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Live cell imaging of signaling and metabolic activities

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ABSTRACT

The interplay of metabolic and signaling processes is prerequisite for the functionality of cells. Any disturbances may have severe consequences, resulting in the development of diseases. However, the complex coordination of metabolism and signaling events makes it difficult to decipher the link between molecular irregularities and pathogenesis. An excellent way to provide more clarity is to see into the living cell and watch cellular processes in real-time, with the add-on of being able to manipulate certain processes. Live cell imaging enables us to do exactly that, with steadily improving spatial and temporal resolution. Modern genetically encoded fluorescent probes in combination with state-of-the-art high-resolution imaging devices have proven themselves as a valuable approach for monitoring, manipulating and ultimately understanding the interaction of cell metabolism and signaling. These probes also represent powerful tools for detecting biomarkers of disease, identifying new drug targets and elucidating drug actions at the cellular to the molecular level.

Contents

Abbreviations: ADP, adenosine-5′-diphosphate; Akt, protein kinase B; AMPK, AMP-activated protein kinase; APG, Asante potassium green; ATP, adenosine-5′-triphosphate; ATPase, adenosine triphosphatase; AXER, ATP/ADP exchanger in the ER membrane; Ca^{2+}, calcium ion; $[Ca^{2+}]$, calcium ion concentration; CAD, conditional aggregation domain; cAMP, cyclic adenosine monophosphate; CFP, cyan fluorescent protein; cGMP, cyclic guanosine monophosphate; Cl^-, chloride ion; COPII, coat protein complex II; cp, circularly permuted; cpFP, circularly permuted fluorescent protein; cpYFP, circularly permuted yellow fluorescent protein; CRAC, Calcium release-activated channel; DAAO, D-amino acid oxidase; eCFP, enhanced cyan fluorescent protein; eGFP, enhanced green fluorescent protein; EMRE, essential mitochondrial calcium uniporter regulator; ER, endoplasmic reticulum; FAD, Flavin adenine dinucleotide; Fe^{2+}, iron ion; FP, fluorescent protein; FRAP, fluorescence recovery after photobleaching; FRET, Förster resonance energy transfer; GECI, genetically encoded Ca^{2+} indicator; GEFP, genetically encoded fluorescent probe; GEPII, genetically encoded potassium ion indicator; GFP, green fluorescent protein; GTP, guanosine-5′-triphosphate; GPCR, G-protein coupled receptor; H_2O_2, hydrogen peroxide; H_3O^+, hydronium ion and; IMS, intermembrane space; IP_3, 1,4,5-inositol triphosphate; IP_3R, 1,4,5-inositol triphosphate receptor; K^+, potassium ion; K_d, dissociation constant; $[K^+]$, potassium ion concentration; $[K^+]_e$, exptracellular potassium ion concentration; LOV, light oxygen voltage; MAM, mitochondria associated membrane; MICU1/2, mitochondrial Ca^{2+} uptake 1 and 2; Mg^{2+}, magnesium ion; mTOR, mechanistic target of rapamycin; Na^+, sodium ion; NAD, nicotinamide adenine dinucleotide; NCKX, sodium ion/calcium ion/potassium ion exchanger; NCX, sodium ion/calcium ion exchanger; NO, nitric oxide; NOS, nitric oxide synthase; O_2, superoxide anion; OH^-, hydroxide ion; PBFI, potassium-binding benzofuran isophthalate; PEMT, phosphatidylethanolamine *N*-methyltransferase; pH_e, extracellular pH; pH_i, intracellular pH; PMCA, plasma membrane calcium ion adenosine triphosphatase; PO_4^{3-}, phosphate ion; PML, promyelocytic leukemia protein; PP2A, protein phosphatase 2A; PRMT1, protein arginine *n*-methyltransferase 1; QUEEN, quantitative evaluator of cellular energy; Rab, Ras-related in brain; RFP, red fluorescent protein; RNS, reactive nitrogen species; ROC, receptor operated calcium ion channel; ROS, reactive oxygen species; RUSH, retention using selective hooks; SBP, steptavidin-binding peptide; SERCA, sarco−/endoplasmic reticulum calcium adenosine triphosphatase; Sirt1, sirtuin 1; SNARE, soluble N-ethylmaleimide-sensitive-factor attachment receptor; SOCE, store operated calcium entry; SR, sarcoplasmic reticulum; Tat, transactivator protein; TCA cycle, tricarboxylic acid cycle; TRIC, trimeric intracellular potassium ion channel; TRPC, transient receptor potential channel; UVR8, ultraviolet-B resistance 8; V-ATPase, vacuolar proton adenosine triphosphatase; VGCC, voltage gated calcium ion channel; VSVG, vesicular stomatitis virus glycoprotein; YFP, yellow fluorescent protein.

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1. Introduction

Here, we review a selection of well-established and novel genetically encoded fluorescent probes (GEFPs) for ions, metabolites, energy equivalents, redox cofactors, reactive oxygen species (ROS) and reactive nitrogen species (RNS), kinase activities, second messenger molecules, and protein trafficking, which allow the high-resolution investigation of signaling and metabolism and, hence, represent highly valuable innovative research tools.

As scientists, we aim at understanding life on the cellular and molecular level. While we comprehend the basic principles of life, we still need to decipher the underlying molecular mechanisms, and their multidirectional orchestration via regulatory processes (Cohen & Harel, 2007; Yewdall, Mason, & van Hest, 2018). Any disturbances on the molecular level can have severe consequences for the cell and the whole organism, and ultimately cause disease (Naylor & Chen, 2010). Thus, cell biological research makes a vital contribution to modern medicine (Asgari, Zabihinpour, Salehzadeh-Yazdi, Schreiber, & Masoudi-Nejad, 2015; Khan, Govindaraj, Meena, & Thangaraj, 2015; Walkley, 1998). Seemingly small changes of e.g. enzymatic activities, protein-protein interactions or ion channel gating may have severe consequences for the cell, and, hence, cause, promote and sustain diseases (Asgari et al., 2015; Onagi et al., 2017). On the other hand, one may be able to provide a remedy by modulating the causative molecular processes, as it has been done for decades by administering drugs, even though their mode of action has often remained poorly understood (Sonner & Cantor, 2013; Zhou, Wang, Lu, & Zhang, 2016). Thus, it is essential to gain mechanistic insights into both the variability and complexity of pathophysiological processes and drug actions on the molecular level to develop novel effective strategies for diagnosis and targeted therapy (Gonzalez-Angulo, Hennessy, & Mills, 2010; Naylor & Chen, 2010; Savoia et al., 2017).

Cells communicate with and respond to their environment via different modes of signaling processes (Brooks, Turkarslan, Beer, Yin Lo, & Baliga, 2011). Information transduction may include the spatially and temporally restricted release and uptake of stable ions (Hansen, Boitano, Dirksen, & Sanderson, 1993; Scemes & Spray, 2012), the generation and degradation of unstable reactive ROS and RNS (Zhang et al., 2016), or protein interactions and modifications (Theillet et al., 2012; Westermarck, Ivaska, & Corthals, 2013). Notably, these molecular processes necessitate a continuous supply of energy which is mainly provided in the form of adenosine-5′-triphosphate (ATP) and guanosine-5′-triphosphate (GTP) (Harris, Jolivet, & Attwell, 2012; Ruiz-Stewart et al., 2004). Many coordinated biochemical reactions, which constitute the cellular metabolism, provide energy equivalents, redox cofactors, biosynthetic intermediates and precursors for posttranslational modifications (DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008; Knorre, Kudryashova, & Godovikova, 2009; Tsuchiya et al., 2010). Both cell signaling events and metabolism are highly interrelated, meaning that different signaling events control cellular metabolism and vice versa (Fig. 1) (Görlach, Bertram, Hudecova, & Krizanova, 2015; Griesser et al., 2017; Rajtik et al., 2017). In line with this assumption, many human diseases, including cancer, cardiovascular diseases, metabolic disorders, and neurodegeneration, seem to be causally linked to both pathophysiological alterations of cellular signaling and metabolism (Asgari et al., 2015; Bezprozvanny, 2010; Costantino, Paneni, & Cosentino, 2016; Silberman, 2018). It is, however, challenging to investigate and comprehensively understand these relations and the underlying malfunctions on the subcellular and molecular level. In this regard, live cell imaging of intracellular molecules using fluorescent protein (FP)-based sensors has the potential to give answers by resolving cellular processes in high temporal and spatial detail (Bischof et al., 2017; Depaoli, Hay, Graier, & Malli, 2018; Eroglu et al., 2017; Vishnu et al., 2014; Waldeck-Weiermair et al., 2012).

Without any doubt, one of the most significant technical developments in cell biology started with the isolation of the green fluorescent protein (GFP) in the early 60s (Shimomura, 2005; Shimomura, Johnson, & Saiga, 1962). Around 30 years later GFP made its breakthrough as a tool for molecular biology, after the successful cloning of the GFP nucleotide sequence, and the expression of the fluorescing recombinant protein in *Escherichia coli* and *Caenorhabditis elegans* (Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994; Inouye & Tsuji, 1994; Prasher, Eckenrode, Ward, Prendergast, & Cormier, 1992). Since then the fluorescent properties of GFP have been improved, and numerous differently colored FPs have been introduced allowing diverse applications (Rodriguez et al., 2017; Shaner, Steinbach, & Tsien, 2005). By using specially designed FP constructs, we can now visualize and monitor cellular processes, such as gene expression (Chalfie et al., 1994), protein translocation (Deponte, 2012), protein-protein interactions (Wilson, Magliery, & Regan, 2004), protein trafficking (Kim, Yuan, Cilia, Khalfan-Jagani, & Jackson, 2002), or organelle dynamics (Westermann & Neupert, 2000).

Moreover, the scope of possible applications of the FP technology has been expanded by a wide range of genetically encoded fluorescent probes (GEFPs). GEFPs usually consist of one (Fig. 2a) or more FPs (Fig. 2b,c) fused to specific selective domains that either bind an analyte of interest (Fig. 2a,b) or are chemically modified by a signaling process (Fig. 2c) (Bischof et al., 2017; Eroglu et al., 2016; Tsou, Zheng, Hsu, Sasaki, & Cantley, 2011; Zhao, Araki et al., 2011; Zhou et al., 2015). Binding of the analyte (Fig. 2a,b) or modifications (Fig. 2c) affect the spectral properties of the FPs and, hence, provide a quantifiable read-out of distinct cell signaling events and metabolic processes (Bischof et al., 2017; Eroglu et al., 2016; Palmer & Tsien, 2006; Tsou et al., 2011). The protein-based GEFPs are introduced into the cell via transient or stable transfection, or transduction of the DNA encoding the desired GEFP (Fig. 3). By adding particular localization sequences to the GEFP gene sequence, the probes can be specifically targeted to different cell parts to investigate local processes, e.g., within mitochondria (Akimzhanov & Boehning, 2011) or at the plasma membrane (Fig. 4) (Simonova, Weissleder, Sergeyev, Vilissova, & Bogdanov, 1999). Most widely used GEFPs are single FP (Fig. 2a) and Förster resonance energy transfer (FRET)-based sensors (Fig. 2b,c). Single FP-based biosensors have the intrinsic capability to change their fluorescent properties depending on the concentration of an analyte, such as in the case of many pH probes (Poburko, Santo-Domingo, & Demaurex, 2011; Tantama, Hung, & Yellen, 2011), or may be fused to a sensor domain, as in the case of, e.g. Ca^{2+} or ATP probes (Yaginuma et al., 2015; Zhao, Araki, et al.,

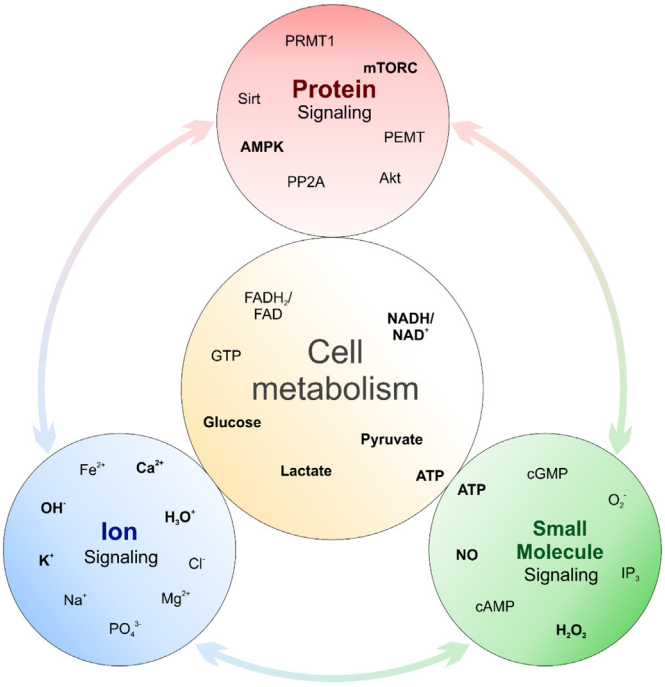

Fig. 1. Schematic demonstration of the crosstalk between cell metabolism, protein-, ion- and small molecule signaling.

2011). FRET-based sensors, on the other side, are composed of a so-called FRET pair of two spectrally overlapping fluorescent proteins and a sensor domain, commonly fused in between the two FPs (Fig. 2b,c) (Marx, 2017). Depending on the proximity of the FPs, energy is transferred from the excited donor (e.g. cyan fluorescent protein (CFP)) to the acceptor FP (e.g. yellow fluorescent protein (YFP)) via FRET (Marx, 2017). Binding of the analyte affects the conformation of the probe, which in turn affects the distance between the FPs, resulting in increased or decreased FRET (Marx, 2017). The FRET principle has proven versatile in combination with numerous possible sensor domains provided by nature (Bischof et al., 2017; Imamura et al., 2009; San Martín et al., 2013; Tsou et al., 2011; Zhou et al., 2015). Moreover, FRET sensors have the significant advantage of being ratiometric, meaning that the read-out of these probes is the ratio of two intensities (Spiering, Bravo-Cordero, Moshfegh, Miskolci, & Hodgson, 2013). One of these intensities increases upon analyte binding while the other one decreases (Fig. 2). This principle allows quantification of the analyte because the ratio is independent of the sensor's expression levels. It also facilitates the detection and correction of measurement artifacts due to bleaching, changes of the focus, and other confounding factors that may affect the fluorescence intensity of a sensor. On the other hand, many single FP-based probes are only intensiometric, which means they can solely measure relative changes because they have only a single fluorescence intensity as the read-out, which is, however, also largely dependent on the number of sensors within a cell. Nevertheless, single FP-based probes surpass FRET sensors in certain aspects, as they are smaller and have a narrower spectral bandwidth, which is advantageous for multispectral imaging with more than one fluorescent sensor (Eroglu et al., 2016; Zhao, Araki, et al., 2011).

The broad pallet of GEFPs for diverse molecules and events can be further expanded by exchanging FPs and/or inserting site-directed or random mutations in the sensor domains to modulate ligand affinity (Eroglu et al., 2016; McCombs & Palmer, 2008; Waldeck-Weiermair et al., 2015; Wu et al., 2013; Zhao, Araki, et al., 2011). The availability of multicolored sensor variants facilitates the combination of different sensors for simultaneous measurements of more than one parameter in a single cell, e.g. Ca^{2+} and pH. A variety of ligand affinities allows choosing appropriate sensors for different ligand concentrations which can be subject to considerable variations and fluctuations.

Despite all these advantages, some important aspects must be considered when using GEFPs and live cell imaging, as described in detail in other reviews (Ettinger & Wittmann, 2014; Specht, Braselmann, & Palmer, 2017). In the context of this review, we want to point out that light itself can affect cellular metabolism (Robertson, Davis, & Johnson, 2013). Hence, it is particularly important to avoid phototoxicity during live fluorescence microscopy (Icha, Weber, Waters, & Norden, 2017; Laissue, Alghamdi, Tomancak, Reynaud, & Shroff, 2017).

2. Cellular Ca^{2+} signals

2.1. Genetically encoded Ca^{2+} indicators (GECIs)

As second messenger, Ca^{2+} is involved in various signaling pathways (Clapham, 2007). The enormous diversity and importance of Ca^{2+} signaling events prompted scientists to develop a wide range of sensors for imaging and quantifying subcellular Ca^{2+} concentrations (Pérez Koldenkova & Nagai, 2013). Both chemical and genetically encoded Ca^{2+} biosensors have been improved continuously, and are available in many colors covering almost the entire spectrum of visible light (Grynkiewicz, Poenie, & Tsien, 1985; Palmer & Tsien, 2006; Waldeck-Weiermair et al., 2015;Wu et al., 2013; Zhao, Araki, et al., 2011). Already before the introduction of Ca^{2+}-sensitive GEFPs, chemical dyes (e.g., Fura, Fluo, Rhod-2, Mag-fura-2) have been used to measure Ca^{2+} within the cytosol, mitochondria, and endoplasmic reticulum (ER) (Jean-Quartier et al., 2012; Verkhratsky & Petersen, 2010). However, chemical Ca^{2+} probes bear a series of disadvantages; mistargeting, toxicity, inhibition of intracellular pathways, and affecting cell functions are common issues with chemical dyes (Smith et al., 2018). Accordingly, genetically encoded Ca^{2+} indicators (GECIs) have become the method of choice for imaging subcellular Ca^{2+} signals (Pérez Koldenkova & Nagai, 2013; Rodriguez-Garcia et al., 2014; Whitaker, 2010). Due to their narrow fluorescence spectra, different GECIs can be combined for simultaneous and multiparametric imaging (Wu et al., 2013; Zhao, Araki, et al., 2011). Genetic engineering provides the opportunity to create sensors with refined features and, particularly, allows subcellular targeting upon fusion with organelle-specific signal peptides (Pendin, Greotti, Lefkimmiatis, & Pozzan, 2017). Strategies including random or directed protein evolution have been used to optimize their Ca^{2+} binding affinity for distinct subcellular compartments (Palmer & Tsien, 2006; Wu et al., 2013; Zhao, Araki, et al., 2011). However, some of the intensiometric GECIs, such as the popular GCamP6, have been improved over decades to yield ultrasensitive and fast responding Ca^{2+} probes that are suitable to record fast (sub)cellular Ca^{2+} transients e.g. during action potentials in neurons in vivo (Chen, Wardill, et al., 2013). Recently, a new variant of GCamP, referred to as GCamP-X, was developed to circumvent some side-effects, which are induced upon the expression of older CGamP versions particularly in excitable cells (Yang et al., 2018).

Based on their operating principle, GECIs fall into three main groups: First, single FP intensiometric GECIs (Nakai, Ohkura, & Imoto, 2001; Wu et al., 2013; Zhao, Araki, et al., 2011), secondly, single FP ratiometric GECIs (Nagai, Sawano, Park, & Miyawaki, 2001; Zhao, Araki, et al., 2011), and thirdly, FRET-based GECIs (Palmer & Tsien, 2006; Waldeck-Weiermair et al., 2015).

Single FP intensiometric GECIs consist of a single FP which is usually a circularly permuted FP (cpFP) (Nakai et al., 2001; Wu et al., 2013; Zhao, Araki, et al., 2011). These sensors show little fluorescence at low [Ca^{2+}], but their fluorescence increases upon Ca^{2+} binding with a high dynamic range (Nakai et al., 2001; Wu et al., 2013; Zhao, Araki, et al., 2011). Since these kinds of Ca^{2+} biosensors emit light with only one band of wavelengths, they are suitable for combined recordings with other fluorescent indicators, thus allowing to visualize the different kinetics in various subcellular regions at once in the same cell (Nakai

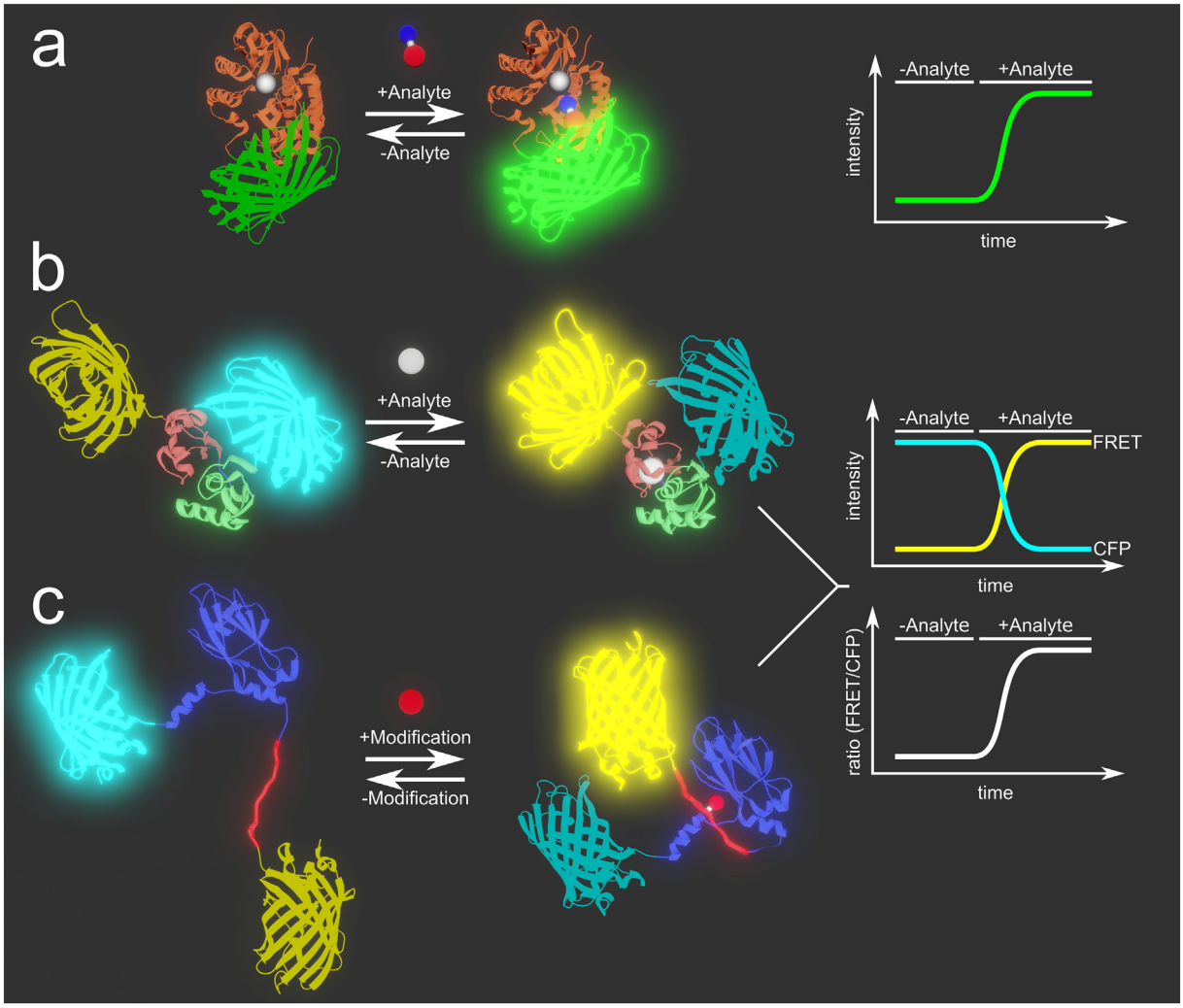

Fig. 2. Schematic illustration of the sensing principles of genetically encoded fluorescent probes (GEFPs).

et al., 2001; Wu et al., 2013; Zhao, Araki, et al., 2011). Because of their non-ratiometric nature, intensiometric GECIs are less suitable for quantifying [Ca^{2+}] under resting conditions (Nakai et al., 2001; Wu et al., 2013; Zhao, Araki, et al., 2011).

Single FP ratiometric GECIs also consist of just one cpFP and can be classified into two groups, emission ratio sensors on the one hand, and excitation ratio sensors on the other hand (Nagai et al., 2001; Wu et al., 2013; Zhao, Araki, et al., 2011). In the case of the former group, excitation with a distinct wavelength requires the collection of the emission at two different wavelengths (Zhao, Araki, et al., 2011). In the case of the latter, excitation with two distinct wavelengths requires the collection of emission light at only one wavelength (Nagai et al., 2001; Wu et al., 2013). Major advantages of these probes are their ratiometric read-out which allows the analysis of basal Ca^{2+} levels, and their high dynamic range upon Ca^{2+} mobilization (Nagai et al., 2001; Wu et al., 2013; Zhao, Araki, et al., 2011). The calibration of ratiometric sensors even allows determining absolute Ca^{2+} concentrations within a cell, which was very important in assessing physiological Ca^{2+} concentrations within the cell and cellular compartments, in particular the ER and mitochondria (McCombs & Palmer, 2008). For more details on the calibration method, we recommend reading the reference literature (McCombs & Palmer, 2008). Another important group of ratiometric Ca^{2+} probes includes FRET-based GECIs which consist of two FPs, a FRET donor and a FRET acceptor (McCombs & Palmer, 2008; Palmer & Tsien, 2006; Waldeck-Weiermair et al., 2015). Collection of the emission at two distinct wavelengths requires excitation of the FRET donor (McCombs & Palmer, 2008; Palmer & Tsien, 2006; Waldeck-Weiermair et al., 2015). Because donor and acceptor FPs are needed for this kind of biosensors, they occupy a broad range of the visible spectrum, rendering the combination with other sensors difficult. Commonly used FRET pairs are based on CFP/YFP or green fluorescent protein (GFP)/red fluorescent protein (RFP) (Palmer & Tsien, 2006; Waldeck-Weiermair et al., 2015).

Notably, the suitability of GECIs for Ca^{2+} imaging in distinct subcellular locales strongly depends on the microenvironment (McCombs & Palmer, 2008). Thus specific GECIs have been developed for the ER, acidic organelles like the Golgi apparatus and endosomes (Suzuki, Kanemaru, & Iino, 2016). We present a selection of state-of-the-art GECIs in Table 1.

2.2. Ca^{2+} signals and metabolism

Cellular Ca^{2+} signaling is fundamentally based on Ca^{2+} gradients that are established and maintained by energy-dependent Ca^{2+} pumps (Papp et al., 2012; Strehler & Treiman, 2004). The continuously working ATPases, the plasma membrane Ca^{2+} ATPase (PMCA) and the sarco—/endoplasmic reticulum calcium ATPase (SERCA), ensure cytosolic Ca^{2+} clearance by pumping Ca^{2+} either to the extracellular space or intracellular Ca^{2+} stores, respectively (Periasamy & Kalyanasundaram, 2007; Thomas, 2009). In addition, the Na^{+}/Ca^{2+}

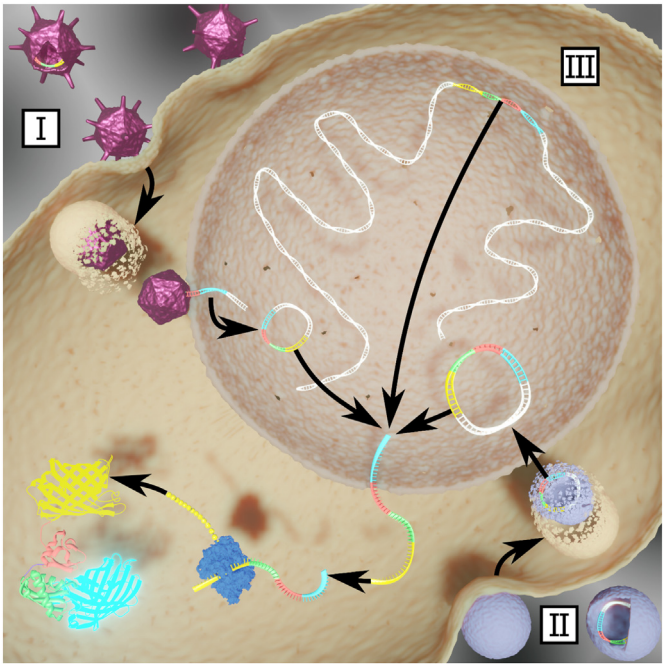

Fig. 3. Representation of different experimental approaches for achieving GEFP expression in a cell of interest.

exchanger (NCX) and the $Na^+/Ca^{2+}/K^+$ exchanger (NCKX) facilitate Ca^{2+} extrusion by exchanging one Ca^{2+} for three Na^+ or cotransport of one Ca^{2+} and one K^+ for four Na^+ (Altimimi & Schnetkamp, 2007; Clapham, 2007; Jeffs, Meloni, Bakker, & Knuckey, 2007). Depending on the cell type, extracellular Ca^{2+} enters the cell via voltage-gated Ca^{2+} channels (VGCCs) (Catterall, 2000), receptor-operated Ca^{2+} channels (ROCs) (McFadzean & Gibson, 2002), transient receptor potential channels (TRPC) (Vazquez, Wedel, Aziz, Trebak, & Putney, 2004), or the Ca^{2+} release-activated channel (CRAC) (Berridge, Lipp, & Bootman, 2000; Prakriya, 2009). Ca^{2+} release from intracellular Ca^{2+} stores is often mediated by G-protein coupled receptors (GPCR) upon binding of a 1,4,5-inositol triphosphate (IP_3) generating agonist that in turn activates the formation of IP_3 and opening of ER located IP_3 receptor (IP_3R) (Mikoshiba, 2007). ER Ca^{2+} release is coupled to the activation of store-operated Ca^{2+} entry (SOCE) (Groschner, Graier, & Romanin, 2017; Malli & Graier, 2017).

Ca^{2+} mobilization affects hundreds of Ca^{2+} binding proteins with different Ca^{2+} affinities, which trigger distinct pathways of Ca^{2+} signaling (Niki, Yokokura, Sudo, Kato, & Hidaka, 1996). Ca^{2+} binding domains such as EF-hand (Lewit-Bentley & Réty, 2000), annexin (Liemann & Lewit-Bentley, 1995), and C2 domains (Nalefski & Falke, 1996) transduce Ca^{2+} signals to protein signaling (Bagur & Hajnóczky, 2017; Clapham, 2007). This sophisticated network of cell type-specific Ca^{2+} signaling events and Ca^{2+} dependent protein composition allows Ca^{2+} to act either globally or locally enabling different cellular processes (Berridge et al., 2000). In this context, Ca^{2+} is a strong regulator of cell metabolism (Williams, Boyman, & Lederer, 2015). Particularly the uptake of Ca^{2+} by mitochondria stimulates the mitochondrial metabolic activity (Williams et al., 2015). Close associations between the ER and the mitochondria, referred to as mitochondria-associated ER membranes (MAMs) (Herrera-Cruz & Simmen, 2017), are necessary for an efficient Ca^{2+} transfer to mitochondria (Rizzuto et al., 2009). Ca^{2+} uptake by mitochondria is accomplished by a regulated protein complex composed of the mitochondrial Ca^{2+} uniporter (MCU) protein (De Stefani, Raffaello, Teardo, Szabò, & Rizzuto, 2011), the essential MCU regulator (EMRE) (Sancak et al., 2013) and mitochondrial Ca^{2+} uptake 1 and 2 (MICU 1/2) (Matesanz-Isabel et al., 2016). Within the mitochondrial matrix, Ca^{2+} stimulates dehydrogenases, such as the pyruvate dehydrogenase, the nicotinamide adenine dinucleotide (NAD)-isocitrate dehydrogenase and the oxoglutarate dehydrogenase (McCormack, Halestrap, & Denton, 1990). Consequently Ca^{2+} within mitochondria boosts ATP production (Denton, 2009; Glancy & Balaban, 2012). However, excess of mitochondrial Ca^{2+} uptake is known to trigger cell death pathways (Giorgi et al., 2012).

3. Cellular K^+ homeostasis and signaling

Both intra- and extracellular potassium ions (K^+) regulate many physiological processes (Palmer, 2015; Palmer & Clegg, 2016). Extracellular K^+ is vital for controlling fluid and electrolyte balance (Terry, 1994). Intracellular K^+ is an indispensable co-factor of many proteins and enzymes (Page & Di Cera, 2006); as free ion, it is required for controlling the cellular membrane potential (Brodie, Bak, Shainberg, & Sampson, 1987) and, hence, the generation of action potentials in excitable cells (Fletcher, 2011). In addition, intracellular K^+ affects organellar membrane potentials (Maedeh Ghasemi, Khodaei, Salari, Eliassi, & Saghiri, 1996; Szewczyk, Jarmuszkiewicz, & Kunz, 2009) and cellular ion homeostasis (Palmer, 2015), which in turn influences intracellular Ca^{2+} signaling (Franchini, Levi, & Visentin, 2004). Furthermore, K^+ itself may act as a signaling ion, a role which has long been underestimated (Eil et al., 2016; Hughes & Cidlowski, 1999).

3.1. Imaging of intracellular K^+ dynamics

The development of several fluorescent K^+ indicators has paved the way for new findings regarding the role of K^+ in physiology and pathophysiology (Depauw et al., 2016; Ding et al., 2017; Jezek, Mahdi, & Garlid, 1990; Kong et al., 2015; Song, Sun, Du, Chen, & Tian, 2017). Similar to Ca^{2+} sensitive chemical dyes, chemical indicators of K^+ have been developed and applied in single living cells for the visualization of K^+ dynamics in real-time (Jezek et al., 1990; Kong et al., 2015; Yaron et al., 2015; Zhou et al., 2011). These probes are mostly based on crown ethers which have been chemically modified to selectively bind and indicate K^+ (Depauw et al., 2016; Jezek et al., 1990; Kong et al., 2015; Song et al., 2017). Nonetheless, these chemical K^+ indicators feature severe disadvantages as compared with GEFPs, most

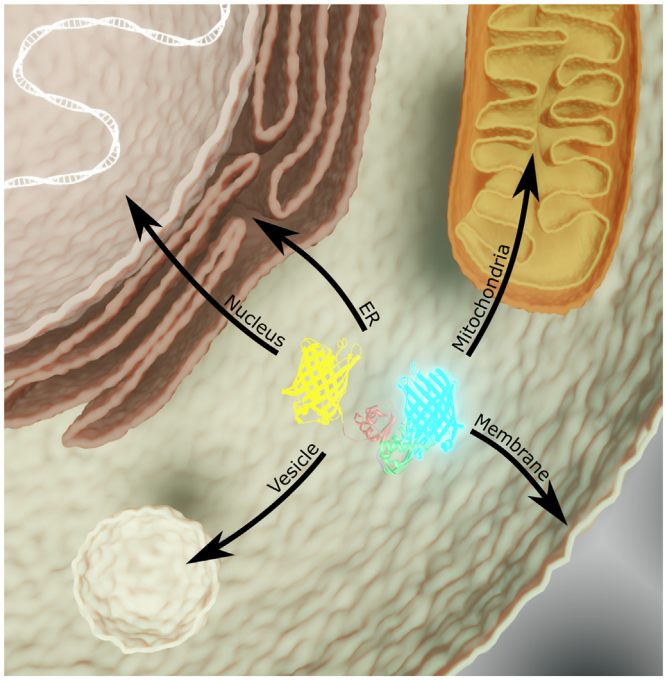

Fig. 4. Schematic representation of different targeting options for GEFPs.

Table 1
Selection of GECIs suitable for subcellular Ca^{2+} imaging.

GECI	Localization	Type	k_D	Dynamic range	λ_{Exc} (nm)	λ_{Em} (nm)	Reference
CAR-GECO1	Cytosol	Intensiometric	490 nM	–	565	620	(Wu et al., 2013)
GEM-GECO1		Ratiometric	340 nM	110	397	455/511	(Zhao, Araki, et al., 2011)
D3cpV		FRET-based ratiometric	600 nM	5.1	430	480/535	(Palmer et al., 2006)
G-GECO1.2-Orai1	Subplasma- membrane	Intensiometric	1.15 μM	23	498	513	(Dynes, Amcheslavsky, & Cahalan, 2016)
LynD3cpV		FRET-based ratiometric	600 nM	5.1	430	480/535	(Palmer et al., 2006)
Cav2.2-TN-XL		FRET-based ratiometric	2.5 μM	1.7	430	480/535	(Tay et al., 2012)
NLS-GCamp2	Nucleus	Intensiometric	146 nM	3.8	487	508	(Bengtson, Freitag, Weislogel, & Bading, 2010)
nucGAP1		Ratiometric	200 nM	–	405/470	510	(Rodriguez-Garcia et al., 2014)
H2BD1cpV		FRET-based ratiometric	–	–	430	480/535	(Giacomello et al., 2010)
mtCAR-GECO1	Mitochondria	Intensiometric	490 nM	27	565	620	(Wu et al., 2013)
mtCamgaroo-2		Intensiometric	5.3 μM	7	480	535	(Griesbeck, Baird, Campbell, Zacharias, & Tsien, 2001)
mito-Case12		Intensiometric	1 μM	12	491	516	(Chang, Niescier, & Min, 2011)
mtGEM-GECO1		Ratiometric	340 nM	110	397	455/511	(Zhao, Araki, et al., 2011)
4mtD3cpV		FRET-based ratiometric	600 nM	5.1	430	480/535	(Palmer et al., 2006)
4mtD1GO-Cam		FRET-based ratiometric	1.53 μM	–	477	510/560	(Waldeck-Weiermair et al., 2012)
OMM-pcmD2-ER	Outer mitochondrialmembrane	Ratiometric	3.14 μM	–	415/494	517	(Csordás et al., 2010)
N33D1cpV		FRET-based ratiometric	–	–	430	480/535	(Giacomello et al., 2010)
GCaMPer	Endoplasmic Reticulum	Intensiometric	400 μM	14			(Henderson et al., 2015)
CatchER		Intensiometric	180 μM	2.3	395/488	510	(Tang et al., 2011)
ER-LAR-GECO1		Intensiometric	24 μM	1.3	561	589	(Wu et al., 2014)
R-CEPIA1er		Intensiometric	565 μM	8.8	562	584	(Suzuki et al., 2014)
Ca-G1-ER		Ratiometric	800 μM	1.8	398/490	510	(Zou et al., 2007)
erGAP3		Ratiometric	489 μM	3	405/470	510	(Navas-Navarro et al., 2016)
D1ER		FRET-based ratiometric	220 μM	–	430	480/535	(Palmer, Jin, Reed, & Tsien, 2004)
D1ERCmR2		FRET-based ratiometric	200 μM	–	480	510/560	(Waldeck-Weiermair et al., 2015)
apoK1-er		FRET-based ratiometric	124 μM	–	430	480/535	(Osibow, Malli, Kostner, & Graier, 2006)
goGAP1	Golgi apparatus	Ratiometric	–	–	405/470	510	(Rodriguez-Garcia et al., 2014)
medialGo-D1cpV		FRET-based ratiometric	27.4	–	430	480/535	(Wong et al., 2013)
GT-YC3.3		FRET-based ratiometric	1.5 μM	–	430	480/535	(Griesbeck et al., 2001)
TiVAMP-GEM-GECO1	Endosomes	Ratiometric	340 nM	110	397	455/511	(Albrecht, Zhao, Nguyen, Campbell, & Johnson, 2015)
D3cpV-KVK-SKL	Peroxisomes	FRET-based ratiometric	1 μM	–	430	480/535	(Drago, Giacomello, Pizzo, & Pozzan, 2008)
Caveolin-1-YC2.1	Caveolae	FRET-based ratiometric	321 nM	–	430	480/535	(Isshiki, Ying, Fujita, & Anderson, 2002)

notably they lack specificity for K^+ over Na^+ (Kong et al., 2015; Minta & Tsien, 1989).

We recently addressed the need for highly specific and sensitive genetically encoded K^+ probes by developing the first Genetically Encoded Potassium Ion Indicators (GEPIIs) (Bischof et al., 2017). Following that, the same K^+-sensing principle was used by others to come up with a red-shifted GEPII and a single FP-based K^+ sensor (Shen et al., 2019). GEPIIs are FRET-based genetically encoded probes suitable for the visualization of cellular K^+ dynamics in living cells (Bischof et al., 2017; Shen et al., 2019). Targeted GEPII variants demonstrated different K^+ concentrations ($[K^+]$) in cellular compartments (Bischof et al., 2017). Interestingly, this study unveiled high $[K^+]$ within the nucleus of different cell types, while $[K^+]$ within mitochondria was found to be lower than in the cytosol (Bischof et al., 2017). Using GEPIIs in combination with a genetically encoded Ca^{2+} indicator (CAR-GECO1) in the pancreatic beta cell line INS-1832/13 allowed the simultaneous visualization of (sub-) cellular Ca^{2+} and K^+ transients upon cell depolarization (Bischof et al., 2017).

In the future, these probes will represent a valuable tool for visualizing cell-specific K^+ dynamics in variable cell types, especially neurons and (cardiac) myocytes (Bischof et al., 2017; Shen et al., 2019). Many open questions regarding the intracellular K^+ distribution and fluctuations can now be addressed using organelle-targeted GEPIIs, for instance regarding the functions of specific K^+ channels that have been found in the inner mitochondrial membrane (Szewczyk et al., 2009; Szewczyk, Olszewska, Augustynek, Laskowski, & Bednaarczyk, 2014). Their involvement in fundamental physiological processes such as ATP generation (Garlid, Dos Santos, Xie, Costa, & Paucek, 2003), apoptosis (Checchetto, Azzolini, Peruzzo, Capitanio, & Leanza, 2018), maintenance of the mitochondrial membrane potential (Garlid & Paucek, 2003; Laskowski et al., 2016) and interorganellar communication (Dakik & Titorenko, 2016) is hypothesized. GEPIIs could now provide more clarity in this regard. Moreover, organelle-targeted GEPIIs, also in combination with patch-clamp experiments, may allow monitoring subcellular K^+ fluxes in response to cell depolarization, which may increase our understanding of organelle membrane ion permeability. K^+ sensitive indicators targeted to the lumen of the ER also seem particularly attractive to examine the so-called trimeric intracellular K^+ channels (TRIC), which are specific K^+ channels within the ER membrane (Pitt et al., 2010; Venturi et al., 2013; Yazawa et al., 2007). As the ER represents the major intracellular Ca^{2+} store, Ca^{2+} fluxes across the ER membrane drastically affect the ER membrane potential (Marhl, Schuster, Brumen, & Heinrich, 1997). TRIC are supposed to enable K^+ counter ion fluxes in order to maintain the ER resting potential (Baylor, Chandler, & Marshall, 1984; Coronado, Rosenberg, & Miller, 1980). Most of the results in this field were obtained using isolated sarcoplasmic reticulum (SR) or ER fractions and patch-clamp techniques (Guo et al., 2013; Pitt et al., 2010; Venturi et al., 2013). Genetically encoded K^+ indicators would represent a significant and promising alternative by enabling ER/SR K^+ measurements in living cells.

3.2. Imaging of extracellular K^+ dynamics

Many electrical and chemical probes have been designed to monitor and study extracellular K^+ concentrations ($[K^+]_e$) (Dror, Bergs, & Rhodes, 1987; Jezek et al., 1990; Octeau, Faas, Mody, & Khakh, 2018; Pioda, Simon, Bosshard, & Curtius, 1970). The dominating approach for the past 45 years has been the usage of microelectrodes, the fastest of which can measure changes in K^+ concentrations at a response time of 27 ms (Messerli, Collis, & Smith, 2009). However, they are restricted in spatial resolution due to the position of the tip of the electrode (Bond, 1994; Pioda et al., 1970). The utilization of microelectrode arrays can partly resolve that problem by increasing the number of microelectrodes in a defined space (Guenat et al., 2006).

Intriguingly, we recently showed that recombinant GEPIIs are functional and applicable for determining $[K^+]_e$ as well (Bischof et al., 2017). The kinetics of GEPIIs have not yet been determined conclusively to allow a comparison with the kinetics of K^+ detection by electrodes. GEPIIs might suffer from speed due to conformational changes that are essential for K^+ sensing. However, more research is needed to determine their actual response time using high-speed fluorescence detection devices. However, the fast on-kinetics of this method make GEPIIs particularly suitable for measuring $[K^+]_e$, as shown by their application in body fluids (urine, serum, and bile) in a high throughput fashion (Bischof et al., 2017). The high selectivity of GEPIIs for K^+ as compared to Na^+ outperforms most chemical sensors (Ashraf et al., 2016; Bischof et al., 2017; Jezek et al., 1990), which is particularly important in the extracellular environment, where the Na^+ concentration is up to 30 times higher than the concentration of K^+. GEPIIs have also been used for measuring $[K^+]_e$ in vivo after incubating the exposed striated muscle of a mouse with the recombinant sensor (Bischof et al., 2017). In vitro, GEPIIs allowed the detection of K^+ release from dying cells, even before any necrosis-specific morphological alterations were visible, thus, providing a GEPII-based cell death assay (Bischof et al., 2017). In future, GEPIIs might be employed for studying the role of K^+ fluxes in animal models, e.g. for the real-time detection of K^+ alterations in veins or activity-driven neuronal K^+ release in the brain.

In addition to GEPIIs, chemical probes like Asante potassium green (APG) (Rimmele & Chatton, 2014) have become available for measuring extracellular K^+ as they also provide a high spatial and temporal resolution. For instance, these sensors were used to detect K^+ changes in the extracellular space of the brain (Bazzigaluppi, Dufour, & Carlen, 2015; Padmawar, Yao, Bloch, Manley, & Verkman, 2005). However, their specificity for K^+ is questionable.

4. Cellular pH

4.1. FP-based pH probes

The maintenance of pH homeostasis is important for every cell as dysregulations are often associated with severe metabolic alterations (Moidunny et al., 2016; Scott, Gebhardt, Mitrovic, Vandenberg, & Dodd, 2011; Trotti et al., 2001). Several genetically encoded pH sensors have been developed to determine pH within different cell compartments (Esposito, Gralle, Dani, Lange, & Wouters, 2008; Mahon, 2011; Matlashov et al., 2015; Poburko et al., 2011; Tantama et al., 2011). These pH sensors consist of fluorescent proteins that show distinct pH sensitivity or stability (Table 2) (Esposito et al., 2008; Mahon, 2011; Matlashov et al., 2015; Poburko et al., 2011; Tantama et al., 2011). Genetically encoded pH sensors are available as single FP- (Mahon, 2011; Matlashov et al., 2015; Poburko et al., 2011; Tantama et al., 2011) or double FP-based sensors (Esposito et al., 2008). The first genetically encoded pH sensors, the so-called pHluorins, were developed in 1998 by Miesenböck et al. (Miesenböck, De Angelis, & Rothman, 1998). They generated two types of sensors, namely ratiometric and ecliptic pHluorins, which both consist of a pH-sensitive GFP variant with specific amino acid substitutions that allow the reversible (de-)protonation of the FP. These probes allowed to visualize and quantify secretory vesicle turnover which is associated with luminal pH changes (Miesenböck et al., 1998).

In 2006, Bizzarri et al. introduced another genetically encoded pH sensor called E^2GFP, providing a ratiometric readout (Bizzarri et al., 2006). E^2GFP was targeted to nucleoli by fusing the sensor with the transactivator protein (Tat) (Tat- E^2GFP) (Bizzarri et al., 2006). Coexpressing Tat- E^2GFP with the promyelocytic leukemia protein (PML) led to a translocation of the Tat- E^2GFP into the PML accumulation bodies (Bizzarri et al., 2006). Therefore this phenomenon could be exploited to determine the pH in PML bodies (Marcello, 2003; Marcello et al., 2001).

Table 2
Selection of genetically encoded probes for intracellular pH measurements.

pH-Sensor	Principle	FP(s)	pKa	λ_{Exc} (nm)	λ_{Em} (nm)	Reference
Ecliptic pHluorin	Intensiometric/ecliptic	GFP	7.1	470	510	(Miesenböck et al., 1998; Sankaranarayanan et al., 2000)
Superecliptic pHluorin						
(Ratiometric) pHluorin	Ratiometric	GFP	7.1	395/475	510	(Miesenböck et al., 1998)
(Ratiometric) pHluorin2				405/485	535	(Mahon, 2011)
mtAlpHi	Intensiometric	GFP	8.5	498	522	(Abad, Di Benedetto, Magalhães, Filippin, & Pozzan, 2004)
E^2GFP	Ratiometric	GFP	6.9/7.5	458/488	500/560	(Bizzarri et al., 2006)
pHlamaleon5	FRET-based ratiometric	CFP/YFP	4.1	430	480/535	(Esposito et al., 2008)
pHlameleon6			5.6			
pH-Lemon	(FRET-based) ratiometric	CFP/YFP	6.3	430/(500)	480/535	(Burgstaller et al., 2019)
pHRed	Ratiometric	RFP	6.6	440/585	610	(Tantama et al., 2011)
SypHer	Ratiometric	YFP	8.7	420/490	535	(Ermakova et al., 2018; Matlashov et al., 2015; Poburko et al., 2011)
SypHer2						
SypHer3s						
pHTomato	Intensiometric	RFP	7.8	550	580	(Li & Tsien, 2012)
pHuji	Intensiometric	RFP	7.7	566	598	(Shen et al., 2014)

In addition to single FP-based pH sensors, double FP sensors have been developed. Among them are pHlameleons (pHlameleon 5, 6 and 7, named after their pKa), which are composed of an enhanced CFP (eCFP) and YFP variants with different pKa (Esposito et al., 2008). The pHlameleons have been characterized in vitro, as well as in situ, where the cytosolic acidification upon lysosomal disruption was measured (Esposito et al., 2008). Recently, pH-Lemon, a sensor based on the design principle of pHlameleons, has been developed (Burgstaller et al., 2019). This ratiometric probe consists of mTurquiose2 fused to EYFP and exhibits a strong pH dependency in the neutral and the acidic range. Targeted pH-Lemon variants thus allowed determining the pH in acidic compartments, such as autophagosomal vesicles or endosomal secretory vesicles via a glycosylphosphatidylinositol (GPI) anchor (Burgstaller et al., 2019). The pH-Lemon-GPI construct can be used to monitor pH alterations throughout the whole anterograde secretory pathway.

To facilitate multispectral imaging of metabolic processes, several red-shifted pH sensors have been developed. In 2011 Tantama et al. presented the genetically encoded sensor pHRed (Tantama et al., 2011). Although pHRed consists of only one FP, it can be exploited as a ratiometric pH indicator, due to its dual excitation peaks (Tantama et al., 2011). In their study, Tantama et al. used cytosolic and mitochondrial pHRed variants as a tool to investigate the link between cellular energy metabolism and pH fluctuations (Tantama et al., 2011). Interestingly, the removal of extracellular glucose resulted in the acidification of both the cytosol and mitochondria (Tantama et al., 2011). They also correlated these pH effects with changes of the ATP/adenosine-5′-diphosphate (ADP) ratio in response to energy stress, by simultaneous imaging of both parameters using pHRed in combination with the ATP/ADP sensor Perceval (Tantama et al., 2011; Tantama, Martínez-François, Mongeon, & Yellen, 2013).

Poburko et al. (2011) generated a ratiometric single FP-based genetically encoded fluorescent pH sensor, named SypHer, which is derived from the H_2O_2 sensor HyPer invented by Belousov et al. (Belousov et al., 2006). The development of SypHer was largely based on the work of Belousov et al. who had already described the pH sensitivity of HyPer and the loss of its H_2O_2-sensing ability upon mutating the H_2O_2-sensing cysteines residues to serines (Belousov et al., 2006). SypHer consists of a pH-sensitive circularly permuted YFP (cpYFP) optimized for sensing pH values between 7 and 10 (Poburko et al., 2011). SypHer, as well as the mitochondrially targeted version mitoSypHer, are among the most frequently used pH sensors for live cell imaging of subcellular pH dynamics (Benčina, 2013; Poburko et al., 2011; Santo-Domingo, Giacomello, Poburko, Scorrano, & Demaurex, 2013). Poburko et al. measured pH changes in response to Ca^{2+} mobilization, using mitoSypHer in combination with the cytosolic Ca^{2+} dye Fura-2 (Poburko et al., 2011).

Later, Matlashow et al. established a brighter variant of SypHer, referred to as SypHer-2 (Matlashov et al., 2015). Using SypHer-2, they monitored activity driven pH changes in the cytosol of cultured neurons, as well as in pre- and postsynaptic termini by respective targeting of the probe (Matlashov et al., 2015). Recently, SypHer3s has been developed, which shows an increased dynamic range and brightness. Ermakova et al. expressed SypHer3s in cultured primary mouse cortex neurons to visualize synaptic boutons and used co-transfection of SypHer3s with a fusion-construct of synaptophysin with tdTomato to measure mitochondrial pH. Furthermore, SypHer3s was successfully applied to visualize pH gradients in different tissues of zebrafish *Danio rerio* larvae (Ermakova et al., 2018).

To overcome the problems with color spectral overlapping of previously used probes, Li et al. generated a new red pH probe called pHTomato (Li & Tsien, 2012). In contrast to pHRed, which is excited at 440 and 585 nm (Tantama et al., 2011), pHTomato is only excited at 550 nm (Li & Tsien, 2012), thus allowing simultaneous imaging with, e.g. GFP-based sensors. Li et al. successfully applied the probe for

monitoring pH changes in synaptic vesicles, which are observed during exocytosis, using SypHTomato that was made by fusing pHTomato to the vesicular membrane protein synaptophysin (Calakos & Scheller, 1994; Li & Tsien, 2012). They also co-expressed SypHTomato with the Ca^{2+} sensor GCamP3 in the same neuron to correlate presynaptic Ca^{2+} transients and neurotransmitter release (Borges-Pereira, Campos, & Garcia, 2014; Li & Tsien, 2012). Another single wavelength red FP-based pH sensor, named pHuji, was found to be six- to sevenfold more pH sensitive than pHTomato and suited for co-imaging with green GEFPs (Shen, Rosendale, Campbell, & Perrais, 2014). In their study, Shen et al. also combined pHuji with the green pH sensor superecliptic pHluorin (Sankaranarayanan, De Angelis, Rothman, & Ryan, 2000) to simultaneously image the endocytosis of two different receptors. It has been discussed that the photo-convertibility of pHuji needs to be considered when using this red fluorescent pH probe (Shen et al., 2014).

4.2. pH changes in the brain

Since neuronal activity leads to pH-alterations in brain tissue, these cells depend on the tight regulation of cellular pH (Ruffin, Salameh, Boron, & Parker, 2014). During neuronal firing, a high level of glutamate is released from brain tissue, which is taken up by astrocytes in order to clear these cytotoxic glutamate concentrations (Danbolt, 2001). Consequently, the uptake of glutamate acidifies the cytosol of astrocytes (Brown & Ransom, 2007). These pH alterations play an essential role in mitochondrial bioenergetics (Burke, 2017). Generally, the mitochondrial matrix is more alkaline compared to the cytosol (Llopis, McCaffery, Miyawaki, Farquhar, & Tsien, 1998; Poburko et al., 2011) due to the activity of the respiratory chain that pumps protons from the mitochondrial matrix into the mitochondrial intermembrane space (Santo-Domingo & Demaurex, 2012), thereby generating a pH gradient, which drives ATP generation via the ATP synthase (Porcelli et al., 2005; Santo-Domingo & Demaurex, 2012). Mitochondria-targeted SypHer (Poburko et al., 2011) reported a matrix pH decrease upon glutamate uptake in astrocytes, thereby abrogating the pH gradient across the inner mitochondrial membrane (Azarias et al., 2011). Mitochondrial acidification was also accompanied by decreased respiration and reduced ROS production (Azarias et al., 2011). Dysregulation or loss of glutamate uptake is associated with severe dysfunctions like amyotrophic lateral sclerosis (Trotti et al., 2001), Alzheimer's disease (Scott et al., 2011) or HIV-associated neurocognitive disorders (Moidunny et al., 2016).

Within synaptic vesicles, acidic pH vacuolar proton ATPases (V-ATPases) generate an acidic pH (Nelson, 1992) that drives the uptake of neurotransmitters into their lumen (Liu, Krantz, Waites, & Edwards, 1999). The acidic vesicles fuse with the synaptic membrane during action potentials and release their cargo into the neutral synaptic cleft (Krapivinsky, Mochida, Krapivinsky, Cibulsky, & Clapham, 2006; Sinning & Hübner, 2013). Based on their finding that the ion channel and kinase TRPM7 is located in the membrane of synaptic vesicles and is important for acetylcholine release (Krapivinsky et al., 2006), the same group aimed at identifying the role of TRP7 in vesicle fusion (Brauchi, Krapivinsky, Krapivinsky, & Clapham, 2008). For this purpose, the genetically encoded single FP sensor pHluorin (Miesenböck et al., 1998) was targeted into the lumen of acetylcholine secreting vesicles to serve as a single-vesicle plasma membrane fusion reporter (Brauchi et al., 2008). Upon release into the synaptic cleft, pHluorin reported the pH change by spontaneous and transient flashes due to enhanced fluorescence of the pH probe under more alkaline conditions. Since knockdown or expression of a non-functional TRPM7 mutant prevented vesicle fusion, they concluded that TRPM7 is important for vesicle fusion events (Brauchi et al., 2008). For similar applications, pHluorin was also fused to different proteins localized within the lumen of synaptic vesicles, including synaptophysin (Kim et al., 2003), synaptotagmin (Dean et al., 2012), the vesicular glutamate transporter 1 (Fernandez-Alfonso & Ryan, 2008), or the vesicular monoamine transporter 1 (Anantharam, Onoa, Edwards, Holz, & Axelrod, 2010). A detailed review focussing on pHluorin variants suitable for imaging synaptic activity is available (Royle, Granseth, Odermatt, Derevier, & Lagnado, 2008).

4.3. pH changes and cancer

Another major field of application for pH sensors is cancer research since dysregulated pH dynamics are a common feature of many cancers (Asgharzadeh et al., 2017). While the extracellular pH (pH_e) is decreased in tumor tissue (Kato et al., 2013), mainly due to lactate secretion from aerobic glycolysis (Apicella et al., 2018; Kato et al., 2013), the intracellular pH (pH_i) is maintained within a pH range of 7.0–7.2 (Zhang, Lin, & Gillies, 2010). The altered pH homeostasis in cancer cells is thought to influence cell metabolism and proliferation (Alfarouk et al., 2014) as well as metastasis (Thews & Riemann, 2019) and tumorigenesis (Swietach, Vaughan-Jones, Harris, & Hulikova, 2014), and might also contribute to radiotherapy and chemotherapy resistance (De Milito & Fais, 2005). On the other hand, there is evidence, that cancer cell behavior can be influenced by re-balancing pH dynamics, e.g. by modulating the activity of ion transporters that regulate pH_i (Dumas et al., 2017; Oosterwijk & Gillies, 2014). Still, many assumptions considering pH_i in cancer have remained speculative and thus require experimental evaluation. As it was done in a study by Shirmanova et al., genetically encoded pH sensors can be used to determine and track pH_i alterations (Shirmanova et al., 2015). Using SypHer2 in monolayer and 3D culture models unveiled decreasing pH_i towards the core of spheroids (Matlashov et al., 2015, p. 2; Shirmanova et al., 2015). Moreover, they observed an overlap of acidic and hypoxic regions within a tumor using SypHer-2 in a xenograft model of cervical cancer (Matlashov et al., 2015; Shirmanova et al., 2015).

In an additional study, Shirmanova et al. explored the mechanism of cell response to the chemotherapeutic drug cisplatin, in the relationship between the pH_i and cell bioenergetics (Shirmanova et al., 2017). They employed SypHer2 for intracellular pH mapping, thereby tracing pH_i changes in the individual cancer cells during cisplatin treatment (Shirmanova et al., 2017). By combining these data with the investigation of the metabolic state, Shirmanova et al. showed that after prolonged therapy, intracellular acidification supports a metabolic reorganization of cells. Interestingly, the cisplatin-induced inhibition of cancer cell growth was accompanied by a metabolic shift from glycolysis towards oxidative metabolism (Shirmanova et al., 2017).

5. ATP and metabolite imaging

Metabolic alterations are a common feature of many diseases and therefore a promising target for therapeutical interventions. GEFPs are valuable, emerging tools in metabolic research. This approach has significant advantages over most classical biochemical methods that typically require cell lysis and represent cell populations rather than individual cells, which might mask certain cell-specific phenomena. In particular, it enables us to monitor and analyze metabolic processes in intact living cells with high spatiotemporal resolution; we can monitor metabolic processes almost in real-time, and localize them on the subcellular level by the proper targeting of a sensor (Depaoli, Hay, et al., 2018; Imamura et al., 2009). Moreover, we can resolve cell-to-cell variations, which may be highly relevant in a physiological context (Depaoli, Hay, et al., 2018).

The applicability of GEFPs for metabolic research will be primarily illustrated by the example of ATP owing to its central role in metabolism as both energy-carrying and signaling molecule. Measuring intracellular ATP may provide important insights into cellular ATP homeostasis – that is ATP production, consumption, and transport – as well as a wide range of processes that depend on energy in the form of ATP. Moreover, one can determine which energy-producing pathways are utilized by different cell types and under specific physiological or

pathophysiological conditions, which is highly relevant in many fields, such as cancer research, neuroscience or immunology.

The most widely used ATP sensors are the so-called ATeams (Table 3) (Imamura et al., 2009). ATeams are FRET-based probes with the ε subunit of the *Bacillus subtilis* F_1F_o ATP synthase as ATP-sensing domain (Imamura et al., 2009). In addition to ATeams, there are also other ATP sensors available. The ratiometric single FP sensor QUEEN (quantitative evaluator of cellular energy) is suitable for quantifying absolute ATP concentrations (Yaginuma et al., 2015). Only recently two groups presented intensiometric single FP-based ATP sensors, which are promising tools for simultaneous imaging approaches with other sensors (Arai et al., 2018; Lobas et al., 2019).

The ratiometric single FP probes Perceval (Berg, Hung, & Yellen, 2009) and the optimized PercevalHR (Perceval high range) (Tantama et al., 2013) measure the intracellular ATP/ADP ratio (Table 3). The ATP/ADP ratio could be particularly informative in tissues with high, fluctuating energy demands, such as skeletal muscles and brain, because here changing ATP concentrations are usually rapidly buffered by ATP production from phosphocreatine and ADP (Schlattner, Tokarska-Schlattner, & Wallimann, 2006).

When Imamura et al. introduced ATeams (Imamura et al., 2009), they presented intriguing data on subcellular ATP distribution and ATP production in HeLa cancer cells. By targeting the ATP probe to different compartments of the cell, they showed that ATP levels in the mitochondrial matrix are significantly lower than in the cytosol and nucleus and that HeLa cells produce ATP primarily via glycolysis (Imamura et al., 2009). Following studies with the ER-targeted ATeam ERAT4.01 (Table 3) (Vishnu et al., 2014) revealed that ATP levels within the ER were again considerably lower than in the mitochondria and that Ca^{2+} release from the ER induced ATP increases within the ER lumen (Vishnu et al., 2014). These ATP elevations were fueled by glycolysis in highly glycolytic cells like the cancerous HeLa cell line on the one hand, and oxidative phosphorylation in cells relying on mitochondrial respiration like the insulin producing beta cell line INS-1 832/13 (Vishnu et al., 2014). This study also suggested using live cell imaging of ER ATP to clarify how the ER is supplied with ATP when the ATP import mechanism and the identity of a possible ATP transporter were still unknown. We still lack understanding of this process (Depaoli et al., 2018), however, a recent study presented a possible ATP transporter, namely the ATP/ADP exchanger in the ER membrane (AXER) (Klein et al., 2018). In this paper, live cell imaging of ER ATP using ERAT4.01 (Vishnu et al., 2014) revealed that depletion of AXER from HeLa cells reduced ATP levels within the ER lumen (Klein et al., 2018).

In our lab, we continued to examine subcellular ATP dynamics in HeLa cells as a model for cancer in more detail, using organelle-targeted ATeams (Depaoli, Hay, et al., 2018; Imamura et al., 2009). Since metabolic alterations are considered as one of the hallmarks of cancer (Hanahan & Weinberg, 2011; Pavlova & Thompson, 2016), we seek a better understanding of cancer metabolism in order to find new possibilities for diagnosis and therapy (Amoedo, Obre, & Rossignol, 2017; Galluzzi, Kepp, Van der Heiden, & Kroemer, 2013). Measuring ATP now provides a meaningful read-out of the metabolic activity of a cell. Our work revealed that mitochondrial ATP levels are most responsive to energy stress compared to other compartments (cytosol, ER) (Depaoli, Hay, et al., 2018), although ATP is produced in the cytosol via aerobic glycolysis ("Warburg effect") (Liberti & Locasale, 2016). Furthermore, we established a protocol for the metabolic profiling of individual cancer and non-cancer cells using mitochondrial ATP measurements, where we determined the contribution of glycolysis and mitochondrial respiration to ATP production, thereby revealing significant differences between different cancer cell types (Depaoli, Hay, et al., 2018).

Like ATP, which is required for most energy-dependent reactions (Harris et al., 2012), NAD is also a central cofactor involved in many enzymatic reactions (Ma et al., 2012), in particular as the major electron carrier in redox reactions (Xiao, Wang, Handy, & Loscalzo, 2018). NAD exists in an oxidized form (NAD^+) and a reduced form (NADH) (Xiao et al., 2018). In the course of glycolysis and the tricarboxylic acid (TCA) cycle, NAD^+ is reduced to NADH (Cantó, Menzies, & Auwerx, 2015). Then NADH is used as an electron donor for the electron transfer chain and, hence, recycled to NAD^+ (Xiao et al., 2018). Thus, similar to ATP, the NADH/NAD^+ redox state is a meaningful readout of the metabolic activity of a cell. Available sensors, as well as the application of GEFPs for that purpose, are described in detail by Zhao et al. (Table 3) (Zhao, Wang, et al., 2016).

GEFPs have also been used for studying the metabolic response to neurotransmission (e.g., Baeza-Lehnert et al., 2018; Díaz-García et al., 2017; Fernández-Moncada et al., 2018; Lerchundi et al., 2018). In the course of neuronal activation high amounts of ATP are required for reestablishing ion gradients, reversing Ca^{2+} changes and recycling vesicles (Ashrafi & Ryan, 2017). However, it is still uncertain how neurons meet their energy demand when they are activated. Baeza-Lehnert et al. chose to address this question by monitoring the early response to neurotransmission using genetically encoded fluorescent metabolite sensors as well as chemical ion sensors (Baeza-Lehnert et al., 2018). By using AT1.03 (Imamura et al., 2009) or PercevalHR (Tantama et al., 2013), respectively, they found that cytosolic ATP levels, as well as cytosolic ATP/ADP ratios, were unaffected by neurotransmission, suggesting a mechanism that couples ATP consumption and production (Baeza-Lehnert et al., 2018). The authors conclude that ATP production was regulated by the Na^+ pump, and not via Ca^{2+} or adenine nucleotide concentrations (Baeza-Lehnert et al., 2018). Furthermore, Baeza-Lehnert et al. showed that glucose and pyruvate fluxes increased in response to neuronal activation with the help of glucose (Takanaga, Chaudhuri, & Frommer, 2008, p. 9) and pyruvate sensors (San Martín et al., 2014) (Table 3). In addition, they found that both glucose and lactate (measured with the lactate sensor Laconic, Table 3) (San Martín et al., 2013) were consumed by resting neurons (Baeza-Lehnert et al., 2018). Altogether, this study impressively shows how we can utilize GEFPs for examining cell physiological processes with high spatiotemporal resolution.

In the context of cellular bioenergetics and ATP production, monitoring mitochondrial biogenesis and turnover as well as mitochondrial morphology and dynamics may be highly relevant as well. The most common way to analyze mitochondrial morphology and dynamics (movement, fusion and fission) is fluorescence microscopy of mitochondria-targeted fluorescent proteins, e.g. mitochondria-targeted GFP (mtGFP) (Marchi, Bonora, Patergnani, Giorgi, & Pinton, 2017; Simula & Campello, 2018). Based on this approach more advanced techniques have been developed for measuring mitochondrial dynamics, including Fluorescence Recovery After Photobleaching (FRAP), the use of photoactivable mtGFP (also known as PAGFPmt assay), and polyethylene glycol (PEG)-based fusion of mtGFP and mtRFP cells (Simula & Campello, 2018). FRAP involves photobleaching of FPs within a certain area of the cell and monitoring the recovery time of the fluorescence which is achieved by the movement of FPs or organelles from the unbleached area. With regard to mitochondria, FRAP experiments can reveal the following aspects, depending on the time scale of recovery (Mitra & Lippincott-Schwartz, 2010): mobility of a fluorescent protein of interest (recovery within milliseconds), mitochondrial continuity (recovery within 2–3 min), mitochondrial movement and dynamics (recovery between 2 and 3 min to about 1 h). Similar information can be obtained using photoactivable mtGFP, which is based on a poorly emitting GFP variant that increases its fluorescence approximately 100-fold when excited at 400 nm (Patterson & Lippincott-Schwartz, 2002). To study mitochondrial movement and dynamics, mtGFP is photoactivated within a certain area of the cell, and its redistribution within the mitochondrial network is monitored (Karbowski, Cleland, & Roelofs, 2014; Molina & Shirihai, 2009). In the PEG-based fusion assay, mtGFP-expressing cells are fused with mtRFP-expressing cells by the addition of PEG to generate a cell with both green and red mitochondria (Legros, Lombès, Frachon, & Rojo, 2002;

Table 3
Selection of genetically encoded probes for visualization of cellular ATP and metabolites.

Sensor	Principle	Analyte	Comments	Reference
ATeams (e.g. AT1.03, mitoAT1.03, ERAT1.03, ERAT4.01, AT1.03NL, ecAT1.03)	FRET based ratiometric	ATP	A wide range of sensor variants is available, optimized, for instance, for the use in specific parts of the cell, in the extracellular space, or in vivo imaging in particular organisms; the sensors are different in terms of e.g. their targeting, ATP binding affinities or FP components	(Chandrasekharan et al., 2019; Conley, Radhakrishnan, Valentino, & Tantama, 2017; Imamura et al., 2009; Tsuyama et al., 2013; Vishnu et al., 2014)
QUEEN	Ratiometric	ATP	Unlike ATeams, QUEEN is insensitive to bacteria growth rate changes, and can thus be used for quantifying absolute ATP concentrations in bacteria; QUEEN is also functional in mammalian cells (unpublished data from our lab)	(Yaginuma et al., 2015)
iATPsnFR	Intensiometric	ATP	Different variants are available; because of their narrow spectral bandwidth iATPsnFRs are suitable for co-imaging experiments with other sensors	(Lobas et al., 2019)
MaLionG/R/B	Intensiometric	ATP	Green, red and blue FP based ATP sensors, targeted to the cytosol or mitochondria, were developed to enable simultaneous imaging	(Arai et al., 2018)
Perceval, PercevalHR	Ratiometric	ATP/ADP ratio	Because of their pH sensitivity, Perceval and PercevalHR are usually co-imaged with a pH sensor (e.g. pHRed) to correct for the pH bias; PercevalHR (high range) is optimized for the ATP/ADP range within mammalian cells	(Berg et al., 2009; Tantama et al., 2013)
GEVALs	Ratiometric	GTP	A series of sensors with a wide dynamic range is available	(Bianchi-Smiraglia et al., 2017)
SoNar	Ratiometric	NADH/NAD^+ ratio	Cytosolic and nuclear variant	(Zhao et al., 2015; Zhao, Wang et al., 2016)
Frex	Ratiometric	NADH	Cytosolic, nuclear and mitochondrial variant; pH sensitive	(Zhao, Jin, et al., 2011)
Peredox	Intensiometric	NADH/NAD^+ ratio; also depends on total NADH	Cytosolic and nuclear variant; tandemly attached mCherry allows normalization to sensor expression levels	(Hung, Albeck, Tantama, & Yellen, 2011)
NADPsor	FRET based ratiometric	$NADP^+$	–	(Zhao, Wang, et al., 2016; Zhao, Zhang, Zhang, Tang, & Ye, 2016)
Apollo-$NADP^+$	FRET based ratiometric	$NADP^+$	–	(Cameron et al., 2016; Cameron & Rocheleau, 2017)
iNAP	Ratiometric	NADPH	–	(Tao et al., 2017)
FLII^{12}Pglu-700μδ6	FRET based ratiometric	Glucose	–	(Takanaga et al., 2008)
Laconic	FRET based ratiometric	Lactate	–	(San Martín et al., 2013)
Pyronic	FRET based ratiometric	Pyruvate	–	(San Martín et al., 2014)
FLIPsuc-4 μ	FRET based ratiometric	Sucrose	Also recognizes other sugars; different variants available	(Lager, Looger, Hilpert, Lalonde, & Frommer, 2006)
FLIPsuc90μΔ1Venus	FRET based ratiometric	Sucrose, trehalose	Improved sucrose sensor, recognizes trehalose with similar affinity	(Kikuta, Hou, Sato, Frommer, & Kikawada, 2016)
FLIPQTV3.0	FRET based ratiometric	Glutamine	Sensors with different affinities available	(Besnard & Okumoto, 2014; Gruenwald et al., 2012)
FLIPEs	FRET based ratiometric	Glutamate	Sensors with different affinities available. Also detects glutamine and aspartate with lower affinity	(Okumoto et al., 2005)
GluSnFR SuperGluSnFR	FRET based ratiometric	Glutamate	SuperGluSnFR is the improved version of GluSnFR	(Hires, Zhu, & Tsien, 2008; Tsien, 2005)
iGluSnFR	Intensiometric	Glutamate	Sensors with different affinities and fluorescent properties are available	(Marvin et al., 2018, 2013)
cpFLIPR	FRET based ratiometric	Arginine	–	(Whitfield et al., 2015)
FLIPK	FRET based ratiometric	Lysine	–	(Ameen et al., 2016)
FLIP-Leu	FRET based ratiometric	Leucine	–	(Mohsin, Abdin, Nischal, Kardam, & Ahmad, 2013)
OGsor-G9	FRET based ratiometric	2-Oxoglutarate/alpha-ketoglutarate	–	(Zhang, Wei, & Ye, 2013)
PII-TC3 PII-TC3-R9P	FRET based ratiometric	2-Oxoglutarate/alpha-ketoglutarate	–	(Lüddecke et al., 2017)
F-mT_PII F-mT_NtcA	FRET based ratiometric	2-Oxoglutarate/alpha-ketoglutarate	–	(Chen, Latifi, Zhang, & Bernard, 2018)
CITs	FRET based ratiometric	Citrate	–	(Ewald, Reich, Baumann, Frommer, & Zamboni, 2011)

Simula & Campello, 2018). Mitochondrial fusion is indicated by the appearance of yellow mitochondria due to the mixing of the green and red FPs. Compared to FRAP and photoactivable mtGFP assays, the PEG-based fusion assay has several disadvantages. It is restricted to cells that can be fused with PEG, the toxicity of PEG might affect a cell's fusion ability, and its suitability for real-time experiments is limited (Karbowski et al., 2014; Molina & Shirihai, 2009, p. 16).

In addition to mitochondrial movement and dynamics, imaging FP-tagged mitochondria may also be applied to assess mitochondrial biogenesis and degradation, as these processes result in the increase or decrease of mitochondrial content and size. However, a number of fluorescent tools have been developed to specifically measure mitochondrial biogenesis and turnover in living cells (Williams, Zhao, Jin, & Ding, 2017), such as mitoTimer (Hernandez et al., 2013) and mt-Keima (Sun et al., 2015, 2017). The mitoTimer construct consists of the fluorescent protein Timer targeted to the mitochondrial matrix. Timer changes its emission from green to red over time in the course of protein maturation (Terskikh, 2000), making mitoTimer an indicator of mitochondrial aging. From mitoTimer data, one can also determine rates of mitochondrial biogenesis and degradation and mitochondrial protein import (Hernandez et al., 2013). Mt-Keima serves as an indicator of mitophagy, the autophagy of mitochondria (Lemasters, 2005, 2014). Keima is a fluorescent protein that changes its color depending on if the environment is acidic (red) or neutral (green) (Kogure et al., 2006). When mitochondria are sequestered to acidic lysosomes in the course of mitophagy, mt-Keima turns red and thus indicates the final stage of mitophagy (Sun et al., 2015, 2017).

6. Imaging ROS and RNS

In the past two decades, it has become apparent that ROS and RNS serve as signaling molecules that regulate cell physiological processes (Forman & Torres, 2001, 2002; Trachootham, Lu, Ogasawara, Valle, & Huang, 2008). In cells, redox signals are primarily transduced by stable ROS such as hydrogen peroxide (H_2O_2) or highly reactive RNS such as nitric oxide (NO) (Holmström & Finkel, 2014; Schieber & Chandel, 2014; Sies, 2017). Thanks to rapid technological advances of novel informative biosensors for redox and NO signaling, we have gained many new insights into the field of redox biology and NO biochemistry in the last years (Belousov et al., 2006; Eroglu et al., 2016; Fan, Chen, & Ai, 2015; Gutscher et al., 2008; Hanson et al., 2004; Kolossov et al., 2011; Østergaard, Tachibana, & Winther, 2004; Sato, Hida, & Umezawa, 2005; Siedler et al., 2014; Sugiura et al., 2015). A variety of genetically encoded redox- and NO biosensors have been developed, which are listed in Table 4. The most prominent ROS sensitive probe is HyPer, which as a yellow single FP-based probe senses H_2O_2 in a ratiometric manner (Belousov et al., 2006). Later, a red-shifted variant was developed, referred to as HyPerRed (Ermakova et al., 2014). Both the yellow and red HyPer variants are sensitive to H_2O_2 due to cysteine residues in the vicinity of the respective chromophores (Belousov et al., 2006; Ermakova et al., 2014). The sensing principle of genetically encoded NO probes (geNOps) is based on a non-heme iron-binding domain, which brings the NO radical close to the FP chromophore (Eroglu et al., 2016). NO binding to such constructs quenches the FP fluorescence (Eroglu et al., 2016). We will now discuss in brief how to use these and other tools (Table 4) for various biological applications.

Genetic biosensors for ROS/RNS in combination with other informative probes are an excellent approach for analyzing the linkage between ROS/RNS mediated signaling pathways and downstream functions such as the modulation of metabolic events. Thanks to their spectral properties, most of the biosensors mentioned in Table 4 are combinable among each other and a variety of other genetic and chemical probes, such as Ca^{2+}, ATP, K^+ or cGMP. Since NO signaling is usually dependent on the second messenger cGMP, combining geNOps and cGMP sensors might be particularly informative. The combination of NO and Ca^{2+} sensors is tempting as well since two of three known nitric oxide synthase

Table 4
Selection of genetically encoded probes for intracellular imaging of ROS and RNS.

Sensor	Principle	Analyte/Status	(Dis)advantage	Reference
rxYFP	Intensiometric	2GSH/GSSG ratio	Prone to pH fluctuations	(Østergaard et al., 2004)
roGFP	Ratiometric	2GSH/GSSG ratio	Kinetic parameters depend on reaction with glutaredoxins	(Hanson et al., 2004)
HyPer/HyperRed	Ratiometric/Intensiometric	H_2O_2	Highly pH sensitive	(Belousov et al., 2006; Ermakova et al., 2014)
rxRFP	Intensiometric	Redox dynamics	Peroxynitrite and superoxide anions may cause artifacts; highly pH sensitive	(Fan et al., 2015)
Grx1-roGFP2	Intensiometric	Glutathione redox potential	Can be used in vivo for rapid detection of small glutathione changes	(Gutscher et al., 2008)
Oba-Q	Intensiometric	Global redox state	Some variants are pH stable and suitable for highly oxidative compartments	(Sugiura et al., 2015)
ECFP-RL7-EYFP	FRET based ratiometric	Thiol/disulfide changes	Not suitable for in vivo imaging	(Kolossov et al., 2011, 2012)
pnGFP	Intensiometric	Peroxynitrite $ONOO^-$	Use of an unnatural amino acid in the FP is associated with difficulties in the expression	(Chen, Ren, Wright, & Ai, 2013)
sNOOOpy	FRET based ratiometric	Nitrite and nitrate	Used to detect byproducts of NO	(Hidaka et al., 2016)
geNOps	Intensiometric/ratiometric	NO	pH sensitive	(Eroglu et al., 2016, 2018)

(NOS) isoforms are Ca^{2+} dependent, namely the neuronal and endothelial NOS (NOS-1 and NOS-3, respectively) (Forstermann & Sessa, 2012; Poulos & Li, 2017). In this way, the tight relationship of agonist-mediated cytosolic Ca^{2+} signals and NO generation in single endothelial cells has been demonstrated (Charoensin et al., 2017; Ermakova et al., 2018; Eroglu et al., 2017). Moreover, it is also widely accepted that NO is a freely diffusible radical that can easily pass biological membranes (Figueroa, Lillo, Gaete, Riquelme, & Sáez, 2013). We reassessed this theory in living cells by monitoring agonist-mediated NO generation in both the cytosol and mitochondria at once, using two differently colored geNOp variants (Charoensin et al., 2017; Eroglu et al., 2016). NO signals were the same in both compartments, confirming the theory of NO membrane diffusibility (Eroglu et al., 2016; Figueroa et al., 2013). In addition to simultaneous imaging approaches, we can even expand the possibilities of redox imaging by controlling and manipulating cell signaling pathways selectively. For that purpose, an increasing number of chemogenetic tools are available that allow cell manipulation in in vitro and in vivo applications (Campbell & Marchant, 2018; Siedler et al., 2014; Steinhorn et al., 2018; Sternson & Roth, 2014). For instance, a yeast-derived D-amino acid oxidase (DAAO) was recently used as endogenous H_2O_2 producer (Steinhorn et al., 2017). Moreover, the combination of this enzyme with the H_2O_2 sensitive biosensor HyPer permitted to simultaneously produce and detect H_2O_2 on the subcellular level as well as in whole organs (Steinhorn et al., 2017, 2018).

7. Imaging second messengers

Second messengers, such as Ca^{2+} — as already described above –, cAMP or cGMP are responsible for transferring signals within the intracellular space. Many important physiological processes (e.g. cell proliferation and differentiation, muscle contraction, neurotransmission) involve the release of second messengers in response to extracellular signals, which then triggers intracellular signal transduction pathways. Measuring second messenger concentrations is thus highly relevant for investigating these processes.

Depending on the cell type, the generation of cAMP may evoke hundreds of different cellular responses based on the activation of cAMP-dependent protein kinase A (PKA) or exchange protein directly activated by cAMP (EPAC) (Johnstone, Agarwal, Harvey, & Ostrom, 2018). As the spatiotemporal progression of cAMP signals is decisive of their effects (Johnstone et al., 2018), GEFPs are particularly helpful to determine how the specificity of these signals is achieved (Zaccolo, 2002). The first cAMP sensors were FRET-based with PKA-derived sensing domains (Zaccolo, 2002). Later, EPAC-based FRET, as well as single wavelength sensors, were developed (Harada et al., 2017; Kitaguchi, Oya, Wada, Tsuboi, & Miyawaki, 2013; Klarenbeek, Goedhart, Hink, Gadella, & Jalink, 2011; Klarenbeek, Goedhart, van Batenburg, Groenewald, & Jalink, 2015; Odaka, Arai, Inoue, & Kitaguchi, 2014; Ponsioen et al., 2004; Tewson, Martinka, Shaner, Hughes, & Quinn, 2016). And more recently, also two PKA-based single wavelength sensors were presented, namely the GFP-based cAMPr sensor (Hackley, Mazzoni, & Blau, 2018) and the RFP-based R-FlincA sensor (Ohta, Furuta, Nagai, & Horikawa, 2018). cAMPr was applied to measure cAMP in mouse embryonic stem cells and neurons differentiated from embryonic stem cells, revealing a strong increase of cAMP after stimulation of the cells with forskolin (Hackley et al., 2018). In the same study, the GFP-based cAMPr sensor was combined with a red-shifted Ca^{2+} sensor to simultaneously monitor cAMP and Ca^{2+} in neurons within an intact fly brain.

Like cAMP, cGMP is also involved in a variety of cellular processes, especially in the nervous and vascular system, but in most other cell types the function of cGMP remains enigmatic (Koesling, Mergia, & Russwurm, 2016). Thus, GEFPs could provide many new insights into cGMP mediated processes. For more detailed information we recommend a recent review which gives a detailed summary of genetically encoded cGMP indicators and their application (Russwurm & Koesling, 2018).

8. Imaging kinase activities

Kinase activation and their subsequent activity represent central events in the cellular response to metabolic signals (López-Camarillo et al., 2011; Paul et al., 1997.; Tsou et al., 2011). A large number of mammalian kinases have been identified and characterized, all directly or indirectly linked to metabolic processes in the cell (Hunter, 1987; Shchemelinin, Sefc, & Necas, 2006). The most prominent examples of energy/metabolism sensing kinases are the AMP-activated protein kinase (AMPK) (Garcia & Shaw, 2017) and the mechanistic target of rapamycin (mTOR) (Saxton & Sabatini, 2017). AMPK itself creates a direct link between cell energy metabolism and ion signaling, as AMPK is sensitive to the cellular adenosine-5′-monophosphate (AMP):ATP ratio and $[Ca^{2+}]$ (Garcia & Shaw, 2017; Heidrich et al., 2010). Genetically encoded sensors allow visualizing the activity of AMPK and other kinases on the level of single living cells, both in situ and in vivo (Konagaya et al., 2017; Tsou et al., 2011). In principle, kinase sensors can be divided into two groups: on the one hand the so-called activation probes, and activity reporters on the other hand (Oldach & Zhang, 2014). Activation probes are themselves substrates of an upstream kinase of interest, reporting specific kinase activation. Activity reporters are direct substrates of the kinase of interest. Generally, these probes consist of a kinase-specific region that changes its conformation upon phosphorylation, which in turn affects their fluorescence (Oldach & Zhang, 2014). One of the most famous and most frequently used kinase activity reporters is the AMPK activity reporter, named AMPKAR (Tsou et al., 2011).

For more details, we refer to informative reviews, focusing on AMPKAR and other genetically encoded kinase probes (Oldach & Zhang, 2014; Sizaire & Tramier, 2017).

9. Imaging protein trafficking

Metabolic and signaling processes both depend on the transport of proteins to the plasma membrane and the extracellular space via the secretory pathway, including essential transport proteins for glucose and ions as well as different receptor proteins (Farhan & Rabouille, 2011). The secretory pathway, in turn, responds to various extra- and intracellular stimuli that also affect cellular metabolism. A prominent example of the interrelation of signaling, metabolism and the secretory pathway is insulin secretion (Alam et al., 2012, p. 1; Eto et al., 1999; Kennedy, Maechler, & Wollheim, 1998; Klec et al., 2019; Merglen et al., 2004; Webb, Akbar, Zhao, & Steiner, 2000).

The entirety of protein trafficking in a cell encompasses an extensive network of anterograde and retrograde transport processes. Due to this complexity, it is a very challenging task to break transport down into uni-directional protein movements. Protein-based fluorescent probes and especially the method of synchronization in transport can help give some answers to crucial questions in this research field.

Unlike K^+ or Ca^{2+} sensors, protein-based probes for imaging protein trafficking are not "sensors" in the classical sense. Instead they represent fluorescently labeled cargo undergoing tightly controlled trafficking triggered by parameters such as a temperature change (Kreis & Lodish, 1986), addition of ligands (Boncompain et al., 2012; Rivera et al., 2000; Rollins et al., 2000) or light (Chen, Gibson, & Kennedy, 2013). These trigger mechanisms enable synchronized entry into the secretory or endosomal pathways.

As described in the preceding chapters, GEFPs enable easy quantification of biomolecules in many cases. The tools for imaging protein trafficking follow a different principle. Namely, the probes undergo a localization change rather than a fluorescence change. Usage of the probes in a quantitative way is possible yet challenging because these observations rely on the transport of the probe itself.

Technically, every fluorescently labeled protein or even a proteinaceous fluorophore undergoes cellular transport processes and reveals information about its steady-state localization. However, a molecule's steady-state localization is typically a combination of rates of trafficking into and away from its visible location(s). In combination with techniques like fluorescence recovery after photobleaching (FRAP) (Nakata, Terada, & Hirokawa, 1998) or photoactivation (Patterson & Lippincott-Schwartz, 2002) viable options for measuring the kinetics of a protein's movement that make up its overall localization have been demonstrated. In this review, however, the focus will lie on tools that follow the premise of synchronizable trafficking; in this technique, a cohort of a probe is released suddenly from one endpoint in a trafficking step so that its rate of accumulation at the other endpoint(s) can be quantitated.

Temperature-controlled trafficking mostly relies on the vesicular stomatitis virus glycoprotein (VSVG) and in particular its temperature-sensitive mutants, VSVG ts045. The attached fluorophore is in most cases a GFP variant. The construct has been used extensively in the past for the live cell-visualization of cellular protein transport, especially between the ER and Golgi (Presley et al., 1997), and the identification and characterization of the involved components (Scales, Pepperkok, & Kreis, 1997). The monomeric VSVG ts045 is retained in the ER at the restrictive temperature of 39.5 °C and released into to the secretory pathway upon switching to the permissive temperature (32 °C) at which it undergoes the folding and trimerization essential for ER exit (Kreis & Lodish, 1986). Although this principle is quite old, it is still applied today in the development of novel imaging approaches (Tie et al., 2016).

Over the years ligand-sensitive variants of tools for studying protein trafficking have emerged. One of the most prominent examples is the application of a conditional aggregation domain (CAD) that forces lattice-like cargo protein oligomerization in the ER and enables the release of the fluorescently tagged cargo into the secretory pathway upon addition of a chemical ligand (Rollins et al., 2000). The conditional aggregation system itself was initially suggested for the controlled release of recombinant proteins in gene therapy (Rivera et al., 2000), however, the principle of the construct proved to be very valuable in protein trafficking research. Besides the investigation of basic transport processes, e.g. in intra- (Volchuk et al., 2000) and post-Golgi dynamics (Jaiswal, Rivera, & Simon, 2009), and secretory machinery such as soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNAREs) (Gordon, Bond, Sahlender, & Peden, 2010), the principle has been widely used in studying physiologically important processes. In a very simple experiment the delivery of a CAD-linked version of the muscarinic M_1 receptor to the plasma membrane has been shown (Sawyer, Ehlert, & Hart, 2006), hinting at the potential significance of these constructs in receptor studies. As described in Sawyer et al., 2006 the introduction of a CAD enables an uncoupled examination of receptor synthesis and its trafficking, respectively (Sawyer et al., 2006).

A different approach using CAD-based synchronizable constructs is to quantitate the rate of appearance of a fluorescent soluble secretory marker, in this case, GFP-human growth hormone, in the extracellular medium (Gordon et al., 2010). This approach estimates the overall rate of the entire secretory pathway, from ER to extracellular medium, an immensely significant parameter. Such an approach was employed to study the effects of alpha-synuclein on constitutive secretion in HeLa cells (Winslow et al., 2010). Alpha-synuclein is the primary structural component of Lewy bodies: intracellular protein aggregates that contribute to Parkinson's disease (Lee & Trojanowski, 2006). Based on the knowledge that alpha-synuclein disrupts ER-to-Golgi-transport in yeast (Cooper et al., 2006) and interacts with Ras-related in brain (Rab) proteins, central regulators of the secretory pathway (Gitler et al., 2008), a CAD-based secretion assay was used successfully to demonstrate that over-expression of Parkinson's-associated mutants of alpha-synuclein significantly slowed secretion of soluble cargo (Winslow et al., 2010). The same principle could be expanded not only to investigate the role of overexpressed or knocked-out proteins involved in pathogenesis, but also to explore treatment options.

A novel approach to optimize ligand-controlled secretion of fluorescent constructs is the Retention Using Selective Hooks (RUSH) system (Boncompain et al., 2012), a framework comprising several applicable constructs working in a "reporter-hook-fashion". A membrane-associated protein fused to streptavidin works as a "hook" keeping the fluorescent reporter containing a streptavidin-binding peptide (SBP) in the donor compartment (e.g. ER). Release of the reporter can be accomplished by adding biotin, which competitively binds streptavidin with higher affinity than SBP. The RUSH system proved to be valuable in the examination of basic protein trafficking processes like coat protein complex II (COPII)-dependent ER export (Stadel et al., 2015). However, ligand-sensitive constructs such as the RUSH assay can be used not only to implicate a particular protein in secretion but also to identify unexpected roles in trafficking-related processes such as ER-plasma membrane junctions (Petkovic et al., 2014) and targeting to postsynaptic membrane protein clusters (Fukata et al., 2013).

Recently, trafficking of fluorescent constructs in the cell has been synchronized by the inclusion of photosensitive oligomerization domains such as the plant ultraviolet-B resistance 8 (UVR8) domain, allowing the release of an aggregated cargo construct from the ER using UVB light pulses (Chen, Gibson, & Kennedy, 2013). The approach appeared promising since a seven-second pulse of light was able to completely photoswitch a VSVG-YFP construct from the retained to the secreted form faster than ligand did for a comparable CAD-based construct. Furthermore, the phototropin light oxygen voltage (LOV) domain was recently employed to trigger reversible tethering between ER and Golgi membranes to study a proposed mechanism of phosphoinositide lipid metabolism occurring at ER/Golgi contact sites (Venditti et al., 2019). Further development of optogenetic probes along these lines is likely to result in numerous other trafficking and protein localization changes to be synchronized and monitored in living cells.

Considering the enormous technological progress made over the past decades in the field of live-cell imaging, these probes will likely help answer long-standing questions about trafficking on the subcellular level and might also soon incorporate super-resolution microscopy techniques. Studying secretory processes has become increasingly important due to their vital role in cell metabolism. The secretory pathway has also been identified as a possible target in tumor cells (Dejeans et al., 2014). Many protein trafficking mechanisms are not understood in detail, and fluorescence-based tracking of cargo has the potential to extend our knowledge on this topic and to reveal a great number of yet unknown pharmacologically important applications.

10. GEFPs in pharmacological research

GEFPs also represent powerful tools for pharmacological research. They may be exploited to investigate the effects of a drug on the cellular to the molecular level, to screen for new drugs and drug targets, as well as to detect biomarkers for various diseases. This can be done on the level of single cells and cell populations, as well as with biological samples (e.g. urine, blood), and in animal models.

GEFPs in combination with fluorescence microscopy are well-suited to test the effects of both established and new pharmacological compounds on the single cell level with subcellular resolution. For instance, measuring cytosolic, mitochondrial and ER Ca^{2+} dynamics, as well as cytosolic and mitochondrial ATP, with GEFPs revealed that the natural polyphenol resveratrol specifically kills cancer cells by inducing a mitochondrial Ca^{2+} overload (Madreiter-Sokolowski et al., 2016). In another study, Ca^{2+} measurements revealed that the endocannabinoid N-arachidonoyl glycine (NAGly) inhibits store-operated Ca^{2+} entry by preventing the interaction of STIM1 and Orai1 (Deak et al., 2013). These examples also illustrate how live cell imaging with GEFPs can

be used to monitor the effects of a drug immediately after its addition to the cells. The high spatial and temporal resolution of the GEFP technology also allows detecting molecular alterations associated with pathologies, which might help to identify new possible drug targets.

For screening purposes it might be more convenient to work with cell populations in a multi-well format and to use a microplate reader for signal detection. The expansion of GEFPs for the use in drug research resulted in the development of specially designed sensors with optimal properties for high-throughput and high-content screening. Chandrasekharan et al., for instance, established a high-throughput real-time in vitro assay for screening of mitochondria targeting cancer drugs, using a mitochondrial redox-sensitive GFP as sensor for mitochondrial damage (Chandrasekharan et al., 2019). Tian et al. exploited the power of FRET-based apoptosis sensors to establish a high throughput drug screening assay which was successfully used to identify new apoptosis-inducing compounds (Tian, Ip, Luo, Chang, & Luo, 2007). The NADH/NAD^+ ratio sensor SoNar was used in a high-throughput screen for agents targeting tumor metabolism (Zhao et al., 2015). Mendelsohn et al. exploited the cytosolic ATeam ATP sensor and fluorescence activated cell sorting (FACS) to screen a CRISPRi library for genes involved in maintaining cellular ATP levels (Mendelsohn et al., 2018). For that purpose, they created a modified, red-shifted ATeam in order to make the sensor more suitable for FACS (Mendelsohn et al., 2018). This represents a recent example of how GEFPs can be exploited to develop innovative assays for high-content pharmacological research.

11. Final remarks

This review gave an overview of genetically encoded fluorescent biosensors, which allow the investigation of cell physiological processes in living cells with high spatiotemporal resolution. We presented sensors for Ca^{2+}, K^+, pH, ATP, and other metabolites, kinase activity, second messengers, ROS and RNS, and protein trafficking, and described the functional principle of different types of biosensors. Furthermore, we brought in remarkable examples of their applications as research tools. By doing so, we want to give scientists a better understanding of the benefits of genetically encoded fluorescent tools for the investigation of metabolic processes and signaling events and help them find biosensors that are suitable for their respective purposes.

Conflict of interests

The authors declare no conflict of interest.

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