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Optimization of Fluorescence Assay of Cellular Manganese Status for High Throughput Screening

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Abstract

The advent of high throughput screening (HTS) technology permits identification of compounds that influence various cellular phenotypes. However, screening for small molecule chemical modifiers of neurotoxicants has been limited by the scalability of existing phenotyping assays. Furthermore, the adaptation of existing cellular assays to HTS format requires substantial modification of experimental parameters and analysis methodology to meet the necessary statistical requirements. Here we describe the successful optimization of the Cellular Fura-2 Manganese Extraction Assay (CFMEA) for HTS. By optimizing cellular density, manganese (Mn) exposure conditions, and extraction parameters, the sensitivity and dynamic range of the fura-2 Mn response was enhanced to permit detection of positive and negative modulators of cellular manganese status. Finally, we quantify and report strategies to control sources of intra- and inter-plate variability by batch level and plate-geometric level analysis. Our goal is to enable HTS with the CFMEA to identify novel modulators of Mn transport.

INTRODUCTION

Manganese (Mn) is an environmentally abundant metal that serves as a critical cofactor for numerous enzymes such as glutamine synthetase, SOD2, and arginase. Despite active research into the physiological regulation of cellular and tissue Mn levels, this process is not fully understood. Mn is believed to primarily enter the central nervous system (CNS) across the capillary endothelium at normal physiological concentrations (1). In addition, at high blood concentrations, transport across the choroid plexus dominates (1–3). Mn transport across the blood-brain barrier involves a diverse set of cellular mechanisms including facilitated diffusion and active transport. Greater understanding of Mn transport has profound clinical relevance, given that Mn has neurotoxic properties that cause debilitating neurological and psychiatric pathologies such as manganism, a condition with features similar to Parkinson's Disease (4). Furthermore, the integral use of Mn in manufacturing sectors such as welding, oil refinement, and glass production increase the potential of excess Mn exposure in industrialized nations (5). In addition, there have been few advances in therapeutics that protect against Mn influx into neurons or promote efflux of the metal once it enters. Although variably effective, chelation therapy with ethylenediaminetetraacetic acid (EDTA) or 4-aminosalicylic acid and treatment with levodopa are currently the only therapeutic interventions for Mn toxicity (6). Despite the critical need to study Mn transport,

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one of the greatest limitations in the study of this process has been the development of novel assays that have the potential to be scaled for high throughput screening (HTS).

Our group has previously developed the Cellular Fura-2 Manganese Extraction Assay (CFMEA) to quantify intracellular Mn concentrations (7,8). CFMEA indirectly measures total cellular Mn content via its quenching of fura-2 fluorescence by Mn^{2+} . The fura-2 isosbestic point for calcium (Ex_{360}/Em_{535}) is used to avoid interference by cellular Ca^{2+} ions. This assay permits the highly accurate assessment of extracted Mn levels over a range of 0.1–10 μM .

The advent of HTS, the interrogation of large chemical libraries in the context of a biological target to identify active compounds, has been a critical advance in the process of drug development and identification of pharmacological candidates for translational applications (9). Although variability within routine laboratory assays can be accounted for by performing technical and biological replicates, reliable single-sample assays are critical for successful high throughput screens. As a result, assays must be optimized for a highly robust yet consistent response. Cell-based assay optimization is multi-factorial; including cell density, exposure time, compound concentration, assay reagent concentration, and extraction volume. The goal of this optimization process is to achieve a Z-factor greater than 0.5 (a statistical parameter that accounts for the signal dynamic range and data variation of signal measurements) (10).

Here our ultimate goal is to apply the CFMEA for HTS to permit the identification of small molecule modifiers of cellular Mn transport and storage. Given its ease of scalability, culture requirements, and previous success measuring Mn homeostasis, we have selected an immortalized murine striatal neuron cell line for these studies. One of the greatest challenges in converting a cellular assay into HTS format is limiting the influence of confounding variables on the output response. Thus, we sought to accomplish three goals: (1) determination of the experimental conditions that elicit approximately 50% quenching of fura-2 signal, (2) characterization of plate-based geometric variability and batch-to-batch variability across varying Mn exposure levels, and (3) statistical evaluation of the suitability of the CFMEA as an HTS assay. By accomplishing these stated objectives, we hope to identify sources of variability inherent in this assay and propose analytical techniques to limit their influence. Furthermore, we hope that the process adapting the CFMEA for HTS will be directly applicable toward developing other cellular plate-based assays.

MATERIALS AND METHODS

Cell Culture Supplies and Reagents

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 $\mu g/ml$ G418 and Penicillin-Streptomycin. Manganese (II) chloride heptahydrate ($MnCl_2 \cdot 7H_2O$), was 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 25 mM HEPES buffer (pH 7.2), 140 mM NaCl, 5.4 mM KCl, and 5 mM 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.

Cell Culture

Murine striatal cell lines, wild-type STHdh^{Q7/Q7}, were grown at 33°C using previously established protocols (7,8,11). Of note, the striatum is amongst brain regions that accumulate the highest levels of Mn (1,4). These cells were plated in a 96-well plate at

varying cellular concentrations cells per 0.32 cm^2 36 hours before Mn treatment and allowed to grow in a 33°C incubator. MnCl_2 was added to HEPES salt exposure buffer the morning of exposure. Total extracted Mn levels were assessed by CFMEA.

Cellular Fura-2 Manganese Extraction Assay (CFMEA)

CFMEA was conducted by adapting a previously published protocol by our laboratory (12). Wild-type $\text{STHdh}^{Q7/Q7}$ were cultured in 96-well tissue plates for 36 hours, media was removed, wells were washed once with $200\mu\text{L}$ PBS and exposed to $100\mu\text{L}$ of varying physiologically and/or toxicologically relevant MnCl_2 concentrations ($0\mu\text{M}$, $25\mu\text{M}$, $100\mu\text{M}$, $500\mu\text{M}$) (1,4,5). After exposure, the media was discarded and cells were washed three times with $200\mu\text{L}$ PBS. Subsequently, the cells were extracted at 33°C for three hours in the indicated volume of ($75\mu\text{L}$, $100\mu\text{L}$, $125\mu\text{L}$) 0.1% Triton X-100 in PBS (PTx) with the indicated concentration of fura-2 ($0.25\mu\text{M}$, $0.5\mu\text{M}$, $0.75\mu\text{M}$).

Quantification of Extracted Manganese

An *in vitro* non-cellular Mn standard curve was generated at $0.75\mu\text{M}$ fura-2 utilizing a sixteen point dataset with samples containing $1\mu\text{L}$ of different 100X stock Mn standards (0 – 100mM) added to $99\mu\text{L}$ of $0.5\mu\text{M}$ fura-2 in PTx and assayed in a 96-well plate ($n=3$). The average raw fluorescence signal values (RFU) of the $0\mu\text{M}$ Mn samples within each independent standard curve were defined as the 100% maximal fluorescence (mean fluorescence_{vehicle} = 100%) for that experiment after background subtraction. Based on this value, a ratio relative to vehicle and expressed as a percentage (% Veh) was calculated for each sample (% Veh = $100 \times (\text{fluorescence}_{\text{sample}} / \text{mean fluorescence}_{\text{vehicle}})$). These plotted values were then used to generate a cubic function using Prism (GraphPad) that related the concentration of Mn in nM (y) to % Veh at each concentration ($y = -11693 * (\% \text{ Veh})^3 + 30939 * (\% \text{ Veh})^2 - 28623 * (\% \text{ Veh}) + 9403.6$), $r^2 = 0.9740$. We then utilized this function to solve for the concentration of Mn extracted in experimental samples using Microsoft Excel.

Statistical Analysis

Calculations of subtracted background levels, % Veh, and solving for Mn concentration were performed in Microsoft Excel. Downstream analyses characterizing variability and calculation of standard deviation (SD) and normalized SD to allow comparisons of variance over different numerical scales are discussed in depth in the Discussion. Z-factor was calculated using the function below as originally described by Zhang *et al.* (10).

$$Z = 1 - (3 * \text{SD}_{\text{concentration1}} + 3 * \text{SD}_{\text{concentration2}}) / |\mu_{\text{concentration1}} - \mu_{\text{concentration2}}|$$

RESULTS

Determination of optimal cellular density and exposure time

In an initial pilot study using 96-well plates to determine the optimal cell density and exposure time for HTS was performed utilizing the wild-type $\text{STHdh}^{Q7/Q7}$ murine striatal cells. Three physiologically relevant Mn concentrations were tested plus a vehicle control ($0\mu\text{M}$, $25\mu\text{M}$, $50\mu\text{M}$, and $100\mu\text{M}$) to determine a condition that produced 50% quenching of the vehicle fura-2 signal at a concentration of $0.5\mu\text{M}$ fura-2. This concentration of fura-2 had been shown in the original assay development of CFMEA to be optimal for quantification of cellular Mn levels (7,8). Concurrently, four different cell counts were tested (5000, 7500, 10000, 20000 cells/well) under each of the exposure conditions (Figure 1). To evaluate the degree of fluorescence quenching all values are normalized to the fluorescence on the vehicle control (% Veh). The data indicate that multiple conditions satisfied our assay goals, such as achieving a 50% Veh signal and non-overlapping of SD between adjacent

concentrations (± 1 SD). However, both conditions were only achieved upon 60-minute exposure of 100 μ M Mn at either 10,000 or 20,000 cells/well (Figure 1). To enhance the scalability of this assay, we utilized the lower cellular density (10,000 cells/well) as it also displayed a smaller degree of experimental error suggesting that higher cell densities may exhibit greater experimental noise. This set-up served as a basis for further optimizations to reduce variability and modulate fura-2 response.

Evaluation of optimal fura-2 concentration, extraction volume, and analysis method

Utilizing the aforementioned cell density and exposure conditions (60-minute, 100 μ M Mn exposure and a plating density of 10,000 cells/well in a 96-well plate), a series of experiments were conducted to evaluate whether well-to-well variability (i.e. changes in fura-2 signal between replicates across plate regions) could be minimized while maintaining a robust response in the dynamic range. Under the hypothesis that optimizing the concentration of fura-2 could enhance the sensitivity of the assay and decrease statistical noise, we sought to explore the impact of concentration changes on variability. Varying the concentration of fura-2 (0.25 μ M, 0.50 μ M, 0.75 μ M) and normalizing the fluorescence quenching by adjusting to % Veh we determined that variance (SD) was minimized with 0.75 μ M fura-2 (Figure 2A). In a similar fashion, we also explored the impact of extraction volume on the sensitivity of CFMEA with the goal of achieving near 50 % Veh while minimizing error. The extraction volume for CFMEA was varied between 75 μ L, 100 μ L, and 125 μ L reported in raw fluorescence values (Figure 2B), indicating the highest signal intensity with 125 μ L. Subsequently, to determine the ideal method to analyze data for HTS using CFMEA, the raw fluorescence values were used to derive % Veh and [Mn] extracted values (Figure 2C, D) respectively (7,8). These results indicated that the higher extraction volume (125 μ L) resulted in slightly greater precision (smaller SD values) across all three quantitative approaches presented here.

Based on the newly optimized fura-2 concentration of 0.75 μ M for HTS, a new *in vitro* (cell-free) standard curve was generated (Supplemental Figure 1) to determine the Mn concentration that yields a 50% signal relative to vehicle to ensure the highest sensitivity for changes in Mn levels. The target sample response of approximately 50% quenching of the fura-2 signal maximizes the detection of both positive and negative modulators of cellular Mn status. This mirrors a common approach utilized in activity based *in vitro* assays that utilize the half-maximal activity concentration, AC_{50} , as an output (13). As a result, for a given increase or decrease in Mn extracted by CFMEA, there would be the greatest change in fluorescence.

Examination of geometric and batch-to-batch variability

Based on the optimal fura-2 concentration of 0.75 μ M, a series of experiments was conducted to evaluate the inherent geometric variability within a plate (horizontal and vertical) and across experimental batches. A batch is defined as the series of plates in which cells were plated, exposed to Mn, and CFMEA performed concurrently. Cells grown in 96-well plates were treated with vehicle, 25 μ M, 100 μ M, and 500 μ M Mn for one hour and analyzed by both raw fluorescence and % Veh (Figure 3A). In order to segregate major sources of geometric variability, cells were plated vertically while Mn exposures and fura-2 extraction buffer were delivered horizontally by multi-channel pipetting (Supplemental Figure 2). In addition, three batches consisting of multiple plates were analyzed independently and are identified here in downstream analyses by batch and plate number (e.g. 1.3 denotes Batch 1, Plate 3). Examination of plate geometric variability by fluorescence revealed pronounced horizontal-related effects, with corresponding decreases and sharp increases within particular plates. For example, plate 2.4 exhibited a decreased level of fluorescence in horizontal rows G-H, while there was a noticeable increase in fluorescence in row G-H of plate 3.4. Analysis by

fluorescence also revealed a striking batch-to-batch variability with Batch 1 exhibiting higher overall levels of fluorescence compared to Batches 2 and 3. Interestingly, this effect was less noticeable as the Mn exposure concentration increased, suggesting that the levels of Mn extracted influence variability of the CFMEA. Based on these observations, we suspect that the primary sources of geometric variability are caused by differences in extraction buffer volume delivery.

The amount of variability observed by analyzing raw fluorescence values guided us to explore whether this phenomenon could be accounted for by normalizing to %Veh (Figure 3A). After reanalyzing by this alternative quantitative methodology, there was a substantial decrease in overall variability. Importantly, this method of analysis appeared to eliminate batch-to-batch variability, but individual plates still retained some horizontal variability. This suggests that differences in the baseline fluorescence of the fura-2 solution prepared for each batch was a major source of variability between batches. In order to further evaluate the change in variability by standardizing to %Veh, we calculated a normalized SD (for %Veh values versus fluorescence values), which adjusted the scale of the SD of each horizontal row to the average SD of all horizontal rows of a given exposure. This was necessary since the magnitude of SD values is directly related to the magnitude of the measurements themselves. By normalizing the mean fura-2 signal for a given exposure condition within each plate to the respective mean fura-2 signal of all rows, of that given exposure condition, across the entire experimental set (all plates, all batches) we could compare the degree of variance independent of differences in the magnitude of the measured values themselves. Specifically, the mean fura-2 signal values for each plate (fluorescence or %Veh) for a given exposure (25 μ M, 100 μ M or 500 μ M Mn) was divided by overall mean signal of every row of every experimental plate at that exposure level, generating a normalized mean signal of each row. Calculation of the SD of these normalized row values for each experimental plate (i.e. the normalized SD) effectively allowed direct comparison of the relative magnitude of experimental variation across all plates irrespective of exposure condition. These average normalized SD values for all rows of a given exposure for each plate were plotted and color coded by batch and compared between analytical methods (fluorescence versus %Veh; Figure 3B). The normalized SD plot revealed that batch-to-batch variability was controlled by quantitative measurements accounting for %Veh. Interestingly, when the dataset was analyzed for vertical-based variability across plate region (left, middle, right column groups), there was substantially less variability across the vertical axis of the plate relative to the horizontal-dependent variability (data not shown). In agreement with the horizontal analysis, a similar pattern of batch-to-batch variability as observed in the vertical-based analysis.

Assessment of HTS adaptability of the CFMEA for HTS

In the final phase of this study, we sought to calculate the Z-factor of each plate to assess whether CFMEA could be utilized for HTS. The Z-factor was calculated between each of the tested concentrations (vehicle, 25 μ M, 100 μ M, and 500 μ M Mn). The optimal scores were the Z-factors between vehicle and 500 μ M Mn, as presented (Table 1). Z-factors were evaluated for HTS based on the following criteria: $Z > 0.5$ was classified as excellent, $0 < Z < 0.5$ as borderline, and $Z < 0$ as poor. Based on these standards, 4 of 11 plates were classified as excellent, 5 of 11 plates as borderline, and 2 of 11 as poor. The high proportion of plates classified as borderline or poor was not unexpected, given that these pilot assays were conducted by manual liquid dispensing. Utilization of an automated pipetting process should substantially reduce variability; thus we sought to explore if controlling for the observed horizontal variation would improve the Z-factors for the plates with poor or borderline Z-factors. As a proof of principle, plate 2.4 which originally had a Z-factor of

-0.66, was separated in two areas (rows A–C and D–H). Using this independent analysis, the Z-factor of each area was improved to 0.42 and 0.45 respectively.

DISCUSSION

Using the described systematic approach we have optimized conditions of the CFMEA for HTS. In this process, we targeted specific technical and theoretical aspects of the assay in an effort to ensure a consistent and robust response to changes in cellular Mn status. Importantly, it must be noted that each of these pilot assays was conducted manually (e.g. manual liquid exchange and plating of cells). Since HTS is conducted using automated robotic delivery and handling systems, we expect the level of overall variability to decrease substantially in subsequent assays and by inference the accuracy in identifying modifiers of cellular Mn status to increase. Specifically, this conservative approach, one with inherently greater error, suggests that Z-factors in the borderline range will likely improve into the excellent category after automation. In addition, we determined that some systematic errors, such as horizontal-dependent variability, could be accounted for by independently analyzing the plate along clear demarcated borders in signal response.

Further analyses explored the sources of geometric variability, changes in signal across vertical regions and across horizontal rows solely due to location and not experimental manipulation. By originally plating cells vertically and delivering all subsequent washes, exposures, and fura-2 extraction buffer horizontally, we have identified potential sources of this variability. The minimal level of vertical variability suggests that differences in cell density and growth were not a major contributor to intra-plate variability. In contrast, the high level of horizontal variability observed could be attributed to differences in the aforementioned deliveries that were administered in a horizontal-specific manner. Thus, we recommend that future HTS adaptation record and carefully design delivery patterns of solutions, permitting detection of sources contributing to geometric variability.

As previously mentioned, HTS does not have the luxury of sample replicates and is performed over a period of weeks to months. As a result, these systemic forms of variation must be minimized during this process and accounted for during downstream analyses. As a final point, all optimizations for HTS are targeted to achieve the threshold Z-factor of 0.5, maximizing confidence in candidate small molecules identified by HTS with this method by decreasing the rate of false positive and false negatives.

Furthermore, we discovered that the quantitative method of analysis is critical to account for batch-to-batch variability. The observed signal variability in raw fluorescence could be attributed to a multitude of factors. However, we suspect that the primary source of this variability is based on liquid delivery and handling, particularly of the fura-2 extraction buffer. Given that the readout of CFMEA is a fluorescent signal detected by a multimode detector, variation in fura-2 delivery could produce quantitative differences in detected fluorescence. Although each experimental batch was prepared independently, the utilized fura-2 was from a single manufacturing lot, making differential fura-2 dye activity unlikely. Additionally, there is the possibility that variation in Mn exposure buffer delivery could have contributed to inter-plate differences in fluorescence intensity. However, the consistent pattern of intra-batch variation across the tested Mn concentrations makes variation in Mn exposure buffer delivery an unlikely culprit given that individual concentrations were composed and delivered independently.

We have previously reported that transforming raw fura-2 fluorescence values into a percentage relative to vehicle could successfully account for signal differences both within and between batches (8). Based on this finding, we recommend standardizing all values

during HTS to the average signal value across each plate. This quantitative method controls for inherent changes in signal across batches caused by changes in cellular growth or a given fura-2 aliquot. This standardization would not limit the ability to identify changes in fluorescence caused by the activity of small molecules within a small molecule library. Another advantage to this approach is that it eliminates the requirement of generating a standard curve for each batch, since the goal of HTS is to identify hits based on clear cut-offs in signal rather than quantitative differences among samples. Given the robustness of this method of analysis, CFMEA is well suited for applications detecting small differences ($[Mn] = 0.1\mu M$) in cellular Mn status, with or without exogenous Mn exposure. The sensitivity of the assay permits detection of moderate changes in Mn status, a property of direct relevance in drug development for compounds of physiological relevance.

Moving forward, we believe that CFMEA has to potential to be a valuable tool in the detection of cellular Mn levels in HTS protocols. We advise investigators seeking to utilize this method to employ a similar approach in validating the parameters (cell density, exposure paradigm, fura-2 concentration) for their cell-type of interest. Subsequently, determination of the Z-factor for the assay will be essential in determining eligibility for HTS robotic automation. Although we have discussed sources of variance in the context of this study, there are other factors that may impact future applications of CFMEA for HTS, such as fura-2 manufacturing lot and storage conditions. Furthermore, investigators should be aware of the potential influence that experimental differences in fura-2 concentration, fluorometric plate reader sensitivity, and detection of Mn quenched fura-2 may have on variability in the context of CFMEA HTS (8).

Thus, we have described an approach broadly applicable toward adapting toxicological assays for HTS: (1) determination of the optimal cellular density, exposure concentrations, and conditions targeting the most dynamic range of the standard curve, (2) adjustment of reagent concentration, extraction volume, and analysis method to minimize error, and (3) performance of a multiple batch assay to compute geometric variation, batch-to-batch variability, and Z-factors. This multi-step process permits the rapid ascertainment of the suitability of a cellular-plate assay for HTS without significant investment of laboratory resources or utilization of automated robotic equipment. Should a given assay achieve Z-factors within the target range, further validations and pilot screens can be pursued with reasonable degree of certainty that HTS will be possible. As more toxicological assays are adapted for this purpose, we hope that drug development, toxicant characterization, and our understanding of underlying physiological responses will be improved significantly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Determination of optimal cellular density and exposure time of CFMEA. Data are presented as % Veh vs. exposure time (min) and sorted by plated cell density and Mn exposure concentration (μM). n=4 for each condition. Arrows indicate exposure conditions that produced approximately 50% quenching of fura-2 signal.

FIGURE 2.

(A) Fura-2 concentration optimization. Data are presented as %Veh vs. concentration of fura-2 (µM) across multiple Mn exposure concentrations (µM), n=8 for each condition. (B-D) Extraction volume optimization using 0.75µM fura-2. (B) Data are presented as raw fluorescence (Fluor.) vs. volume of fura-2 (µL) across multiple Mn exposure concentrations (µM). n=24 for each condition. (C) Extraction volume optimization (%Veh). Data are presented as %Veh vs. volume of fura-2 (µL) across multiple Mn exposure concentrations (µM). n=24 for each condition. (D) Extraction Volume Optimization (Mn). Data are presented as concentration of Mn extracted (nM) vs. volume of fura-2 (µL) across multiple Mn exposure concentrations (µM). n=24 for each condition. (A-D) Bracketed bars indicate selected fura-2 concentration and extraction volume data for subsequent downstream assays.

FIGURE 3.

(A) Examination of plate-based geometric and batch-to-batch variability. Data are presented in three-dimensional surface plots showing raw fluorescence/percent relative to vehicle (Y-axis), by each batch/plate number (X-axis; for example 1.2 is plate 2 of batch 1), by the eight horizontal row positions of each plate (Z-axis; rows A–H). Coloration within the surface plot indicates demarcated value ranges along the Y-axis for clarity. Separate graphs were generated for each Mn exposure (μ M) and analysis method (Fluorescence vs. %Relative to Vehicle). $n=8$ for each condition. (B) Impact of analysis method on normalized SD. Data are presented as normalized SD vs. analysis method (Fluorescence vs. %Relative

to Vehicle). Separate plots were generated for each Mn exposure (μM). n=8 for each condition.

TABLE 1

Batch	Plate	Z-Factor	HTS Quality
1	1	0.73	Excellent
1	2	0.73	Excellent
1	3	0.36	Borderline
2	1	0.27	Borderline
2	2	0.40	Borderline
2	3	0.50	Excellent
2	4	−0.66	Poor
3	1	0.47	Borderline
3	2	0.51	Excellent
3	3	0.38	Borderline
3	4	−0.93	Poor