

# Chapter 3

## Assessment of Extracellular ATP Concentrations

Lucia Seminario-Vidal, Eduardo R. Lazarowski, and Seiko F. Okada

### Abstract

Most cells release ATP to the extracellular milieu. Extracellular ATP plays important signaling roles by activating a score of broadly distributed cell surface purinergic receptors (purinoceptors). Biological responses regulated by purinergic receptors include neurotransmission, smooth muscle relaxation and contraction, epithelial cell ion transport, inflammation, platelet activation, immune responses, cardiac function, endocrine and exocrine secretion, glucose transport, and cell proliferation. ATP concentrations at the cell surface, and consequently the magnitude of purinergic receptor stimulation, reflect a well-controlled balance between rates of ATP release and extracellular metabolism. Given the broad spectrum of responses triggered by extracellular ATP, there is a growing interest in accurately assessing the concentrations of this nucleotide at the cell surface. In this chapter, we discuss the use of the luciferin/luciferase-based reaction to measure extracellular ATP concentrations with high sensitivity. Protocols are adapted to assess ATP levels either in sampled extracellular fluids or in situ at the cell surface. Although our focus is on studies of ATP release from epithelial cells, protocols described here are applicable to practically all cell types.

**Key words:** ATP release, extracellular ATP, ecto-ATPase, luciferase, protein A-luciferase, luciferin.

**Abbreviations:** 6 × His: hexa-histidine, ALU: arbitrary light unit, ARL-67156: 6-*N,N*-diethyl- $\beta,\gamma$ -dibromomethylene-D-ATP,  $\beta,\gamma$ -metATP:  $\beta,\gamma$ -methylenadenosine 5'-triphosphate, BSA: bovine serum albumin, DMEM: Dulbecco's modified eagle's medium, ebselen: 2-phenyl-1,2-benzisoxaselenazol-3(2H)-one, FBS: fetal bovine serum, HBSS: Hank's balanced salt solution, HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, MEM: minimum essential medium, PBS: phosphate-buffered saline, RT: room temperature, SPA-luc: *Staphylococcus* protein A-fused luciferase.

---

### 1. Introduction

ATP is an essential component of living cells. ATP is the major source of energy in most biosynthetic processes, participates as co-factor or activator of numerous enzymatic reactions, and is a

building block of nucleic acid chains. In addition, ATP is released from cells in a regulated manner to accomplish autocrine and paracrine functions via the activation of cell surface purinergic receptors (purinoceptors) (1). Purinoceptors consist of three widely distributed families: P2X, P2Y, and P1 receptors. Purinoceptor-mediated responses include cell proliferation, migration, differentiation, embryonic development, wound healing, restenosis, atherosclerosis, ischemia, turnover of epithelial cells in skin and visceral organs, inflammation, neuroprotection, and cancer (1). P2X receptors are ligand-gated cation channels. They include seven molecularly defined species (P2X<sub>1</sub>–P2X<sub>7</sub>), all of which are selectively activated by ATP, but not by other nucleotides (2). The P2Y receptor family includes eight G protein-coupled receptors: the P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> receptors. The P2Y<sub>2</sub> receptor is potently activated by ATP (and UTP). The P2Y<sub>11</sub> receptor is selectively activated by ATP, whereas the P2Y<sub>12</sub> and P2Y<sub>13</sub> receptors are most potently and selectively activated by ADP. P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>14</sub> receptors are activated selectively by uridine nucleotides and UDP-sugars (1). The P1 receptor family (A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> adenosine receptors) is activated by the nucleoside adenosine, the final product of ATP dephosphorylation.

Cellular ATP is released both in the absence of external stimuli and in response to physiological stimuli. Levels of extracellular ATP are controlled by a complex array of nucleotide-metabolizing cell surface enzymes, which include ecto-nucleotidases of the ecto-nucleotide triphosphate diphosphohydrolase (eNTPDase) and ecto-nucleotide pyrophosphatase (eNPP) families, 5'-nucleotidase (5'-NT), non-specific phosphatases, and transphosphorylating enzymes, such as nucleoside diphosphokinase and adenylyl kinase (3). Thus, extracellular ATP concentrations and, consequently, ATP, ADP, and adenosine actions on purinergic receptors, are dynamically regulated via cellular release and extracellular metabolism of ATP (4).

Given the physiological importance of purinergic signaling, there is an increased interest in assessing nucleotide concentrations on the surface of cells and tissues and in understanding the mechanisms of cellular ATP release. Numerous approaches have been developed in recent years to assess extracellular levels of ATP and other nucleotides (reviewed in (5)). Several factors complicate the accurate measurement of extracellular ATP concentrations. For example, it is difficult to assess ATP concentrations in the physiologically relevant unstirred film covering the cell surface. Moreover, robust ATP release occurs in response to mechanical stress; thus, experimental maneuvers (cell wash, sampling, transporting the cell dishes) often result in artifacts. Finally, rapid hydrolysis of released ATP may compromise the relevance of ATP measurements.

In this chapter, we will describe approaches to measure ATP concentrations in sampled, diluted extracellular fluids, as well as in cell surface thin films (**Fig. 3.1**). We will focus on epithelial cells as examples; however, these methods are applicable to all cell types.

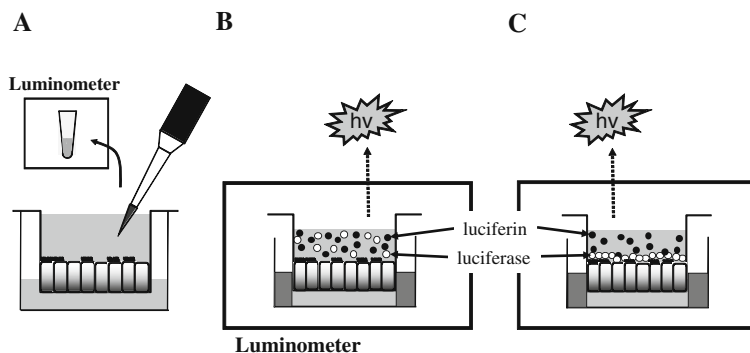


Fig. 3.1. Off-line and real-time approaches to measure extracellular ATP concentrations. Extracellular ATP concentrations can be measured by off-line luminometry of sampled extracellular fluids (**A**), or on-line luminometry using either soluble luciferase dissolved in medium covering the cells (**B**) or cell –surface-attached luciferase (**C**). ATP concentrations detected by each method in different volumes are illustrated in **Figs. 3.2 and 3.3**.

### 1.1. Measuring ATP Concentrations in Sampled Fluids: Off-Line Bioluminescence Detection

This section describes a protocol that uses the luciferin/luciferase-based reaction (*see Note 1*) to quantify ATP concentrations in samples obtained from cell culture-conditioned media. In epithelial and endothelial cells, robust ATP release can be triggered by mechanical stimuli such as shear stress, stretch, compression, and hypotonicity-induced cell swelling (4, 6–9). Here, we will use hypotonicity-induced ATP release as an example. ATP release can also be measured after inhibition of ATP metabolism. Commonly used inhibitors of ecto-nucleotidase activities are  $\beta,\gamma$ -methylenadenosine 5-triphosphate ( $\beta,\gamma$ -metATP), 6-*N,N*-diethyl- $\beta,\gamma$ -dibromomethylene-D-ATP (ARL-67156), and 2-phenyl-1,2-benziselenazol-3(2H)-one (ebselen) (4, 8–11). Levamisole has been used to inhibit alkaline phosphatase activity present on epithelial cells (4). In this example, we obtained maximal inhibition of ATP metabolism in A549 cell cultures by using a cocktail of  $\beta,\gamma$ -metATP and ebselen. After stimulation of the cells and/or inhibition of ATP metabolism, the conditioned media are analyzed for ATP concentration. Briefly, samples are collected gently to minimize unwanted mechanical release of ATP, boiled to abolish ATPase activities potentially present in the extracellular solution, and transferred to the dark chamber of a luminometer. The luciferase/luciferin cocktail is added by an automatic injector, and the resulting luminescence is recorded (**Fig. 3.1A**).

The methodology described here is applicable to ATP measurements in tissue extracts, biological fluids, bacterial cultures, in vitro enzymatic reactions, etc.

## 1.2. Real-Time, Cell Surface Measurement of Extracellular ATP

In this section, we describe methods for real-time measurement of ATP by using cell surface-bound luciferase (**Fig. 3.1C**), and will compare this method with measurements obtained with soluble luciferase (**Fig. 3.1B**). The protocols below are designed for measuring luminal ATP concentrations on polarized epithelial cells; however, they are also applicable to measuring extracellular ATP concentrations of non-polarized cells grown on culture plates. Cell surface-binding luciferase can be engineered by fusing luciferase to cell surface-binding constructs, e.g., *Staphylococcus* protein A (4, 10, 12), biotin, or lectins, and allows the assessment of ATP concentrations near the cell surface. Soluble luciferase assesses the average ATP concentrations in the medium (from the cell surface to the surface of the bathing solution) and, when used in a small volume, reflects near-cell surface ATP concentrations (**Figs. 3.2 and 3.3**). For real-time assessment of ATP concentrations, cultures (either non-polarized or polarized) are placed directly in the luminometer.

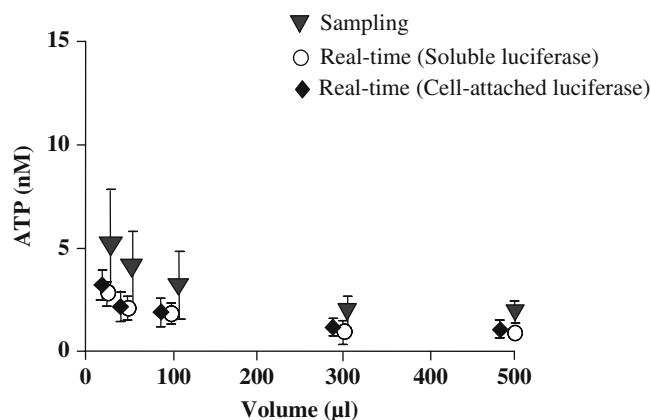


Fig. 3.2. Basal ATP concentrations on the cell surface. ATP concentrations in varied luminal volumes on resting human bronchial cells were measured by off-line luminometry (as in **Fig. 3.1A**, grey triangle), or by real-time measurement with luciferase dissolved in bulk (as in **Fig. 3.1B**, open circle), and attached to the cell surface (as in **Fig. 3.1C**, solid diamond). Values are mean  $\pm$  SEM of four Transwells/subject established from three different subjects. No major differences in basal ATP concentrations were observed with these approaches. Reprinted with permission from JBC, vol. 281, no. 32, pp. 22992–23002 (2006).

## 2. Materials

All reagents should be of the highest purity available and maintained free of bacterial contamination to avoid ATP degradation. Use of aerosol-protected tips is strongly recommended to avoid reagent cross-contamination.

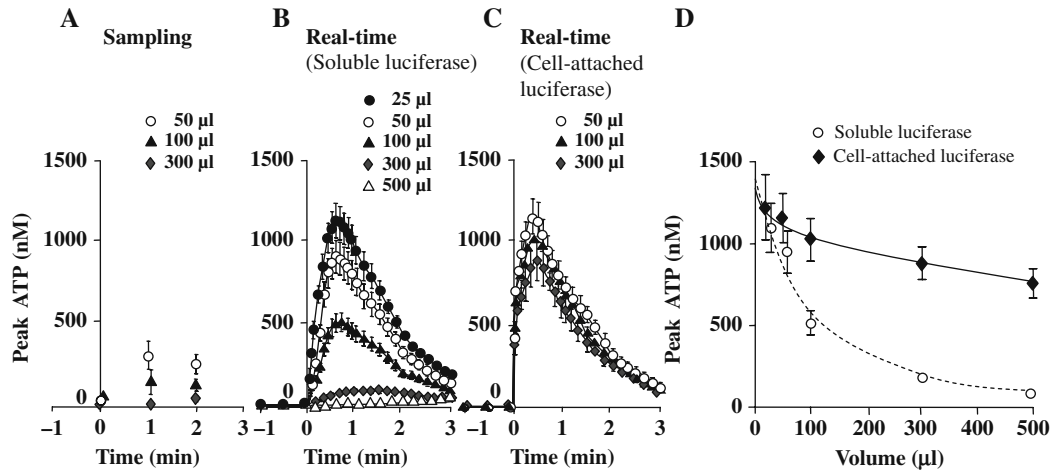


Fig. 3.3. Hypotonicity-induced ATP release. Primary human bronchial epithelial cultures were exposed to luminal 33% hypotonic challenge at  $t = 0$ . ATP concentrations were measured by off-line luminometry (A), real-time luminometry with soluble luciferase (B), and real-time luminometry with cell surface-attached luciferase (C). Varied luminal volumes were applied on 12-mm Transwells, as indicated. D: Summary data illustrating peak ATP concentrations as measured by soluble luciferase (open circle) and cell-attached luciferase (solid diamond) in varied luminal volumes. In (A)–(C), values are mean  $\pm$  SEM of 3–4 Transwells/subject established from three different subjects. In diluted solutions (100–500  $\mu$ l), ATP concentrations measured at the cell surface (C) are higher than those measured in bulk (B) or by sampling (A). However, ATP concentrations in small volumes (25–50  $\mu$ l) were similar between cell-attached luciferase detection and soluble luciferase detection (D). Reprinted with permission from JBC, vol. 281, no. 32, pp. 22992–23002 (2006).

## 2.1. Measuring ATP Concentrations in Sampled Fluids: Off-line Bioluminescence Detection

### 2.1.1. Cell Culture

### 2.1.2. Stimulation of ATP Release by Hypotonic Swelling

### 2.1.3. Inhibition of ATP Metabolism

Experiments illustrated in this section are performed with A549 cells (ATCC # CCL-185) seeded on 24-well multiwell plastic plates (BD Falcon). Cells are grown on Dulbecco's modified eagle's medium (DMEM) with high glucose (D-Glucose: 4.5 g/L), supplemented with 10% fetal bovine serum (FBS), 60  $\mu$ g/ml (100 IU/mL) penicillin, and 100  $\mu$ g/mL streptomycin.

1. Hypotonic solution: 1.2 mM  $\text{CaCl}_2$ , 1.8 mM  $\text{MgCl}_2$ , and 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.4. Store at 4°C.
2. Control (isotonic) solution: 154 mM NaCl (0.9% NaCl solution), 1.2 mM  $\text{CaCl}_2$ , 1.8 mM  $\text{MgCl}_2$ , and 25 mM HEPES, pH 7.4. Store at 4°C.
1. Ebselen: 10 mM in dimethyl sulfoxide (DMSO), aliquoted, and stored at  $-20^\circ\text{C}$ .
2.  $\beta,\gamma$ -metATP: 100 mM in water, aliquoted, and stored at  $-20^\circ\text{C}$ .

In A549 cell cultures, we obtain maximal inhibition of ATP metabolism by using a cocktail containing 300  $\mu$ M  $\beta$ , $\gamma$ -metATP and 30  $\mu$ M ebselen (*see* Fig. 3.4).

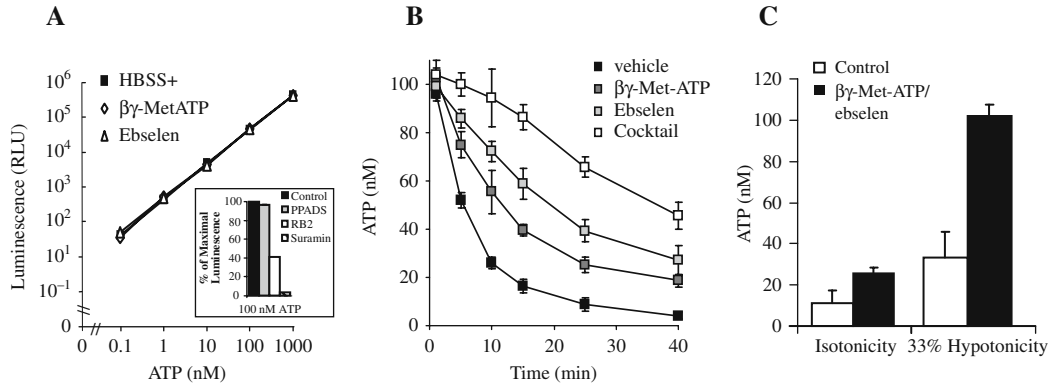


Fig. 3.4. Effect of pharmacological reagents on ATP detection. **(A)** *Luciferase activity is not affected by ATPase inhibitors, but decreases in the presence of some purinoceptor antagonists.* Calibration curves of ATP were performed in HBSS+ alone, or supplemented with 300  $\mu$ M  $\beta$ , $\gamma$ -metATP, or 30  $\mu$ M ebselen. *Inset:* 100 nM ATP was prepared in HBSS+ alone, or containing 100  $\mu$ M pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), 100  $\mu$ M reactive blue 2 (RB2), or 100  $\mu$ M suramin. Values are the mean  $\pm$  SEM of three separate experiments,  $n = 3$ . **(B)** *Effect of ecto-ATPase inhibitors on ATP hydrolysis in A549 cells.* Lung alveolar A549 cells were incubated for the indicated times at 37°C with 300  $\mu$ l HBSS+ containing 100 nM ATP (vehicle), or 100 nM ATP and 300  $\mu$ M  $\beta$ , $\gamma$ -metATP, or 100 nM ATP and 30  $\mu$ M ebselen, or 100 nM ATP and  $\beta$ , $\gamma$ -metATP and ebselen combined. Samples were collected and luminescence recorded as described in the Methods section. Values are the mean  $\pm$  SEM of two separate experiments,  $n = 4$ . **(C)** *Measurements of extracellular ATP concentrations are underestimated in the absence of ecto-ATPase inhibitors.* Confluent A549 cells grown on 24-well plates were incubated at 37°C for 5 min with 300  $\mu$ l HBSS+ in the absence (control) or in the presence of 300  $\mu$ M  $\beta$ , $\gamma$ -metATP and 30  $\mu$ M ebselen. Cultures were subsequently treated for 5 min with isotonic solution or 33% hypotonic challenge. Samples were collected and luminescence recorded as described in the Methods section. Values are the mean  $\pm$  SEM of 2 separate experiments,  $n = 6$ .

#### 2.1.4. Luminometry Reagents

Several commercial brands of luminometers are available. The protocol described below was adapted for a Berthold AutoLumat luminometer, which is configured to process 180 test tubes at a time (*see* Note 2).

1. 4X LUMI solution: 6.25 mM MgCl<sub>2</sub>, 0.63 mM ethylenedinitrilotetraacetic acid (EDTA), 75  $\mu$ M dithiothreitol (DTT), 1 mg/mL bovine serum albumin (BSA), and 25 mM HEPES, pH 7.8. Filter and store sterile at 4°C.
2. Luciferase from *Photinus pyralis* (Sigma) is dissolved at 0.5 mg/mL in 4X LUMI solution and stored in 30  $\mu$ l aliquots at -20°C.
3. Luciferin (BD PharMingen) is dissolved at 10 mg/mL in water and stored in 100  $\mu$ l aliquots, protected from light at -20°C.
4. Hank's balanced salt solution (HBSS) supplemented with 1.2 mM CaCl<sub>2</sub> and 1.8 mM MgCl<sub>2</sub> (HBSS+). HBSS+ is filtered sterile and stored at 4°C. 25 mM HEPES, pH 7.4, is added freshly prior to experiments (*see* Note 3).

5. 5 mL clear polystyrene or glass test tubes (e.g., Sarstedt).
6. ATP stock solution (e.g., 100 mM, GE Healthcare) stored at  $-20^{\circ}\text{C}$ .

## 2.2. Real-Time, Cell Surface Measurement of Extracellular ATP

1. Cells can be grown on plastic dishes (for non-polarized cells) or Transwells (for polarized cells) 3.5 cm or less in diameter.
2. Luciferase (Sigma)
3. Luciferin (BD PharMingen)
4. *Staphylococcus* protein A-fused luciferase (SPA-luc; modified from the original construct provided by Dr. George Dubyak, Case Western Reserve University; *see* **Note 4** and (4) for purification protocols)
5. Blocking solution: PBS containing 1% BSA (PBS/BSA)
6. Anti-keratan sulfate antibody (mouse IgG2b, Chemicon, Temecula, CA)
7. Buffer: HBSS+ buffered with 10 mM HEPES (HBSS/HEPES). HBSS+ can be replaced with other nutrient-containing solutions (e.g., DMEM, MEM, F12).
8. Luminometer with a real-time measurement function, e.g., TD-20/20 (Turner Biosystems, Sunnyvale, CA).

---

## 3. Methods

### 3.1. Measuring ATP Concentrations in Sampled Fluids: Off-Line Bioluminescence Detection

#### 3.1.1. Preparation of Samples

1. Grow lung epithelial A549 cells in 24-well plastic plates (surface area of  $2\text{ cm}^2$ ) until confluence (*see* **Note 5**).
2. Rinse confluent cultures gently twice with HBSS+ to remove cell debris and serum components present in the medium.
3. Pre-incubate cells in HBSS+ for 1 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a tissue culture incubator. To minimize unwanted mechanically induced ATP release during sampling, cell cultures should be covered sufficiently with media (e.g., 250  $\mu\text{L}$  for each well of a 24-well plate).
4. Expose cell cultures to reagents and/or stimuli, as described in **Fig. 3.4**.
5. Collect up to 100  $\mu\text{L}$  of the cell bathing medium into 1.5-mL microcentrifuge tubes placed on ice.
6. Heat samples for 2 min at  $98^{\circ}\text{C}$  to inactivate potential nucleotidase activities.
7. Store samples at  $-20^{\circ}\text{C}$  until bioluminescence measurements.

## 3.1.2. Quantification of ATP

This protocol assumes the use of a LB953 AutoLumat luminometer (Berthold, Wildbad, Germany), but can be modified to other luminometers by following the manufacturer's instructions (Figs. 3.4 and 3.5).

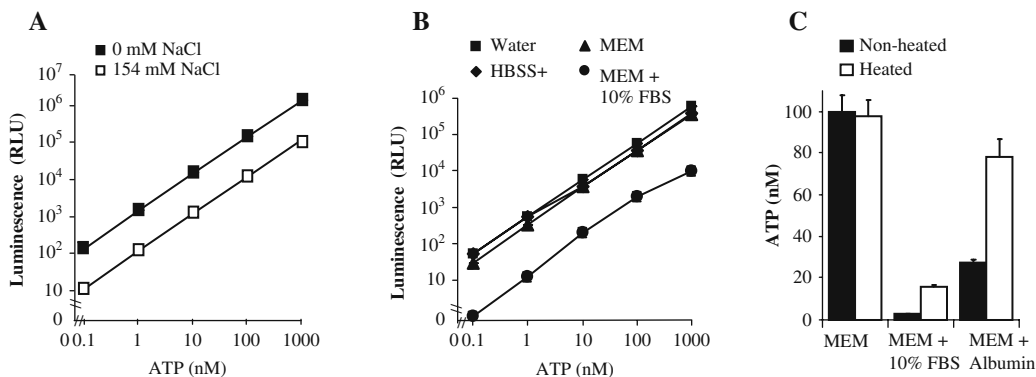


Fig. 3.5. Quantification of ATP using luciferin/luciferase. **(A)** Anions greatly interfere with the luciferase reaction. Calibration curves of ATP were performed by adding 30  $\mu$ L of the indicated ATP concentrations to 300  $\mu$ L H<sub>2</sub>O or 154 mM NaCl. Values are the mean  $\pm$  SEM of two separate experiments,  $n = 3$ . **(B)** Serum components decrease ATP detection. ATP was diluted at the indicated concentrations in H<sub>2</sub>O, HBSS+, MEM, or MEM supplemented with 10% FBS. A 30- $\mu$ L aliquot was collected and added to a 5-mL test tube containing 300  $\mu$ L of water. Values are the mean  $\pm$  SEM of two separate experiments,  $n = 4$ . **(C)** Albumin and other serum components affect ATP detection. 100 nM ATP was prepared in MEM, MEM supplemented with 10% FBS, or MEM supplemented with 4 g/dL human albumin, and incubated at RT for 10 min. Samples were heated at 98°C for 2 min (except non-heated controls) prior to ATP measurements.

1. Prepare the luciferin/luciferase cocktail freshly by adding one aliquot of luciferase and luciferin stock solutions (described in Materials) to 12.5 ml 4X LUMI solutions at room temperature (RT), protected from light. Final luciferin and luciferase concentrations in 4X LUMI are 265  $\mu$ M and 1.2  $\mu$ g/ml, respectively.
2. Place the luciferin/luciferase solution in the injector port of the luminometer. Prime the injector line following the manufacturer's instructions.
3. Prepare an ATP calibration curve (e.g., up to 1,000 nM ATP, *see Note 6*) in the same solution/media used for incubations with cells.
4. Add 30  $\mu$ L of each sample to a 5 mL test tube containing 300  $\mu$ L H<sub>2</sub>O (*see Note 7*).
5. Transfer the test tubes to the dark chamber of the luminometer and proceed with the luciferin/luciferase injection and bioluminescence recording, i.e., arbitrary light units (ALUs), as instructed by the manufacturer.
6. Determine ATP concentration in the sample by intersecting sample ALU values with the calibration curve ALU values (*see Notes 8 and 9*).



### 3.2. Real-Time, Cell Surface Measurement of Extracellular ATP

#### 3.2.1. Attachment of *Staphylococcus Protein A-Fused Luciferase (SPA-Luc)* to Cell Surface (see Note 10)

1. Wash the surface (apical, if polarized cells are used) of cell cultures with phosphate-buffered saline (PBS),  $3 \times$ .
2. Incubate the (apical) surface with 50  $\mu$ L (for cultures of 12 mm diameter) of blocking solution for 30 min on ice. If polarized cells are used, keep the basolateral surface immersed in medium.
3. Replace the blocking solution with a solution containing the designated primary antibody (see Note 11). For primary airway epithelial cells, use 50  $\mu$ L of 10  $\mu$ g/mL (i.e., 1:300) anti-keratan sulfate antibody in PBS/BSA. Incubate for 1 h on ice.
4. Wash  $3 \times$  with PBS.
5. Incubate with 0.5 mg/mL purified SPA-luc (see Note 4) for 1 h at 4°C in the dark. SPA-luc will bind to the Fc domain of the antibody attached to the cells, as indicated in Step 3.
6. Wash carefully  $3 \times$  with PBS. Replenish the apical surface with ATP assay solution (e.g., HBSS/HEPES). Keep cultures in the dark at RT for 30 min to equilibrate the extracellular ATP concentrations.

#### 3.2.2. Measurement of Cell Surface ATP Concentrations Using SPA-luc

1. Place a SPA-luc-bound cell culture in the Turner TD-20/20, add soluble luciferin (150  $\mu$ M final, to the apical solution for polarized cultures) and close the lid. When a Transwell is used, place it in a chamber (or a dish) containing HBSS/HEPES to cover the basolateral side (Fig. 3.1B, C). Assays are typically performed at RT (see Note 12).
2. Record baseline luminescence (arbitrary light unit, ALU) every minute with 5–10 s integration time, according to manufacturer's instructions. Monitor ALU until baseline luminescence is achieved (see Note 13). Baseline luminescence is usually achieved within 5–30 min and represents basal ATP concentrations (see Fig. 3.3).
3. To assess stimulated ATP release, add stimuli (e.g., pharmacological reagents, hypotonic challenge, etc.) and record the ALU. ALU integration time needs to be optimized, as well as the frequency of recording, for each experiment. For example, when airway epithelial cells are challenged with 33% hypotonicity, H<sub>2</sub>O (a half volume of the initial luminal volume) is added to the luminal solution at  $t = 0$ . The ALU is recorded for 5 min; every 0.2 s for the first minute, then every 10 s (with 4-s integration time) for the next 4 min. A typical time-course of ATP concentrations is shown in Fig. 3.3.
4. At the end of each assay, an ATP–luminescence relationship (calibration curve) is generated to calculate ATP concentrations. Known concentrations of ATP are added to the luminal liquid in a stepwise manner (e.g., 1 nM added twice, 10 nM added twice, then 100 nM added twice – for the accuracy of

the calibration curve, adding each concentration twice is recommended), and increases in ALUs recorded each time (*see* **Note 14**)

### 3.2.3. Measurement of Cell Surface ATP Concentrations Using Soluble Luciferase

1. Wash the surface of cultures with PBS,  $3 \times$ .
2. Add HBSS/HEPES (0.5–1 ml for non-polarized 3.5 cm cultures. Bilaterally for polarized cultures – 1 cc and 25–500  $\mu$ l to luminal and serosal side, respectively, when 12-mm Transwell is used). Equilibrate the cultures in an incubator (37°C and 5% CO<sub>2</sub>) for 1 h.
3. Add luciferase ( $\sim 0.8 \mu\text{g}/\text{cm}^2$  culture surface) and luciferin (150  $\mu\text{M}$ ) to the luminal buffer, and start the measurement as described in Methods 3.2.2.

---

## 4. Notes

1. Firefly luciferase catalyzes the following reaction:
 
$$\begin{aligned} \text{D-luciferin} + \text{ATP} + \text{luciferase (L)} &\rightarrow \text{L(luciferyl-adenylate)} \\ &+ \text{pyrophosphate} \\ \text{L(luciferyl-adenylate)} + \text{O}_2 &\rightarrow \text{L(oxy luciferin* ; AMP)} + \text{CO}_2 \\ \text{L(oxy luciferin* ; AMP)} &\rightarrow \text{L(oxy luciferin ; AMP)} + \text{photon} \\ \text{L(oxy luciferin ; AMP)} &\rightarrow \text{L} + \text{oxy luciferin} + \text{AMP} \end{aligned}$$
2. Many luminometers are configured as microplate readers. Sample volume and luciferase-luciferin cocktail should be modified to fit the volume of an individual well, following the manufacturer's instructions.
3. Minimum essential medium (MEM), DMEM, or several other culture media (without serum) are equally effective as HBSS+, and could be used as an alternative in the sample preparation assay. Avoid using media supplemented with ATP, such as Medium 199.
4. SPA-luc fused to a hexa-histidine ( $6 \times \text{His}$ ) tag (4) is purified over a Ni<sup>2+</sup>-chelating column. The  $6 \times \text{His}$  tag is cleaved by Tobacco-Etch virus (TEV) protease after purification. For detailed purification protocols, *see* (4).
5. Seeding density of  $1 \times 10^5$  A549 cells/well will provide confluent cultures at 24 h.
6. Under the conditions described, a linear ATP concentration:-luminescence relationship is observed in the range of 0.1–1000 nM ATP. This ATP concentration range covers ATP concentrations detected in the bulk extracellular medium of most cell cultures.

7. Media or other saline-based solutions (e.g., 0.9% NaCl or PBS) contain anions that interfere with the luciferase reaction [Fig. 3.5A and (13, 14)], decreasing the sensitivity of the assay. Therefore, we recommend using water as the diluting agent to achieve the highest sensitivity in the assay.
8. The luciferase reaction is inhibited by components present in cell culture media, e.g., anions [Fig. 3.5B and (14)]. Moreover, phosphatases and other components present in FBS-supplemented and hormone-supplemented media (e.g., BEGM or SAGM, Lonza Walkersville, MD) affect ATP availability for the luciferase reaction. Albumin-bound ATP can be dissociated by heating the sample at 95°C for 2 min (Fig. 3.5C).
9. All test drugs added to the cells should be tested for potential interference with luciferase activity [Fig. 3.4 and (14)].
10. The principle of SPA-luc attachment to cell surface is as follows. First, bind an antibody to cell surface molecules; next, attach protein A (of SPA-luc) to the Fc domain of the antibody. It is important to choose an antibody that protein A is capable of binding; for example, protein A strongly binds to total IgG, IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>3</sub>, but exhibit weak or no binding to IgG<sub>1</sub>, which is the most common class of monoclonal antibodies.
11. For primary human airway cells, lectins and monoclonal antibodies against keratan sulfate or MUC1 served as SPA-luc attachment molecules (4). For mouse Bac-1.2F5 macrophages, monoclonal antibodies against CD45.2 or H-2 K<sup>d</sup> major histocompatibility complex (MHC) class I; for human platelets, monoclonal antibodies against CD41 or anti-HLA-ABC served as SPA-luc attachment molecules (12). For cell types in which finding an endogenous antigen on the cell surface for sufficient antibody attachment is difficult, antigens can be overexpressed [e.g., CD14 (10)]. However, the effect of antigen overexpression on ATP release and metabolism needs to be addressed.
12. Though it is ideal to perform ATP release assays at a physiological temperature (37°C), luciferase activity is dramatically decreased above 30°C (15). Being aware that some ATP release pathways (e.g., exocytosis) might be suppressed at low temperatures, assays can be carried out at RT. It is critical to maintain pH of the assay solution on cells (which contains luciferin and luciferase) at 7.0–7.4 (15) by including 25 mM HEPES (pH 7.4).
13. Experimental maneuvers, such as changing and adding luminal solutions and transferring Transwells, cause robust ATP release from cells. Baseline ATP concentrations are achieved after such artifactually released ATP is hydrolyzed by endogenous ecto-ATPases, usually within 5–30 min of incubation.

14. The sensitivity of luciferin-luciferase reactions may vary among assays; thus, an ATP–ALU relationship should be generated for each assay. The end products of luciferin-luciferase reaction (e.g., pyrophosphate, oxyluciferin) inhibit the luciferase reaction. However, when sufficient amounts of luciferin and luciferase are included at the beginning of the assay, the assay sensitivity is typically maintained for at least 30 min on cells.

## References

1. Burnstock, G. (2006) Purinergic signaling. *Br J Pharmacol* **147** (Supple 1), S172–S181.
2. North, R. A. (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82**, 1013–1067.
3. Zimmermann, H. (2000) Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 299–309.
4. Okada, S. F., Nicholas, R. A., Kreda, S. M., Lazarowski, E. R., and Boucher, R. C. (2006) Physiological regulation of ATP release at the apical surface of human airway epithelia. *J Biol Chem* **281**, 22992–23002.
5. Lazarowski, E. R., Shea, D. A., Boucher, R. C., and Harden, T. K. (2003) Release of cellular UDP-glucose as a potential extracellular signaling molecule. *Mol Pharmacol* **63**, 1190–1197.
6. Gatof, D., Kilic, G., and Fitz, J. G. (2004) Vesicular exocytosis contributes to volume-sensitive ATP release in biliary cells. *Am J Physiol Gastrointest Liver Physiol* **286**, G538–G546.
7. Boudreault, F., and Grygorczyk, R. (2004) Cell swelling-induced ATP release is tightly dependent on intracellular calcium elevations. *J Physiol* **561**, 499–513.
8. Button, B., Picher, M., and Boucher, R. C. (2007) Differential effects of cyclic and constant stress on ATP release and mucociliary transport by human airway epithelia. *J Physiol* **580**, 577–592.
9. Kreda, S. M., Seminario-Vidal, L., Heusden, C. V., and Lazarowski, E. R. (2008) Thrombin-promoted release of UDP-glucose from human astrocytoma cells. *Br J Pharmacol* **153**, 1528–1537.
10. Joseph, S. M., Buchakjian, M. R., and Dubyak, G. R. (2003) Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *J Biol Chem* **278**, 23331–23342.
11. Kreda, S. M., Okada, S. F., van Heusden, C. A., O’Neal, W., Gabriel, S., Abdullah, L., Davis, C. W., Boucher, R. C., and Lazarowski, E. R. (2007) Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol* **584**, 245–259.
12. Beigi, R., Kobatake, E., Aizawa, M., and Dubyak, G. R. (1999) Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. *Am J Physiol* **276**, C267–C278.
13. Lundin, A. (2000) Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. *Methods Enzymol* **305**, 346–370.
14. Taylor, A. L., Kudlow, B. A., Marrs, K. L., Gruenert, D. C., Guggino, W. B., and Schwiebert, E. M. (1998) Bioluminescence detection of ATP release mechanisms in epithelia. *Am J Physiol* **275**, C1391–C1406.
15. DeLuca, M., and McElroy, W. D. (1978) Purification and properties of firefly luciferase. *In Methods Enzymol* **57**, 3–15.

Bioluminescence

Methods and Protocols

Rich, P.B.; Douillet, C. (Eds.)

2009, XII, 268 p. 60 illus., 29 illus. in color., Hardcover

ISBN: 978-1-60327-320-6

A product of Humana Press