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Novel high-throughput assay to assess cellular manganese levels in a striatal cell line model of Huntington's disease confirms a deficit in manganese accumulation

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Abstract

In spite of the essentiality of manganese (Mn) as a trace element necessary for a variety of physiological processes, Mn in excess accumulates in the brain and has been associated with dysfunction and degeneration of the basal ganglia. Despite the high sensitivity, limited chemical interference, and multi-elemental advantages of traditional methods for measuring Mn levels, they lack the feasibility to assess Mn transport dynamics in a high-throughput manner. Our lab has previously reported decreased net Mn accumulation in a mutant striatal cell line model of Huntington's disease (*STHdh*^{Q111/Q111}) relative to wild-type following Mn exposure. To evaluate Mn transport dynamics in these striatal cell lines, we have developed a high-throughput fluorescence-quenching extraction assay (Cellular Fura-2 Manganese Extraction Assay - CFMEA). CFMEA utilizes changes in fura-2 fluorescence upon excitation at 360 nm (Ca²⁺ isosbestic point) and emission at 535 nm, as an indirect measurement of total cellular Mn content. Here, we report the establishment, development, and application of CFMEA. Specifically, we evaluate critical extraction and assay conditions (e.g. extraction buffer, temperature, and fura-2 concentration) required for efficient extraction and quantitative detection of cellular Mn from cultured cells. Mn concentrations can be derived from quenching of fura-2 fluorescence with standard curves based on saturation one-site specific binding kinetics. Importantly, we show that extracted calcium and magnesium concentrations below 10 μ M have negligible influence on measurements of Mn by fura-2. CFMEA is able to accurately measure extracted Mn levels from cultured striatal cells over a range of at least 0.1 μ M – 10 μ M. We have used two independent Mn supplementation approaches to validate the quantitative accuracy of CFMEA over a 0 μ M – 200 μ M cellular Mn-exposure range. Finally, we have utilized CFMEA to experimentally confirm a deficit in net Mn accumulation in the mutant HD striatal cell line versus wild-type cells. To

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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conclude, we have developed and applied a novel assay to assess Mn transport dynamics in cultured striatal cell lines. CFMEA provides a rapid means of evaluating Mn transport kinetics in cellular toxicity and disease models.

Keywords

Manganese; Huntington's disease; High-throughput assay; Metal transport; Fura-2

1. Introduction

Manganese (Mn) is an essential ubiquitous trace element required for normal growth, development and cellular homeostasis (Erikson et al., 2005). In humans and animals, Mn functions as a required cofactor of several enzymes necessary for neuronal and glial cell function, as well as enzymes involved in neurotransmitter synthesis and metabolism (Butterworth, 1986, Erikson and Aschner, 2003, Hurley and Keen, 1987). Despite its essentiality in multiple metabolic functions, excessive levels of Mn exposure via occupational routes such as farmers exposed to Mn-based pesticides, industrial welders and miners (Dobson et al., 2004, Olanow, 2004, Roth and Garrick, 2003), and dietary intake, which include high concentrations of Mn in drinking water and long-term parenteral nutrition (Ensing, 1985), can accumulate in the brain and have been associated with dysfunction of the basal ganglia system (Aschner et al., 2007). We have previously reported decreased net Mn accumulation in a mutant *STHdh*^{Q111/Q111} cell line model of Huntington's disease (HD) relative to wild-type *STHdh*^{Q7/Q7} cells by graphite furnace atomic absorption spectrometry (GFAAS). Decreased Mn accumulation correlates with a concomitant decrease in vulnerability to Mn cytotoxicity (Williams et al., 2010a, Williams et al., 2010b). Furthermore, we have demonstrated a striatal specific decrease in net Mn uptake following systemic Mn exposure in a mouse model of HD (Williams et al., 2010a, Williams et al., 2010b). Given the decreased net Mn uptake in both cellular and mouse models of HD following Mn exposure, we sought to decipher the transport mechanism underlying decreased net Mn accumulation in mutant *STHdh*^{Q111/Q111} cells. Available methods to measure Mn levels in cultured cells and tissues include GFAAS, radioactive trace assays and inductively coupled plasma mass spectrometry (ICP-MS). Although these techniques include multi-elemental analysis, excellent specificity, extremely high sensitivity and limited chemical interference, they are not feasible to assess Mn transport kinetics in a high-throughput manner. This is mainly due to the large number of cells required, duration of experimental analysis and cost of analysis. Therefore, we sought to develop a high-throughput assay that enables rapid and efficient assessment of Mn transport kinetics and mechanism underlying mutant Mn deficit. After a literature search aimed at guiding the development of the high-throughput Mn assessment assay, we were intrigued by the relationship between Mn and the ratiometric calcium fluorophore fura-2, specifically, the Mn quenching properties of fura-2.

The development and utilization of the fluorescent calcium (Ca^{2+}) indicator, fura-2, by R.Y. Tsien and colleagues for both the determination of intracellular Ca^{2+} concentrations and its regulation by extracellular stimuli has improved our understanding of calcium signaling events and dysfunction in neurodegenerative diseases, including HD (Cobbold and Rink, 1987, Grynkiewicz et al., 1985, Lim et al., 2007, Oliveira et al., 2006, Tang et al., 2003, Tsien and Malinow, 1991). Fura-2 is the most commonly used 1,2-bis (o-aminophenoxy) ethane -N,N,N',N'-tetraacetic acid (BAPTA) based metal-binding fluorophore for microscopy of individual loaded cells. The spectral properties of these fluorophores change upon binding to Ca^{2+} ions and are modeled after the octacoordinate binding sites of the Ca^{2+} selective chelator ethylene glycol tetraacetic acid (EGTA). In comparison to other Ca^{2+}

indicators such as quin-2, fura-2 has a larger fluorophore that slightly increases its wavelengths and makes it compatible with glass microscope optics (Tsien and Pozzan, 1989). Upon Ca^{2+} binding to fura-2, the excitation spectrum shifts about 30 nm to shorter wavelengths. Hence, the ratiometric fluorescence intensity measurements obtained from the $F_{340/380}$ nm excitation pair (ratio of fluorescence yield following excitation at 340 nm over excitation at 380 nm) is considered to be a good measure of intracellular calcium concentration, and is unperturbed by variable dye content or cell thickness (Grynkiewicz et al., 1985). The green emission of fura-2 does not usually shift with calcium binding and peaks between 505 – 520 nm (Tsien, 1989).

Published data examining the influence of metal ions on fura-2 suggest an interaction between endogenous metal ions and fura-2 loaded cells by either quenching (Mn^{2+} , Cu^{2+} , Fe^{2+}) or increasing (Zn^{2+} , Cd^{2+}) fura-2 fluorescence (Grynkiewicz et al., 1985, Snitsarev et al., 1996). Consequently, other studies have utilized fluorescence quenching assays to assess intracellular metal ion concentration and transport in loaded cells and demonstrated a rapid and time-dependent quenching of fura-2 and calcein fluorescence following Mn^{2+} , Fe^{2+} , Co^{2+} , and Cu^{2+} exposure (Forbes and Gros, 2003, Grynkiewicz et al., 1985, Merritt et al., 1989, Picard et al., 2000, Snitsarev et al., 1996, Xu et al., 2009). However, all these studies were conducted on single cultured cells, which limit high-throughput measurements of Mn levels. We reasoned that a high-throughput manganese extraction based fura-2 assay might enable efficient and accurate measurements of Mn levels in cultured cells and allow for assessment of Mn dynamics in cellular models. Importantly, fura-2 has a Ca^{2+} isosbestic point at 360 nm, a wavelength at which fura-2 fluorescence emission properties are independent of Ca^{2+} concentration. Interestingly, Snitsarev and colleagues reported that Mn^{2+} effectively quenches fura-2 fluorescence, even at the Ca^{2+} isosbestic wavelength (Snitsarev et al., 1996). The aim of the study was to determine if a cellular fura-2 manganese extraction assay (CFMEA) could accurately measure Mn content in MnCl_2 exposed striatal cells and detect the Mn transport deficit previously reported in a HD striatal cell line model.

2. Materials and Methods

2.1. Chemicals, reagents, and cell culture supplies

Cell culture media and supplements were obtained from Mediatech (Manassas, VA) unless indicated. Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, and Sigma, St. Louis, MO), L-glutamine, 400 $\mu\text{g}/\text{ml}$ G418 and Penicillin-Streptomycin. Calcium (II) chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), cadmium (II) chloride heptahydrate ($\text{CdCl}_2 \cdot 7\text{H}_2\text{O}$), cobalt (II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), copper (II) chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), iron (II) chloride (FeCl_2), magnesium (II) chloride tetrahydrate ($\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$), manganese (II) chloride heptahydrate ($\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$), nickel (II) chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), lead (II) chloride (PbCl_2), zinc (II) chloride (ZnCl_2) were from Alfa Aesar (Ward Hill, MA). Ultra-pure fura-2 salt (cell-impermeable, ENZ-52007) was obtained from ENZO Biochem (New York, NY). The HEPES salt exposure buffer consisted of 25mM HEPES buffer (pH 7.2), 140mM NaCl, 5.4mM KCl, and 5mM D-glucose (Sigma). 1X ultra-pure phosphate-buffered saline (PBS), pH 7.4, without calcium and magnesium was used for post Mn exposure washes. Triton X-100 and sodium dodecyl sulfate (SDS) were obtained from Sigma.

2.2. Cell-free fura-2 assays

Cell-free system was composed of the indicated stock metal ions and fura-2 in either PBS with 0.1% Triton X-100 (PTx) or PBS with 0.8% SDS and assayed in a 96-well assay plate. After 5 seconds orbital shake, fura-2 fluorescence was measured at the Ca^{2+} isosbestic point

of Ex₃₆₀ (bandwidth of filter = ± 35 nm) and Em₅₃₅ (bandwidth of filter = ± 25 nm) with a Beckman coulter DTX 880 multimode plate reader using multimode analysis software (version 3.2.0.6) and top read settings.

2.3. Saturation binding curve and mathematical modeling of Mn-fura-2 interaction

Saturation binding curve experiments were performed using a ten point Mn-fura-2 curve with samples containing 1 μ L of different 100X stock Mn standards (0 – 100 mM) added to 99 μ L of 0.5 μ M fura-2 in PTx and assayed in a 96-well assay as described in section 2.2. The average raw fluorescence signal values (RFU) of the 0 μ M Mn samples within each independent standard curve were defined as the 100% maximal fluorescence for that experiment after background subtraction. RFU of each well were then normalized as percent maximal fluorescence (%_{MAX}) to this value. A one-site specific saturation-binding curve with Hill slope could be fit to log₁₀ Mn concentration (x-axis) and either %_{MAX} (y-axis) or RFU (y-axis) by non-linear regression analysis using graphpad prism software (version 5.0b). As utilization of %_{MAX} rather than RFU was found to control better for the experimental variation, we preferred use of %_{MAX} for experimental determination of extracted Mn concentrations. To permit back calculations of extracted Mn levels from experimentally determined %_{MAX} values we used the trend line command in Microsoft Excel to fit power (Mn concentration = $A \cdot (\%_{MAX})^B$) and logarithmic (Mn concentration = $A \cdot \ln(\%_{MAX}) + B$) equations to the standard curve data and calculated binding curve from non-linear regression analysis. Power curves were used for %_{MAX} values less than 50% and logarithmic curves for values greater than 50%, on either side of the inflection point of the saturation-binding curve plotted with Mn concentration (x-axis) and %_{MAX} (y-axis). These mathematical models used for calculating extracted Mn concentration routinely fit the saturation binding curves and standards data with r^2 values greater than 0.995.

2.4. Cell culture

The striatal cell lines – wild-type *STHdh*^{Q7/Q7} and mutant *STHdh*^{Q111/Q111} were a generous gift from Marcy Macdonald, PhD (Massachusetts General Hospital, Boston, MA, USA) and Gail Johnson, PhD (University of Rochester, Rochester, NY, USA) and grown at 33°C (Cattaneo and Conti, 1998, Milakovic and Johnson, 2005, Trettel et al., 2000, Williams et al., 2010a). Briefly, wild-type *STHdh*^{Q7/Q7} and mutant *STHdh*^{Q111/Q111} cells were plated in a 96 well plate at 8,000 cells per 0.32 cm² respectively the evening before treatment and allowed to grow in a 33°C incubator. MnCl₂ was added to the complete culture media or HEPES salt exposure buffer the morning of exposure. Total extracted Mn levels were assessed by CFMEA.

2.5. Cellular fura-2 manganese extraction assay (CFMEA)

Wild-type *STHdh*^{Q7/Q7} and mutant *STHdh*^{Q111/Q111} were cultured in 96 well tissue plates and exposed to varying MnCl₂ concentrations. After exposure, the media was discarded and cells quickly washed three times with 200 μ L PBS. The cells were extracted at 33°C for 1 hour in 100 μ L PTx with 0.5 μ M fura-2. Changes in fura-2 fluorescence caused by Mn quenching were measured on a plate reader as described in section 2.2. Extracted Mn concentrations from cultured cells were calculated using the logarithmic and power Mn standard curves described in section 2.3 with the %_{MAX} of each sample. Experimental sample %_{MAX} was determined by normalizing the RFU of each Mn-exposed sample with the RFU of vehicle-only samples (untreated), which was defined as the experimental 100% maximal fluorescence signal.

2.6. Mn-supplementation (Mn-spike) method

Wild-type *STHdh*^{Q7/Q7} cells were cultured and plated as described above in the methods and materials (section 2.5). Briefly, cells were plated in a 96 well tissue culture plate and exposed to 0, 50, 100, and 200 μM MnCl_2 for 4 hours in complete culture media. Cells were washed three times with 200 μL PBS and extracted at 33°C for 1 hour in 100 μL PTx buffer containing 0.5 μM fura-2 followed by measurements of fura-2 fluorescence as described in the methods and materials (section 2.2). Cell-extracts were quickly supplemented (i.e. “spiked”) with Mn and changes in fura-2 fluorescence re-measured at the aforementioned wavelengths (post-supplement). Mn concentrations in the cell extracts (pre and post-Mn spike) were computed by substituting the %_{MAX} of each experimental well after background subtraction for pre and post Mn spike measurements into a generated Mn standard curve. The difference in Mn levels in each well between pre and post Mn-spike (difference) was calculated. Alternatively wild-type striatal cells were exposed to 100 μM MnCl_2 and extracted in known concentrations of Mn spiked fura-2 containing extraction buffers (0 μM , 250 μM , 500 μM , 1000 μM MnCl_2). Both cellular and spiked Mn levels in the cultured cell-extracts were measured by CFMEA. The difference in Mn levels in each well between unspiked and Mn-spiked fura-2 containing extraction buffers in unexposed and Mn-exposed cultured cells (measured difference) was calculated by substituting the %_{MAX} of each experimental well after background subtraction for unspiked and Mn spiked measurements into a generated Mn standard curve. The measured difference between known and expected Mn concentrations was compared.

2.7. Statistical Analysis

Two-way analysis of variance (ANOVA) and student’s t-tests were performed using graphpad prism software analysis. Standard deviations of the mean for the change in net Mn uptake above basal levels were calculated by appropriate propagation of uncertainty calculations for subtraction of sample means, with significant differences between vehicle and Mn exposed wild-type by non-overlap of the standard deviation.

3. Results

3.1. Optimization of buffer, temperature, and detergent for CFMEA

To establish the basic CFMEA conditions, we first compared buffer conditions for detection of Mn-fura-2 interaction and found no significant difference between PBS and HEPES buffers (Fig. 1A). Previous studies using fura-2 AM loading in single cells exhibited a temperature-dependent subcellular localization of fura-2 (Margaroli et al., 1987, Plieth and Hansen, 1996, Poenie et al., 1986). Hence, we sought to determine the optimal temperature for detection of Mn quenched fura-2 fluorescence. Using a cell-free system, with varying MnCl_2 concentrations and fura-2, we observed a minimal influence of temperature on the Mn-fura-2 interaction (Fig. 1B). Finally, we evaluated the ideal detergent concentration for maximum Mn extraction and detection by fura-2 from a striatal cell line following a 100 μM MnCl_2 exposure. Maximal extraction was indicated by the greatest degree of fura-2 fluorescence quenching (i.e. decrease in RFU relative to the fura-2 containing detergent-buffer alone). After 45 minutes MnCl_2 exposure, cells were extracted in different concentrations of SDS or Triton X-100 in PBS for 1 hour at 33°C. RFU of fura-2 after the cell extraction (Mn extraction) and the baseline control (RFU of fura-2 containing buffer prior to extraction) were plotted for the different concentrations of detergent (Fig. 1C and D). In the absence of detergent, fluorescence quenching was not observed, indicating the post-exposure PBS washes were sufficient to remove fura-2 detectable extracellular Mn. As little as 0.001% Triton X-100 or 0.01% SDS was sufficient to extract Mn from Mn-exposed cells. Although 0.01% SDS exhibited the greatest quenching following extraction of Mn-exposed cells, we selected 0.1% Triton X-100 in PBS for subsequent Mn extraction

experiments in the cultured striatal cell line based on its maximal quenching and relatively minor changes in baseline RFU compared with SDS. SDS exhibited a dramatic phasic response in average RFU following Mn extraction of exposed cells. Moreover, we observed a concentration-dependent influence of both detergents on the baseline control fura-2 fluorescence. Specifically, SDS exhibited ~170 to 250% increase in control baseline RFU between 0.001 and 1% SDS. Triton X-100 showed a 10 to 20% increase in control baseline RFU at concentrations below 0.1 and 30% decrease at 1% Triton X-100. Furthermore, we observed an increase in control and Mn-exposed baseline fluorescence for both detergents at concentrations above 1% (data not shown). These results elucidated the optimal buffer, temperature, and detergent concentration for CFMEA in the striatal cell line.

3.2. Relationship between fura-2 concentration and Mn detection

In order to explore the optimal fura-2 concentration for detection of Mn, we utilized the aforementioned cell-free system with PTx and 0.5 μ M fura-2. Owing to the sigmoidal nature of the fura-2 fluorescence response to Mn, we defined the optimal detection range for Mn by fura-2 to be between 10% to 85% the maximal baseline control fura-2 fluorescence. The detection range for Mn by fura-2 was explored by examining concentration-response curves for Mn at three fura-2 concentrations (0.05, 0.5, and 2 μ M), and then fitting one-site specific binding curves to RFU values and Mn concentration by non-linear regression (Fig. 2). Specifically, 0.05 μ M and 2 μ M fura-2 accurately detected extracted Mn concentrations between about 50 nM – 5,000 nM and 300 nM – 10,000 nM respectively while 0.5 μ M fura-2 detected Mn concentrations between about 70 nM – 10,000 nM. Although 0.05 μ M fura-2 was capable of detecting lower Mn concentrations (50 nM), the lower absolute RFU values led to an inferior signal to noise ratio. Based on the signal to noise ratio, detection range, and expected concentration of Mn in cellular extracts, we selected 0.5 μ M fura-2 as the optimal concentration for detection of extracted Mn from striatal cell lines.

3.3. Quantitative relationship between Mn concentration and fura-2 fluorescence

To generate a Mn-fura-2 standard curve for back calculation of total extracted Mn levels from cultured cells, we utilized the cell-free system described in the methods and materials (section 2.3). Eight independent Mn-fura-2 standard curves generated over an extended period of time exhibited a fairly consistent relationship between absolute RFU values and Mn concentration across experiments, with higher variability in the absolute values of the low Mn concentration samples (Fig. 3A). To minimize this variability, we averaged the RFU values of the 0 μ M Mn samples within each independent standard curve and defined this value as the 100% maximal fluorescence for that experiment. RFU of each well were then normalized as %_{MAX} to this value and plotted as %_{MAX} (y-axis) by non-linear regression analysis (Fig. 3B). Importantly, we observed significantly less experimental variability in the standard curves when represented as %_{MAX} rather than RFU (y-axis). To explore the binding relationship between Mn and fura-2 in our standard curves, we generated a one-site specific saturation-binding curve with Hill slope as discussed in methods and materials (section 2.3), which tightly fit four averaged independent cell-free system experiments (Fig. 3C). Binding curve data was fit to exponential and logarithmic curves to enable back calculation of extracted Mn levels from experimentally determined %_{MAX} values. These curves could be generated alongside Mn-exposure experiments to accurately quantify extracted Mn levels, and consistently demonstrated nearly identical Mn-fura-2 saturation binding curves and equations (Fig. 3B).

3.4. Effect of metal ions on fura-2 fluorescence at Ex₃₆₀

Previous studies investigating the effect of metal ions on fura-2 fluorescence have demonstrated that other metal ions influence fura-2 fluorescence (increase or decrease) at the F_{340/380} excitation/emission wavelengths (Forbes and Gros, 2003, Grynkiewicz et al.,

1985, Merritt et al., 1989, Picard et al., 2000, Snitsarev et al., 1996). In addition, Snitsarev and colleagues have shown that Zn^{2+} , Ca^{2+} , and Mn^{2+} influence fura-2 fluorescence between 300 nm and 400 nm excitation wavelengths (Snitsarev et al., 1996). To evaluate the potential of other metal ions to influence CFMEA, we examined the effect of other metal ions on our fura-2 cell-free system. Specifically, we generated $\text{Ex}_{360}/\text{Em}_{535}$ concentration-response curves for different metal ions with 0.5 μM fura-2 in PTx buffer. The influence of these metal ions on fura-2 fluorescence was measured and fitted by nonlinear regression analysis to a one-site competitive binding curve (Fig. 4). We determined the approximate $\%_{\text{MAX}}$ at saturated metal binding of fura-2 (B_{max}) and half maximal effective concentration (EC_{50}) values for each metal ion from the one-site binding curves (Table 1). Here, we show that while Mn^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , and Cd^{2+} quenched fura-2 fluorescence at varying concentrations, Pb^{2+} , Ca^{2+} , and Mg^{2+} had no effect on fura-2 fluorescence at the Ca^{2+} isosbestic wavelength. Furthermore, we observed an increase in fura-2 fluorescence in the presence of Zn^{2+} . This suggests a need to control for the influence of some of these intracellular metal ions on CFMEA.

3.5. Influence of metal ions on CFMEA

Although our experiments above and previous experimental reports suggest that intracellular metal ions could influence fura-2 fluorescence, it was unclear whether the concentrations of abundant endogenous cellular metal ions (Grynkiewicz et al., 1985) would competitively influence Mn quantification by fura-2 in CFMEA. Therefore, we assessed the competitive influence of the two most abundant cellular metal ions, Ca^{2+} and Mg^{2+} , on the Mn-fura-2 interaction at the Ca^{2+} isosbestic wavelength of 360 nm using a cell-free system with varying concentrations of either CaCl_2 or MgCl_2 at different MnCl_2 levels. We observed no competitive influence of either Ca^{2+} or Mg^{2+} ions on Mn induced fura-2 quenching at concentrations of 10 μM or lower for all tested Mn concentrations (Fig. 5A and B). However, we observed a competitive quenching effect on fura-2 fluorescence with increasing Ca^{2+} concentrations above 10 μM Ca^{2+} for all tested Mn concentrations (data not shown). Given the considerable affinity of Co^{2+} , Cu^{2+} , and Fe^{2+} for fura-2, we examined the possible competitive interference of the aforementioned metal ions on the Mn-fura-2 interaction at the Ca^{2+} isosbestic wavelength of 360 nm using a cell-free system with varying concentrations of either CoCl_2 , CuCl_2 , and FeCl_2 at different MnCl_2 levels. We observed a concentration-dependent quenching effect of all three metals on fura-2 fluorescence at the Ca^{2+} isosbestic wavelength of 360 nm. However, there was no significant competitive influence of Co^{2+} concentrations below 1 μM on Mn induced fura-2 quenching at 0.05 μM and 0.2 μM MnCl_2 . Importantly we observed minimal competitive quenching influence of the differently tested Co^{2+} concentrations on 2 μM and 20 μM MnCl_2 concentrations (Fig. 5C). Moreover, we observed a similar effect of Cu^{2+} on Mn-fura-2 interaction (data not shown). In addition, Fe^{2+} demonstrated a minimal additive quenching effect on Mn-Fura-2 interaction at all the tested concentrations (Fig. 5D).

3.6. Validation of CFMEA by Mn supplementation

To determine if other metal ions present in cultured striatal cells influence the specificity and accuracy of CFMEA, we validated the accuracy of Mn determination by a Mn supplementation test (Mn spike method A). This method measures Mn levels in extracts of MnCl_2 exposed cultured cells before (pre) and after (post) the addition of a fixed concentration of Mn to the cell extract. We reasoned that if metal ions other than Mn, present in the cultured cells, have a significant influence on measured Mn concentrations, then the different concentration of Mn and other ions in the cell-extracts following exposure would impede our ability to accurately measure a known quantity of Mn spiked into the samples ("post" minus "pre"). Upon comparison of measured Mn levels in the cultured striatal cell-extracts following MnCl_2 exposure, pre, and post-Mn spike, we observed a

concentration-dependent increase in measured Mn levels in pre and post Mn-spiked conditions. However, there was no statistically significant difference in the calculated concentration of supplemented Mn measured over the full range of MnCl₂ exposure conditions. This data suggests that CFMEA is capable of accurately measuring Mn concentrations in extracted cultured cells over a two log₁₀ scale concentration range of extracted Mn (Fig. 6A and B). Conversely, we validated the accuracy of CFMEA by an alternative Mn supplementation method (Mn spike method B). This method measures Mn levels in cultured cells extracted in differently spiked (0 μM, 250 μM, 500 μM, and 1000 μM MnCl₂) fura-2 containing extraction buffer. We reasoned that if endogenous cellular metal ions other than Mn present in the cultured cells would competitively influence measured Mn concentrations, then CFMEA would be incapable of accurately measuring both the known concentration of Mn spiked into the fura-2 containing extraction buffer and cellular Mn levels. Upon comparison of the difference in Mn levels in each well containing cell-extract between unexposed and Mn-exposed cultured cells extracted in unspiked or Mn-spiked (measured difference) fura-2 containing extraction buffers, we observed an increase in measured Mn levels under all Mn-spiked conditions. Moreover, we observed no statistically significant difference between the known and measured Mn concentration difference at all the tested Mn spike concentrations (Fig. 6C and D). The findings in both Mn spike methods (A and B) suggest that CFMEA is proficient in accurately and specifically measuring Mn concentrations in both Mn spiked extracted cultured cells and fura-2 containing extraction buffer.

3.7. Mn accumulation deficit in mutant HD striatal cell line

To determine if CFMEA is capable of detecting a net Mn accumulation deficit in mutant striatal cells previously identified by GFAAS (Williams et al., 2010a, Williams et al., 2010b) we exposed cultured wild-type and mutant striatal cells to different MnCl₂ concentrations for 27 hours in culture media. After this Mn exposure, we performed CFMEA. We observed a concentration-dependent increase in net Mn uptake in both wild-type and mutant cells (Fig. 7). A two-way ANOVA showed a main effect of Mn-exposure ($F_{(4, 170)} = 91.60$, $p < 0.0001$) and genotype ($F_{(1, 170)} = 1221$, $p < 0.0001$), as well as exposure by genotype interaction effect was seen ($F_{(4, 170)} = 50.11$, $p < 0.0001$). Furthermore, *post-hoc* analysis by *t*-test found that mutant cells had a statistically significant decrease in net accumulated Mn compared to wild-type cells at all exposures ($p < 0.0001$).

4. Discussion

We have developed a high-throughput fluorescence quenching assay, CFMEA, by establishing the critical extraction and assay conditions (e.g. extraction buffer, temperature, and fura-2 concentration) required for quantitative detection of extracted Mn from cultured striatal cell lines by fura-2. The assay utilizes changes in fura-2 fluorescence as an indirect read-out of intracellular Mn concentrations. In contrast to the typical usage of fura-2 following cell loading, this high-throughput and cost-effective assay extracts intracellular Mn in cultured striatal cells following MnCl₂ exposure and allows for rapid assessment of Mn dynamics. In this assay, cultured cells are thoroughly washed following Mn exposure and intracellular Mn is extracted by detergent solubilization in the presence of fura-2. Fluorescence is then measured at Ex₃₆₀/Em₅₃₅. We have shown that a Mn standard curve, based on saturation one-site specific binding kinetics, can be generated to enable back calculation of Mn concentration from Mn quenching of fura-2 fluorescence. We have also examined the influence of other metal ions on CFMEA at this Ca²⁺ isosbestic wavelength (Ex₃₆₀/Em₅₃₅). In addition, we have validated the accuracy of CFMEA Mn quantification and utilized this assay to confirm a net Mn accumulation deficit in a striatal cell line model of HD.

We report that the ideal detergent concentration for Mn extraction from the striatal cell lines is 0.1% Triton X-100 (Fig. 1C). Furthermore, detergent was required for detection of fura-2 quenching by CFMEA, suggesting that extracellular Mn is not a significant component of the Mn detected by this assay. We observed minimal influence of Triton X-100 on baseline fura-2 fluorescence relative to SDS. In addition, the unexpected biphasic increase in fura-2 RFU of both baseline control and cell extracts from 0 to 1% SDS may possibly be due to an interaction between SDS and fura-2. Interestingly, previous experimental evidence suggests that SDS can exist either as monomers or micellar aggregates in aqueous solutions depending on the total concentration, ionic strength, and temperature (Reynolds and Tanford, 1970). Moreover, increasing total concentration of SDS at a given ionic strength impedes the ability to measure increases in monomer concentration above the critical micelle concentration (CMC) (Reynolds and Tanford, 1970). The phasic Mn quenching observed in both SDS and Triton X-100 at higher concentrations might be due to alterations in the CMC of both detergents used for Mn extraction in the cultured striatal cells.

The generation of a Mn-fura-2 standard curve enabled back calculation of Mn levels in cultured striatal cells. However, we observed experimental variability in RFU between independent standard curves generated over an extended period of time. This variability may be due to experimental differences in fura-2 concentration, fluorometric plate reader sensitivity, and detection of Mn quenched fura-2. Thus, the use of %_{MAX} to calculate Mn levels in cell extracts may improve the accuracy of Mn determination across experiments. We observed agreement in the quantitative relationship between Mn concentration and fura-2 fluorescence in the eight independent standard curves, which had a tight fit to a one-site specific binding curve with Hill slope (Figure 3). This data argues for the possibility of using a single Mn-fura-2 standard curve for multiple independent experiments to facilitate high throughput analysis.

Owing to the published effect of metal ions on fura-2 fluorescence at the $F_{340/380}$ excitation ratio wavelengths (Forbes and Gros, 2003, Grynkiewicz et al., 1985, Merritt et al., 1989, Picard et al., 2000, Snitsarev et al., 1996), we examined and demonstrated a similar influence of Mn^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Ca^{2+} , Mg^{2+} , Pb^{2+} , and Zn^{2+} on fura-2 fluorescence in our cell-free system at Ex_{360}/Em_{535} (Figure 4 and Table 1) and published data that used single cell loaded fura-2 AM at $F_{340/380}$ nm excitation ratio (Coyle et al., 1994, Grynkiewicz et al., 1985, Hughes, 1987, Snitsarev et al., 1996). However, in contrast to published evidence of an increase in fura-2 fluorescence by Cd^{2+} at the $F_{340/380}$ ratio (Grynkiewicz et al., 1985, Snitsarev et al., 1996), we observed a quenching effect at high Cd^{2+} concentrations with an EC_{50} of 0.3 μM (Figure 4 and Table 1). In addition, we observed an unexpected fura-2 quenching effect (~10%) with Ca^{2+} concentrations above 10 μM , regardless of the concentration of Mn present in the cell-free system (data not shown). It is possible that the competitive quenching effect of Ca^{2+} may be due to detection limits of the plate reader or changes in fura-2 and Mn binding stoichiometry that would induce molecular crowding of fura-2 by Ca^{2+} ions and decrease fura-2 fluorescence. Furthermore, we demonstrated that not only do Co^{2+} and Cu^{2+} quench fura-2 fluorescence in a concentration-dependent manner, but also they can competitively interfere with Mn-fura-2 interaction at concentrations above 1 μM (Figure 5C and data not shown). Although Fe^{2+} quenched fura-2 in a concentration-dependent manner, it had minimal influence on the Mn-fura-2 interaction (Figure 5D). However, we did not observe any significant influence of basal cellular metal ions on the quantified Mn levels in the striatal cell extracts by the CFMEA assay. This may be due in part to the fact that cells were extracted in a large volume of fura-2 detergent-based extraction buffer (100 μL) that reduced the concentration of endogenous metal ions that might otherwise influence the assay. However, CFMEA may fail to provide accurate measurements of cellular Mn levels following exposure to mixtures of toxicants containing multiple fura-2 binding metals if levels reach concentrations high

enough to influence fura-2 fluorescence. On the other hand, given the robust quenching of Cu^{2+} , Co^{2+} , Ni^{2+} , and Fe^{2+} on fura-2, it is likely that CFMEA could be adapted for studies examining the cellular uptake of these other metals.

We observed approximately 5% - 10% quenching effect of endogenous metal ions from fura-2 extracts of untreated cells (data not shown). To account for the potential effect of endogenous metal ions on fura-2 fluorescence, we defined the fluorescence of untreated cells as the 100% maximal fluorescence rather than the cell-free fura-2 fluorescence value for the experiment. The Mn-spike validation assays (Mn-spike methods A and B) strongly suggest a minimal influence of endogenous metal ions on the accuracy of CFMEA. Specifically, the Mn-spike methods revealed that irrespective of the basal metal levels in cells exposed to different MnCl_2 concentrations or extracted in different Mn-spiked fura-2 containing extraction buffer, CFMEA is capable of accurately measuring a known quantity of supplemented Mn over a wide range of measured Mn concentrations (Fig. 6A – D). In addition, the Mn-spike method B demonstrated that Mn spiking of the fura-2 containing extraction buffer before cellular Mn extraction did not saturate the fura-2 metal binding sites.

It is possible that the extraction conditions and fura-2 concentration of CFMEA could be modified and applied to rapidly assess different metal ion levels and dynamics in other cells and animal tissues. Furthermore CFMEA has the potential to provide Mn kinetic measurements (i.e. B_{max} , EC_{50} , K_m) in cells and tissues. This approach has the potential to extend our understanding of Mn transport kinetics and homeostasis. Finally, we have utilized CFMEA to independently confirm a deficit in net Mn accumulation in a mutant HD cell model (Fig. 7). Importantly, we are utilizing CFMEA in conjunction with pharmacological agents, metal ions, and small molecule inhibitors of known Mn transporters or channels to dissect the molecular mechanism underlying the mutant Mn transport deficit (*manuscript in preparation*). In conclusion, we have developed, optimized, validated and applied a functional high-throughput fluorescence-quench extraction assay (CFMEA) to accurately assess Mn levels in a striatal cell line. Importantly, CFMEA provides a rapid means to evaluate Mn transport kinetics in cellular toxicity and disease models.

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Fig 1. Optimization of buffer, temperature and detergent for CFMEA

(A) No significant difference in two commonly used buffers (PBS and HEPES) on Mn-fura-2 fluorescence quenching. (B) Minimal influence of temperature (15, 28, and 33°C) on Mn-fura-2 fluorescence quenching. Data is represented as RFU (y-axis) versus transformed log₁₀ Mn concentration on a linear scale (x-axis). A one-site specific saturation-binding curve with Hill slope was fit to log₁₀ Mn concentration (x-axis). N=3; 8 wells/experiment. Mean levels are indicated as ± standard deviation for (A) and (B). Fura-2 concentrations for (A) and (B) are 0.05 μM and 0.5 μM respectively. To evaluate the optimal detergent concentration required for intracellular Mn extraction from cultured striatal cells following MnCl₂ exposure, Mn was extracted in different concentrations of (C) Triton X-100 or (D) SDS in PBS containing 0.5 μM fura-2. Arrow (red) indicates the maximum intracellular Mn extracted by each buffer. Data is represented as RFU of baseline control (fura-2 containing buffer with indicated concentration of detergents) and extracts of Mn exposed cells (Mn-extraction). N=3; 4 wells/experiment. Mean levels are indicated as ± standard deviation.

Fig 2. Optimal fura-2 concentration for CFMEA

The detection range for Mn concentration by fura-2 was explored by examining concentration-response curves for Mn at three fura-2 concentrations (0.05, 0.5, and 2 μ M) in a cell-free system. Dotted lines represent the estimated Mn detection range of each fura-2 concentration and were chosen based on an arbitrary 10 to 85 % maximal fluorescence intensity range. The width of arrowhead line also indicates fura-2 detection range. Mn-fura-2 concentration-response curves are analyzed by nonlinear regression to fit one-site specific binding curves with Hill slope, and plotted as RFU, top row, or normalized to unbound fura-2 (% $_{\text{MAX}}$), bottom row, versus transformed $\log_{10}$ Mn concentration on a linear scale for each fura-2 concentration. Approximate optimal Mn detection concentrations (based upon the 10%-85% range) for each fura-2 concentration are provided below each graph. N=3; 8 wells/experiment. Mean levels are indicated as $\pm$ standard deviation.

Fig. 3. Mn-fura-2 standard curves

Mn-fura-2 standard curves were generated using a cell-free system with 0.5 μ M fura-2 in PTx at Ex₃₆₀/Em₅₃₅. (A) Eight independent fura-2 standard curves were generated over an extended period of time (> 1 year). Data is plotted as RFU versus log₁₀ transformed Mn concentration on a linear scale. (B) The same curves as shown in (A) were plotted instead as %_{MAX} versus log₁₀ transformed Mn concentration on a linear scale. Data in (A) and (B) were used to fit one-site specific binding curves with Hill slope (dashed lines). Mean values for (A) and (B) are indicated as $\pm$ standard deviation, N=3; 8 wells/experiment. (C) A one-site specific binding curve with Hill slope (black, equation indicated on plot) was fit by non-linear regression to %_{MAX} values obtained from four independent cell-free experiments (blue). The 95% confidence interval of the calculated binding curve constants are indicated on the plot. N=4; 4 wells/exposure condition. Mean levels are indicated as $\pm$ standard deviation.

Fig 4. Effect of metal ions on fura-2 fluorescence at Ex₃₆₀/Em₅₃₅

Ex₃₆₀/Em₅₃₅ concentration-response curves were generated for 10 different divalent metal cations with 0.5 μM fura-2 in PTx. The influence of these metal ions on fura-2 fluorescence was measured and fitted by nonlinear regression analysis to a one-site competitive binding curve. Mn concentrations were transformed to log₁₀ Mn concentration versus normalized response curves and represented as transformed log₁₀ Mn concentration on a linear scale. N=3; 8 wells/exposure condition. Mean levels are indicated ± standard deviation.

Fig. 5. Influence of metal ions on CFMEA

To examine the competitive interference of metal ions that have considerable affinity for fura-2 on CFMEA, we utilized 0.5 μM fura-2 in PTx and different concentrations of (A) CaCl₂ (B) MgCl₂, (C) CoCl₂ and (D) FeCl₂ with or without different concentrations of MnCl₂. N=3; 8 wells/exposure condition. Mean levels are indicated ± standard deviation.

Fig. 6. Validation of CFMEA by Mn-supplementation

Mn-spike methods accurately measured supplemented Mn levels in cultured wild-type neuronal cells. **(A)** Wild-type striatal cells were exposed to MnCl₂ and cellular Mn levels measured by CFMEA. Cell-extracts were quickly supplemented/spiked with Mn (~285 nM final Mn concentration) and changes in fura-2 fluorescence re-measured (post-supplement). **(B)** The difference in Mn levels in each well between pre and post Mn-spike (difference) was calculated. N=3; 4 wells/exposure condition. Mean levels are indicated ± standard deviation. (*) Indicates a significant concentration-dependent net Mn uptake (p < 0.0001, post-hoc t-test) in wild-type striatal cell lines. **(C)** Wild-type striatal cells were exposed to MnCl₂ and extracted in 0 μM, 250 μM, 500 μM, and 1000 μM MnCl₂ spiked fura-2 containing extraction buffers and total Mn levels assessed by CFMEA. **(D)** The difference in Mn levels in each well between unspiked and Mn-spiked (measured difference) fura-2 containing extraction buffers was calculated and compared to the known and expected Mn concentration. N=4; 4 wells/exposure condition. Mean levels are indicated ± standard deviation.

Fig. 7. CFMEA confirms net Mn accumulation deficit in mutant HD striatal cell line
 Wild-type STHdh^{Q7/Q7} (black) and mutant STHdh^{Q111/Q111} (white) striatal cell lines were cultured in a 96 well tissue culture plate and exposed to different MnCl₂ concentrations for 27 hours in culture media. Mutant striatal cell lines exhibited statistically significant decrease in net accumulated Mn compared to wild-type striatal cell lines. (*) Indicates a significant difference at the tested MnCl₂ concentrations (*post-hoc t-test* $p < 0.0001$) in net Mn uptake between wild-type and mutant striatal cell lines. N=3; 6 wells/exposure condition. Mean levels are indicated $\pm$ standard deviation.

Table 1Effect of metal ions on fura-2 at Ex₃₆₀

Metal	Fluorescence (Ex ₃₆₀ , Em ₅₃₅) nm	EC ₅₀ (μM)	% Fluorescence at Bmax
Cd ²⁺	Decrease	0.3	70%
Co ²⁺	Decrease	0.7	0%
Cu ²⁺	Decrease	0.02	5%
Fe ²⁺	Decrease	80	0%
Mn ²⁺	Decrease	0.3	0%
Ni ²⁺	Decrease	4	15%
Zn ²⁺	Increase	~100	120%
Ca ²⁺	No effect *	N/A	N/A
Mg ²⁺	No effect	N/A	N/A
Pb ²⁺	No effect	N/A	N/A

~ Indicates approximated value extrapolated from the one-site competition binding curve.

* Represents decrease effect on fura-2 fluorescence at concentrations above 10 μM.