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Abstract. Several studies demonstrated that the cyclic adenosine monophosphate (cAMP), an important second messenger, is involved in the mechanism of low-level laser irradiation (LLLI) treatment. However, most of these studies obtained the cAMP level in cell culture extracts or supernatant. In this study, the cAMP level in living cells was measured with bioluminescence resonance energy transfer (BRET). The effect of LLLI on cAMP level in living cells with adenosine receptors blocked was explored to identify the role of adenosine receptors in LLLI. The results showed that LLLI increased the cAMP level. Moreover, the rise of cAMP level was light dose dependent but wavelength independent for 658-, 785-, and 830-nm laser light. The results also exhibited that the adenosine receptors, a class of G protein-coupled receptor (GPCR), modulated the increase of cAMP level induced by LLLI. The cAMP level increased more significantly when the A_3 adenosine receptors (A_3R) were blocked by A_3R antagonist compared with A_1 adenosine receptor or A_{2a} adenosine receptor blocked in HEK293T cells after LLLI, which was in good agreement with the adenosine receptors' expressions. All these results suggested that measuring the cAMP level with BRET could be a useful technique to study the role of GPCRs in living cells under LLLI. [DOI: 10.1117/1.JBO.20.5.051029]

Keywords: low-level laser irradiation; cyclic adenosine monophosphate; HEK293T cells; adenosine receptor; bioluminescence resonance energy transfer.

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1 Introduction

Low-level laser irradiation (LLLI) treatment has been studied for several decades. Much research has demonstrated that LLLI can be used to promote wound healing,[1,2] and pain relief,[3,4] and to treat various diseases such as recurrent herpes simplex infection[5] and inflammatory conditions.[6–8] Although the effects of LLLI have been proven to be positive, some studies imply that the effect of LLLI is still controversial. The controversy is probably due to two factors: (1) the complexity of choosing the optical parameters such as wavelength, fluence, and power density; and (2) the mechanisms of LLLI are still not fully understood.[9]

A number of studies have investigated the cellular or molecular mechanisms of LLLI.[10–12] Mitochondrion is considered widely as one of the most important organelles, which plays a central role in LLLI.[13,14] The photon energy absorbed by the cytochrome c oxidase in mitochondrion, the primary photoacceptor, could be transformed to other forms of energy such as chemical energy.[15] In addition to energy transforming, mitochondrial signaling is also crucial in LLLI. Mitochondrial signaling is an information channel between the mitochondrial respiratory chain and nucleus.[13] This signaling could be modulated through mitochondrial membrane potential, generation of reactive oxygen species, calcium flow, cyclic adenosine monophosphate (cAMP), and so on.[13,16] The cAMP, an important second messenger, can regulate many kinds of biological processes including cell division,[17] axon regeneration,[18–20] learning,[21] memory,[22] and metabolism.[23]

Several studies proved that the cAMP is involved in the mechanisms of LLLI. Karu et al.[24] pioneered the measurement of cAMP content in Chinese hamster fibroblasts under light irradiation. Hu et al.[25] measured the cAMP level of the cell extracts by lysing and found that He-Ne laser irradiation stimulates cell proliferation, which is relative to the increase of the mitochondrial membrane potential, adenosine triphosphate (ATP), and cAMP. Lima et al.[26] got the cAMP level of supernatant of alveolar macrophages and also found that LLLI raises the cAMP level in acute respiratory distress syndrome. Zungu et al.[27] studied the effect of LLLI on the cAMP level in normal and wounded cells. The results showed that LLLI significantly enhanced the cAMP level in wounded cells but not in normal cells. Recently, Wu et al.[28] investigated the physiological effects of LLLI on the proliferation and osteogenic differentiation of human periodontal

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ligament (hPDL) cells and pointed out that the cAMP is an important regulator of the LLLI effects on hPDL cells. They also demonstrated that LLLI suppresses NF-kB transcriptional activity in human adipose-derived stem cells by increasing the cAMP level.[29] However, most of these studies obtained the cAMP level in nonphysiological conditions (cell culture extracts or supernatant).

We are interested in exploring cAMP plasmid assay for measuring the cAMP level in living cells with bioluminescence resonance energy transfer (BRET). The assay uses genetically encoded biosensor variants with cAMP binding domains fused to mutant forms of luciferase. Upon binding to cAMP, conformational changes occur that promote large increases in light output.[30–32] This can be utilized to study the function of G protein-coupled receptors (GPCRs) because the cAMP signal pathway involves the GPCR. Adenosine receptors, a class of GPCR, are activated by adenosine to take part in the physiologies, such as wound healing,[33–35] inflammation,[36,37] and pain,[38,39] which are closely relevant to low-level laser therapy. To the best of our knowledge, few studies characterize the modulation of adenosine receptors on the cAMP dynamics induced by LLLI.

In this study, we measured the cAMP level of human embryonic kidney cell line 293T (HEK293T) cell with BRET. The effects of LLLI on the cAMP level in living cells were explored. Moreover, the roles of adenosine receptors in the modulation of cAMP dynamics induced by LLLI were also investigated.

2 Materials and Methods

2.1 *Chemicals*

The cAMP plasmid, cAMP reagent stock solution, and FuGENE HD transfection reagent were obtained from Promega (Madison, Wisconsin). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, Missouri).

2.2 *Cell Culture and Transfection*

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under humidified air containing 5% CO_2. The experiment of HEK293T cells transfection was performed according to the cAMP assay technical manual. In brief, HEK293T cells were harvested and suspended at a density of 1.5×10^5 cells/ml in growth medium (90% DMEM and 10% FBS) and were seeded into 96-well flat bottom plates by 100 μl/well. Thus, the number of cells in the plate is 1.5×10^4 cells/well. The plates were placed into a 37°C culture incubator with 5% CO_2 overnight. The cAMP plasmid was diluted to a final concentration of 12.5 ng/μl in Opti-MEM I reduced-serum medium (Invitrogen). Then, FuGENE HD transfection reagent (6 μl) was added to the diluted cAMP plasmid (160 μl) and mixed carefully by gentle pipetting. The complex (166 μl) was sufficient for 20 wells. After incubating for 15 min at room temperature, 8 μl of complex per well was added to 96-well plates and gently mixed without disturbing the cell monolayer. The 96-well plates were incubated for 24 h in a 37°C culture incubator with 5% CO_2. The medium in 96-well plates was removed carefully and replaced with 100-μl equilibration medium. The equilibration medium contains 88% CO_2-independent medium (Invitrogen), 10% FBS, and 2% cAMP reagent stock solution. The 96-well plates were incubated for 2 h at room temperature or until a steady-state basal signal was obtained.

2.3 *Laser Irradiation*

Three LQC laser diode modules (658, 785, and 830 nm, Newport Corporation, Irvine, California) were chosen as the light sources for LLLI. The laser beam was expanded by a lens and then reflected by a mirror to irradiate the cells in 96-well plates. The laser output was measured before and after laser irradiation by a laser power meter (Coherent, Wilsonville, Oregon) to check the stability of the laser output. The laser power densities on the irradiation surface were 20, 10, and 40 mW/cm^2 for 658-, 785-, and 830-nm laser diode modules, respectively. Different light doses were obtained by adjusting the irradiation duration. Laser irradiation was performed in a dark room at room temperature.

2.4 *Measurement of cAMP Level*

A microplate fluorescence reader (Mithras LB940, Berthold, Germany) was utilized to measure the luminescence of cAMP reagent, which indicates the level of cAMP in living cells. The counting time was set as 1 s for obtaining a good signal-to-noise ratio. Adenosine receptors' antagonists and other reagents were automatically injected into each well with injectors.

2.5 *Detection of Adenosine Receptors Using Laser Scanning Confocal Microscope*

HEK293T cells were fixed with 4% paraformaldehyde for 1 h at room temperature. After being washed, cells were permeabilized with 0.3% Triton X-100 in 0.1 M phosphate buffer for 10 min. Cells were blocked in normal goat serum for 30 min and incubated with primary antibodies (1:20) overnight at 4°C. Then, cells were washed and incubated with Fluorescein Isothiocyanate-conjugated secondary antibodies (1:50) for 2 h at room temperature. At last, cells were mounted with Ultra Cruz TM Mounting Medium (sc-24941, Santa Cruz) and imaged with a laser scanning confocal microscope (Leica SP8, Germany).

2.6 *Measurement of the Temperature Distribution Induced by Laser Irradiation*

The temperature distribution was measured with an infrared camera (Varioscan 3021, Germany). The temperature resolution is 0.03 K.

3 Results and Discussion

3.1 *Characteristics of the cAMP Reagent*

To confirm the transfection and to obtain the cAMP reagent's characteristics, we continuously monitored the cAMP luminescence signals of the blank control group (without transfection), control group (transfection), and forskolin group (transfection and adding forskolin to increase the cAMP level). There was no luminescence signal in the blank control group due to no transfection (data not shown). The luminescence signal of the control and forskolin groups was normalized by the initial value to reduce the influence of differences among wells. It is interesting that the normalized luminescence intensity of the control group increased for a while and then decreased

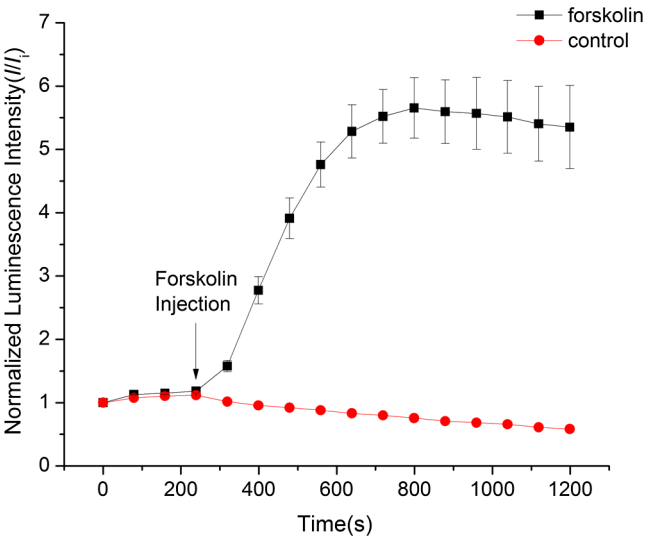

Fig. 1 The normalized luminescence intensity changed with time. I_i represents the initial luminescence intensity. Forskolin was injected into wells by injector automatically. The final concentration of forskolin was 10 μM. The results were presented as the mean $\pm$ SD, $n = 6$.

gradually, as shown in Fig. 1. This is due to the differences of temperature between the environment and the microplate fluorescence reader. An increasing in temperature can decrease the luminescence intensity of the cAMP reagent, because the distance between the donor and acceptor will increase when the temperature rises. The temperature in the microplate fluorescence reader was higher than the room temperature. Therefore, the normalized luminescence intensity decreased when the 96-well microplate was placed in the microplate fluorescence reader. We noted that the change of the luminescence intensity with temperature was reversible (data not shown). To verify the validity of the measurement, further forskolin, which can activate adenylate cyclase to increase the cAMP level, was automatically injected into each well. The normalized luminescence intensity increased rapidly and up to sixfold after the injection of 10-μM forskolin. These indicated that the transfection experiment was carried out in the correct way and that the system was sensitive for measuring the cAMP level in living cells.

3.2 *Temperature Distribution Induced by Laser Irradiation*

The cAMP reagent is temperature sensitive. To check the contribution of the laser irradiation on temperature change, a high-resolution infrared camera was used to measure the temperature distribution. The temperature at the center of the irradiation area, indicated by "+" in Fig. 2, increased gradually during laser irradiation. However, the maximum increment of the temperature was only 0.6 K [Fig. 2(b)] after being irradiated for 3 min by 40 mW/cm^2, 830-nm laser light. Moreover, the temperature decreased when irradiation was stopped. It returned to the initial value quickly [Fig. 2(d)]. Therefore, the contribution of LLLI on the temperature variation could be neglected.

3.3 *cAMP Dynamics After Laser Irradiation*

In order to study the effect of laser irradiation on the dynamics of cAMP, we recorded the cAMP level every 30 s after the laser irradiation. The laser irradiation experiments were performed using an 830-nm laser diode providing a 40 mW/cm^2 power density. Two groups of samples were irradiated: one for 30 s and another for 60 s. A control group did not receive any laser irradiation, and its cAMP level was also measured at the corresponding time points for the other two groups. All the samples (all three groups) were put on the same 96-well plate, so that they were always at the same temperature and humidity conditions during the experiment. After the irradiation, the samples were placed in the microplate fluorescence reader and the cAMP signal levels were then monitored continuously as a function of time. The results are shown in Fig. 3. The normalized luminescence intensity of the control group decreased gradually similar to what is shown in Fig. 1. Unlike the control group, the

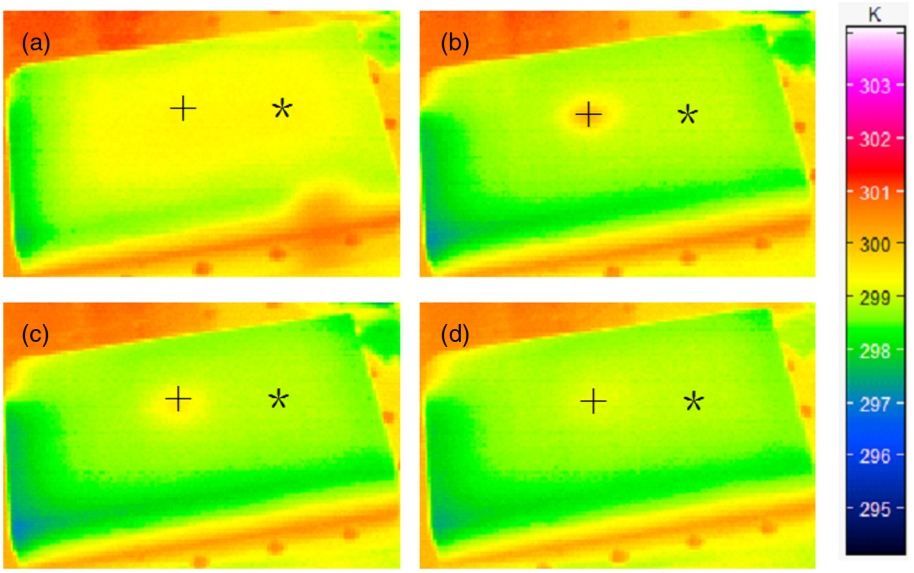

Fig. 2 Temperature variation induced by laser irradiation: (a) before laser irradiation; (b) after irradiating for 3 min; (c) 1 min after irradiation was stopped; and (d) 3 min after irradiation was stopped. Laser parameters: 830 nm and 40 mW/cm^2. + indicates the irradiation spot, and * indicates the nonirradiation area.

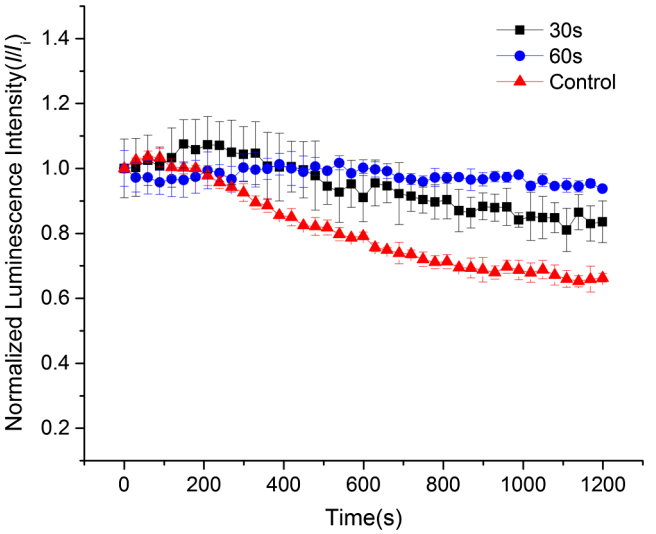

Fig. 3 Laser irradiation inhibited the cAMP level. I_i represents the initial luminescence intensity. The wavelength of the laser is 830 nm, and the power density is 40 mW/cm^2. Irradiation duration was 30 and 60 s, respectively. The results were presented as the mean ± SD, $n = 6$.

cellular cAMP level of the irradiation groups remained largely unchanged. This indicates that the LLLI could raise the cAMP level, which is consistent with previous reports.[26,29]

The increase of cAMP level may be due to the increase of ATP induced by LLLI. Numerous studies demonstrated that LLLI can enhance the ATP synthesis.[40–44] ATP can be hydrolyzed sequentially to adenosine diphosphate (ADP), adenosine monophosphate (AMP), and finally adenosine.[45] Increasing ATP and adenosine could raise the cAMP level indirectly by activating the adenylate cyclase.[45]

For studying the effect of laser wavelength on the modulation of the cAMP level, 658-, 785-, and 830-nm lasers were used to irradiate the cells. As the power densities are different between

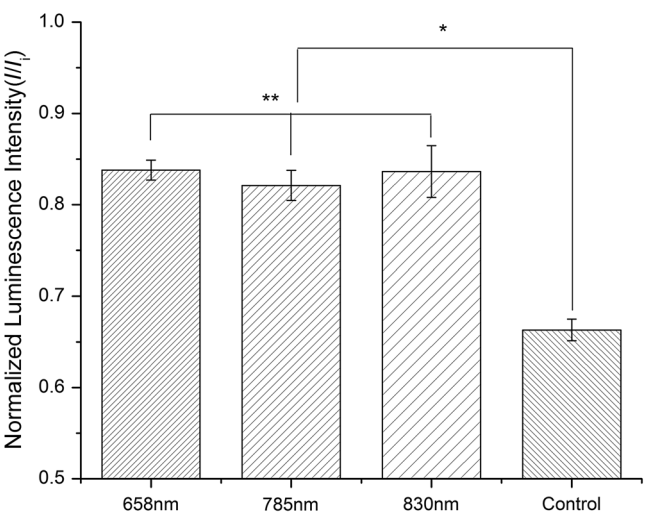

Fig. 4 The effect of laser wavelength on the modulation of cAMP level. There were significant differences between the control group and any of the three laser irradiation groups for the cellular cAMP level at 20 min after laser irradiation ($*p < 0.01$). There were no significant differences among the three laser irradiation groups ($**p > 0.05$). The light doses of the three lasers are the same, 456 mJ. The results were presented as the mean ± SD, $n = 6$.

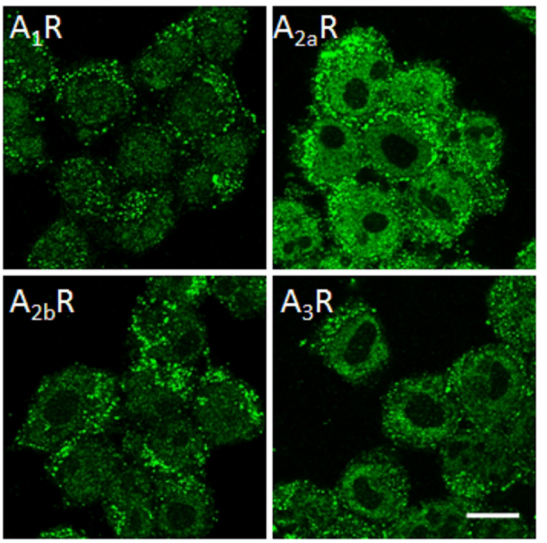

Fig. 5 Images of adenosine receptors (A_1R, $A_{2a}R$, $A_{2b}R$, and A_3R) on HEK293T cells by confocal microscopy. Scale bar = 10 μm.

the three lasers, which are 20, 10, and 40 mW/cm^2, to obtain the same light dose, we set the irradiation duration as 1, 2, and 0.5 min, respectively. The normalized luminescence intensities after laser irradiation were compared to evaluate the wavelength effect. Figure 4 clearly shows that the normalized luminescence intensities at 20 min after laser irradiation were greater than that of control group ($*p < 0.01$). However, there were no significant differences among the three laser irradiation groups ($**p > 0.05$).

3.4 *Role of Adenosine Receptors in the Modulation of cAMP*

There are four subtypes of adenosine receptors, which are A_1R, $A_{2a}R$, $A_{2b}R$, and A_3R. Immunofluorescence was used to check the expressions of the four subtypes of adenosine receptors on HEK293T cells. Figure 5 shows that all the four subtypes of

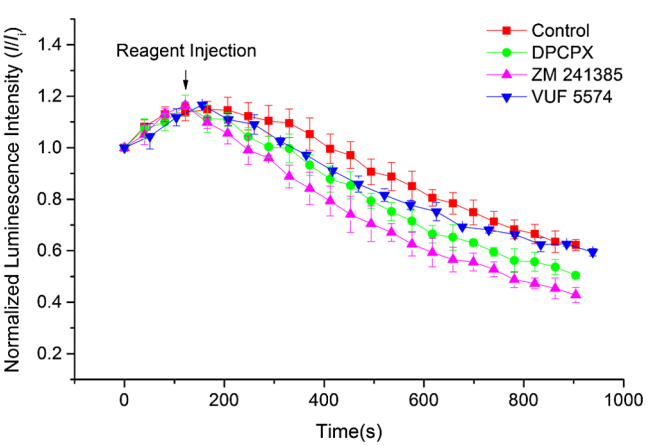

Fig. 6 Effect of adenosine receptors' antagonists on the cellular cAMP level. There was no difference between the control group and the VUF 5574 group at the end of measurement. There were differences between the control group and DPCPX and ZM 241385 groups. The results were presented as the mean ± SD, $n = 6$. DPCPX, A_1R antagonist; ZM 241385, $A_{2a}R$ antagonist; and VUF 5574, A_3R antagonist.

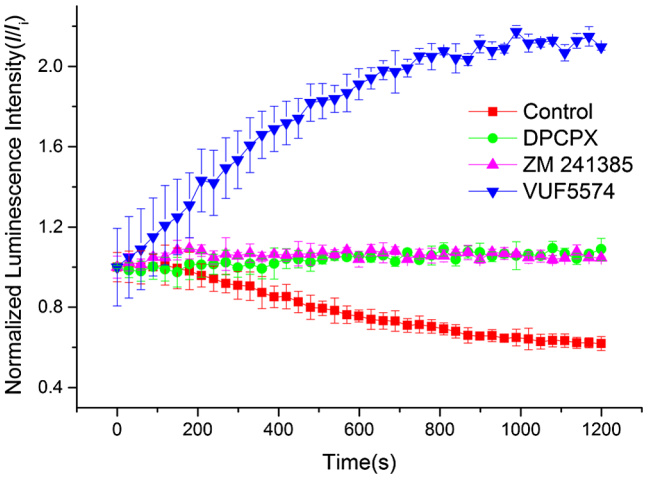

Fig. 7 Effects of adenosine receptors' antagonists on the increase of the cAMP level in HEK293T cells irradiated by laser. The concentrations of these reagents were all 10μM. The results were presented as the mean $\pm$ SD, $n = 6$. DPCPX, A_1R antagonist; ZM 241385, $A_{2a}R$ antagonist; and VUF 5574, A_3R antagonist.

adenosine receptors expressed on HEK293T cells. However, their expressions were different. $A_{2a}R$ had the strongest expression, whereas A_1R had the weakest expression. $A_{2b}R$ and A_3R had similar expressions.

To explore the role of adenosine receptors on the increase of cAMP level induced by laser irradiation, we applied the A_1R, $A_{2a}R$, and A_3R antagonists before laser irradiation. We first studied the effects of the receptor antagonists alone on the cAMP level. A_1R antagonist (DPCPX), $A_{2a}R$ antagonist (ZM 241385), and A_3R antagonist (VUF 5574) were automatically injected into the 96-well plate. There were no significant differences between the VUF 5574 group and the control group at the end of measurements, while there were a few differences between the DPCPX group, ZM 241385 group, and the control group, as shown in Fig. 6.

Then, the cells applied with adenosine receptor antagonists for 20 min were irradiated by an 830-nm laser for 3 min per well. The power density was 40 mW/cm^2. As shown in Fig. 7, laser irradiation could increase the cAMP level of the three groups of HEK293T cells injected with DPCPX, ZM 241385, and VUF 5574 compared with the control group. There were no differences between the groups of DPCPX and ZM 241385; however, laser irradiation increased the cAMP level of the VUF 5574 group more than DPCPX or ZM 241385 group. These results were in good agreement with the adenosine receptors expressions on HEK293T cells. The four subtypes of adenosine receptors are GPCRs with seven transmembrane domains. A_1R and A_3R preferably interact with members of the G_i family and inactive adenylate cyclase to decrease the production of cAMP, whereas $A_{2a}R$ and $A_{2b}R$ are coupled to the G_s family and stimulate adenylate cyclase to increase the production of cAMP.[46] Since A_1R was scarce in the HEK293T cells, when the A_3R was blocked by the antagonists VUF 5574, the increasing adenosine induced by LLLI would activate the $A_{2a}R$ and $A_{2b}R$ to stimulate the adenylate cyclase to raise the cAMP level.

4 Conclusions

This study revealed that LLLI could increase the cAMP level in living cells. The rise of the cAMP level was in evidenced by a dose-dependent and wavelength-independent manner for the 658-, 785-nm, and 830-nm laser lights. The adenosine receptors took part in the modulation of the cAMP level in living cells under LLLI. These results suggest that measuring the cAMP level with BRET could be a useful technique to study the role of GPCRs in living cells under LLLI.

Acknowledgments

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