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# Supramolecular Metal Displacement allows On-Fluorescence Analysis of Manganese(II) in Living Cells

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## Abstract

Due to the importance of $Mn^{2+}$ ions in biological processes, it is of growing interest to develop protocols for analysis of $Mn^{2+}$ uptake and distribution in cells. A supramolecular metal displacement assay can provide ratiometric fluorescence detection of $Mn^{2+}$, allowing for quantitative and longitudinal analysis of $Mn^{2+}$ uptake in living cells.

Manganese functions in both catalytic and structural roles in diverse proteins such as the photosynthetic apparatus and the enzyme superoxide dismutase.[1] It plays important roles in gene expression via Mn-binding transcription factors, and metalloregulatory proteins are involved in metal-specific homeostasis.[2] Its roles in virulence[3] and oxidative stress[4] have been reviewed. Additionally, $Mn^{2+}$ is used widely as a versatile tool for biological studies at the cellular and organ level. Its similarity to calcium and efficient quench of intracellular dyes have facilitated the measurement of ion channel flux.[5] The high spin of $Mn^{2+}$ renders it an excellent MRI contrast agent, the only metal ion other than gadolinium(III) approved for clinical use. Moreover, paramagnetic $Mn^{2+}$ ions have been shown to accumulate in neural cells by uptake through voltage-gated calcium channels,[6] enabling Mn-enhanced MRI (MEMRI) for mapping neural activity in mice.[7–9] In light of the wide occurrence and broad utility of $Mn^{2+}$ in living systems, it is of interest to develop analytical tools for its detection. The goal of this study was to develop an on-fluorescence method for the quantitation of $Mn^{2+}$ in cells that may eventually be adapted to tissue assays.

Fluorescent probes have proven highly useful for cell biology studies. Chelating dyes such as Quin-2 and Fura-2 have been used to measure $Ca^{2+}$ in different cellular compartments.[10,11] The membrane permeable dye Mag-fura-2 has been used to quantify ER $Ca^{2+}$ levels.[12] Fluctuation of $Ca^{2+}$ levels associated with cellular transitions have been measured.[13] Several available probes developed for $Ca^{2+}$ are quenched by $Mn^{2+}$.[10] Some on-fluorescence probes for $Mn^{2+}$ are available (e.g., Calcium Green, Invitrogen), but they generally show poor selectivity, particularly against $Ca^{2+}$. We are not aware of the availability of ratiometric fluorescent probes for intracellular $Mn^{2+}$.[14,15]

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Supplementary Information available. Additional experimental data including control experiments on A549 and HEK cell lines and statistical analysis.

Supramolecular strategies for analyte detection have drawn increasing attention for the solution of a variety of detection, quantification, and imaging problems.[16] We reported previously a two-metal, two-dye displacement assay to ratiometrically monitor $Cu^{2+}$ ions in aqueous solution.[17] We applied a similar strategy to the development of systems for naked-eye detection of $Mn^{2+}$.[18] Recently, Lippard and coworkers reported an on/off MRI/fluorescence displacement system.[19] In this paper, we utilize our metal-displacement assay to visualize $Mn^{2+}$ concentrations both in solution and in living cells.

Similarly to the previous method, two commercially available dyes were employed, calcein blue (CB) and fluozin-1 (Fz1). Initially, $Cd^{2+}$ was chelated by the stronger ligand CB and the formed complex was strongly fluorescent ("on"), while free ligand Fz1 gave weak fluorescence ("off"). Added $Mn^{2+}$ competes with $Cd^{2+}$ for CB, and quenches CB. In the new equilibrium state, $Cd^{2+}$ forms a complex with ligand Fz1 whose fluorescence is consequently turned "on". Solution experiments indicated that the metal displacement assay was compatible for use with $Mn^{2+}$. With an equimolar solution of $Cd^{2+}$, CB, and Fz1, strong blue and weak green fluorescence was observed with excitation at 350 and 493 nm, respectively (Figure 1). As $Mn^{2+}$ was added, the blue fluorescence diminished and the green fluorescence increased proportionately. Over the concentration range shown, the 433 nm fluorescence was quenched by 12-fold, while that at 518 nm increased by 7-fold. Since complexation of $Mn^{2+}$ quenched CB and $Cd^{2+}$ caused Fz1 to show increased fluorescence quantum yield, the observed behavior is consistent with the expectation that $Mn^{2+}$ competes with $Cd^{2+}$ for complexation of the stronger chelator (CB), liberating $Cd^{2+}$ to form a complex with Fz1. Interestingly, a large excess of $Mn^{2+}$ compared with concentrations of the $Cd^{2+}$ and the two dye molecules did not result in quenching at 518 nm, indicating that $Mn^{2+}$ did not compete effectively with $Cd^{2+}$ for chelation by Fz1 (Figure 1c). This conclusion was supported by a direct competition experiment (Figure S4) in which addition of 10 μM $Mn^{2+}$, $Cd^{2+}$ and Fz1 showed only slightly diminished fluorescence compared to $Cd^{2+}$ and Fz1 alone, implying a strong preference for $Cd^{2+}$. This is a significant advantage for this system with $Mn^{2+}$ compared to $Cu^{2+}$, where excess $Cu^{2+}$ quenched green fluorescence.

The system was less sensitive to $Mn^{2+}$ as compared with $Cu^{2+}$ as a result of the relative binding constants of the chelating dyes with $Mn^{2+}$ and $Cd^{2+}$ ($5.9 \times 10^6$ vs. $7.1 \times 10^7$ $M^{-1}$ for CB). Lower association of $Mn^{2+}$ compared with $Cu^{2+}$ would be expected as predicted by the classic Irving-Williams series. Association constants of $Mn^{2+}$ and $Cd^{2+}$ were determined by titration; in addition, a direct competition experiment for $Mn^{2+}$ and $Cd^{2+}$ for CB indicated a 7.5-fold preference for $Cd^{2+}$, in contrast to the very strong association of $Cu^{2+}$.[17] Fortunately, with 10 μM concentrations of the two dyes and $Cd^{2+}$, ratiometric behavior was observed over a range from 5–1000 μM.

Metal ion response screening for the same system was analyzed. A solution containing 10 μM Fz1, 10 μM CB, 10 μM $Cd^{2+}$, 10 μM metal ion was titrated with 50 μM $Mn^{2+}$. $Na^+$, $Cs^+$, $Mg^{+2}$, $Li^+$ did not interfere with fluorescence (Figure S1). The biologically important metal, $Zn^{2+}$, does compete with $Mn^{2+}$ and could be a potential source of background fluorescence in certain applications. Fluorescence intensity at 493 nm and at 350 nm was studied at several pH values. In the range of pH from 6.1 to 7.6 (Figure S6), Fz1 response is not dependent on pH, and its fluorescence intensity in both trials is consistent, whereas CB fluorescence intensity increases slightly with pH.

The method was next adapted to detect intracellular $Mn^{2+}$. We examined the displacement assay approach in Human Embryonic Kidney cells (HEK 293-F, fast growing variant) and an HEK cell line that stably over-expressed Divalent Metal Transporter 1 (*DMT-1*)[20] obtained from the Garrick Laboratory.[21] Cell permeable acetoxymethyl (AM) esters of CB and Fz1 (CBAM and Fz1AM) were employed, as these neutral derivatives cross the cell

membrane and are then hydrolyzed to cell impermeant CB and Fz1 by intracellular esterases within one hour.[22,23] An excess of CBAM compared to Fz1AM, was required as a result of the reduced take-up of CBAM which has only two AM esters as purchased commercially while Fz1AM has three and can therefore cross the cell membrane more easily. In experiments where $Mn^{2+}$ supplementation was used, cells were first incubated with $Mn^{2+}$, followed by the two dyes, and finally the $Cd^{2+}$.

Previous $^{54}Mn^{2+}$ uptake experiments performed on HEK cells showed minimal uptake without the expression the metal transport protein DMT-1.[21] Supplementation of HEK and *DMT-1* cells with 5 μM $Mn^{2+}$ yielded very little green fluorescence in HEK cells while *DMT-1* cells showed strong enhancement of fluorescence in the green channel, and diminished fluorescence in the blue channel (Figure 2). Anticorrelated green and blue fluorescence was heterogeneously distributed within the field of view (Figure S3). The ability of this method to resolve cellular, and potentially subcellular, localization of Mn[24–25] promises distinct advantages over other techniques. Control experiments were consistent with the operation of the displacement mechanism. In *DMT-1* cells treated with only Fz1AM and $Mn^{2+}$, absence of $Cd^{2+}$gave no fluorescent response (Figure S8). Cells treated with only CBAM enhanced with $Cd^{2+}$ but not $Mn^{2+}$(Figure S9). Treatment with CBAM and FzAM showed quenched CB with $Mn^{2+}$ and enhanced CB with $Cd^{2+}$(Figure S10). These experiments demonstrate that the fluorescence enhancement is well above background, and that $Zn^{2+}$ does not significantly interfere at the micromolar $Mn^{2+}$ concentrations examined here..

To fully realize the utility of our system for live imaging of cellular $Mn^{2+}$, real-time uptake of $Mn^{2+}$ was analysed in the *DMT-1* cells (Figure 3). Cells were incubated with the standard amount of CBAM, Fz1AM, and $Cd^{2+}$. $Mn^{2+}$ was then added and images were collected every twenty min over a period of 100 min. Fluorescence changes plateaued after 80 min (Figure S11). consistent with $^{54}Mn$ assays in cell pellets.[21] Blue fluorescence from CB correspondingly decreased over the entire time course.

The intracellular supramolecular assay responded quantitatively to varying concentrations of $Mn^{2+}$ (Figure 4, S12). Blue and green fluorescence intensities shifted over a range of 0–50 mM extracellular $Mn^{2+}$ in *DMT-1* cells. In a multiwell experiment, each concentration of $Mn^{2+}$ from 0 to 50 μM gave a decrease in blue fluorescence by a factor of 3 and an increase in green fluorescence by a factor of 11, consistent with a saturable system (Figure 4). A double reciprocal plot shows a linear relationship as expected for the DMT1 transporter for both green and blue average fluorescence intensities (Figure S13). These data again fit very well with previous $^{54}Mn^{2+}$ uptake experiments.[21]

Mn sensitivity was also explored in an additional cell line. The human lung carcinoma A549 cell line[26–27] behaved similarly to control HEK cells, giving signal only with high $Mn^{2+}$ exposure (Figure S14). Transient transfection with a DMT1 expressing plasmid improved sensitivity to $Mn^{2+}$ by several orders of magnitude in transfected cells (Figures S15). These data suggest that, in multiple cell types, $Mn^{2+}$ uptake in the cells is a tightly regulated process, which normally allows for very little free intracellular $Mn^{2+}$ without active uptake by DMT1.

Supramolecular displacement assays have found widespread interest in recent years,[16] but there have been few applications demonstrated in a cellular context. There are many challenges to the application of such methods in biological systems, including solubility, cell permeability, potentially variable subcellular localization of components, biocompatibility, orthogonality of the chemistry to intracellular receptors and metals, among others. The present assay requires a metal exchange reaction to occur in living cells and in preference to

other potentially competing processes. The $Mn^{2+}$ ion must find the Cd(CB) complex, undergo an exchange reaction, and the $Cd^{2+}$ must then find the Fz1 dye to form a complex without the metals being sequestered by endogenous metal receptor sites and without intracellular metal ions blocking access by the $Mn^{2+}$ or $Cd^{2+}$. As demonstrated by controls, without delivery of all components at relevant concentrations, no green fluorescence can be observed. Despite these challenges, these experiments were successful in reporting intracellular $Mn^{2+}$ in two different cell lines, and offers hope that other chemical displacement methods may also be effective in cellular systems.

Although our results presented here represent a significant advance in terms of demonstration of supramolecular displacement and $Mn^{2+}$ studies in live cells, there are several potential areas for improvement of this method for analysis of $Mn^{2+}$. Modification of CB with chelating moieties that would provide more selective association of $Mn^{2+}$ would improve quenching efficiency of the blue dye and would increase sensitivity. Also, linking the two dyes together covalently could guarantee delivery of equivalent concentrations to the cells and simplify the calibration required for quantitative studies.[28] Use of a more widely biocompatible reporter ion could permit a wider scope of application.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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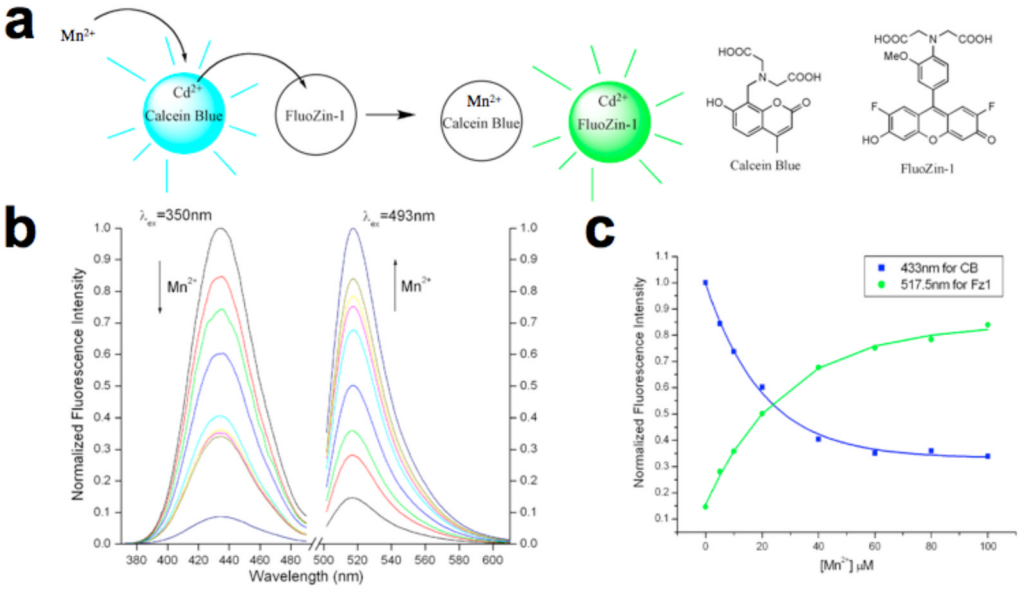


**Figure 1.**

Reporter metal displacement assay. (**a**) Reaction scheme. (**b**) Normalized fluorescence response for the titration of aqueous solution (pH=7.2) containing 10 μM CB, 10 μM Fz1, 10 μM $Cd(ClO_4)_2$, 50 mM HEPES and 100 mM $KNO_3$ with 0, 5, 10, 20, 40, 60, 80, 100 and 1000 μM $MnCl_2$. (**c**) Normalized fluorescence intensity at 433 nm (CB) and 517.5 nm (Fz1), as a function of $Mn^{2+}$ concentration.

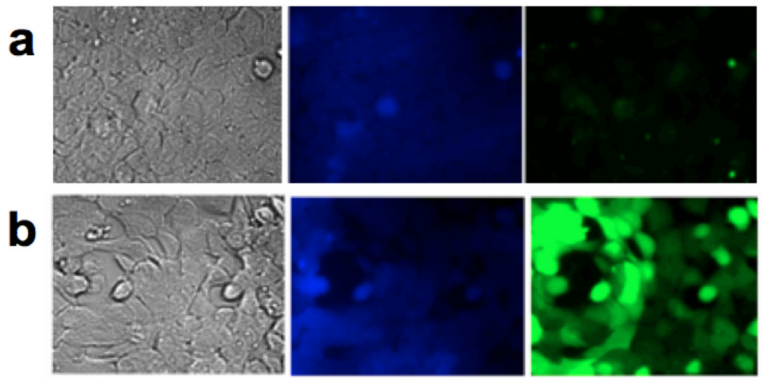


**Figure 2.**

HEK and *DMT-1* cells treated with: 5 μM $Mn^{2+}$, 3.6 μM Fz1AM, 26.6 μM CB-AM and 10 μM $Cd^{2+}$. (**a**) HEK cells show blue fluorescence but little green fluorescence, suggesting minimal $Mn^{2+}$ present in the cell. (**b**) *DMT-1* cells under the same conditions show lower blue fluorescence with bright green fluorescence indicative of markedly increased $Mn^{2+}$ uptake.

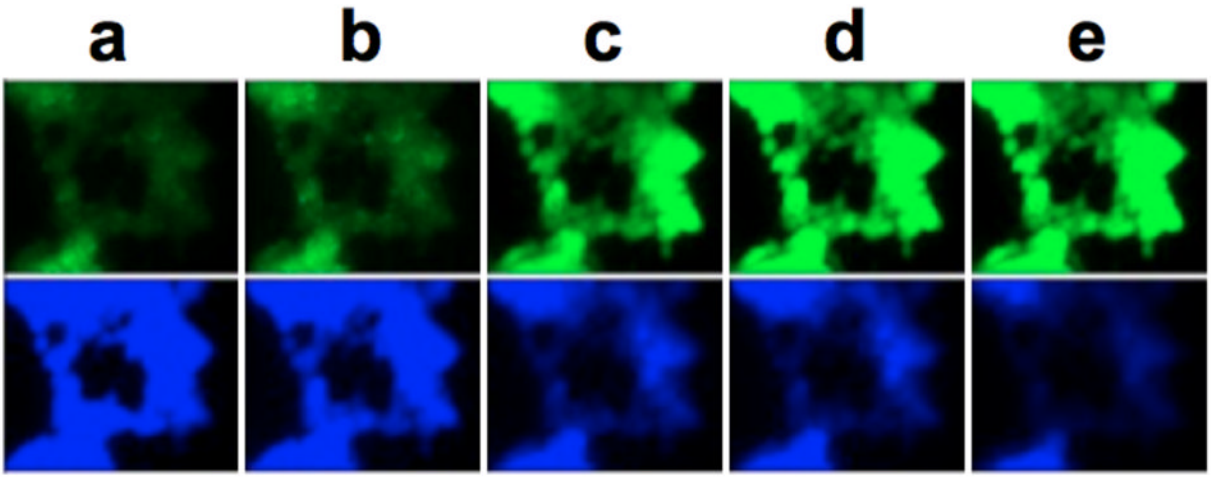


**Figure 3.**

Uptake of $Mn^{2+}$ over time monitored using fluorescence. Pictures were taken upon addition of 3.6uM Fz1-AM, 26.6 uM CB-AM, and 10 uM Cd(II) followed by 5 uM $Mn^{2+}$ at: (a) 20 min (b) 40 min (c) 60 min (d) 80 min and (e)100 min.

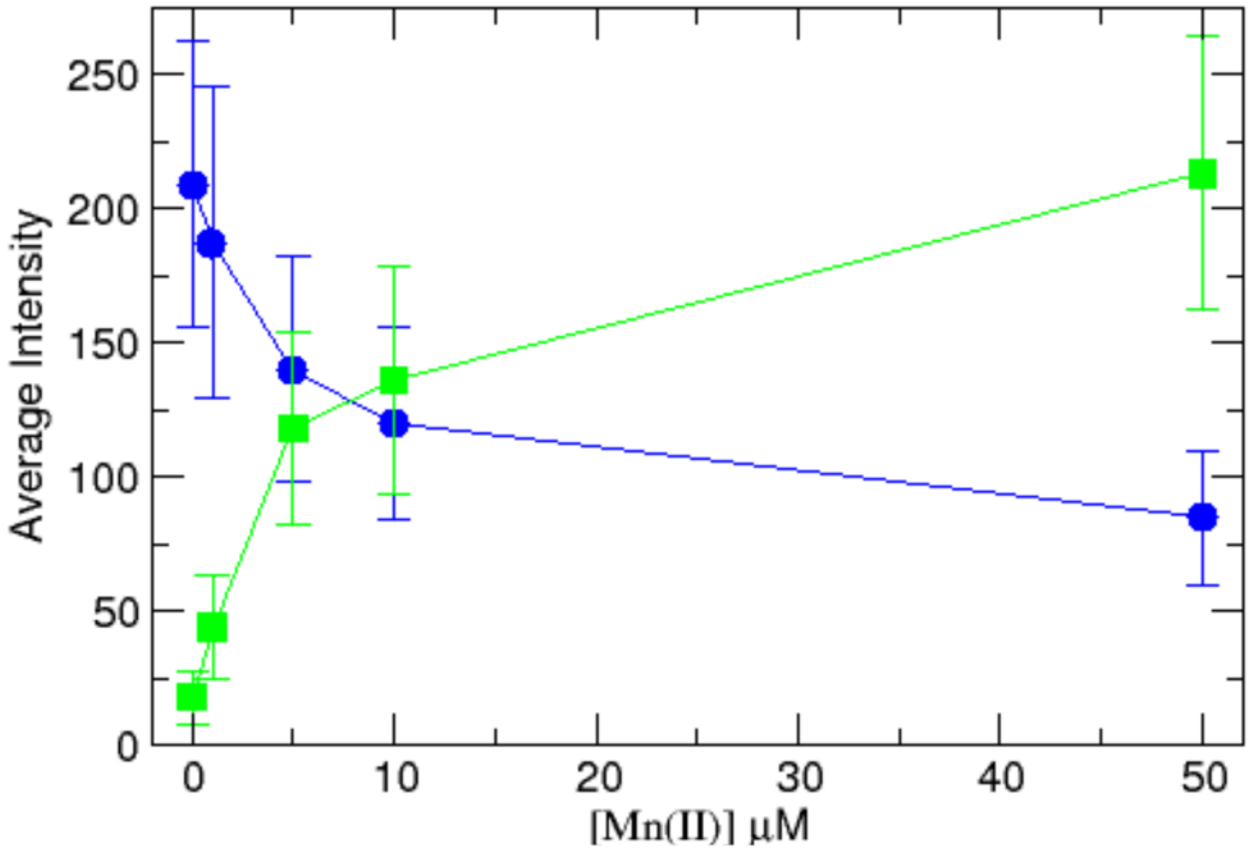


**Figure 4.**
Plot of fluorescent intensities as a function of $Mn^{2+}$ concentration in *DMT-1* cells.