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Recent Development of Two-Photon Fluorescent Probes for Bioimaging

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Abstract.
Fluorescent probes are essential tools for studying biological systems. The last decade has witnessed a particular interest in the development of two-photon excitable probes, owing to their advantageous features in tissue imaging compared to the corresponding one-photon probes. This review summarizes various types of two-photon probes that have been applied to bioimaging, categorized by the principles in the probe design and the target analytes, which would provide a basis for the future development of novel two-photon probes to tackle important biological issues.

Introduction
Understanding the molecular interactions in biological systems is of fundamental importance. Various types of assay and imaging tools have been developed for studying diverse biological processes. Among these tools, fluorescence methods have received great attention as they enable sensitive in vivo detection and imaging by relatively simple operations. Fluorescent probes with desirable sensing properties (selectivity, sensitivity, bioimaging capability, etc) are essential for the investigation of molecular interactions and thus have been widely used as diagnostic, monitoring, and analytical tools in biochemical, medical, and environmental research areas as well as in industry. Development of microscopy technology combined with various fluorescent probes has kept up with the bioscience growth. Along with conventional one-photon confocal microscopy, two-photon microscopy (TPM) based on the two-photon excitation combined with laser scanning technology has received increasing interest in recent years for its several advantageous features in tissue imaging where scattering of light becomes a serious issue.

Two-photons that arrive simultaneously at a spot combine their energies to excite the molecules there; therefore, two-photon excitation probability diminishes dramatically outside the focal point. This “localization of excitation” allows excitation to a tiny focal volume (~1 femtoliter), which is a distinct feature of two-photon excitation microscopy compared with one-photon excitation microscopy. The focal point is scanned throughout the desired region of the sample to offer very high resolution with 3D images. The use of two-photon excitation at near-infrared wavelength of 700-1100 nm, so called the biological optical window, also enables tissue imaging at increased depths (down to 1 mm). Furthermore, as TPM uses NIR light with focussed excitation capability, it causes less photo-damage and photobleaching to tissues than the corresponding OPM. These promising features of TPM for live tissue imaging in turn have inspired a quest for fluorescent molecules (fluorophores) with enhanced two-photon absorption property.

The efficiency of two-photon absorption property is expressed by two-photon absorption cross-section (TPACS, \( \sigma \)) in units of \( \text{GM} \) (1 GM = \( 10^{-50} \text{ cm}^4 \text{sec} \)) in recognition of Göppert-Mayer who theoretically predicted the two-photon excitation process. The brightness of a two-photon fluorophore is represented by the product of the fluorescence quantum yield (\( \Phi_F \)) and TPACS value, so-called the two-photon action cross-section value (\( \sigma \Phi_F \)). Fluorophores with very low action cross-sections can still be used for two-photon imaging. Two-photon excitable dyes with high action cross-sections, however, allow use of a lower laser power to reduce the probe photobleaching and to enhance cellular viability. Most common fluorescent dyes have \( \sigma \Phi_F \) values in the range of 1–300 GM. Generally, a large \( \sigma \Phi_F \) value can be obtained by conjugating electron donating (D) and accepting (A) substituents through \( \pi \)-bridges (\( \pi\pi \)). Most two-photon excitable fluorophores can be classified into several types depending on the spatial arrangement of D and A groups: dipolar, quadrupolar, and octupolar types. All of these dyes are designed to maximize the degree of intramolecular charge transfer upon excitation and the transition dipole moment.

In recent years many two-photon excitable fluorescent probes have been developed by introducing an analyte-specific binding or a reaction site to a two-photon excitable dye in such a way that the binding interaction can cause fluorescence signal change. Quadrupolar and octupolar two-photon dyes can show large TPACS values; however, they have relatively large molecular size which can make them less soluble in water. Thus, in most cases, additional functionalization is necessary to enhance the solubility. Also, owing to symmetrical nature of the quadrupolar and octupolar dyes, the corresponding probes have more than two binding sites. In this case, a multiple equilibrium result upon binding more than one analyte, which can complicate the quantification process based on the fluorescence change that is dependent on the binding event as well as the structural change. A variety of two-photon probes have been developed, which contain small aromatic cores (naphthalene, fluorene, styrene, porphyrin, coumarin, fluorescein, rhodamine, naphthylimide, quinoline, metal complexes (boron complex, platinum complex, lanthanide complexes), and hydrocarbon diradical species. Two-photon probes based on...
even larger platforms such as quantum dots, dendrimers, and polymers are known. Some of these two-photon excitable dyes have been used for immunoassay, cell staining, in vivo blood vessel staining, brain tissue staining, and cancer imaging. In this feature article, we focus on dipolar dye-based two-photon probes that have been applied to bioimaging. Known two-photon probes can be classified into three groups based on the signalling mode: (1) "always-on", (2) "turn-on" or "turn-off", and (3) "ratemetric". The always-on probes are suitable for staining purposes such as cell staining, protein labelling, and histology analysis. The definition of turn-on is arbitrary in scientific community; however, in general sense it is acceptable to use the terminology when the signal enhancement is more than ten times. The turn-on probes are preferred over the turn-off probes in terms of sensitivity and resolution. The ratemetric probes give fluorescence change in a ratiometric manner, which allows quantification free from environmental parameters such as pH, polarity, temperature, emission collection efficiency, effective cell thickness in the optical beam, and changes in the excitation intensity. The ratiometric fluorescent change of probes may be obtained two ways, either by inducing the intramolecular charge transfer (ICT) or by the fluorescence resonance energy transfer (FRET) through molecular interactions with analytes.

Most of two-photon probes can be also classified into two categories according to their sensing mechanism: (1) reaction-based and (2) coordination-based probes. The reaction-based or reactive probes undergo chemical conversion upon interaction with analytes and produce fluorescence change. Although in most cases it involves an irreversible process, the reaction-based approach complements the conventional supramolecular approach in that it can provide a complete selectivity toward the target analyte by employing an analyte-specific chemical conversion. Also, through the reaction-based approach we can realize turn-on sensing of certain analytes that apt to quench fluorescence (such as amine, carboxylate, and paramagnetic metal ions) in the conventional supra-molecular approach. The coordination-based two-photon probes are well developed for metal cations by introducing the metal-binding ligands to two-photon excitable dyes.

It is necessary to note typical sensing principles that are widely used to develop two-photon dyes. Three approaches are used to induce fluorescence change in the reaction-based approach (Scheme 1).

The first one is to modulate the ICT state in dipolar dyes by chemical conversion; dipolar dyes have ICT excited states that are sensitive to the electronic perturbation. The second one is to alleviate the photo-induced electron transfer (PET) process that quenches fluorescence of the dye, by chemical conversion, which restores or light up fluorescence. The third one is to transform a latent fluorescent molecule into an fluorescent one, which usually involves a significant structural change. In all cases, the key issue is how to install a reactive site to a two-photon dye in such a way that the target analyte can selectively induce the desired chemical conversion. Various ingenious approaches have been disclosed in the last decade, as seen in the following examples of two-photon probes. In the case of coordination-based approach, it is common to modulate the PET quenching process from a heteroatom-based (mostly nitrogen) ligand by metal coordination, which, in principle, is impossible to extend to sense anions.

In this feature article, we introduce selected examples of various two-photon fluorescent probes with bio-imaging applications, categorized by the two sensing modes.

1. Probing analytes through chemical conversion (reactive probes)

Various chemical probes have been developed based on the receptor–analyte noncovalent interactions such as hydrogen bonding, π-π, hydrophobic and electrostatic interactions. Also, chemical probes based on the metal-coordination bonding have been developed for securing effective binding affinity in aqueous media. However, it is only in recent years to arrest a surge of attention toward the reaction-based fluorescence probes, which involve the use of highly selective and usually irreversible chemical reactions coupled with signal transduction. Such probes in most cases provide higher selectivity with larger spectroscopic change than the chemical probes based on the noncovalent interactions, owing to the large structural changes from the chemical conversion.

In this section, the reaction-based two-photon probes for biothiols, a few gaseous molecules, enzymes and metal ions are described.

H$_2$S (Hydrogen sulfide). Hydrogen sulfide (pK$_a$ = 6.9 in 0.01–0.1 M at 18 °C) exists with its hydrosulfide anion (HS$^-$) under physiological conditions. H$_2$S has emerged as the third gasotransmitter along with NO and CO. H$_2$S appears to be involved in diverse physiological processes including modulation of neuronal activity, regulation of insulin release, smooth muscle relaxation, induction of angiogenesis, suppression of inflammation, protection of cells against oxidative stress, and reduction of the metabolic rate. To understand its role in physiology, it is crucial to monitor the H$_2$S level in living systems.

For that purpose, a variety of fluorescence probes have been developed in recent years, mainly based on the reaction-based approach by utilizing the strong nucleophilicity and tandem reaction capability of H$_2$S. Representative one-photon excitation probes are aryl azides (Scheme 2), copper complexes of azamacrocycles, and H$_2$S-specific Michael acceptors.
Following the azidofluorescein probe of Chang and co-workers, Cho and Peng groups introduced two-photon excitable aryl azide probes 1 and 2 based on the fluorene and benzopyran dyes, respectively. Nonfluorescent azidofluorene 1 underwent reduction by H$_2$S to produce the corresponding arylamine that is strongly fluorescent (turn-on; $\lambda_{ex/TP} = 750$ nm, $\lambda_{em} = 550$ nm, $\sigma = 302$ GM; HEPES buffer). Probe 1 was used to image H$_2$S in the living tissues at 90–190 $\mu$m depth under biologically relevant conditions. In a similar manner, probe 2 also reacted with H$_2$S to give the corresponding amine that emitted fluorescence (turn-on; $\lambda_{em} = 670$ nm, $\lambda_{ex/TP} = 820$ nm, $\sigma = 50$ GM; DMSO) (Scheme 2). Cho and co-workers later reported a related sensing system, probe 3, which was used to reveal reduction in mitochondrial H$_2$S production in Parkinson’s disease gene knockout astrocytes (ratiometric; $\lambda_{ex/TP} = 750$ nm, $\lambda_{em/probe} = 420$ nm, $\lambda_{em/product} = 500$ nm; $\sigma = 14$ GM (probe), 63 GM (product); HEPES buffer). Such aryl azide type probes can show a good level of selectivity to H$_2$S over competing biothiols (GSH in particular) under biologically relevant concentrations, but by sacrificing reactivity (> 1 h for signal saturation).

Scheme 2. Turn-on sensing of H$_2$S through reduction of aryl azides (in the box: the original sensing scheme), and two-photon probes.

NO (Nitric oxide). Nitric oxide (NO) is an important cellular signalling molecule associated with a variety of physiological and pathological processes in living organisms. NO is involved in the vascular smooth muscle relaxation and thus acts as a powerful vasodilator. Also, NO plays a key role in synaptic activity in the brain and host immune system defense. Dysfunction in the NO production pathways can cause various diseases such as ischemia, inflammation, cancer and neurodegenerative disorders. In order to understand its roles in biology, it is crucial to monitor NO in intact tissues. Accordingly, many fluorescent probes have been developed mainly based on or by mimicking the NO-promoted chemical conversion of o-diaminobenzene to the corresponding benzotriazole, a sensing scheme originally developed by T. Nagano and co-workers (Scheme 3).

Cho and co-workers introduced o-diaminobenzene moiety onto two-photon excitable acedan dye to generate probe 4. Probe 4 is nonfluorescent because of PET quenching from the o-diamo groups. Upon treatment with NO, probe 4 produced the corresponding benzotriazole with strong fluorescence restored (turn-on; $\lambda_{ex/TP} = 750$ nm, $\lambda_{em} = 502$ nm, $\sigma = 170$ GM; PBS buffer). With probe 4, they were able to selectively detect NO over other competing reactive nitrogen and oxygen species (RNS, ROS) including nitroxy1 (HNO). The probe was able to detect NO down to 5 nM level in a wide pH range. Furthermore, probe 4 enabled real-time monitoring of NO in living tissues at 100–180 $\mu$m depth for a long period (1200 s).

Jin and co-workers reported a naphthalene derivative 5 containing both an o-diaminobenzene moiety and a lysosome-targetable morpholine group. Upon treatment with NO, probe 5 produced the corresponding benzotriazole that emitted strong fluorescence (turn-on; $\lambda_{ex/TP} = 840$ nm, $\lambda_{em} = 550$ nm, $\sigma = 210$ GM; PBS buffer). Again, o-diaminobenzene 5 showed high selectivity to NO over other ROS and RNS species, with lysosomal NO targeting property. For the first time, probe 5 was used to detect endogenous NO that was captured in lysosomes of macrophage cells.

Recently, Liu and co-workers reported a quinoline-based two-photon fluorescent probe 6, which emitted at a longer wavelength compared with that of the acedan based probes (turn-on; $\lambda_{ex/TP} = 810$ nm, $\lambda_{em} = 535$ nm, $\sigma = 52$ GM; PBS buffer). The majority of known probes belong to one-photon excitable probes. Recently, Cho and co-workers reported acedan-based two-photon probe 7, which contained an aryl boronate reaction site. H$_2$O$_2$ oxidatively cleaved the arylboronates in 7 to give the corresponding phenol that was unstable and underwent a sequential bond cleavage to generate 4-methylenecyclohexa-2,5-dienone, carbon dioxide, and $N$-methyl-acedan. This conversion results in a ratiometric fluorescence change because acedan in its carbamate form also emits fluorescence with a hypochromic shift from that of the
acedan (ratiometric; $\lambda_{ex/TP} = 750$ nm, $\lambda_{em/probe} = 450$ nm, $\lambda_{em/product} = 500$ nm; $\sigma = 12$ GM (probe), 45 GM (product); HEPES buffer). Probe 7 detected endogenous H$_2$O$_2$ in a brain tissue. In a further study, they developed probe 8 that contained mitochondria-localizing triphenylphosphonium moiety.\(^{58}\)

**Scheme 4.** Fluorescent sensing of H$_2$O$_2$ through oxidative cleavage of an aryl boronate (in the box: the original sensing scheme), and ratiometric two-photon probes.

**Biothiols.** Biological thiols or biothiols such as glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and hydrogen sulfide (H$_2$S) play key roles in biological systems.\(^{59}\) These thiol compounds are known to be associated with infectious hepatitis, cancers, Alzheimer’s and Parkinson’s diseases, and HIV/AIDS.\(^{60}\) The development of fluorescent probes for biothiols have been pursued mainly through the reaction-based approach by exploring thiol’s unique chemical reactivity: (1) conjugate (Michael) addition to $\alpha,\beta$-unsaturated carbonyl compounds, (2) nucleophilic substitution of activated sulfonates, $\text{N}$-selenides, and electron deficient heterocycles, (3) disulfide exchange, (4) tandem aldehyde and enone addition, (5) demetallation of copper complexes.\(^{69}\) Cho and co-workers developed acedan derivative 9 as a two-photon excitable probe for biothiols, by adopting the thio–disulfide exchange reaction.\(^{70}\) Upon treatment of biothiols, probe 9 underwent the thiol–disulfide exchange reaction to generate the acedan (2-mercaptoethyl)carbamate, which further underwent a fragmentation step to generate $N$-methyl-acedan. The two-photon action spectra of 9 showed fluorescence enhancement in the presence of thiols (turn-on; $\lambda_{ex/TP} = 780$ nm, $\lambda_{em} = 500$ nm, $\sigma = 113$ GM; MOPS buffer). Probe 9 detected thiols in live cells and tissues at 90–180 $\mu$m depth, without noticeable interference from other biologically relevant species (Scheme 5).

The formation of thiazolines from aryl aldehydes was also used to develop two-photon probes for biothiols. Zhang et al. and Yang et al. developed aryl aldehydes 10 and 11 as two-photon probes for cysteine, respectively (Scheme 6).\(^{71}\) The chemical conversion resulted in turn-on fluorescence change, owing to ICT change.

Ahn and co-workers evaluated an arylsulfonyl azide system as two-photon probes for biothiols.\(^{72}\) Although related arylsulfonyl azides were reported as fast-reacting probes for H$_2$S, it was found that those arylsulfonyl azides do not discriminate H$_2$S from other biothiols under biologically relevant concentrations (turn-on; $\lambda_{ex/TP} = 750$ nm, $\lambda_{em} = 445$ nm; PBS buffer). A phosphonium containing probe 12 derived from 6-(methylamino)naphthalene-2-sulfonyl azide was found to be biocompatible, hydrolytically stable, water soluble, fast-responsive, and capable of two-photon imaging of biothiols in mitochondria (Scheme 7). The reduction of the azido group by biothiols resulted in turn-on fluorescence change, plausibly as the PET process from the azido group was disabled.

![Scheme 5. A two-photon probe that senses biothiols through the thiol–disulfide exchange.](image)

**Scheme 5.** A two-photon probe that senses biothiols through the thiol–disulfide exchange.

![Scheme 6. Two-photon probes that sense Cys/Hcy through thiazoline formation.](image)

**Scheme 6.** Two-photon probes that sense Cys/Hcy through thiazoline formation.

![Scheme 7. A two-photon probe that senses Cys/Hcy through reduction of arylsulfonyl azide.](image)

**Scheme 7.** A two-photon probe that senses Cys/Hcy through reduction of arylsulfonyl azide.

**Monoamine oxidase (MAO).** MAO A and MAO B are two isoforms of MAO. MAOs play a crucial role in regulating tissue levels of amine neurotransmitters and dietary amines. MAO inhibitors have been developed for the treatment of neurological disorders, such as Parkinson’s disease and schizophrenia.\(^{73}\)

Known fluorescent sensing systems for MAO include luciferin–luciferase, resorufin and quinazolinone derivatives, which are based on one-photon fluorophores. Ahn and co-workers developed compounds 13 (R = H, Me) as two-photon probes for MAOs for the first time, by applying the known reaction-based sensing scheme.\(^{74}\) The amine functionality in 13 underwent enzymatic oxidation to the iminium ion, which underwent hydrolysis followed by $\beta$-elimination to generate the phenol intermediate which, in turn, underwent fast cyclization to form the $\pi$-extended iminocoumarin (Scheme 8). It is notable that the $\pi$-extended iminocoumarin showed improved two-photon properties compared to acedan: a higher quantum yield, a longer absorption wavelength maximum and a larger TPACS value ($\Phi_p = 0.63$, $\lambda_{em} = 585$ nm, $\lambda_{abs} = 446$ nm, $\lambda_{ex/TP} = 900$ nm, $\sigma = 180$ GM; EtOH) (acedan: $\Phi_p = 0.52$, $\lambda_{abs} = 365$ nm, $\sigma = 151$ GM; EtOH). The improved photophysical properties of the $\pi$-extended iminocoumarin and its analogues are promising for two-photon imaging of tissues where autofluorescence become significant in addition to the shallow penetration issue when excited at a shorter wavelength.

Probes 13 were used to two-photon fluorescent imaging of MAOs...
in C6 glioma cells (MAOs not expressed) and chromaffin cells (MAOs expressed), in the presence and absence of MAO inhibitors (Moclobemide for MAO A, Pargyline for MAO B). Yao and co-workers reported a MAO B-selective two-photon probe 14 and its use to fluorescently image MAO B activities in mammalian cells, flies, mice and patients during Parkinson’s disease development. Furthermore, they assessed MAO-B activities in proteome lysates prepared from B lymphocytes and also from fibroblasts, both derived from PD patients. Importantly, they found elevated MAO B activities only in B lymphocytes, not in fibroblasts. They concluded that the difference in MAO-B activity profiles between control and PD patients are cell-type-specific. This finding hints that the detection of MAO B activity in blood with such MAO probes may provide a rapid diagnosis of PD.78

Scheme 8. Two-photon probes that sense MAOs by enzymatic oxidative cleavage of acedan-derived aminopropyl ethers (13) and aminopropyl carbamates (14).

Tyrosinase. Zhou and co-workers reported an acedan derived 3-(4-hydroxyphenyl)-1-methylurea as the first two-photon probe for tyrosinase.79 Tyrosinase is a copper-containing enzyme that catalyzes the hydroxylation of phenol to catechol and also the conversion of catechol to the corresponding o-quinone which eventually forms melanin. Thus, upon treatment with tyrosinase probe 15 was converted to the corresponding o-quinone, which further underwent hydrolysis to generate the N-methyl-acedan fluorophore (Scheme 9) with turn-on fluorescence change ($\lambda_{\text{ex/TP}}$ = 770 nm, $\lambda_{\text{em}}$ = 503 nm; PBS buffer). This probe was used to image tyrosinase activity in living cells by TPM.80

Scheme 9. A two-photon probe that senses tyrosinase through oxidative cleavage.

Phosphatase. Yao and co-workers reported fluorescently quenched activity-based probes for enzymes. One of the probes contained a photo-caged substrate for tyrosine phosphatase (PTP), a two-photon excitable fluorophore, and a quencher (probe 16). UV irradiation of the probe (photo-uncaging) could expose the phenyl phosphate moiety to PTPs, causing hydrolysis and subsequent fragmentation into the quinodimethane and the quencher moieties. A phosphatase removes a phosphate group from its substrate, a reverse process of kinase activity; both activities play key roles in many signal transduction pathways.81 The quinodimethane intermediate could be further captured by bionucleophiles, to afford fluorophore-tagged products (Scheme 10).82 This sensing scheme was demonstrated by in vitro fluorescence assay using purified four PTPs or PTP-expressing cell lysates. Upon uncaging and enzymatic treatment of PTPB, the presence and absence of MAO A, Pargyline for MAO B).

Scheme 10. An activatable two-photon probe for tyrosine phosphatase.

The same group extended the fluorescently quenched activity-based labelling approach to the SNAP-tag technology,83 this time employing a two-photon excitable acedan dye.84 Yao and co-workers further devised aryl (o-nitrobenzyl) phosphate 17 as a two-photon probe for phosphatase. The probe enabled them to imaging of phosphatase activities in both live mammalian cells and Drosophila brains.85 Probe 17 containing cell-penetrating peptides (CPPs) (Scheme 11) was used to organelle- and tumour cell-specific imaging of phosphatase activities in cells and, furthermore, in Drosophila brain with >100 $\mu$m detection depth by TPM. This result shows an advantageous feature of two-photon probes over the one-photon probe.86
Accordingly, a great number of methods for the detection of cathepsin activities in endolysosomal compartments of the mammalian lysates form HepG2 cells; gradual increase in the fluorescence intensity was observed, as the quencher part was enzymatically cleaved. The probes were further used to live cell imaging to detect endogenous cysteinyli cathepsin activities in endolysosomal compartments of the HepG2 cells by TPM.

**Scheme 12. Ativatable two-photon probes for cathepsin L.**

**Mercury ions.** Mercury ions are highly poisonous and widespread pollutant. Mercury ions can damage DNA, impair mitosis, and disrupt the central nervous and endocrine systems. Accordingly, a great number of methods for the detection of mercury ions have been developed. The reaction-based approach that utilizes mercury ion-specific reactions has emerged as a powerful approach to the development of highly selective and sensitive fluorescent probes.

As mentioned in the introduction, two-photon probes based on dipolar D-π-A dyes can give fluorescence change when the ICT excited states are perturbed. Thus, structural change in the donor and acceptor functional groups that can induce significant fluorescence signal change has become a useful sensing scheme for the development of two-photon probes for metal species which can interact with the functional groups. On the basis of this strategy, thioacetals 19 and 20 were concurrently developed as two-photon probes for mercury ions by Zhou and Ahn groups, respectively (Scheme 13).

Deprotection of the thioacetals promoted by mercury ions generated acedan. Interestingly, Zhou’s cyclic acetal 19 responded to Hg\(^{2+}\) very slowly with turn-on fluorescence change, whereas Ahn’s acyclic thiaoctetal 20 showed very fast response (within a few minutes) with a ratiometric fluorescence change. Thus, probe 20 itself showed strong fluorescence at 428 nm in pH 7.4 HEPES buffer containing 1% acetonitrile; this peak gradually decreased upon addition of Hg\(^{2+}\) with simultaneous increase of a fluorescence band at 525 nm (under excitation at 330 nm). Two-photon fluorescence imaging of probe 20 in the absence and presence of Hg\(^{2+}\) ions in B16F10 mouse skin cancer cells were successfully carried out using emission filters (430–480 nm for the probe and 500–550 nm for the presence of Hg\(^{2+}\)) under excitation at 730 nm.

**Scheme 13. Two-photon probes that sense Hg\(^{2+}\) through hydrolysis of dithioacetals.**

**Fluoride.** Fluoride is added to drinking water to promote dental health in some countries (fluorination), which, however, may cause dental fluorosis. An acute intake of fluoride can cause gastric and kidney disorders, skeletal fluorosis, and urolithiasis in humans. Many fluorescent probes for fluoride have been developed, and most of them are based on hydrogen bonding interactions between fluoride and NH hydrogen, boron–fluoride complex formation, and fluoride-mediated desilylation. Among them, the desilylation strategy seems to be suitable for the development of fluoride probes for biological applications, because silyl ethers are generally not cytotoxic, stable, and inert in cellular environments. The fluoride mediated desilylation approach was originally devised by Swager and co-workers. The widespread use of fluoride and its biological effects obviously demand a fluorescent probe with bioimaging capability.

Kim and co-workers developed a silyl enol ether containing a naphthalimide dye as a fluoride probe, which showed a ratiometric response to fluoride along with two-photon excitable property. However, owing to very slow response (3–8 h in 8:2 CH\(_3\)CN–HEPES buffer) and poor solubility in aqueous media, the probe was not applied to two-photon bioimaging.

Ahn and co-workers developed a fast-reacting two-photon probe for fluoride, probe 21, which provided the π-extended iminocoumarin (Scheme 8) through the fluoride-specific desilylation reaction (Scheme 14). As demonstrated in other probes, the desilylation sensing scheme was highly selective, and hence various other anions and biothiols such as cysteine caused little inference. Probe 21 was used to fluorescently image fluoride in B16F10 cells (turn-on; \(\lambda_{ex,TP} = 880\) nm, \(\lambda_{em} = 600\) nm; HEPES buffer). Furthermore, the probe enabled two-photon imaging of fluoride in a whole body of zebrasfish with spatial and time-dependent manner for the first time.

**Scheme 14. A two-photon probe that senses F through desilylation followed by cyclization.**
2. Probing of analytes through coordination or binding

Along with the reaction-based approach, the coordination-based sensing approach has also been enjoyed by chemists particularly for the development of fluorescent probes for metal cations, because metal–ligand binding interactions provide strong affinity even in aqueous media. The most popular sensing mechanism used in sensing metal ions is the modulation of PET process by employing heteroatom-containing ligands (amine, sulphur, and carboxylate). In this case, probes are usually at a quenched state owing to the PET process from the heteroatom lone pair but become fluorescent upon binding metal ions, which process blocks the PET quenching (Scheme 1).

Among various target metal ions, biologically essential metal ions such as Na⁺, Ca²⁺, Mg²⁺, Zn²⁺, and Cu⁺ have received much attention in search of fluorescent two-photon probes. Less commonly, heavy metal species such as Ni²⁺, Hg²⁺, and Cd²⁺ have been targets of two-photon probes. In contrast to metal ions, two-photon probes for biologically important anionic species such as ATP, DNA, and RNA are very limited at present, plausibly because it is much more challenging to selectively recognize these negatively charged and complex species in aqueous media in the fluorescence turn-on mode than the case of metal ions.

25 Metal ions

The development of two-photon probes for the biologically essential metal ions has been pioneered by Cho group.⁹⁷ By introducing the known metal binding ligands to two-photon excitable acedan dye and its derivatives, Cho group has developed two-photon probes for the detection and bioimaging of Ca²⁺, Mg²⁺, Na⁺, and Zn²⁺. The listed probes structures show that all the ligands contain heteroatoms that can quench fluorescence of the dye through the PET process; upon binding metal ions, this PET process is blocked and thus fluorescence signals are restored, establishing turn-on type sensing in all the cases. As excellent review articles on two-photon metal probes have been reported by Cho group,⁹⁸ in this feature article we will briefly overview some of notable features observed in two-photon bioimaging.

26 Calcium ion (Ca²⁺)

Calcium ion, one of the most widespread second messengers, plays an important role in signal transduction pathways associated with neurotransmitter release, muscle contraction, and fertilization. Calcium ion levels in mammals are tightly regulated by controlled release from bone and intracellular organelles (mitochondria and endoplasmic reticulum), at roughly 10 μM level of intracellular concentration but with 10- to 100-fold dramatic increase during cellular functions.⁹⁹

Fluorescent probes for calcium ions are developed by combining a metal chelate and a dye indicator. A typical chelate used for calcium ion is BAPTA (1,2-bis(4,5-(2-amino-phenoxo)ethane-N, N, N′, N′-tetraacetic acid),¹⁰⁰ which has high selectivity over competing magnesium ion. Typical one-photon probes for calcium ion include Fura-2, Indo-1, Fluo-3, Fluo-4, and Calcium Green-1.¹⁰¹ To take advantages of two-photon dyes in bioimaging, Cho and co-workers developed a series of acedan–BAPTA compounds 22–24 as two-photon probes for calcium ion (Scheme 15).¹⁰²

These probes selectively responded to calcium ion in the fluorescence turn-on mode, as the metal binding to BAPTA blocks the PET quenching process. Probes 22 and 23 showed Φₐₚₐ values of 90–110 GM at 780 nm (two-photon absorption maximum) in the presence of Ca²⁺ in buffered solutions. The probes detected dynamic levels of intracellular free Ca²⁺ in living cells and tissues (down to ~150 μm depth) without interference from other metal ions. Using probe 22b, they visualized the intracellular calcium ion waves in cultured astrocytes using TPM. Furthermore, they confirmed that calcium waves propagate by the astrocytic process to the soma, from TPM imaging experiments for astrocytes in hypothalamic slices of a rat labelled with probe 23b. Probe 23e was used to TPM imaging of near-membrane Ca²⁺.

Magnesium ion (Mg²⁺). As the fourth-most-abundant metal ion in cells, magnesium ion is involved in diverse cellular functions. For example, ATP, the main source of energy in cells, is indeed used in the Mg²⁺-bound form. Also, the triphosphate moiety of DNA and RNA involved in all enzymatic processes is stabilized by association with Mg²⁺. Magnesium deficiency in human has been associated with cardiovascular disease, diabetes, high blood pressure, anxiety disorders, migraines, osteoporosis, and cerebral infarction.¹⁰³ Accordingly, a number of one-photon fluorescent probes for magnesium ion have been developed including Fura-
mag-2 and Magnesium Green; 104 however, most of them show a higher affinity for Ca2+. Owing to this limit, those probes can be used to sense Mg2+ in cells where the resting level of Ca2+ is below 1 µM. 105 Cho and co-workers developed a two-photon probe for Mg2+ by introducing a known Mg2+-ligand, o-aminophenol-NN,N2-triaceitic acid (APTRA), to the acedan fluorophore (AMg1, 25). 106 AMg1 showed turn-on response to Mg2+ in Tris buffer solution (10 mM, pH 7.05). Although AMg1 showed a higher Kd value to Mg2+(1.4±0.1 mM) over Ca2+(9.0±0.3 µM), the probe detected the intracellular free Mg2+ in the mM range with little interference from Ca2+ because the intracellular Mg2+ concentration (0.1–6.0 mM) is much higher than that of Ca2+(10 nm–1 µM). AMg1–Mg2+ complex showed a 7-fold larger TPACS value of 125 GM at 870 nm than that of Fura-mag-2–Mg2+ complex. AMg1 was used to detect the labile Mg2+ in endogenous stores in live tissues at 100–300 µm depth by TPM. Another turn-on type two-photon fluorescent probe developed by the same group is benzocoumarin 26, which provides a β-diketone binding site to Mg2+. 107 This probe showed a similar level of dissociation constants to Mg2+(1.3 mM) and Ca2+(3.6 mM), allowing two-photon imaging of free Mg2+ in endogenous stores at 100–300 nm depth, without interference from Ca2+. The TPACS value of 26-Mg2+ complex was determined to be 107 GM at 880 nm.

Sodium ion (Na+). Sodium ion is necessary for regulation of the electrolyte level, cation transport, blood volume and pressure, and muscle volume, osmotic pressure and pH, nerve conduction, and muscle and heart contraction. 108 The intracellular free [Na+] is in the range of 5–30 mM, and that of extracellular [Na+] is >100 mM. In a resting cell, Na+ influx is regulated by Na+/Ca2+ exchanger, Na+/H+ exchanger, and Na+ channels, whereas the efflux is mediated by Na+/K+-ATPase (sodium pump). 109 Cho and co-workers developed a two-photon fluorescent probe for Na+ by incorporating known Na+-binding 1,7-diaza-15-crown-5 moiety into acedan fluorophore (probe 27). 110 Probe 27–Na+ complex showed a TPACS value of 95 GM at 780 nm in pH 7.0 MOPS buffer, a higher value than those of the commercially available probes, Sodium Green and SBFI. Probe 27 was used to image the Na+ change in astrocytes upon addition of Ouabain, a steroid hormone that increases the cytosolic free Na+, to modulate brain excitability. 111 A typical ligand for Zn2+ is 1,10-phenanthroline (phen) that was independently developed by Nagano and Lippard DGEP-(N,N-di(2-picolylethylene-diamine) and its analogues, which were selected upon synaptic activation to modulate brain excitability. 112 A typical ligand for Zn2+ is 1,10-phenanthroline (phen) that was independently developed by Nagano and Lippard DGEP-(N,N-di(2-picolylethylene-diamine) and its analogues, which were selected upon synaptic activation to modulate brain excitability. 113 Zinc ion (Zn2+). Zinc ion is found in hundreds of enzymes (carbonic anhydrase, carboxypeptidase, etc) and proteins (zinc fingers), 112 serving as structural ions and plays ubiquitous biological roles such as in the metabolism of RNA and DNA, signal transduction, gene expression, and apoptosis. Most zinc ions are in the brain, muscle, bones, kidney, and liver. In the brain, a few millimoles of intracellular free Zn2+ ions stored in the presynaptic vesicles, which are released upon synaptic activation to modulate brain excitability. 115 A typical fluorescent dendritic probe is DPEN (N, N-di(2-picolylethylene-diamine) and its analogues, which were independently developed by Nagano and Lippard groups, respectively. 116 Numerous fluorescent probes for Zn2+ have been developed by incorporating DPEN ligands to fluorophores such as rhodamine 119 and fluorescein dyes. 120
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Zn2+ complex showed a TPACS value of 90 GM at 780 nm and 5 times, along with significant TPACS values in the presence of 20 high photo-stability, pH insensitivity at pH>7, and short response appreciable water solubility, cell permeability, high selectivity, two-photon fluorescent probes for Zn2+. These probes showed Also, reported are two-photon fluorescent probes for Zn2+ ion which reduced residual fluorescence of probes (33).123 In addition, simple acedan derivatives that contain the DPEN moiety on the electron-acceptor part (34).124 styrene-based probe (35)125 and quinolone-based probe (36)126 are reported as two-photon fluorescent probes for Zn2+. These probes showed appreciable water solubility, cell permeability, high selectivity, high photo-stability, pH insensitivity at pH>7, and short response times, along with significant TPACS values in the presence of Zn2+.

Copper ion (Cu2+). Copper is an essential trace metal found in all living organisms in the oxidized Cu2+ and reduced Cu+ states.127 It is used as a cofactor by many cytosolic, mitochondrial, and vesicular oxygen processing enzymes, including cytochrome-C oxidase, copper/zinc superoxide dismutase, and metallothionein.128 Copper ion (Cu+).

Nickel is used in various industrial and consumer products; stainless steel, magnets, coinage, rechargeable batteries, and special alloys.134 The toxicity of nickel is low but excess nickel accumulation can cause lethal effects in aquatic ecosystems. The polluted water by Ni ions can effects on human as well as living species like fish.135

can occur through enhanced spin–orbit coupling or competing energy and electron transfer processes.130

Chang and co-workers developed a Cu+-selective fluorescent probe based on an azatetrahia receptor.131 Cho and co-workers introduced this moiety into an acedan fluorophore to develop the corresponding two-photon probe 37 (Scheme 17).132 37-Cu+ complex showed a TPACS value of 67 GM at 750 nm, with the maximum emission wavelength at 500 nm and 4-fold fluorescence enhancement. The probe was insensitive to pH in the biologically relevant pH range, and was used to detect Cu+ in live cells and living tissues at 90–220 μm depth by TPM.


Cho and co-workers developed acedan-derived two-photon probe 30a for Zn2+,121 which showed turn-on fluorescence change. 30a–

Zn2+ complex showed a TPACS value of 90 GM at 780 nm and the maximum emission wavelength at 500 nm (Scheme 16). Probe 30a was predominantly located in the cytosolic compartments, and thereby could detect Zn2+ ion in live cells without interference from the membrane-bound probes. In further studies, p-methoxy-modified DPEN moieties were developed, which reduced residual fluorescence of probes (30c and 31).122 Also, reported are two-photon fluorescent probes for Zn2+ ion with a triphenylphosphonium group that targets mitochondria (32 and 33).123 In addition, simple acedan derivatives that contain the DPEN moiety on the electron-acceptor part (34).124 styrene-based probe (35)125 and quinolone-based probe (36)126 are reported as two-photon fluorescent probes for Zn2+. These probes showed appreciable water solubility, cell permeability, high selectivity, high photo-stability, pH insensitivity at pH>7, and short response times, along with significant TPACS values in the presence of Zn2+.

Scheme 17. Two-photon probes for Cu+ (37), Hg2+ (38), Ni2+ (39, 40), and Cd2+ (41, 42).

Mercury ion (Hg2+). As mentioned in the reaction-based probes for Hg2+, mercury species are highly toxic; hence, efforts to develop efficient detection and analysis tools for them have witnessed a great number of fluorescent probes. Only a few fluorescence probes, however, showed two-photon imaging capability such as the ratiometric probe 20 developed by Ahn and co-workers. Cho and co-workers developed acedan-based two-photon probes 38 for Hg2+ by incorporating the sulphur-bridged 5-crown ether ligand that was developed by C. J. Chang (Scheme 17).133 Probe 38 showed turn-on response to Hg2+; and 38-Hg2+ complex showed a TPACS value of 78 GM at 750 nm with the maximum emission wavelength at 500 nm. Probe 38 was used to imaging of Hg2+ in live cells and fish organs at 80–170 μm depth by TPM, with little interference from other metal ions.

Nickel ion (Ni2+). Nickel is used in various industrial and consumer products; stainless steel, magnets, coinage, rechargeable batteries, and special alloys.134 The toxicity of nickel is low but excess nickel accumulation can cause lethal effects in aquatic ecosystems. The polluted water by Ni ions can effects on human as well as living species like fish.135

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A number of fluorescent probes for Ni\(^{2+}\) ions have been developed based on BODIPY,\(^{136}\) coumarin,\(^{137}\) and other dyes.\(^{138}\) Cho and co-workers developed an acedan based two-photon fluorescence probes by incorporating the Ni\(^{2+}\) binding CTEA ligand (N,N-bis[2-(carboxyethyl)methylthioethyl]amine) (39, Scheme 17).\(^{40}\) Ni\(^{2+}\)-complexes of 39 and 40 showed TPACS values of 32 and 90 GM at 750 nm, respectively. Probe 40 showed 26-fold-enhanced fluorescence in the presence of Ni\(^{2+}\); it was also influenced by other factors such as ligand (Scheme 17).\(^{41}\) Cd\(^{2+}\) complex showed a TPACS value of 200 GM at 720 nm, with the maximum emission wavelength at 510 nm. Guo and co-workers developed an acedan-based two-photon probe for Cd\(^{2+}\) (probe 41, Scheme 17).\(^{42}\) Cd\(^{2+}\)-complex showed a TPACS value of 109 GM at 740 nm, with the pH dependent.\(^{143}\) Liu and co-workers reported a selective two-photon probe for Cd\(^{2+}\) (probe 42, Scheme 17)\(^{43}\) towards Cd\(^{2+}\) with a variety of colorimetric and fluorescent probes.\(^{149}\) The "luciferin-luciferase" bioluminescence assay is available, together with the maximum emission wavelength at 520 nm. Probe 42 was successfully applied to intracellular imaging of Cd\(^{2+}\) in live HepG2 cells by TPM.

**Biophosphates**

**ATP.** Adenosine triphosphate (ATP) is perceived as the "molecular unit of currency" for intracellular energy transfer in the living cells; it transports chemical energy within the cells for metabolism.\(^{146}\) The average concentration of ATP in normal and tumour cells was determined to be 2.5 and 3.1 mM, respectively.\(^{147}\) To name a few, the glycolysis, the Krebs cycle, transporting activities of proteins, modulating ion channels and activating signalling cascades are the fundamental activities of life that rely on ATP energy.\(^{148}\) For the analysis of ATP, a commercialized "luciferin-luciferase" bioluminescence assay is available, together with a variety of colorimetric and fluorescent probes.\(^{149}\) The bioluminescence assay poses limitation in the case of in vivo imaging of ATP. Also, the known fluorescent probes are only useful for one-photon microscopy.\(^{150}\)

Ahn and co-workers developed a two-photon fluorescence probe for ATP/ADP based on mononuclear zinc complex and acedan fluorophore (43, Scheme 18).\(^{151}\) Zn\(^{2+}\)-complex 43 was found to be chemically stable and non-fluorescent in solution (\(\Phi_T = 0.02\)) but became highly fluorescent in the presence of ATP (2 equivalents; \(\Phi_T = 0.546, 1.0 \times 10^{-5}\) M \(\text{H}_2\text{SO}_4\)) and ADP. The competitive binding of Zn\(^{2+}\)-complex 43 to ATP/ADP seemed to be responsible for the emission change. A comparison of the binding constants and fluorescence enhancement factors of Zn\(^{2+}\) complex toward ATP (6.2 \(\times\) 10\(^6\) M\(^{-1}\); 8.9-fold), ADP (3.2 \(\times\) 10\(^6\) M\(^{-1}\); 7.0-fold), AMP (5.0 \(\times\) 10\(^5\) M\(^{-1}\); 1.9-fold), and pyrophosphate (PPi, 4.0 \(\times\) 10\(^5\) M\(^{-1}\); 2.8-fold) suggested that the fluorescence change was also influenced by other factors such as \(\pi-\pi\) stacking interactions between the nucleobase and the naphthalene ring, in addition to the binding constant.

![Scheme 18. Schematic illustration for the binding mode between Zn(DPA) complex 43 and ATP.](image)

Upon addition of ATP to Zn\(^{2+}\) complex 43 in pH 7.4 HEPES buffer (10 mM; containing 1% CH\(_3\)CN), green fluorescence increased with a shift in the maximum emission wavelength from 553 to 573 nm under excitation at 370 nm (at \(\lambda_{\text{ex}}\) of acedan). Probe 43 was biocompatible, permeable to cell membrane, able to detect ATP lower than 1.0 \(\mu\)M level, and allowed two-photon imaging of ATP/ADP in live cells for the first time. Ahn and Jung further used ATP probe 43 to obtain higher quality of fluorescence images of ATP in lung, spleen, liver, and spinal cord tissues, compared to commercial quinacrine dihydrochloride.\(^{152}\)

**DNA.** The detection of nucleic acid by fluorescent probes has become increasingly important for a variety of analytic and diagnostic applications.\(^{153}\) For such applications, a probe with a higher affinity for DNA than for other cellular components is required.\(^{154}\) Also, a large increase in quantum yield should be induced by the binding event. So far, a few fluorescent probes evaluated with one-photon fluorescence microscopy are known.\(^{155}\) Feng and co-workers reported a carbazole-based pyridinium salt 44 as two-photon probe for double-stranded DNA (dsDNA), which showed high sensitivity and fluorescence turn-on response (Scheme 19).\(^{156}\) Intercalation of pyridinium salt 44 between dsDNA base pairs served clear TPM images in HeLa cells with specific subcellular localization without interference from other materials.

**RNA.** RNA is a ubiquitous family of large biological molecules that perform multiple vital roles in the coding, decoding, regulation, and expression of genes.\(^{157}\) Thus, fluorescent probes for RNA in nuclei and cytoplasm are essential tools in biochemistry and biomedicine.\(^{158}\) For instance, fluorescence imaging of RNA in parasites increases parasite detection, improves the spatial and temporal resolution of the parasite under drug treatments, and resolves morphological changes in an individual cell.\(^{159}\) But, DNA staining dyes cannot give additional information regarding the parasite stage affected by the drug and a better indication of the drug’s action mode.\(^{160}\) Li and co-workers screened a large combinatorial library of fluorescent styril molecules (1336 compounds) to find several RNA-selective probes including 44 and 45.\(^{161}\) Song and co-workers reported indole-based mono- and di-cationic salts such as a V-shaped 45 (Scheme 19), which possessed higher affinity to
RNA over DNA. These compounds exhibited marginal fluorescence enhancement upon binding to RNA. RNA-bound showed a small TPACS value (20 GM at 800 nm) with the maximum emission wavelength at 550 nm.

Scheme 19. Two-photon probes for DNA (44) and RNA (45).

**Conclusion**

Owing to the several advantageous features of two-photon microscopy in fluorescence imaging of tissue, a variety of two-photon fluorescent probes have been developed in recent years. Various two-photon fluorescent probes with bio-imaging capability are overviewed in this review categorized by the sensing modes: the reaction-based and coordination- or binding-based approaches. As demonstrated by Cho and co-workers with various examples, the coordination-based sensing approach is particularly useful in the development of two-photon fluorescent probes for metal cations (Ca²⁺, Mg²⁺, Na⁺, Ca²⁺/Mg²⁺, Zn²⁺, Cu²⁺, Hg²⁺, and Ni²⁺) where the photo-induced electron-transfer process from the heteroatom (mostly nitrogen) lone-pair electrons of the coordinating ligand was utilized to induce turn-on fluorescence change. For neutral and anionic species (RSI, MAOs, tyrosinase, phosphatase, F⁻, ATP/ADP, DNA, and RNA), the reaction-based approach has proven to be promising in the development of two-photon fluorescent probes for realizing high selectivity and turn-on or ratiometric fluorescence change. The use of chemical conversions applies to enzyme catalysed reactions, leading to the so-called activity-based probes. The fluorescently quenched activity-based probes, notably the modular system of quencher–enzyme-substrate–fluorophore offers a general platform of two-photon enzyme probes, as demonstrated by Yao and co-workers. As shown in the examples, biologically essential metal ions such as Ca²⁺, Mg²⁺, Na⁺, Zn²⁺, and Cu²⁺ have received much attention in contrast to other metal cations including heavy metal species such as Ni²⁺, Hg²⁺, and Cd²⁺. As one-photon fluorescent probes for other metal cations are abundant, obviously the corresponding two-photon probes are expected to appear in near future. On the other hand, it is a more challenging but demanding task to develop two-photon probes for biologically important enzymes, nucleosides (ATP, ADP, AMP, c-GMP, etc) and nucleic acids (DNA and RNA) by securing high selectivity among the competing biomolecules. Efforts toward this direction will be greatly recognized by researchers in biological and biomedical science areas. Also, it should be noted that most of the two-photon probes essentially belong to the intensity-based probes, which may limit their use in quantification of analytes in biological systems because the intensity of fluorescence is susceptible to the environmental parameters such as pH, polarity, temperature, and several instrumental parameters. In this regards, more efforts to develop ratiometric two-photon probes (the ICT or FRET based) are necessary even though it requires creative modulation of ICT geared with the binding or the conversion by analytes or synthetic elaboration (for the FRET based).

In addition, most of the two-photon probes are based on acedan and its derivatives that show the maximum absorption wavelength around 370 nm or so, rather in the short wavelength region, which cause limited light penetration as well as substantial autofluorescence and photo-bleaching in tissue imaging under the two-photon excitation conditions. Thus, there is a strong need for new two-photon excitable dyes that can be excited at longer wavelength (~900 nm or greater). In this regard, the linear benzocoumarin derivatives developed by Ahn and co-workers are promising for the development of two-photon probes. Surely, other types of novel two-photon fluorescent probes are also expected to enter the stage in coming years as more chemists become interested in this area.

Finally, a number of other issues should be considered in designing two-photon probes, which include solubility in aqueous media, cell permeability, toxicity, organelle specificity, in addition to chemical stability and photo-stability. Most of the two-photon probes described in this review have an adequate solubility (1–10 µM in aqueous buffer) for biological studies solubility in aqueous media.

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**Notes and references**

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