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Advances in Imaging of Understudied Ions in Signaling: A Focus on Magnesium

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Metal ions in signaling: looking beyond Calcium

Cell signaling —the processes by which cells relay information from their environment to the intracellular machinery—is essential for cells to adjust to changes at the organismal and tissue level and produce a coordinated physiological response. Metal ions play a prominent role in these processes. Calcium(II), in particular, is widely recognized as a ubiquitous second messenger that impacts almost every aspect of cell physiology, ranging from cell motility to cell death.[1] Fully validated examples of other divalent main group cations and *d*-block metals in a signaling role, however, are much more difficult to pinpoint. This gap stems from a combination of pervasive conceptual and methodological limitations.

Conceptually, *d*-block metals have been traditionally considered to play structural and catalytic roles as static cofactors in proteins, thus controlling basal metabolic activity in the cell. The fraction of the total metal content that is “free” or, more fittingly, kinetically available is maintained at very low levels to prevent cytotoxicity, especially for redox-active metals that may otherwise generate harmful reactive oxygen species. This fact seems paradoxical with the occurrence of large transients in free ions first thought to be required for signaling. A recent account by Maret [2*] discusses how such transients can be generated for a tightly bound metal such as Zn^{2+}. For example, either release from subcellular compartments or mobilization of the bound pool upon chemical modification of thiol-based metal binding sites by small redox-reactive species (e.g. nitric oxide action on metallothioneins) can change the levels of labile ion. Analogous models could be invoked for the mobilization of other tightly bound *d*-block metals, consistent with their unique bioinorganic chemistry. For these, it is clear that the term “free” metal does not apply in the same way as with Ca^{2+}, and that changes in metal *availability* must be considered instead. With these considerations in mind, the possibility of *d*-block metal signaling has started to gain traction [3*], although whether the cations are involved as effectors of canonical signaling pathways (e.g. Ras/MAPK), regulators of second messengers (Ca^{2+}), or acting as signal carriers of their own, is still matter of debate in many systems.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The notion of magnesium playing a role as a signaling ion has been considered for decades [4], but is still plagued by similar conceptual challenges to other metals at the other end of the concentration spectrum. The basal levels of intracellular free Mg^{2+} are relatively high—Mg^{2+} is the most abundant divalent cation in the cell—leaving little room for large transients [5,6*]. Furthermore, the extra-and intracellular concentrations of this metal are similar, raising questions about the feasibility of large fluxes in the absence of marked concentration gradients across membranes (though an electrochemical gradient is present). Yet an important, but often ignored, aspect of Mg^{2+} biology is that the main species comprising the "bound" pool are polyphosphates (e.g. MgATP) whose concentrations are highly dynamic. As such, localized changes in metal availability triggered by processes that alter the rate of synthesis or conversion of these species, altering the free to bound ratio of the metal, are possible. Moreover, based on the well-tuned binding affinity of some Mg^{2+}-regulated proteins such as kinases and phosphatases, it is possible that relatively small transients—much smaller than previously recognized—are sufficient to generate a downstream effect. A bona fide sensor protein capable of carrying forward the effect of a Mg^{2+} signal, however, has remained elusive [7].

Full validation of a signaling event requires the demonstration of transient changes in the availability of the signaling species, completed with the identification of a source and a target. Changes in the free or available metal pools can be detected with small molecule fluorescent indicators that bind to kinetically accessible metals without disrupting the total cellular buffer. Armed with the right indicators, fluorescence microscopy can be a powerful tool for the study of metal signaling, providing the spatial resolution required to visualize the transient mobilization of intracellular metal cations in response to stimuli, combined with the temporal resolution to distinguish acute transients with reversible downstream effects from the chronic changes leading to long-term regulation. We present herein the most recent advances in the development of fluorescent probes and imaging techniques for understudied ions in cellular signaling, discussing remaining challenges and opportunities in the field. The reader is directed to excellent literature that discusses Zn^{2+} [2*,8] and Cu^{+} signaling [3*,9], and presents recent advances in the detection of labile $Fe^{2+/3+}$ [10,11], which are not covered here in detail. Instead, we focus the discussion on Mg^{2+}, an ion that is most often overlooked but that exemplifies the kind of paradigm shift posed by metal signaling beyond calcium.

Magnesium, a controversial second messenger:

In marked contrast with its heavier Group II metal congener, magnesium has received little attention in the context of signaling. A possible role for this metal in signal transduction was first investigated with regards to the cellular response to insulin, and the proposal that Mg^{2+} ions act as second messengers in this context has been out in the field since the 1970s [12]. But this notion has been met with skepticism. Evidence for a connection between hypomagnesemia and glucose-stimulated insulin response has been contradictory [13–15] and the relevant mechanistic details have remained elusive.

Within the last decade, the idea of Mg^{2+} participating directly in signal transduction was re-examined by Lenardo and coworkers, who demonstrated that this cation meets the criteria—originally delineated based on cAMP [16]—to be designated as a second messenger in the

context of T-cell activation. Their mechanistic proposal invokes an influx of Mg^{2+} through MagT1 in response to antigen receptor stimulation, leading to activation of phospholipase C-γ (PLCγ1) and changes in Ca^{2+} influx [17]. This mechanism has since been revised and refined [18**,19] to account for the fact that MagT1 functions as an accessory subunit of the oligosaccharyl-transferase, OST—and perhaps not as a magnesium transporter, after all—playing a role in Mg^{2+}-dependent glycosylation of NKG2D (also known as killer cell lectin-like receptor K1) and thus affecting the function of cytotoxic immune cells [19]. The basic features of the model, however, are thought to remain, though the source of the observed cytosolic Mg^{2+} transient upon T-cell activation is unclear.

More recently, Oka and coworkers demonstrated an increase in cytosolic Mg^{2+} upon γ-aminobutyric acid (GABA) receptor stimulation in young neurons. The transient was proposed to originate from mobilization of Mg^{2+} from mitochondria, and it was shown to activate the mammalian Target of Rapamycin (mTOR) and transcription factor CREB, independent of Ca^{2+}, thus suggesting a role of Mg^{2+} as a second messenger in early stages of neuronal development (Figure 1) [20**].

Imaging cellular free Mg^{2+}:

The selectivity problem: One of the most important challenges in the study of Mg^{2+} in a signaling context is disentangling its role from that of Ca^{2+}. The complex interplay between the two ions has been difficult to examine given the poor selectivity of currently available molecular tools for detection of the former. Specifically, most small molecule fluorescent sensors for the hard Mg^{2+} ion bear metal recognition moieties rich in carboxylate groups, which also bind other hard biologically-relevant divalent cations leading to interferences in cellular imaging [21]. The aminophenol triacetic acid (APTRA) moiety, most commonly used in commercial Mg^{2+} indicators, is notorious for this limitation [22].

Recent efforts toward the design of better, more selective chelators [23,24**] have ushered important advances in fluorescent sensor development. The groups of Mizukami and Kikuchi developed the MGQ series of sensors (Figure 2) based on a novel 2,8-dicarboxyquinoline metal binding moiety that exhibits good selectivity for Mg^{2+} over Ca^{2+} and is well tuned to the low millimolar physiological concentrations of Mg^{2+} (e.g. $K_{D\ Mg}{}^{2+}$= 0.24 mM for MGQ-2) [24,25]. Both green and red-emitting derivatives exhibit a turn-off fluorescent response, less desirable for imaging applications. Nevertheless, combination with a turn-on sensor of a different color enabled ratiometric imaging of Mg^{2+} extrusion with enhanced sensitivity in cells overexpressing CNNM4, a Mg^{2+} transporter [25]. Highly selective, ratiometric imaging based on individual small molecule probes remains an important unmet need in the field, particularly relevant to proper quantification of Mg^{2+} transients.

Sensors based on β-dicarbonyl chelators, including the KMG series [30–32,40,41] and related compounds [33,35] (Figure 2), also show an excellent metal selectivity profile. Due to their low denticity, however, these compounds easily form ternary complexes with Mg^{2+}-bound biomolecules [42*], encumbering the distinction of free—available for signaling—versus bound metal. In recent work, Murata *et al* [41] used a near infrared-emitting member of the KMG family in parallel to a genetically encoded MgATP indicator to monitor rapid

changes in magnesium upon mitochondria uncoupling. This combined imaging approach ruled out a major direct effect of MgATP on the fluorescence output of the small molecule. Similar approaches are likely necessary for the unequivocal study of free Mg^{2+} with these indicators in systems in which the highly dynamic pools of biomolecule-bound Mg^{2+} may change. Oka and coworkers capitalized on a combination of green and mitochondria-targeted, red-emitting members of the KMG family, namely KMG-104 and KMG-301, respectively, to show GABA-induced release of Mg^{2+} from mitochondria leading to activation of the mTOR and CREB signaling pathways in the maturation of neural networks. This work constitutes the most recent demonstration that Mg^{2+} participates in signal transduction, with a clearly identified source and molecular target [20**].

Looking for the source of ion mobilization—Despite mounting evidence toward Mg^{2+} acting as a second messenger, questions remain regarding the identity of the cellular stores that act as sources of the free metal, especially relevant to mobilization in a signaling context. Mitochondria have been found to perform this function [20,31,43], but studies have also shown that the pool of biomolecule-bound Mg^{2+}, including the highly abundant pool of MgATP available in many compartments, could be a major contributor to intracellular changes in free Mg^{2+} under certain circumstances [39*]. The development of targeted fluorescent sensors is crucial for probing organelle-specific levels of metals and investigating patterns of ion accumulation and mobilization. Through favorable combinations of lipophilicity and positive charge, mitochondria targeting of Mg^{2+} indicators can be readily achieved [28*,32]. But more general design strategies, suitable for targeted imaging of other compartments of choice, remain scarce.

Buccella and coworkers have tackled the challenge of organelle-targeted Mg^{2+} detection by developing a hybrid system that combines a small molecule fluorescent sensor, Mag-S-Tz, and a HaloTag fusion protein that acts as intracellular directing group [36*]. The small molecule fluorophore is installed on the protein via *in situ* fluorogenic reaction with a dual reactive chloroalkane ligand. The fluorogenic conjugation step is key to enabling selective activation of fluorescence in the chosen subcellular locale, minimizing spurious signal from fluorophores that remain trapped in undesired compartments and compromise spatial resolution. In experiments done with HEK 239T cells, the hybrid system enabled comparison of the relative levels of free Mg^{2+} in various organelles, showing differences in basal concentrations of the compartments as well as the strengths and generality of this approach for potentially probing sources and destinations of intracellular metal trafficking. Another important advantage of hybrid protein-small molecule sensing designs is their increased intracellular retention compared to the freely diffusing small molecule counterparts. This feature, demonstrated by the groups of Kikuchi and Mizukami with a HaloTag-conjugated Magnesium Green derivative, MGH [37], enables imaging of ions over long periods without loss of signal, thus facilitating the distinction of short term signals versus long term changes involved in chronic regulation.

Quantifying the amplitude of a signal: a major challenge across metals—Quantification of the magnitude of a transient—the amplitude of a signal—remains a major challenge for most metals. Ratiometric detection, typically based on two wavelengths of

fluorescence emission or excitation, normalizes the signal and minimizes the effect of most analyte-independent factors that affect the fluorescence output in microscopy experiments. Reversible, ratiometric metal-responsive indicators are thus preferred when quantitative rather than qualitative information is sought about the target metal.

Genetically encoded, entirely protein-based FRET sensors are ideal candidates for ratiometric detection of ions with subcellular resolution. This class of sensors has been used extensively to study the roles of Ca^{2+} and Zn^{2+} in cell signaling [44,45]. For Mg^{2+}, however, the scarcity of protein domains that (i) bind the metal with the right affinity and selectivity, (ii) exhibit a large conformational change upon metal binding, and (iii) retain *in cellulo* the binding properties optimized in vitro, have been major roadblocks. Merkx and coworkers reported MagFRET, the first genetically-encoded Mg^{2+} sensor, based on modified human centrin-3 (HsCen3) for magnesium recognition [38]. Though MagFRET-1 showed favorable properties in vitro, it was unresponsive to procedures known to alter cellular Mg^{2+} levels, possibly due to unanticipated interactions of the probe with other proteins in the cellular milieu. Most recently, Maeshima and coworkers reported MARIO, a new genetically-encoded Mg^{2+} indicator that incorporates the cytosolic domain of the *E.coli* Mg^{2+} transporter CorA for metal binding, flanked by an ECFP/Venus FRET pair for ratiometric detection [39*]. A nuclear-targeted variant was employed to monitor relative Mg^{2+} levels during mitosis in the first—and only—successful example of fluorescence detection of changes in cellular free Mg^{2+} by a genetically encoded magnesium indicator in live cells. Metal quantification based on calibration of fluorescence ratio from dual wavelength Mg^{2+} indicators, however, is still primarily achieved with less selective small molecule indicators and remains to be demonstrated with the newer genetically encoded counterparts. Advances toward this goal are important for assessing the amplitude of the Mg^{2+} transients, which are notoriously difficult to quantify over the background of a high basal concentration.

Conclusions and Perspective: Advances in imaging technologies combined with the development of increasingly selective, sensitive fluorescent indicators have revolutionized the study of the cell biology of metals. Fluorescent indicators developed by Roger Tsien, such as fura-2 [46], were instrumental in attaining our current understanding of calcium signaling, and they established much of the foundation for the design of tools that are now ushering the re-evaluation of the roles of other metals in biology. Furnished with the right indicators, fluorescence microscopy is particularly well suited to reveal in real time the transient changes in metal availability required for signaling to take place. Furthermore, the high resolution offered by fluorescence imaging with *targeted* indicators can deliver information on the spatial localization of such signals and help uncover intraor extracellular sources of ion mobilization, thus providing a more complete picture of the information flow.

As exposed herein through the lens of magnesium, the study of metals in signaling roles is pushing the design of a better toolbox to track metals at the sub-cellular level. Each metal cation brings unique parameters and technical challenges to the development of fluorescent indicators. Yet the final requirements are similar for all, and include well-tuned affinity, high selectivity for the target cation over others, ability to quantify transient changes in ion concentration, and controllable localization to enable visualization of patterns of metal accumulation and translocation to and from subcellular compartments. Undoubtedly, much

progress has been made in various fronts. Careful molecular design, based on consideration of the unique coordination chemistry of each metal, has enabled the development of indicators with sufficient selectivity to probe, at last, transients of Mg^{2+} without the interference from Ca^{2+} fluxes. New genetically encoded indicators have provided access to metals on specific cellular compartments of interest. Finally, hybrid indicators are filling some of the gaps left by small molecules and protein-based ones, combining some of the best features of both classes. But a number of challenges still remain, and reliable quantification is perhaps at the top of the list.

The notion of *d*-block metals and magnesium playing a role in signaling represents a clear departure from the current paradigm in which these metals are viewed exclusively as controlling basal metabolic activity. This shift forces us to rethink the basic mechanistic aspects of information transfer and to question the magnitude of a transient required to carry a signal. In principle, the vast differences in the concentrations of the different metals—and of the affinities and concentrations of their binding partners, which ultimately determine both metal availability and the concentration range at which a downstream effect can be triggered—open the possibility of multiple ions operating in parallel, carrying different signals with virtually no crosstalk. Probing the molecular aspects of such model poses an exciting challenge for chemists and biologists alike, presenting a fertile ground for innovation in tool design.

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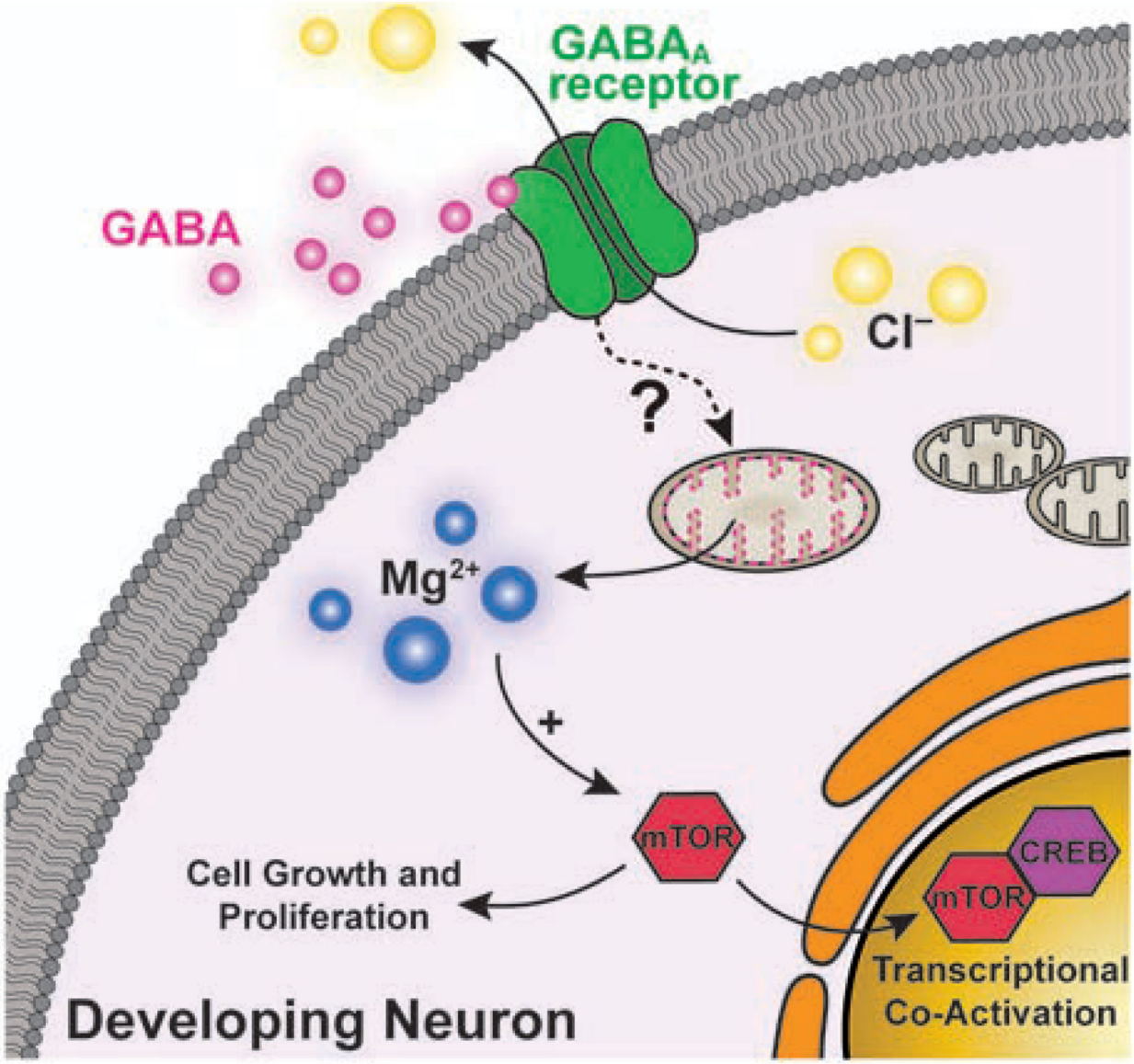

Figure 1.

Schematic representation of the proposed mechanism for the GABA-induced, Mg^{2+}-mediated activation of mTOR in developing neurons. Stimulation of GABA receptors is shown to elicit the release of mitochondrial Mg^{2+} into the cytosol by an unidentified mechanism. An increase in $[Mg^{2+}]_{cyto}$ leads to the activation of mTOR and CREB, which activates transcriptional programs toward dendritic growth.

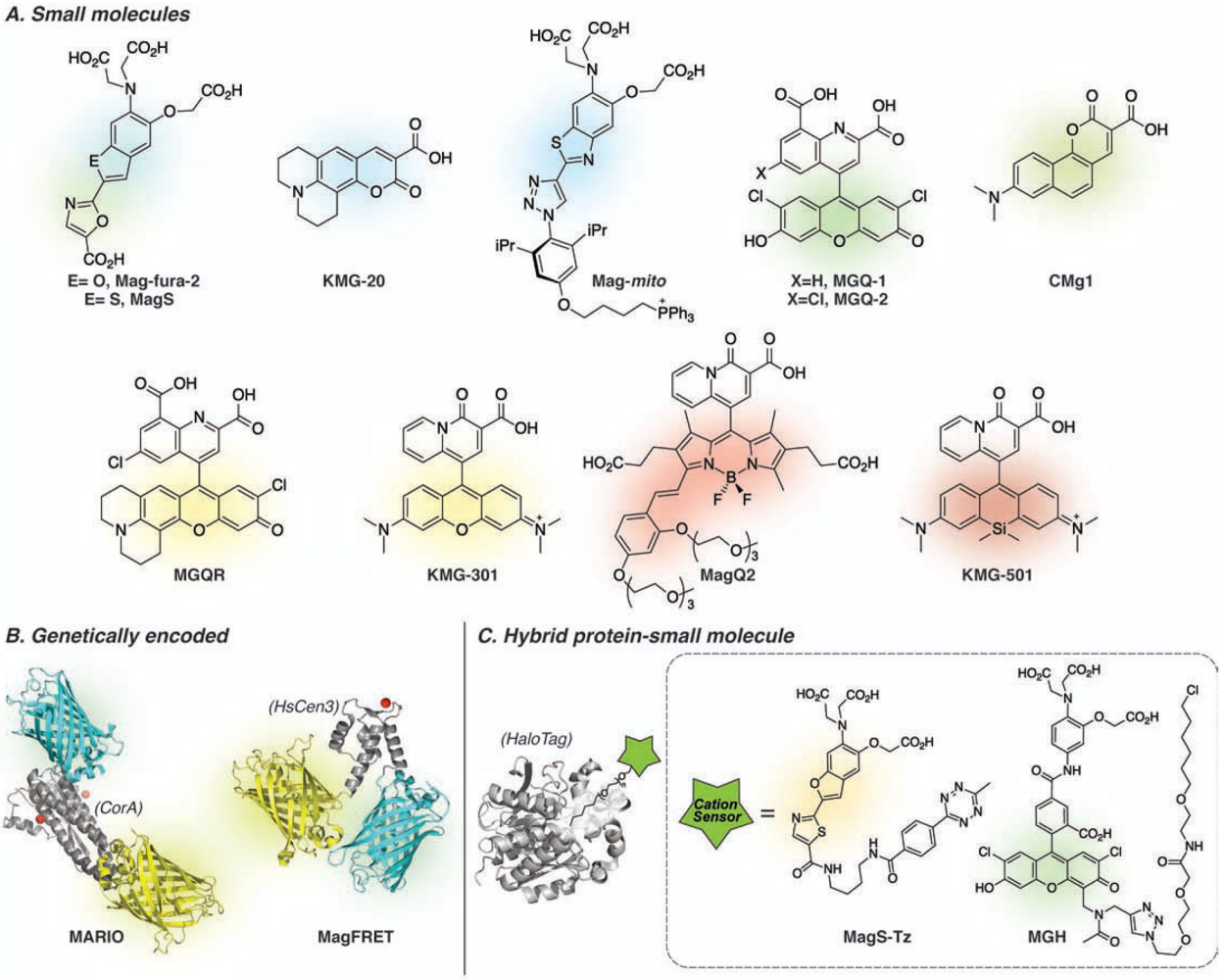

Figure 2.

Selected fluorescent indicators used for imaging cellular free Mg^{2+}. (A) Small molecule indicators. (B) Genetically encoded, all-protein-based indicators. MARIO is comprised of the metal binding cytoplasmic domain of CorA (PDBID: 2HN2), flanked by ECFP (PDBID: 2YDZ) and Venus (PDBID: 1MYW) fluorescent proteins; MagFRET contains the N terminus of sCen3 as metal binding domain (drawn based on HsCen2, PDBID: 2GGM) flanked by Cerulean (PDBID: 2Q57) and Citrine (PDBID: 1HUY). (C) Hybrid indicators comprised of a small molecule sensing component and a protein carrier. The basal concentration of free Mg^{2+} in most cells is 0.5–1.0 mM, requiring indicators with low affinity for maximum sensitivity under physiological conditions.

Table 1.

Spectroscopic properties of selected fluorescent indicators for imaging free Mg^{2+}.

Probe	Absorption λ_{max} (nm)	Fluorescence Emission λ_{max} (nm)	$\Phi_{(free)}$, $\Phi_{(bound)}$	$K_{D, Mg^{2+}}$ 25 °C (mM)	$K_{D, Mg^{2+}}$ 37 °C (mM)	Ref.
Small molecules						
Mag-Fura-2	369, 330[a]	511, 491[a]	0.24, 0.30	1.9[b]	1.5	[26,27]
Mag-mito	356, 330[a]	495, 482[a]	0.42, 0.25[c]	6.7	N.R.	[28*]
Mag-S	396, 350[a]	572, 547[a]	0.17, 0.30	3.2	1.97	[29]
KMG-20	425, 445[a]	485	0.63	N.R.	10	[30]
KMG-104	504	523	N.R., 0.02	2.1	N.R.	[31]
MGQ-1	515	536	0.36, <0.01	N.R.	0.14	[24**]
MGQ-2	516	536	0.33, <0.01	N.R.	0.27	[24**]
MGQR	561	588	0.29, <0.01	N.R.	0.29	[25]
KMG-301	563	590	N.R., 0.15	4.5	N.R.	[32]
MagQ2	600	634	0.0099, 0.34	1.51	N.R.	[33]
KMG-501	663	684	0.004, 0.05	3.2	N.R.	[34]
CMg1	820, *880*[d]	559	0.29, 0.28	1.7	N.R.	[35]
Hybrid Sensors						
Mag-S-Tz	404, 358[a]	595	0.1, 0.24	3.1	N.R.	[36*]
MGH	515	538	0.19, 0.56	N.R.	1.3	[37]
Genetically Encoded						
MagFRET-1	Cerulean, Citrine			0.15[e]	-	[38]
MARIO	ECFP, Venus			7.2	N.R.	[39*]

[a] Ratiometric sensor; two values represent absorption or emission maxima in the metal-free and -bound form, respectively.

[b] Dissociation constant reported at 22 °C.

[c] Quantum yields determined on reference, non-targeted analogue.

[d] Two-photon absorption maxima.

[e] Temperature not specified *N.R.* = not reported.