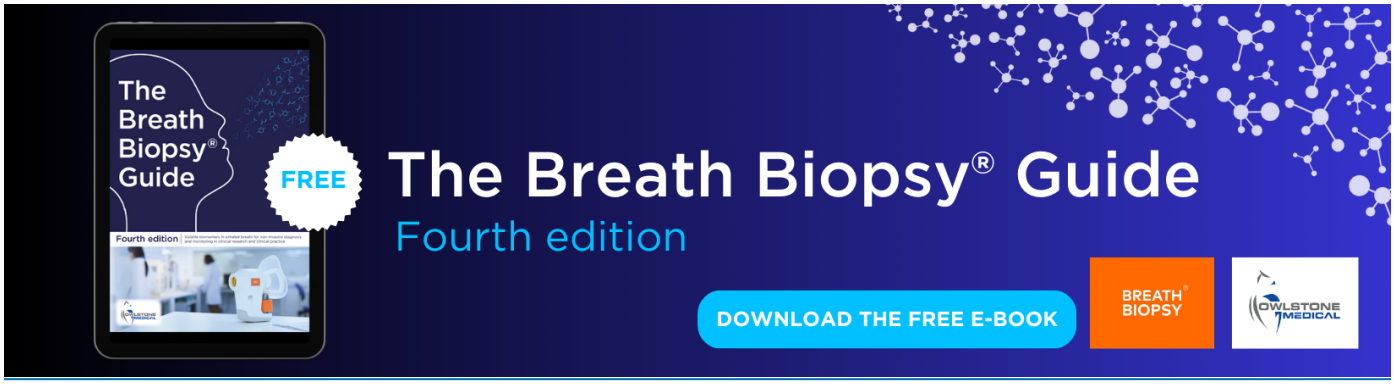
PAPER

Recent progress in developing fluorescent probes for imaging cell metabolites



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Abstract

Cellular metabolites play a crucial role in promoting and regulating cellular activities, but it has been difficult to monitor these cellular metabolites in living cells and in real time. Over the past decades, iterative development and improvements of fluorescent probes have been made, resulting in the effective monitoring of metabolites. In this review, we highlight recent progress in the use of fluorescent probes for tracking some key metabolites, such as adenosine triphosphate, cyclic adenosine monophosphate, cyclic guanosine 5′-monophosphate, Nicotinamide adenine dinucleotide (NADH), reactive oxygen species, sugar, carbon monoxide, and nitric oxide for both whole cell and subcellular imaging.

1. Introduction

Metabolites are essential components in biological systems, and as such, play a significant role in all biological processes [1, 2]. For example, numerous cellular metabolites, such as adenosine triphosphate (ATP), NAD^+/NADH, amino acids, and sugars have been found to be essential in cell physiology and signalling pathways [3]. Meanwhile, the abnormal fluctuation of cell metabolites, such as redox state and nitrogen could result in inflammation and diseases in living organisms [4, 5]. Therefore, analysis of these metabolites would provide deeper insight into both physiological and pathophysiological processes. Metabolomics studies metabolites, the substrates and products of metabolism that drive important cellular functions, such as energy generation and storage, signalling, and apoptosis [5–7]. It has been applied for assessing changes of comparing cell line mutants, drug discovery, toxicology, natural product discovery, studying global effect of genetic manipulation, cancer, and nutrition [8–10]. Despite the importance of metabolomics, it is much less developed than genomics or proteomics because it is much more difficult to detect and quantify metabolites, which vary widely in speciation and concentration, but have only subtle structural differences. Lack of effective metabolomic methods, particularly for *in situ* and real-time detection *in vivo* is a major barrier to our full understanding of physiological and pathophysiological processes.

A common analytical method for most cellular metabolites is mass spectrometry, which is often combined with liquid chromatography [11, 12]. While the mass spectrometry is extremely powerful in detecting numerous metabolites simultaneously, it cannot distinguish between isomers and enantiomers, making it difficult to identify common metabolites such as L-amino acids versus D-amino acids and anomers of sugars [13]. It also can be difficult for living cell analysis of metabolites because the location and concentration of metabolites in living cells can change quickly in response to different signals [14, 15]. Therefore, there is a need to develop a complementary method for rapid, sensitive, and selective detection and quantification of metabolites in living cells and *in vivo*.

Fluorometric assays are such a complementary approach for analysing cell metabolites, which is based on the presence of fluorescence tags or probes [1, 16]. In general, the major advantages of fluorescence analysis of cell metabolites include high sensitivity, capabilities for performing time-based studies of concentration, experiments that are

non-destructive to the cell, and high-throughput detection [17]. While some metabolites can be directly analysed in individual cells by autofluorescence via their intrinsic fluorescent compounds, only a very limited number of the metabolites display autofluorescence [18]. To overcome this limitation, fluorescent probes which combine a molecule that can bind these small-molecule metabolites and a fluorophore can be introduced in cells [1, 19–22].

After introducing the fluorescent probe into cells, a fluorescence microscope can be used to visualise cellular metabolites within their sub-cellular location using different fluorescence imaging analysis methods to achieve deeper understanding of biological processes [23]. Over the past decades, numerous fluorescent probes have been developed and they can be categorized into two types: whole cell and subcellular imaging. In this review, we highlight recent advances in the past 10 years in fluorescent probes for imaging metabolites in either whole cells or subcellular locations, focusing mainly on representative examples of small molecule fluorophore probes, nanomaterial based fluorescent probes, and light-up (aptamer/dye) fluorescent probes. In particular, we discuss the strengths and limitations as well as some new trends in the development with illustrative examples.

2. Whole cell imaging

Cellular metabolite fluctuations are a common feature of many diseases and therefore a promising target for diagnostics and therapeutical interventions. Therefore, a large number of fluorescent probes have been developed to image the cellular metabolites and their applications for the whole cell imaging has been the most extensively studied and widely used in biological studies. (Examples are listed in table 1, figure 1 and table 2.)

2.1. Fluorescent probe for cellular ATP detection

ATP is one of the most important cellular metabolites because it is the primary energy currency in living organisms and plays critical roles in many biological processes. Many efforts to develop fluorescent probes have been made over the last several decades to visualize ATP in living cells. These probes have been developed using both direct and indirect detection mechanisms from a variety of physical formats, such as small organic indicators, nanomaterials, and fluorogenic probes

Magnesium Green is one of the best small organic indicators, developed by Leyssens *et al* in 1996, for indirectly detecting ATP hydrolysis [97]. Most of the intracellular ATP are complexed with Mg^{2+}, while ADP has lower affinity for magnesium ions than ATP. Therefore, hydrolysis of MgATP can lead to an increase in free Mg^{2+} concentration and subsequent increase in Magnesium Green fluorescence. Shin *et al* then applied Magnesium Green to indirectly visualize ATP in hair cells [24]. The fluorescence of Magnesium Green could be excited with illumination in visible range, reducing the phototoxicity. However, Magnesium Green is not a ratiometric probe, showing a simple increase in fluorescence increase upon binding Mg^{2+}, which makes it a challenge to use in quantitative studies. To directly image cellular ATP, quinacrine, another small molecule probe, stains peptide-bound ATP found in high concentrations in intracellular granules [98]. Researchers have been using quinacrine to image ATP release in endothelial and epithelial cells [25–27]. In addition, Pak *et al* developed an imidazolium-based, ratiometric fluorescent probe for ATP with a pyrene excimer clamp [28, 29]. This fluorescent probe will form a pyrene-adenine-pyrene sandwich via π–π stacking when it binds with ATP. Thus, the probes were applied to monitor the decrease of ATP levels in HeLa cells upon addition of an ATP synthase inhibitor (oligomycin).

Aptamers are short, single stranded DNA or RNA oligonucleotides capable of specific, high-affinity molecular binding. Aptamers are widely used in studying small-molecule metabolites, which can be engineered to detect metabolite such as ATP in the nanomolar to millimolar ranges [99, 100]. However, cell permeability and oligonucleotide degradation by nucleases hinder their use in cell imaging [101, 102]. To solve this problem, nanoparticles have been used to deliver and protect aptamers from degradation by nuclease in cells. For example, Qiang *et al* employed a carboxyfluorescein (FAM)-labelled DNA aptamer, which binds to ATP, and polydopamine nanosphere to create a biosensor for protecting the aptamer and quenching its fluorescence [30]. The aptamer released when adding ATP to system. Zheng *et al* constructed an aptamer nano-flare, that can directly quantify ATP in living cells [31, 103]. The aptamer nano-flares were composed of a gold nanoparticle core, which is functionalized with a dense monolayer of aptamers. However, these fluorescence probes employed an 'always on' design, which lacks target-activatable nature, will inevitably result in a high-background and low signal-to-noise ratio [32, 33, 104]. To overcome the problem, Zheng *et al* designed a fluorescence resonance energy transfer (FRET)-based DNA nanoprism with a split aptamer design for ATP sensing in living cells [32]. The nanoprism showed high cellular permeability and successfully realised 'FRET-off' to 'FRET-on' sensing of ATP in living cells [32]. Moreover, Zhao *et al* developed an upconversion nanoparticle conjugated with a photocleavable linker (PC linker) modified ATP aptamer sensors, which can detect ATP in living cells in a conversion luminescence-activatable manner [33]. One disadvantage of this aptamer-based fluorescence probe is that the aptamers are selective for ATP over other nucleotides (GTP, CTP, and UTP), but cannot distinguish between adenine derivatives (ATP, ADP, and AMP) [105].

Table 1. Examples of fluorescent probes (FP) for ATP visualization.

Type	Name	Detection mechanism	Reference
Small molecule FP	Magnesium green	The detection of hydrolysis of MgATP;	[24]
	Quinacrine	Fluorescent dye that binds peptide-bound ATP found in intracellular granules;	[25–27]
	Pincer-like benzene-bridged sensor	Form pyrene-adenine-pyrene sandwich via π–π stacking when it binds with ATP;	[28, 29]
Nanomaterial based FP	P1/PDANS	Fluorophore labelled ATP aptamer release from PDANS when binds with ATP;	[30]
	Aptamer Nano-Flares	Gold nanoparticle core functionalized with a dense monolayer of ATP aptamers;	[31]
	DNA TP nanoprobes	ATP binds to split ATP aptamer on the DNA triangular prism (TP) to induce the fluorescent change;	[32]
	Apt-Act/UCNPs	Upconversion nanoparticle conjugated with PC linker modified ATP aptamer sensor;	[33]
Genetically encoded FP	ATeam	ATP binding of F_1F_0ATP synthase causes an increase in FRET between a CFP and a YFP;	[34]
	QUEEN	ATP binding causes a change in the excitation spectrum of a cpEGFP;	[35]
	MaLion series	ATP binding of F_1F_0ATP synthase causes an increase fluorescence of fluorescent protein;	[36, 37]
	PercevalHR	ATP binding causes a change in the excitation spectrum of circularly permuted yellow fluorescent protein (cpVenus), and measuring cellular ATP/ADP ratio;	[20, 38, 39]

Although both small organic indicators and aptamer-based fluorescent probes have been widely used in imaging studies, they still face a challenge during sample preparation because of the need to introduce exogenous reagent by cell penetration or cell loading. On the contrary, the genetically encoded indicators such as fluorescent protein-based are in part or wholly encoded imaging reagents by a specific gene sequence. The most recently developed fluorescent protein-based probes are capable of undergoing FRET. These probes typically composed of a donor fluorescent protein and an acceptor fluorescent protein that are separated by an analyte binding domain. When binding with the analytes, this domain undergoes a conformational change that changes the distance between two fluorescent proteins, resulting in a change of FRET efficiency. Tsuboi *et al* reported ATP-sensitive K^+ (K_{ATP}) channels fused to a cyan and yellow fluorescent protein FRET pair (ECFP–EYFP) for imaging ATP concentration changes in HEK-293 cells [106]. Imamura *et al* generated a series of FRET-based probes for ATP named 'ATeam', in which the ε subunit of the *Bacillus subtilis* F_1F_0ATP synthase acts as the ATP sensing domain [34]. Yaginuma *et al* reported a ratiometric single fluorescent protein probe called 'QUEEN' (quantitative evaluator of cellular energy) to quantify absolute ATP concentrations [35]. Recently, intensiometric single fluorescent protein probes developed by Aria *et al* and Lobas *et al* enabling the simultaneous visualization of cellular ATP dynamics [36, 37].

Since the absolute ATP, ADP, and AMP can fluctuate, the ratio of ATP/ADP ratio can be a more reliable indicator of cellular energy status. The ratiometric single fluorescent protein probe 'Perceval' developed by Berg *et al* and improved version 'Perceval HR' has been used for measuring cellular ATP/ADP ratio [20, 38]. Zala *et al* also used the Perceval to measure ATP/ADP ratio in neurons and found out that mitochondrial trafficking is dependent on mitochondrial ATP but not glycolysis [39].

2.2. Fluorescent probes for intracellular second messengers

Cyclic adenosine monophosphate (cAMP) is a second messenger of many G protein-coupled receptors (GPCRs) and regulates cAMP-dependent kinase (PKA) and the exchange protein activated by cAMP (Epac) to participate in cellular metabolism. The first cAMP fluorescent probe (FICRhR) for cellular imaging was reported by Adams *et al*, which was a FRET-based probe utilizing dissociation of purified regulatory and catalytic subunits of PKA, sensing the cellular cAMP by microinjection [49]. Later, the

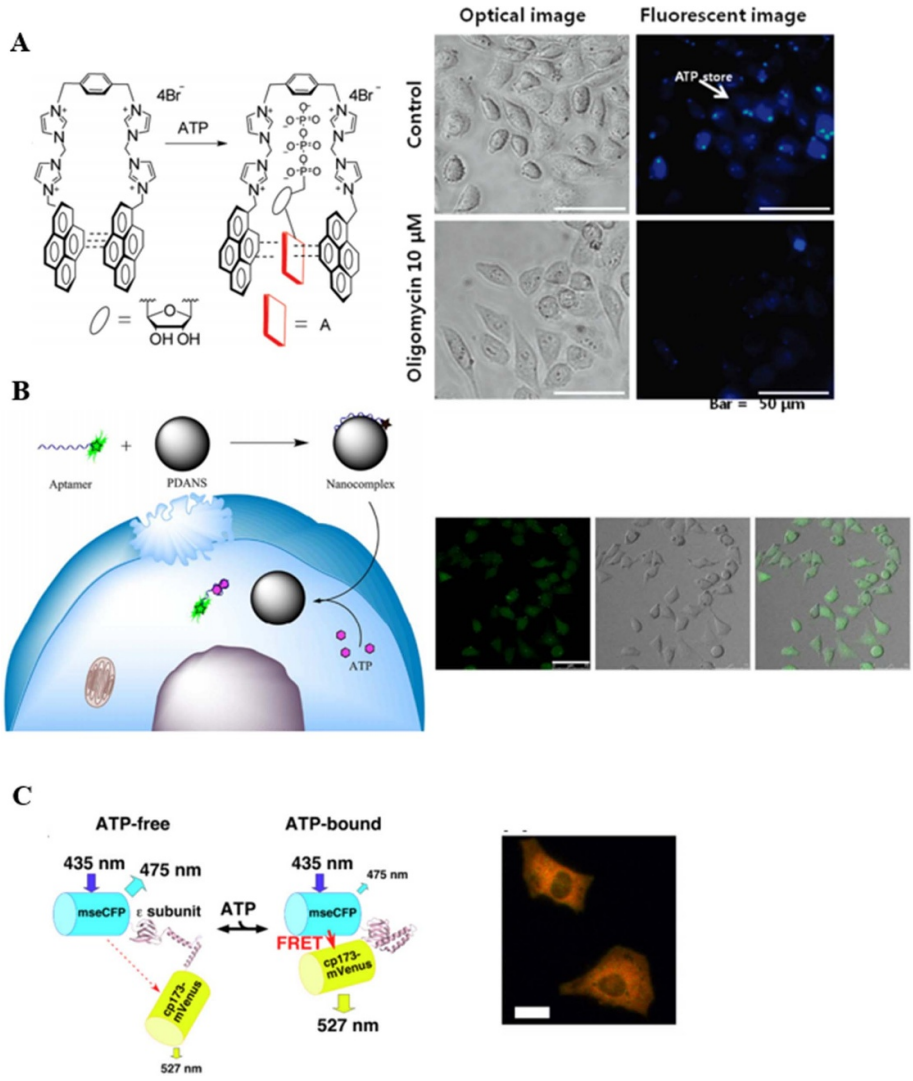

Figure 1. Examples of FP for ATP visualization. (A) Small molecule FP; (B) nanomaterial based FP; (C) genetically encoded FP. Reprinted with permission from [29, 30, 34]. Reprinted with permission from [29]. Copyright (2009) American Chemical Society. Reprinted with permission from [30]. Copyright (2015) American Chemical Society. Reproduced from [34]. CC BY 4.0.

FICRhR probe was applied to investigate the link between cAMP and diverse biological activities and to monitor cAMP levels in the processes of stimulated *Aplysia* neurons [50, 51]. Moreover, the FICRhR probe was also introduced into single cells within brain slice preparations by perfusable patch pipettes [52]. However, the requirement for invasive loading of PKA holoenzyme in this method limits its applications. To solve this limitation, researchers developed genetically encoded versions of FICRhR, which could be introduced into cell by a routine transfection. Zaccolo *et al* developed FICRhR-like genetically encoded probe, which was composed of enhanced blue fluorescent protein-labelled type 2 regulatory subunits of PKA and GFP-labelled catalytic subunits [53]. Subsequently, many labs have also carried out optimization studies on this probe [54–57]. DiPilato *et al* constructed a fluorescent probe to monitor cellular cAMP dynamics and Epac activation by sandwiching the full-length Epac1 between cyan and yellow mutants of GFP [58]. Over the years, multiple labs developed and improved FRET-based fluorescent probe for cAMP with different characteristics regarding sensitivity, kinetics, and dynamic ranges.

As an alternative to the FRET-based methods, single-wavelength methods were developed, which the cAMP binding domain was fused to only one fluorophore. Tewson *et al* first developed a single-wavelength intensiometric cAMP probes cADDis [59], then Moore *et al* optimized the probe that cADDis fused with a 5HT6 receptor and mCherry to target cilia and measure the cAMP ratiometrically [60]. Recently, Kellenberger *et al* first developed a RNA-based fluorescent probe for cyclic di-AMP (cdiA, is also a second messenger in Gram-positive bacteria, some Gram-negative bacteria, and Archaea) by fusing of Spinach2 aptamer to ligand-binding domains of cdiA riboswitches, visualizing intracellular cdiA levels in live *Listeria monocytogenes* strains [61].

The other important second messenger is cyclic guanosine 5′-monophosphate (cGMP), which participates in many physiological processes in

Table 2. Examples of fluorescent probes (FP) for other metabolites visualization.

Type	Name	Target	Detection mechanism	Reference
Small molecule FP	EOS	Glutamate	Glutamate binds of AMPA causes a fluorescence increase of small molecule dye;	[40]
	PTMs	H_2O_2	Based on the oxidation of boronate esters;	[16, 41]
	PFBS		Based on C–S bond cleavage of perfluoro-benzyl sulfonates;	[42]
	NBzF		Based on oxidation-induced C–C bond cleavage of benzils;	[43]
	SO3 H-APL		Based on C–N bond cleavage of anilines;	[44]
	DPPEA-HC		Based on oxidation of phosphorous	[45]
	ANRP	CO	Anchored to cell membrane and sense the CO by a metal palladium-catalysed reaction;	[46]
	DAF	NO	Based on the reaction with NO to furnish fluorescent triazole derivatives;	[47]
Nanomaterial based FP	C_3N_4 Nanoribbons	Citrate	Quenched by $Cu2^+$ and then recovered by the addition of citrate;	[48]
Genetically encoded FP	FICRhR	cAMP	Based on the dissociation of purified regulatory and catalytic subunits of PKA;	[49–57]
	ICUE1		Form Epac1-cyan-YFP sandwich when it binds with cAMP;	[58]
	cADDis		cAMP binding of the regulatory region causes a large conformational change in EPAC 2;	[59, 60]
	cdiA		cdiA riboswitches fused with Spinach2 aptamer;	[61]
	cGES	cGMP	cGMP binding domain from PDEs;	[62, 63]
	cGi		cGMP binding domains from PKG and from PDEs;	[64]
	CGY		cGMP binding of PKGs induced fluorescent change;	[65]
	c-di-GMP-I	c-di-GMP	c-di-GMP riboswitches fused with Spinach aptamer	[66–68]
	Bc RNA		c-di-GMP riboswitches fused with two FP genes;	[69]
	DNB sensor		c-di-GMP binding aptamer fused into DNB, Broccoli as the ;	[70]
	Frex	NADH	NADH binds to Rex subunit causes the conformational change of cpFP;	[71]
	Peredox	$[NAD^+]/[NADH]$	The competition between NADH and NAD^+ for binding to Rex subunit causes the conformational change of two FP;	[72, 73]
	Rex YFP		Different binding affinity of NAD^+ and NADH to nucleotide-binding domains of each Rex subunit;	[74]
	SoNar		Binding of NAD^+ or NADH to Rex subunit both induces changes in protein conformation and fluorescence;	[75, 76]

(Continued.)

Table 2. (Continued.)

Type	Name	Target	Detection mechanism	Reference
	GluSnFR/FLIPE	Glutamate	Glutamate binds to GltI causes the conformational change of two FP;	[77–82]
	FLIPQ-TV	Glutamine	Glutamate binds to GlnH causes the conformational change of two FP;	[82]
	FLIP family	Sugar	Sugar binds to PBPs causes an increase in FRET between two FP;	[83–89]
	roGFP/roGFP2	ROS	ROS binds to the surface-exposed cysteine;	[90]
	CY-RL5	Redox state	Binds to redox linker 5 (RL5) causes an increase in FRET between two FP;	[91]
	COSer	CO	CO binds to CO sensitive heme protein causes the conformational change of cpYFP;	[92]
	sGC	NO	Combining endogenously expressed guanylate cyclase with a FRET-based cGMP indicator;	[93]
	OGsor	2OG	Binds to 2OG-binding domain GAF causes an increase in FRET between two FP;	[94]
	CIT	Citrate	Binds to citrate-binding domain CitA causes an increase in FRET between two FP;	[95]
	Lactate sensor	Lactate	Binds to lactate-binding domain of bacterial transcription factor LldR causes an increase in FRET between two FP;	[88]
	Lapronic	Lactate/pyruvate	Different binding affinity of lactate and pyruvate to the binding domains of transcriptional factor LutR subunit;	[96]

mammals. cGMP can be used to regulate effectors such as cGMP-specific phosphodiesterases (PDEs), cGMP-dependent protein kinases (PKGs) and cyclic nucleotide-activation ion channels. Therefore, the visualization of intracellular cGMP is critical for understanding of cGMP signalling pathway. Several different cGMP binding domains have been used as sensing units in genetically fluorescent probes. For example, the binding domain of Cygnus in cGMP energy transfer sensors (cGES) was from PDEs [62, 63] and cGMP indicators (cGi) [64] were from PKGs and PDEs. These binding domains were used to separate donor fluorescent protein and acceptor fluorescent protein. Moreover, Sato *et al*, reported a fluorescent probe named CGY-del1 for cGMP that contained PKG fused to single fluorescent protein [65]. Honda *et al* optimized the selectivity for cGMP and eliminated the constitutive kinase activity of the binding domain to reduce the disturb from the probe [107, 108].

Breaker's group first reported the Cyclic di-GMP riboswitch (named c-di-GMP-I) [66] in eubacteria and then discovered another c-di-GMP riboswitch termed c-di-GMP-II in the *Clostridium difficile* [67]. Kellenberger *et al* designed two different probes for live cell imaging of c-di-GMP and cyclic AMP-GMP by fusing the Spinach aptamer to variants of a natural GEMM-I riboswitch (c-di-GMP-I), demonstrating the ability to change specificity of the RNA-based probes by taking advantage of rational mutations to the ligand binding domain instead of by inserting distinct aptamers [68]. Zhou *et al* discovered three new c-di-GMP riboswitches (Bc3, Bc4, and Bc5 RNA), which were fused between the two fluorescent protein genes *amcyan* and *turbofp* [69]. Recently, Wu *et al* designed a ratiometric RNA probe that comprised of dinitroaniline-binding aptamer (DNB)-based sensing domain and Broccoli domain to quantify the intracellular c-di-GMP concentration by DNB-to-Broccoli fluorescence ratio [70].

2.3. Fluorescent probes for intracellular NAD pools

Nicotinamide adenine dinucleotide (NAD) is a central cofactor involved in many enzymatic reactions, especially as a major electron carrier in redox reactions [109–111]. NAD exists in two forms, the

oxidized form NAD^+ and the reduced form NADH [111]. NAD^+ can be reduced to NADH in the process of glycolysis and in the tricarboxylic acid (TCA) cycle [112]. NADH can be re-oxidized back to NAD^+ in the electron transfer chain [111]. Meanwhile, NAD can also be phosphorylated to NADP via NAD kinases [111]. The NAD^+/NADH redox couple is served as a regulator of cellular energy metabolism of glycolysis and mitochondria oxidative phosphorylation [113, 114]. While $NADP^+$, together with its reduced form NADPH, maintain redox balance and support the biosynthesis of fatty acids and nucleic acids [114]. Therefore, similar to ATP, the NAD pool plays an important role in cellular energy balance, which is determined by the ratio of NAD^+/NADH and $NADP^+$/NADPH. Nowadays, the well-developed fluorescent probes for cellular NAD pool imaging are genetically encoded fluorescent probes. Zhao *et al* first inserted the circularly permuted fluorescent proteins (cpFPs) into NADH sensing domain (Rex) subunit to sense the NADH [71]. To date, there are several genetically encoded fluorescent probes that can detect NAD^+/NADH ratios: Peredox [72, 73], Rex YFP [74], and SoNar [75, 76]. Peredox and Frex family probes were based on inserting a cpYPs into the Rex dimer between its subunits, detecting the NAD^+/NADH ratios via Rex intersubunit interactions. While Rex YFP and SoNar were based on integrating a circularly permuted yellow fluorescent proteins (cpYFPs) into the loop between nucleotide-binding domains of each Rex subunit.

2.4. Fluorescent probes for intracellular amino acids

Glutamate plays a critical role in amino acid metabolism, participating not only signal transduction, but also regulating nitrogen circulation together with glutamine and 2-oxoglutarate [115–117]. The glutamate-sensing fluorescent reporter (GluSnFR) [77–79] and fluorescent indicator protein (FLIP) for glutamate [80–82] were the primary genetically encoded fluorescent probes, which fused the glutamate periplasmic binding protein (PBP) GltI (also known as ybeJ) to enhanced cyan fluorescent protein and a yellow fluorescent protein Citrine [118] or Venus [119]. Marvin *et al* also reported an intensity-based GluSnFR (iGluSnFR), fusing the binding protein GltI to cpFPs cGFP [120, 121]. Namiki *et al* developed a small molecule fluorescent probe, which consists of the mutated glutamate receptor GluR2 subunit of an alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and a small molecule fluorescent dye, termed EOS [40]. Gruenwald *et al* studied another pivotal amino acid, glutamine, measuring the glutamine concentration in living cells by an array of FLIPQ-TV sensors with different affinities [82]. Okada *et al* applied bacterial PBPs to construct FRET-based probes, taking advantage of PBPs' structure to expand the dynamic range of the probes [122].

2.5. Fluorescent probes for intracellular sugar

Sugar metabolism is involved in many types of metabolic reactions in living organisms. The PBPs have been used as the substrate-binding element of protein-based fluorescent probes by linking to fluorescent protein. These fluorescent probes are named as FLIP family probes (which are also FRET-based probes) due to the hinge-bend movement of probes leading to FRET response. Frommer *et al* used PBPs to design a series FLIP probes to monitor the intracellular distribution of many sugars including glucose, galactose, maltose, ribose, arabinose, sucrose, lactate, and trehalose [83–89]. Since the intracellular sugar concentrations and distribution were directly related to carbohydrate metabolism, these FLIP probes have been broadly applied in many areas, such as the food, pharmaceutical, and biofuel industries. Ballerstadt *et al* reported a fluorescent affinity hollow fibre probe for transdermal glucose monitoring, which consisted of the dyed beads (Safranin O and Pararosanilin) and Alexa488-Con A (Alexa fluor 488 labelled Concanavaline A) inside a hollow fiber dialysis membrane [123]. Then Heo *et al* developed a probe based on fluorescent hydrogel fibers for long-term monitoring of glucose *in vivo* [124]. Although the use of protein-based fluorescent probes in metabolite research and bioprocess visualization has progressed, there is still a need to develop new probes for other critical sugar metabolites.

2.6. Fluorescent probes for intracellular redox state

Reactive oxygen species (ROS) refers to a series of key oxygen (O_2) metabolites, including hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), hypochlorous acid (HClO), superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), ozone (O_3), and organic peroxides [125–132]. The changes of intracellular ROS will influence the redox equilibrium, resulting in macromolecular damage and is implicated in various diseases such as atherosclerosis, diabetes, neurodegeneration, cancer, and aging [126]. Numerous fluorescent probes have been developed to image the intracellular ROS or redox states. The genetically encoded fluorescent probes for sensing ROS or redox states were taking advantage of existing a sensing domain from naturally occurring protein structures by linking to fluorescent proteins. For example, redox-sensitive GFP with two surface-exposed cysteines close to the chromophore was used to monitor the molecule's own redox states [90, 133, 134]; redox-sensitive polypeptide-flanked CFP/YFP leads to FRET signal changes response to different redox states [91]; the environmentally sensitive fluorescent protein (Venus) was fused with responsive domain of the transcriptional regulatory protein OhrR to visualize organic

hydroperoxides, which constantly generate cellular stress [135]. The other types of fluorescent probe were based on small molecules. Taking H_2O_2 as an example, the majority of H_2O_2 probes were based on the oxidation of boronate esters [16, 41]. Other H_2O_2 sensing reactions cover C–S bond cleavage of perfluoro-benzyl sulfonates [42], oxidation-induced C–C bond cleavage of benzils [43], C–N bond cleavage of anilines [44], and direct oxidation of phosphorous [45], selenium [136, 137] and tellurium [138]. Most of the small molecule-based fluorescent probes for redox states have been extensively reviewed [16, 139–142]. Readers wishing to further advance this field would be advised to read recent discussion from Tampieri and Lu in order to address which ROS are present in solution [143, 144].

2.7. Fluorescent probes for intracellular other significant metabolites

Carbon monoxide (CO) is generally regarded as a toxicant or pollutant. Nevertheless, more and more studies suggest that CO, like NO, functions as an essential second messenger [145]. Wang *et al* reported a genetically encoded fluorescent probe COSer for monitoring intracellular CO by fusing a dimeric CO-sensitive heme protein to cpYFP [92]. Xu *et al* reported a novel cell membrane-anchored fluorescent probe ANRP, which complexed ANR (a cell membrane-anchored fluorophore designed by grafting a positive charged ammonium group onto a long and linear hydrophobic Nile Red molecule.) with palladium, monitoring the release of CO from living cells [46]. Sato *et al* have reported a novel cell-based fluorescent probe to visualize picomolar levels of NO release from living cells, made by combining endogenously expressed guanylate cyclase with a FRET-based cGi [93]. In addition, the small molecule-based fluorescent probes for NO were well-developed. The most common method involves the use of *o*-Diamino aromatics under aerobic conditions, which firstly reported by Nagano's group [47]. In the presence of O_2, *o*-Diamino aromatics could react with NO to furnish fluorescent triazole derivatives [146–148]. Other fluorescent probes are summarized in several reviews [149, 150]. 2-Oxogluatarate (2OG) is another metabolite that plays an important role in metabolism and also serves as a signalling molecule in various organisms. Zhang *et al* reported FRET-based genetically encoded fluorescent probe for detecting 2OG in real-time; results showed the probe's dynamic range appeared identical to the physiological range observed in *Escherichia coli* [94]. Citrate is also a critical metabolite in various biological systems, such as mitochondrial energy generation, inflammatory response, blood coagulation, and cytosolic biomacromolecular synthesis [48, 151]. Ewald *et al* developed FRET-based genetically encoded fluorescent probe for citrate; they optimized peptide linkers to achieve an optimal change ratio and modified the citrate-binding pocket to obtain a probe with the proper affinity for the application [95]. Hu *et al* also reported another fluorescent probe, which was based on carbon nitride nanoribbons for visualizing intracellular citrate anion [48]. Lactate also plays metabolic and signalling roles in healthy tissues. As the fluctuation of lactate level is associated with inflammation, hypoxia/ischemia, neurodegeneration, and cancer, visualizing intracellular lactate levels has diagnostic and therapeutic applications [88]. San Matín *et al* reported a genetically encoded FRET lactate probe that discriminates lactate flux in different cells; results showed T98G glioma cells have 3–5-fold higher rate of lactate production than normal cells [88]. Recently, Galaz *et al* developed a genetically encoded FRET probe Lapronic for imaging lactate/pyruvate ratio in living cells' cytosolic and mitochondria matrix, allowing the assessment of glycolytic/oxidative metabolism with a straightforward fluorescent readout [96].

3. Subcellular imaging

Sub-cellular organelles are specialized subunit within cells, which are usually enclosed by their own lipid bilayer. The main eukaryotic organelles include nucleus, mitochondria, lysosome, endoplasmic reticulum (ER), and Golgi apparatus. All of these organelles play a critical and indispensable role in cellular processes [152, 153]. The dynamic fluctuations of intracellular metabolites in subcellular microenvironments determine cellular metabolism, homeostasis, signal conduction, and immunity; abnormal levels of the sub-cellular metabolites can cause disorders, which are associated with various major diseases [154–156]. Monitoring intracellular metabolites in subcellular structures is therefore important for bioanalysis and related drug discovery. Organelle fluorescent probes mainly contain three domains: localizing group, fluorophores, and recognition domain [154, 157, 158]. Fluorescent probes with diverse localizing groups can be localized in specific organelles by utilizing different physiochemical properties of diverse organelles [159]. After reaching the subcellular location of interest, they can subsequently target or react with diverse metabolites by recognition domain and further make detectable signal changes via different response mechanisms. Herein, we summarized the fluorescent probes for monitoring the fluctuation of metabolites with subcellular accuracy.

3.1. Fluorescent probes for imaging mitochondrial metabolites

Mitochondria, the double-membrane constructed organelles and the primary compartments for intracellular respiration in most eukaryotes, regulate energy generation, calcium circulation, protein synthesis, cell proliferation, division, and death pathways

[160]. Abnormal metabolite levels in mitochondria may lead to mitochondrial dysfunction, which is related to neurodegenerative disease, malignant cancers, and cardiac diseases. Thus, fluorescent probes that specifically accumulate in mitochondria play critical roles in monitoring the mitochondrial functions and investigating various mitochondrial disease [161–166]. During mitochondrial respiration, proton pumps in the mitochondrial inner membrane transport protons into the mitochondrial membrane space, resulting in a highly negative mitochondrial transmembrane potential (MMP, approx. −180 mV) [167, 168]. Therefore, most fluorescent probes for mitochondria attract the negative potential of the mitochondrial membrane by cations. The fluorescent probes with intrinsic or post-functionalized cationic aromatic structures can be applied to image mitochondria. Delocalized lipophilic cations (DLCs) have been shown to possess the ability to localize mitochondria [169–171]. Typical DLCs ligands include triphenylphosphonium (TPP), quinoline derivatives, and positive charged pyridine; rhodamine and cyanine are the common fluorophores for the design of probes for mitochondria imaging (figure 2) [156, 172–178]. Except for MMP based fluorescent probes, mitochondria transport proteins have also been developed for targeting. The localizing ligands, such as peptides and pyruvate, possess the affinities for specific mitochondrial protein, which have been used for designing the probes for imaging mitochondria [160, 179–181].

ATP is primarily produced in mitochondria and the fluctuation of ATP will lead to, or is caused by, mitochondria dysfunction. Thus, it is essential to monitor the ATP in and around the mitochondria. Until now, several methods have been developed. The genetically encoded fluorescent probes for imaging mitochondrial ATP are based on fusing a mitochondrial localizing sequence to a fluorescent protein gene. Imamura *et al* fused a duplex of the mitochondrial localizing signal of cytochrome c oxidase subunit VIII to the N terminus of ATeams indicator, which made the indicator localized to the mitochondria properly [34]. Then, Depaoli *et al* applied this mitochondria-localizing ATeams indicator to investigate the dynamic of mitochondrial ATP pools in response to acute glucose removal, glucose substitution, as well as mitochondrial toxins [185]. Over the past decades, only few small molecule-based fluorescent probes developed for localizing mitochondrial ATP and based on MMP. Srivastava *et al* first developed a photoinduced electron transfer based molecular scaffolds/fluorescent probes that can monitor mitochondrial ATP [186]. Wang *et al* then developed a multisite-binding, switchable fluorescent probes ATP-Red 1 to monitor mitochondrial ATP levels [187]. Tan *et al* reported a fluorescent probe named Mito-Rh to real time monitor mitochondrial ATP, which was constructed by an ATP-sensitive fluorophore rhodamine, ATP reaction site diethylenetriamine and mitochondria-localizing site TPP [182]. Recently, Ren *et al* reported a novel ratiometric fluorescent probe Rh6G–ACFPN for quantitatively detecting the mitochondrial ATP levels [188]. Several nanoparticles have been developed to delivery fluorescent probes to mitochondria. Deng *et al* developed zeolitic imidazole frameworks to encapsulate the ATP sensitive fluorophore Rhodamine B, monitoring mitochondrial ATP fluctuation during cellular glycolysis and apoptosis [189]. Liu *et al* developed yellow emissive single-layered graphene quantum dots with dual recognition sites including π-conjugated single sheet to sense ATP and positively charged site to localize in mitochondria [183]. Recently, our group applied positively charged nanoparticles called DQAsomes to deliver a PC ATP aptamer sensor to mitochondria for spatiotemporally controlled monitoring of mitochondrial ATP fluctuation [184]. This approach kept the fluorescent probe inactive before reaching the mitochondria and can be activated by light to detect ATP.

To make sure cellular energy supply, diverse electron-transport chain (ETC) reactions for ATP synthesis are performed in mitochondria. The electrons leaked from ETC can react with oxygen molecules to generate $O_2^{\bullet-}$ and transformed into H_2O_2, $ONOO^-$, $^{\bullet}OH$, $^1O_2$ *et al* under the catalysis of diverse enzyme [173–175, 190, 191]. These metabolites can not only maintain the mitochondria redox homeostasis, but also regulate the cellular function. Various of fluorescent probes for mitochondria imaging have been developed for visualizing ROS levels during the cell processes. For genetically encoded fluorescent probes, the mitochondria-localizing sequences are fused to ROS sensitive fluorescent protein sequences so that the expressed protein probes can respond to mitochondrial ROS. Hyper is a classic family of genetically encoded probes for H_2O_2, which consists of circularly permuted YFP (cpYFP) inserted into the regulatory domain of the prokaryotic H_2O_2-sensing protein OxyR. Researchers developed hyper probes by fusing the mitochondria-localizing sequences so that the probes can localize to the mitochondria matrix [192, 193] and mitochondria intermembrane space [194] of HEK and other cells. Wang *et al* employed adenovirus-mediated gene transferred to express ROS sensitive cpYFP in the mitochondrial matrix of cultured adult cardiac myocytes using the cytochrome C oxidase subunit IV (COX IV) localizing sequence (mt-cpYFP) [195]. TPP modified small molecule fluorescent probes have been widely applied in mitochondria specific metabolites imaging. For example, MF-DBZH [190] and HKSOX-1 probes [196] for sensing $O_2^{\bullet-}$; PMN-TPP [197], RMClO-2 [198] and RSTPP [199] probes for sensing HOCl; MNAH probe [200] for sensing $^1O_2$. Other cationic groups are widely used in the design of probes for imaging

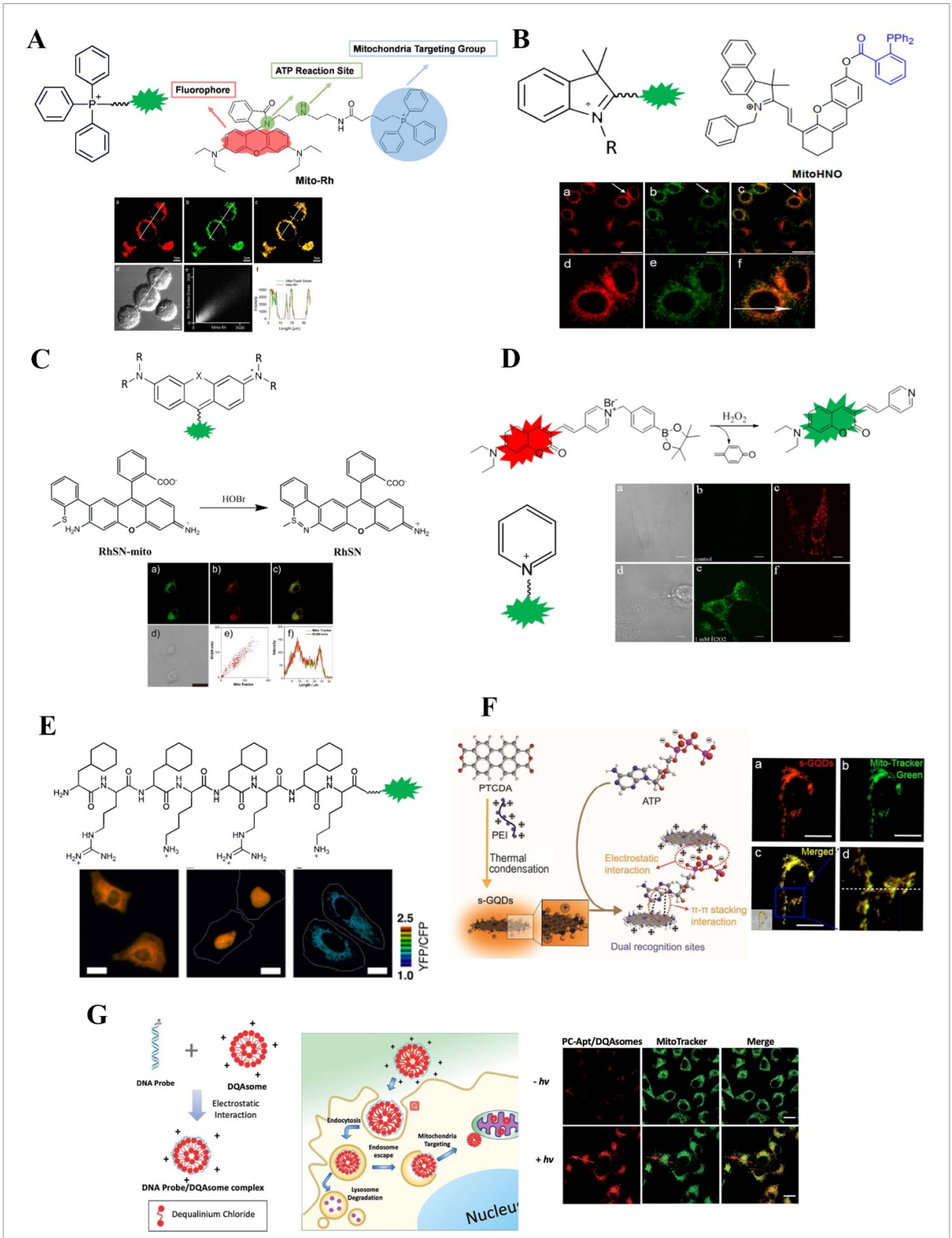

Figure 2. Integration of common groups and their application examples for mitochondrial metabolite probes design: (A) TPP cation. Reprinted with permission from [182]; (B) indolium cation. Reprinted with permission from [178]; (C) rhodamine cation. Reprinted with permission from [175]; (D) pyridinium cation. Reprinted with permission from [176]; (E) mitochondrial localization sequence. Reprinted with permission from [34] and selection nanomaterials for visualizing mitochondrial metabolites: (F) s-GQDs. Reprinted from [183]; (G) DQAsome. Reprinted with permission from [184]. Reprinted with permission from [182]. Copyright (2017) American Chemical Society. Reprinted from [178], Copyright (2016), with permission from Elsevier. Reprinted with permission from [175]. Copyright (2017) American Chemical Society. Reprinted from [176], Copyright (2018), with permission from Elsevier. Reproduced from [34]. CC BY 4.0. Reproduced from [183] with permission of The Royal Society of Chemistry. Reproduced from [184] with permission of The Royal Society of Chemistry.

mitochondria, such as cyan [201–203] and rhodamine [175, 198, 199, 204–206], which are also served as the fluorophore for the probe. In addition, peptides have also been developed for the design of fluorescent probes for imaging mitochondria [207]. TPP group has also been used in fluorescent nanoparticle probes. For example, Gong *et al* reported a mitochondrial oxidative stress amplifier to image GSH, MitoCAT-g,

which consists of carbon-dot-supported atomically dispersed gold (CAT) with further surface modifications of TPP and cinnamaldehyde [208].

NADH is the important hydrogen carrier during TCA cycle (TCA cycle), and can release more energy to cells. Mitochondria-localizing sequences have also been used in genetically encoded fluorescent probes for mitochondrial NADH imaging. The genetically encoded NADH sensors, such as Frex, Peredox, RexYFP, and SoNar, are already genetically introduce to mitochondria by fusing the mitochondria-localizing sequences [74, 209–211].

Owing to the crucial biological function of mitochondria, fluorescent probes for imaging mitochondrial metabolites have been widely developed and employed. Nevertheless, most of the localizing ligands are lipophilic cationic structures, which may reduce the MMP. Furthermore, the fluorescent probes can easily leak out or become untargeted due to the MMP lost during various stimulations and cannot work precisely in these situations. Although genetically encoded fluorescent probes are able to anchor to mitochondria covalently with high selectivity and avoid the fluorescent probe leakage or poor targeting, we cannot guarantee the gene transfection efficiency, and the repeated washing steps may cause the change of mitochondrial microenvironment. In addition, the process of gene transfection makes the genetically encoded fluorescent probes difficult to apply in *in vivo* imaging. As we know, there are thousands of biomolecules in mitochondria, such as mitochondrial DNA, RNA, enzymes, ions, lipids, and amino acids. So, *in situ* imaging and measurement of specific mitochondrial metabolites at ultralow concentrations using fluorescent probes remains a challenge. A lot of reported fluorescent probes only work when cells are stimulated by various chemical agents or abundant of exogenous analytes are added. There are still relatively few probes can detect basal concentrations, which limits the further explorations of physiological and biological functions of these metabolites. Ratiometric fluorescent probes are promising for eliminating these interferences, but only limited ratiometric fluorescent probes for mitochondria imaging have been developed until now.

3.2. Fluorescent probes for imaging nuclear metabolites

The nucleus is the crucial organelle that serves as the container of the majority of DNA in cells, maintaining the integrity of genes and regulating the gene expression to control the cell activity [212, 213]. The nucleus is enveloped by a double-layered membrane containing hundreds of nuclear pores and ribosomes. Some small molecules and ions can permeate via the nuclear membrane freely. Meanwhile, the large biomacromolecules, such as RNA and ribosomes, can transit through the nuclear pores by energy related pathway. Owing to the large amount of DNA in nucleus, small cationic fluorescent probes with two or more cationic centres and hydrophobic planar aromatic structure can be used to target to the minor grooves in negatively charged DNA double-strands to accomplish the nuclear localizing, such as commercialized Hoechst dye or DAPI [212, 214]. Dickinson *et al* reported another nucleus-localizing ligand modified probe, arylboronate based fluorescent probe, for imaging the nuclear H_2O_2 [215]. Moreover, modifying the fluorescent probes with nuclear localization signal (NLS) peptides enables the probes to bind to importins and further delivering into nucleus through nuclear pores (figure 3(A)) [213]. For example, Wen *et al* developed a peptide conjugated small molecule probes based on 1,8-naphthalimide and boric acid ester for imaging nuclear H_2O_2 [216]. Meanwhile, protein tagging is also a promising strategy for the development of fluorescent probes for imaging nucleus. The protein tagging ligands used for imaging nucleus include SNAP-tag [217, 218], HaloTag [219], coumarin [220] and HaloRT ligand. Imamura *et al* reported FRET-based genetically encoded fluorescent probe fused with the SV40 large T-antigen sequence to imaging the nuclear ATP [34]. However, nanomaterials are rarely used in the imaging of nuclear metabolites due to size limitation for nanomaterials to localize in nucleus [221].

Several reports have shown that the modification of NLS peptides to molecules or nanomaterials can be sufficient to drive to the fluorescent probe to the nucleus specifically, and using this method, nucleus-localizing cancer therapy has been broadly studied. However, many fluorescent probes need long incubation time for positioning to nucleus due to the low targeting efficiency. Meanwhile, it is difficult to directly conjugate the probes with nucleus-localizing molecular scaffolds or NLS peptides, probably due to the minor structural alterations which can reduce the affinity of localizing ligands [154, 158]. Protein tagging appears to be an ideal way for positioning the probes to almost any organelles including the nucleus. But the synthesis process is difficult and complex, and the loss of solubility of probes after binding to the substances may hamper the application of protein tagging probes or genetically encoded fluorescent probes. In addition, non-specific or un-tagged fluorescent probes need to be washed out repeatedly before imaging to lower the background fluorescence, which can change the nucleus microenvironment of cells. Therefore, the development of novel ultrasensitive fluorescent probes for imaging nuclear metabolites should be given more attention.

3.3. Fluorescent probes for imaging lysosomal metabolites

The lysosome is the main digestive compartment in cells, where many macromolecules are delivered for degradation [159]. The main characteristics of

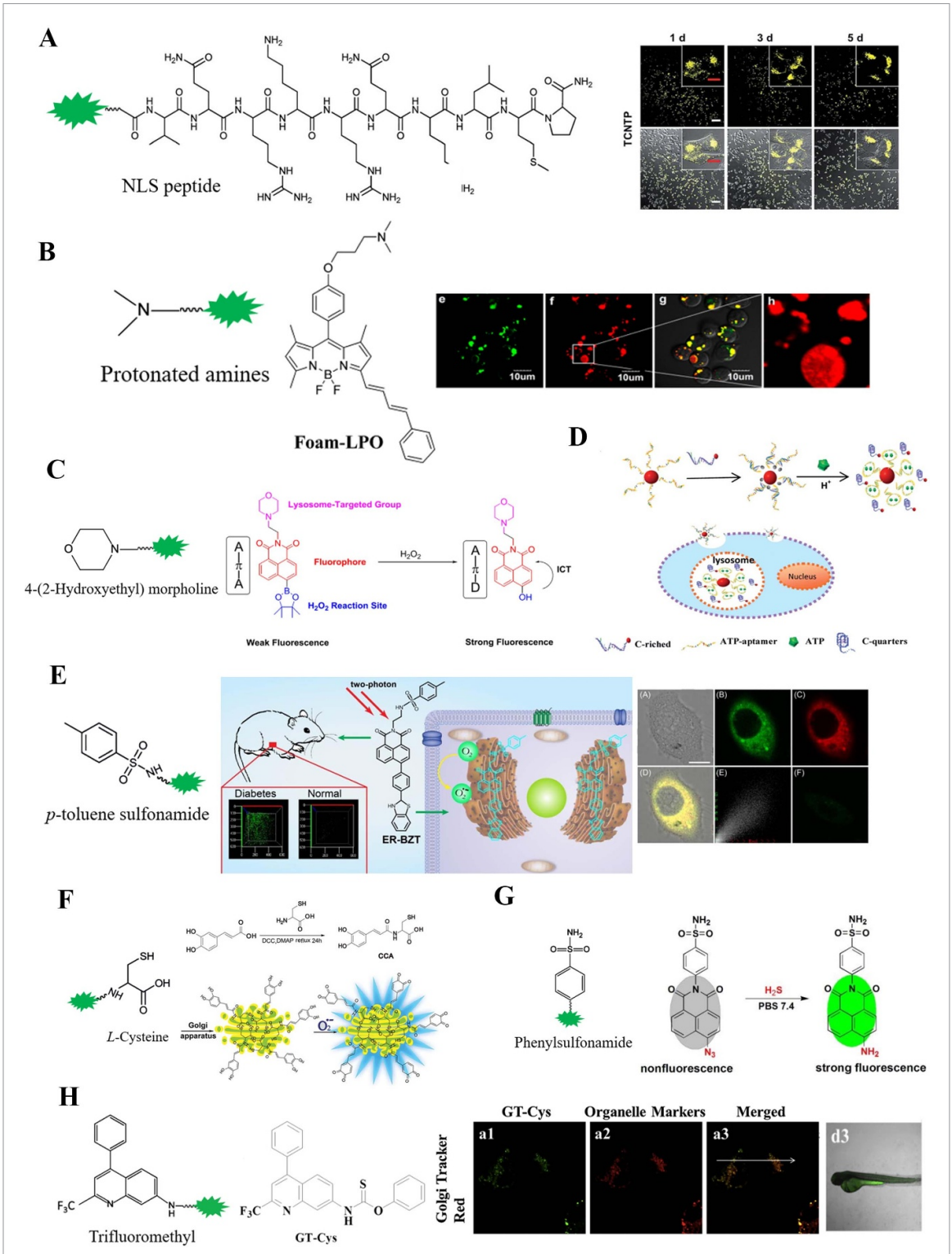

Figure 3. Integration of typical groups and their application examples for (A) nucleus; (B)–(D) lysosome; (E) ER; (F)–(H) Golgi apparatus localized fluorescent probes. Reprinted with permission from [213, 222–228]. Reproduced from [213] with permission of The Royal Society of Chemistry. Reprinted with permission from [222]. Copyright (2015) American Chemical Society. Reprinted from [223], Copyright (2016), with permission from Elsevier. Reproduced from [224] with permission of The Royal Society of Chemistry. Reprinted from [225], Copyright (2017), with permission from Elsevier. Reproduced from [226] with permission of The Royal Society of Chemistry. Reproduced from [227] with permission of The Royal Society of Chemistry. Reprinted from [228], Copyright (2020), with permission from Elsevier.

lysosomes are acidic microenvironment (pH ∼ 5.0) and an abundance of hydrolases [158]. Most importantly, lysosomes are responsible for foreign substance scavenging, digestion, and autophagy [229–231]. As the importance of lysosomes have been understood, more and more fluorescent probes for imaging lysosomal metabolites have been developed.

Similarly, the genetically encoded fluorescent probes localized in lysosome by fusing the lysosome localizing sequences. For example, McCue *et al*

reported a genetically encoded fluorescent probes fused with lysosomal resident membrane protein LAMP1 to image lysosomal Ca^{2+} [232]. Other types of fluorescent probes are usually modified with lipophilic amines for driving probes into lysosomes. The protonated amines in lysosomes are membrane permeable so that the lysosomes can entrap the probes selectively (figure 3(B)) [222, 233]. The lysosome-localizing ligands morpholine has been broadly applied in the development of lysosome-localizing small molecule fluorescent probe for imaging the lysosomal ATP, H_2O_2, HOCl, NO, and HNO (figure 3(C)) [223, 234–240]. The other popular localizing ligand *N,N*-dimethylethylenediamine has been used for imaging lysosomal NO and H_2S [241, 242]. Recently, Jun *et al* reported a ratiometric two-photon fluorescent probe Lyso-ATP for imaging lysosomal ATP by changing the core into rhodamine 6G and introducing a BODIPY at the end of the tetraamine chain, which showed the lysosome fusion process [243]. Nanomaterials have also been developed for imaging the lysosomal metabolites. For example, Jin *et al* constructed a nanoflare composed of AuNP, i-motif and ATP aptamer to image lysosomal ATP (figure 3(D)) [224]. The fluorescent carbon dot developed by Geng *et al* was also used in imaging the lysosomal ATP [244].

Although, in recent years, the development of fluorescent probes for imaging lysosomal metabolites has been made significant progress. There are still remaining certain limitations. For instance, the localizing principle of most reported fluorescent probes are based on trapping lipophilic amines and cannot be used to differentiate between endosomes, autophagosomes, autolysosomes and other acidic organelles. Furthermore, these localizing ligands are toxic to living cells because they can cause the alkalization of lysosome microenvironment, making them unsuitable for long-term tracing. While the genetically encoded fluorescent probes need to be washed out to lower the background fluorescent before imaging, which may also influence the lysosome microenvironment. Meanwhile, the fluorescent of some probes can be quenched owing to the acidic lysosomal microenvironment. The emission wavelengths of most of the reported probes are located in the visible region, which hinder the application in deep tissue imaging due to the poor penetration capability. These shortcomings greatly limit the development and application of lysosome-localizing fluorescent probes. Therefore, it is still a challenge to develop the fluorescent probes for imaging lysosomal metabolites with strong anti-interference capability, outstanding selectivity, and high sensitivity.

3.4. Fluorescent probes for imaging the metabolites in ER

The ER can be divided into rough ER and smooth ER based on whether they contain ribosomes. The rough ER is responsible for protein synthesis, while the smooth ER is mainly in charge of lipid and carbohydrate metabolism and calcium signalling. During these biological processes, the metabolites like ATP, ADP, ROS, NO, HNO, and H_2S are essential for ER functions. Once the homeostasis of metabolites in ER is out of control, it can cause several types of disorder in ER. Therefore, the development of ER-localizing fluorescent probes and tracing of the fluctuation of metabolites become a new strategy to study the metabolism process in ER.

The ER-localizing sequences have been fused to genetically encoded fluorescent probes. Vishnu *et al* reported ER-localizing ATeam ERAT4.01 to record the ER ATP changes in real-time, revealing that the ATP levels within the ER were significantly lower than in the mitochondria and that Ca^{2+} release from the ER induced ATP increase within ER lumen [245]. Then this ER-localizing probe had also been applied in imaging ATP depletion of ATP/ADP exchanger in ER membrane [246]. The most popular ER-localizing ligand for small molecule fluorescent probes is *p*-toluene sulphonamide, such as ER-BZT for $O_2^{\bullet -}$ (figure 3(E)) [225], ER-ClO for HOCl [247], ER-Nap-NO for NO [248], and Na-H_2S-ER for H_2S [249].

Over the past decades, a series of fluorescent probes have been developed for imaging specific metabolites selectively in ER. However, the targeting principle of these fluorescent probes still remains unknown, which hamper the development of ER-localizing fluorescent tools and the understanding of physiological and biological functions of different metabolites. Although the conjugation of ER-localizing dyes seems feasible, a slight change of molecular charge and bulk may influence the targeting capability. Meanwhile, the protein tagging methods can also be used to drive the fluorescent probes to ER, but the synthetic difficulty and complexity make this probe need to be further improvement. In addition, the emission wavelength of most of the probes are also localized in visible region, which hinder the *in vivo* application. Until now, probes for imaging and quantification of *in situ* metabolites in ER are still rare, so the development of this kind of probe could speed up the understanding of biological functions of ER.

3.5. Fluorescent probes for imaging the metabolites in Golgi apparatus

The Golgi apparatus is another crucial intracellular organelle for the modification, storage and transportation of carbohydrates, lipid and proteins. Modified and labelled cargoes in Golgi apparatus will be transferred to the final destinations, such as lysosomes and cytoplasmic membrane, and further exert the biological functions [158, 159, 226]. Notably, the stress-signalling overload in Golgi apparatus can result a series of disorders and further lead diverse diseases.

However, the probes for imaging the metabolites in Golgi apparatus have not been well developed.

The developed Golgi apparatus-localizing genetically encoded fluorescent probes, by fusing amino acids 1–60 of the human galactosyl transferase truncated at position 60 in its luminal domain to N terminus of sensing domain, have been used to image Zn^{2+} in Golgi apparatus [250]. However, it has not been applied to the imaging of other metabolites in Golgi apparatus. Owing to a large quantity of cysteine residue recognition receptors in Golgi apparatus, the localizing ligand of some small molecule fluorescent probes are containing *L*-cysteine, such as CCA for $O_2^{\bullet-}$ (figure 3(F)) [226] and SF-1 for HOCl [251]. Recently, Zhu's group reported two small molecule fluorescent probes for sensing H_2S in Golgi apparatus, one is containing a phenylsulfonamide moiety as a localizing group and a 1,8-Naphthalimide moiety as a sensing group (figure 3(G)) [227], the other one has a trifluoromethyl moiety as a localizing group and quinoline as a sensing group (figure 3(H)) [252]. They also applied the trifluoromethyl moiety and thiobenzoate moiety to image the cysteine in Golgi apparatus [228].

Due to the lack of effective localizing ligand, fluorescent probes for imaging the Golgi apparatus have not received much attention. Although *L*-Cysteine has been reported to be a promising localizing ligand, we still need to do the further investigation of universality. With the development of the enzyme-activated probes, they will have the potential to apply in the Golgi apparatus-localizing fluorescent probes.

4. Summary and prospective

Metabolites serve critical roles in biology and any imbalance or fluctuation of these metabolites may result in diseases such as cancer, diabetes, obesity and neurodegeneration [2, 253]. The detecting and imaging cellular metabolites can help us to understand their roles in cellular metabolisms under both physiological and pathological conditions, thereby providing a powerful basis for diagnosis and treatment of these diseases. The development of fluorescent probes has made a remarkable contribution to biology, making it possible to observe the biochemical process directly inside living cells and sub-cellular organelles [254, 255]. Therefore, fluorescent probes for imaging subcellular metabolites have attracted much attention. Based on various localizing ligands, recognition groups, and fluorophores, many subcellular localized probes have been developed and applied in different areas, such as monitoring inflammation, diabetes, depression, and cancers. The development of these fluorescent probes has also greatly promoted our understanding of molecular mechanisms of different biological processes. In this review, we summarize the fluorescent probes for imaging metabolites in whole cells and subcellular systems. Localization, detection, and response principles are also discussed. Even though many fluorescent probes have been developed and applied, there are still several challenges in their application.

Although researchers have made extensive efforts in the development of fluorescent probes for imaging metabolites with subcellular accuracy during past decades, the development of fluorescent probes for whole cell imaging are significantly more than that of fluorescent probes for subcellular imaging. The main reasons for this limitation are the lack of efficient subcellular localizing ligands and potential toxic effects. For example, the most widely used mitochondria-localized ligand TPP can decrease MMP due to the cationic feature, and the TPP-modified fluorescent probes can leak out when the MMP is lost; the lysosome-localized ligand morpholine can cause the alkalization effect. Otherwise, the strategies that can efficiently localize sensors in other organelles, such as ER, Golgi apparatus, or the nucleus, are still rare, which severely limits the understanding of biological processes in these sub-cellular organelles. In addition, this limitation also affects the development of genetically encoded fluorescent probes for imaging the subcellular metabolites. Therefore, it is very important to develop the reliable localizing strategies and novel non-toxic localizing ligands for imaging the metabolites in subcellular organelles.

Furthermore, many of the cellular metabolites discussed here are present in ultralow concentration, sensitive to the environment, and have a short lifetime. Most reported probes failed in tracing the metabolites due to the poor sensitivity and/or specificity. Therefore, novel fluorescent probes for imaging the subcellular metabolites with lower detection limit and higher specificity are required. The promising solutions for these limitations are to explore higher-throughput probe screening systems, novel detection mechanisms, and accurate theoretical calculation methods. In addition, the fluorescent probes for imaging the subcellular metabolites with signal amplification may also contribute to improving detection sensitivity in future.

The signal-noise ratio is an important parameter in fluorescent imaging, because there are autofluorescent biomolecules in living cells, and they are emissive when exposure to the laser irradiation, that will interfere with the imaging resolution. Moreover, the emission wavelength of most reported fluorescent probes is in the visible region, hindering the application in background free *in vivo* imaging. Therefore, it is critical to develop fluorescent probes with high quantum yields, long lifetime, outstanding photostability and deep-tissue penetration capability. The fluorescent probes that can promote two or more photon excitations in near infrared emissions are promising for designing the novel probes.

Many different diseases are closely related to abnormal concentration or fluctuation of

metabolites. In addition, the communication between metabolites from different sub-cellular organelles is also a promising area for investigators to explore the understanding of metabolism.

Over the past decades, considerable progress has been made to the development of fluorescent probes to image metabolites in whole cell and subcellular. The progress is mainly in the area of the improving the imaging resolution, specificity, sensitivity and better explanation for molecular mechanisms in diverse biological processes. However, several challenges remain as described in the above paragraphs. Given the tremendous progress that has been made so far, we are confident that researchers in the field will be able to meet these challenges to develop more fluorescent probes with higher performance to enhance our understanding of metabolisms that play significant roles on all biological processes.

Data availability statement

No new data were created or analysed in this study.

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