, cannot detect the spatial and temporal localization of ATP or monitor ATP on a single cell level. Recently, a fluorescence-based ATP biosensor, named Perceval, was utilized to monitor ATP flux in single cells, but displayed imperfect specificity to ATP (Berg et al. 2009). Here, we have developed a single cell Enhanced Acceptor Fluorescence (EAF)-based ATP biosensor to visualize ATP in real time. This biosensor utilizes a modified mimic of the ε-subunit of the *Bacillus subtilis* F_0F_1 synthase (Imamura et al. 2009) coupled to the EAF fluorophore pairs, GFP and YFP (Calleja et al. 2003).

Cancer cells are unique. The metabolic machinery and the ATP-generating pathways limit the function of cancer cells (Israelsen and Vander Heiden 2010). Warburg (see Vander Heiden et al. 2009) first demonstrated that the metabolism of cancer cells, even under normoxia, is characterized by an increase in the ratio of cytoplasmic glycolysis to mitochondrial glucose oxidation. Although the precise mechanisms for this still remains largely unknown, this metabolic switch may confer cancer cells their proliferative advantage (Locasale and Cantley 2012). Due to the complexity of cancer metabolism, a better understanding of the ATP-regulating machinery is required. We monitored the efficacy of the EAF-based ATP biosensor in single glioblastoma multiform (GBM) cancer cell lines and detected differential ATP flux in a heterogeneous GBM cancer stem cell population.

Materials and methods

Cell lines

Mammalian cell lines were from ATCC (Manassas, VA). U87 and U87-EGFRvIII (also referred to U87vIII) GBM cell lines were gifts from the UCLA Brain Tumor Translational Resource. Cell lines were cultured and maintained in DMEM (Cellgro) supplemented with 5 % (v/v) FBS and penicillin and streptomycin at 100 U/ml in a humidified 5 % CO_2 incubator at 37 °C. All cells were cultured as adherent monolayers.

Total ATP measurements

Utilizing recombinant firefly luciferase and its substrate D-luciferin, total cellular ATP levels was quantified. Briefly, cells were grown in 96-well plates and following treatments, cells were washed and incubated with digitonin (30 μM) for 20 min to permeabilize cells membranes at 37 °C. Cells were then subjected to both luciferase and D-luciferin. This assay is based on a simple luciferase reaction, which requires ATP as a cofactor to produce luminescent light. Luminescence was monitored on a luminometer (emission maximum 560 nm). Utilizing standards of known ATP concentration, cellular ATP levels was correlated to luminescence levels.

EAF-based ATP biosensor construction

Bacillus subtilis ε-subunit cDNA was synthesized by GeneScript. The synthesized cDNA also has Valine-9, Leucine-42, Phenylalanine-67, and Leucine-78 mutations to disrupt a hydrophobic patch for interaction with the γ-subunit of ATP synthase. The cDNA was either genetically linked via PCR ligation (*N*-terminus) to yellow fluorescence protein (YFP) and (*C*-terminus) to green fluorescence protein (GFP) and inserted into pRSET-B vector (Invitrogen) for purification purposes or inserted into the pAcGFP1-C vector (Clontech) to allow for a mammalian expression vector containing humanized codons. Enhanced Acceptor Fluorescence (EAF), an alternative approach to traditional Fluorescence Resonance Energy Transfer (FRET), was detected via excitation of the construct at the donor emission at 480 nm and monitoring acceptor emission at 520 nm.

Purification of EAF-based ATP sensor

Stellar competent *E. coli* (Clontech) carrying the ATP sensor plasmid was cultured in LB medium at 37 °C for 3 h. Protein expression was then induced by adding 10 μM IPTG followed by overnight incubation at 24 °C. Cells were collected by centrifugation, suspended in buffer containing 100 mM sodium phosphate [pH 8.0], 200 mM NaCl, and 10 mM imidazole, and then disrupted by ultrasonication. Cell-free extract, obtained by centrifugation at 10,000×*g* for 60 min, was applied to a Ni–NTA column (Qiagen), the protein was eluted by increasing the imidazole to

200 mM. Purified protein was stored at −20 °C for further use.

Characterization of EAF-based ATP sensor in vitro

Protein concentration was determined at 515 nm. The fluorescence of purified proteins was investigated in PBS using a spectrofluorometer at 37 °C. To obtain the fluorescence spectra, GFP was excited at 480 ± 20 nm with the emission from 460 to 600 nm being scanned. To measure the time course of YFP emission change and EAF, GFP was excited at 480 nm and emission at 520 nm was monitored by adding varying concentrations of ATP at specific time points. Specificity of the sensor was determined via monitoring changes of EAF in the presence of varying concentrations of both UTP and ADP.

Confocal microscopy

Cells were plated on a laminin-coated glass-bottom dish and transfected with plasmid coding the EAF-based ATP biosensor using FuGENE6 transfection reagent (Roche Diagnostics). Between one and two days after transfection, cells were subjected to confocal imaging. Wide-field observations of the cells were performed on a Nikon TE2000-PFS inverted fluorescence microscope. The exposure times were 500 ms for GFP and YFP images. Cells were maintained on a microscope at 37 °C with a continuous supply of 95 % air/5 % CO_2 by using a stage-top incubator. Image analysis was performed using ImageJ. YFP emission saturation was calculated by dividing pixel-by-pixel a YFP image with a GFP image.

Statistical analysis

Results are shown as mean ± SEM. Student *t* test was utilized for all other experimental correlations. $*p < 0.05$, $**p < 0.01$ was considered statistically significant.

Results

To monitor ATP on a single cell level and in real-time in vitro, we have developed a genetically-encoded enhanced acceptor fluorescence (EAF)-based ATP sensor. The sensor utilizes a modified mimic of the ε-subunits of the *B. subtilis* F_0F_1 synthase. This ε-subunit was chosen for its ability to bind ATP without hydrolyzing it (Imamura et al. 2009; Stephenson and Hoch 2001). Additionally, this EAF-based ATP biosensor also utilizes EAF fluorophore pairs capable of enhanced acceptor fluorescence (Calleja et al. 2003). Unlike traditional fluorescence resonance energy transfer (FRET) fluorophore pairs, we utilized the fluorophores, GFP and YFP. Both GFP and YFP are excited at similar wavelengths and, upon ATP binding, the sensor will undergo EAF, emitting an enhanced fluorescence signal. The EAF-based ATP sensor was genetically linked to GFP at the *C*-terminus and YFP at the *N*-terminus. Additionally, four hydrophobic amino acid residues forming a hydrophobic surface were replaced by hydrophilic residues to retard non-specific hydrophobic interactions with other cellular proteins, such as the F_0F_1 synthase (Fig. 1a). To characterize the EAF-based ATP biosensor in vitro, protein expression of the EAF-based ATP sensor plasmid was induced in *E. coli* and purified. EAF emission spectrum was determined by spectrofluorometry. The purified EAF-based biosensor was excited at 480 nm and emission was monitored from 500 to 600 nm. EAF donor, GFP, displayed a single emission peak at 509 nm, and the EAF acceptor, YFP, displayed an emission peak at 527 nm (Fig. 1b). As expected, excitation of the donor fluorophore resulted in emission from the acceptor, due to overlapping spectra. Upon ATP binding and donor excitation, acceptor emission was enhanced due to additional energy transfer from the donor. This EAF signal was detected in the presence of low (10 nM) levels of ATP (Fig. 1c). Addition of up to 5 mM ADP (Fig. 1c) or UTP (data not shown) had little effect on the EAF signal. To monitor EAF specificity, the ATP-based biosenor coupled only to GFP was monitored and exhibited no EAF and only a single GFP spectra (Fig. 1d).

We also visualized ATP levels in single U87 glioblastoma blastoma multiform (GBM) cancer cell lines and U87vIII (epidermal growth factor receptor [EGFR] overexpressing GBM) cell lines expressing the EAF-based ATP sensor. EAF acceptor was excitated with the 488 nm laser line and monitored via confocal microscopy. Both U87 and U87vIII show strong EAF signal in the cytosol. Additionally, increased EAF was observed in the U87vIII cell line when compared to U87 cells (Fig. 2a). U87 and U87vIII cells expressing the EAF-based ATP sensor

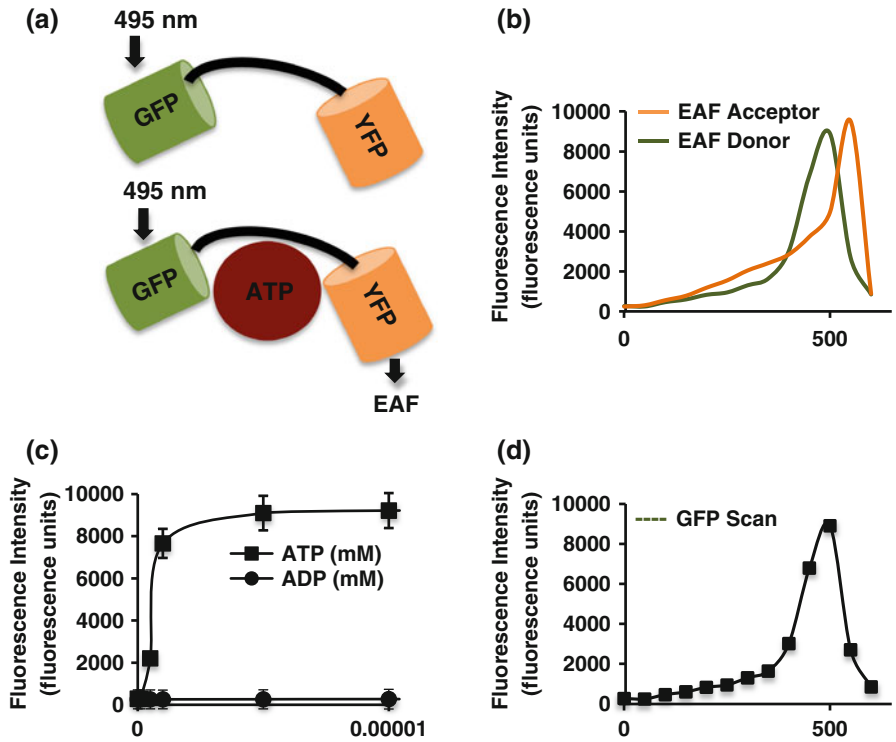

Fig. 1 Enhanced acceptor fluorescence (EAF)-based Single Cell ATP Biosensor. **a** Schematic drawing of the ATP sensor. The *Bacillius subtilis* PS3 ε-subunit flanked by the fluorophores, GFP and YFP. Excitation at 495 nm results in enhanced acceptor emission from the YFP when ATP is bound due to greater transfer of donor energy. **b** Purified EAF-based sensor was excited at 480 nm and emission was monitored from 500 to 600 nm. EAF donor, GFP, displayed a single emission peak at 509 nm, and the EAF acceptor, YFP, displayed an emission peak at 527 nm. **c** EAF signal was detected in the presence of low (10 nM) levels of ATP. Addition of up to 5 mM ADP had little effect on EAF of the sensor. **d** Purified EAF-based sensor coupled to GFP was excited at 480 nm and emission was monitored from 500 to 600 nm, and only displayed a single emission peak at 509 nm

were also quantified for EAF signal via spectrofluorometric analysis. U87vIII cells exhibited a significant increase in EAF signal when compared to U87 cells (Fig. 2a). To further analyze the role of this increase EAF signal in U87vIII and to observe real time modulation of ATP in single living cells, U87 and U87vIII cells expressing the EAF-based ATP biosensor were treated with 2-deoxyglucose (2DG), an inhibitor of glycolysis. Addition of 2DG dramatically decreased EAF signal in both U87 and U87vIII cells, indicating a decrease in cytosolic ATP levels when glycolysis is inhibited (Fig. 2b). Utilizing a modified recombinant firefly luciferase and its substrate D-luciferin, total cellular ATP levels were also quantified to confirm specificity of the EAF-based ATP biosensor (Fig. 2c).

Next we visualized ATP in GBM cancer stem cell lines, GBM39 and GBM6 expressing the EAF-based ATP biosensor. Both GBM39 and GBM6 cells show strong cytosolic EAF signal. GBM6 cells show significantly larger EAF signal compared to GBM39 cells, possibly due to the activation of metabolic regulator, PTEN, present in GBM39 cells, and not in GBM6 cells. Several studies have implicated PTEN in regulating cytosolic ATP in several cancers. Pseudocolor saturation treatment of captured images was applied to highlight time-and treatment-dependent changes in EAF signal (Fig. 2d). Interestingly, confocal images of both GBM6 and GBM39 cell populations expressing the EAF-based ATP sensor show differential EAF signal. Within a population of heterogeneous GBM cells, several varying degrees of EAF were detected in real time under normal conditions (Fig. 2d). The EAF-based ATP sensor potentially detects differentiating ATP flux behavior in heterogeneous cell populations.

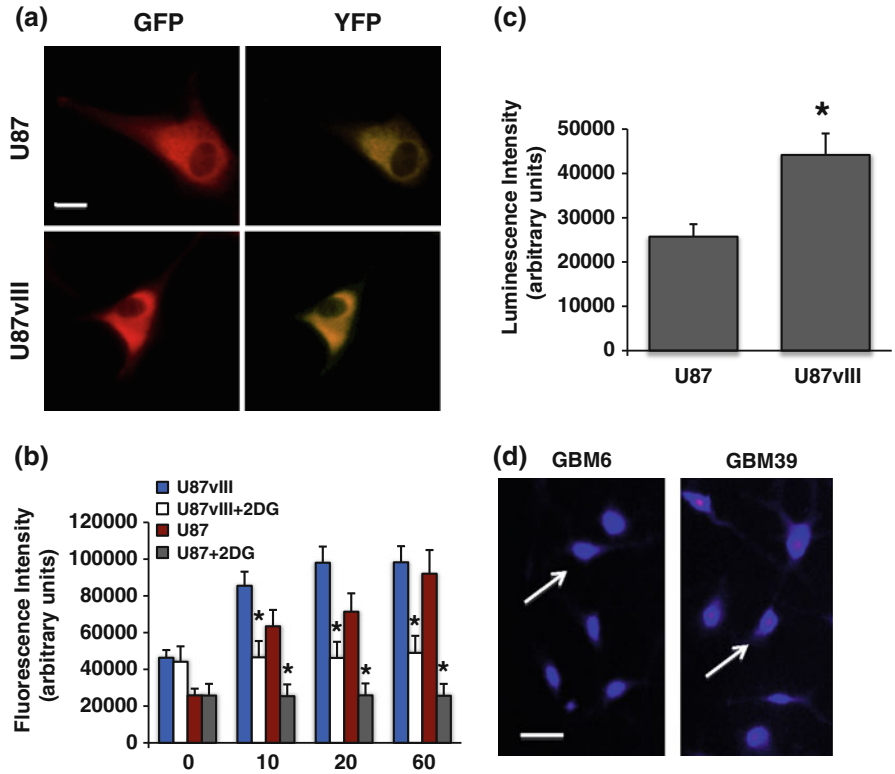

Fig. 2 FRET-based ATP sensor monitors cytosolic ATP on a single cell level. **a** U87 and U87vIII GBM cells lines expressing the EAF-based ATP sensor were monitored via confocal microscopy upon excitation with the 488 nm laser line. *Scale bars* = 40 μm. **b** U87 and U87vIII cells expressing the EAF-based ATP sensor was treated with 2-deoxyglucose (2DG) and EAF signal was monitored. Data represent the mean ± SEM of five independent experiments (statistical significance was assessed by students *t* test, where $*p < 0.05$). **c** Whole cells ATP levels of U87 and U87vIII cells were monitored using a modified luciferin/luciferase-based ATP assay. Data represent the mean ± SEM of five independent experiments (statistical significance was assessed by students *t* test, where $*p < 0.05$). **d** Confocal microscopy of GBM39 and GBM6 cancer stem cell heterogeneous cell population expressing the EAF-based ATP sensor. *Scale bars* = 20 μm

Discussion

Cancer metabolism is a complex phenomenon, with many of the processes and the regulation of the metabolic machinery in cancers still an enigma. A better understanding of cancer cell metabolism will greatly improve development of target therapeutics and, in particular, will provide a better understanding of differential cancer cell metabolism in heterogeneous tumor populations. Our novel EAF-based ATP sensor monitored cellular changes in cytosolic ATP in vitro in both GBM U87 and U87vIII cells. The sensor's functionality and sensitivity provide real time analysis of cytosolic ATP on a single cell level. Additionally, the sensor can be manipulated to monitor ATP flux in mitochondria, nuclei and the ER. In summary our results identifies novel therapeutic target in GBM and a novel single cell ATP biosensor; a tool that will have substantial influence on future translational and biomedical studies.

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