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Fluorescence imaging of metal ions implicated in diseases

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Metal ions play an important role in various biological processes, their abnormal homeostasis in cells is related to many diseases, such as neurodegenerative disease, cancer and diabetes. Fluorescent imaging offers a unique route to detect metal ions in cells *via* a contactless and damage-free way with high spatial and temporal fidelity. Consequently, it represents a promising method to advance the understanding of physiological and pathological functions of metal ions in cell biology. In this highlight article, we will discuss recent advances in fluorescent imaging of metal ions by small-molecule sensors for understanding the role of metals in related diseases. We will also discuss challenges and opportunities for the design of small-molecule sensors for fluorescent detection of cellular metal ions as a potential method for disease diagnosis.

Introduction

Over the past decades, the abnormal homeostasis of metal ions in cells/tissues has been related to several diseases, such as neurodegenerative disease,[1] cancer[2] and diabetes.[3] Although their exact roles in disease pathologies remain unclear, a steadily growing number of diseases have been characterized with metal ion imbalance. In the diseased state, metal homeostasis is believed to be disrupted, resulting in poor control of potentially toxic metal ions.[4] Abnormal accumulation of transition metal ions, such as copper, zinc and iron ions, has been observed in brain tissues with neurodegenerative diseases.[5,6] Elevated copper ion levels have been shown to link to a variety of tumor and cancers.[7] Irregular zinc ion concentrations have

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also been identified in cancers[8] and diabetes.[9] For example, the concentration of mobile zinc ions decreases considerably during the development of prostate cancer.[10] Consequently, the identification and quantification of metal ions, ideally in a native physiological environment in tissues, cells, or even at the level of individual organelles and subcellular compartments,[11] has become critical for understanding these diseases and developing diagnosis methods.[12]

Since Tsien's pioneering work using fluorescent Ca^{2+} sensors to study Ca^{2+}-involved biochemistry in cells and tissues,[13] fluorescent imaging has evolved to become an essential tool for investigating the roles of metal ions in biology systems.[14–16] Over the last thirty years, chemists have developed a variety of sensing mechanisms like photoinduced electron transfer (PET) and fluorescence resonance energy transfer (FRET) and established several molecular design criteria for making fluorescent metal sensors.[17] Hence, various cell-permeable fluorescent sensors for different types of metal ions have been developed, by adopting different molecular recognition components and sensing mechanisms.

In this themed issue of *Chem. Soc. Rev.*, various fluorescent sensors have been reviewed, and organized according to their target metal ions. In this highlight, the required functionalities of small-molecule sensors have been firstly examined, with an objective to understand the roles of metal ions in disease development and diagnosis. Following that, we have surveyed recent progress in fluorescent imaging of metal homeostasis related to various diseases, such as neurodegenerative disease, cancer, and diabetes. We have then discussed challenges and opportunities for designing fluorescent metal ion sensors with enhanced performance.

Functionality of fluorescent sensors

Metal ions play an important role in various cellular processes, such as proliferation, differentiation, and apoptosis.[18] Yet, their intracellular distributions and dynamic changes are largely unknown. It remains challenging to quantify metal ion concentrations and detect the associated dynamics, which is critical for understanding different disease states.

To meet these objectives, fluorescent sensors should possess a few functionalities, including a high selectivity for target metal ions, a large dynamic range for *in situ* quantification of ion concentrations, and organelle-targetable ability to describe cellular distributions of metal ions.

To image intracellular metal homeostasis, genetically encoded fluorescent sensors feature highly, ascribing to a few advantages, such as extremely good selectivity, easy incorporation into cells, high region-specificity, controlled concentration and long imaging stability over days.[19] However, there are also several disadvantage associated with this method, such as complicated manipulation, potential interference with the local system, and relatively weak fluorescence changes. In contrast, small-molecule fluorescent sensors with improved functionalities afford small sizes and versatile sensing strategies, thus representing promising tools in disease studies.

Selectivity

In nature, the coordination chemistry of metal ion–protein complexes forms an important foundation for metal ion discrimination. Accordingly, *O*, *N* or *S*-containing ligands have been mostly used for metal ion recognition in artificial fluorescent sensors. The specificity and stability of the resulted complexes depend on the properties of both the metal ion and the ligand. To design a selective complexation ligand, several factors should be considered, including donor atom preference, size and preorganization of the polydentate ligand, and complex geometry.[20] For example, sulfur-containing donors [*i.e.*, thioethers (R_2S)] prefer soft metal ions, such as Cu^{+}, whereas oxygen donors (*i.e.*, carboxylates and phenolates) are often used to detect hard metal ions, such as Fe^{3+}.[20]

In general, polydentate ligands show greater complex stability than monodentate, a polydentate ligand can thus be designed to bind metal ions selectively, according to the preferred binding geometry with a metal ion. More information on selective ligand design can be found in several comprehensive reviews,[20–22] while a few representative examples are shown in Fig. 1.

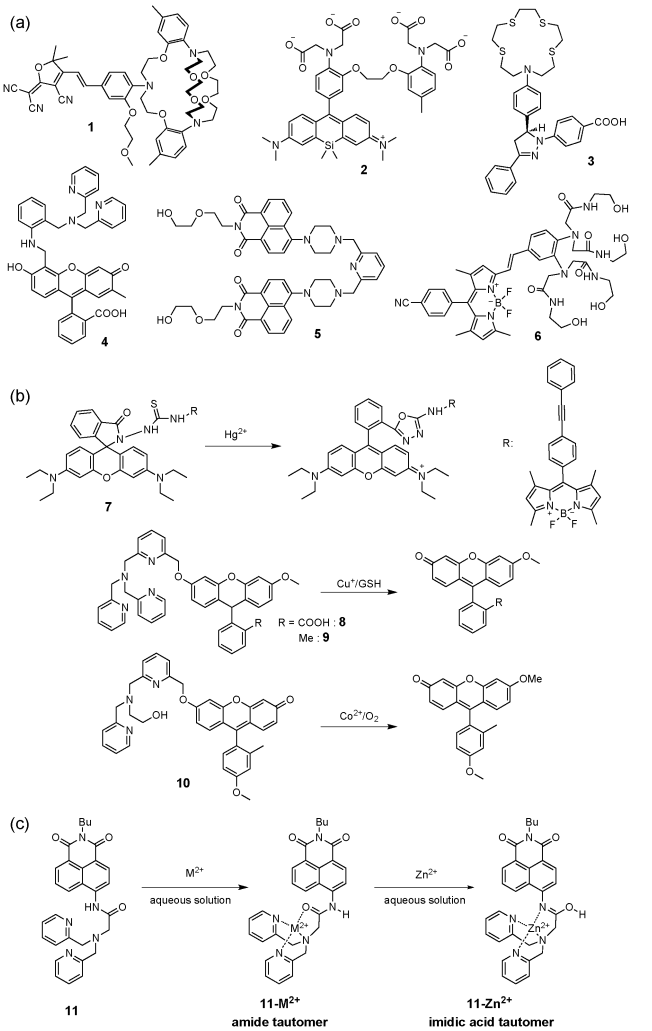

Fig. 1 Representative types of receptors for selective binding of metal ions. (a) Coordination-based, (b) reaction-based, (c) tautomerization-based.

In Fig. 1a, a macrobicyclic cryptand incorporating an aniline group in **1** was designed as a selective K^+ receptor over Na^+.[23] Fluorescent sensor **1** has been used to image intracellular K^+ distributions over a large concentration range. Iminodiacetic acid and derivatives such as bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were used to construct fluorescent Ca^{2+} sensors. A BAPTA-based near-infrared (NIR) sensor **2** using Si–rhodamine as the fluorophore has been adopted to image Ca^{2+} in brain slices with a high fluorescence off/on ratio of over 1000.[24] An azatetrathiacrown ligand in **3** was reported firstly in 2005 to bind Cu^+ selectively over Cu^{2+} and other metal ions.[25] For zinc ions, di-2-picolylamine (DPA) in **4**[26] has been used as the most popular receptor to develop fluorescent Zn^{2+} sensors.[27] For heavy metal ions, non-cyclic multi-*N*-coordination ligands in **5**[28–30] and **6**[31] displayed high selectivity for Hg^{2+} or Cd^{2+}, respectively.

In another strategy, a reaction-based approach known as chemodosimeter has also been developed. This type of sensors usually exhibit extremely high selectivity, since they rely on the occurrence of specific chemical reactions with metal ions. The mostly used reaction to construct metal sensors is the spiroring-opening of xanthenes.[32] In **7** (Fig. 1b), the ring-opening reaction induced by Hg^{2+} triggered the FRET process from BODIPY to rhodamine.[33] Another recently developed reaction for designing metal sensors concerns the metal-catalyzed cleavage of the C–O fluorescein–ether bond,[34] such as in the cases of **8**, **9**[35] and **10**[36] (Fig. 1b). Owing to the catalysis nature, this type of sensor can detect a trace amount of metal ions down to the ppb level. Nevertheless, they are not suitable for imaging metal homeostasis, because these reactions are often irreversible.

It is also worth highlighting a nature-inspired molecular modification strategy to improve the metal ion selectivity of small-molecular sensors, *via* structure transformation induced by the tautomerization of a peptide bond. For example, an amide-containing DPA receptor in **11** displayed excellent selectivity to Zn^{2+} due to the specific tautomerization of the amide induced by zinc ions (Fig. 1c). The excellent performance of **11** suggests that constitutional dynamic chemistry and adaptive chemistry[37] represents a promising route to design new fluorescent sensors with high selectivity.

Dynamic range

The concentrations of cytosolic metal ions vary in various cells and at different disease states (even in the same cells). A precise measurement of metal ion concentrations is important for assessing the impact of metal ion deviations from their normal levels on cellular functions. As a result, fluorescent sensors should ideally possess a large dynamic range in order to quantify the highly dynamic changes of metal ion concentrations.

There are two aspects to be considered. The first one concerns the responding range of a sensor, which depends on its affinity with metal ions. A sensor can detect the concentration variations of metal ions only when its apparent dissociation constant is near the target concentration of metal ions. However, at higher or lower concentration regions, the fluorescence intensity of the sensor remains saturated or unaffected, rendering it ineffective in detecting metal ion concentrations. To cope with this problem, one possible strategy is to develop and collectively use a series of sensors, each of which possess varied metal ion affinities and hence work at different concentration ranges.[38]

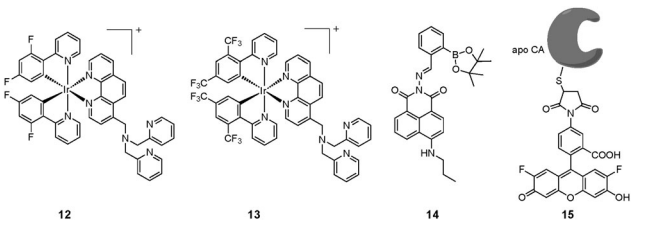

Fig. 2 Fluorescence lifetime imaging sensors for Zn^{2+} (sensors **12** and **13**) and Cu^{2+} (sensors **14** and **15**).

Another important consideration for quantifying metal ions regards the calibration of the fluorescent intensity on the concentration of the sensor itself. Different from genetically encoded sensors, the concentration of small-molecule sensors cannot be controlled. Two methods have thus been developed to deal with this problem: ratiometric detection allows signal rationing and has been widely applied in metal ion imaging and quantification,[14–16] fluorescence lifetime imaging microscopy (FLIM), which is also independent of sensor concentration, has recently been used to image Zn^{2+} (sensors **12**[39] and **13**[40]) and Cu^{2+} (sensors **14**[41] and **15**,[42] Fig. 2).

Organelle-targetable

The subcellular distribution of metal ions is highly heterogeneous. In view of the fact that many diseases are organelle phenotypic, it is essential to precisely define the identity of the cellular compartments being measured, when quantifying metal ions.[43] Although it is difficult for a fluorescent sensor to obtain a full map of metal ion distributions across the entire cell,[44] it is reasonable to unload and fix fluorescent sensors at a targeted cellular compartment by direct targeting or serendipitous localization. More information about factors governing the localization of molecular sensors can be found in a recent review.[15]

By attaching a sub-cellular targetable group, fluorescent sensors are able to detect metal ions in specific regions of a cell. For example, lipophilic delocalized cations, such as phosphonium ions or positively charged rhodamine derivatives, promote the accumulation of sensors in mitochondria. With the direction of triphenylphosphonium (TPP), sensors **16**[45] and **17**[46] are able to image Cu^+ and Zn^{2+} in the mitochondria, respectively (Fig. 3a). An exemplary sensor **18**[47] uses a cholesterol moiety for attaching to the cell membrane and allows the detection of changing Zn^{2+} concentrations in a localized region. In another recent study, 2-morpholinoethylamine has been reported as a lysosome-targeted group, making **19**[48] a lysosomal Zn^{2+} sensor.

However, it is challenging for these small anchors alone to achieve sufficient accuracy in organelle targeting. To attain more accurate organelle localization, one attractive approach is to introduce a genetically encoded component to small-molecular sensors by transfection, viral transduction, or other transgenic technologies. This approach typically relies on the use of protein

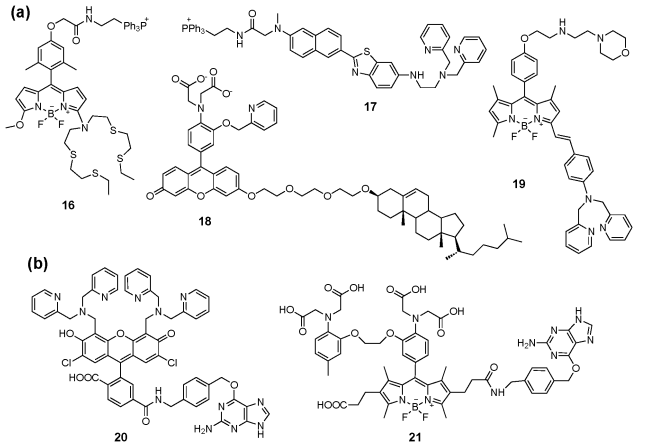

Fig. 3 Organelle-targetable sensors with (a) sub-cellular targetable groups, (b) SNAP tags.

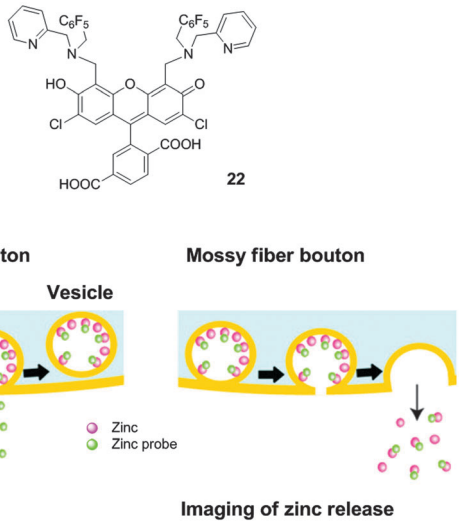

Fig. 4 Molecular structures of sensor **22** and a model for presynaptic imaging at mossy fibre termini using **22**.

or peptide tags.[49] Among various tags, SNAP is the only one used for constructing organelle targetable metal sensors at the present stage. The first example, sensor **20**,[50] is essentially a combination of zinc sensor **ZP1** with a SNAP-tag to image zinc in mitochondria and Golgi. Later, SNAP-tag was also incorporated in Ca^{2+} sensor **21**.[51]

Imaging in diseases

Historically, the development of fluorescent sensors for metal ion imaging started with the fluorescent stain of Zn^{2+} in human plasma by 8-hydroxyquinoline in 1968.[52] Living cell imaging of metal ions began with the Ca^{2+} sensor Quin2 by Roger Tsien in the early 1980s.[13] In the field of metal homeostasis investigations at various disease states, Zn^{2+} and Cu^{+} have attracted much more attention than other metal ions, probably owing to the relatively mature design of fluorescent sensors for these two types of ions.[14] To this end, recent work on zinc homeostasis imaging has demonstrated strong potential in prostate cancer diagnosis.[53,54]

Neurodegenerative diseases

The crucial role of metal ions in neurodegenerative diseases has been studied for many years. Metal ions such as copper, zinc and iron ions have been identified as molecular aggregation modulators of some specific proteins that are directly linked to neurodegenerative diseases. In addition, altered metal homeostasis in the brain has been suggested as a possible cause for most of the neurodegenerative diseases. However, the detailed biochemical mechanisms regarding the involvement of metal ions in neurodegenerative diseases are still largely unknown. Among fluorescent imaging of various metal ions, fluorescent Zn^{2+} imaging in the brain has been the hottest topic in the past decade.[15] In a recent study, Khan *et al.* reported a two-photo fluorescent sensors **22** to image Zn^{2+} dynamics at a single mossy fiber termini of dentate gyrus neurons in adult mouse hippocampal slices.[55] This membrane-impermeant fluorescent sensor was loaded into presynaptic vesicles in hippocampal mossy fiber termini upon KCl-induced depolarization, which triggered subsequent endocytosis and vesicle restoration. Local tetanic stimulation decreased the Zn^{2+} signal observed at individual presynaptic sites, indicating the release of Zn^{2+} from the vesicles (Fig. 4).

Cancers

Due to its close association with diseases, altered metal homeostasis is a potentially useful indicator for early diagnosis of diseases, disease progression monitoring, and drug activity tracking. Among various metal ions, previous research has identified Zn^{2+} as an excellent biomarker for prostate cells,[10] fluorescent Zn^{2+} imaging has been performed for the early diagnosis of prostate cancer.[53,54] Lippard *et al.* used the Zn^{2+} sensor ZPP1 (**23**[56]) to image Zn^{2+} levels in the prostate cancer.[53] A significant fluorescence decrease in prostate cancer cells was observed, because the concentrations of Zn^{2+} drop dramatically during the early stage of prostate cancer. In contrast, the Zn^{2+}-binding induced green fluorescence intensity remained little changed in healthy prostate cells. Moreover, changes associated with mobile zinc ions in prostate were also monitored during the progression of prostate cancer in mice (Fig. 5b). The substantially decreased fluorescence in the mouse model of the prostate cancer suggests that fluorescent Zn^{2+} imaging could potentially be used for early detection and progression tracking of the prostate cancer. The same group recently reported a new Zn^{2+} sensor **24** with improved properties used in differentiating prostate cancer cells from healthy prostate cells.[54] The introduction of a TPP group allows the successful delivery of this sensor to the mitochondria. The Zn^{2+}-induced hydrolysis reaction of the acetyl groups ensures the excellent selectivity for Zn^{2+} over other metal ions. Sensor **24** represents an improved reaction-based version of metal ion sensor, since Zn^{2+} detection is a reversible coordination process (Fig. 5a). The hybrid approach combining metal-dependent coordination and reaction-based assistance affords a promising option for the development of new metal ion sensors with enhanced properties.

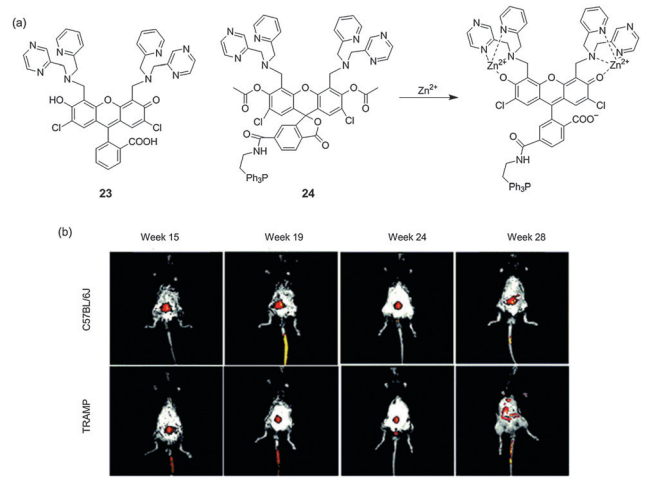

Fig. 5 (a) Molecular structures of sensors **23** and **24**. (b) *In vivo* detection and monitoring of prostate cancer by epifluorescence whole-body optical imaging. A whole-body epifluorescence optical imaging of 15-, 19-, 24-, and 28-week-old TRAMP (bottom) and C57BL/6J (top) mice, 30 min after tail-vein injection of **23** (2.5 μmol kg^{-1} if one assumes an average weight of 20 g for a 15-week-old mouse). In TRAMP mice, consistent with prostate cancer progression, there was an overall reduction in prostate-associated fluorescence with increasing age beginning at 19 week. In contrast, the signal in the C57BL/6J mice remained the same (n = 4). Reprinted by permission of the American Association for Cancer Research, ref. 52.

Diabetes

Understanding insulin secretion at pancreatic islet β cells is of great interest for its importance in treating diabetes.[57] Insulin is stored in secretory vesicles as a solid hexamer bound with two Zn^{2+} ions per hexamer. During exocytosis the Zn^{2+}–insulin complex dissolves and dissociates to release free Zn^{2+} and insulin. Therefore, detection of Zn^{2+} efflux could help to monitor insulin secretion. Kennedy *et al.* firstly used fluorescent zinc sensor Zinquin to measure Zn^{2+} efflux from pancreatic β-cells.[58] Following this work, several other zinc sensors, such as FluoZin-3,[59] Rhod-Zin-3[60] and ZnAF-2,[61] were also applied to image the insulin release process. However, these sensors lack micro-localization abilities, thus displaying compromised sensitivity of detecting local Zn^{2+} release near the plasma membrane. Recently, a plasma membrane-targetable fluorescent sensor **25** has been developed to monitor Zn^{2+} release from cultured β cells and intact pancreatic islets after stimulation by high glucose.[62] The two dodecyl side-chains quickly integrate into the outer cell membrane and allow **25** to anchor to the extracellular side of the cell membrane (Fig. 6). When exposed to a high glucose level and imaged by **25**, the rat pancreatic islets β-cells do not exocytose Zn^{2+} homogenously. Rather, only a subpopulation of clustered β-cells exhibit robust secretion at any given time. These secretory clusters of β-cells were scattered throughout the islet along with other β-cells that show much weaker secretory activities. It has also been observed that Zn^{2+} release occurs in both homologous cell–cell contacts (β–β) and heterologous (β–α) cell–cell contacts. In contrast, Zn^{2+} release is rare at other sites within the cell clusters. In future, more accurate location techniques, like protein tags, may help to further increase the spatial resolution of imaging Zn^{2+} release from pancreatic islet β cells.

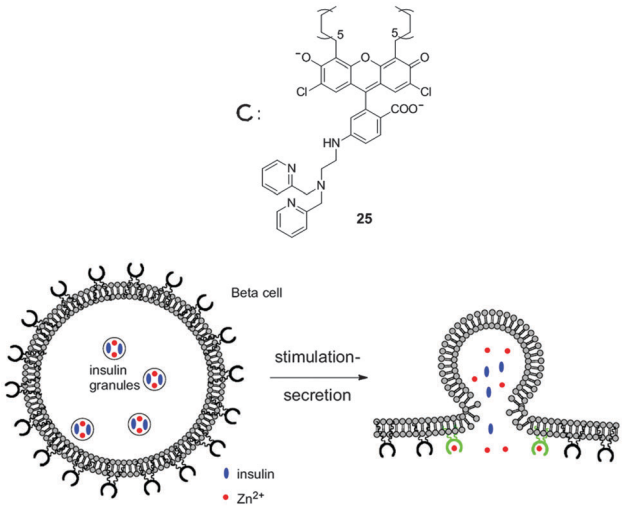

Fig. 6 Action mode of **25** for reporting local Zn^{2+} elevation at the membrane surface during exocytotic insulin granule fusion.

Conclusions and outlook

Fluorescent sensors of metal ions are important tools to assist the further development of biochemical and biomedical science. These sensors are able to detect various metal ions both *in vitro* and *in vivo*, demonstrating strong potential in elucidating the roles of metal ions in disease development and treatment. Current efforts on fluorescent sensors have been focused on improving sensor sensitivity and selectivity, expanding the family of detectable metal ions, developing new sensing mechanisms, shifting the sensor excitation and emission spectra towards the NIR region, and so forth. However, current research is still limited by the numbers and types of diseases under investigation.

It is expected that the future development of metal ion sensors will become more interdisciplinary and biomedical application driven, which requires a close collaboration between chemists and biologists. Furthermore, although challenging, it will be interesting and useful to image metal homeostasis in the whole organism in order to get a complete understanding about the functionalities of metal ions. In addition, more efforts should be directed to less explored areas. For example, fluorescent sensors for Ni^{2+} and Co^{2+} are still rare, in spite of their important biochemical and biomedical roles.[4,63] Finally, currently the achievable spatial resolutions of standard fluorescence microscopies are still relatively low for precise organelle localization. However, recent development of super-resolution fluorescence microscopies has created unprecedented new possibilities for fluorescent sensors.[64] A rapid development in the research of fluorescent metal ion sensors is thus anticipated in the coming years.

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