Chapter 7

Assessing Extracellular ATP as Danger Signal In Vivo: The pmeLuc System

Francesco Di Virgilio, Paolo Pinton, and Simonetta Falzoni

Abstract

Inflammation is the key pathophysiological response triggered by noxious agents in multicellular organisms. Central to inflammation is detection of exogenous or endogenous danger signals by immune cells. Extracellular ATP is a ubiquitous danger signal released during septic or sterile inflammation. The development of reliable techniques to measure extracellular ATP in vivo has become an urgent need in inflammation studies after the discovery that the most potent plasma membrane receptor responsible for NLRP3 inflammasome activation is an ATP-activated receptor, P2RX7. Here we describe an easy bioluminescence technique for the measurement of extracellular ATP in vivo.

Key words Extracellular ATP, Plasma membrane luciferase, Luciferin, Luminescence, Luminometry

1 Introduction

Bioluminescence is a natural phenomenon, due to chemical emission of light (chemiluminescence) by living organisms, conserved in many different species (bacteria, protists, fungi, insect, several marine organisms) with the notable exception of higher terrestrial organisms. This process yields photons as a consequence of an exergonic reaction catalyzed by a family of enzymes (e.g., luciferases) that oxidize a photon-emitting substrate. Luciferase (Luc), mainly derived from the firefly *Photinus pyralis* or from the coelenterate *Renilla reniformis*, has been long used as an in vitro assay to measure ATP [1]. Firefly luciferase is a 62 kDa protein belonging to the adenylating enzyme superfamily. In the presence of magnesium ions, molecular oxygen, and ATP, luciferase catalyzes oxidation of the substrate D-luciferin (LH_2) accompanied by light emission in the green to yellow region ($\lambda max = 560$ nm). The reaction proceeds through three main steps:

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1. Formation of the intermediate Luc-D-luciferyl adenylate (LH_2-AMP), with release of inorganic phosphate (PPi):

$$\text{Luc} + \text{LH}_2 + \text{ATP} \rightarrow \text{Luc} \cdot \text{LH}_2 - \text{AMP} + \text{PPi}$$

2. The intermediate complex Luc-D-luciferyl adenylate is oxidized by molecular oxygen with the formation of an excited enzyme-oxyluciferin-AMP complex and the release of carbon dioxide (CO_2):

$$\text{Luc} \cdot \text{LH}_2 - \text{AMP} + \text{O}_2 \rightarrow \text{Luc} \cdot \text{AMP} \cdot \text{Oxyluciferin} + \text{CO}_2$$

3. In the final step, energy loss from the excited complex produces photon emission and dissociation of the complex:

$$\text{Luc} \cdot \text{AMP} \cdot \text{Oxyluciferin} \rightarrow \text{Luc} + \text{Oxyluciferin} + \text{AMP} + \text{h}\nu$$

Photon emission is then recorded with a luminometer. A luminometer is made of photon-collecting apparatus (low-noise photomultiplier tube) and a computer station equipped with a software to allow data storage and analysis. The photomultiplier tube can be replaced by a high sensitive photo camera. Automated plate readers for luminescence (as well as fluorescence) measurement can also be used. Bioluminescence measurement in small animals is usually done with total body luminometers. Widespread use of bioluminescence techniques has made a tremendous impact in immunology and cancer where it has been used to investigate gene expression and track cancer cells in living animals. Luciferase reporter gene cloned downhill to many different promoters or fused in frame with the genes of interest allows to monitor the transcriptional activity of countless stimuli or to detect gene activation in many different pathophysiological conditions [2]. Alternatively, luciferase transfection has allowed investigation of intracellular ATP levels under many different metabolic conditions. Thanks to the possibility to fuse the luciferase gene in frame with leader sequences targeting various intracellular compartments, it has also become possible to monitor intra-organelle ATP changes [3]. Total body luminometry allows luminescence recording and analysis in those experimental settings where luciferase is used either as an in vivo intracellular reporter for cell tracking or as a probe to measure extracellular ATP. To perfect bioluminescence measurement of extracellular ATP, we have engineered a chimeric *firefly luciferase* selectively expressed on the outer aspect of the plasma membrane, and therefore named plasma membrane luciferase (pmeLUC) (Fig. 1) [4–6]. PmeLUC expression allows extracellular ATP monitoring in the extracellular compartment, notably in the close vicinity of the plasma membrane, thus pmeLUC-transfected cells can be used as in vivo reporters of the extracellular ATP concentration [7–9].

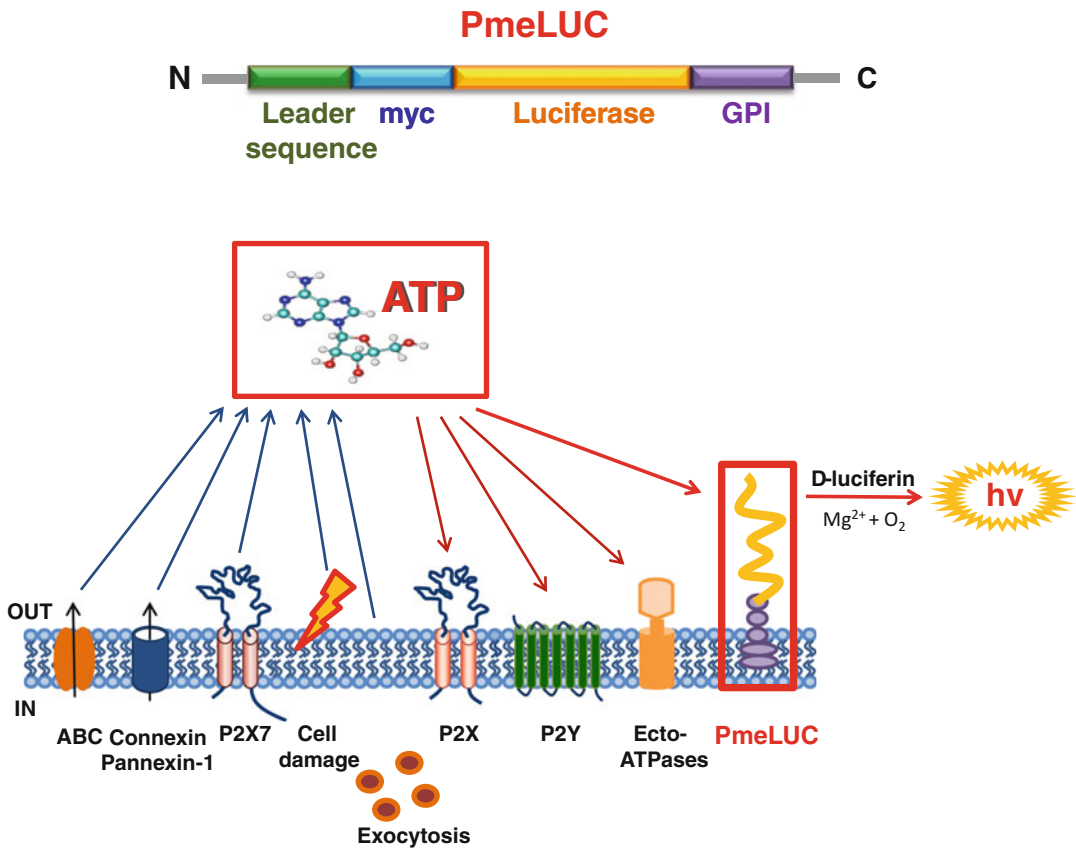

Fig. 1 Membrane topology of pmeLUC. The pmeLUC construct is made of the full-length coding sequence of luciferase (*yellow*) inserted in frame between the N-terminal leader sequence (*green*) and the C-terminal GPI anchor (*violet*) of the folate receptor. A c-myc tag (*light blue*) is added in frame for tracking purposes. The pmeLUC protein is targeted and localized to the extracellular aspect of the plasma membrane (from Falzoni et al., ref. 6)

2 Materials

2.1 Cells

Any cell type suitable to be transfected can be used as reporter of the extracellular ATP concentration with pmeLUC. We have used human HEK293 embryonic kidney cells, mouse CT26 colon carcinoma cells, mouse B16 melanoma cells, and many others. We will describe here the basic protocol for HEK293 cells.

2.2 Cell Culture

1. Cell media: Dulbecco's modified Eagle's medium (DMEM)-F12 medium containing 0.5 mM sodium pyruvate and 15 mM sodium bicarbonate, supplemented with 2 mM L-glutamine, 10 % heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3 Engineering of pmeLUC

1. pGL3 plasmid (Promega)
2. *Pst*I, *Not*I, *Xho*I, and *Xba*I restriction enzymes

3. pBSK+ vector (Stratagene)
4. *Pst*I fragment encoding the N-terminal leader sequence (26 aa) of the human folate receptor fused with a c-myc tag (10 amino acids)
5. *Pst*I fragment encoding the GPI anchor protein of the human folate receptor (28 amino acids)
6. pcDNA3 expression vector (Life Technologies)
7. *Leader-myc* forward primer with *Xba*I site (underlined):
   1AF forward: 5′-GGT CTA GAG GAG AGC CAC CTC CT-3′
8. *Leader-myc* reverse primer without stop codon, with *Pst*I site (underlined):
   1BR reverse: 5′-GGC TGC AGC AGG TCC TCC TCG CT-3′
9. Luciferase forward primer with *Pst*I site (underlined):
   LucPstF forward: 5′-CCC TGC AGA TGG AAG ACG CCA AAA ACA TAA AGA AAG C-3′
10. Luciferase reverse primer with *Pst*I site (underlined) and without stop codon:
    LucPstR reverse: 5′-GCT GCA GCC ACG GCG ATC TTT CCG CCC TTC TTG G-3′
11. 10× T4 ligase buffer: 200 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 50 mM DTT, 500 μg/ml BSA, 10 mM ATP
12. T4 Ligase
13. Xl1-Blue *E. coli*
14. LB agar
15. Ampicillin
16. DNA extraction kit

2.4 Transfection

1. Complete cell culture medium (*see* Subheading 2.2).
2. HEK293 cells.
3. 2.5 M $CaCl_2$ solution in distilled water and filter sterilize. Store in 500 μl aliquots at −20 °C.
4. 2× HEPES-buffered saline (HBS-2X) solution: 280 mM NaCl, 50 mM HEPES base, 1.5 mM Na_2HPO_4, pH 7.12 with 0.5 N NaOH. Filter sterilize and store in 5 ml aliquots at −20 °C.
5. Phosphate buffered saline (PBS): 137 mM NaCl, 4.3 mM Na_2HPO_4 $7H_2O$, 1.4 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.
6. TRIS/EDTA (TE) buffer: 10 mM Tris–Cl pH 7.4, 1 mM EDTA pH 8.0 in filter-sterilized distilled water.
7. 10 cm Petri dishes for tissue culture.
8. 10 ml conical tubes.
9. 40 μg pmeLUC plasmid, resuspend in 450 μl of aqueous TE solution.

10. 40 μg of pcDNA3 empty vector, resuspend in 450 μl of TE solution.
11. G-418.

2.5 Immunofluorescence

1. 13 mm glass coverslips
2. 24-well cell culture plates
3. 0.01 % poly-L-lysine solution in water
4. PBS (*see* Subheading 2.4)
5. 2 % gelatin solution, type B, in distilled filter-sterilized H_2O
6. Anti c-myc-SC-40 monoclonal antibody (Santa Cruz Biotechnology)
7. Texas Red-conjugated goat anti-mouse IgG
8. FITC-conjugated anti-mouse antibody
9. Prolong Gold® antifade reagent (Life Technologies) or other suitable antifade reagents
10. 4 % paraformaldehyde solution in PBS
11. 10× solution Cell Fix™ (BD Biosciences)

2.6 In Vitro Extracellular ATP Measurement

1. 12-well cell culture plate.
2. DMEM-F12 medium.
3. 15 mg/ml D-luciferin stock solution in sterile PBS. Allow the luciferin solution to sit for a minimum of 15 min with gentle agitation prior to make 1 ml aliquots. Protect from light and store aliquots at −80 °C.
4. 100 mM adenosine-5′-triphosphate disodium salt (ATP) in 0.1 M Tris-base solution at pH 7.5. Store in 0.5 ml aliquots at −80 °C.

2.7 In Vivo Extracellular ATP Measurement

1. D-Luciferin.
2. Complete HEK293 cell culture medium (*see* Subheading 2.2).
3. RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.
4. Isofluorane.
5. Syringe and 27-gauge needle.
6. 500 units apyrase from potato reconstitute in 1 ml of PBS buffer. Store in 50 μl aliquots at −80 °C.
7. PmeLUC-transfected HEK293 cells (HEK293-pmeLUC).
8. OVCAR-3 (human ovary carcinoma cell line) (*see* **Note 3**).
9. MZ2-MEL (human melanoma cell line) (*see* **Note 3**).
10. Athimic nude mice (*nude/nude* mice) 5–6 weeks old (Harlan Laboratories).

11. Total body luminometer (we use Perkin-Elmer Caliper IVIS 100 System™, but other compatible equipment is also suitable).
12. Apparatus for inhalatory anesthesia.
13. C57BL/6 or Balb/c 20 day old mice, weighing 12–14 g.
14. Dextran sulfate sodium salt powder from *Leuconostoc* spp. (MW > 500,000): dissolve 5 g of DSS in 100 ml of filtered drinking water, adjust the pH at 8.5. Store at 4 °C.

3 Methods

3.1 Engineering of pmeLUC

1. Firefly luciferase is amplified from the pGL3 plasmid (Promega) using the following primers: 5′-CCC TGC AGA TGG AAG CAA AAA ACA TAA AGA AAG G3′ (corresponding to the sequence encoding luciferase amino acids 1–9; *Pst*I site underlined) and 5′-GCT GCA GCC ACG GCG ATC TTT CCG CCC TTC TTG G3′ (corresponding to a 542–549 of luciferase cDNA without the stop codon; *Pst*I site underlined).
2. Thermal profile: denaturation at 95 °C for 30 min, hybridization at 62 °C for 30 min, polymerization at 72 °C for 2 min, repeat for 37 cycles.
3. Amplify the *leader-myc* sequence with primers described above using the following thermal profile: denaturation at 95 °C for 30 min, hybridization at 55 °C for 30 min, polymerization at 72 °C for 2 min, repeat for 32 cycles.
4. Transfer the PCR product to a pBSK+ vector, digest with *Pst*I, and insert in the right frame between a *Pst*I fragment encoding the N-terminal leader sequence of the human folate receptor (26 aa) fused with myc tag (10 aa) and a *Pst*I fragment of the GPI anchor protein (28 aa) to generate pmeLUC.
5. The whole final construct is excised by a *Not*I/*Xho*I or *Xba*I digestion and cloned into a pcDNA3 expression vector. For this purpose, a ligation reaction is performed with recombinant DNA and expression vector with a stoichiometric balance of 3:1 (Fig. 2).
6. The ligation buffer is made with 2 μl Buffer T4 ligase 10×, implemented with 1 μl T4 DNA ligation enzyme (2 U/μl) reconstituted in deionized water up to 20 μl to obtain the pmeLUC plasmid.
7. 100 ng of plasmid DNA is used to transform one aliquot of Xl1-Blue *E. coli* competent cells by thermic shock: 42 °C for 45 s followed by 2 min on ice.

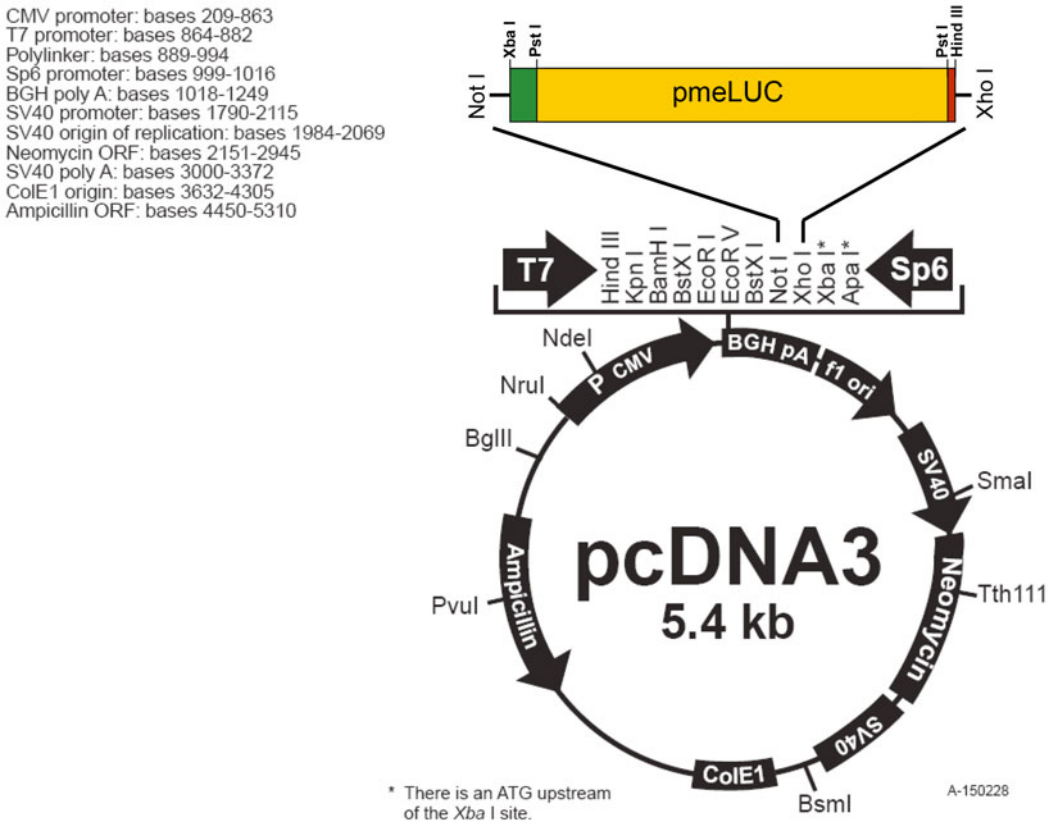

Fig. 2 Map of the pmeLUC plasmid

8. Thereafter, the bacterial cell suspension is cultured overnight at 37 °C in LB agar in the presence of 100 μg/ml ampicillin.
9. The day after, the bacterial cell suspension is precipitated by centrifugation at 3000 × *g* for 20 min.
10. DNA is extracted with DNA extraction kit from Qiagen as per manufacturer instruction.

3.2 Cell Transfection

1. Plate HEK293 (3×10^6 cells/plate) into 10-cm tissue culture Petri dish 24 h before transfection. Rinse and add fresh DMEM F12 medium 4 h before transfection.
2. Prepare tube 1 solution (DNA-Ca) as follows: add 50 μl of $CaCl_2$ solution to 40 μg pmeLUC-pcDNA3 plasmid resuspended in 450 μl of aqueous (TE solution or DNAse/RNAse-free H_2O).
3. Prepare tube 2 solution: 500 μl HBS-2X solution.
4. Add the DNA-Ca solution (tube 1) dropwise with a Pasteur pipet to tube 2 and vortex immediately for a few seconds. Allow the precipitate to sit 30 min at room temperature.

5. Add the precipitate to the HEK293 cell culture plate, mix gently, and incubate overnight.
6. The day after remove medium, wash cells twice with 10 ml of warm (37 °C) PBS, and add 10 ml of complete DMEM-F12. For stable transfection, allow HEK293 cells to double twice before plating in selection medium.

3.3 Selection of Stably Transfected Cell Clones

1. For stable transfection, the cell culture is supplemented with 0.8 mg/ml of the antibiotic G 418 three days after transfection and kept in its continuous presence for 2 weeks.
2. Thereafter, positive clones are obtained by limiting dilution: 100 cells are resuspended in 10 ml of 0.2 mg/ml G418-supplemented DMEM-F12 medium; 100 µl of this cell suspension are added to each well of a 96-well cell plate. Place the cell plate in a 5 % CO_2, humidified incubator at 37 °C.
3. Check clones with a phase contrast microscope at 20× magnification after 5–6 days. Check each well and mark those well that contain only one colony.
4. Transfer colonies to a T-25 culture flask in 0.2 mg/ml G418-supplemented DMEM-F12 medium and wait for colony growth.

3.4 In Vitro Validation of Transfected Cells by Immunofluorescence

1. 13 mm glass coverslips are placed into single wells of a 24 well culture plate.
2. Add 200 µl of poly-L-lysine to each well for coverslip coating and incubate 1 h at room temperature.
3. Rinse coverslips twice with sterile H_2O. Allow coverslips to dry completely and sterilize under UV light for at least 4 h.
4. Plate 2×10^4 HEK293 pmeLUC cells onto each coverslip. Allow cells to adhere and fix with formaldehyde, 4 % in PBS, for 30 min.
5. Rinse coverslips three times with PBS.
6. Incubate coverslips for 30 min with 0.2 % gelatin in PBS to block nonspecific binding sites.
7. Immunostaining is carried out for 1 h at 37 °C with a commercial monoclonal Sc-40 antibody against the c-myc epitope tag diluted at 1:100 in 0.2 % gelatin-supplemented PBS.
8. Discharge the antibody solution, add fresh PBS, and incubate for 5 min. Repeat washing three times, for a total of 15 min.
9. Immunodetection is carried out using Texas Red-conjugated goat anti-mouse IgG diluted 1:50 in PBS supplemented with 0.2 % gelatin. Leave the coverslips in this solution for 1 h at room temperature in the dark.

10. Discharge the secondary antibody, add fresh PBS, and incubate for 5 min. Repeat washing three times, for a total of 15 min.
11. After immunostaining, cells are mounted with a drop of Prolong Gold antifade reagent and analyzed with a fluorescence microscope at 60× or 100× magnifications, e.g., a Zeiss LSM 510 confocal microscope.
12. HEK293 cells, mock transfected with the empty pcDNA3 vector are used as a negative control.

3.5 In Vitro Validation of Transfected Cells by FACS Analysis

1. Resuspend 1×10^6 HEK293-pmeLUC cells in 1 ml of PBS.
2. Incubate HEK293-pmeLUC cells for 1 h at 4 °C with monoclonal Sc-40 antibody against the c-myc epitope tag diluted at 1:100.
3. Rinse two times with cold PBS.
4. Incubate cells at 4 °C for 1 h with a FITC-conjugated anti-mouse antibody at a 1:50 dilution in PBS. Protect from light.
5. Rinse two times with cold PBS.
6. Fix cells with 100 µl of 10× Cell Fix™ diluted in 0.9 ml of cold PBS.
7. Acquire fluorescence with a flow cytometer (e.g., Becton Dickinson BD FACSscan), and analyze data with BD Cell Quest software. HEK293 cells, mock- transfected with the empty pcDNA3vector, are used as a negative control (*see* **Note 1**).

3.6 Analysis of pmeLUC Expression and Function by In Vitro Luminescence Recording with a Total Body Luminometer

1. Seed 7×10^4 HEK293-pmeLUC cells into 12-well plates (Becton Dickinson Biosciences) in DMEM-F12 medium. Allow to adhere overnight in a 5 % CO_2, 37 °C, humidified incubator.
2. The following day, add D-luciferin sodium salt (*see* **item 3**, Subheading 2.6) to each well (8 µl, 120 µg/well), and, 3 min later, start luminescence acquisition in a luminometer (e.g., IVIS 100). Set acquisition time and binning at 1 min/plate and 4, respectively.
3. Build a calibration curve by consecutive additions of increasing (from 0.001 to 1 mM) ATP concentrations to one or more wells. Start a new acquisition after each ATP addition.
4. As a control, add 5 U apyrase to one or more wells and start acquisition. Apyrase addition should cause a large luminescence decrease (*see* **Note 2**).
5. A region of interest (ROI) is manually selected on each well. Keep the area of ROI constant and record the intensity as photons per seconds.

3.7 Analysis of pmeLUC Expression and Function by In Vitro Luminescence Recording with a Plate Reader Luminometer

1. Plate the HEK293-pmeLUC cells as above.
2. Add 8 μl of D-luciferin solution (*see* **item 3**, Subheading 2.6) to each well.
3. Add increasing ATP concentrations solution to consecutive wells (calibration).
4. Place the plate in the plate reader luminometer and start the acquisition. Set the counting time at 7 s/well. Triplicates are recommended.
5. Record luminescence in counts per second (CPS) and express as function of ATP concentration.

3.8 In Vivo Analysis of Extracellular ATP

PmeLUC-transfected reporter cells can be used to monitor the extracellular ATP concentration in several experimental settings. In healthy mice, the extracellular ATP concentration is negligible, i.e., in the low nanomolar range, and therefore below pmeLUC detection limit. Thus, best examples of extracellular ATP measurements by pmeLUC are given in experimental models of inflammation and cancer.

3.9 Measurement of Extracellular ATP Levels in a Model of Experimental Colitis Induced with Dextran Sulfate

1. Inject mice (C57Bl/6 or Balb/c) i.p. with 2×10^6 HEK293-pmeLUC cells in 200 μl of DMEM-F12 the day before starting DSS administration to record basal peritoneal ATP levels.
2. Gently massage the abdomen to allow pmeLUC cell distribution throughout the peritoneum.
3. Fifteen minutes later, inject each mouse i.p. with 150 mg/kg D-luciferin (3 mg/mouse) of D-luciferin-containing PBS in a final volume of 200 μl. Wait 15 min to allow luciferin distribution throughout the mouse tissue.
4. Anesthetize mice with isofluorane (2 % in 1 L/min oxygen).
5. Place the animals (abdominal view) into a total body luminometer (e.g., IVIS 100 System™). Set acquisition time and binning at 3 min/acquisition and 4, respectively. Up to three mice each time can be acquired at the same time.
6. For quantification select manually a region of interest (ROI) around peritoneum. Record ROI luminescence intensity as photons per seconds.
7. Analyze data with the Living Image software.
8. The day after, supplement mice drinking water with 5 % DSS (dextran sulfate solution) for at least 7 days.
9. Feed control mice with regular, DSS-free, drinking water.
10. Replace drinking water, with or without DSS, daily.
11. Check mice wellness daily: measure body weight and inspect stools to monitor rectal bleeding (indications of colitis).

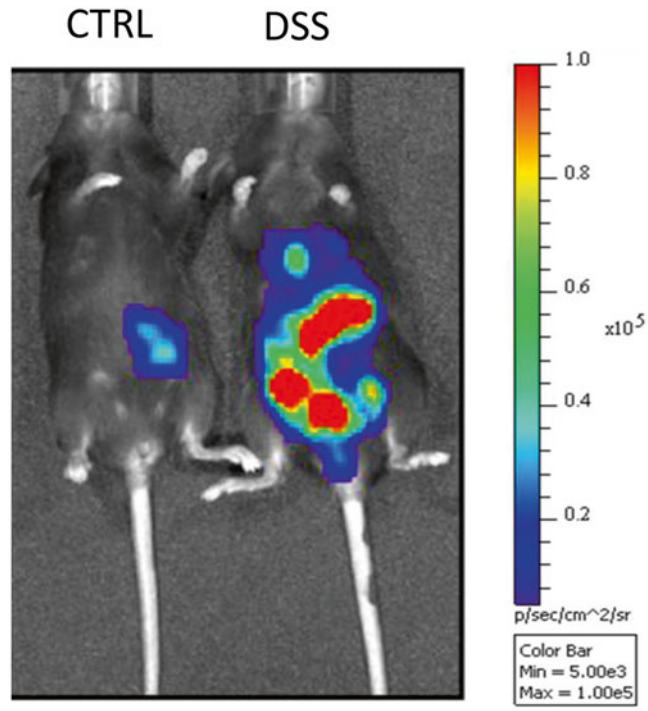

Fig. 3 Detection of experimental colitis by HEK293-pmeLUC cell inoculated i.p. in DSS-treated C57Bl/6 mouse. Mouse on the right received DSS and HEK293-pmeLUC cells. Mouse on the *left* (control) received only HEK293-pmeLUC cells

12. Repeat **steps 1**–7 every 2 days for at least 10 days.
13. Colitis shows as an increased luminescence emission of the colon (Fig. 3).

3.10 Measurement of the ATP Content of the Tumor Microenvironment in a Model of Peritoneal OVCAR-3 Ovarian Carcinoma

1. Resuspend 1.5×10^6 OVCAR-3 cells in 200 µl of RPMI.
2. Inject i.p. into *nude/nude* mice the OVCAR-3 cell suspension (200 µl in each mouse) with a syringe fitted with a 27-gauge needle. A minimum of five mice is recommended.
3. Wait 20 days for tumor growth.
4. Inject i.p. in each mouse 2×10^6 HEK293-pmeLUC cells in 200 µl of DMEM-F12.
5. Twenty-four hours later, i.p. inject each mouse with 200 µl of D-luciferin-containing PBS (150 mg/kg D-Luciferin, 3 mg/mouse). Wait 15 min and anesthetize the mice with isofluorane (2 % in 1 L/min oxygen).
6. Place the animals (abdominal view) under continuous anesthesia into the IVIS 100™ Luminometer. Set acquisition time and binning at 3 min/acquisition and 4, respectively. Up to three mice can be acquired at the same time.

7. Repeat **steps 5** and **6** every 2 days for at least 16 days.
8. For quantification, select manually a region of interest (ROI) including the tumor area, and record ROI luminescence intensity as photons per seconds.
9. Analyze data with Living Image software (*see* **Note 4**).
10. Inoculate HEK293-pmeLUC cells into healthy *nude/nude* mice as a control (*see* **Note 5**).

3.11 Measurement of the ATP Content of the Tumor Microenvironment in a Model of MZ2-MEL Melanoma

1. Resuspend 8×10^6 MZ2-MEL cells in 200 μl of RPMI.
2. Inject into *nude/nude* mice the MZ2-MEL suspension i.p. (200 μl in each mouse) with a syringe fitted with a 27-gauge needle. A minimum of five mice is recommended.
3. Inject the cell suspension subcutaneously (s.c.) in the right dorsal hip of each mouse with a syringe fitted with a 27-gauge needle.
4. Wait 10–15 days for tumor growth, or until tumor mass has reached a size of 1.5×1.5 cm.
5. Inject 2×10^6 HEK293-pmeLUC cells in 200 μl of DMEM-F12 into the tumor mass.
6. As a control, inject 2×10^6 HEK293-pmeLUC cells into the contralateral (left) dorsal hip (healthy tissue) of each mouse.
7. Inject each mouse i.p. with 150 mg/kg D-luciferin (3 mg/mouse).
8. Fifteen minutes after, anesthetize the mice with isofluorane (2 % in 1 L/min oxygen).
9. Place the animals (dorsal view) into the luminometer. Set acquisition time and binning at 3 min/acquisition and 4, respectively.
10. Up to three mice at the same time can be acquired.
11. Repeat **steps 6** and 7 every 2 days for at least 10 days.
12. For quantification, select manually a region of interest (ROI) including the tumor area. Draw ROI with the same size area also on the left dorsal hip (healthy tissue is used as a negative control). Record ROI luminescence intensity as photons per seconds (Fig. 4).
13. Analyze data with Living Image software.

3.12 HEK293-pmeLUC Cell Validation with Apyrase

1. Induce a melanoma tumor as describe above (*see* Subheading 3.11).
2. When tumor mass has reached a size of 1.5×1.5 cm, inject 2×10^6 HEK293 pmeLUC cells (resuspended in 200 μl of DMEM-F12) into the tumor mass.

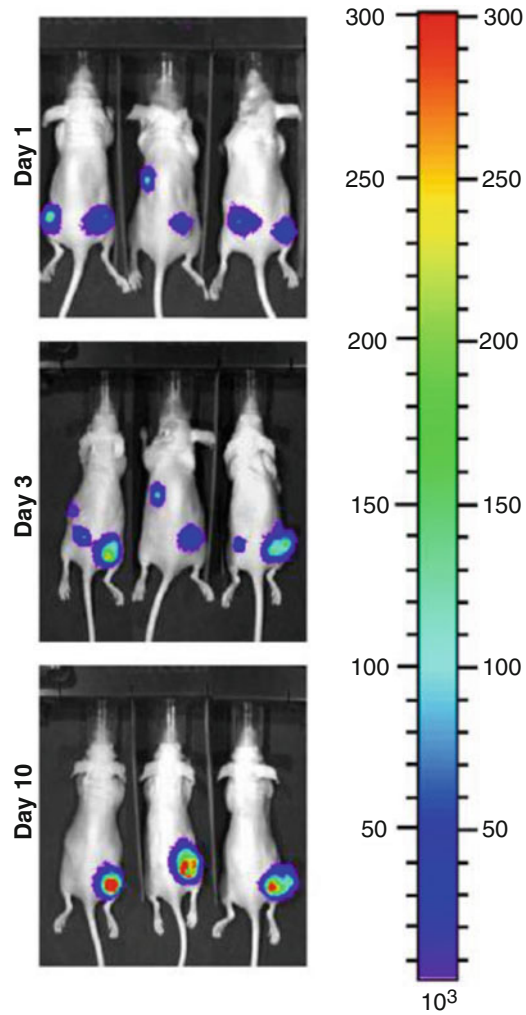

Fig. 4 Detection of the ATP concentration in the microenvironment of MZ2-MEL tumor intra-mass injected with HEK293-pmeLUC cells (from Pellegatti et al., ref. 5)

3. Four days later, inject into the tumor mass 20 U of apyrase dissolved in 100 μl of sterile PBS. Inject sterile PBS alone (vehicle) in control tumor-bearing mice.
4. Acquire and measure luminescence as described in Subheading 3.11.

4 Notes

1. To enhance plasma membrane expression of the pmeLUC construct, cells can be overnight incubated in the presence of 1 mM DTT, or kept at room temperature (21 °C) for 2 h

before immunostaining or luminescence recording. These treatments do not affect luciferase activity, and maximize pmeLUC surface expression by enhancing transport to the plasma membrane and slowing recycling.

2. Apyrase, a cell-impermeant ATP-hydrolyzing enzyme, is used as a control of the extracellular location of pmeLUC probe.
3. OVCAR-3 and MZ2-MEL cell lines are cultured in RPMI 1640 with L-glutamine, Na-pyruvate, and $NaHCO_3$ (Sigma Aldrich) supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells are cultured in Falcon T75 cell culture flasks and split once they reach 80–90 % confluence by treatment with 0.025 % Tripsin and 0.025 % EDTA in PBS buffer.
4. In tumor-bearing mice luminescence is very bright shortly after pmeLUC inoculation, because of the ATP-rich inflammatory environment within the peritoneal cavity. Then the signal progressively attenuates and localizes to discrete abdominal foci (metastases). Postmortem analysis and luminometry reveal numerous light-emitting metastasis on the abdominal wall. Moreover, histologic analysis of metastases with anti-luciferase Abs shows that HEK293 pmeLUC cells infiltrate tumor masses and report the ATP concentration in the tumor microenvironment.
5. Control mice: Healthy nude mice were injected i.p. with 2×10^6 HEK293 pmeLUC cells and monitored for 3 months. No significant luminescence emission can be detected over this extended time span. This underlines that in healthy tissue the ATP concentration in the extracellular environment is very low, certainly below the threshold for detection by HEK293 pmeLUC (1–5 μM).

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