# MRT Letter: Expression of ATP Sensor Protein in *Caenorhabditis elegans*

JUN-ICHI KISHIKAWA,[1] MAKOTO FUJIKAWA,[2] HIROMI IMAMURA,[3] KAYO YASUDA,[4] HIROYUKI NOJI,[3] NAOAKI ISHII,[4] SHOHEI MITANI,[5] AND KEN YOKOYAMA[1,2]*

[1]*Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan*
[2]*ATP-Synthesis Regulation Project, International Cooperative Project, Japan Science and Technology Agency, National Museum of Emerging Science and Innovation, Tokyo, Japan*
[3]*Institute of Scientific and Industrial Research, Osaka University, Osaka, Japan*
[4]*Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa, Japan*
[5]*Department of Physiology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan*

*KEY WORDS* *Caenorhabditis elegans*; metabolism, ATP; FRET

*ABSTRACT* Adenosine 5′-triphosphate (ATP) is the major energy currency and is involved in many biological processes. The ATP-monitoring system for cells in animals can be helpful to study the relationship between energy metabolism and biological processes. The fluorescent ATP biosensor ATeam (ATP indicator based on Epsilon subunit for Analytical Measurements), which has been reported to monitor ATP levels in cultured cells on the basis of fluorescence resonance energy transfer (FRET), was introduced into nematodes by microinjection and UV-irradiation method. To confirm whether ATeam functions as an ATP sensor in nematode cells, the authors measured FRET of ATeam in cells of transgenic nematode. The ATeam was expressed in target cells in nematode. In vulva cells, ATP levels in the cytosol were higher than those in mitochondria. ATeam also sensed ATP level change in cultured cells from the transgenic nematode. These experiments indicated that ATeam is available for detection of changes in ATP levels in nematode cells. *Microsc. Res. Tech. 75:15–19, 2012.* 

## INTRODUCTION

In all living organisms, adenosine 5′-triphosphate (ATP) is used as a ubiquitous energy currency, as well as an intra- and extracellular signaling molecule (Ashcroft, 2005; Finger et al., 2005). ATP is predominantly generated by glycolysis in the cytosol and oxidative phosphorylation in mitochondria (Yokoyama and Imamura, 2005; Yoshida et al., 2001). The balance between them is affected by the environment for growth, such as nutrient and oxygen availability (Feng and Levine, 2010; Xie and Wang, 1996).

Even though ATP is an important molecule, changes in intracellular ATP levels have remained unclear. In particular, it has been very difficult to determine the difference and dynamics of ATP levels in every cell of the body because conventional ATP quantification methods are based on cell extract analysis and can only report the averaged ATP concentration of a collection of cells. Therefore, to our knowledge, precise observation of the change of ATP levels of multicellular organism in vivo has not been reported. Recently, Imamura et al. (2009) reported a series of fluorescence resonance energy transfer (FRET)-based ATP biosensors, called ATeam (ATP indicator based on Epsilon subunit for Analytical Measurements). ATeam is composed of genetically linked cyan florescent protein (CFP) and yellow fluorescent protein (YFP) each at either the N- or the C-terminus of the ϵ subunit, which was derived from *Bacillus subtilis* $F_oF_1$–ATP synthase. Upon ATP binding, the ϵ subunit undergoes a large conformational change and the physical distance between CFP and YFP is shortened. The ATeam FRET efficiency, which can be monitored as YFP/CFP emission ratio, changes in a manner dependent on ATP levels. ATeam has been used to compare ATP levels in different cellular compartments and to monitor the dynamics of ATP levels at the single cell level in real time.

In this study, the authors introduced ATeam into *Caenorhabditis elegans* and evaluated the availability of ATeam for measurement of ATP levels of each tissue in nematodes.

## MATERIALS AND METHODS

### Strain and Culture of Nematodes

All *C. elegans* strains were derived from the wild-type Bristol strain N2, and were maintained using standard techniques (Evans, 2006).

### Preparation of Transgenic Lines

DNA injection into the *C. elegans* germline was carried out as described by Evans (2006). The plasmids, pPuncAT, and pPuncCoxAT were used for ATeam expression in worm muscle cells. The ATeam gene,

Makoto Fujikawa has equal contribution to the first author.

*Correspondence to: Ken Yokoyama, Faculty of Life Sciences, Kyoto Sangyo University, Motoyama, Kamigamo, Kita-ku, Kyoto 603-8555, Japan. E-mail: yokoken@cc.kyoto-su.ac.jp

Abbreviations: ATeam, ATP indicator based on Epsilon subunit for Analytical Measurements; ATP, adenosine 5'-triphosphate; CFP, cyan florescent protein; 2DG, 2-deoxyglucose; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein

Received 6 April 2011; accepted in revised form 8 September 2011
DOI 10.1002/jemt.21103
Published online 28 October 2011 in Wiley Online Library (wileyonlinelibrary.com).

AT1.03, which has the highest $K_d$ value for ATP (3.3 mM) among ATeams, was connected to the *unc-54* promoter, which promotes specific protein expression in body and vulval muscle cells. For pPuncCoxAT, the mitochondrial signal sequence was fused to the 5′ region of the ATeam gene. For expression of DsRed protein, a variant of red fluorescent protein, in pharyngeal muscle cells, the plasmid pFX_DsRedxT_myo was co-injected as a transformation marker. The total DNA concentration of the injected mixture was 100 ng/μL. The resultant transformants were subjected to UV-irradiation for insertion of the transgene into worm chromosomes, and crossed with wild-type cells to stabilize them as described earlier (Mitani, 1995).

### Cell Culturing

Worm eggs were prepared by the bleaching method (Evans, 2006) and washed four times with egg buffer (118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES pH 7.3). The eggs were re-suspended in 30% sucrose in egg buffer. The worm carcasses were centrifuged and precipitated. Then, the eggs were collected by centrifugation and washed twice with egg buffer. The eggs were re-suspended in 2 mg/mL chitinase in egg buffer and incubated for 30 min at room temperature. Subsequently, the treated eggs were passed through a 27-gage needle syringe to peel-off the eggshell. The egg suspension was filtered through a 5-μm PAS syringe filter (PALL). The cells were seeded onto a glass-bottomed dish at 100,000 cells/$cm^2$ in L15 medium supplemented with FBS (10%) and 15 mg/mL sucrose and cultivated for several days.

### Measurement of FRET of ATeam in Nematodes

The observation system for fluorescence of ATeam in nematode cells is described in the previous article (Imamura et al., 2009). The nematodes were sandwiched between two cover glasses to immobilize them during observation. Nematodes were illuminated using a 75-W xenon lamp through 12.5 and 25% neutral density filters. Fluorescence emission from ATeam was imaged using a cooled charge-coupled device camera (ORCA-AG; Hamamatsu Photonics); the exposure times were 500 ms for CFP and YFP images. Cells were maintained on a microscope at 25°C. Image analysis was performed using MetaMorph (Molecular Devices). The YFP/CFP emission ratio was calculated by dividing an YFP image with a CFP image pixel-by-pixel.

## RESULTS

### Expression of ATP Sensor Protein ATeam in Nematode Cells

To carry out the direct measurement of ATP levels in a single cell of a living nematode, the authors expressed the ATeam biosensor in nematode cells. Transgenic nematodes expressing ATeam biosensor in either cytosol or mitochondria in both body and vulval muscle cells were generated as described in Materials and Methods. The ATeam-transgenic lines (four lines for cytosol and two lines for mitochondria) showed similar properties to wild-type N2, including lifespan, body size, and swimming cycles. The expression of ATeam in either cytosol or mitochondria target cells of L1 nematodes was directly confirmed by observation using fluorescence microscopy, as shown in Figure 1A. Unfortunately, intrinsic fluorescence in intestines of adult worms significantly overlapped with ATeam fluorescence in body muscle cells (see Fig. 1B). Thus, the authors measured the YFP/CFP ratio of ATeam in vulval cells, where intrinsic fluorescence is negligible. As shown in Fig. 1B, both CFP and YFP fluorescence in vulval cells were clearly observed in both cytosol and mitochondria. In L4 stage nematodes, the cytosolic YFP/CFP ratio was 1.98 ± 0.13. Interestingly, the mitochondrial YFP/CFP ratio appeared to be lower than that in cytosol, 1.68 ± 0.24. This is consistent with the results in cultured mammalian cells, where ATP levels in mitochondria are significantly lower than in cytosol (Imamura et al., 2009). In senescent nematodes, it was difficult to measure the YFP/CFP ratio accurately in both cytosol and mitochondria, and even in vulval cells, because intrinsic fluorescence in the cells significantly increased. In day 8 nematodes, the YFP/CFP ratio in both cytosol and mitochondria was found to be roughly equal to that in L4. The FRET in mitochondria of day 8 nematode was slightly lower than that in cytosol.

### FRET of ATeam in Primary Culture Cells of Nematode

To investigate whether ATeam can be used to report ATP levels in nematode cells, the authors treated the ATeam-transgenic nematodes with 2-deoxyglucose (2DG) and cyanide, inhibitors that are commonly used to alter ATP levels in culture cells (Imamura et al., 2009). The treated nematodes appeared to exhibit reduced movement but no significant change in ATeam FRET could be observed in either cytosol or mitochondria of nematode vulval cells (data not shown). The authors do not know the precise reason why the ATeam in nematode cells responded poorly to treatment with these inhibitors, as has been observed in cultured cells. One possible reason is that the nematode cuticle may act as a barrier for entry of sufficient inhibitors into the target cells. Therefore, to evaluate the function of ATeam in nematode cells, the authors prepared primary culture cells from the transgenic nematode embryo that express ATeam in cytosol. As shown in Fig. 2A, the cells expressing DsRed (colored as red) or ATeam (colored as blue) were easily identified under a fluorescent light field. After treatment with 2DG and KCN for 10 min, a dramatic change of FRET in cytosol was observed (Fig. 2B). The FRET in treated cells decreased to half of the level observed in nontreated cells, suggesting that the ATP levels in the cytosol of the target cells decreased owing to 2DG–KCN treatment. Conversely, the fluorescence intensity of DsRed in pharynx cells was not affected by the same treatment. Taken together, it can be concluded that ATeam in nematode cells senses the ATP levels as observed in cultured mammalian cells (Imamura et al., 2009).

## DISCUSSION

The authors directly investigated ATP levels in nematode cells using the fluorescent ATP biosensor ATeam. The authors expressed the ATeam in nematodes using an *unc-54* promoter, which induces protein expression predominantly in the body and vulval muscle cells. ATeam was clearly expressed in target cells in L4 stage nematodes (Fig. 1). The authors prepared primary cul-

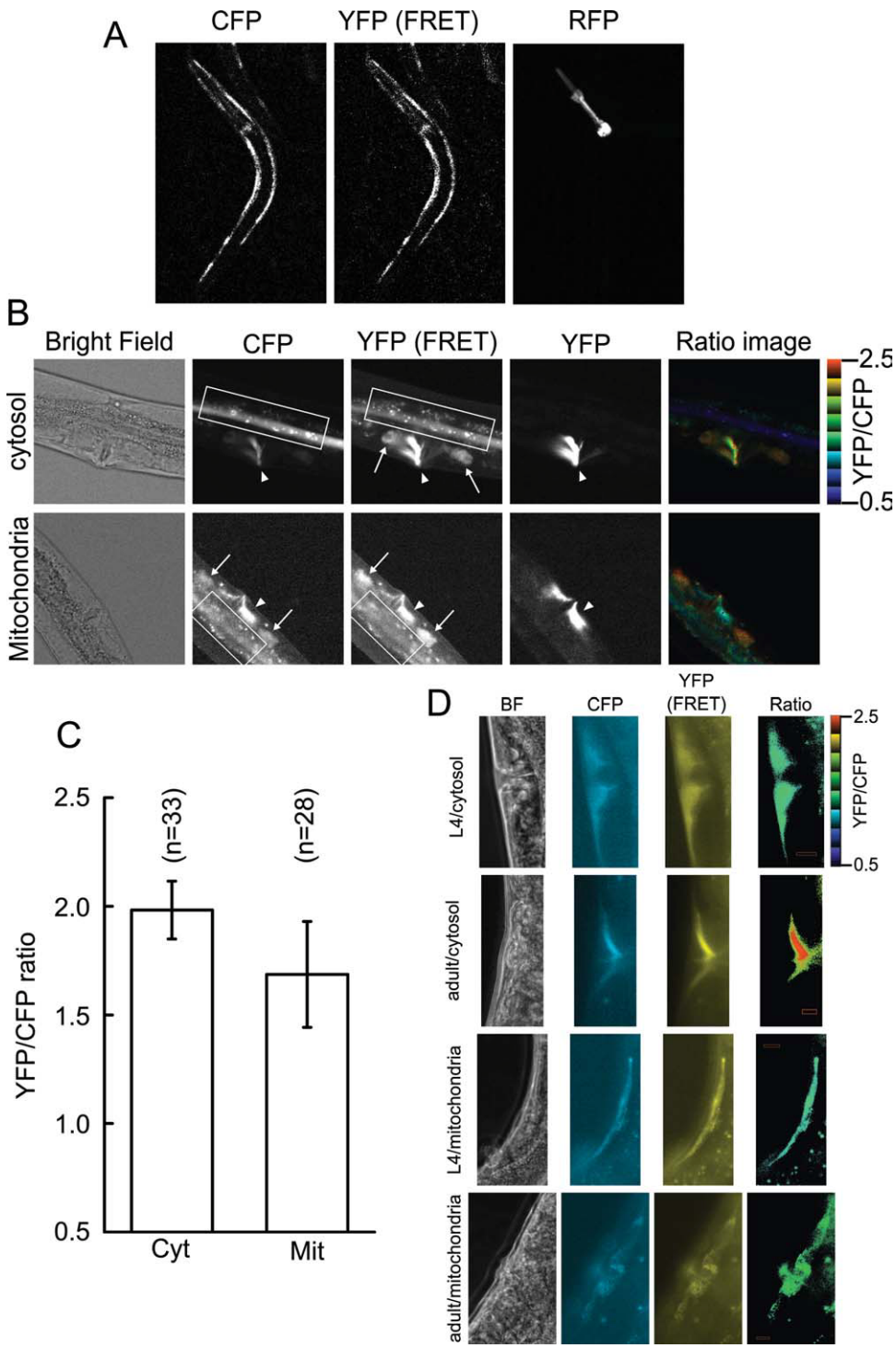


Fig. 1. Expression of ATeam protein in nematode cells. (**A**) Overall images for L1 nematode of CFP (435/485), YFP (FRET) (435/538), and DsRed (540/570) from left. The numbers shown in parentheses are excitation/emission wavelengths, respectively. (**B**) The images of the transgenic L4 nematode. ATeam was expressed in the cytosol and mitochondria of vulval muscle cells (arrowhead). Left column: bright field, fluorescence of CFP, YFP (FRET), YFP (495/538), and ratiometric pseudo-color images. The intrinsic fluorescence in intestine is indicated by arrows and boxes. (**C**) Comparison of YFP/CFP emission ratio between cytosol and mitochondria of vulval muscle cells. The ratio was calculated from the fluorescent images. The number of images used for calculating the ratio is indicated in the column. Error bars represent standard deviation of ratios. $P$-value $<0.001$, Student's $t$-test. (**D**) The images of the transgenic 8-day-old nematode. Left column: bright field, fluorescence of CFP, YFP (FRET), and ratiometric pseudo-color images. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

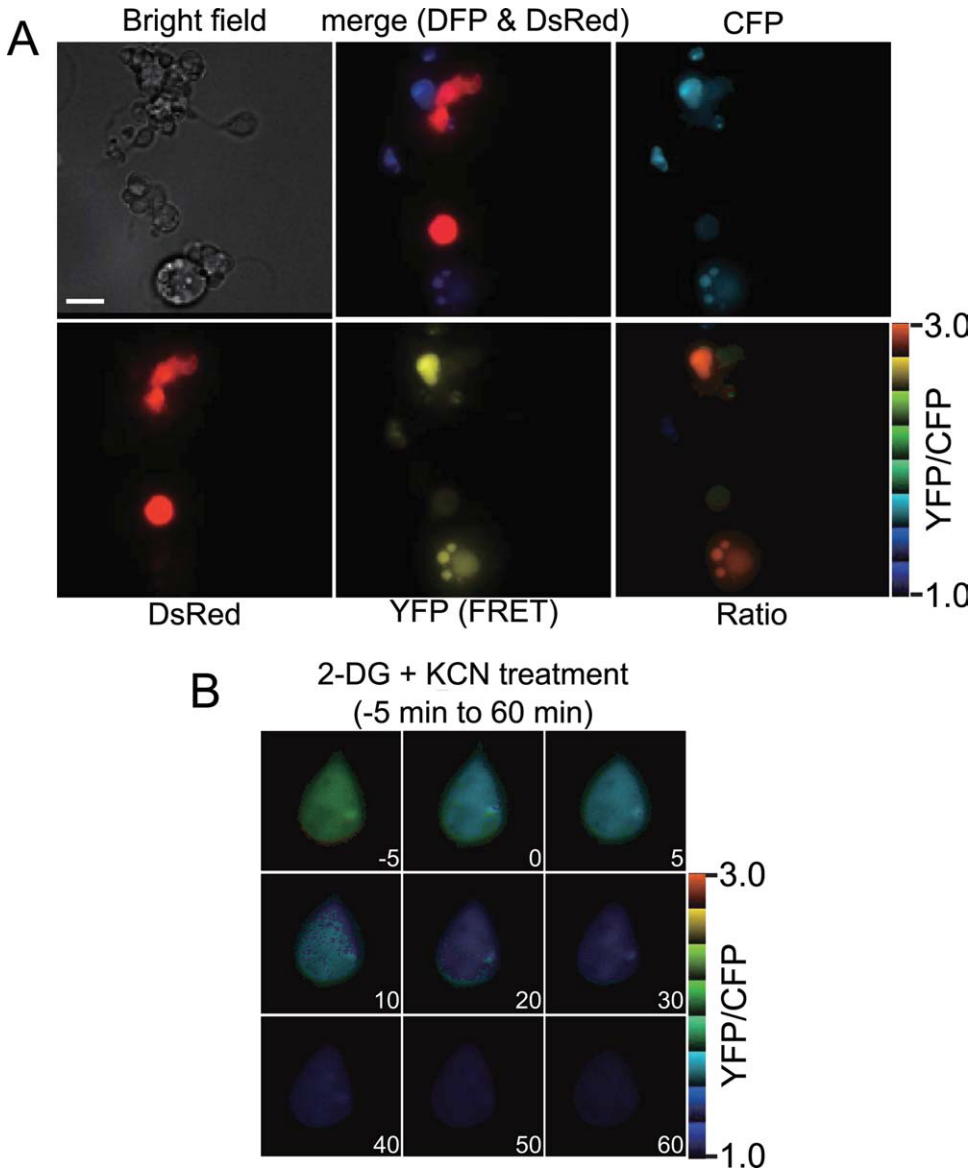


Fig. 2. Expression and sequential monitoring of ATeam in nematode cultured cells. (**A**) Wide-field images of nematode cultured cells expressing ATeam in cytosol (scale bar, 10 μm). The nematode embryos expressing transgenes of DsRed in the pharynx and ATeam in muscle cells were separated in terms of cells by chitinase treatment. The separated cells were seeded onto a dish containing culture medium and cultured for 2 days. (**B**) Sequential images of YFP/CFP emission ratio of nematode cultured cell expressing ATeam. Inhibitors of glycolysis (2DG, 2-deoxyglucose) and oxidative phosphorylation (KCN, potassium cyanide) were added at time = 0 (min). Elapsed time (min) after addition of the inhibitors is shown at the bottom right of the cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ture cells of the transgenic nematode to evaluate the performance of ATeam in nematode cells. Indeed, the FRET of ATeam in nematode cytosolic cells was apparently reduced in response to treatment with both 2DG and KCN, in which ATP synthesis had been blocked by both glycolysis and oxidative phosphorylation (Fig. 2B). This indicates that the ATeam biosensor can be applied to nematode as well as to mammalian cells.

The authors observed a clear expression pattern of ATeam in L4 young adult nematode. However, ATeam fluorescence decayed with increasing stages of aging and was disturbed by intrinsic fluorescence, which also increases during aging. In conclusion, it is difficult to measure fluorescence of ATeam in body muscle cells. Thus, the authors chose to measure ATeam FRET in the vulva, which contains a cluster of eight muscle cells. ATeam fluorescence could be observed without interference by intrinsic fluorescence in intestines in day 8 nematodes. As shown in Fig. 1B, the ATP levels of the cytosol in vulval cells were apparently higher than those in the mitochondria, as is the case in Hela cells (Imamura et al., 2009). In contrast, no sign of changes in ATP levels due to aging was observed. It was reported that the affinity of ATeam against ATP at

25°C is much higher than that at 37°C. Thus, it is possible that the FRET signal of ATeam does not respond to ATP level change due to aging at 25°C. Further studies are required to investigate age-related changes in ATP levels in several kinds of tissue cells.

In this study, the authors introduced ATeam into *C. elegans* and detected the difference of ATP level between cytosol and mitochondria in their body muscle cells. It was also observed that ATeam FRET changed by treatment of respiration and glycolysis inhibitors in *C. elegans* primary culture cells. These results indicate that ATeam can potentially be an useful tool for monitoring of cellular ATP level in vivo. Thus, these studies will help to shed light on the relationship between ATP production/consumption, that is, energy metabolism and its regulatory systems.

## ACKNOWLEDGMENTS

We thank Hitomi Moriya for technical advice and support relating to *C. elegans* manipulations. We also thank Yoshiko Hori and Masako Mori for technical assistance.

## REFERENCES

Ashcroft FM. 2005. ATP-sensitive potassium channelopathies: Focus on insulin secretion. J Clin Invest 115:2047–2058.

Evans TC. 2006. Transformation and microinjection. In: The *C. elegans* Research Community, editors. WormBook, doi/10.1895/wormbook. 1.7.1. Available at: http://www.ncbi.nlm.nih.gov/books/NBK19648/.

Feng Z, Levine AJ. 2010. The regulation of energy metabolism and the IGF-1/mTOR pathways by the p53 protein. Trends Cell Biol 20:427–443.

Finger TE, Danilova V, Barrows J, Bartel, Dianna L, Vigers AJ, Stone L, Hellekant G, Kinnamon SC. 2005. ATP signaling is crucial for communication from taste buds to gustatory nerves. Science 310:1495–1499.

Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H. 2009. Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. Proc Natl Acad Sci USA 106: 15651–15656.

Mitani S. 1995. Genetic regulation of mec-3 gene expression implicated in the specification of the mechanosensory neuron cell types in *Caenorhabditis elegans*. Dev Growth Differ 37:551–557.

Xie L, Wang DI. 1996. Energy metabolism and ATP balance in animal cell cultivation using a stoichiometrically based reaction network. Biotechnol Bioeng 52:591–601.

Yokoyama K, Imamura H. 2005. Rotation, structure, and classification of prokaryotic V-ATPase. J Bioenerg Biomembr 37:405–410.

Yoshida M, Muneyuki E, Hisabori T. 2001. ATP synthase – A marvellous rotary engine of the cell. Nat Rev Mol Cell Biol 2:669–677.