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Imaging Adenosine Triphosphate (ATP)

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Abstract

Adenosine triphosphate (ATP) is a universal mediator of metabolism and signaling across unicellular and multicellular species. There is a fundamental interdependence between the dynamics of ATP and the physiology that occurs inside and outside the cell. Characterizing and understanding ATP dynamics provides valuable mechanistic insight into processes that range from neurotransmission to the chemotaxis of immune cells. Therefore, we require the methodology to interrogate both temporal and spatial components of ATP dynamics from the subcellular to organismal levels in live specimens. Over the last several decades, a number of molecular probes that are specific for ATP have been developed. These probes have been combined with imaging approaches, particularly optical microscopy, to enable qualitative and quantitative detection of this critical molecule. In this review, we survey current examples of technologies that are available to visualize ATP in living cells and identify areas where new tools and approaches are needed to expand our capabilities.

Keywords

ATP; imaging; energy metabolism; purinergic signaling; fluorescence; sensors; biosensors

INTRODUCTION

Significant efforts have been made over the decades to directly visualize adenosine triphosphate (ATP) in living systems. ATP is a molecule at the center of metabolism and signaling both inside and outside the living cell, and its universal importance in biology reaches well beyond its most familiar role as an energy metabolite.

In energy transduction, ATP hydrolysis provides a thermodynamic driving force for cellular chemistry (Westheimer, 1987; Kamerlin *et al.*, 2013). It remains ambiguous why ATP evolved such a central and universal role (Plattner and Verkhatsky, 2016), but it is clear that energy-dependent reactions are critical for numerous cellular processes from the maintenance of neuronal membrane potential (Magistretti and Allaman, 2015) to organelle transport (Zala *et al.*, 2013) to nucleocytoplasmic translocation (Dzeja *et al.*, 2002). In fact, hydrolysis of other nucleotides, such as GTP, and other phosphate metabolites can also

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provide a driving force for specific energy-dependent processes. Despite the energetic contribution of these alternatives, ATP still plays a central role through the action of nucleotide diphosphate kinases and enzymes that create a phosphotransfer network involved in the distribution of bioenergetics (Shugar, 1996; Dzeja and Terzic, 2003).

Beyond energy transduction, ATP plays a central role in signaling. ATP serves as a phosphate-group donor for substrate activation in metabolic reactions and as the coenzyme for a large number of kinases. In the regulation of protein function, ATP can serve as both a phosphate- and an adenylyl-group donor for post-translational modifications. ATP is also required for the biosynthesis of cyclic adenosine monophosphate (cAMP), which is a critical second messenger in signal transduction. Furthermore, ATP itself can serve as a bona fide signaling ligand for ATP-sensitive or purinergic ionotropic and G-protein coupled receptors. With its vast repertoire of biological functions, the spatial and temporal dynamics of ATP broadly affect both intracellular and extracellular processes that determine normal physiology as well as pathological states. To this end, we briefly review examples demonstrating the importance of ATP in brain energy metabolism and inflammation before surveying a number of ATP detection and imaging methods that are currently available.

Intracellular ATP in Metabolism and Signaling: Brain energy metabolism

In the healthy brain, ATP-consuming processes that are involved in electrochemical signaling impose significant energy demands, which is reflected in the high rates of cerebral glucose and oxygen consumption (Harris *et al.*, 2012; Howarth *et al.*, 2012; Magistretti and Allaman, 2015). The dynamics of brain energy metabolism are complex, in part, because ATP production must respond to these energy demands in an activity-dependent manner. For example, neuronal excitability relies on sufficient energy production to maintain neuronal ion gradients and membrane potentials in the face of continuous action potential generation and signaling in the brain. ATP production must also support ancillary processes associated with neurotransmission such as biosynthesis of neurotransmitters, vesicle loading, and axonal transport, to name just a few (Howarth *et al.*, 2012). Many open questions remain concerning the precise mechanisms by which fuel utilization and ATP production are coordinated in the brain, both within cells and between cells. For example, while ATP production is typically viewed as requiring efficient mitochondrial respiration in neurons, aerobic glycolysis occurs under physiological conditions in certain regions of the brain such as the parietal cortex and prefrontal cortex, especially during development (Vaishnavi *et al.*, 2010; Goyal *et al.*, 2014). Observation of regional aerobic glycolysis may be related to the compartmentation of metabolism between neurons and glia. It has been hypothesized that astrocytes support neuronal activity by performing aerobic glycolysis and shuttling lactate to neurons for mitochondrial respiration and ATP generation (Bélanger *et al.*, 2011). Furthermore, the complex morphology of dendritic arbors and elongated axons requires local ATP generation for synaptic function, which may be provided by motile glycolytic machinery or mitochondria (Zala *et al.*, 2013; Rangaraju *et al.*, 2014).

Because of the high numbers and activities of ATP-consuming processes in the brain, bioenergetics fundamentally influence cognition and behavior. As a result, the physiology underlying activity-dependent ATP production is closely linked to and ultimately dictates the

utility of methods such as ^{18}F -fluorodeoxyglucose positron emission tomography, magnetic resonance imaging (MRI) of cerebral metabolic rates, and blood oxygen level dependent functional MRI of cerebral blood flow (Raichle and Mintun, 2006). These methods have, in turn, demonstrated the vulnerability of the brain to metabolic insult. Bioenergetic deficits occur in aging, injury, and neurological diseases such as Alzheimer's and Parkinson's diseases (Johnson *et al.*, 2012; Surmeier *et al.*, 2012). In brain ischemia and stroke, for example, attenuated cerebral blood flow causes loss of glucose and oxygen availability, intracellular ATP depletion, and collapse of ion gradients within minutes (Dreier and Reiffurth, 2015). Additionally, ischemic injury results in the release of ATP to the pericellular space, initiating an extracellular cascade of events, including purinergic signaling, chemotaxis of immune cells, and inflammation (Pedata *et al.*, 2015).

Extracellular ATP in Metabolism and Signaling: Inflammation

Leakage of intracellular ATP into the pericellular space can result from disruption of the plasma membrane, which may be precipitated by mechanical damage or necrotic cell death. In addition to this unregulated release, cells can promote ATP secretion through the expression of protein channels that connect the cytoplasm to the extracellular space, as has been observed in cells undergoing apoptosis and in activated inflammatory immune cells (Figure 1). In both cases, the rapid ATP egress is driven by the gradient from high intracellular ATP concentration to low extracellular ATP concentration. If extracellular ATP levels become significantly elevated, activation of membrane bound nucleotide receptors on neighboring cells incites a strong proinflammatory response (Idzko *et al.*, 2014). In this context, extracellular ATP acts as a damage associated molecular pattern (DAMP) molecule and is interpreted by immune cells as a danger signal (Eltzschig *et al.*, 2012).

The receptors activated by ATP (Figure 1), which are broadly expressed on immune and other cells, are members of the P2 class of receptors for extracellular nucleotides and are classified into two families, P2Y and P2X (Burnstock and Boeynaems, 2014). The P2Y family of receptors are G-protein coupled receptors (GPCRs), and eight members have been identified to date. P2X receptors are ligand-gated ion channels. The P2 receptors vary in their responsiveness to a range of nucleotides, with P2Y receptors being optimally responsive to endogenous nucleotides such as ATP, ADP, UTP, etc. depending on the receptor subtype and species. On the other hand, ATP is the preferred endogenous agonist for all subtypes of P2X receptors.

The P2X₇ receptor (P2X₇R) is the most studied member from the ionotropic P2X receptor family, and its activation by ATP is an important proinflammatory signal for a number of immune cells, including macrophages, microglia, and dendritic cells (Di Virgilio, 2007). ATP binding to P2X₇R causes channel opening and membrane depolarization via Na⁺ and Ca²⁺ influx and K⁺ efflux. P2X₇R activates the NLRP3 inflammasome, which is a multiprotein assembly that enables the caspase-1 catalyzed release of the proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18. In addition, the P2X₇ receptor undergoes a conformational change that widens the channel to allow passage of larger molecules, including ATP. However, the mechanism and role of channel widening are debated, and it is not necessary for NLRP3 activation as it has been shown that a decrease in intracellular

potassium is necessary and sufficient (Muñoz-Planillo *et al.*, 2013). P2X₇R activation and the resulting IL-1 β signaling has been shown to be important in bacterial pathogen clearance by macrophages (Coutinho-Silva and Ojcius, 2012) and cancer antigen presentation to lymphocytes by dendritic cells (Ghiringhelli *et al.*, 2009). However, when dysregulated or activated inappropriately this pathway contributes to a number of inflammatory disorders, including inflammatory bowel disease (Kurashima *et al.*, 2012) and asthma (Müller *et al.*, 2011).

From the metabotropic P2Y receptor family, P2Y₂R has been shown to be an important mediator of inflammation and active in the recruitment of immune cells to sites of infection and wound healing (Elliott *et al.*, 2009). ATP released from damaged or infected cells that binds P2Y₂R on leukocytes acts as a chemoattractant to draw in neutrophils and macrophages, which can then phagocytose the ailing cells and help resolve the inflammation. Interestingly, neutrophils have been shown to use directional, autocrine ATP signaling as a mechanism to amplify their migration response to chemoattractants. Specifically, Chen and coworkers reported that stimulation with the chemoattractant N-formyl-Met-Leu-Phe (FMLP) causes human neutrophils to release ATP that is localized near the stimulus (Chen *et al.*, 2006). The released ATP then binds P2Y₂Rs expressed on the neutrophil cell surface and promotes chemotaxis along the FMLP gradient. Neutrophils from mice deficient in P2Y₂R showed significantly impaired chemotaxis.

The proinflammatory signaling of extracellular ATP is terminated by nucleotidases active in the interstitial fluid as well as the membrane-bound ectonucleotidases CD39 and CD73 (Figure 1) (Idzko *et al.*, 2014). CD39 catalyzes the conversion of ATP first to ADP and then to AMP, and it can also accept ADP as a substrate for conversion to AMP. Subsequently, CD73 converts AMP to adenosine, and thus a decrease in extracellular ATP results in an increase in extracellular adenosine. Extracellular adenosine can engage P1 receptors, of which there are four subtypes, A₁, A_{2A}, A_{2B}, A₃. Whereas ATP signaling generally upregulates the immune response, signaling at adenosine receptors is associated with the dampening and resolution of inflammation. Adenosine signaling is terminated by cellular uptake of adenosine through equilibrative nucleoside transporters 1 and 2 (ENT1, ENT2). Following cellular uptake, adenosine is metabolized to either AMP by adenosine kinase or to inosine by adenosine deaminase.

Examples from brain energetics and inflammation illustrate why the “how, when, and where” of ATP production and consumption clearly impact physiology. These examples also highlight the importance of studying the spatial and temporal dynamics of ATP. Because ATP levels can respond rapidly and locally to physiological changes, real time imaging in live specimens is essential for: (1) fully capturing how changes in ATP levels are distributed throughout cells and tissues, and (2) providing experimental access to dissect the mechanisms and machinery responsible for ATP’s roles in metabolism and signaling.

METHODS FOR DETECTION AND IMAGING ATP

A number of well-established and newly developed methods can measure ATP, though there are far fewer methods that can image ATP in live specimens. Chemical and physical

methods, ranging from liquid chromatography to mass spectrometry (Khlyntseva *et al.*, 2009), can offer superb specificity. For example, extracellular ATP has been measured using enzyme-coated platinum microelectrodes (Kueng *et al.* 2004; Llaudet *et al.* 2005) or optical fibers (Wang *et al.*, 2013). However, these methods typically lack spatiotemporal resolution or lack compatibility with live specimens. Chemical and physical methods that involve de-proteinization to measure intracellular concentrations also release bound forms of adenine nucleotides (Harris *et al.*, 1973). This type of sample preparation results in measurements of total rather than free ATP and ADP concentrations that may skew equilibrium and energy status estimates (Veech *et al.*, 1979; Mörikofer-Zwez and Walter, 1989; Tantama *et al.*, 2013). As an alternative to instrumental analysis, molecular probes that are specific to free ATP offer the opportunity for real-time analysis with greater resolution or compatibility with live specimens or both. Thus, we present examples of both non-imaging and imaging methods for detecting ATP, in order to contrast their advantages.

A number of non-imaging approaches to ATP measurement have been developed. For example, in an electrophysiological approach, the ligand sensitivity of P2X receptors can be exploited because, as discussed, they are ATP-gated cation channels and thus produce ATP-dependent currents. When P2X receptors are expressed in the plasma membrane of PC12 cells (Praetorius and Leipziger, 2009) or HEK-293 cells (Hayashi *et al.*, 2004), patch-clamp electrophysiology in outside-out or whole-cell mode can be used to detect ATP in proximity to the electrode-immobilized membrane or cell. This technique has been used to study ATP release from a variety of cells; for example, Hazama *et al.* were able to measure ATP concentrations released from pancreatic β cells in real time by measuring P2X current amplitudes via whole-cell patch-clamp (Hazama *et al.*, 1998). Also exploiting an electrophysiological approach, nucleotide-calibrated measurements of ATP-sensitive potassium (K_{ATP}) channel currents have been used to calculate submembrane concentrations of ATP and test for local gradients in COSm6 monkey kidney cells and in *Xenopus* oocytes (Gribble *et al.*, 2000). In a contrasting non-imaging spectroscopic approach, Vancraenenbroeck recently published a sensor based on malonyl-coenzyme A synthetase, an enzyme that undergoes a conformational change upon ATP binding. Two molecules of tetramethylrhodamine are conjugated to the synthetase in a manner such that ATP binding causes a substantial increase in fluorescence intensity, with high selectivity for ATP and micromolar sensitivity (Vancraenenbroeck and Webb, 2015). These and other non-imaging methods provide important means to quantify ATP, and in many cases are ideal for assaying cells or extracts with high sensitivity and high throughput; however, non-imaging methods are unable to provide the same high level of spatial and time-resolved information content achieved with imaging methods.

Several methods to image ATP have been developed over the past many decades (Figure 2) (Table 1). While the ideal approach would be quantitative and compatible with non-invasive, probe-free visualization of ATP in live specimens with high spatial and temporal resolution, a single method satisfying these criteria does not yet exist. Magnetic resonance spectroscopy techniques that exploit phosphorous magnetization transfer can non-invasively quantify ATP *in vivo*, but practical considerations, such as long acquisition times per voxel and the limited availability of the instrumentation to many biologists, have limited its applicability to imaging (Du *et al.*, 2008; Chaumeil *et al.*, 2009; Befroy *et al.*, 2012). Instead, methods to

visualize ATP primarily rely on optical microscopy paired with molecular probes, which provide signal readouts that are specific for ATP. These molecular probes have been developed using a variety of physical formats from small organic indicators to nanoparticles, and they exploit both indirect and direct ATP detection mechanisms.

Magnesium Green, for example, is a magnesium-sensitive small organic fluorophore that can be used to indirectly detect ATP hydrolysis (Leyssens *et al.*, 1996). The majority of intracellular ATP is complexed with divalent magnesium ions; however, ADP has a lower affinity for magnesium ions relative to ATP. Thus, hydrolysis of MgATP causes an increase in free magnesium ion concentration and subsequent increase in Magnesium Green fluorescence. By invoking binding equilibrium, Magnesium Green has been used in non-imaging studies to determine the ATP-ADP exchange rate through the mitochondrial adenine nucleotide transporter in isolated mitochondria (Chinopoulos *et al.*, 2009) and permeabilized cells (Kawamata *et al.*, 2010). In an imaging study exploiting fluorescence confocal microscopy with isolated hair cells loaded with Magnesium Green, Shin *et al.* indirectly visualized a higher contribution of creatine kinase activity to ATP generation in hair bundles versus their soma (Shin *et al.*, 2007). While sensitive to ATP hydrolysis, Magnesium Green has the disadvantage that it is not perfectly specific, as it has moderate affinity for calcium. On the other hand, Magnesium Green is advantageous over other magnesium indicators because its fluorescence can be excited with illumination in visible range, reducing phototoxicity compared to indicators such as Mag-Fura-2 and Mag-Indo-1, which require ultraviolet (UV) excitation. However, Magnesium Green shows a simple increase in fluorescence intensity upon binding magnesium ions without a ratiometric shift in excitation or emission peaks. Thus, unlike ratiometric probes, the Magnesium Green signal is also dependent on dye concentration, making it challenging to use for quantitative studies because loading efficiency and photobleaching can cause variations in signal unrelated to changes in ATP hydrolysis (Leyssens *et al.*, 1996).

In order to directly visualize ATP pools, fluorescent analogues can be used as tracers once loaded into the tissue, cell, or organelle of interest. Analogues can be synthesized by conjugating fluorescent groups such as a methylantraniloyl (mant) or a coumarin (deac) to the nucleobase, ribose or phosphate groups of ATP. The analogues mant-ATP and deac-ATP have been extensively used to study kinase and ATPase activities in solution (Fili and Toseland, 2014). For example, mant-ATP has been used to monitor vesicular ATP release from dopaminergic neurons (Ho *et al.*, 2015), and deac-ATP has been used to study the kinetics of myosin Va movement on actin using total internal reflection fluorescence (TIRF) microscopy (Sakamoto *et al.*, 2008). Analogues of ATP modified with Cy3 or BODIPY fluorescent dyes are also available, which shift excitation bands into the visible range to reduce phototoxicity.

Additional synthetic ATP analogs have been developed to detect ATP hydrolysis. These analogs exploit Förster-type resonance energy transfer (FRET) between a donor fluorophore covalently attached to a γ -phosphate group and an acceptor fluorophore linked to the base or ribose (Hardt *et al.*, 2013). Cleavage of the phosphodiester bond allows the donor and acceptor to diffuse away from one another, causing a drastic loss of FRET and an increase in donor fluorescence. Hacker *et al.* have used such an analog to monitor ATP consumption in

real time during ubiquitin activation by UBA1, a human E1 enzyme (Hacker *et al.*, 2013). Similarly, a FRET-based ATP analogue with the organic fluorophore Sulfo-Cy3 dye linked to the γ -phosphate and a quencher linked to the ribose C2 position was used by Gutiérrez Acosta *et al.* to study acetone degradation in *D. biacutus* cell extract (Gutiérrez Acosta *et al.*, 2014). While these FRET-based ATP analogues have not yet been used for imaging, in principle they could be used similarly to mant-ATP. It is important to recognize with these ATP analogues that the fluorescent dye group can be of equivalent or greater mass than ATP itself. Therefore, the covalent modification could change the behavior of the analogue in unpredictable ways, and functional assays are critical to validate the use of such analogues.

Small molecule sensors for ATP as an analyte have also been reported. For example, quinacrine is a fluorescent acridine derivative that stains peptide-bound ATP found in high concentrations in intracellular granules (Irvin and Irvin, 1954; Bodin and Burnstock, 2001). In this manner, quinacrine has been used to image vesicular ATP release in endothelial and epithelial cells (Bodin and Burnstock, 2001; Feranchak *et al.*, 2010; Akopova *et al.*, 2012). Alternatively, Pak *et al.* developed an imidazolium based ratiometric sensor for ATP with a pyrene excimer clamp. When ATP binds to this sensor, it forms a pyrene-adenine-pyrene sandwich by π - π stacking. Formation of the complex results in an increase in pyrene emission at 375 nm and a decrease in emission at 487 nm. This pyrene-based sensor was used in HeLa cells to monitor the decrease in ATP levels upon addition of the ATP synthase inhibitor oligomycin and upon the hydrolysis of ATP to ADP by apyrase (Pak *et al.*, 2015).

Aptamers are single-stranded DNA or RNA oligonucleotides which can be easily modified to attain high-affinity and specificity for their targets. Aptamers are extensively used to study small molecule metabolites (Paige *et al.*, 2012; Feng *et al.*, 2014) and are analyzed by variety of methods such as fluorescence spectroscopy (Sun *et al.*, 2010; Park *et al.*, 2015b; Wang *et al.*, 2015; Song *et al.*, 2016) electrochemistry (Mukherjee *et al.*, 2015; Zhao *et al.*, 2015), surface plasmon resonance (Park *et al.*, 2015a) and colorimetry (Huo *et al.*, 2016). Aptamers can be engineered to detect ATP in nanomolar to millimolar ranges; however, cell permeability and degradation issues limit their use in live-cell imaging (Wang *et al.*, 2014). To overcome this problem, nanoparticles have been used to deliver and protect aptamers from degradation by DNases and RNases in cells while modifying their fluorescent properties. For example, Qiang *et al.* reported a polydopamine nanosphere-linked aptamer hybrid that protects the aptamer and quenches its fluorescence (Qiang *et al.*, 2015). Addition of ATP releases the aptamer resulting in an increase in the fluorescence. The aptamer is highly selective and sensitive, detecting ATP in 0.01–2mM range. Changes in ATP concentration could be measured in HeLa cells upon treatment with oligomycin or Ca^{2+} . Similarly, nanoparticles have been used to construct nanoflares (Zheng *et al.*, 2009) to image ATP. Aptamers bound to gold nanoparticles are hybridized with fluorescent DNA strands that are quenched by proximity to the nanoparticle. Binding of ATP to the aptamers releases the fluorescent strands, and the increase in fluorescence can be used to quantitate ATP in live cells (Zheng *et al.*, 2009; Torabi and Lu, 2014). One drawback of these biosensors is that the aptamers have been engineered for adenine selectivity, which can make it challenging to distinguish between adenine derivatives (Ozalp *et al.*, 2010). To overcome this drawback, Sassanfar *et al.* synthesized RNA aptamers specific for ATP (Sassanfar and Szostak, 1993),

and Ozalp *et al.* (2010) developed DNA aptamers selective for ATP (apparent K_D : 3.2 mmol l^{-1}) and ADP (apparent K_D : 4.4 mmol l^{-1}).

While both small organic indicators and aptamer-based biosensors have found utility in imaging studies, they pose a challenge to sample preparation because they require cell penetration or cell loading of the exogenous reagent. In simple model systems, such as monolayer cell cultures, introduction of these ATP imaging reagents can typically be achieved by sustained incubations, electroporation, or microinjection. However, these preparatory requirements limit their application in many cell types and complex or thick tissues. In contrast to these technologies, genetically-encoded indicators are protein-based reagents that are in part or wholly encoded by an appropriate gene sequence. Thus, genetically-encoded imaging reagents offer the advantage of being compatible with a wide variety of specimens when the appropriate gene transfer or expression method is available, such as transfection reagents, viral transduction, and tissue-specific expression in transgenic mice. Recent examples of genetically-encoded ATP imaging reagents have utilized luciferases and fluorescent proteins.

Luciferases are used to produce bioluminescence in order to visualize cellular activities as well as track cell populations *in vivo*. Firefly luciferase and its exogenously-supplied substrate luciferin can be used to measure ATP because the chemiluminescent reaction is ATP dependent. Adenylation of luciferin by luciferase activates the substrate for conversion to oxyluciferin, and thus the resulting luminescence is proportional to ATP concentration (Manfredi *et al.*, 2002; Lundin, 2014). Currently, commercial and academic efforts have produced a variety of luciferases that have been engineered and codon-optimized from different species for bioluminescence imaging in live samples (Thome *et al.*, 2010; Hall *et al.*, 2012), but the ATP-dependent firefly and beetle luciferases are most commonly used to measure ATP (Manfredi *et al.*, 2002; Lundin, 2014). Branchini *et al.* have recently developed a luciferase with higher activity and quantum yield by fusing the N-terminal domain of the *Photinus pyralis* luciferase joined to the C-terminal domain of the *Luciola italica* luciferase. The chimeric luciferase was further optimized to show 3-fold higher sensitivity in live cells compared to the commercial Luc2 variant (Branchini *et al.*, 2015). Mutagenesis has also produced luciferase variants with red-shifted luminescence (Branchini *et al.*, 2010). While these emission variants typically exhibit lower luminescence yields, they offer the advantage that red luminescence suffers less tissue absorbance, making them good reporters for *in vivo* imaging (Liang *et al.*, 2012). There are also important caveats to the quantitation of ATP by luciferase. For example, optimization is critical because of the inherent dependence on oxygen concentration and the often underappreciated complication of product inhibition as well as inhibition by cellular factors or pharmacological agents (Leitão and Esteves da Silva, 2010; Lundin, 2014).

Despite difficulties with absolute quantitation, luciferase has been used monitor ATP changes in variety of cells such as HEK-293 cells, cardiomyocytes and neurons (Brovko, 2010). Bell *et al.* (2007) were able to use wild-type luciferase inside the cell to monitor differences in intracellular ATP responses in mitochondria and the cytosol of cardiomyocytes after stimulation. Free cytosolic and membrane-tethered luciferase have also been used to study the possibility of compartmentalization of submembrane ATP and control

of K_{ATP} channel activity in pancreatic β -cells and hypothalamic neurons (Kennedy *et al.*, 1999; Ainscow *et al.*, 2002). In detection of intracellular ATP, luciferase is introduced into mammalian cells by gene transfection, microinjection or viral vectors, though Lee *et al.* developed luciferase fused to a protein transduction domain to facilitate direct transport of the luciferase protein into the cell (Lee *et al.*, 2012). Even with genetic encoding, however, the exogenous luciferin substrate must be supplied and must penetrate tissues and cells. While this has proven achievable in live cells and animals, there is still the possibility of variable substrate access and cell-type specific toxicity which must be taken into account (Rangaraju *et al.*, 2014). In the detection of extracellular ATP, luciferase has also been used to study ATP release by tethering the enzyme to the extracellular face of the plasma membrane via conjugation to primary IgG antibodies that can bind to surface antigens, via streptavidin-biotin tags, and via glycosylphosphatidylinositol (GPI) lipid anchors (pmeLuc) (Praetorius and Leipziger, 2009). For example, HEK-293 cells stably transfected with pmeLuc could detect micromolar levels of ATP in the tumor microenvironment while extracellular ATP was undetectable in healthy tissues (Pellegatti *et al.*, 2008). The luciferase-luciferin system can offer a low-background, low-toxicity method to monitor ATP; however, low luminescence limits its applicability in real time ATP imaging. Bioluminescence imaging typically requires long exposure times that limit spatiotemporal resolution, and in some applications it can require specialized equipment (Bell *et al.*, 2007; Brovko, 2010). For example, Furuya *et al.* were able to improve the temporal resolution to 100-ms by using a cooled electron multiplying charge coupled device (EMCCD) camera coupled with an image intensifier to compensate for low photon emission rates (Furuya *et al.*, 2014). They used it along with low-magnification, high numerical aperture objectives to monitor ATP release from a single cell with 10 nM detection sensitivity. Furthermore, interference from the sample matrix can occur due to inhibitory levels of various anions and salts, ion channel inhibitors, and P2 receptor antagonists (Praetorius and Leipziger, 2009).

An alternative to luciferase-based detection is the use of fluorescent protein-based ATP sensors. Fluorescent protein-based sensors do not require addition of an exogenous luciferin substrate, and they are convenient due to ease of manipulation at the DNA level and subsequent ease of expression in cells and *in vivo*. For example, as an alternative to the electrophysiological approach (Gribble *et al.*, 2000), ATP level changes in HEK-293 cells have been visualized by imaging the ATP-dependent conformational change of K_{ATP} channels fused to an ECFP-EYFP cyan and yellow fluorescent protein FRET pair (Tsuboi *et al.*, 2004). Imamura *et al.* developed a family of sensors named the ATeams that are also based on FRET between a donor fluorescent protein and acceptor fluorescent protein. In the original ATeams the ϵ subunit from the F₀F₁-ATP synthase is linked to the CFP and YFP FRET pair (Imamura *et al.*, 2009). The ϵ subunit is a 14 kDa protein subunit, composed of a N-terminal β -barrel and 2 C-terminal helices. It undergoes a large conformational change upon binding of ATP, which is the mechanism by which the ϵ subunit regulates F₀F₁ ATPase activity based on intracellular ATP levels. The ATeam sensors exhibit apparent dissociation constants ranging from 7.4 μ M to 3.3 mM, with at least 10- to 100-fold greater selectivity for ATP over ADP, and response kinetics on the timescale of seconds.

The ATeam sensors have been used to study ATP changes in bacterial cells, neurons, and a number of different cell types in different species (Imamura *et al.*, 2009; Toloe *et al.*, 2014).

In unicellular organisms, Maglica *et al.* (2015) used ATeam sensors to monitor antibiotic induced cell death by single cell tracking of ATP levels in *Mycobacterium smegmatis*, demonstrating its potential as an important technology in drug discovery for antibiotic screening and mechanism of action imaging assays. In multicellular organisms, Ozawa *et al.* (2015) found that glycolysis dependent ATP production was necessary for lamellipodia formation in podocytes. In contrast, the cortical subcellular region of podocytes produces ATP by both glycolysis and oxidative phosphorylation in mitochondria. These metabolic studies demonstrate how the ATeam sensors can provide important insight into podocyte cell biology, potentially advancing our understanding of the role of metabolic dysfunction in chronic kidney diseases (Ozawa *et al.*, 2015).

When imaging fluorescent ATP sensors that excite and emit in the 400 nm to 500 nm spectral range, it is important to consider metabolism-dependent changes in autofluorescence background. For example, autofluorescence of flavins such as flavin adenine dinucleotide (FAD) have been long used to visualize metabolic changes in live samples and continues to be used as a “label-free imaging” option (Quinn *et al.*, 2013; Jahn *et al.*, 2015). This background could convolute fluorescent signals when using sensors at low expression levels, but at moderate sensor expression this is less likely because the lower extinction coefficient ($\sim 10,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$) and fluorescence quantum yield (< 0.01 to 0.06) of FAD (Valle *et al.*, 2012). In order to mitigate problems with spectral overlap with FAD autofluorescence and to generate spectral diversity for multi-sensor imaging, Nakano *et al.* (2011) replaced the CFP and YFP in the original ATeam sensors with a different FRET pair. They used GFP (cp173-mEGFP) and OFP (mKOK) to generate a red shifted ATeam named GO-ATeam. This allowed them to image Ca^{2+} level changes, using Ca^{2+} sensors that are excited with UV illumination, simultaneously along with ATP levels in HeLa cells. GO-ATeam is also more stable to acidification, an important advantage because metabolic stress can cause a drop in intracellular pH (Nakano *et al.*, 2011). Subsequently, Rueda *et al.* used GO-ATeam to image ATP depletion in presence and absence of Ca^{2+} upon NMDA exposure in neurons (Rueda *et al.*, 2015).

Zadran *et al.* developed an ATP sensor using a particular type of fluorescent protein FRET pair that can exhibit enhanced acceptor fluorescence. In their approach, a mutated variant of the ϵ subunit of *B. subtilis* F_0F_1 -ATP synthase is coupled to GFP and YFP. Both GFP and YFP are excited at the same wavelength, and ATP binding results in enhanced YFP acceptor fluorescence signal as a result of increased FRET. Zadran’s sensor was shown to detect as low as 10 nM ATP with high specificity (Zadran *et al.*, 2013), and subsequently, this sensor was used to monitor ATP flux in tumors and during transition to metastatic behavior (Zadran *et al.*, 2014).

Yaginuma *et al.* recently developed the QUEEN family of sensors, which are related to the ATeam sensors, but with a different architecture that uses a circularly permuted fluorescent protein (cpFP). In the QUEEN architecture, a cpFP is inserted between 2 α helices of the F_0F_1 ATP synthase ϵ subunit with linkers. Two variants were designed with apparent affinities of 7 μM and 2 mM, called QUEEN-7mu and QUEEN-2m, respectively (Yaginuma *et al.*, 2014). Using these sensors, they were able to quantify ATP distribution in individual *E. coli* cells.

Using a circular permutation strategy, Tantama *et al.* (2013) reported an ATP-to-ADP ratio sensor, PercevalHR, which is an improved version of the original Perceval (Berg *et al.*, 2009). The PII family protein GlnK1, which can bind MgATP and ADP with high affinity, was modified with a cpFP insertion within a loop at the nucleotide binding site. MgATP binding causes a conformational change that alters the chromophore environment of the cpFP. As a result of the protein engineering, the sensor has two peaks in its excitation spectra at approximately 420 nm and 500 nm. MgATP binding increases the fluorescence intensity with 500 nm excitation while ADP binding increases the fluorescence intensity with 420 nm excitation. These nucleotide-dependent spectral features enable excitation ratiometric imaging, which offers the significant advantage of concentration independence that normalize the signal for protein expression and reduces artifacts from photobleaching. The fast association and dissociation rates as well as the high dynamic range of the sensor at physiologically relevant ATP-to-ADP ratio ranges make PercevalHR useful for studying energy metabolism in cells (Tantama *et al.*, 2013). Although the Perceval sensors exhibit some pH sensitivity, the ATP response can be deconvoluted from changes in sample pH using a pH sensor and experimental calibration (Tantama *et al.*, 2011; Tantama and Yellen, 2014). Using Perceval to measure ATP-to-ADP ratios in neurons, Zala *et al.* showed that mitochondrial trafficking is dependent on mitochondrial ATP, but not glycolysis (Zala *et al.*, 2013). Using PercevalHR, Rueda *et al.* (2015) studied NMDA stimulation-dependent decreases in ATP-to-ADP ratio in neurons deficient in the SCA_{MC}-3 mitochondrial calcium-dependent MgATP-phosphate exchanger.

Finally, luciferase and fluorescent protein technologies have been combined in ATP sensors that exploit bioluminescence resonance energy transfer (BRET). These BRET sensors have been developed to improve brightness and red-shifted, luciferase-based imaging (Chu *et al.*, 2016). Saito *et al.* developed an ATP sensor, Nano-lantern (ATP1) in which a split *Renilla* luciferase (Rluc8) is modified with the ϵ subunit of the F₀F₁-ATP synthase and Venus (Saito *et al.*, 2012). ATP binding results in complementation and reconstitution of the active Rluc8, which can efficiently transfer energy to Venus. It has an apparent ATP affinity of 0.3 mmol l⁻¹ and is useful for imaging in tissues with high autofluorescence, and conveniently, Rluc8 does not consume any ATP in its chemiluminescent reaction, simplifying the detection mechanism and interpretation. Using this sensor, Saito *et al.* (2012) were able to visualize ATP increases in the mesophyll of leaf cells after light irradiation. Borghei *et al.* used firefly luciferase to develop a red shifted ATP sensor. They generated a fusion protein with firefly luciferase (X5) and the fluorescent protein, mCherry. Although the low quantum yield of mCherry (0.22) results in a weak signal, they could detect an ATP dependent increase in emission at 600 nm by mCherry (Borghei and Hall, 2014). Presumably, replacement of mCherry with a brighter red fluorescent protein would improve signal strength. Importantly, Rangaraju *et al.* used a synaptically-targeted engineered luciferase-mCherry sensor (Syn-ATP) to achieve ratiometric measurements of activity-dependent ATP consumption and production, although the Syn-ATP ratiometric signal was not obtained through BRET (Rangaraju *et al.*, 2014).

Currently, there are a wide variety of ATP detection and imaging reagents that can be used to visualize both intracellular energy metabolism and extracellular purinergic signaling. The choice of technologies depends on the biological process under study, the specimen format,

and any limitation of the imaging instrumentation. While we have presented several examples, it is beyond the scope of this review to evaluate all detection and imaging technologies for ATP here, and other examples have been reviewed elsewhere (for example see Praetorius and Leipziger, 2009; Khlynsteva *et al.*, 2009).

DISCUSSION AND CONCLUSIONS

While a number of ATP imaging technologies currently exist, there are still gaps in our ability to fully probe ATP metabolism and signaling. Quantitative imaging could be improved by engineering sensors with (1) a variety of ATP affinity ranges, (2) faster ATP binding and response kinetics, (3) higher brightness and contrast ratios, (4) selectivity for ATP hydrolysis products, and (5) spectral color variation. Engineering sensors with different affinity ranges is important because ATP concentrations vary widely from nanomolar extracellular levels in some tissues to millimolar concentrations in cytosolic pools. Likewise, engineering sensors with faster kinetics is important to capture ATP concentration changes that may occur on the timescale of milliseconds, such as during the initial phases of vesicular ATP release. Improving the brightness of fluorescent sensors and the contrast ratio between unbound and ATP-bound states will improve signal-to-noise ratios, improve quantitation, and can improve spatial and temporal resolution in practice. Engineering sensors with selectivity for ATP hydrolysis products would expand the toolbox to include ADP, AMP, and adenosine as measurable analytes. Similarly, engineering sensors with spectral color variation will enable simultaneous imaging of multiple sensors, providing a means to directly correlate ATP with, for example Ca^{2+} signaling and kinase activities. Finally, engineering spectral variants with luminescence and fluorescence in the farred and infrared spectral ranges will ultimately enable ATP imaging to be achieved with specificity and subcellular resolution over subsecond to lifetime timescales in live animals.

With current technologies and future improvements, there is great potential to study ATP with a systems biology perspective of energy metabolism and purinergic signaling. Continued use of these methods to image activity-dependent bioenergetics will improve our mechanistic understanding of neurotransmission (Tantama *et al.*, 2013; Rangaraju *et al.*, 2014). Furthermore, bioenergetic deficits have been linked to a number of aging-related neurodegenerative diseases such as Huntington's and Parkinson's diseases, and use of imaging approaches will aid the study of neurodegenerative mechanisms (Surmeier *et al.*, 2012; Zala *et al.*, 2013; Rangaraju *et al.*, 2014). Across cell types, these technologies can probe metabolic and mitochondrial function at the single-cell level, which is becoming important for understanding diseases such as cancer in which links to metabolism are increasingly being found (Mayers and Vander Heiden, 2015). Ultimately, in both healthy and disease state biology, live-cell imaging is becoming an integral approach, and our growing ability to quantitatively visualize ATP dynamics will allow us to link phenomenology and mechanism.

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ABBREVIATIONS

BRET	bioluminescence resonance energy transfer
cpFP	circularly-permuted fluorescent protein
Cy	cyanine
DAMP	damage associated molecular pattern
deac	diethylaminocoumarin
EMCCD	electron multiplying charge coupled device
ENT	equilibrative nucleoside transporter
FMLP	N-formyl-Met-Leu-Phe peptide
FRET	Förster-type resonance energy transfer
mant	(2′/3′-O-(N-Methylanthraniloyl)
NMDA	N-methyl-D-aspartate
TIRF	total internal reflection fluorescence

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Figure 1.

Extracellular ATP. (A) Mechanical damage of the cell membrane can cause uncontrolled ATP release whereas ATP release from apoptotic or inflammatory cells is mediated by transmembrane channels. (B) Extracellular ATP and ADP act as signaling molecules by binding P2 receptors, which are classified into 2 families based on their structure. P2Y receptors are GPCRs whereas P2X receptors are nucleotide-gated ion channels. Activation of P2Rs is generally proinflammatory. Extracellular ATP and ADP are converted to adenosine by the action of the ectonucleotidases CD39 and CD73. Adenosine signaling can occur through four structurally related GPCRs and generally leads to decreased inflammation. Adenosine is cleared from the extracellular space by the equilibrative nucleoside transporters 1 and 2 (ENT1, ENT2).

Figure 2.

Tools for ATP Visualization. (A) Quinacrine is a fluorescent dye that can stain vesicular ATP. (B) Mant-ATP is a fluorescent analogue. (C) Nanoflare using an ATP aptamer attached to a gold nanoparticle. In the absence of ATP, a competitor strand that is conjugated to a fluorescent dye is bound to the aptamer, but fluorescence is quenched by proximity to the gold. When ATP binds, the competitor strand is released and fluorescence is de-quenched. (D) Luciferase chemiluminescence requires a luciferin substrate and ATP. (E) The ATeam sensors use the F_1F_0 ATP synthase ϵ subunit as an ATP binding domain attached to a FRET pair of fluorescent proteins. (F) The Perceval sensors use the PII family protein GlnK as an ATP binding domain attached to a circularly permuted fluorescent protein. For (A) – (D) the sensor or a substrate must be exogenously supplied and loaded into cells or tissue. For (E) – (F) the sensors are completely genetically-encoded.

Table 1

Examples of tools for adenosine triphosphate (ATP) visualization.

Technology	Detection Mechanisms	Imaging Parameters
Quinacrine ^a	Fluorescent dye that binds peptide-bound ATP found in intracellular granules; intensimetric *	Fluorescence $\lambda_{\text{ex}} = 420\text{--}488\text{ nm}$; $\lambda_{\text{em}} = 490\text{--}510\text{ nm}$
Mant-ATP ^b	Fluorescent ATP analogue that can be used as a tracer of ATP pools; intensimetric	Fluorescence $\lambda_{\text{ex}} = 356\text{ nm}$; $\lambda_{\text{em}} = 428\text{--}448\text{ nm}$
ATP Aptamer Nanoflare ^c	ATP binding to DNA aptamers bound to a gold nanoparticles causes release of Cy5 conjugated “reporter” strand whose fluorescence “turns-on” upon release; intensimetric	Fluorescence (Dye Dependent) Cy5: $\lambda_{\text{ex}} = 649\text{ nm}$; $\lambda_{\text{em}} = 666\text{ nm}$
Luciferase ^d	Chemiluminescent enzyme metabolizes its substrate luciferin using ATP, resulting in ATP-dependent luminescence; intensity-based signal	Chemiluminescence (substrate dependent) D-luciferin: $\lambda_{\text{em}} = 560\text{ nm}$
Syn-ATP ^e	Luciferase-mCherry bioluminescence resonance energy transfer (BRET) sensor targeted to neuronal synapses; both luminescence and red fluorescence; ratiometric	Chemiluminescence D-luciferin: $\lambda_{\text{em}} = 560\text{ nm}$ Fluorescence $\lambda_{\text{ex}} = 587\text{ nm}$; $\lambda_{\text{em}} = 610\text{ nm}$
ATeam ^f	ATP binding causes an increase in Förster resonance energy transfer (FRET) between a CFP and a YFP; ratiometric	CFP Donor Fluorescence $\lambda_{\text{ex}} = 435\text{ nm}$; $\lambda_{\text{em}} = 475\text{ nm}$ YFP Acceptor Fluorescence $\lambda_{\text{ex}} = 515\text{ nm}$; $\lambda_{\text{em}} = 527\text{ nm}$ FRET $\lambda_{\text{ex}} = 435\text{ nm}$; $\lambda_{\text{em}} = 527\text{ nm}$
QUEEN ^g	ATP binding causes a change in the excitation spectrum of a circularly-permuted green fluorescent protein (cpEGFP); ratiometric	A-Band Fluorescence $\lambda_{\text{ex}} = 400\text{ nm}$; $\lambda_{\text{em}} = 513\text{ nm}$ B-Band Fluorescence $\lambda_{\text{ex}} = 494\text{ nm}$; $\lambda_{\text{em}} = 513\text{ nm}$
PercevalHR ^h	ATP binding causes a change in the excitation spectrum of a circularly-permuted yellow fluorescent protein (cpVenus); ratiometric	A-Band Fluorescence $\lambda_{\text{ex}} = 420\text{ nm}$; $\lambda_{\text{em}} = 515\text{ nm}$ B-Band Fluorescence $\lambda_{\text{ex}} = 500\text{ nm}$; $\lambda_{\text{em}} = 515\text{ nm}$

* Intensimetric sensors exhibit a change in intensity without a drastic change in spectrum: for luminescence a single emission wavelength is monitored, and for fluorescence, a single excitation (λ_{ex}) wavelength and emission wavelength (λ_{em}) pair is monitored. Intensimetric signals depend not only on ATP but also can be affected by dye concentration, expression level, and photobleaching. Ratiometric signals obtain two readouts that are divided to provide a normalized response. Ratiometric signals are advantageous because they normalize for dye concentration and expression level and reduce signal drift from photobleaching. A-Band fluorescence: excitation of the protonated chromophore form of the fluorescent protein. B-Band fluorescence: excitation of the deprotonated chromophore form of the fluorescent protein; CFP, cyan fluorescent protein; Cy5, cyanine dye; YFP, yellow fluorescent protein.

^a (Akopova *et al.*, 2012);

^b (Ho *et al.*, 2015);

^c (Zheng *et al.*, 2009);

^d (Manfredi *et al.*, 2002);

^e (Rangaraju *et al.*, 2014);

^f (Imamura *et al.*, 2009);

^g (Yaginuma *et al.*, 2014);

^h (Tantama *et al.*, 2013)