EMERGING METHODS AND TECHNOLOGIES

Approaches to monitor ATP levels in living cells: where do we stand?

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ATP is the most universal and essential energy molecule in cells. This is due to its ability to store cellular energy in form of high-energy phosphate bonds, which are extremely stable and readily usable by the cell. This energy is key for a variety of biological functions such as cell growth and division, metabolism, and signaling, and for the turnover of biomolecules. Understanding how ATP is produced and hydrolyzed with a spatiotemporal resolution is necessary to understand its functions both in physiological and in pathological contexts. In this review, first we will describe the organization of the electron transport chain and ATP synthase, the main molecular motor for ATP production in mitochondria. Second, we will review the biochemical assays currently available to estimate ATP quantities in cells, and we will compare their readouts, strengths, and weaknesses. Finally, we will explore the palette of genetically encoded biosensors designed for microscopy-based approaches, and show how their spatiotemporal resolution opened up the possibility to follow ATP levels in living cells.

Introduction: ATP production and functioning of the ATP synthase

ATP (or adenosine triphosphate) is a key and universal energy molecule with the ability to store and transport energy. This is achieved thanks to the phosphoanhydride bonds between the $\alpha$-$\beta$ phosphate groups, and between the $\beta$-$\gamma$ ones. These are defined as high-energy bonds, because their hydrolysis is energetically favorable in both intracellular and extracellular environments [1,2]. The term ‘high-energy bond’ to define phosphoanhydride bonds is a shortcut first appeared in literature in the 70s, because of the high free energy ($G$) of the ATP hydrolysis reaction. Indeed, this type of bonds does not have intrinsically high energy levels, but their rupture in a particular molecular environment such as the living cell releases a large amount of energy. The products formed after an ATP hydrolysis reaction have a lower free energy

Abbreviations
AAC, ADP/ATP carrier; ADP, adenosine diphosphate; ANT, Adenine nucleotide translocator; ARSeNL, ATP detection with a Ratiometric mScarlet-NanoLuc sensor; ATEAM, adenosine 5’-triphosphate indicator based on Epsilon subunit for Analytical Measurements; BRET, bioluminescence resonance energy transfer; CFP, cyan fluorescent protein; CyPD, cyclophilin D; EAF-ATP, enhanced acceptor fluorescence-based ATP biosensor; ECL, electrochemiluminescence; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FMN, flavin mononucleotide coenzyme; FRET, Förster’s resonance energy transfer; GCE, glassy carbon electrode; iATPSnFR, intensity-based ATP-sensing fluorescent reporter; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; $\Delta\Psi$ m, mitochondrial membrane potential; MAlion, Monitoring aTP Level intensiometric turn-on indicator; MCF, mitochondrial carrier family; mPPT, mitochondrial permeability transition pore; MRS, magnetic resonance spectroscopy; PBA, phenylboronic acid; P$\text{i}$, inorganic phosphate; PIC, phosphate carrier; PolyP, inorganic polyphosphate; QD, quantum dots; QUEEN, quantitative evaluator of cellular energy; R6G, rhodamine 6G; ROS, reactive oxygen species; SPG-7, spastic paraplegia 7; VDAC, voltage-dependent anion channel; VNUT, vesicular nucleotide transporter.
content, hence the term of ‘high-energy bond’ to describe phosphoanhydride bonds [3].

From a biological point of view, the energy released from the hydrolysis of the phosphoanhydride bonds is essential to almost all cellular processes and it is consumed to perform a various number of reactions as anabolism [4], ion transport [5], synaptic communication [6,7], and muscular contraction [8]. Overall, cellular activities can be seen as energy-consuming processes, where ATP is the most common energy source [2,9]. In addition to its energy-providing role, ATP is also a key molecule involved in cell signaling [10], and particularly in purinergic signaling [9]. In this context, ATP has the ability to be recognized by purinergic receptors and plays key roles in inflammation [11,12], coagulation, cell proliferation, synaptic transmission, and cell permeability, among other roles [13–15].

From a structural point of view, there are significant electrostatic repulsions between the negatively charged phosphate groups of the ATP molecule. As a result, ATP tends to undergo spontaneous hydrolysis. Because of this feature, ATP (as well as ADP, adenosine diphosphate) forms a complex with the magnesium ion (Mg$^{2+}$) within the cell. The Mg$^{2+}$-ATP complex reduces the electrostatic repulsions within the ATP molecule, due to the positive charge of the Mg$^{2+}$ ion that partially neutralizes the negative charge of oxygen. This makes the Mg$^{2+}$-ATP complex less prone to spontaneous hydrolysis and thus more stable in the cell than ATP in its free form [16] (Fig. 1).

Due to its particular structure in terms of size and charge [17], the transport of ATP across the cell membrane and the intracellular membranes requires specific transporters [18,19]. Generally speaking, transporters are ATP/ADP carriers, which allow the import of ATP and the export of ADP inside a particular compartment [20,21]. Transporters can be found at the level of mitochondria, of the endoplasmic reticulum, of the Golgi apparatus, and of peroxisomes. Of note, the vesicular transport of ATP by the vesicular nucleotide transporter (VNUT) has also been described, acting mainly in the brain and in the adrenal gland [22].

The transport of ATP across mitochondrial membranes is essentially ensured by mitochondrial carriers (MCF). Three major classes of MCF have been described: the ADP/ATP carrier (AAC or the Adenine Nucleotide Translocator, ANT), localized at the inner mitochondrial membrane and allowing the export of ATP by importing ADP [23–26]; the ATP-Mg/Pi carrier, which allows the exchange of ATP-Mg$^{2+}$ for HPO$_4$$^{2-}$ between the cytosol and the mitochondrial matrix [27,28]; and the AMP/ATP carrier, found exclusively in plant mitochondria and allowing the export of ATP and the import of AMP [29].

The mechanisms of ATP transport are intimately linked with the membrane potential of the corresponding organelles or of the secreting vesicles. In mitochondria, the membrane potential ($\Delta \Psi_m$) is not only essential for MCFs to orchestrate the translocation of nucleotides, but also for energy production, and for the quality control of mitochondria by mitophagy [30]. The mitochondrial membrane potential is maintained by the activity of the electron transport chain and the reverse activity of the ATP synthase, which will be described in the next sections. Interestingly, a noncorrelation between $\Delta \Psi_m$ and ATP production has been described to occur during axonal elongation processes [31]. Although $\Delta \Psi_m$ is essential for the production of mitochondrial ATP, these recent data indicate that it should not be used as a direct readout of the quantity of ATP produced by the organelles.

![Fig. 1. Chemical representation of ATP.](image-url)
Due to the multiple and pivotal roles of ATP, the eukaryotic cell established multiple pathways to synthesize this essential molecule. The energy-providing biomolecules are carbohydrates, lipids, and proteins, and are mostly obtained with food intake. These molecules allow ATP to be synthesized by different catabolic pathways such as glycolysis, lipolysis, and proteolysis, respectively. Each of these pathways converges on a common pathway: oxidative phosphorylation, which is the main route for aerobic ATP synthesis [32]. In the sections below, we will focus on the comparison between the bacterial and eukaryotic complexes orchestrating oxidative phosphorylation, and on structural insights of the ATP synthase.

**Oxidative phosphorylation**

Oxidative phosphorylation is the process of coupling the oxidation of electron-donor molecules—NADH, H⁺ and FADH₂—with the phosphorylation of ADP into ATP. This process takes place at the level of the inner mitochondrial membrane, within the mitochondrial respiratory chain. Functionally speaking, the mitochondrial respiratory chain can be divided into the electron transport chain module (complexes I to IV) and the ATP synthase (complex V) (Fig. 2).

The electron transport chain is constituted of four large protein complexes (I, II, III, and IV) and two membrane shuttles, coenzyme Q and cytochrome c (Fig. 2) [33]. Complex I, also called NADH dehydrogenase, is a complex of approximately 1000 kDa and is composed of 45 protein subunits, 1 flavin mononucleotide coenzyme (FMN, similar to FAD), and 8 iron-sulfur clusters. It catalyzes the transfer of electrons from NADH, H⁺ to coenzyme Q, while translocating protons across the inner mitochondrial membrane into the intermembrane space [34,35]. Complex II, also named succinate dehydrogenase, is a complex of approximately 140 kDa and is composed of 4 protein subunits, 1 FAD, 3 iron-sulfur clusters, and 1 single heme group. It catalyzes the transfer of electrons from succinate to coenzyme Q10 [35]. Complex III, named cytochrome c reductase, is a complex of approximately 250 kDa and is composed of 11 subunits, 1 iron-sulfur cluster, and 3 cytochromes (1 cytochrome c₁ and 2 cytochromes b). It catalyzes the transfer of electrons from coenzyme Q to cytochrome c, and it translocates protons across the inner mitochondrial membrane into the intermembrane space [35]. Complex IV, also named cytochrome c oxidase, is a complex of approximately 200 kDa and is composed of 13 subunits, 3 copper atoms, and 2 heme groups, also known as cytochrome a and cytochrome a₃. It catalyzes the transfer of electrons from cytochrome c to oxygen, and it translocates protons across the inner mitochondrial membrane into the intermembrane space.

![Fig. 2. Schematic representation of the respiratory chain. In bacteria, the respiratory chain is located in the cytoplasmic membrane (left panel, italics) and in the inner mitochondrial membrane in eukaryotes (right panel, italics). It is composed of an electron transport chain that creates a proton gradient in the periplasmic space for bacteria, and in the mitochondrial intermembrane space for eukaryotes. Then, the proton gradient is translocated through the ATP synthase to the periplasmic space in bacteria, or to the mitochondrial matrix in eukaryotic cells. This translocation allows the rotation of the ATP synthase at the structural level, and ATP will be converted from an ADP + inorganic phosphate (Pi) reaction.](image)
space [35]. Last, the shuttles function as follows: Coenzyme Q, also called CoQ or ubiquinone, is a liposoluble electron carrier and transports both electrons and protons [36], while cytochrome c is a water-soluble protein, which carries electrons exclusively [37].

In mitochondria, the respiratory chain complexes were shown to assemble into supercomplexes called respirasomes [38], with the following composition: (complex I)\textsubscript{1}–(complex III)\textsubscript{2}–(complex IV)\textsubscript{1,2} or (complex III)\textsubscript{2}–(complex IV)\textsubscript{1,2}. In addition, the association of multiple respirasomes into a megacomplex, composed by (complex I)\textsubscript{2}–(complex III)\textsubscript{2}–(complex IV)\textsubscript{2} has been described [38] (Fig. 3). The association of the respiratory chain complexes in respirasomes or megacomplexes has many advantages [39]. First, complex I was shown to have an increased stability when it is integrated into a supercomplex. Second, respirasomes produce less reactive oxygen species (ROS) than individual complexes, since the ROS formation sites are less accessible. Third, the catalytic activity of the complexes forming the respirasome was shown to be greater in terms of proton transfer and electron transport rates. In light of this feature of supercomplexes and unlike complexes I, III, and IV, complex II does not participate in the formation of supercomplexes. This is most likely due to its inability to transfer protons from the mitochondrial matrix to the intermitochondrial space. [39–41]. Last, the link between the ultrastructural characteristics of mitochondria and their overall number appears to be tissue-specific, and optimized to better respond to in situ energy demands [42,43]. Similarly, the quantity and the distribution of supercomplexes and megacomplexes inside mitochondria were shown to dynamically adapt to the type of tissue, as well as to physiological and metabolic conditions [44–50].

In summary, the role of these first four complexes is to oxidize electron-donor molecules such as NADH, H\textsuperscript{+} and FADH\textsubscript{2}, which are derived from the Krebs cycle, and which are powered by the catabolism of carbohydrates, lipids, and proteins. The electrons are then transferred to O\textsubscript{2}, thereby making this process aerobic. This successive oxidation of donor molecules creates a proton gradient, allowing the phosphorylation of an ADP molecule into ATP by the ATP synthase complex. The ATP synthase (complex V) can also arrange in supramolecular complexes as dimers and tetramers (Fig. 3). The clustering of multiple ATP synthase complexes triggers local folding events on the inner mitochondrial membrane, commonly known as mitochondrial cristae [53,54]. We will deepen the description of this ATP factory in the next paragraph [9].

**ATP synthase**

The process of ATP synthesis by oxidative phosphorylation is a phenomenon already existing in bacteria, which have a membrane respiratory chain very similar to the mitochondrial one (Fig. 2). This similarity between the bacterial and mitochondrial respiratory chains became one of the pillars of the theory of primary endosymbiosis, which explains the origin of mitochondria in eukaryotic cells as the result of the endocytosis of bacteria by a primitive eukaryotic cell.

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**Fig. 3.** Organization of electron transport chain and ATP synthase inside mitochondria. A megacomplex, [(complex I)\textsubscript{2}–(complex III)\textsubscript{2}–(complex IV)\textsubscript{1}], is the result of the association of supercomplexes (or respirasomes) [(complex I)\textsubscript{1}–(complex III)\textsubscript{2}–(complex IV)\textsubscript{1}] or [(complex I)\textsubscript{2}–(complex III)\textsubscript{2}–(complex IV)\textsubscript{1}]. ATP synthase (complex V) has the ability to form dimers or tetramers inside mitochondria. Dashed lines: representative view of the ‘bifurcated’ electron flow within the supercomplex. One electron is transferred to cytochrome c, and the other is recycled back to complex I [51,52].
It is thought that during evolution, this primitive eukaryotic cell maintained an endosymbiotic relationship with bacteria, which then transformed into mitochondria approximately between 1.5 and 2 billion years ago [58]. In support of this theory, the main 'energy currency' unit of bacterial cells is inorganic polyphosphate (polyP). PolyP is a polymer of few to several hundred phosphate molecules linked by phosphoanhydride bonds, similar to ATP (Fig. 1) [59]. This molecule provides energy and phosphate storage, and it contributes to the conversion of AMP into ADP, and from ADP into ATP [60,61]. PolyP is also found in mammalian cells, and its production is directly linked to mitochondrial respiration in the activation and/or formation of the mitochondrial permeability transition pore described below [59,62,63]. Although the endosymbiotic theory is becoming increasingly controversial [64–66], the first analyses of the ATP synthase were carried out on the bacterial one due to its similarity with the human cognate complex, the fast replication rates of bacteria, and the ease of introducing modifications to the bacterial genome [67].

ATP synthase is a large complex of about 600 kDa, and it consists of three parts: the F0 unit, the F1 unit, and the peripheral stalk. The F0 unit is embedded in the inner mitochondrial membrane (or the bacterial plasma membrane), and it is responsible for proton translocation (Fig. 4). This unit rotates within the membrane upon its interaction with protons, hence its nickname ‘rotor’. F0 also forms a central stalk, which connects it to the F1 unit. The F1 unit protrudes in the mitochondrial matrix (or the bacterial cytoplasm) and is the static unit of the ATP synthase or ‘stator’.

From a functional point of view, F1 is responsible for the phosphorylation of ADP into ATP. In addition to a direct connection between F0 and F1 by the central stalk, the two units are also connected by a peripheral stalk. The peripheral stalk further stabilizes the complex and allows to keep the entire ATP synthase complex stable and static during the rotation of the c-ring and within the hexamer $a_3$-$b_3$ [68] (Figs 4 and 5).

From a structural point of view, the bacterial and mitochondrial ATP synthases show high similarities in their F0, F1, and peripheral stalk units. The bacterial F0 unit is composed of subunits a-c9-15, the F1 unit is

![Fig. 4. Structural comparison of the bacterial and mitochondrial ATP synthase machinery.](image)

**Left panel:** The bacterial ATP synthase consists of a rotor part F0 (yellow, orange), forming the proton channel by subunit a and c9-15. F0 also carries a central stalk (pink), composed of the $\gamma$ and $\varepsilon$ subunits. The static part F1 is formed by the peripheral stalk (purple) and the catalytic headpiece (blue). The peripheral stalk is constituted by the b, b', and $\delta$ subunits, and the catalytic headpiece by the $a_3$-$b_3$ subunits (blue). **Right panel:** Mitochondrial ATP synthase consists of rotor part F0 (yellow, orange), forming the proton channel by subunit a and c9-15, in addition to subunits e, g, f, A6L, j, and k. As in bacteria, F0 also carries a central stalk (pink) composed of the $\gamma$, $\delta$, and $\varepsilon$ subunits. The F1 is formed by the peripheral stalk (purple) and the catalytic headpiece (blue). The peripheral stalk is composed of the b, d, F6, and OSCP subunits, and the catalytic headpiece is constituted by the $a_3$-$b_3$ subunits (blue). Note: The mitochondrial subunit $\delta$ corresponds to the bacterial subunit $\varepsilon$, whereas the mitochondrial $\varepsilon$ subunit does not exist in bacteria.
composed of subunits \(\alpha_3\beta_3\gamma\epsilon\), and the peripheral stalk is composed of subunits \(b_2\delta\) [67]. Similarly, the mitochondrial F0 unit is composed of subunits \(a\epsilon_8\epsilon_{15}\), the F1 unit is composed of subunits \(a\beta_3\gamma\delta\epsilon\), and the peripheral stalk is composed of subunits OSCP-\(b_2\)-d-F6. In addition to the differences observable between the two peripheral stalks, the mitochondrial ATP synthase is composed of some additional subunits—e, g, f, A6L, j, and k—which are associated with the F0 part [69,70] (Fig. 4).

Being structurally very similar, the bacterial and mitochondrial ATP synthases synthesize ATP on the same principle, the rotational catalysis (Fig. 5). As introduced above, the electron transport chain causes an efflux of protons toward the mitochondrial intermembrane space (or bacterial periplasmic space), thereby creating a proton gradient. Through the F0 unit of ATP synthase, the flux of protons is then re-internalized from the mitochondrial intermembrane space to the mitochondrial matrix (or bacterial cytoplasm). The rotation of F0, including the central stalk that is in the core of the F1 unit, results in conformational changes of the \(\alpha_3\beta_3\) hexamer. The nucleotide-binding sites are located at the interface of \(\alpha\) and \(\beta\) subunits. It is at the level of the \(\beta\) subunits that ATP is directly synthesized, thanks to the presence of a catalytic site within each subunit. The \(\alpha\) subunits do not play an active role in catalysis, but they participate in the regulation of the ATP synthase activity [70]. Each of the \(\beta\) subunits has a given conformation (Fig. 5): the first is the loose conformation, also called \(\beta_{\text{DP}}\) because it binds ADP and Pi; the second is the tight conformation or \(\beta_{\text{TP}}\), because it forms a bond between ADP and Pi and synthesizes ATP. The last is the open conformation, also called \(\beta_{\text{E}}\) or empty, because it releases the newly formed ATP molecule. These different conformations work in synergy to synthesize or hydrolyze ATP, and at each time point, each of the \(\beta\) subunits is occupied by a nucleotide [71]. Of note, a recent study reported that the \(\beta\) subunit does not undergo successive rotations of 120°, but a series of rotations of 80° + 40° [72]. This would lead to six distinct conformations, and not only three as documented so far.

Dimers of ATP synthase have the capacity to form a mitochondrial permeability transition pore (mPTP), also known as mitochondrial megachannel (MMC)
The OSCP and c subunits of ATP synthase are the main proteins involved in this structure. The OSCP subunit, in association with cyclophilin D (CyPD), leads to the opening of the mPTP, while the c-subunit forms a voltage-sensitive channel leading to cytosolic calcium overload [75–77]. Although the composition of the mPTP is becoming increasingly clear, the exact protein associations within this structure are still under intense investigation [78,79]. Beyond its association with CyPD, recent studies highlighted that mPTP can associate with mitochondrial proteins such as ANT, the outer membrane voltage-dependent anion channel (VDAC), the phosphate carrier (PiC), and spastic paraplegia 7 (SPG-7) (Fig. 6) [80–82]. The ATP synthase / PiC / ANT complexes were also shown to assemble into the so-called ‘Mitochondrial ATP synthasome’, a structure coordinating the entry of P_i and ADP into mitochondria with the synthesis of ATP via the ATP synthase [83–85].

The mechanisms leading to the opening or closing of the mPTP are well described [78]. Among the factors stimulating the opening of the PTP, there are elevated concentrations of calcium or elevated concentrations of reactive oxygen species (ROS), free fatty acids, the reduction of the transmembrane potential, and the binding of CyPD on the OSCP subunit of the ATP synthase [78,86–88]. Among the factors inhibiting the opening of the mPTP, there are alterations in nucleotides or magnesium concentrations, coenzyme Q10, and binding of cyclosporin A (CsA) or CyPD [78,89,90]. Brief and reversible openings of the mPTP were shown to participate in calcium homeostasis within mitochondria, and they allow the bidirectional passage of small molecules of less than 0,3 kDa. However, when the opening of the mPTP is long, it becomes irreversible and causes the bidirectional passage of molecules up to 1,5 kDa. As a consequence, mitochondria become waterlogged and swell, lose their internal architecture, and release pro-apoptotic factors leading to cell death by apoptosis and necroptosis [78,79,91]. This mechanism can also be used by tumor cells to increase their resistance to cell death by desensitizing the mPTP to calcium and ROS. In this light, the mPTP became a prominent target for anticancer strategies [92].

To summarize, the entire electron transport chain and ATP synthase therefore form a powerful and efficient combination for ATP production, both in bacteria and in eukaryotic cells. Given that the intracellular functions of ATP are critical for cell physiology and in pathological conditions, monitoring ATP levels in cells has been used for decades as readout of cellular functionality. First analyzed using biophysical and biochemical approaches, the raise in microscopy-based techniques has deepened our understanding of the many roles of ATP in the cell.

In the section below, we will first describe the most employed biochemical and biophysical assays to monitor ATP levels in a quantitative or qualitative way. Second, we will review the panel of microscopy-based tools and approaches to monitor cellular and

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[Fig. 6. Composition of the mPTP. The ATP synthase can arrange in dimers and associate with partners such as cyclophilin D (CyPD), adenine nucleotide translocase (ANT), the outer membrane voltage-dependent anion channel (VDAC), the phosphate carrier (PiC), and spastic paraplegia 7 (SPG-7) to form the mitochondrial permeability transition pore (mPTP).]
extracellular ATP levels at the single-cell level and with spatiotemporal resolution.

**Biochemical and biophysical approaches to measure cellular and extracellular ATP levels**

**Nuclear magnetic resonance spectroscopy**

NMR is a technique based on the property of specific atoms to have an odd number of protons and/or neutrons. This results in a nonzero nuclear spin ($I$). Atoms, such as $^1$H, $^{13}$C, $^{19}$F, and $^{31}$P, have an $I$ value equal to $\frac{1}{2}$, making them comparable to magnetic dipoles. When these atoms are subjected to a magnetic field, they absorb an electromagnetic wave and resonate by emitting an energy peak detectable with a NMR spectrometer [93]. Since ATP, ADP, and AMP molecules contain a significant proportion of H and P atoms, they behave as magnetic dipoles and it is possible to detect them using this approach [94–96] (Fig. 7).

The quantitative nuclear magnetic resonance spectroscopy (qNMR) is a variant of NMR allowing, as the name indicates, to quantitatively measure one or more molecules simultaneously [97]. On a qNMR spectrum, the peak area is directly proportional to the number of atoms. The concentration of the molecules analyzed can be determined by using an internal standard (as TMSP or Na$_2$HPO$_4$) of known concentration previously added to the sample. By using $^1$H and $^{31}$P qNMR ($^1$H-NMR and $^{31}$P-NMR), Lian et al. were able to simultaneously monitor ATP, ADP, and AMP [96]. The main advantage of this method is the perfect linearity in a given range: 0.1–100 mM for $^1$H-NMR, and 1–75 mM for $^{31}$P-NMR. qNMR turned out to be particularly useful in pathological situations where cytoplasmic ATP is massively released into the circulation [98]. However, this technique has some limitations: first, the detection range is lower than the concentration of physiological ATP present in cells and tissues. Furthermore, this technique has a low temporal resolution, as it requires an extended time frame—approximately of 14 h—to complete the detection of a specific spectrum. In addition, and although extremely precise in estimating the quantity of specific components, qNMR requires significant amounts of...

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**Fig. 7.** Principle of nuclear magnetic resonance. The spectrometer consists of a magnet and several coil systems. The sample is placed in a superconducting magnet (or electromagnet), in which a continuous magnetic current (magenta) is generated, creating a $B_0$ magnetic field of up to 25 Tesla [99]. Then, a radiofrequency oscillator generates an alternating magnetic current (cyan) $B_1$ at the frequency $\nu_1$, called resonance frequency, of 0.1 to 960 MHz [100,101]. A radioelectromagnetic wave is thus generated (purple). The analyzed atom absorbs this radioelectromagnetic wave and is capable to resonate. It will then emit energy peaks that can be captured on an NMR spectrum thanks to a radiofrequency transmitter (orange), which detects and recovers the radiofrequencies, and transmits them to a computer. On this spectrum, the reference (peak at 0 ppm on the x-axis) corresponds to the internal standard, which is a molecule with a known resonance frequency and which can be used as a reference. This reference compound (TMSP) has very low toxicity and an overall chemical inertia, and emits a single peak on the spectrum. Since ATP, ADP, and AMP molecules contain a significant proportion of H and P atoms, their relative signals on the NMR spectrometer will differ from the one of TMSP.
biological materials (e.g., total cell extracts) to provide reliable readouts on the concentration of nucleotides in physiological conditions. Last, NMR requires very expensive equipment that prevents its thorough diffusion in every laboratory interested in analyzing ATP levels.

**High-performance liquid chromatography and liquid chromatography coupled to tandem mass spectrometry**

HPLC is a widespread technique, which has been widely used since the 1990s. Its purpose is to separate molecules within a complex sample according to specific features such as polarity [102]. This method is based on a mobile phase, which is constituted of the sample to analyze, previously resuspended in a solvent with a given polarity. The mobile phase then passes through a column, which constitutes the stationary phase. The molecules in the sample will be separated thanks to their different affinities with the column: those with more affinity will be retained on the stationary phase, while the others will be washed out. As a result, the molecules will be separated and will exit the column at different times. At the exit of the stationary phase, the molecules will pass through a detector for analysis, thus generating a chromatogram (Fig. 8). Globally, this approach allows to identify the nature of the molecules analyzed and their absolute quantity [102].

HPLC has thoroughly been used to determine ATP, ADP, and AMP levels in different types of samples, like in plant primary cells [103], in conventional mammalian cell lines such as MCF7 and MDA436 [104], or in primary cells such as human and mouse platelets [105]. The sensitivity of HPLC in detecting nucleotides is in the order of µmol; therefore, HPLC analyses on biological samples require large amounts of cells and a complete extraction of cell contents. This is a potential caveat, as complete extractions can lead to the loss of nucleotides due to hydrolysis. To counteract this limitation, Bhatt et al. have developed an ion-pairing HPLC method coupled with fluorescence detection to quantify ATP, ADP, and AMP in primary astrocytes [106]. In ion-pairing HPLC, the stationary phase consists of an ion-pairing column, and an ion pair reagent is added to the mobile phase to increase or decrease the retention time of partly ionized organic analytes in the column, according to the type of molecule to analyze. In addition, the method to detect nucleotides at the exit of the column benefits from the sensitivity of fluorescence (nmol/pmol range). Nucleotides undergo a derivatization by the transformation of adenine-based nucleotides into 1,N6-etheno derivatives, which gives them fluorescent-like properties and protects them from hydrolysis during cell extraction. This method relies on an excitation wavelength at 280 nm and an emission wavelength at 410 nm, specific for ethenoadenine compounds. Unlike conventional HPLC, ion-pairing HPLC is extremely precise and capable to monitor the levels of nucleotides at the pmol scale. However, it still requires complete cell extraction and a modification step to make ATP, ADP, and AMP fluorescent. Compared with NMR, HPLC is a fast (the retention time is about 20 min [106]), automated, highly reproducible and very accurate method to identify nucleotides in total cellular extracts. As for NMR, this technique is very expensive, thereby hindering its diffusion as a gold-standard method to quantify nucleotides in living samples.

The liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) combines the ability to separate different molecules by liquid chromatography as in HPLC, and the ability to detect and identify molecules of interest by measuring their mass using mass spectrometry. Briefly, mass spectrometry is based on the ionization of molecules of interest (generally by electrospray ionization, ESI; or MALDI) and their sublimation [107]. Once ionized, the molecules enter an

![Fig. 8. Principle of high-performance liquid chromatography.](image)
acceleration zone in which they acquire a specific speed, which directly depends on their mass \((m)\) and charge \((z)\). Then, the molecules are sorted according to their \(m/z\) ratios in an analyzer, which is constituted by an empty column of air preserving the respective speed of each molecule against friction forces. Once the molecules have gone through the analyzer, an ion detector detects the molecules individually and generates a spectrum (Fig. 9).

The LC-MS/MS method benefits from the advantages of LC to separate molecules according to their polarity, while MS/MS allows to determine their quantity with high precision. Recent studies used LC-MS/MS for the simultaneous quantification of ATP and other small metabolites such as 2,3-diphosphoglycerate, \(\text{NAD}^+ / \text{NADH}, \text{H}^+\), or short chain acyl-CoAs \([108,109]\). Therefore, this method makes it possible to obtain the concentration of ATP and that of other molecules with very different biochemical properties, at the same time and faster than the previous approaches (retention time is between 2 min \([109]\) and 10 min \([108]\), according to the setup used). However like HPLC, LC-MS/MS requires expensive instruments and sample preparation can be cumbersome.

**Respirometry**

Respirometry (or oxygraphy) is an indirect, qualitative-only method to measure the efficiency of mitochondrial ATP production, although it is a convenient and less expensive technique than NMR and HPLC to measure ATP levels.

As described above, the electron transport chain creates a proton gradient by consuming oxygen, which in turn allows the ATP synthase to rotate and produce ATP from ADP+P\(_i\) (Fig. 2). Oxygen consumption can directly be linked to ATP production: the more the cells produce ATP, the more oxygen they consume \([110]\). The principle of oxygraphy is to modulate the respiratory chain using selective drugs and metabolites known for their capacity to activate or inhibit its activity, and to measure the corresponding flux of oxygen. The most common technologies to perform respirometry are the high-resolution Oxygraph-2k (O2k; Oroboros Instruments, Austria), and the sensitive, high-throughput Seahorse XF Extracellular Flux Analyzer (Seahorse XF, Seahorse Bioscience Inc.). These setups were shown to perform efficiently on isolated mitochondria, cultured cells, and tissues \([111–113]\), and their output is complementary \([111]\). Overall, the sample is placed in a specific support before undergoing the sequential injection of different drugs and / or metabolites. The cocktail of drugs used in an oxygraphy experiment classically contains oligomycin, FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), and a mix of antimycin/rotenone, while succinate, ADP, and cytochrome c are the most commonly used metabolites (Fig. 10).

Oligomycin is an antibiotic that inhibits the ATP synthase by associating with the F0 subunit and

![Fig. 9. Principle of mass spectrometry for ATP quantification. First, the sample is prepared by integrating it into a matrix and placed inside a spectrometer. The ion source (nitrogen laser) irradiates the sample and generates heating, which converts the sample from a solid state to a gaseous state. Then, this irradiation causes collision events between the molecules of the sample and the matrix. These events will trigger the transfer of protons from the matrix to the sample, thus leading to ionization. The sample passes through an acceleration zone, which is negatively charged, and the molecules of the sample therefore acquire a certain speed, which is inversely proportional to the mass \((m)\) or charge \((z)\) of the molecule. Then, the molecules enter the analyzer, which will separate the molecules according to their \(m/z\) ratio. At the exit of the analyzer, the molecules of interest will be detected and processed by a computer to generate a spectrum.](https://example.com/fig9.png)
prevents the backflow of protons to the mitochondrial matrix [114]. This results in low or no oxygen consumption, and thus no ATP production. FCCP is a weak and lipophilic acid, which acts as an ionophore and an uncoupling agent. Its lipophilic properties allow FCCP protonation in the intermembrane space. Then, FCCP freely crosses the inner mitochondrial membrane and returns to the mitochondrial matrix. Once there, it deprotonates itself before re-crossing freely the inner mitochondrial membrane and being re-protonated in the intermembrane space. Because of these protonation/deprotonation cycles on the FCCP molecule, the protons bypass the ATP synthase almost completely. Oxygen consumption is therefore maximized, but there is almost no ATP synthesis by the ATP synthase [115]. Last, antimycin is an antibiotic blocking the electron transfer at complex III [116]. It is often used in combination with rotenone, an insecticide blocking electron transfer at complex I [117]. The antimycin/rotenone mix blocks the overall electron transfer of the respiratory chain, and as a consequence, the electrons cannot be transferred to oxygen. As a result, the proton gradient no longer exists, and ATP synthesis by the ATP synthase stops (Fig. 10).

In conclusion, NMR, HPLC, and LC-MS/MS allow the direct quantification of global ATP levels from cell extracts and serum samples previously denatured.
Recently developed techniques such as phosphorous-31 ($^{31}$P)-magnetic resonance spectroscopy ($^{31}$P MRS) and magnetic resonance imaging (MRI) allow for ATP quantifications in complex biological samples and in vivo, although the acquisition time to complete one reaction remains excessively long, and the spatial resolution poor [118,119]. Conversely, oxygraphy allows to work on intact samples without prior denaturation, but the measurement of ATP production is inferred from oxygen levels, and remains therefore indirect. Overall, these are widely employed, robust and reproducible methods. However, these approaches are hardly compatible with real-time analyses and do not provide a sufficient spatiotemporal resolution. Indeed, phosphorylation of ADP to ATP occurs in seconds, while any analysis performed with the methods described above occurs in minutes or hours [120]. This is the reason why the development of tools based on microscopy represented a significant advance in the field. As described below, these tools allow to detect different pools of ATP (i.e., organellar, cellular, and extracellular) with high spatiotemporal resolution, and they improved our understanding of the mechanisms of ATP production at the single-cell level.

Microscopy-based tool approaches to measure cellular and extracellular ATP levels

In the last years, huge efforts have been made to develop tools capable of detecting ATP levels not only in living cells, but also at the subcellular level and with sufficient spatiotemporal resolution.

In the sections below, we will explore the probes using electroluminescence, chemiluminescence, and bioluminescence to estimate ATP levels, with a particular focus on fluorescence-based biosensors. Fluorescence-based biosensors are built on three selective properties of fluorophores: (a) the quantum yield of each fluorophore, that is, the ratio between the number of photons emitted and the number of photons absorbed; (b) specific excitation and emission wavelengths ($\lambda$) that allow to use probes individually or in combination; and (c) the specific lifetime of their excited state, that is, the time between the excitation of a molecule of fluorophore and its return to the fundamental state [121,122] (Fig. 11).

Electrochemiluminescence-based tools

Electrochemiluminescence (ECL) is a technique initiating with a step of electron transfer to the surface of an electrode. Several studies used this highly performing and sensitive technique to detect and quantify ATP [123–125]. Liu et al. developed an ECL ATP sensor, which turns on or off when ATP is bound or unbound, respectively [123] (Fig. 12). This method is based on the use of quantum dots (QDs), aptamers, and DNAzyme. QDs, also called 'artificial atoms', are semiconductor nanostructures capable of collecting electrical charges and emitting light according to their size. The QDs are attached to an electrode (glassy carbon electrode, GCE) and then linked to a DNA sequence (hereby called DNA1). The DNA1 is partially complementary with the aptamer, a synthetic oligonucleotide capable of binding a specific ligand such as ATP. The DNA1 allows the aptamer to be linked to the QDs. The complex ‘QDs–DNA1–Aptamer’ forms an aptasensor, and it will synergistically work with the signal probe called DNAzyme, an

Fig. 11. Principle of fluorescence and excitation/emission spectra of fluorophores. Fluorescence is the phenomenon of photon emission by a fluorophore. Left panel: a fluorophore is excited, it absorbs photons and changes from a stable (or fundamental) state to an unstable (or excited) state. To return to the fundamental state, the fluorophore emits photons at a given wavelength, resulting in fluorescence emission. Right panel: Each fluorophore is excitable and emits at a particular wavelength ($\lambda_{\text{excitation}}$/$\lambda_{\text{emission}}$, respectively). This constitutes the excitation/emission spectra of the fluorophore.
oligonucleotide capable of performing a specific chemical reaction. Here, the DNAzyme is carried by a gold nanoparticle and consists of a second DNA sequence (hereby called DNA2), which is also partially complementary to the aptamer and associated with a hemin molecule, a porphyrin playing the role of an electron-binding cofactor. This DNA2–hemin association then forms a secondary structure called G-quadruplex, and it constitutes the signal probe. If there is no ATP, the aptasensor is linked to the signal probe, which is able to reduce O2 into OH− and leading to a drastic decrease in the emission of ECL. Conversely, the aptamer preferentially binds to the ATP molecule when it is present. As a result, the aptamer and the signal probe detach from the QD-modified electrode, allowing a significant ECL emission at 610 nm.

This method of detecting ATP was originally validated on human serum samples, as first-generation QDs necessitated a liquid medium for better results. Recently, the use of QDs has become more diffused, and although their use in medicine is still largely unexplored, in vivo analyses using mouse models seem promising [126,127]. This ECL-based technique seems to be very sensitive to detect ATP levels (detection at nanomolar scale), and the use of quantum dots for live cell imaging is more and more widespread [128]. However, this technique should be further optimized to use it in single-cell imaging applications, and to obtain a better spatiotemporal resolution in living samples.

**Chemoluminescence-based tools**

Chemoluminescence-based tools or chemosensors are based on the chemical properties of ATP itself, which is capable of carrying out interactions of different nature (covalent, electrostatic, hydrogen bonds, etc.) with its substrates. This is due to the chemical structure of ATP, containing three negatively charged phosphate groups, an aromatic adenosine, and a molecule of ribose with several hydroxyl groups. The vast majority of chemosensors are single-wavelength ratiometric sensors, therefore requiring a spectrometer to quantify the presence of ATP [122].

The most significant chemosensors are based on rhodamine, a small organic molecule that emits fluorescence due to its xanthene nucleus ($\lambda_{em} = 558$ nm). The rhodamine-based probe called Rh6G-NH-PBA consists of rhodamine 6G (Rh6G), a diethylenetriamine group (NH), and a phenylboronic acid group (PBA) (Fig. 13). In the absence of ATP, the probe does not

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**Fig. 12.** Functioning mode of an electrochemiluminescence-based ATP probe. This type of luminescence is initiated by an electron transfer. The quantum dot is fixed to an electrode (GCE) and then linked to the aptasensor. The aptasensor consists of a DNA sequence (DNA1) and a synthetic oligonucleotide (aptamer) capable of binding ATP. The DNA1 is partially complementary with the aptamer, a synthetic oligonucleotide capable of binding a specific ligand such as ATP, and the signal probe. The signal probe is a DNAzyme, which consists of a second DNA sequence (DNA2, also partially complementary with the aptamer) and a gold nanoparticle associated with a hemin molecule. **Left panel:** If there is no ATP, the aptasensor is linked to the signal probe, which is able to reduce O2 into OH− and leading to a drastic decrease in the emission of ECL. **Right panel:** If ATP is present, the aptamer will preferentially bind it. This will detach the signal probe from the aptamer, and it will allow a significant ECL emission.
emit light thanks to its closed ring structure. Conversely, three specific interactions will take place in the presence of ATP: 1) covalent interactions between the rhodamine molecule and the ribose group; 2) \(\pi-\pi\) interactions between the xanthene nucleus of rhodamine and adenine; and 3) electrostatic interactions between the amino groups of the rhodamine molecule and the phosphate group of ATP. These interactions open the ring and allow rhodamine light emission [131].

The use of small organic molecules such as Rh6G allows not only to obtain an excellent selectivity for ATP over ADP and AMP, but also to monitor mitochondrial ATP fluctuations in live cells [122]. However, some biophysical properties of this probe still remain to be elucidated. It has been previously shown that rhodamine B, which is a lipophilic cation, accumulates in the mitochondrial matrix [130], and it has been suggested that this accumulation may result from a combination of the negative mitochondrial membrane potential, the high viscosity of the organelle, and ATP concentration in mitochondria [131]. Although rhodamine-based probes turned out to be a real advance in monitoring of mitochondrial functions and in particular to determine ATP levels [132,133], they also show very important limitations for live-cell imaging, as aggregation, quenching, and low photosensitivity [134].

Genetically encoded ATP biosensors

We will now explore biosensors based on specific fluorescent properties such as FRET (Förster’s resonance energy transfer), BRET (bioluminescence resonance energy transfer), and intensiometric / ratiometric single-fluorophore probes. In general, these tools rely on the conformational changes of a bacterial protein, the \textit{Bacillus subtilis} ATP synthase subunit \(\epsilon\) (Fig. 14).
This subunit corresponds to the mitochondrial ATP synthase subunit δ (Fig. 4), and it has been thoroughly used because of its many advantages. First, it is capable of binding ATP without hydrolyzing it [135–137]. Furthermore, the ϵ subunit can bind ATP with higher specificity than ADP, GTP, UTP, and CTP [136]. Then, it undergoes detectable conformational changes when binding to ATP (Fig. 14) [138,139]. Using proteins of bacterial origin to engineer sensors is a commonly used approach [140], as it potentially avoids the establishment of nonspecific interactions with endogenous mammalian proteins. In the case of the ϵ subunit, a mutated version with humanized codons has been used in sensors instead of its normal counterpart, and this disrupts a hydrophobic patch required for the interaction with the γ-subunit of the endogenous mitochondrial ATP synthase [138]. In light of these considerations and improvements, the use of the *Bacillus subtilis* ϵ subunit of the ATP synthase seems to be a good strategy to study intracellular ATP fluctuations.

**FRET-based biosensors to measure cellular and extracellular ATP levels**

FRET microscopy relies on the transfer of energy between a donor/acceptor fluorescent pair. The energy transfer occurs only if the donor and acceptor are less than 10 nm apart, and if the emission spectrum of the donor fluorophore overlaps the excitation spectrum of the acceptor fluorophore [143]. This technique is often used to study the physical interaction between two different proteins, and protein oligomerization (intermolecular FRET), or conformational changes of a given protein (intramolecular FRET). Intramolecular FRET can be used to develop biosensors (Fig. 15).

A large panel of fluorescent FRET-based ATP biosensors has already been developed. Nowadays, the most diffused probes to monitor ATP in single cells are the ATEAM biosensors (adenosine 5'-triphosphate indicator based on Epsilon subunit for Analytical Measurements). Originally developed by Imamura et al., the ATEAM biosensor comprises the ϵ subunit of the bacterial ATP synthase within a cyan (mscCFP, λex/λem = 435/475 nm) and yellow (cp173-mVenus, λex/λem = 515/527 nm) donor/acceptor pair at the N and C termini, respectively [138]. This biosensor allows the monitoring of intracellular ATP in living HeLa cells with subcellular resolution: ATP was detected in the cytoplasm, nucleus, and the mitochondria by adding an organelle-specific localization signal to the biosensor. Taking advantage of organelle-specific variants of ATEAM, steady-state
ATP levels in mitochondria of HeLa cells turned out to be lower than those detected in the cytoplasm or in the nucleus [138]. This suggests that either ATP synthesis by the ATP synthase is low, and/or ATP/ADP exchange rates are rapid in this model. Human skin fibroblasts expressing a mitochondrially targeted ATEAM showed nonuniform fluorescence ratio signals [144], illustrating the differential capability of organelles to produce energy within the same mitochondrial network. In this model, the biosensor also uncovered the contribution of glycolysis to the maintenance of mitochondrial ATP levels [144]. More recently, the ATEAM probe has been used to compare ATP levels in the cytosol, at mitochondria and at the ER under metabolic stress. By using the 535/480 fluorescence ratio, the amount of ATP was reported to be higher in the cytosol (ratio ~ 3) than at mitochondria (ratio ~ 2.3), or at the ER (ratio ~ 1.5) [145]. Interestingly, it has been reported that ATEAM can undergo glycosylation events in other cellular compartments such as the ER and Golgi, and that this post-translational modification makes it incapable of detecting ATP at these locations [144,146]. However, there is no consensus in the literature on this point, and ATEAM appeared to report on ER ATP levels in an unperturbed manner in subsequent studies [147,148].

Unfortunately, the sensitivity of the donor/acceptor pair to the acidic pH of the lysosomes prevents its use and the estimation of ATP production at this compartment [144]. In a second version of the ATEAM biosensor, called GO-ATEAM, the original donor and acceptor fluorophores were replaced by a green (cp173-mEGFP, \( \lambda_{ex}/\lambda_{em} = 470/510 \) nm) and orange (mKO\( \epsilon \), \( \lambda_{ex}/\lambda_{em} = 551/560 \) nm) fluorescent pair. This pair allows to have a more stable signal under acidic pH conditions, and a less phototoxic excitation wavelength than the one used to excite CFP in case of prolonged observations [149,150].

Further rounds of optimization of the ATEAM biosensor resulted in ATEAM1.03NL, where the introduction of mutations in the N-term and C-term domains of the \( \epsilon \) subunit allows the monitoring of intracellular ATP levels at 25°C. This allowed the use of the ATEAM biosensor in in vivo/ex vivo models such as Drosophila melanogaster and Caenorhabditis elegans [141].

By modifying the donor/acceptor pair, an EAF-ATP biosensor (enhanced acceptor fluorescence-based ATP biosensor) was engineered. It consists of the \( \epsilon \) subunit of bacterial ATP synthase as in the ATEAM biosensors, where the C-term and N-term domains are grafted with green (GFP, \( \lambda_{ex}/\lambda_{em} = 488/507 \) nm) and yellow (YFP, \( \lambda_{ex}/\lambda_{em} = 513/527 \) nm) fluorescent proteins, respectively. These fluorophores can be excited at similar wavelengths, which allows the emission of an enhanced fluorescence signal. This biosensor was used to monitor cytoplasmic ATP levels in cells lines derived from glioblastoma to better understand the metabolic switch from mitochondrial oxidative phosphorylation to cytoplasmic glycolysis—the Warburg effect—in this model [151].

Last, a re-engineering of the ATEAM biosensor was recently carried out to monitor extracellular ATP. The ecATEAM3.10 biosensor was expressed on the cell surface thanks to a PDGFR transmembrane anchor, with a CFP/YFP donor/acceptor FRET pair (CFP, \( \lambda_{ex}/\lambda_{em} = 456/480 \) nm; YFP, \( \lambda_{ex}/\lambda_{em} = 513/527 \) nm) [147]. As introduced above, ATP is also a key signaling molecule of purinergic signalization, and this biosensor represents a useful tool to better understand the dynamics of this nucleotide as a messenger in the context of synaptic communication.

### Bioluminescence and BRET-based tools to measure cellular and extracellular ATP levels

In bioluminescence, the emitted light is produced by an enzymatic reaction triggered by a luciferase. In the presence of \( O_2 \), ATP, and \( Mg^{2+} \), luciferase oxidizes luciferin, its substrate, into oxyluciferin (Fig. 16). This oxidation causes the emission of photons, and the resulting emission spectra are generally comprised between 546 nm and 618 nm according to the type of luciferase used [152]. Therefore, the capacity of luciferin to emit light is linked to the available amount of ATP in the surrounding environment. Light emission can thus be used to determine the concentration of ATP in plasma, mitochondrial isolates, or tissue extracts (global extraction of nucleotides can be performed by using perchloric acid) [153,154]. By adding a localization signal to the luciferase, it is also possible to target cell subcompartments (mitochondria, cell membrane, nucleus, etc.) [146].

Rangajaraju et al. have developed a luciferase-based enzymatic system capable of monitoring presynaptic ATP concentrations [155]. This luciferase-based tool is

![Luciferase](https://via.placeholder.com/150)

**Fig. 16.** Principle of the luciferase reaction in luciferase-based ATP assays. In the presence of luciferin, oxygen, and ATP, the luciferase generates oxyluciferin, AMP, pyrophosphate anion, \( CO_2 \), and photon emission (light).
called Syn-ATP, and it consists of synaptophysin, a major synaptic vesicle protein, fused with luciferase and mCherry. The use of synaptophysin allows the targeting of the probe to nerve terminals, and the use of mCherry helps to determine the total amount of luciferase by performing a luminescence / fluorescence ratio. The Syn-ATP biosensor is a useful tool for researchers interested in exploring synaptic functions: the more the ATP is consumed, the more the bioluminescence levels—and therefore the synaptic activity—are significant.

These types of solutions for ATP quantification are widely used and commercialized by several companies. However, this method has some notable caveats. First, certain luciferases have the capacity to produce ATP from the pool of ADP present in the sample, thereby showing poor specificity for the ATP produced after the oxidation of luciferin [156]. In addition, the enzymatic activity of luciferases can be perturbed by cellular inhibitors and by other cellular activities, which may reduce their capacity to perform bioluminescence [157].

To overcome these problems, the bioluminescence property of luciferase-like enzymes is used to develop biosensors based on the BRET phenomenon. BRET is based on the energy transfer between a bioluminescent molecule and a fluorophore. In the case where the luciferase and the fluorophore are close—less than 10 nm apart—the energy derived from a luciferase reaction excites the fluorophore [158]. BRET can be used to study the interactions between two different proteins or protein oligomerization (intermolecular BRET), or the conformational changes of a protein (intramolecular BRET). In this light, BRET-based biosensors rely on intramolecular BRET to monitor ATP (Fig. 16). In such probes, the chosen luciferase generally does not require the consumption of endogenous ATP to oxidize luciferin into oxyluciferin (Fig. 17), and this does not perturb endogenous signaling pathways.

Yoshida et al. developed a BRET version of the ATEAM biosensor (a FRET-based biosensor described earlier) called BTEAM. This probe consists in the ATP binding domain of the $\epsilon$ subunit of bacterial ATP synthase flanked by Venus (yellow fluorescent protein, $\lambda_{em} = 528$ nm) at the N-ter, and a NanoLuciferase at the C-ter, which is an enzyme not relying on endogenous ATP to catalyze its reaction. This biosensor was shown to detect intracellular ATP in the cytoplasm.
(Cyt-BTEAM) or in the mitochondrial matrix (Mit-BTEAM) in live HeLa cells [159].

Following an analogous approach, Saito et al developed another variant of the ATEAM biosensor, called the Nano-lantern ATP biosensor. This sensor combines BRET and complementation of split luciferase (CSL) [160], and it is built by placing Venus at the N-term of the sensor, while the \( \epsilon \) subunit of the bacterial ATP synthase is inserted within two portions of the Nano-Luciferase. When a molecule of ATP binds to the sensor, it allows the reconstitution of a full luciferase, and a BRET reaction between Venus and the NanoLuciferase can occur. This biosensor was validated on plant leaves to study ATP dynamics in the cytoplasm and in chloroplasts.

A third BRET-based ATP biosensor designed by Min et al, called ARSeNL (ATP detection with a Ratiometric mScarlet-NanoLuc sensor), consists of the \( \epsilon \) subunit of the bacterial ATP synthase inserted between an mScarlet (red fluorescent protein, \( \lambda_{em} = 594 \text{ nm} \)) and a NanoLuciferase [161]. This biosensor was shown to detect ATP levels in a HEK293A cell line and in mice. The major advantage of this biosensor is that it provides a stable and robust signal within animal tissues, and it seems to be particularly useful to study ATP levels in tumors [162].

BRET-based biosensors present many advantages. First, light emission comes from the enzymatic reaction triggered by the addition of the luciferase substrate, which eliminates the need for an external exciting light source (laser). This avoids the generation of autofluorescence and phototoxicity, two phenomena potentially harmful for live cells [163]. Moreover, BRET is extremely sensitive because this phenomenon occurs only when luciferase and the fluorophore are close [158]. For measurements on steady-states levels of ATP, this technique can be very useful and easy to perform, especially since luciferin is capable to cross the cellular membrane almost instantaneously, and it is not harmful to cell physiology [146,164].

However, BRET also shows an intrinsic limitation due to the luciferin itself. Indeed, some drug excipients such as cyclodextrin were shown to reduce bioluminescence levels inside cells [165]. This is a potential issue for studies focusing on drug development, since luciferin might interfere with the substance tested. For all these reasons, the creation of new luciferin variants, luciferases, and acceptor fluorophores is a field under intense investigation [166,167]. In addition, the number of cells, and the concentration of enzymes and of substrates can severely limit the quantification of BRET efficiency. As the light emitted is directly proportional to the amount of ATP in the cell, it is necessary to precisely quantify the initial number of cells. It is also necessary to ensure that the transfection of luciferase for BRET-based biosensors is homogeneous in cells, as the higher the concentration of the biosensor, the higher the amount of the emitted light. Finally, the amount of substrate to integrate into the medium to achieve a maximal detection of ATP must be carefully optimized.

Single-fluorophore ratiometric and intensiometric biosensors

Another class of biosensors is based on single fluorescent proteins, which exhibit a fluorescence signal or an increase in fluorescence emission at their corresponding wavelengths. This increase occurs only when the molecule of interest, in this case ATP, binds to the sensor and changes its conformation (Fig. 18). In these probes, the most common fluorophores are circularly permuted fluorescent proteins. Indeed, circularly permuted fluorophores have their N and C termini directly fused with a peptide linker, thereby creating...
new terminal ends close to the chromophore. As a result, the slightest change in conformation is easily detected by the chromophore, which changes its fluorescence properties [168]. Within this class of probes, the QUEEN biosensor (Quantitative Evaluator of cellular ENEergy) is a genetically encoded, ratiometric fluorescent ATP indicator that allows to quantify global ATP levels inside bacteria and yeast [169]. This biosensor consists of a single circularly permuted enhanced GFP ($\lambda_{ex}/\lambda_{em} = 400–494/513$ nm) inserted between 2 $\alpha$-helices of the $\epsilon$ subunit of the bacterial ATP synthase. The integration of circularly permuted enhanced GFP into a flexible region of a sensory domain, as the $\epsilon$ subunit, allows to sense slight changes in its conformation. When ATP binds to the biosensor, we observe a shift of the 400/494 nm ratio, which is proportional to the concentration of ATP. Yaginuma et al [169] compared the capacity of the QUEEN biosensor to report on ATP concentration levels with classical luciferase ATP assays in bacterial cells. They showed that the results obtained with the QUEEN biosensor are almost equal to those obtained from bioluminescence luciferase assays.

Second, another ATP biosensor based on fluorescence intensity is iATPsnFR (intensity-based ATP-sensing fluorescent reporter), which allows to monitor extracellular and cytosolic ATP in HEK293 and U373 cells [170]. iATPsnFR consists in the insertion of a circularly permuted superfolder GFP ($\lambda_{ex}/\lambda_{em} = 488/525$ nm) between 2 $\alpha$-helices of the $\epsilon$ subunit of the bacterial ATP synthase, as in the QUEEN biosensor. When ATP binds to the biosensor, a rapid increase in fluorescence occurs. However, iATPsnFR shows a modest pH sensitivity, which can be potentially problematic for mitochondria or other subcellular compartments that undergo significant pH variations.

Third, the intensiometric MaLion family of biosensors (Monitoring aTP Level intensiometric turn-on indicator) was used to follow cytosolic, mitochondrial, and nuclear ATP levels in HeLa cells, in primary adipocytes and in Caenorhabditis elegans [171]. The MaLion family of biosensors consists of MaLionR, MaLionG, and MaLionB, three constructs based on the fusion of the $\epsilon$ subunit of the bacterial ATP synthase with red ($\lambda_{ex}/\lambda_{em} = 565/585$ nm), green ($\lambda_{ex}/\lambda_{em} = 505/522$ nm), and blue ($\lambda_{ex}/\lambda_{em} = 373/446$ nm) fluorophores, respectively. These probes present several advantages: as for iATPsnFR, MaLionB and MaLionG were fused at their N-terminal with nuclear and mitochondrial targeting sequences for organelle-specific ATP estimations. In addition, the higher the concentration of ATP, the greater the fluorescence emission is. Last, all the probes of the family show a very low pH sensitivity.

Last, the Perceval biosensor family are ratiometric biosensors based on the estimation of the ADP/ATP ratio with live-cell imaging. They are composed of a bacterial regulatory protein, GlnK1, linked to circularly permuted Venus ($\lambda_{ex}/\lambda_{em} = 405–490/530$ nm). Similar to the $\epsilon$ subunit of the bacterial ATP synthase, GlnK1 undergoes a conformational change when binding ATP. This change induces a change of the 490/405 nm ratio of cpVenus, which is proportional to ATP concentration levels. Conversely, no conformational change is observed upon ADP binding. This biosensor was validated in live mammalian cells and in yeast, and demonstrated its usefulness to determine the energy status and metabolic profiles of cells [172–175].

Overall, there is therefore a multitude of tools in the field of quantitative microscopy to measure ATP levels with high selectivity both in cells and in vivo. Depending on the desired detection method, but also on the model available (i.e., live cells, cell extracts, animal models), it is now possible to find the most suitable sensor for any desired application (Table 1). On the contrary, rounds of optimization remain to be done in order to extend the use of these tools for diagnostic purposes.

Thanks to the thorough characterization of the probes presented in this section, it is increasingly clear that genetically encoded biosensors present several advantages. First, they allow to work on living cells and at the single-cell level with an exquisite spatiotemporal resolution. While canonical fluorescence has a resolution limit of approximately 250 nm, FRET and BRET phenomena occur only when fluorophores are less than 10 nm apart [143,159,176]. Furthermore, the use of circularly permuted fluorophores for ratiometric and intensiometric sensors allows to detect subtle changes in ATP production, thanks to the particular position of their chromophores within the sensors. In addition, their analysis can be performed on standard microscope setups [143].

Although genetically encoded biosensors provide an excellent spatiotemporal resolution, they also present significant drawbacks. Among them, it is worth mentioning their nonlinearity [138] and their saturation at physiological ATP levels [175]. It should also be noticed that biosensors can act as metabolite buffers, if a sensor with a low $K_d$ is expressed at a high level to detect a substrate that is present at low concentrations [144,177,178]. In addition, the great majority of the fluorophores used to build biosensors are influenced by pH changes. This is why new pH-resistant fluorophores are under development, aiming to combine the benefits of standard excitation and emission.
wavelengths, sufficient brightness, optimal quantum yield, and a rapid maturation time together with the capacity of resisting to low pH. Last, it should be kept in mind that autofluorescence artifacts and phototoxicity in long-term experiments could potentially affect results obtained with all fluorescent-based probes.

### Table 1. Comparison of genetically encoded ATP biosensors

| Biosensor | Application | Fluorophores (λex/λem; nm) or reporters | Validation done in: | Optimization/Biological application | \(K_0\) |
|-----------|-------------|----------------------------------------|---------------------|--------------------------------------|--------|
| ATEAM [138] | FRET | mseCFP (435/475); cp173-mVenus (515/527) | HeLa | Optimization of the original ATEAM sensor: less phototoxic excitation wavelength for prolonged observations | 3.3 mM |
| GO-ATEAM [149] | FRET | cp173-mEGFP (470/510); mKOx (551/560) | HeLa | | 7.1 mM |
| ATEAM1.03NL [141] | FRET | mseCFP (435/475); cp173-mVenus (515/527) | Drosophila melanogaster; Caenorhabditis elegans | Optimization of the original ATEAM sensor: monitoring of intracellular ATP at 25°C | 1.8 mM |
| EAF-ATP [151] | FRET | GFP (488/507); YFP (513/527) | U87 and U87vIII | Optimization of the original ATEAM sensor: emission of an enhanced fluorescent signal | Not specified |
| ecATEAM3.10 [147] | FRET | CFP (456/480); YFP (513/527) | N2A, HEK293, and SK-MEL-5 | Optimization of the original ATEAM sensor: monitoring of extracellular ATP | 12 μM |
| Syn-ATP [155] | Bioluminescence | mCherry (587/610); Luciferase | Hippocampal neurons | Monitoring of synaptic activity | 2 mM |
| BTEAM [159] | BRET | NanoLuciferase; Venus (λem = 528) | HeLa, Cos7, HepG2, HEK293A, PC12, B16F10 | Optimization of the original ATEAM sensor: does not require the use of a laser as excitation source | 0 to 10 mM |
| Nano-lantern [160] | BRET and CSL | Split-NanoLuciferase; Venus (λem = 528) | Living animals and plants | Optimization of the original ATEAM sensor: usable for high-throughput drug screening and single-cell tracking | 0.3 mM |
| ARSeNL [161] | BRET | NanoLuciferase; mScarlet (λem = 594) | HEK293A and mice | Optimization of the original BTEAM sensor | 1.1 mM |
| QUEEN [169] | Ratiometric | Circularly permutated enhanced GFP (400-494/513) | Bacterial cells | Optimization of the original ATEAM sensor: monitoring of ATP compatible with bacterial growth rate | QUEEN-7 μM |
| iATPSnFR [170] | Intensiometric | Circularly permutated superfolder GFP (488/525) | HEK293 and U373 | Monitoring of ATP in the extracellular space | 3 mM |
| MaLion [R, G and B] [171] | Intensiometric | Red (565/585); Green (505/522); Blue (373/446) | HeLa, primary adipocytes, and Caenorhabditis elegans | Low pH sensitivity | MaLionG: 1.1 mM; MaLionR: 0.34 mM; MaLionB: not specified |
| Perceval [172] | Ratiometric | Circularly permuted Venus (405-490/530) | Mammalian cells and yeast | Monitoring of the ADP/ATP ratio | 0.3 mM |
CONCLUSIONS AND PERSPECTIVES

As presented here, there is a wide range of tools available to detect and quantify ATP levels (Table 2). Historically, the first techniques implemented were biophysical approaches, such as NMR, HPLC, and LC-MS/MS. These techniques have a very precise read-out and allow the detection of ATP levels in denatured samples that require high quantities of starting material. However, these analyses can hardly be carried out in conventional diagnostic and/or research laboratories, mainly due to the high cost of the equipment needed to perform them. There are also biochemical techniques such as respirometry, which can indirectly measure the amount of ATP produced within mitochondria by linking ATP production to oxygen consumption in live intact cells or mitochondrial isolates. Although the possibility of using live cells is a great advance to study the concentration of ATP in its physiological environment, respirometry does not provide insights on its spatiotemporal variations.

In this light, the arrival of luminescent-based sensors (electrochemiluminescence, chemiluminescence, bioluminescence, fluorescence) has revolutionized research on ATP. The biggest benefit of these tools is that the spatiotemporal resolution of ATP levels at the single-cell scale can finally be achieved. Since ATP is largely synthesized within mitochondria, the use of an ATP synthase subunit as a means of sensing ATP is particularly convenient.

FRET-based biosensors appear to be the most promising class of probes, as they possess a superior spatiotemporal resolution thanks to the FRET phenomenon and provide reliable quantitative information on subcellular ATP levels. However, they need to be stably or transiently integrated in the genome of the targeted cell. Chemosensors and electrochemosensors do not require an integration, but they show low specificity since the sole parameter of mitochondrial membrane potential turned out to be insufficient for their mitochondrial targeting. Single-wavelength-based biosensors are biosensors with on/off readouts, but they were shown to present a fluorescent background, which makes it nontrivial to determine the off state. However, they are suitable to detect large variations in ATP production. Finally, BRET-based biosensors have a spatiotemporal resolution similar to FRET probes, and do not require an external light source (i.e., a laser) to trigger the luciferin-luciferase reaction. In contrast to the large body of literature demonstrating the phototoxic effects of laser excitations and

| Techniques for monitoring ATP | Type of samples | Readout | Spatiotemporal resolution | Equipment | Detection time | Sensitivity |
|------------------------------|-----------------|---------|---------------------------|-----------|---------------|-------------|
| NMR [94–96]                 | Cell extracts, serum, plasma | Quantitative | No | NMR spectrometer | ≈ 14 h | mM |
| HPLC [103–106]             | Cell extracts, serum, plasma | Quantitative | No | Chromatograph | ≈ 20 min | pmol |
| LC-MS/MS [108,109]         | Cell extracts, serum, plasma | Quantitative | No | Mass spectrometer | < 10 min | nmol |
| Respirometry [110]          | Intact cells, mitochondrial fractions | Qualitative | No | Oxygraph | ≈ 90 min | % |
| Electrochemiluminescent Sensors [123] | Serum | Quantitative/Qualitative | No | Spectrometer, electrodes | ≈ 5 min | nmol |
| Chemoluminescent Sensors [122,129] | Intact cells | Quantitative/Qualitative | Yes | Spectrometer or confocal microscope | ≈ 15 s | mM |
| Bioluminescent Sensors [146,153,154] | Cell extracts, plasma, mitochondrial fractions | Quantitative/Qualitative | No | Spectrometer or luminometer | < 5 min | pmol |
| BRET sensors [159–161]      | Intact cells | Quantitative/Qualitative | Yes | Spectrometer, luminometer or fluorescence microscope | < 20 min | mM |
| Single-wavelength sensors [169–171,175] | Intact cells | Quantitative/Qualitative | Yes | Spectrometer or fluorescence microscope | < 1 min | µM |
| FRET sensors [138,141,147,149–151] | Intact cells | Quantitative/Qualitative | Yes | Spectrometer or fluorescence microscope | < 1 min | µM |
wavelengths on cell viability and the response of organelles to irradiations, there are very few studies exploring the effects of luciferin on cell physiology. Unless the toxic effects of luciferin are formally ruled out in future studies, FRET-based probes currently represent the best and most extensively characterized sets of probes to monitor ATP in living cells and with spatiotemporal resolution.

Defects in ATP levels and/or in ATP processing are commonly found in a variety of pathologies such as mitochondrial pathologies [179–185], chronic glaucoma [186–189], irritant dermatitis [190–192], HIV-associated neurocognitive disorders (HAND) [193,194], vascular pathologies [195–201], neurodegenerative pathologies [202–206], and cancer [207–211]. Beyond the interest of ATP as a biomarker in disease paradigms, monitoring its levels is also a valuable tool to determine the degree of contamination of surfaces in healthcare-related environments [212–216]. Overall, the need for innovative and sensitive techniques to monitor ATP levels is a major challenge for therapeutic purposes. To develop new treatments, or to improve the existing ones by limiting their side-effects [217], a deeper understanding of how metabolic homeostasis is altered in pathological conditions is mandatory. Given that cell metabolism is dynamic and highly compartmentalized, approaches that allow to simultaneously visualize different subcellular pools of ATP will certainly be of extreme interest in the near future. This will be important not only to unravel new metabolic regulations within the cell, but also to visualize the consequences of metabolic dysfunctions in patient-derived samples. In this light, robust fluorescence-based approaches combine an exquisite spatiotemporal resolution, a quantitative readout, and a high sensitivity requiring relatively low amounts of biological material. All these features are a driving force for the implementation of drug screening strategies, with the final goal of gaining a system-level understanding of metabolic alterations and therapeutic solutions for personalized medicine.

Conflict of interest
The authors declare no conflict of interest.

Author contributions
SLN participated in the conceptualization of this review and wrote the original draft. GB coconceptualized the manuscript, supervised, reviewed and finalized the manuscript.

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