PROTOCOL

Use of luciferase probes to measure ATP in living cells and animals

Giampaolo Morciano[1,4], Alba Clara Sarti[1,4], Saverio Marchi[1], Sonia Missiroli[1], Simonetta Falzoni[1], Lizzia Raffaghello[2], Vito Pistoia[3], Carlotta Giorgi[1], Francesco Di Virgilio[1] & Paolo Pinton[1]

[1]Section of Pathology, Oncology and Experimental Biology, Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy. [2]Laboratorio di Oncologia, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) G. Gaslini, Genoa, Italy. [3]Immunology Area, Ospedale Pediatrico Bambino Gesù, Rome, Italy. [4]These authors contributed equally to this work. Correspondence should be addressed to P.P. (paolo.pinton@unife.it) or F.D.V. (francesco.divirgilio@unife.it).

Published online 6 July 2017; doi:10.1038/nprot.2017.052

ATP, the energy exchange factor that connects anabolism and catabolism, is required for major reactions and processes that occur in living cells, such as muscle contraction, phosphorylation and active transport. ATP is also the key molecule in extracellular purinergic signaling mechanisms, with an established crucial role in inflammation and several additional disease conditions. Here, we describe detailed protocols to measure the ATP concentration in isolated living cells and animals using luminescence techniques based on targeted luciferase probes. In the presence of magnesium, oxygen and ATP, the protein luciferase catalyzes oxidation of the substrate luciferin, which is associated with light emission. Recombinantly expressed wild-type luciferase is exclusively cytosolic; however, adding specific targeting sequences can modify its cellular localization. Using this strategy, we have constructed luciferase chimeras targeted to the mitochondrial matrix and the outer surface of the plasma membrane. Here, we describe optimized protocols for monitoring ATP concentrations in the cytosol, mitochondrial matrix and pericellular space in living cells via an overall procedure that requires an average of 3 d. In addition, we present a detailed protocol for the *in vivo* detection of extracellular ATP in mice using luciferase-transfected reporter cells. This latter procedure may require up to 25 d to complete.

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INTRODUCTION

ATP is the universal and most important intermediate of cellular energy transactions and, in addition, is a ubiquitous extracellular messenger involved in many pathophysiological responses[1]. Accumulation of extracellular ATP is particularly relevant at inflammatory and tumor sites, where this nucleotide acts at P2 receptors to trigger the release of inflammatory cytokines, supports cell growth and may also precipitate cell death. Degradation of extracellular ATP by plasma membrane ecto-ATPases (epitomized by CD39) generates adenosine, a powerful anti-inflammatory, immunosuppressive mediator[2]. Although ATP release was previous thought to primarily occur as a consequence of cell death, it is now generally acknowledged that nonlytic release frequently occurs and is possibly the main mechanism of ATP release. Plasma membrane pathways for ATP release are generally found in all cell types; these include pathways that involve pannexins and connexins, anion transporters and various purinergic receptor subtypes (e.g., the P2X7 receptor)[3]. In addition, ATP can be accumulated in secretory vesicles, and therefore it can be released via classical secretory exocytosis[3]. ATP is an ideal extracellular messenger because its concentration in the interstitium of healthy tissues is lower than 200 nM (and thus ATP signaling has a very high signal-to-noise ratio), it is a highly charged species (and therefore easily diffusible in aqueous environments), it is quickly degraded (to terminate ATP signaling) and it activates specific plasma membrane receptors (P2 receptors). There is strong evidence that modulation of extracellular ATP concentration has marked effects on cell growth, cell motility and cell viability *in vitro* and *in vivo*[4]. It is believed that the ability to monitor changes in extracellular ATP concentrations in a variety of pathophysiological settings will shed light on the pathogenesis of serious chronic inflammatory diseases and cancer[4]. However, a major obstacle to understanding the pathophysiological role of ATP is the lack of reliable probes to directly measure its concentration *in vitro* and *in vivo*. Plasma-membrane-targeted luciferase (pmeLUC) was designed to fill this deficit.

Development of the protocol

The most sensitive and reliable technique for measuring ATP in isolated living cells and in whole animals is based on the bioluminescent luciferin–luciferase reaction. Luciferase, an enzyme that chemically generates light as a byproduct of the oxidation of the small-molecule substrate D-luciferin, is derived from the North American firefly *Photinus pyralis*. The reaction catalyzed by luciferase in the presence of magnesium ions implicates the conversion of D-luciferin into oxyluciferin, producing a flash of yellow–green light proportional to the amount of ATP present, with a peak emission at 560 nm, according to the following equation[5]:

$$\text{ATP} + \text{luciferin} + O_2 \text{ ----------> oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

Therefore, the introduction of luciferase and luciferin into living cells or whole animals generates light that can be captured by sensitive detectors, such as counting bioluminometers or highly sensitive photocameras. Dynamic analysis of luciferase activity in live human embryonic kidney (HEK293) cells revealed that the transport of D-luciferin across the cell membrane is essentially instantaneous and that the relative light units measured in intact cells are proportional to those found in the cell lysates[6].

In conjunction with the use of specific signal peptides, luciferase transferred to pcDNA3.0 plasmids has been used to target distinct intracellular organelles and has been developed as a reporter of free ATP. Using the mitochondrial targeting sequence for subunit VIII of cytochrome c oxidase (COX8), luciferase has been successfully targeted inside the mitochondrial matrix[7–10]. Wild-type

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luciferase contains a short C-terminal amino acid sequence that acts as a peroxisomal targeting signal[11]. Substitution of the terminal leucine (position 550) with valine abolishes the localization of luciferase at peroxisomes but maintains its properties, thereby allowing the measurement of free ATP inside the cytoplasm[12]. The same mutant has been fused with the NLS (nuclear localization sequence) portion of the large T antigen to localize the luciferase enzyme inside the nucleus[13]. Interestingly, the energy required for chromatin remodeling might originate from poly-ADP-ribose, which is directly produced inside the nucleus, rather than from the diffusion of ATP from mitochondria in the cytosol[14]. This is simply one example underlining the importance of studying ATP dynamics inside organelles in a highly specific manner.

Di Virgilio and co-workers originally developed a luciferase probe targeting the external side of the plasma membrane in 2005 (ref. 15). The firefly luciferase was engineered to incorporate an N-terminal leader sequence and a C-terminal glycosyl phosphatidylinositol (GPI) anchor of the folate receptor. A c-myc tag was inserted close to the N terminus to aid in visualization of pmeLUC expression with c-myc-specific monoclonal antibodies (mAbs). This chimeric protein, pmeLUC, targets and localizes to the outside of the plasma membrane with the catalytic site facing the extracellular environment and is therefore able to sample the ATP content of the pericellular space[15].

Before this, Dubyak and co-workers[16] had developed a protocol based on luciferase bound to the cell surface via the immunoglobulin G (IgG) binding domain of *Staphylococcus aureus* protein A (a construct named proA-luc), which allowed binding to IgG adsorbed on the surface of target cells. The probe engineered by Di Virgilio and co-workers was an improvement on this, as it was the first to allow generation of cell lines intrinsically equipped with a genetically encoded sensor of extracellular ATP. Stable cell lines transfected with pmeLUC have been used to monitor extracellular ATP concentrations in different *in vivo* models[17–21].

Overview of the protocol

Intracellular ATP synthesis is compartmentalized in different subcellular regions, such as the cytoplasm[22], the nucleus[14] and mitochondria[23,24]. Therefore, understanding how ATP levels might change in distinct cellular regions depending on specific stimuli and signals, or variations in the metabolic status could provide pivotal details on the physiology of the cell and its metabolic regulation, as well as on the role of unbalanced ATP homeostasis in different pathological contexts. The *in vitro* luciferin–luciferase assay is a relatively simple method that comprises cell transfection and expression of a luciferase probe targeted to a given organelle and the detection of the yellow–green light by a luminometer; these flashes are due to the conversion of D-luciferin (a substrate supplied by the operator during the measurement) into oxyluciferin, which is proportional to the amount of ATP present. It is known that increased mitochondrial Ca^{2+} accumulation induced by agonist stimulation triggers activation of the mitochondrial metabolic machinery, which in turn boosts ATP production in the mitochondria and, accordingly, ATP levels in the cytosol[7]. Therefore, not only the basal mitochondrial ATP content but also Ca^{2+}-stimulated ATP synthesis can be measured.

The genetically encoded probe pmeLUC (**Fig. 1**) is inserted into a vector (usually pcDNA3) suitable for transfection into mammalian cells. The pcDNA3/pmeLUC plasmid is not commercially available but is provided freely by us. The first step in the procedure is the generation of cell clones stably transfected with pmeLUC (pmeLUC cells). PmeLUC-transfected stable cell clones can be generated from virtually any mammalian cell line[15,17,25,26]. However, most experiments that we have carried out have used pmeLUC-transfected HEK293 cells (HEK293-pmeLUC). It is always critical to verify pmeLUC expression on the cell plasma membrane by immunofluorescence or immunocytochemistry and by titrating luminescence emission of pmeLUC cell cultures by sequential additions of ATP in the presence of added luciferin. Luminescence emission can be monitored using a standard plate

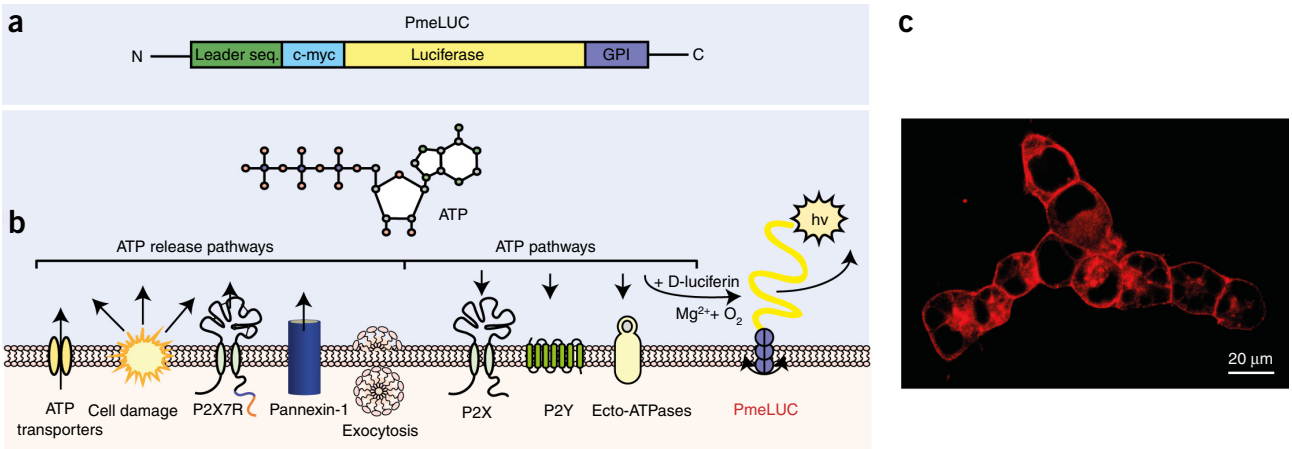

Figure 1 | Schematic rendition of pmeLUC, plasma membrane localization and pathways for ATP release. (**a**) PmeLUC structure showing the full-length luciferase coding sequence inserted in-frame between the N-terminal leader sequence (26 aa) and the C-terminal GPI anchor (28 aa) of the folate receptor. The c-myc tag is also shown. (**b**) PmeLUC is targeted and localized to the outer side of the plasma membrane, thus allowing the measurement of extracellular ATP at discrete plasma membrane sites facing the pericellular environment. Cellular release of ATP is thought to occur via several mechanisms, including (1) ATP-binding cassette (ABC) transporters[56]; (2) cell damage; (3) the P2X7 receptor[57]; (4) gap junctions and/or pannexin hemichannels[56,58]; and (5) vesicular exocytosis, perhaps involving lysosomes[59]. Extracellular ATP acts as a signaling molecule by binding P2 receptors. P2Y receptors are G-protein-coupled receptors (GPCRs), whereas P2X receptors are nucleotide-gated ion channels. (**c**) Immunofluorescence staining with anti-c-myc mAbs showing plasma membrane localization of pmeLUC in HEK293 cells. hv, yield light. **a**,**b** adapted with permission from Pellegatti *et al.*[15], American Society for Cell Biology.

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reader (e.g., PerkinElmer Wallac Victor Multilabel Counter) or luminometer (e.g., PerkinElmer Caliper IVIS or similar).

For *in vivo* experiments, reporter pmeLUC-transfected cells are amplified *in vitro*, and a cell suspension is made to the final desired concentration. Isoflurane-anesthetized mice are inoculated intraperitoneally (i.p.) with luciferin, and after 5–10 min, the relevant anatomical site is inoculated with an aliquot of the cell suspension. Pme-LUC-transfected cells maintain their light-emitting properties for several days after *in vivo* inoculation, therefore allowing changes in extracellular ATP concentration to be followed for an extended period of time. However, because luciferin is quickly cleared into the urine, it must always be i.p. injected before luminescence acquisition.

Advantages and limitations of the protocol

There are a number of advantages of this method:

- As mentioned earlier, mammalian cells lack chemiluminescent proteins, and thus the background photon emission assay is virtually zero.
- The excited oxyluciferin emits light with great efficiency[27] and the extremely high signal-to-noise ratio offers substantial advantages over fluorescence-based imaging approaches, avoiding important untoward effects such as photobleaching, phototoxicity and autofluorescence.
- The approach reported here is a simple method that is highly sensitive and rapid when a substrate is administered to cells, thus providing real-time results in only a few minutes, as compared with conventional methods, which take days.
- It is a low-cost procedure.
- Given that luciferase can be compartmentalized in different subcellular organelles, it represents a reliable technique for measuring ATP in living cells.
- pmeLUC allows ATP to be measured in the extracellular space near the plasma membrane, which is generally difficult to assess using soluble probes and reagents. Thus, ATP concentrations can be monitored at the most relevant physiological sites—i.e., in close proximity to the ATP transport systems, the relevant receptors (P2 receptors) and the degrading systems (ecto-nucleotidases). For *in vivo* measurements, this is even more critical because pmeLUC may allow for ATP measurements in protected environments such as inflammatory or tumor microenvironments.

However, there are also limitations to luciferase-based techniques. The key limitations are as follows:

- Luciferase is a genetically encoded reporter. Therefore, a critical requirement of the luciferase–luciferin system is that the cell type under investigation must be amenable to transfection.
- Basal ATP content is dependent on luciferase expression levels; thus, an immunoblot assay with an antibody against luciferase (mouse monoclonal firefly luciferase antibody; Thermo Fisher Scientific) is mandatory to verify its intracellular expression.
- Light emission from luciferase is hampered by the intrinsic structural properties of luciferin (D-luciferin), thus causing low bioluminescence output. To overcome this problem, mutant firefly luciferases that use aminoluciferins with higher efficiency have been generated[28], leading to more intense and persistent light output[29,30].
- ATP concentration and light emission are directly proportional, but the light-emitting luciferase reaction depends not only on ATP but also on the amount of molecular oxygen and the exogenous delivery of luciferin. In addition, it cannot be excluded that other ATP-consuming enzymes might affect ATP levels, but this should be a common variable of all screened samples.
- ATP levels, as measured by this protocol, are not generally expressed as concentrations but as luminescence signals (counts per second, cps). To convert cps to the actual ATP concentration, a calibration curve must be used (see below).

TABLE 1 | Summary of the most common techniques for measuring ATP *in vitro* and *in vivo*: advantages and disadvantages.

Technique	Use (detection method)	Advantages	Disadvantages	Ref.
Firefly-luciferase-targeted probes	*In vitro/in vivo* (luminescence)	Sensitivity, high affinity, can be compartmentalized in different subcellular organelles, low noise	Transfection, detection depends on luciferase expression	7,8,15,60
Aptamer-based methods	*In vitro* (mainly fluorescence)	High sensitivity, easy synthesis, low cost, enhanced chemical stability	Cell lysis	61
FRET-based ATP indicators	*In vitro* (fluorescence)	Can be compartmentalized in some subcellular organelles, uses ratiometric probe, the affinity versus ATP can be modulated	Transfection, various physical changes in the molecular system can alter the fluorescent properties of the probes	38
HPLC-based methods	*In vitro* (UV, fluorescence)	Additional measure of ADP and AMP levels, transfection not required	Cell lysis, low stability of ion-exchange columns, reproducibility	12,62
Synthesized fluorescent dye	*In vitro* (colorimetric, fluorescence)	Transfection not required	Low sensitivity	63
Perceval	*In vitro* (fluorescence)	Stronger signal, ability to monitor the ATP:ADP ratio	High pH sensitivity	42

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TABLE 2 | Organelle-specific luciferase chimeras.

Chimera	Intracellular localization	Strategy
mtLUC	Mitochondrial matrix	Addition of DNA fragments encoding a mitochondrial presequence and the hemagglutinin HA1 tag[7] (and the **Supplementary Data** for details)
cytLUC	Cytosol	Replacement of the leucine at position 550 with a valine, destroying the natural peroxisomal targeting sequence[7]
pmLUC	Subplasma membrane	Fusion of cytLUC downstream of the second external arm of the Ht1a receptor, a G-linked protein[64]
pmeLUC	Outer side of the plasma membrane	CytLUC is fused in frame with a fragment encoding the complete N-terminal leader sequence of the human folate receptor and a c-myc tag, and a C-terminal fragment of the GPI anchor protein (see ref. 15, **Fig. 1** and the **Supplementary Data** for details)
nucLUC	Nuclear	Fusion of cytLUC downstream of the SV-40 large T antigen nuclear localization signal[13]

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- PmeLUC luminescence can be calibrated *in vitro* by adding known ATP amounts to the cell culture. To determine the *in vivo* ATP concentration, the investigator must rely on *in vitro* calibration performed with the same pmeLUC-transfected cell type used for *in vivo* experiments.
- For *in vivo* measurements, luminescence depends not only on the tissue distribution of the pmeLUC-expressing cells and on the ATP concentration, but also on the amount of molecular oxygen and the exogenous delivery of luciferin. This might be critical to extracellular ATP measurement in tissues and organs that might be ischemic or hypoxic.

Applications of the protocol

The most valuable feature of this reporter system is its extreme versatility, as indicated by its use in a wide variety of applications ranging from gene expression to protein–protein interaction studies, from investigations of T lymphocyte subset expansions to analysis of tumor progression, from *in vitro* cell viability assays to *in vivo* cell tracking and for measuring intracellular or extracellular ATP content (see refs. 31–34 for details). PmeLUC-transfected cells, in addition to the applications described in the present protocol, have also been used to measure ATP release during graft-versus-host reactions[21], allergic dermatitis[20] and autophagy-associated immunogenic cell death[17,19].

Alternative methods

In addition to luciferase-based assays, several other methods for ATP detection are currently available (**Table 1**).

Different fluorimetric/colorimetric/luminescent commercial ATP determination kits are widely used; these kits can detect ATP with very high sensitivity. However, these applications do not work in intact cells but rather require cell harvesting, cell lysis and other procedures aimed at extracting the intracellular ATP content; such processes could lead to inaccurate measurements because of the rapid loss of cytoplasmic ATP. Other well-developed approaches for measuring ATP in cellular lysates or extracellular fluids are based on pyruvate kinase and adenylate kinase activity[35,36].

The most convincing alternative method for the real-time monitoring of ATP production in living cells involves the use of Förster resonance energy transfer (FRET)-based ATP indicators[37,38]. The sensor termed ATeam is based on the use of the epsilon subunit of the bacterial *Bacillus subtilis* F_0F_1-ATPase—which possesses important features, such as small size (14 kDa) and high specific binding to ATP—as an ATP-sensing probe[38]. The targeting of ATeam to different subcellular regions of HeLa cells revealed that the mitochondrial ATP content is substantially lower than the nuclear and cytoplasmic ATP content[38]. Moreover, the introduction of ATeam into the NS5A coding region of the hepatitis C virus (HCV) replicon showed the formation of specific ATP-enriched dot-like structures in HCV-replicating cells[37]. Upgraded versions of ATeam have been developed in order to increase its relative ATP affinity[38], improve its dynamic range (ATeam K206A)[39] or generate a red-shifted version (GO-ATeam)[40]. However, the ATeam probe suffers from substantial pitfalls, including (i) a lower dynamic range than that of the luciferase–luciferin system; (ii) the risk that it might buffer ATP, and therefore alter its homeostasis, when expressed at high levels; and (iii) as ATeam is inactivated by glycosylation, it cannot be used to monitor ATP in other subcellular compartments, such as lysosomes, the Golgi apparatus and the endoplasmic reticulum, where in addition the acidic luminal pH probably interferes with the FRET signal[41].

The fluorescent ATP biosensor Perceval is a genetically encoded reporter of the ATP/ADP ratio consisting of circularly permuted monomeric Venus (cpmVenus) inserted into GlnK1, a bacterial protein that regulates ammonia transport[42]. As compared with firefly luciferase, Perceval offered some advantages, including a stronger signal that allows measurement with a traditional epifluorescence microscope and a canonical charge-coupled device (CCD) camera, and the ability to monitor the energy status of a cell based on the competitive binding of ATP and ADP, whereas luciferase depends solely on ATP hydrolysis reaction[42]. However, Perceval displays a high pH sensitivity, making its fluorescence signal difficult to interpret, and becomes saturated at a low ATP/ADP ratio (ATP/ADP <5), thereby limiting its effectiveness in mammalian cells, which can reach ATP/ADP ratios of ~100 under healthy conditions. To avoid this latter pitfall, a new version of Perceval, named PercevalHR[43], has been developed. PercevalHR is able to sense large changes in ATP/ADP ratios and is compatible with other fluorescent probes in multichannel imaging experiments[44]. To date, no report of Perceval targeting to different intracellular regions has been published.

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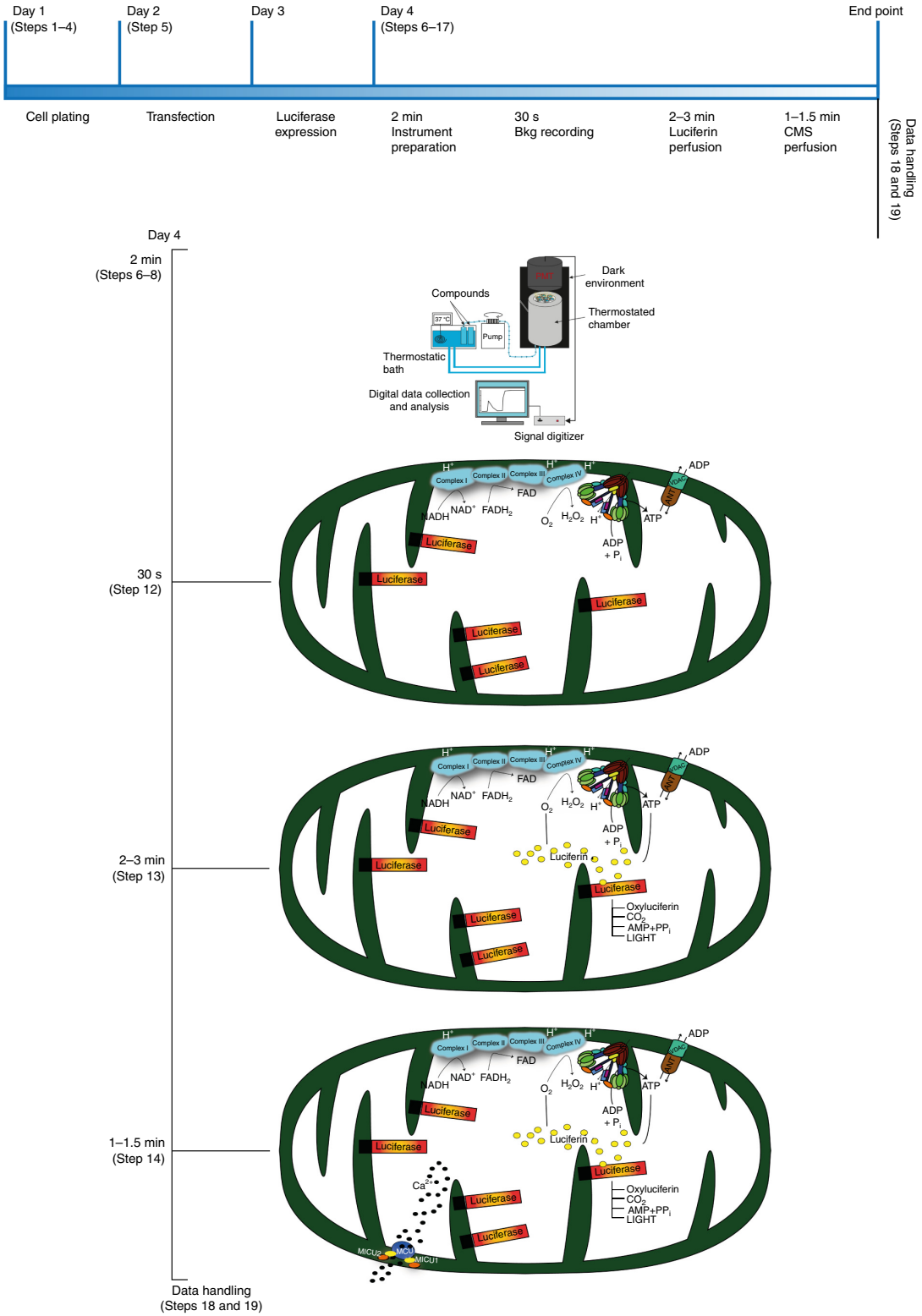

Figure 2 | Schematics of the *in vitro* protocol. Overview of the *in vitro* protocol. *In vitro* experimental workflow takes generally 3–4 d from cell preparation (day 1; Steps 1–4) to data handling (Steps 18 and 19). Cell plating is followed by transfection (or infection/electroporation, depending on the cell type being analyzed; Step 5) of luciferase constructs. In this scheme, mitochondrial luciferase (mtLUC) expression must proceed for 24–48 h before it is detectable at the luminometer. The three-phase acquisition starts with 30 s of background recording in modified KRB solution (Step 12). Then, the cell population is perfused by luciferin and in 2–3 min luciferase catalyzes light production, reaching a plateau after reacting with intracellular ATP and oxygen-producing oxyluciferin, CO_2, AMP and pyrophosphates (Step 13). The bottom panel shows the calcium-dependent ATP production by mitochondria (Step 14). CMS perfusion allows the activation of the calcium signal pathway; calcium released from stores (e.g., from the endoplasmic reticulum) enters mitochondrial organelles through the MCU complex (composed of MCU in blue, MICU1 in yellow and MICU2 in orange, at the inner mitochondrial membrane and in the intermembrane space) and promotes an increased amount of ATP production by complex V. CMS: calcium-mobilizing solution. PMT, photomultiplier tube.

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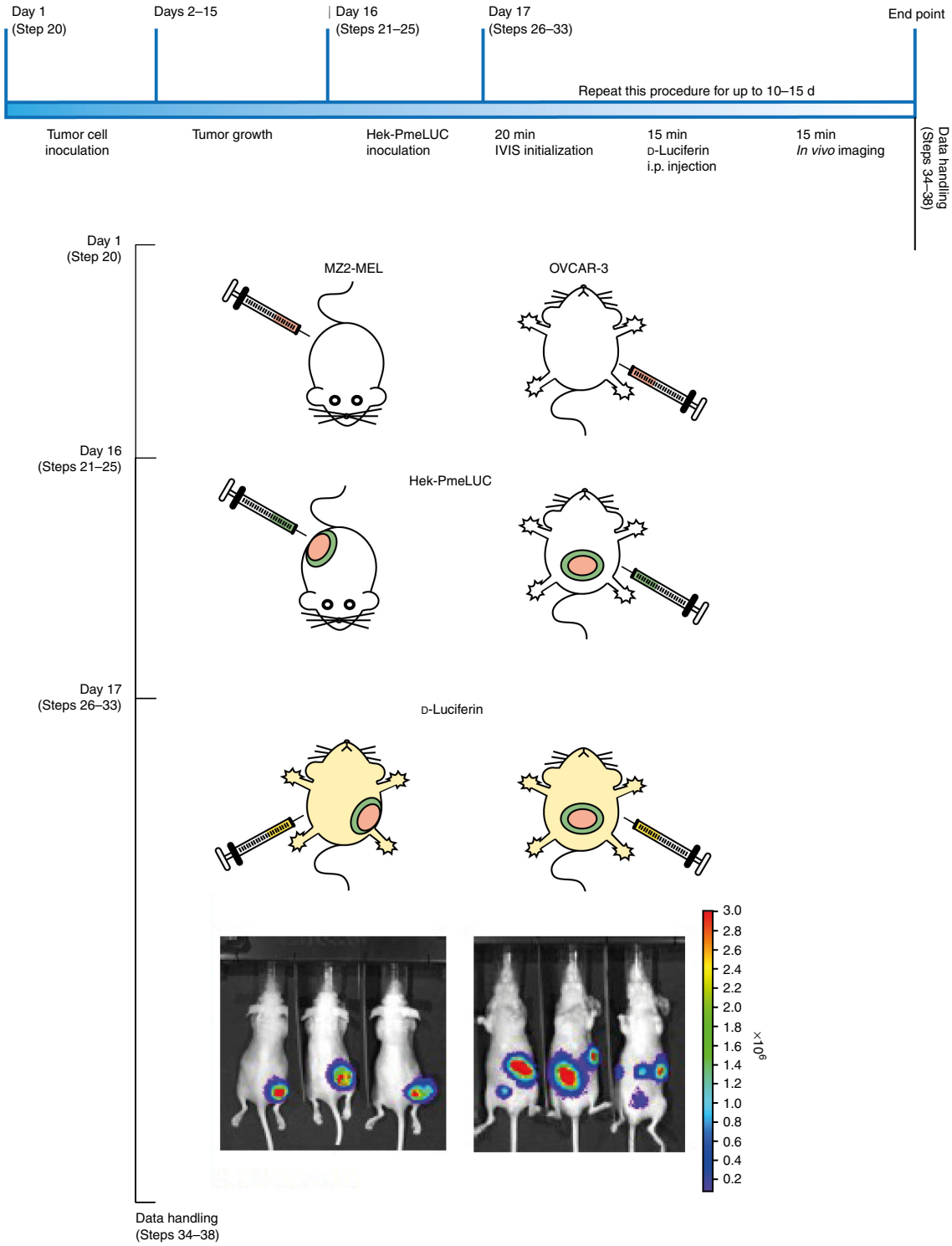

Figure 3 | Schematics of the *in vivo* protocol. Day 1: Athymic nude (nude/nude) mice are inoculated with MZ2-MEL melanoma cells (s.c. in the right hip) or OVCAR-3 cells (i.p.) (Step 20A and 20B). Tumor establishment takes ~2 weeks. After 16 d, mice are inoculated with a stable HEK293-pmeLUC cell clone, either directly into the tumor mass (MZ2-MEL melanoma) or i.p. (OVCAR-3 carcinoma) (Steps 23–25). Then, after a pause of at least 24 h to allow HEK293 cell re-distribution, on day 17, the Live Image software is started. It is important to wait until the instrument is ready for operation. Fifteen minutes before image acquisition, the anesthetized tumor-bearing mice are injected i.p. with D-luciferin. Anesthetized mice are then placed into the light-tight chamber of the IVIS Imaging System and imaged. 3–4 mice can be imaged at the same time. ROIs are drawn around the tumor sites and quantified as photons/s using the Living Image software. Mice are imaged every 2 d for up to 15–20 d (Steps 26–33). Strict animal care procedures were in accordance with institutional guidelines and in compliance with national and international laws and policies (European Economic Community Council Directive 86/109, OJL 358, 1 Dec 1987 and NIH Guide for the Care and Use of Laboratory Animals). We routinely use 5- to 6-week-old nude/nude mice weighing 18–25 g purchased from Harlan Laboratories (Udine, Italy), and housed in sterile cages. Luminescence is expressed as photons/sec/cm^2/sr (the number of photons per sec that leave a square centimeter of tissue and radiate into a solid angle of 1 steradian (sr)).

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Box 1 | Adenoviral infection procedures ● TIMING 2–3 d

Recombinant nonreplicative adenoviruses are useful for inducing protein expression in cell lines that have low transfection efficiency with the liposome method. After entering cells, the virus is not able to integrate into the host genome; a CMV promoter drives the firefly luciferase expression. Here, we provide some additional information so that this protocol may be applied under any experimental conditions.

Additional reagents

- mtLUC- or cytLUC-expressing adenovirus
- Hexadimethrine bromide, polybrene (Sigma-Aldrich, cat. no. H9268)

Additional procedures

The following step should be performed instead of that in the main Procedure:

- At Step 5, infect cells in 200 µl of medium by adding 2 µl of virus and 5 µg/ml of polybrene. Add 300 µl of fresh medium after incubation for 4 h at 37 °C.

! CAUTION Virus handling requires the following of appropriate safety procedures. For further details on this topic, refer to ref. 65.

▲ CRITICAL STEP Using polybrene enhances adenoviral transduction[66].

? TROUBLESHOOTING

■ PAUSE POINT After infection, wait for 36–48 h before proceeding to Step 6 of the main Procedure.

Extracellular ATP is usually measured by adding luciferase and luciferin to cell cultures or to the cell supernatants. This is a sensitive and selective *in vitro* assay, but it is highly impractical for *in vivo* or real-time measurements. In addition, assaying the cellular supernatants provides little information on the ATP concentration in the pericellular space. Different techniques have been developed over the past several years to sample extracellular ATP (or ATP release) in interstitial fluid, or in close proximity to the outer surface of the plasma membrane. In 2005, Dale and co-workers described a microelectrode ATP biosensor that allows monitoring of levels of extracellular ATP in tissue slices and live animals during physiological activity[45]. Detection of ATP is based on the use of microelectrodes functionalized with glycerol kinase and glycerol. Conversion of glycerol to glycerol 3-phosphate in the presence of ATP generates hydrogen peroxide, which is detected at the electrode. The current generated is directly related to the ATP concentration. The major drawback of this method is that the insertion of the electrode into a tissue causes injury, and therefore by itself increases the extracellular ATP levels. In 2007, Corriden *et al.*[46] proposed an assay based on a tandem enzyme reaction driven by hexokinase and glucose-6-phosphate dehydrogenase (G6PD), which, in the presence of ATP and glucose, convert NADP to NADPH at an equimolar ratio. Accumulation of NADH is proportional to the ATP concentration, and can be measured by fluorescence microscopy (excitation at 340±30nm and emission at 460±50nm). Schneider *et al.*[47] used atomic force microscopy with a scanning tip coated with the ATPase-containing S1 myosin subfragment to measure extracellular ATP released near the plasma membrane. Finally, Hayashi *et al.*[48] used the current generated by the opening of an ATP-gated plasma membrane

Box 2 | Evaluation of luciferase expression in cells by immunoblotting ● TIMING 2 d

The transfection (or infection) efficiency among cells and/or the experimental conditions could greatly vary and are influenced by many factors, such as the transfection method chosen, the viability and confluency of the cells and the quality and quantity of the nucleic acid used. In agreement with this statement, performing an immunoblot assay with an antibody against luciferase is suggested to confirm that protein levels are similar among various experimental conditions.

Additional reagents

- Mouse monoclonal firefly luciferase antibody (Thermo Fisher Scientific, cat. no. MA1-80225)
- Most common reagents for protein electrophoresis

Additional equipment

- ImageQuant LAS4000 (GE Healthcare) or similar

Additional procedures

To use cells for an immunoblot assay, the following steps should be performed in parallel to those in the main Procedure:

- With Step 4, lyse the whole cell sample, and quantify the extracted proteins following the preferred method. Load at least 10 µg of protein on a precast SDS-PAGE gel, and incubate the nitrocellulose-membrane-transferred proteins with the antibody against luciferase, according to the manufacturer's instructions.
- With Step 5, detect the luciferase band at 62 kDa using horseradish peroxidase (HRP)-secondary antibody, and acquire the signal using the ImageQuant LAS4000.

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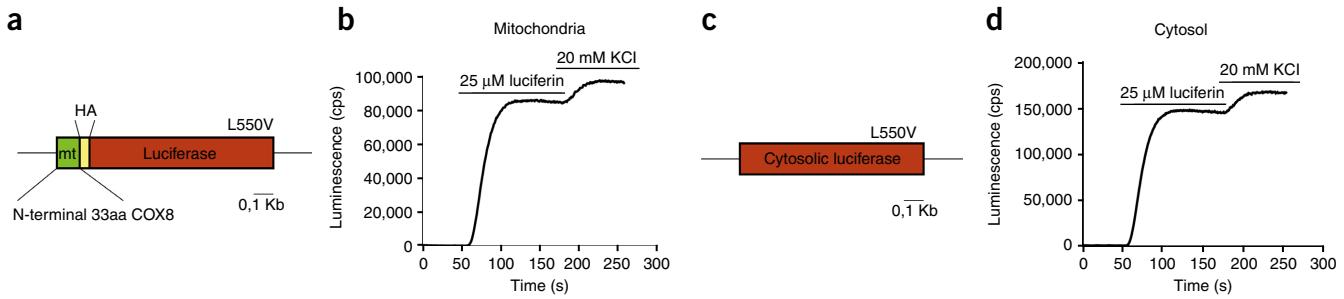

Figure 4 | ATP kinetics in mitochondrial and cytosolic compartments. (**a**) Diagram of the mitochondrial luciferase DNA. A mitochondrial presequence encoding 33 aa of the N-terminal COX8 protein is shown in green and the hemagglutinin HA1 tag that precedes the luciferase nucleotide sequence (red) is shown in yellow. This mitochondrial construct derives from the cytosolic luciferase (**c**), characterized by a substitution of the terminal leucine (position 550) with valine in the wild-type sequence that abolishes its localization at peroxisomes, maintaining its properties. (**b**) An example of mitochondrial ATP kinetics in the HL-1 cell line. (**d**) An example of cytosolic ATP kinetics depicting the increased ATP signal in this organelle as compared with mitochondria.

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ion channel as a readout of ATP release. Briefly, a responder cell was transfected with the P2X2 receptor, patched on a patch-clamp and placed near an ATP-releasing target cell.

Experimental design

The *in vitro* protocol described here (Steps 1–19) measures ATP in the HL-1 cell line (mouse cardiac muscle cell line; patent no. EP0956341 A2) and in the pericellular space of HEK293 cells (HEK293-pmeLUC). The use of HEK293-pmeLUC cells to sample the ATP concentration in the microenvironment of ovarian carcinoma tumors (generated by OVCAR-3 cell inoculation) and melanoma tumors (generated by MZ2–MEL cell inoculation) is also described in Steps 20–38. Because of the variety of intracellular ATP-containing compartments and the need to sample extracellular ATP, different targeting sequences were added to modify wild-type cytosolic luciferase, giving rise to nucLUC, mtLUC (mitochondrial luciferase), pmLUC and pmeLUC constructs, as detailed in **Table 2** and the **Supplementary Data**. Although this library of constructs is well expressed in cells, it is not mammalian-codon-optimized; thus, here we suggest a link to a comprehensive usage tool that allows for their optimization in any host (http://www.genscript.com/codon-opt.html). **Figure 2** provides an overview of the key stages of the protocol for measuring ATP within different intracellular compartments in living cells (Steps 1–19), whereas **Figure 3** shows the stages for measuring ATP *in vivo* in animals (Steps 20–38). These figures summarize cell preparation and transfection, instrument setup, ATP level measurements and data handling. For *in vitro* measurements, once the researcher has chosen the luciferase construct of interest and the appropriate method of transfection (primary cultures are notably difficult to transfect, and often require electroporation or infection with adenoviral vectors; see **Box 1** for details), he/she must then make two fundamental decisions regarding (i) what luciferin concentration to perfuse (Steps 13 and 14) and (ii) how to assess intracellular ATP production (see Step 14 and ANTICIPATED RESULTS section). For intracellular ATP measurement, luciferin should be used in a concentration ranging from 20 to 200 µM, as proportionality between luminescence values and perfused luciferin is maintained only when these concentrations are used. Furthermore, although a range of concentrations can be used, we suggest using the lowest luciferin concentration that produces a reasonably strong signal. The *in vitro* protocol ends with backup data (Steps 16 and 17) and data handling (Steps 18 and 19) procedures following the instructions provided in the Procedure and in the figures.

The luciferase–luciferin system for measuring ATP strongly depends on luciferase expression in cells; therefore, the detection of its levels upon transfection of different experimental samples and before data analysis and discussion is mandatory (**Box 2**). ATP assays are often reported in light units (**Figs. 4** and **5**), and any interpretation to yield concentration values requires a calibration curve that can developed by adding known ATP amounts to LUC-expressing cells under strictly controlled (temperature, pH, divalent ion concentration, serum content of the culture medium) experimental conditions (see below).

In vivo. Unlike the use of mtLUC and cytosolic firefly luciferase DNA (cytLUC) previously described (Steps 1–19), the genetically encoded probe pmeLUC (**Fig. 1**) has been used to measure ATP in the vicinity of the outer aspect of the plasma membrane in a variety of mouse and human cell types, as well as *in vivo*[15,18]. A c-myc tag has been inserted close to the N terminus to allow visualization of pmeLUC expression with c-myc-specific mAbs (**Fig. 1** and **Box 3**); moreover, an *in vitro* validation of the probe can be performed before its use in an *in vivo* experimental setting (**Box 4**). For *in vivo* ATP measurement, reporter cells stably expressing pmeLUC are prepared 3 d before the complete establishment of the *in vivo* tumor (Steps 21 and 22) and then inoculated through either i.p. or intramass injection (Steps 23–25). Alternatively, the tumor cells themselves can be transfected with pmeLUC and inoculated into the host[17] (not shown in this Procedure). As reporter cell, any easy-to-transfect cell type can be used. A reference calibration curve can be built *in vitro* by adding known ATP amounts to the pmeLUC-expressing cells. The *in vitro* calibration curve run for each given pmeLUC-transfected cell can also be used to roughly estimate the *in vivo* extracellular ATP levels (see **Box 4**, steps 7–9). For *in vivo* measurements of ATP within the pericellular space, the first and most important step is checking pmeLUC expression and localization (**Box 3**). Once an appropriate expression of pmeLUC has been verified (**Box 3**), the size of the inoculum of the pmeLUC-expressing cells must be chosen, usually 2×10^6 cells. PmeLUC cells are then injected into the relevant anatomic site—i.e., into the peritoneum or into the tumor mass (Step 25).

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Luciferin should be i.p. injected at a concentration of 150 mg/kg. Furthermore, it is important to consider that luciferin is almost completely cleared from the mouse body within ~2–3 h[49]. Be sure that the mice are fully anesthetized (i.e., completely immobile) during luminescence acquisition to avoid blurry and misplaced images. Finally, and importantly, always carefully check mouse well-being, and stop the protocol and ask for veterinary advice if signs of distress due to tumor growth and/or experimental manipulations appear.

In vivo measurement of extracellular ATP has always been exquisitely demanding because of the inherent difficulty of measuring a quickly degradable chemical species in a compartment that is difficult to access. Furthermore, the investigator must understand absolutely that many factors, mouse manipulation and diet included, may cause an increase in extracellular ATP levels, and may therefore affect data reproducibility. However, because of high permeability of luciferin across tissues, expression of the plasma-membrane-tethered luciferase (catalytic site outside) allows ATP sampling in close proximity to the cell surface, even in anatomically protected compartments. As for most *in vivo* measurements, luminescence can be affected by several factors. Therefore, it is mandatory to use a sufficiently large number of animals (five or more) for each given experimental condition and to perform the appropriate controls—i.e., acquisition of images from noninjected sites to establish background luminescence levels and injection of apyrase at the site of pmeLUC-transfected cell inoculation to check for specificity of luminescence emissions. Apyrase is a potato ecto-nucleotidase with a strong ATP-hydrolyzing activity. Abrogation of pmeLUC luminescence upon apyrase addition is a generally accepted proof of the ATP specificity of the reaction. The *in vivo* extracellular ATP concentration can be estimated only indirectly by performing an *in vitro* calibration by adding known amounts of ATP to a monolayer of cells from the same batch used for the inoculum. For calibration purposes, luminescence increases must be recorded with the IVIS setup used for *in vivo* experiments (**Box 4**). If the pmeLUC signal is not responsive to apyrase injection, this usually means that pmeLUC is not properly expressed on the cell surface (i.e., it has been retained within the proteosynthetic patyhway or even diverted to the cytoplasm) and therefore is not accessible to extracellular apyrase. In this case, it is advisable to check pmeLUC expression and, if necessary, to try to enhance plasma membrane localization (see the Troubleshooting section), and to start a new experiment. An ROI should be carefully drawn around the luminescent spot on the mouse body, and luminescence emissions should be recorded for at least 3–5 min. Draw an ROI of the same size over an area far from the luminescent spot for background measurement. Luminescence is expressed as radiance—i.e., photons/s in each pixel summed or integrated over the ROI area ($cm^2 \times 4\pi$). Subtraction of background luminescence allows measurement of the specific ATP-dependent luminescence, and therefore estimation of the ATP concentration of the tissue interstitium (**Fig. 3**)[18]. It is important to repeat luminescence measurement at at least 2 d intervals for 15–20 d from the inoculum to monitor HEK293-pmeLUC cell re-distribution within the tumor and throughout peri-tumor tissues. The use of pmeLUC-transfected reporter cells (HEK293-pmeLUC) allows real-time measurement of the ATP concentration in the tumor microenvironment.

Measurement of intracellular ATP in living cells. Mitochondrial ATP production and its cytosolic accumulation can be evaluated by either perturbing Ca^{2+} resting conditions, with the generation of changes in intracellular Ca^{2+} concentration triggering ATP synthesis, or analyzing the contribution of the single oxidative phosphorylation (OXPHOS) pathway. For the first case, the Ca^{2+}-mobilizing solution (CMS) must be chosen with consideration of the cell type because receptors capable of activating Ca^{2+} flux could differ in type and expression depending on the cell model. For the second case, the use of oligomycin or N,N′-dicyclohexylcarbodiimide (DCC) (two known, potent ATP synthase inhibitors

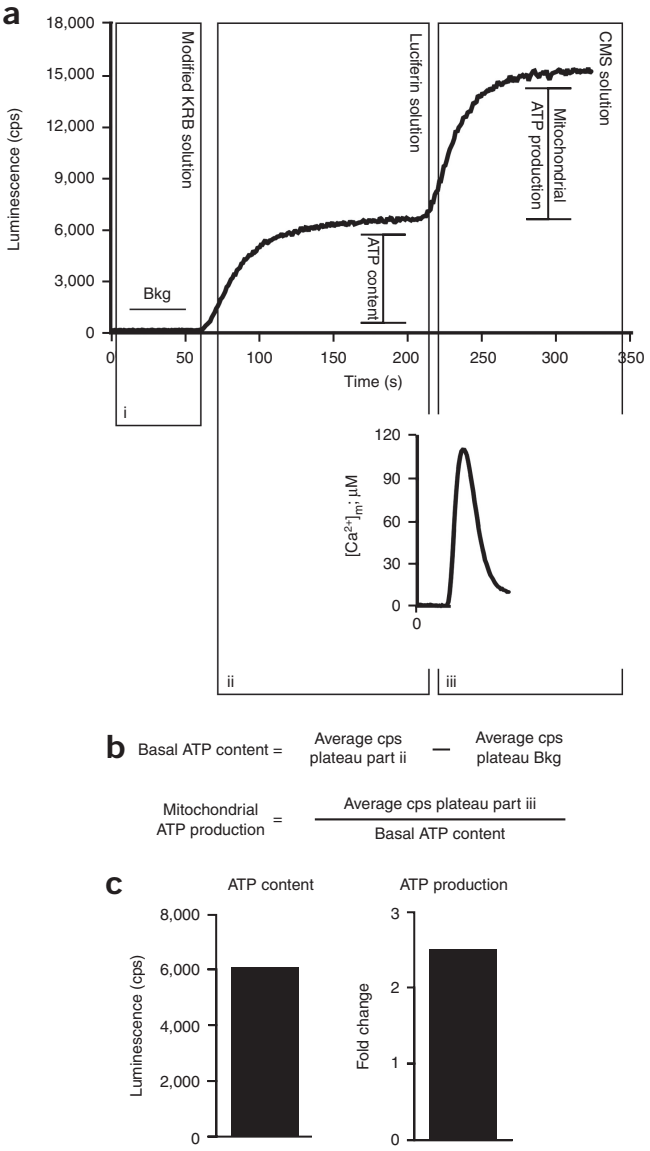

Figure 5 | Calculation of basal ATP content and mitochondrial ATP production. (**a**) A mitochondrial ATP kinetics is used as an example to describe the Anticipated Results section. It shows the background recording (i), and the basal-induced (from 60 to 210 s, ii) and calcium-induced (from 211 to 320 s, iii) ATP measurements. The mitochondrial calcium uptake after CMS perfusion that assists and triggers mitochondrial ATP production is shown at the bottom of panel **a**. *y* axis = calcium concentration; *x* axis = time. (**b**) Suggested math formulas for calculating basal ATP content and ATP production; formulas refer to panel **a**. (**c**) Final representation of the data; graphs are derived from data in panel **a**. Bkg, background; cps, counts per s; m, mitochondrial.

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Box 3 | *In vitro* validation of pmeLUC-transfected HEK293 cells by immunofluorescence with anti c-myc-SC-40 monoclonal antibody ● TIMING ~3 d

Transfection efficiency is greatly affected by many variables, such as the method of choice, cell viability and confluency, and the quality and quantity of the nucleic acid used. Immunofluorescence staining with an anti-c-myc mAb (**Fig. 1**) is recommended to check for pmeLUC expression and localization.

Additional reagents

- Anti c-myc-SC-40 mAb (Santa Cruz Biotechnology, cat. no. 9E10)
- Texas-Red-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, cat. no. sc-2781)

Additional reagent setup

Triton X-100, 0.1% (vol/vol) in PBS. Store it at RT (25 °C) for up to 1 m.
Blocking buffer (0.2% gelatin in PBS). To prepare 25 ml, add 2.5 ml of 10× PBS and 2.5 ml of gelatin to 20 ml of dH2O and mix well.
Antibody dilution buffer. anti c-myc-SC-40 mAb antibody 1:100 dilution in 0.2% gelatin-containing PBS. Texas-Red-conjugated goat anti-mouse IgG, 1:50 dilution in 0.2% gelatin-containing PBS. Antibody solutions should be combined at this point.
! CAUTION Any time a primary or secondary antibody is used, it is necessary to titrate the antibody to identify the optimal dilution yielding the strongest specific signal with the lowest background.

Additional equipment

A standard inverted fluorescence microscope equipped with high-numerical-aperture lenses and a tetramethylrhodamine isothiocyanate (TRIC) filter set; see below:

Menu	Setting
'Illumination settings'	Switch on the laser and wait 30 min for it to warm up.
'Excitation filter'	Select the dedicated filter set to detect Texas Red fluorescence.
'Microscope control'	Select the 63× oil-immersion objective from the list.
'Experiment manager'	Excitation filter, 560 (nm); Emission filter, 610 (nm).

Additional procedures

The following steps should be performed in parallel to those in the main Procedure (Steps 23–33):

Coverslip preparation

1. Coat circular glass coverslips (25-mm diameter) with 0.02% gelatin in PBS for 2 h at 37 °C and place one coverslip into each well of a six-well plate (BD Falcon, cat. no. 353046).
2. Wash the coverslips twice with PBS and let them dry for several minutes.
3. Rinse the coverslips twice by adding 2 ml of sterile PBS to each well.

Cell monolayer preparation

4. Plate 2 × 10^4 cells in each well of a six-well plate and let them adhere overnight.
▲ CRITICAL STEP The amount of plated cells must be optimized for each given cell type.
5. Wash cells once with 1× PBS, and incubate with 4% formaldehyde in PBS for 30 min. All steps are carried out at room temperature.
? TROUBLESHOOTING
■ PAUSE POINT Fixed cells can be stored in PBS at 4 °C for up to a week.
6. Rinse coverslips three times with PBS.
7. Permeabilize cells by dispensing 1 ml of 0.1% (vol/vol) Triton X-100 into each well, and incubate the plate at RT for 10 min.
8. Remove permeabilizing solution and add 2 of ml blocking buffer. Incubate at room temperature for 1 h.

Primary antibody staining for fluorescence microscopy

9. Dilute the primary antibody in blocking buffer.
10. Place the inverted coverslips, cells down, onto a drop of blocking buffer.
11. Carry out hybridization for 1 h at 37 °C.

Staining with secondary antibody

12. Dilute the secondary antibody in blocking buffer.
13. Incubate the coverslip with the secondary antibody for 1 h at room temperature in a light-tight box.
▲ CRITICAL STEP Cover the cell monolayer evenly with the secondary antibody solution.
14. Wash the cells three times with PBS for a total of 15 min.

Coverslip mounting and image acquisition

15. Mount coverslips with Prolong Gold antifade reagent on a glass microscope slide. Allow each slide to dry for at least 1 h in a light-tight box, and analyze for fluorescence emissions.
▲ CRITICAL STEP Press the coverslip gently onto the slide, using tweezers to make sure that the cell monolayer is evenly in focus.
? TROUBLESHOOTING

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Box 4 | *In vitro* analysis of pmeLUC expression and activity: luminescence recording from pmeLUC-transfected HEK293 (HEK293-pmeLUC) cells with a total body luminometer for small animals (PerkinElmer IVIS) ● TIMING ~ 1 d

If the researchers must validate or test pmeLUC expression and its *in vitro* activity before using it in *in vivo* experiments, they can apply step-by-step the protocol outlined below.

Cell preparation

1. Harvest HEK293-pmeLUC cells with trypsin–EDTA for 5 min at 37 °C and resuspend the cells in complete DMEM-F12 medium.
2. Count the cells with a hemocytometer. Centrifuge the cells at 200*g* for 5 min at RT, remove the medium and resuspend the cells in complete DMEM-F12 medium at a concentration of 7 × 10^4 cells per ml. Gently agitate the cell suspension to avoid cell aggregates.
3. Plate 1 ml of cell suspension in a 24-well plate and allow the cells to adhere at 37 °C in a 5% CO_2 humidified incubator.

■ **PAUSE POINT** After plating, wait for 24 h.

Initialization of the IVIS luminometer ● TIMING ~ 0 min

4. The following day, initialize the IVIS system by starting the Living Image software and clicking on 'Initialize IVIS system' in the system control panel.
5. Wait until the 'Temperature Square' display in the IVIS System Control turns from red to green. When the temperature is locked to –90 °C, as indicated by the green square, the instrument is ready for operation.
6. Set the acquisition from the 'Acquisition Control Panel' as follows: put a check mark on the 'Luminescent' option and select the following parameters: 'Exposition Time', 1 min; 'Binning', 4; 'F/STOP', 1. Put a check mark on the 'Photograph option, Auto exposition'. Put a check mark on 'Overlay'. Select option 'D' from the 'Field of View' list.

Measurement of ATP-dependent luminescence ● TIMING ~15 min

7. Add 8 µl of a 15 mg/ml D-luciferin sodium salt solution to each well. Wait for 3 min.
8. Click the 'Acquire' button. Once acquisition has been completed, the Living Image software automatically co-registers images in the 'image window' to generate an overlay image of the photographic image (in grayscale) and the luminescent image (in pseudocolors).
9. Build a calibration curve by adding increasing ATP concentrations (from 1 µM to 1 mM) to HEK293-pmeLUC cells plated in the 24-well plate (at least triplicate samples for each ATP concentration).

▲ **CRITICAL STEP** Gently stir each well soon after ATP addition to evenly mix the ATP.

10. Click the 'Acquire' button to record ATP-dependent luminescence emissions.

? TROUBLESHOOTING

11. Optional control: add 5 U of apyrase to each well and start a new acquisition. Apyrase quickly hydrolyzes extracellular ATP and therefore should obliterate luminescence emissions.

Image processing and data analysis. ● TIMING ~15 min

12. Open the stored images using the Living Image software.
13. Draw a circular ROI around each well on the ATP calibration curve image using the free-draw method.
14. Select the type of data to include in the table from the ROI measuring table. In this case, select 'Total Flux' (i.e., radiance, photons/s) emitted from each pixel summed or integrated over the ROI area (cm^2 × 4π) and 'St. dev. Radiance' (standard deviation of the pixel radiance inside the ROI).
15. Export the data in a spreadsheet-compatible format (i.e., Microsoft Office Excel), and report the data for each ROI in a single column.
16. Subtract the background value for each column at every given imaging time.
17. Plot the corrected luminescence values obtained by adding the known concentration of ATP for each sample as a function of the final concentration of ATP.
18. Draw the best smooth curve through these points to construct the standard curve. Calculate the trend line equation based on your standard curve data.
19. Use your equation to determine the luminescence (total flux) of your sample as a function of ATP concentration.

targeting the C subunit of the F_O region of ATP synthase[50]) dissolved in luciferin solution as a perfusion buffer following ATP basal content detection is critical to defining the OXPHOS and, hence, the glycolytic contribution in the experimental setting.

Controls. The only suggested control for the Procedure is an immunoblot assay (**Box 2**) to assess the levels of transfected luciferase, as the signal recorded mainly depends on luciferase expression inside cells.

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MATERIALS

REAGENTS

- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- Potassium chloride (KCl; Fluka, cat. no. 60128)
- Potassium phosphate, monobasic (KH_2PO_4; Sigma-Aldrich, cat. no. P0662)
- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$; Sigma-Aldrich, cat. no. M5921)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Glucose (Sigma-Aldrich, cat. no. G7528)
- Calcium chloride, 1 M ($CaCl_2$; Fluka, cat. no. 10043-52-4)
- Sodium hydroxide (NaOH; Fluka, cat. no. 71691)
- Sodium phosphate, dibasic (Na_2HPO_4; Sigma-Aldrich, cat. no. 255793)
- Milli-Q Direct System water (Millipore, http://www.merckmillipore.com/IT/it/product/Milli-Q-Direct-Water-Purification-System,MM_NF-C85358#ordering-information)
- Ethanol (Sigma-Aldrich, cat. no. 02860)
- Beetle luciferin, potassium salt (Promega, cat. no. E1605)
- Cytosolic firefly luciferase DNA (cytLuc, available from our lab)
- Mitochondrial firefly luciferase DNA (mtLuc, available from our lab)
- Ca^{2+}-perturbing agents
- N,N′-dicyclohexylcarbodiimide (DCC; Sigma-Aldrich, cat. no. 36650)
- Gelatin (Sigma-Aldrich, cat. no. G1890)
- Lipofectamine 2000 (Thermo Fisher Scientific, cat. no. 11668019)
- Adherent cultured cell lines. The anticipated results described in this protocol have been obtained using HL-1 cells (Sigma-Aldrich, cat. no. SCC065). However, the Procedure has been successfully tested in PC3, HEK293T, DU145, HeLa, CHO and breast cancer cells, as well as in mouse embryonic fibroblasts and human adult fibroblasts **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic and they are not infected with mycoplasma.
- ***In vivo* experiments only**
- EDTA (Sigma-Aldrich, cat. no. E5134)
- Tris–HCl (Sigma-Aldrich, cat. no. 33742)
- Dithiothreitol (Merck, cat no 1.11474.0005)
- ATP disodium salt (Roche, cat. no. 10 519 987 001)
- ProLong Gold Antifade mountant (Thermo Fisher Scientific, cat. no. P10144)
- Triton X-100 (Sigma-Aldrich, cat. no. T9284)
- Paraformadehyde solution (Santa Cruz, cat. no. CAS 30525-89-4)
- Isofluorane-IsoFlo (Abbott Animal Health, cat. no. 103287025)
- Apyrase (Sigma-Aldrich, cat. no. A6535)
- FBS (Life Technologies, cat. no.10270)
- RPMI-1640 (Sigma-Aldrich, cat. no. R6504-10L)
- DMEM-F12 (Sigma-Aldrich, cat. no. D8900-10L)
- Trypsin–EDTA (Euroclone, cat. no. ECB 3052D)
- Penicillin–streptomycin solution 100× (Euroclone, cat. no. ECB3001D)
- Adherent cell lines: extracellular ATP with PmeLUC can be measured with any cell line (e.g., CT26-PmeLUC mouse colon carcinoma and mouse B16-pmeLUC melanoma cells). However, the present protocol was developed using human HEK293-pmeLUC cells (Sigma-Aldrich, cat. no. 85120602-1VL). Human ovarian carcinoma OVCAR-3 and human M2Z-Mel melanoma cells, which can be purchased from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium), are used for *in vivo* ATP sampling in the tumor microenvironment
- Mice: we have used Hsd:Athymic Nude-Foxn1nu mice (Envigo, cat. no. 498761). For this procedure, a minimum of five mice weighing 18–25 g is recommended **! CAUTION** Any experiments involving live mice must conform to relevant institutional and national guidelines and regulations. All experiments involving animals were reviewed and approved by the Italian Ministry of Health.

EQUIPMENT

- Parafilm (Sigma-Aldrich, cat. no. P7793)
- 1.5-ml Microcentrifuge tubes
- 50-ml Tubes
- Aluminum foil
- Glass coverslip, 13-mm diameter, thickness between 0.16 and 0.19 mm (Thermo Fisher Scientific)
- Peristaltic pump (Elettrofor Scientific Instruments)
- Temperature-controlled bath (Elettrofor Scientific Instruments)
- Windows-based PC (a custom-made setup could also be implemented on Mac or Linux-based workstations)
- Light measuring: custom-made luminescence reader (aequorinometer) or any similar single-photomultiplier tube (PMT), photon-counting head (Hamamatsu, model no. C8855-01), digitizer counting unit (Hamamatsu, model no. C9744) or high-voltage power supply (Hamamatsu, model no. C9525). Alternatively, many other devices can be purchased, such as a GloMax Discover System (Promega), a Lucetta Luminometer (Lonza), a LUMIstar Omega Plate Reader (BMG Labtech) or a Luminoskan Ascent Microplate (Thermo Fisher Scientific)

***In vivo* experiments only**

- XGl-8 gas anesthesia system
- Total-body luminometer (PerkinElmer, model no. Caliper IVIS 100, or other compatible equipment)
- 27-gauge syringe needle, 1/2 inch (BD, cat. no. 305109)
- Syringe filters (for sterilization), 0.45 µm (Corning, cat. no. 431220)
- Hemocytometer, 0.0025 mm^2 (Labor Optik, Neubauer)
- Manual caliper (Vetrotecnica)

REAGENT SETUP

DMEM DMEM-F12 medium containing 0.5 mM sodium pyruvate and 15 mM sodium bicarbonate is supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, and 1% penicillin–streptomycin solution (see Reagents list). Store the medium at 4 °C for up to 1 month.

TRIS–EDTA TRIS–EDTA (TE) buffer is 10 mM Tris–HCl and 1 mM EDTA at pH 7.4 in filter-sterilized dH_2O. Dissolve 121.14 mg of Tris–HCl and 37.24 mg of EDTA in 100 ml of Milli-Q water. Adjust the pH to 7.4 with HCl, and then bring the final volume to 100 ml. Sterilize by filtration and store the solution at room temperature (RT, 25 °C) for up to one month.

15 mg/ml D-Luciferin stock solution Reconstitute 1 g of D-luciferin with 66 ml of sterile PBS. Allow the luciferin solution to sit for a minimum of 15 min with gentle agitation before making 1-ml aliquots. Aliquots can be

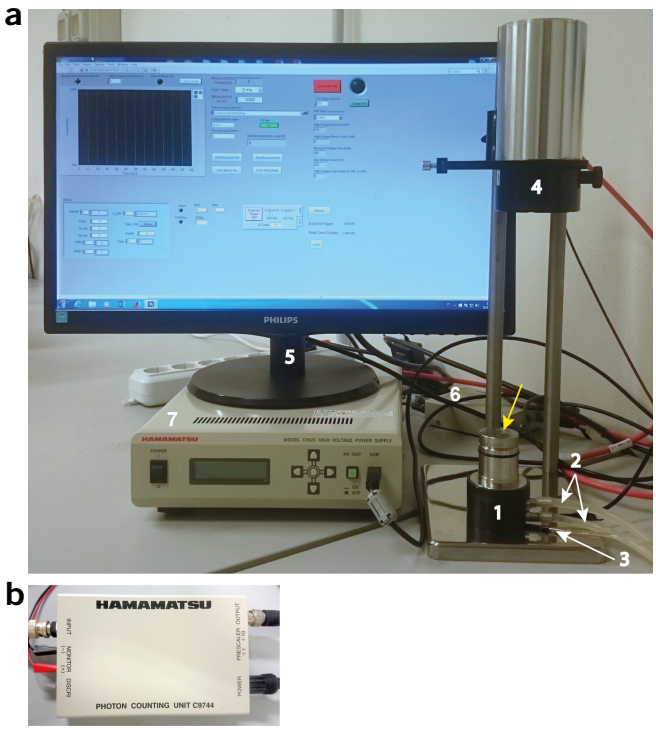

Figure 6 | The luminometer. (**a**) The system comprises a perfusion chamber above a hollow metal cylinder (1), which is temperature-controlled by a couple of tubes (2) connected to a thermostatic group and is continuously perfused with solutions (3) via a peristaltic pump. The coverslip with cells that is lying in the chamber (yellow arrow) is placed 2 mm from the surface of a high-performance photomultiplier tube (4). The photon counts are then transferred to a computer (5) via the photon-counting head (**b**) and a digitizer unit (6). The instrument is supported by a high-voltage power supply (7).

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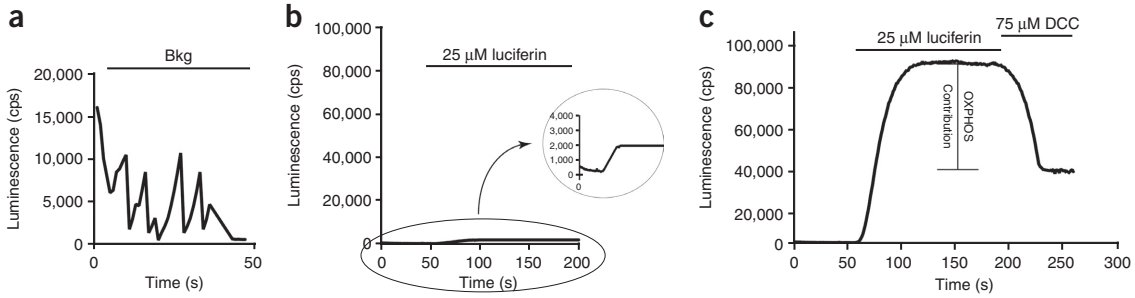

Figure 7 | OXPHOS contribution in ATP synthesis. (**a**) An example of an experiment that was not properly set up, in which the background signal shows high noise and instability. (**b**) An example of kinetics in which luciferase did not produce luminescence, expressed as cps. (**c**) An alternative method for assessment of the OXPHOS contribution in ATP synthesis. The cell population in luciferin solution was perfused with high concentrations of DCC. Inhibition of ATP synthase promotes a decrease in ATP detection, and hence cps, revealing the OXPHOS contribution in that cell line.

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stored at −80 °C for up to 6 months. ▲ **CRITICAL** Avoid multiple freeze–thaw cycles and protect the solution from light.

100 mM ATP stock solution Dissolve 1 g of ATP with 16.5 ml of 0.1 M Tris–HCl base solution at pH 7.5. Make aliquots from the stock solution. Aliquots can be stored at −80 °C for up to 6 months. ▲ **CRITICAL** Avoid multiple freeze–thaw cycles.

Krebs–Ringer buffer Krebs–Ringer buffer (KRB) is 135 mM NaCl, 5 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4{\cdot}7H_2O$, 20 mM HEPES and 5.5 mM glucose, pH 7.4. Dissolve 7.89 g of NaCl, 0.373 g of KCl, 0.054 g of KH_2PO_4, 0.247 g of $MgSO_4{\cdot}7H_2O$, 4.76 g of HEPES and 1 g of glucose in <1,000 ml of Milli-Q water. Adjust the pH to 7.4 with NaOH; then, bring the final volume to 1,000 ml. Store the buffer at 4 °C for up to 3 d.

Modified KRB Add 0.5 ml of 1 M $CaCl_2$ to 500 ml of KRB. Store the buffer at 4 °C for up to 3 d. ▲ **CRITICAL** This solution should be prepared in a polypropylene cylinder, as glass containers can bind Ca^{2+}, which could produce imprecise experimental conditions.

PBS, 1× PBS is 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in <1,000 ml of Milli-Q water. Adjust the pH to 7.4 with NaOH; then, bring the final volume to 1,000 ml. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 p.s.i. (1.05 kg/cm^2). Store the solution at RT for up to 1 week.

0.02% gelatin coating solution Prepare a 0.02% gelatin solution in Milli-Q water, and sterilize it by autoclaving for 20 min at 15 p.s.i. (1.05 kg/cm^2). Store the solution at 4 °C for up to 1 month. ▲ **CRITICAL** Allow the gelatin solution to completely liquefy at 37 °C; coat the culture surface with 0.10.2 mg/cm^2 gelatin; and allow the surface to dry for at least 2 h before introducing cells and medium.

10 mM Luciferin stock solution Luciferin, delivered in powder form, is stable when stored undissolved at 80 °C but labile once dissolved. Luciferin is usually dissolved at 10 mM in PBS, pH 7.4, and stored at 80 °C for long periods of time. Dissolve 0.064 g of luciferin in 20 ml of PBS and divide the stock solution into 1-ml aliquots. ▲ **CRITICAL** This solution should be prepared away from light and bubbled with argon to remove oxygen. The vial should then be sealed over an argon atmosphere. ▲ **CRITICAL** Avoid freeze–thaw cycles.

25 µM Luciferin solution Add 125 µl of the luciferin stock solution to 50 ml of modified KRB. The solution volume to be prepared depends on the cell type, the number of samples to be analyzed and the instrument settings. ▲ **CRITICAL** Protect the solution from light, and store it at 37 °C for the whole duration of the experiment. ▲ **CRITICAL** Once the experiment is over, discard thawed luciferin aliquots.

Ca^{2+}-mobilizing solution (CMS) Dissolve the Ca^{2+}-perturbing agent in a 25 µM luciferin solution. Different cell types require different agents[7]; the most common agents and concentrations are listed in ref. 51. ▲ **CRITICAL** Freshly prepare the CMS; do not store the solution.

75 mM DCC stock solution Dissolve 0.077 g of DCC in 5 ml of pure ethanol. Store the solution at −20 °C for up to 1 month.

! CAUTION DCC is a mutagenic compound and is thus harmful to health; use adequate protection.

75 µM DCC solution Add 50 µl of 75 mM DCC stock solution to 50 ml of 25 µM luciferin solution. ▲ **CRITICAL** Freshly prepare this solution; do not store it.

70% Ethanol solution Prepare 500 ml of 70% ethanol by combining 150 ml of Milli-Q water with 350 ml of pure ethanol. ▲ **CRITICAL** Ethanol evaporates quickly, so we recommend sealing the cap when not in use.

! CAUTION Ethanol is dangerous to human health if inhaled.

EQUIPMENT SETUP

Luminescence reader for *in vitro* experiments Photons emitted during the experimental procedure can be detected and suitably recorded using a luminometer (**Fig. 6**).

In this protocol, the system comprises a perfusion chamber above a hollow metal cylinder, which is temperature-controlled by a water jacket and is continuously perfused with solutions (see PROCEDURE) via a peristaltic pump. The coverslip with cells that is lying in the chamber is placed a few millimeters from the surface of a high-performance photomultiplier tube.

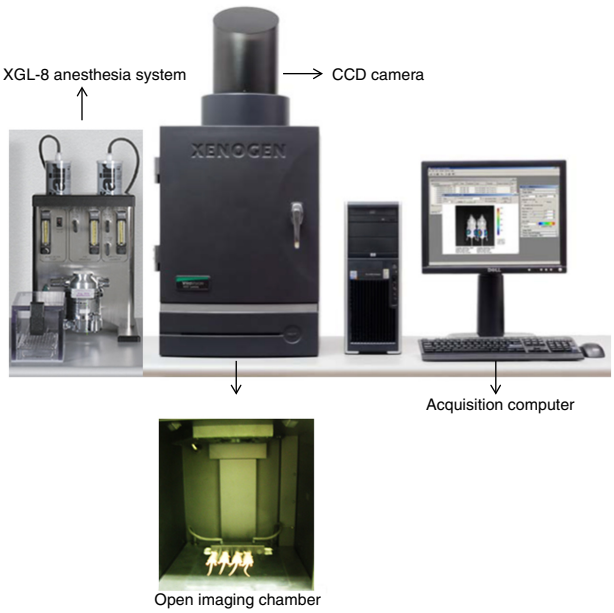

Figure 8 | The *in vivo* imaging system. The *in vivo* imaging system is a real-time system enabling acquisition of biologically relevant events within milliseconds. This IVIS system can be used for *in vivo*, *ex vivo* and *in vitro* imaging studies and includes a highly sensitive camera, a dark imaging chamber to minimize incident light and software to quantify the results. A CCD camera (cryogenically cooled to −90 °C) minimizes system noise while maximizing sensitivity. The system is connected to a gas anesthesia unit that allows continuous administration of isoflurane gas for anesthetizing animals during imaging. It has the capacity to monitor and image up to three mice simultaneously, in addition to imaging cell culture plates.

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The photon counts are then transferred to a computer via the photon-counting head and a digitizer unit. The following components are used: photon-counting head (Hamamatsu, model no. C8855-01); digitizer counting unit (Hamamatsu, model no. C9744); high-voltage power supply (Hamamatsu, model no. C9525); perfusion chamber (Elettrofor Scientific Instruments); Elettrofor thermostatic bath set to 37° C (Elettrofor Scientific Instruments, model no. GT-80/P); and peristaltic pump (Elettrofor Scientific Instruments).

The luminometer is composed of an upper part containing a highly sensitive photomultiplier arranged in a metal dark box in order to protect it and the temperature-controlled chamber from light exposure. The chamber (2-mm height and 265-µl volume) houses the cell sample seeded on a 13-mm-diameter coverslip during ATP measurement and is placed in close proximity to the photomultiplier. Cells are continuously perfused with temperature-controlled solutions in a water bath at 37 °C. A peristaltic pump allows for liquid perfusion[51]. The output signal is recorded and converted to kinetics via Hamamatsu Photonics software and is then ready for further analysis. A typical recording is shown in **Figure 7**.

***In vivo* imaging system** The *in vivo* imaging system (IVIS) consists of a CCD camera and an imaging chamber. The CCD camera has the following features: low dark current, low-noise electronic readout for extremely low-background images, thermoelectrical cooling and 16-bit digitization. The imaging chamber is a highly specialized device consisting of an imaging chamber housing, a heated movable platform, an autofocusing lens system with f/stop control and sample illumination LEDs. All the components are motorized and computer-controlled. In this protocol, the device described was acquired from PerkinElmer Caliper (IVIS) and equipped with Living Image Software (see **Fig. 8**). However, similar performance can be achieved using other *in vivo* imaging systems, such as an FXPro system from Carestream Health[52]. A comparison of currently available *in vivo* optical systems for bioluminescence imaging can be found in Cool *et al.*[53].

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PROCEDURE

▲ **CRITICAL** Steps 1–19 describe the *in vitro* procedure; Steps 20–38 describe the *in vivo* procedure.

***In vitro* experiment: cell preparation and transfection** ● **TIMING ~3 d**

1| *Cell growth*. Coat circular glass coverslips (13-mm diameter) with 0.02% gelatin for 2 h at 37 °C.

▲ **CRITICAL STEP** Sterilize glass coverslips under UV light for 30 min before use.

2| Wash the coverslips twice with PBS and let them dry for several minutes.

▲ **CRITICAL STEP** Maintain sterile conditions.

3| Seed 100,000 cells onto the circular glass coverslips for single-sample luminescence measurements.

4| Allow the cells to grow until they reach 80% confluence.

▲ **CRITICAL STEP** Optimize plating densities for each specific cell line.

5| *Transfection*. Transfect cells with 1 µg of vector encoding firefly luciferase per coverslip by using the Lipofectamine 2000 protocol provided by the manufacturer. Incubate the cells for 24–36 h post transfection.

▲ **CRITICAL STEP** Choose the appropriate transfection reagent and optimized protocol for the particular cell line being used. Alternatively, generate a stable cell line using standard protocols. We suggest using 1 µg/cm^2 of vector encoding firefly luciferase for liposomes or an electroporator and 3 µg/cm^2 for Ca^{2+}-phosphate.

Initialization of the luminometer ● **TIMING ~3–5 min**

! **CAUTION** Avoid exposing the photon-counting head to a direct light source; keep the light source turned off or the shutter closed when not in use.

▲ **CRITICAL** In Steps 6–8, we list some simple and basic rules to follow for correct use of the experimental equipment.

6| Before (and after) each experiment, wash the plastic tubes connecting the perfusion chamber extensively with Milli-Q water and 70% ethanol. Ensure that both the tubes and the perfusion chamber contain appropriate buffer when the coverslip is placed inside.

7| Ensure there are no air bubbles in the system and that the perfusion chamber is carefully sealed to avoid buffer leakage.

8| Ensure that all the systems are at 37 °C before starting the experiment to ensure a proper cellular response.

Measurement recording and data backup ● **TIMING ~5–6 min**

9| *Measurement of basal ATP content*. Remove a 13-mm coverslip containing cells from the incubator, rinse twice with 1 ml of modified KRB to remove residual medium and transfer the coverslip to the temperature-controlled (37 °C) perfusion chamber.

? TROUBLESHOOTING

10| Start perfusing the cell monolayer with modified KRB using a peristaltic pump (standard flow rate: 2.5 ml/min).

11| Continue to perfuse the cells constantly, place the chamber in the dark box in close proximity to the surface of the photon-counting head (**Fig. 6**) and start recording.

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12| Measure the background signal by recording luminescence values for at least the first 30 s to obtain a stable basal signal.
? TROUBLESHOOTING

13| Pause the perfusion and change from modified KRB to the luciferin solution to record the basal ATP content. After luciferin is added, light emission (and thus cps values) rapidly increases and reaches a plateau within ~2–3 min.
▲ **CRITICAL STEP** You must stop the perfusion before changing the solution to avoid the formation of air bubbles.
? TROUBLESHOOTING

14| *Measurement of mitochondrial ATP production*. Wait until the cps values remain unchanged for ~15–30 s. Pause the perfusion, change from the luciferin solution to the CMS solution and resume the perfusion. Under these conditions, the light emission (and therefore the cps values) rapidly increases and reaches a second plateau within ~1–1.5 min. Wait until the cps values remain unchanged for ~20–30 s. (See Experimental design section for a second method of assessing ATP production.)
? TROUBLESHOOTING

15| Stop the analysis system. First, remove the perfusion chamber from the photon-counting head; then, remove the 13-mm coverslip from the chamber groove. Reseal the chamber, and wash the entire system extensively with Milli-Q water, 70% ethanol and modified KRB solution to avoid nonspecific signals.
▲ **CRITICAL STEP** After each data recording, save the file to avoid losing kinetics.

16| *Backup data*. Export results in a spreadsheet-compatible format for primary data analysis.

17| Open exported files using a spreadsheet software (e.g., Microsoft Office Excel), and arrange each luminescence recording in a single column.

Data handling ● TIMING ~2–3 min
18| To calculate the basal ATP content for both the cytosolic and mitochondrial compartments, estimate the average of the luminescence values reached in the first plateau generated during sample perfusion with luciferin solution. Obtain a ratio by dividing this value by the average of the luminescence values recorded during the background step (Step 12; **Fig. 5b**,**c**).
! CAUTION As the basal ATP content is dependent on the abundance of transfected luciferase, performing an immunoblot assay with an antibody against luciferase is recommended (**Box 2**).

19| To calculate the amount of ATP produced by mitochondria, divide the average of the luminescence values reached in the second plateau generated after the addition of the CMS solution by the luminescence values of the first plateau (**Fig. 5b**,**c**).

***In vivo* experiment: establishment of *in vivo* model ● TIMING ~30 d**
20| Follow option A to establish peritoneal OVCAR-3 ovarian carcinoma and option B to establish subcutaneous MZ2–MEL melanoma.
! CAUTION All animal experiments must conform to the relevant governmental and institutional guidelines and regulations. Always carry out *in vivo* experiments under close supervision by an expert veterinarian appointed by your institution. For this procedure, a minimum of five mice weighing 18–25 g is recommended.
(A) Establishment of peritoneal OVCAR-3 ovarian carcinoma ● TIMING ~ 20 d
(i) Grow OVCAR-3 cells in complete RPMI-1640 medium.
(ii) Harvest cells by a 5-min incubation at 37 °C in trypsin–EDTA, and suspend the cells in complete RPMI-1640 medium.
(iii) Count cells with a hemocytometer. Centrifuge the cells at 200*g* for 5 min at RT, remove the medium and resuspend in RPMI-1640 without serum at a concentration of 7.5×10^6 cells per ml.
(iv) Gently agitate the cell suspension to avoid cell aggregates. Place the mouse on a rigid stage, keep the mouse with its head down and gently massage the abdomen in such a way as to push the intestinal loops toward the diaphragm. This maneuver prevents the cells from being inoculated into the intestinal loops. Inoculate 0.2 ml of the cell suspension i.p. into each mouse (1.5×10^6 cells) with a 27-gauge needle.
(v) After tumor cell inoculation, wait 15–20 d to allow tumor growth within the peritoneal cavity. During this period, proceed to the next step to prepare cells ready for injection into the mice when the tumors reach an appropriate size.

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(B) Establishment of a subcutaneous MZ2-Mel melanoma ● TIMING ~ 15 d

(i) Grow MZ2-MEL cells in complete RPMI-1640 medium.

(ii) Harvest MZ2-MEL cells by a 5-min incubation with trypsin–EDTA at 37 °C, and suspend the cells in complete RPMI-1640 medium.

(iii) Count the cells with a hemocytometer. Centrifuge the cells at 200*g* for 5 min at RT, remove the medium and resuspend in FBS-free RPMI-1640 at a concentration of 40 × 10^6 cells per ml.

(iv) Gently agitate the cell suspension to avoid cell aggregates. Inoculate 0.2 ml of the cell suspension (40 × 10^6 cells) subcutaneously (s.c.) in the dorsal hip of a *nude/nude* mouse with a syringe fitted with a 27-gauge needle.
▲ **CRITICAL STEP** Insert the needle parallel to the skin to avoid injection of the cell suspension into the muscle.

(v) Check tumor growth with a manual caliper. A tumor mass will be palpable at the site of injection 10–15 d post inoculum. Usually at this time point, the tumor has reached a size of ~1.5 × 1.5 cm and is ready for injection with the reporter HEK293-pmeLUC cells. During the period of tumor growth, proceed to the next step to prepare cells ready for injection into the mice when the tumors reach an appropriate size.
▲ **CRITICAL STEP** Carefully monitor tumor size daily with the caliper.

Cell preparation and transfection with pmeLUC ● TIMING ~3 d

▲ **CRITICAL STEP** This step should be performed 3 d before the end of the establishment of the *in vivo* tumor (Step 20A/20B).

21| *Cell growth*. Harvest exponentially growing HEK293 (or alternatively selected) cells 24 h before transfection and plate at a density of 2 × 10^6 cells in 10-cm tissue culture Petri dishes in appropriate medium. Incubate cell cultures at 37 °C for 20–24 h in a 5% CO_2 humidified incubator. Change the medium 1 h before transfection.
▲ **CRITICAL STEP** Optimize plating densities for each specific cell line.

22| *Transfection*. Transfect cells with 40 µg of pmeLUC-pcDNA3 per Petri dish by using a standard calcium phosphate transfection protocol (e.g., as described in ref. 54). After transfection, incubate cells for 24–36 h.
▲ **CRITICAL STEP** Choose the appropriate transfection reagent and optimize the protocol for the particular cell line being used. Alternatively, generate a stably transfected cell line according to standard protocols. We suggest using 1 µg/cm^2 of pmeLUC for liposome or electroporator transfection.

Preparation of the tumor-bearing mouse for *in vivo* imaging ● TIMING ~1 d

23| Harvest HEK293-pmeLUC cells by 5-min incubation at 37 °C with trypsin–EDTA and suspend them in complete DMEM-F12 medium.

24| Count cells with a hemocytometer. Centrifuge the cells at 200*g* for 5 min at RT, remove the medium and suspend in PBS buffer at a concentration of 10^7 cells per ml.

25| Gently agitate the cell suspension to avoid cell aggregates. Using a 27-gauge needle, inoculate the mice i.p. with 0.2 ml of the cell suspension (2 × 10^6 cells). After HEK293-pmeLUC i.p. injection, wait at least 24 h to allow cell distribution throughout the abdomen.
▲ **CRITICAL STEP** Gently massage the abdomen to allow HEK293-pmeLUC cell distribution throughout the abdomen.
▲ **CRITICAL STEP** Be sure to inject the cell suspension into the center of the tumor mass to allow an even diffusion of HEK293-pmeLUC cells throughout the tumor mass.

IVIS setup ● TIMING ~20 min

26| Start the Living Image software and click 'Initialize IVIS system' in the system control panel.

27| Wait until the 'Temperature Square' display in the IVIS System Control turns from red to green. When the temperature is locked to –90 °C, as indicated by the green square, the instrument is ready for operation.

Anesthetization of the mouse and *in vivo* imaging of extracellular ATP using HEK293-pmeLUC cells ● TIMING ~30 min

28| Inject the tumor-bearing mouse i.p. with 0.2 ml of luciferin-containing PBS (150 mg/kg luciferin, 3 mg/mouse).
▲ **CRITICAL STEP** Gently massage the abdomen to allow luciferin distribution throughout the abdomen.

29| Wait for 15 min.

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30| Anesthetize the mouse in an anesthesia chamber by delivering an oxygen mixture containing 1–2% isofluorane at a rate of 1 liter/min.
! CAUTION Xenogen recommends using the XGI-8 GAS anesthesia system when imaging small animals. This system supplies a controlled amount of isofluorane to the imaging chamber and continuously reduces the buildup of isofluorane in the chamber.
▲ CRITICAL STEP Test the level of anesthesia by checking that the mouse has smooth respiration and contractions of hind legs are absent. If respiration is uneven and hind-leg contractions are present, anesthesia is too heavy and the isoflurane concentration in the chamber should be reduced.

31| Place the mouse (abdominal view) under continuous anesthesia into the light-tight IVIS Luminometer Imaging Chamber. Be sure that the mouse snout is placed well into the isoflurane-delivering mask in the imaging chamber.
▲ CRITICAL STEP Verify that the mouse is centered on the stage before acquiring the image by placing a check mark in the 'LIVE' check box from the 'Control Panel'. The monitor displays a photographic image of the mouse. If the subject is centered properly, proceed with the imaging.

32| Start the acquisition from the 'Acquisition Control Panel' as follows. First put a check mark on the 'Luminescent' option and select the following parameters: 'Exposition Time', 3 min; 'Binning', 4; 'F/STOP', 1. Put a check mark on the 'Photograph' option, 'Auto exposition'. Put a check mark on 'Overlay'. Select option 'D' from the 'Field of View' list. Click the 'Acquire' button. Once acquisition has been completed, the Living Image software automatically co-registers images in the 'image window' to generate an overlay image of the photographic image (in grayscale) and the luminescent image (in pseudocolors).
▲ CRITICAL STEP Acquire at least three replicates of luminescence images to allow for variability due to measurement errors or inhomogeneity of luciferin distribution in the body.
? TROUBLESHOOTING

33| Repeat Steps 30–32 every 2 d for at least 16 d.
▲ CRITICAL STEP Check for tumor growth and mouse health conditions each day, and if signs of distress appear, stop the protocol and ask for veterinary advice.

Image processing and data analysis ● TIMING ~20 min
34| Open stored images with the Living Image software.

35| With the free-draw method, draw an ROI, usually of round shape, around luminescent sites. Draw an ROI of one or more nonluminescent areas of the same size as the luminescent spots to record background emissions.

36| Select type of data to include in the table from the 'ROI Measuring' table, in this case 'Total Flux' (the radiance, photons/s) in each pixel summed or integrated over the ROI area ($cm^2 \times 4\pi$) and 'SD of the Radiance' (standard deviation of the pixel radiance inside the ROI).

37| Export the data in a spreadsheet-compatible format (i.e., Microsoft Office Excel) and report the data for each ROI in a single column.

38| Subtract background emission from every column at each given imaging time.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
9 and **Box 1**	Cells detach from the coverslip	Low cell adhesion	Prepare a selective coating (depending on cell type) or reconstitute in DMEM supplemented with 1% FBS

(continued)

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TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
12	High noise during Bkg recording (>1,000 cps)	Photon-counting head has been exposed to light	Continue recording until the signal detected does not decrease. Restart recording
		Buffer perfusion to cell population is not optimal	Stop recording and perfusion. Control chamber perfusion, check for the presence of, and remove, air bubbles
	Progressive reduction of basal recording	Cells are detaching	Reduce the speed of the pump or seed fewer cells in each well
13	Luminescence signal (cps) does not increase	Luciferase transfection (or infection) was not efficient	Change or improve the transfection (or infection) method
		DNA used was a low-quality plasmid	Transfect (or infect) using an increased quantity of DNA or the change luciferase plasmid
		Low number of cells	Increase the cell number
		Luciferin perfusion is not optimal	Increase luciferin concentration and/or assess its perfusion
14	Luminescence signal (cps) does not increase	Luciferin perfusion is not optimal or CMS was prepared improperly	Luciferin solution and CMS should be prepared in one step and divided for all parts of each experiment and for all replicates
		Cell samples are based on a mainly glycolytic metabolism	Measure only the ATP basal content or change cell line
Box 3 (steps 5 and 15), **Box 4** (step 10)	Weak luminescence emission	Low plasma membrane expression	Incubate cells overnight in the presence of 1 mM dithiothreitol (DTT), or keep at RT for 2 h before luminescence recording
32	No luminescence	D-luciferin was not properly injected	Repeat D-luciferin injection, taking care that luciferin is injected into the peritoneal space and not into the bowel
	No overlay between the photograph and the luminescence image	Involuntary displacement of the mouse	Open the imaging chamber, center the mouse and start a new acquisition immediately
	Scattered luminescence	Animal started moving because anesthesia wore off	Open the imaging chamber, anesthetize the mouse again and start a new acquisition immediately

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● TIMING

***In vitro* experiment**

Steps 1–5, cell preparation and transfection: 1 d for Steps 1–4 and 1–2 d for Step 5 (this step is highly dependent on the pause points required based on the cell type and transfection method used)

Steps 6–8, initialization of the luminometer: ~3–5 min

Steps 9–17, measurement recording and data backup: ~5–6 min

Steps 18 and 19, data handling: ~2–3 min

***In vivo* experiment**

Step 20, establishment of *in vivo* model: ~30 d

Steps 21 and 22, cell preparation and transfection with pmeLUC: ~3 d

Steps 23–25, preparation of the tumor-bearing mouse for *in vivo* imaging: ~1 d

Steps 26 and 27, IVIS setup: ~20 min

Steps 28–33, anesthetization of the mouse and *in vivo* imaging of extracellular ATP using HEK293-pmeLUC cells: ~30 min

Steps 34–38, image processing and data analysis: ~20 min

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Box 1, adenoviral infection procedures: 2–3 d
Box 2, evaluation of luciferase expression in cells by immunoblotting: 2 d
Box 3, *in vitro* validation of pmeLUC-transfected HEK293 cells by immunofluorescence with anti c-myc-SC-40 monoclonal antibody: 3 d
Box 4, *in vitro* analysis of pmeLUC expression and activity: luminescence recording from pmeLUC-transfected HEK293 cells with a total body luminometer for small animals: 1 d

ANTICIPATED RESULTS

Two fundamental concepts must be understood when applying this protocol. First, the luminescence peak detected by the luminometer depends on, and is proportional to, luciferin perfusion when the concentration of luciferin ranges from 20 to 200 μM. The signal precisely reveals the ATP concentration in a given subcellular compartment and represents a reliable and sensitive technique for experimentation in living cells. Second, in the absence of an autocalibration method to perform ATP measurements (see Limitations in INTRODUCTION), the final results should be discussed after evaluating the luciferase expression levels inside cells (**Box 2**).

This protocol helps the researcher to measure ATP produced or stored in different organelles in a wide variety of living cell lines (**Table 2**), with ATP levels represented as cps. The cps value represents the luminescent signal detected by the luminometer at a given time during the experiment. The trend of the curve obtained by a representative experiment could be ideally divided into three different parts, as illustrated in **Figure 5a**.

The first part of the kinetics (i) is the background (Bkg) recording, which is measured for up to 30 s in modified KRB solution. If the basal signal has high noise and/or is not stable (illustrated in **Fig. 7a**), then the experiment is not properly set up; in such cases, please refer to troubleshooting guide (**Table 3**, Step 12) for details. In section ii, the perfusion of luciferin solution to the cell population activates luciferase, which catalyzes light production in the presence of ATP, Mg^{2+} and O_2, and hence increases the signal detected (cps), producing a sigmoidal-shaped curve. Light emission reaches a plateau in several seconds or a few minutes (based on the cell type) and indicates the ATP content in a given organelle. Thus, within certain limits (previously discussed), higher concentrations of ATP will correspond to higher signal. At this time point, the researcher is able to determine the final ATP concentration, which is expressed as the subtraction of the mean of the plateau reached in (**Fig. 5a**) (i) from the plateau of **Figure 5a** (ii) (**Fig. 5b**). If the cps value does not increase upon luciferin administration (or if the cps value reaches only low levels; **Fig. 7b**), problems such as an inefficient transfection method or a low-quality luciferase plasmid must be addressed. For further details, please refer to the troubleshooting table (**Table 3**, Step 13).

If the aim of the experiment was to measure ATP produced by mitochondria, the researcher can continue to record data without pause points by performing step iii of **Figure 5**. Ca^{2+} signals are coupled to a mitochondrial metabolic response[55]; specifically, mitochondrial Ca^{2+} accumulation triggers ATP synthesis in mitochondria, as well as in the cytosol[7]. Moreover, the amount of ATP synthesis depends on the presence of oxidative substrates, particularly in glycolytic cells (e.g., HeLa cells), where the priming signal (Ca^{2+} elevation in mitochondria) is much smaller than that in primary cells (e.g., fibroblasts). Thus, the perfusion of cells by CMS promotes mitochondrial ATP synthesis and thus a further increase in cps value (iii in **Fig. 5**). ATP production should be expressed as the ratio between the plateau reached upon Ca^{2+}-dependent stimulation and that reached in part ii (**Fig. 5b**).

A second way to assess the contribution of OXPHOS to intracellular ATP production is treatment with potent mitochondrial ATP synthase inhibitors such as oligomycin and DCC[50], as briefly mentioned in **Figure 7c**.

Traces obtained from data handling can help the researcher to realize when an experiment is not properly set up. Thus, **Figure 7a**,**b** show two examples of the most common negative results; please also refer to the Troubleshooting guidelines.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS P.P. is grateful to C. degli Scrovegni for continuous support. P.P. is supported by the Italian Ministry of Education, University and Research (COFIN no. 20129JLHSY_002, FIRB no. RBAP11FXBC_002 and Futuro in Ricerca no. RBFR10EGVP_001), the Italian Cystic Fibrosis Research Foundation (no. 19/2014) and the Telethon Foundation (no. GGP15219/B). P.P. and C.G. are supported by local funds from the University of Ferrara and the Italian Ministry of Health, as well as by the Italian Association for Cancer Research (AIRC nos. IG-18624 and MFAG-13521). F.D.V. is supported by grants from the Italian Association for Cancer Research (no. IG 2016 Id.18581), the Ministry of Health of Italy (no. RF-2011–02348435) and EU-COST Action (no. BM 1406). L.R. is funded by 'Cinque per mille dell'IRPEF–Finanziamento della ricerca sanitaria'.

AUTHOR CONTRIBUTIONS G.M., A.C.S., S. Marchi, S. Missiroli, S.F., L.R., V.P., C.G., F.D.V. and P.P. contributed extensively to the writing of this paper. G.M. and A.C.S. performed the experiments, analyzed the data and generated the figures.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the online version of the paper.

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