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Cellular Fura-2 Manganese Extraction Assay (CFMEA)

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Manganese; High-throughput assay; Metal transport; Fura-2

Assessment of Cellular Manganese Levels and Transport kinetics

Manganese (Mn) is an indispensable ubiquitous trace element required for normal development, growth, and cellular homeostasis (Erikson *et al.*, 2005). In addition, Mn functions as an important cofactor for several Mn-dependent enzymes that are appropriate for neuron 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, Mn can accumulate in the brain at high concentrations via long-term occupational and dietary exposures, which result in dysfunction of the basal ganglia system (Aschner *et al.*, 2007). Other available methods (e.g. atomic absorption spectroscopy, inductively coupled mass spectrometry, and radioactive trace assays) used to measure cellular Mn levels in cultured cells and tissues provide excellent specificity, multi-elemental analysis, limited chemical interference, and extremely high sensitivity. However, they are not practical to measure cellular Mn levels and transport dynamics in a high-throughput manner, mainly due to the cost of analysis, considerable number of cells required, and duration of sample processing and experimental analysis. The fluorescent calcium (Ca²⁺) indicator, Fura-2, utilized for both the assessment of intracellular Ca²⁺ concentrations under normal conditions and cellular dysfunction that is induced by intrinsic and extrinsic mechanisms has improved our understanding of neurodegenerative diseases (Cobbold and Rink, 1987; Gryniewicz *et al.*, 1985; Lim *et al.*, 2007; Oliveira *et al.*, 2006; Tang *et al.*, 2003; Tsien *et al.*, 1991). Experimental evidence suggests a differential influence of various metal ions on Fura-2 fluorescence. Specifically, Mn has been reported to bind and quench (decrease) Fura-2 fluorescence at a wide-range of excitation wavelengths (Snitsarev *et al.*, 1996). Importantly, Mn quenches Fura-2 at the Ca²⁺ isosbestic point (360 nm), an excitation wavelength at which Fura-2 fluorescence properties are independent of the Ca²⁺ concentrations (Snitsarev *et al.*, 1996). Based on the Mn quenching properties of Fura-2 at the Ca²⁺ isosbestic point, we developed a high-throughput assay, Cellular Fura-2 Manganese Extraction Assay (CFMEA), as an indirect measurement

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of total cellular Mn content (Kwakye et al, manuscript under review). CFMEA enables rapid and efficient quantitative assessment of cellular Mn levels in cultured mammalian cells in a 96 well tissue culture plate (or other plating formats), allowing high-throughput cellular Mn analysis. Data from CFMEA can be used to elucidate cellular Mn transport kinetics and dynamics.

This unit will describe methods for measuring cellular Mn uptake and transport dynamics in an immortalized murine striatal cell line and primary cortical astrocytes using CFMEA (see Basic Protocol 1). However, the method is equally adaptable to other cultured mammalian cells. An ultra sensitive fluorescent nucleic acid stain for quantification of double-stranded DNA (dsDNA) in solution, Quant-iTTM PicoGreen, has been utilized for normalization of Mn concentration in the cultured cells following Mn (II) chloride (MnCl₂) exposure (see Basic Protocol 2). However, depending on the cell type and density, other methods such as protein determination assays or cell counts may also be used for normalization. Methods are described for rapidly stopping Mn uptake and transport processes at specified times, extraction and quantification of cellular Mn content, and normalization to dsDNA concentration. Each of the above protocols requires basic understanding of cell culture and the fluorophores for which they are designed.

BASIC PROTOCOL 1. MEASUREMENT OF CELLULAR MN LEVELS

This protocol describes a procedure for measuring cellular Mn content in Mn exposed cells cultured in 96 well tissue culture plates. The cells used in this protocol are an immortalized murine striatal cell line (Trettel *et al.*, 2000; Williams *et al.*, 2010; Williams *et al.*, 2010) and primary murine cortical astrocytes (Aschner *et al.*, 1992), but the protocol is adaptable to any cultured mammalian cell type. We have used this protocol to compare Mn uptake and accumulation between wild-type and mutant striatal cells (Kwakye *et al.*, 2010, manuscript under review). Two critical points for the accurate determination of cellular Mn content in cultured cells are: (1) Rapid washes of cells following Mn exposure are necessary to remove plasma membrane associated and extracellular Mn before cellular Mn extraction or sample processing; (2) exposure of cells to mixtures of toxicants containing multiple Fura-2 binding metals may interfere with CFMEA. The first point is addressed by washing cells cultured in 96 well tissue culture plates three times with 200 μ L ultra-pure phosphate-buffered saline (PBS) following MnCl₂ exposure, prior to extraction of cellular Mn. The second point can be addressed by testing the influence of other metal ions on Fura-2 at the Ca²⁺ isosbestic point, at excitation 360 nm (Ex₃₆₀ nm) and emission at 535 nm (Em₅₃₅ nm). In addition, cellular Mn can be extracted in a relatively large volume to reduce the concentration of endogenous cellular metal ions that may alter the accuracy of CFMEA. The following protocol may be used for accurate measurement of cellular Mn uptake, accumulation, and efflux kinetics in any cultured mammalian cell types at extracted Mn concentrations between at least 100 nM and as high as 10 μ M using 0.5 μ M Fura-2 in PBS with 0.1% Triton X-100. The extraction conditions and Fura-2 concentration for CFMEA can be modified to extend the range of measurable Mn levels in either direction (Kwakye *et al.*, 2010, manuscript under review). Furthermore CFMEA has the potential to provide Mn kinetic measurements (i.e. B_{max}, EC₅₀, K_m) by acquiring concentration and time dependent measurements of cellular Mn content in exposed cultured cells. This approach has the potential to extend the understanding of Mn transport kinetics and homeostasis.

MATERIALS

Chemicals and Supplies

Dulbecco's modified Eagle's medium (DMEM) (Catalog # D6546; Sigma) – Store at 4°C

10% fetal bovine serum (Catalog # S11150; Atlanta Biologicals) – Store at -20°C

100x GlutaMAX[™] - I (Catalog # 35050; Mediatech) – Store at -20°C

400 $\mu\text{g/ml}$ G418 (Catalog # G64000; Research products international corp.) – Store at -20°C

Penicillin-Streptomycin (10,000 I.U./mL Penicillin, 10,000 $\mu\text{g/mL}$ Streptomycin) (Catalog # 30-002-CI; Mediatech) – Store at -20°C

MEM Non-essential Amino Acid Solution (100x) (Catalog # M7145; Sigma) – Store at 4°C

0.05% Trypsin (Catalog # 253000-54; Invitrogen) – Store at -20°C

Manganese (II) chloride tetrahydrate ($\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$) (Catalog # M3634; Sigma) – Store at room temperature

1x ultra-pure PBS, pH 7.4, without calcium and magnesium (Catalog # 21-040-CV; Mediatech) – Store at room temperature

0.1 % Triton X-100 (Catalog # T8787; Sigma) – Store at room temperature

1 mM ultra-pure cell-impermeable Fura-2 pentapotassium salt (Catalog # 21025; ENZO) solution in Dimethyl sulfoxide (DMSO) (see recipe) – Store at -20°C in the dark

1 M HEPES salt exposure buffer (pH 7.2, see recipe), (Catalog # 25-060-CI; Mediatech) – Store at room temperature

1 M Tris-HCl (Catalog # 9310; OmniPur) (see recipe) – Store at room temperature

0.5 M Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (Catalog # E5134, Sigma) – Store at room temperature

500 mL Sterile Polystyrene; Non-Pyrogenic bottle (Corning)

Sterile Pasteur glass pipette

1.5 ml graduated, DNase and RNase free, autoclavable polyethylene microcentrifuge tubes

15 ml sterile, cell culture tested, polypropylene conical centrifuge tubes

50 ml sterile, cell culture tested, polypropylene conical centrifuge tubes

Equipment

Hemocytometer or automated cell counter (e.g. Cellometer[™] Auto T4, Nexcelom Bioscience)

Multichannel pipette (Thermo Scientific)

Beckman coulter DTX 880 multimode plate reader (multimode analysis software, version 3.2.0.6)

Beckman coulter DTX 880 multimode excitation filters: Excitation of 360 nm (bandwidth of filter = ± 35 nm) and excitation of 485 nm (bandwidth of filter = ± 20 nm)

Beckman coulter DTX 880 multimode emission filter: Emission of 535 nm (bandwidth of filter = ± 25 nm)

Computer with a current version of Microsoft Excel

Cell culture facility – including sterile laminar flow hoods and tissue culture incubators.

Plate cultured cells

- 1 Aspirate culture media from cultured cells grown in a 10 cm² tissue culture dish or 25 cm² flask with a sterile Pasteur glass pipette.
- 2 Add 5 mL PBS to tissue culture dish or flask and swirl for 1 – 3 seconds. Aspirate PBS with a sterile Pasteur glass pipette.
- 3 Add 1 mL 0.05% Trypsin to cultured cells and place tissue culture dish or flask in a tissue culture incubator set at the appropriate environmental controls and for the optimal duration of the cultured cell-type.

Gently swirl tissue culture plate or flask to obtain an even spread of trypsin on the cultured cells before incubation. The optimal duration required for trypsinization of cultured cells is cell-type specific.

- 4 Add 9 mL of culture media containing serum to the cells to inactivate the trypsin.
- 5 Pipette culture media to completely dissociate the cells.
- 6 Transfer 10 – 20 µL of the cells onto a manual or automated hemocytometer and count the number of cells.
- 7 Plate ~ 5,000 – 40,000 cells per 0.32 cm² in a 96 well tissue culture plate using 100 µL of culture media per well the evening before treatment. Place 96 well tissue culture plate in a tissue incubator until ready for Mn exposure.

To achieve efficient plating and minimal variability in cell density between wells, a multichannel pipette is recommended when plating in a 96 well plate. Total volume in each well of the 96 wells should not exceed 200 µL.

MnCl₂ exposure of cells

- 8 Add 10 µL of different 1000x MnCl₂ stock solutions (see recipe) to 9.90 mL of culture media, HEPES exposure salt buffer, or Krebs Ringer buffer (see recipe) the morning of exposure and pipette to mix.
- 9 Aspirate culture media from cultured cells with a sterile Pasteur glass pipette or a multichannel pipette.
- 10 Add 100 µL of freshly prepared MnCl₂ solutions to cultured cells.

For exposures in which HEPES salt exposure buffer, Krebs Ringer buffer, or an alternative serum free buffer is used, wash cultured cells two times with 200 µL of the same exposure buffer prior to exposing cultured cells to MnCl₂. It is important to include a control for measurement of basal Mn levels in cultured cells to provide a baseline measure of Fura-2 fluorescence (defining the 100% fluorescence value for the experiment) as well as to evaluate the influence of basal endogenous metal ions on Fura-2 fluorescence.

- 11 Place cells in tissue culture incubator for the preferred duration of exposure.

Cellular Mn extraction from cultured cells

- 12 Aspirate media or exposure buffer from cultured cells with a sterile Pasteur glass pipette or multichannel pipette.

It is important to use new multichannel pipette tips or sterile Pasteur glass pipette for each well in the plate. This will avoid cross contamination between wells.

- 13 Wash cells three times with 200 μ L PBS.
Ensure that residual PBS on cultured cells is completely removed after the final PBS wash.
- 14 Make a final concentration of 0.5 μ M Fura-2 from a 1 mM ultra-pure cell-impermeable Fura-2 stock solution (see recipe) in PBS with 0.1% Triton X-100 (extraction buffer). For example: To make 20 mL of 0.5 μ M Fura-2 extraction buffer, add 10 μ L of 1 mM ultra-pure cell-impermeable Fura-2 stock solution to 19.99 mL PBS with 0.1% Triton X-100 in a 50 mL polypropylene conical centrifuge tube. Invert tube 4 – 6 times to mix at room temperature.
Make up enough volume of Fura-2 containing extraction buffer for both the extraction of cellular Mn from cultured cells and generation of Mn-Fura-2 standard curve. Cover polypropylene conical centrifuge tube containing Fura-2 with foil or keep in the dark to protect from light. Fura-2 reagent is susceptible to photodegradation.
- 15 Add 100 μ L of Fura-2 containing extraction buffer to each well that contains cultured cells.
Do not discard the remaining extraction buffer. Use it to generate a cell-free Mn-Fura-2 standard curve.
- 16 Place cells in tissue culture incubator for at least 1 hour to extract cellular Mn.
Protect Fura-2 from light during cellular Mn extraction.

Generation of cell-free Mn-Fura-2 standard curve

- 17 Add 1 μ L of MnCl_2 stock concentrations (0 – 100 mM) (see recipe) to 99 μ L of Fura-2 containing extraction buffer in a 96 well plate to generate a ten-point standard curve, as described in Table 1.
- 18 Add 100 μ L of PBS with 0.1 % Triton X-100 (without Fura-2) in the ten-point standard curve plate, as indicated in Table 1.

Measure Mn concentration in cell-extracts and cell-free system

- 19 Place 96 well plate containing cells in a Beckman coulter DTX 880 multimode plate reader (or similar device) and shake (orbital) for 5 seconds.
- 20 Measure changes in Fura-2 fluorescence in each well at the Ca^{2+} isosbestic point at Ex_{360} (bandwidth of filter = ± 35 nm), Em_{535} (bandwidth of filter = ± 25 nm), and integration time of 200 ms with the indicated plate reader using multimode analysis software (version 3.2.0.6) and top read settings.
Other fluorometric plate readers with similar methods are likely acceptable for CFMEA measurements.
- 21 Place cell-free Mn-Fura-2 standard curve plate in the Beckman coulter DTX plate reader and shake (orbital) for 5 seconds.
- 22 Measure changes in Fura-2 in each well at the Ca^{2+} isosbestic point at Ex_{360} (bandwidth of filter = ± 35 nm), Em_{535} (bandwidth of filter = ± 25 nm), and

integration time of 200 ms with the indicated plate reader using multimode analysis software (version 3.2.0.6) and top read settings.

Calculation of cell-free Mn-Fura-2 standard curve using Microsoft Excel

- 23 Define the average raw fluorescence signal values (RFU) of the 0 μ M Mn cell-free sample within the standard curve as the 100% maximal fluorescence for that experiment after background (obtained from step # 18) subtraction.
- 24 Normalize the RFU of each well in the cell-free Mn-Fura-2 standard curve plate as percent maximal fluorescence (%_{MAX}) to the 100% maximal fluorescence value.
- 25 Plot log₁₀ scale of Mn concentration (y-axis) and %_{MAX} (x-axis) by non-linear regression analysis using Microsoft Excel (Fig. 1).
- 26 Calculate the binding curve from non-linear regression analysis using the trend line command in Microsoft Excel to fit power (Mn concentration = $A \cdot (x)^B$) and logarithmic (Mn concentration = $A \cdot \ln(x) + B$) equations to the standard curve data (Fig. 1).

For the power trend line equation,

$$A = \text{EXP}(\text{INDEX}(\text{LINEST}(\text{LN}(y), \text{LN}(x), , , 1, 2)))$$

$$B = \text{INDEX}(\text{LINEST}(\text{LN}(y), \text{LN}(x), , , 1, 2))$$

$$x = \%_{\text{MAX}}$$

For the logarithmic trend line equation,

$$A = \text{INDEX}(\text{LINEST}(y, \text{LN}(x)), 1)$$

$$B = \text{INDEX}(\text{LINEST}(y, \text{LN}(x)), 1, 2)$$

$$x = \%_{\text{MAX}}$$

Power curve is used for %_{MAX} values less than 50% and logarithmic curve for values greater than 50%, on either side of the inflection point of the saturation-binding curve plotted with Mn concentration (y-axis) and %_{MAX} (x-axis) (Fig. 1).

Calculation of extracted cellular Mn concentration using Microsoft Excel

- 27 Subtract background (obtained from step # 18) fluorescence from each well containing cell-extract.
- 28 Average and normalize the RFU values of unexposed cell-extracts. Define this value as 100% maximal fluorescence of the untreated cell-extracts.
For example, average RFU of unexposed cell-extracts (Y) = (A + B + C + D) / 4
where, A, B, C, and D are RFU values from four independent wells containing unexposed cell-extracts.
100% maximal fluorescence of the untreated cell-extracts = (Y / Y) * 100
- 29 Normalize the RFU of each Mn exposed well containing cell-extracts as %_{MAX} to the 100% maximal fluorescence value of the untreated cell-extract described in step # 28 (Fig. 2A).

For example, %_{MAX} of Z = (Z / Y) * 100

Z is the RFU value of a Mn exposed cell-extract and Y is the average RFU of unexposed cell-extracts.

- 30 Calculate %_{MAX} for each well containing cell-extracts in the 96 well tissue culture plate.
- 31 Make an 'IF' function that contains the generated Mn-Fura-2 standard curve power (Mn concentration = $A \cdot (x)^B$) and logarithmic (Mn concentration = $A \cdot \ln(x) + B$) trend line equations using Microsoft Excel. For example: Mn concentration = IF(%_{MAX} = 94.3, 0, (IF(%_{MAX} > 50, (($A \cdot \ln(\%_{MAX}) + B$))), ($A \cdot 6 \cdot (\%_{MAX})^B$))).

Based on our mathematical modeling of Mn-Fura-2 interaction by a one-site specific binding curve with hill slope, we recommend capping the minimal detectable Mn concentration (which asymptotically approaches 100% maximal fluorescence) to define anything above 94.3% maximal fluorescence as 0 μ M Mn. This eliminates experimental errors that can occur along the asymptotical part of the standard curve and potentially decrease the accuracy of measured extracted Mn levels in the cultured cells. The 'IF' function allows Microsoft Excel to automatically choose which trend line equation to use for calculation of extracted cellular Mn levels in cultured cells based on their %_{max} values.

- 32 Substitute %_{MAX} value for each cell-extract well into the 'IF' function to calculate the concentration (nanomolar (nM)) of Mn levels in the cell-extracts (Fig. 2B).

BASIC PROTOCOL 2. NORMALIZATION OF CELLULAR MN LEVELS BY QUANT-IT™ PICOGREEN REAGENT

Protein detection methods (for example, Lowry and Bradford assays) have been extensively used to measure protein levels in cultured cells and animal tissues. However, their sensitivity and accuracy depends on the number of cells required for minimum detection, and are influenced by interfering agents (e.g. detergents and amino acid concentrations).

Unfortunately, some cultured cells do not show sufficient cell density in the 96 well plate format to be detected by these traditional protein assays. Therefore, we provide here a protocol to normalize cellular Mn levels to double-stranded DNA (dsDNA) instead. This protocol describes a procedure using Quant-iT™ PicoGreen dsDNA reagent, to measure dsDNA from cultured cell-extracts post CFMEA analysis. Quant-iT™ PicoGreen dsDNA reagent is an ultrasensitive asymmetrical fluorescent nucleic acid dye that has previously been used to quantify the concentration of dsDNA in solution (Ahn *et al.*, 1996; Enger, 1996; Seville *et al.*, 1996). The free dye fluoresces upon binding to dsDNA, but not RNA. Thus, alterations in RNA levels in cultured cells do not influence the sensitivity of the PicoGreen assay. Quant-iT™ PicoGreen reagent is capable of quantifying at least 25 pg/mL dsDNA with a standard fluorometric plate reader (Molecular Probes, 1996). The long stability of PicoGreen to photobleaching enables longer exposure times and assay flexibility (Ahn *et al.*, 1996). We have modified and utilized the Quant-iT™ PicoGreen dsDNA assay to measure dsDNA in cell-extracts following MnCl₂ exposure. This method enables the normalization of extracted cellular Mn concentration by dsDNA levels in cell-extracts following MnCl₂ exposure (Fig. 4)

MATERIALS

Chemicals

1x TE buffer (see recipe) – Store at room temperature

5 µg/mL Deoxyribonucleic acid sodium salt, from salmon testes (Catalog # D1626; Sigma) (see recipe) – Store at 4°C

1 mL Quant-iT PicoGreen dsDNA Reagent (Catalog # P7589; Molecular Probes) – Store at –20°C in the dark.

Equipment and Supplies

15 ml sterile, cell culture tested, polypropylene conical centrifuge tubes

Beckman coulter DTX 880 multimode plate reader (multimode analysis software, version 3.2.0.6) or similar device

Beckman coulter DTX 880 multimode excitation filter: Excitation of 485 nm (bandwidth of filter = ± 20 nm) and emission of 535 nm (bandwidth of filter = ± 25 nm)

Prepare DNA standard curve and measure dsDNA concentration

- 1 Prepare 1x TE buffer from the 20x TE buffer (see recipe).
To make 50 mL 1x TE buffer, add 5mL of 10x TE buffer to 45 mL sterile distilled DNase-free water). 50 mL is sufficient for 625 assays.
- 2 Add 20 µL of salmon testes dsDNA standards (0 – 10 µg/mL) (see recipe) to at least 3 independent wells for the seven tested concentrations in a 96 well plate (see Table 2.)
- 3 Make 1:400 dilution of the PicoGreen reagent in 1x TE buffer.
Prepare enough PicoGreen solution for both salmon DNA standard curve and cell-extracts. For best results, PicoGreen working solution should be used within few hours of its preparation.
- 4 Add 80 µL of working PicoGreen solution to each well containing 20 µL DNA standard in the 96 well plate (see Table 2.)
- 5 Pipette to mix and incubate at room temperature for 5 minutes and protect from light.
Avoid air bubbles in wells when mixing. Air bubbles may interfere with fluorescence readings. Should air bubbles occur, use fresh micropipette tips to remove them.
- 6 Add 20 µL PBS in 0.1% Triton-X-100 and 80 µL 1x TE buffer into triplicate or more wells in the same 96 well DNA standard curve plate (see Table 2.).
- 7 Place 96 well plate in the Beckman coulter DTX 880 multimode plate reader (or similar device) and shake (orbital) for 5 seconds at ~30°C
- 8 Measure PicoGreen fluorescence in each well at Ex₄₈₅ (bandwidth of filter = ± 20 nm), Em₅₃₅ (bandwidth of filter = ± 25 nm), and integration time of 200 ms with the indicated plate reader using multimode analysis software (version 3.2.0.6) and top read settings.
- 9 Subtract background (20 µL PBS in 0.1% Triton-X-100 and 80 µL 1x TE buffer) as described in step # 6, basic protocols 2, from each of the salmon testes dsDNA PicoGreen fluorescence tested in the standard curve.
- 10 Plot average PicoGreen RFU of the DNA standards (y-axis) and their concentrations (x-axis) by nonlinear regression analysis using Microsoft Excel (Fig. 3).

- 11 Use the standard linear regression analysis in Microsoft Excel to obtain a linear equation ($\text{PicoGreen Fluorescence} = A \cdot (x) + B$) to the standard curve data; where A is the coefficient, rate and slope of the trend line, x is the unknown concentration of dsDNA in the cell-extract, and B is the y-intercept or where the line crosses the y-axis.

Measure DNA concentration in cell-extracts

- 12 Transfer 20 μL of post-CFMEA cell-extract into a 96 well plate and add 80 μL of 1:400 PicoGreen solution in 1x TE buffer.

Ensure that new micropipette tips are used to transfer 20 μL cell-extract from each well. This would avoid contamination of DNA and decrease variability between experimental wells. Cell-extracts are stable for up to ~ 3 months after CFMEA analysis if tightly sealed with Parafilm and stored at 4°C before DNA normalization by PicoGreen assay. To enable efficient data export and analysis, it is advisable to use the same 96 well plate format used in the original cell plating format prior to MnCl_2 exposure when transferring the post-CFMEA cell-extracts into a new 96 well plate.
- 13 Incubate cell-extracts containing PicoGreen at room temperature for 5 minutes. Protect from light.
- 14 Place the plate in the Beckman coulter DTX 880 multimode plate reader (or similar device) and shake (orbital) for 5 seconds.
- 15 Measure PicoGreen fluorescence in each well at excitation Ex_{485} (bandwidth of filter = ± 20 nm), Em_{535} (bandwidth of filter = ± 25 nm), and integration time of 200 ms with the indicated plate reader using multimode analysis software (version 3.2.0.6) and top read settings.
- 16 Subtract background (20 μL PBS in 0.1% Triton-X-100 and 80 μL 1x TE buffer) from each well.
- 17 Calculate the concentration of dsDNA in each cell-extract using the linear equation generated from the salmon DNA standard curve ($\text{PicoGreen Fluorescence} = A \cdot (x) + B$)

For example: To calculate the concentration of DNA in cell-extract per well using the above linear equation ($\text{PicoGreen Fluorescence} = A \cdot (x) + B$), rearrange the equation to the following: Concentration of DNA in extract per well $[x] = (\text{PicoGreen Fluorescence} - B) / A$.

Representation of cellular Mn levels after measurement of dsDNA concentration

- 18 Divide the calculated concentration of extracted Mn levels in each well (e.g. femtomoles, picomoles, nanomoles, micromoles, millimoles) to its respective dsDNA concentration (e.g. nanogram, microgram, milligram).

For example, if the calculated concentrations of Mn and dsDNA in well # A1 containing cell-extracts 250 femtomoles per 100 μL and 0.2 μg per 100 μL respectively, then the final concentration of cellular Mn level in well # A1 $(W) = 250 \text{ femtomoles per } 100 \mu\text{L} / 0.2 \mu\text{g per } 100 \mu\text{L}$. Thus, $W = 125 \text{ femtomoles of Mn per } \mu\text{g of dsDNA}$.

- 19 Average the normalized concentration of Mn in each well based on the concentration of MnCl_2 exposure and represent the data as 'n' moles of Mn per 'v' of dsDNA (y-axis) and concentration of MnCl_2 exposed to cells ('b' molar) by Microsoft Excel regression analysis (Fig. 4).

where, 'n' is the number of moles, 'b' is the molarity of MnCl_2 , and 'v' is the mass of dsDNA.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of HEPES salt exposure, Krebs Ringers or similar buffers used for cellular Mn exposure.

Culture media—It is important to note that other standard tissue culture medias could be used for MnCl_2 exposures.

430 mL DMEM

50 mL fetal bovine serum (10% final concentration)

5 mL 200 mM L-Alanyl-L-Glutamine

5 mL G418 (400 $\mu\text{g}/\text{ml}$ final concentration)

5 mL Penicillin-Streptomycin

5 mL MEM Non-essential Amino Acid Solution (100x)

Prepare culture media in a sterile 500 mL Sterile polystyrene; non-pyrogenic bottle. Gently swirl bottle to mix and store at 4°C for up to 2 weeks.

HEPES salt exposure buffer

25 mM HEPES

140 mM NaCl (Catalog # S7653; Sigma)

5.4 mM KCl (Catalog # 7300; OmniPur)

5 mM D-glucose (Catalog # G7528; Sigma)

Adjust pH to 7.4 with 1 M HCl or 1 M NaOH as needed

Prepare buffer in advance and store in a 1-liter polycarbonate bottle at 4°C for up to 2 weeks.

Krebs Ringer Buffer (KRB)

20 mM HEPES

135 mM NaCl

5 mM KCl

0.4 mM KH_2PO_4 (Catalog # P0662; Sigma)

1.8 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Catalog # C7902; Sigma)

1 mM $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ (Catalog # 63138; Fluka BioChemika)

5.5 mM D-glucose

Adjust pH to 7.4 with 1 M HCl or 1 M NaOH as needed

Prepare 1x buffer in advance and store in a 1-liter polycarbonate bottle at 4°C for up to 2 weeks.

Cell-impermeable Fura-2 salt solution, 1000x

Prepare a 1 mM (1000x) stock solution of ultra-pure cell-impermeable Fura-2 salt (ENZO Biochem) solution in Dimethyl sulfoxide (Sigma). Aliquot Fura-2 stock solution into 1.5 ml polyethylene microcentrifuge tubes at 10 μ L per tube. Store in tightly sealed tubes at –20°C for up to 6 months. Protect from light and moisture.

Manganese (II) chloride solutions, 1000x

Stock solution: Prepare a 1000X stock solution as follows:

Add 98.96 mg of $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ powder to 5 mL of water in a 15 ml polypropylene conical centrifuge tube to prepare 100 mM MnCl_2 stock solution. Seal tubes tightly and invert 8 – 10 times to mix. Perform serial dilutions of 100 mM MnCl_2 stock solution to obtain 1 mL of 50 mM, 10 mM, 5 mM, 1 mM, 500 μ M, 100 μ M, 50 μ M, 10 μ M, and 5 μ M MnCl_2 stock solutions. Seal tubes tightly and store at room temperature for up to 1 month.

For example: To perform serial dilutions of 100 mM MnCl_2 stock solution to obtain 1 mL of 50 mM MnCl_2 , add 500 μ L water to 500 μ L of 100 mM MnCl_2 stock solution in a 1.5 ml polyethylene microcentrifuge tube. Seal tube tightly and invert 4 – 6 times. Consequently, to make 1 mL of 10 mM MnCl_2 stock solution from a 50 mM MnCl_2 stock solution, add 200 μ L of the freshly prepared 50 mM MnCl_2 to 800 μ L of water. Seal tube tightly and invert 4 – 6 times to mix. These serial dilutions can be performed to obtain the aforementioned concentrations of MnCl_2 stock solutions.

Salmon Testes dsDNA (100 μ g/mL stock)

Prepare a 100 μ g/mL stock solution of salmon testes dsDNA in 1x TE buffer. For example: add 1 mg of salmon testes dsDNA to 10 mL of PBS with 0.1 % Triton X-100 in a 15 ml polypropylene conical centrifuge tube and pipette to mix. Make sure the dsDNA is completely dissolved before proceeding to the next step. Perform serial dilutions of the 1000 μ g/mL (final concentration of stock solution) salmon testes dsDNA in PBS with 0.1 % Triton X-100 to obtain 10 μ g/mL, 5 μ g/mL, 2.5 μ g/mL, 1.25 μ g/mL, 0.625 μ g/mL, 0.3125 μ g/mL, 0.15625 μ g/mL, and 0.078125 μ g/mL stock DNA concentrations. Store at 4°C temperature for up to 3 months.

TE Buffer, 10x

Prepare a 10x Tris-HCl-EDTA (TE) buffer from 1 M stock of Tris-HCl (pH 7.5) and 0.5 M stock of EDTA (pH 8.0) with distilled DNase-free water. Add 10 mL of 1 M Tris-HCl (pH 7.5) per liter and 2 mL of 500 mM EDTA (pH 8.0) in a 15 ml polypropylene conical centrifuge tube to obtain a final concentration of 10 mM and 1 mM, respectively. Adjust pH to 7.5 with 1 M HCl or 1 M NaOH as needed. Pipette (4 – 6 times) to mix and store at room temperature for up to 1 month. Dilute 10x TE buffer in distilled DNase-free water to obtain 1x TE buffer (final working concentration) before use in experiments.

Prepare 1 M Tris (crystallized free base) in distilled DNase-free water and adjust pH to 7.5 using HCl. EDTA can be prepared in water and is not soluble until pH reaches 8.0. Use vigorous stirring and moderate heat (if desired).

COMMENTARY

Analysis of Mn levels in biological samples

Mn levels in biological samples can be measured by numerous analytical methods including graphite furnace atomic absorption spectroscopy (GFAAS), electrothermal atomic absorption spectrometry (EAAS), inductively coupled plasma mass spectrometry (ICPMS), spectrochemical emission, spectrophotometry, photometry, fluorometry, and neutron activation analysis (NAA) (Baruthio *et al.*, 1988). However, GFAAS and ICPMS are the two most commonly used Mn detection methods for assessment of cellular Mn levels in biological samples. Although GFAAS and ICPMS provide multi-elemental analysis, limited chemical interference, good precision and accuracy, and extremely high sensitivity, they are not feasible for rapid and high-throughput measurement of Mn uptake, cellular storage, efflux, and Mn transport kinetics (i.e. B_{max} , EC_{50} , K_m) in cultured mammalian or any other cell types. This is mainly due to the cost of analysis, considerable number of cells required for transport kinetic studies, duration of sample processing, and experimental analysis. Therefore, we sought to develop a rapid, inexpensive, accurate, and reliable high-throughput assay that enables efficient assessment of Mn uptake, cellular accumulation, efflux, and transport kinetics in cultured cells.

Background information on calcium detection by the Fura-2 fluorophore

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 sodium and potassium salts of Fura-2 exist as either cell-impermeable probes (Fura-2) or the acetoxymethyl (AM) ester derivative of Fura-2 (Fura-2 AM). 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, which slightly increases its wavelength 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). 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. Previous published experimental evidence has elucidated an interaction between endogenous metal ions and Fura-2 loaded cells at the Ex_{340}/Em_{380} nm excitation/emission wavelengths. Specifically, while Mn^{2+} , Cu^{2+} , and Fe^{2+} quench Fura-2, Zn^{2+} and Cd^{2+} increase Fura-2 fluorescence. (Grynkiewicz *et al.*, 1985; Snitsarev *et al.*, 1996). In addition, we have demonstrated that Mn^{2+} , Cu^{2+} , Co^{2+} , and Fe^{2+} quench Fura-2 fluorescence in a cell-free system at the Ex_{360}/Em_{535} nm excitation/emission wavelengths (Kwakye *et al.*, 2010, manuscript under review). Given the similarities of Fura-2 quenching by the indicated metal ions, it is likely that CFMEA could be adapted for studies examining the cellular uptake and transport kinetics of these other metals in cultured mammalian or any other cell types.

Development of Cellular Fura-2 Manganese Extraction Assay (CFMEA)

Other studies have utilized fluorescence quenching assays to assess intracellular metal ion concentration and transport in Fura-2 or calcein loaded cells 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 transport

studies were conducted on individual cultured cells, which limit high-throughput measurements of Mn^{2+} , Fe^{2+} , Co^{2+} , or Cu^{2+} levels. Given the limitations of these quenching assays, we reasoned that development of an inexpensive high-throughput cell-extraction based Fura-2 assay would enable efficient and accurate measurements of cellular Mn uptake levels and transport kinetics in a large number of cultured cells and cellular models. Interestingly, there had been previously reported evidence that Mn^{2+} effectively quenches Fura-2 fluorescence at a wide range of excitation wavelengths, including the Ca^{2+} isosbestic wavelength at 360 nm (Snitsarev *et al.*, 1996). Given the unique relationship between Mn and Fura-2, specifically, the Mn quenching properties of Fura-2 at the Ca^{2+} isosbestic wavelength at 360 nm, we have developed a high-throughput assay, CFMEA, as an indirect measurement of total cellular Mn content in biological samples. We have utilized CFMEA to evaluate a concentration-dependent difference in cellular Mn uptake between wild-type and mutant immortalized mouse striatal cell line model of HD (Kwakye *et al.*, 2010, manuscript under review) and primary astrocytes (Kwakye *et al.*, manuscript in preparation). Furthermore, we have validated and confirmed the precision and accuracy of cellular Mn measurement by CFMEA of Mn-exposed cultured cells by GFAAS (Kwakye *et al.*, manuscript in preparation).

CRITICAL PARAMETERS AND TROUBLESHOOTING

This unit describes the analysis of cellular Mn levels in cultured cells following exposure to MnCl_2 . Although the CFMEA conditions described in basic protocol 1 have been optimized to assess cellular Mn uptake levels in an immortalized murine striatal cell line and primary astrocytes following MnCl_2 exposure, it is worth noting that identical or slightly modified optimization parameters of CFMEA could be adapted to measure cellular Mn levels and transport kinetics in a variety of cultured mammalian cell types. Newly developed methods for precise and accurate measurements of cellular Mn uptake and Mn transport dynamics must take into account not only the sensitivity and detection limit of the assay, but concerns that are specific to the feasibility, cost, and efficiency. The general concerns about the accuracy of CFMEA to measure cellular Mn uptake levels and transport kinetic measurements include the relationship between Fura-2 concentration and Mn detection, extraction conditions, influence of membrane-bound and extracellular Mn, and the effect of metal ions on Fura-2 fluorescence at Ex_{360} nm.

Relationship between Fura-2 concentration and Mn detection

Owing to the sigmoidal nature of the Fura-2 fluorescence response to Mn (Fig. 1.), we defined the optimal detection range of Mn by Fura-2 to be between 10% and 85% of the maximal baseline control Fura-2 fluorescence (Fig. 1.). However, we observed that changes in the concentration of Fura-2 alter the Mn detection window. Specifically, we demonstrated that 0.05 μM and 2 μM Fura-2 accurately detect extracted Mn concentrations between 50 nM – 6,000 nM and 300 nM – 10,000 nM respectively (Kwakye *et al.*, 2010, manuscript under review). Given the unique dependency of Fura-2 concentration on Mn detection limits and the expected cellular Mn levels in cultured cells following MnCl_2 exposure, it is important to examine the optimal Fura-2 concentration required for precise and accurate detection of extracted cellular Mn detection if the expected cellular Mn concentrations are below or above the recommended Mn detection window (between 10% and 85% of the maximal baseline control Fura-2 fluorescence). This could be achieved by first making an estimate of the maximum concentration of Mn that could possibly be extracted from the cultured cells following MnCl_2 exposure for the preferred duration. Second, a cell-free system could be utilized, consisting of different Fura-2 concentrations in the extraction buffer (99 μL) and varying concentrations of MnCl_2 (1 μL) to generate Mn-Fura-2 curves at the Fura-2 Ca^{2+} isosbestic wavelength at 360 nm. The final concentrations of Mn used to generate the Mn-Fura-2 standard curves must span the expected cellular Mn concentration

following MnCl_2 exposure. Finally, the recommended Mn detection window by Fura-2 should be applicable to any generated Mn-Fura-2 standard curves. However, if the experimenter decides to modify the recommended detection window, it is important that the newly defined Mn detection window obtained by extrapolating the minimum and maximum $\%_{\text{MAX}}$ values on the Mn-Fura-2 standard curve do fit within the optimal range of the sigmoidal Mn-Fura-2 standard curve.

Optimal detergent and buffer for extraction of cellular Mn

We evaluated and optimized the ideal detergent, buffer, temperature, and cellular Mn detection required for CFMEA using a cell-free system and cell-extracts from an immortalized murine striatal cell line (Kwakye *et al.*, 2010, manuscript under review). In addition, we applied these optimized assay conditions to assess cellular Mn levels in cultured murine striatal and primary astrocyte cells following MnCl_2 exposure. Specifically, we observed that 0.5 μM Fura-2 in 0.1% Triton X-100 with PBS is the optimal Fura-2 and detergent concentrations, and buffer conditions for maximum detection of cellular Mn levels. Furthermore, we showed that changes in experimental temperature conditions do not influence the specificity and accuracy of CFMEA (Kwakye *et al.*, 2010, manuscript under review; Kwakye *et al.*, manuscript in preparation). It is likely that the aforementioned optimized parameters could be applied to measure cellular Mn uptake and transport kinetics in other cultured cells. However, if the cultured cells used for the experiment contain a higher than usual lipid content which may alter the efficiency of detergent extraction, then the following experiment would have to be conducted using cell-extracts from such cultured cells to optimize the detergent conditions for maximal cellular Mn extraction and detection by CFMEA. Specifically, increasing concentrations of one or more of the commonly used detergents (Triton X-100, Sodium dodecyl sulfate, Tween 80, Digitonin) could be used to extract cellular Mn from the cultured cells following MnCl_2 exposure at the Fura-2 Ca^{2+} isosbestic wavelength at 360 nm as described in basic protocol 1. Importantly, it is critical that cultured cells are washed thoroughly (at least three times with 200 μL PBS or the preferred buffer) to remove plasma membrane-bound and extracellular Mn following MnCl_2 exposure and before cellular Mn extraction.

Quantitative relationship between Mn concentration and Fura-2 fluorescence

We explored the quantitative relationship between Mn and Fura-2 using a cell-free Mn-Fura-2 standard curve and demonstrated that the fluorescence yield relationship between Fura-2 and Mn concentration follow a Michaelis-Menten one-site specific saturation-binding kinetics when represented as $\%_{\text{MAX}}$ Fura-2 values (y-axis) and Mn concentration (x-axis) curve, quantified using Graphpad Prism or an alternative curve fitting program (Kwakye *et al.*, 2010, manuscript under review). However, to enable back calculation of Mn concentrations from changes in Fura-2 fluorescence using this Mn-standard curve, it is necessary to switch the axes of the Mn-standard curve and represent $\%_{\text{MAX}}$ Fura-2 values (described in basic protocol 1) and Mn concentration on the x and y-axes respectively. The binding curve data obtained when the axes are switched tightly fit to exponential and logarithmic curves generated using Microsoft excel regression analysis. This enables a direct back calculation of extracted Mn levels from experimentally determined $\%_{\text{MAX}}$ values (Fig. 1). Conversely, other curves (polynomial, linear, etc) may also be used to model the mathematical relationship between Mn and Fura-2 depending on the range of Mn concentrations used to generate the Mn-Fura-2 standard curve, which will allow accurate calculations of extracted cellular Mn concentrations. Importantly, it is necessary to generate Mn-Fura-2 standard curves alongside Mn-exposure experiments to accurately quantify extracted cellular Mn levels. Furthermore, it is critical to make enough of the Fura-2 containing extraction buffer for use in both the extraction of cellular Mn from cultured cells and the cell-free Mn-Fura-2 standard curve. This will decrease any variability that may be

caused by experimental variation in the concentration of Fura-2 during preparation of the extraction buffer. Although we observed that the absolute Fura-2 RFU values exhibit a fairly consistent relationship with Mn concentration across experiments, low Mn concentration samples (1 – 100 nM) used to generate Mn-Fura-2 standard curves between independent experiments demonstrated moderate variability (Kwakye *et al.*, 2010, manuscript under review). This might be due to low sensitivity of the plate reader at those concentrations. To minimize this variability, it is important to average the RFU values of the 0 μ M Mn samples within each independent standard curve and define this value as the 100% maximal fluorescence for that experiment. In addition, normalizing the RFU of each well as a %_{MAX} of the 100% maximal fluorescence value for representation on the Mn-Fura-2 standard curve decreases experimental variability.

Influence of metal ions on Fura-2 fluorescence at Ex₃₆₀

We evaluated the potential of other metal ions and abundant cellular metal ions to influence CFMEA using Ex₃₆₀/Em₅₃₅ concentration-response curves, which were generated using Fura-2 containing extraction buffer and different metal ions such as Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Cd²⁺, Pb²⁺, Ca²⁺, and Mg²⁺ (Kwakye *et al.*, 2010, manuscript under review). We also determined the approximate %_{MAX} values at half maximal effective concentration (EC₅₀) values and the saturated binding of Fura-2 (B_{max}) for each of the indicated metal ions using one-site specific binding curves. We demonstrated that while Zn²⁺ increased Fura-2 fluorescence (~20%) at an EC₅₀ of ~100 nM, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, and Cd²⁺ quenched Fura-2 fluorescence at varying concentrations. In addition, Pb²⁺, Ca²⁺, and Mg²⁺ had no effect on Fura-2 fluorescence at the Ca²⁺ isosbestic wavelength (Kwakye *et al.*, 2010, manuscript under review). Given the differential influence of endogenous metal ions on CFMEA at Ca²⁺ isosbestic point, it is necessary to ensure that endogenous metal ions in the cultured cells do not significantly influence the baseline Fura-2 fluorescence of untreated cultured cells. This can be examined by comparing the average RFU value of the untreated cultured cells to the 0 μ M Mn sample for the Mn-Fura-2 standard curve after background subtraction. The relationship between the number of cultured cells per well plated in the 96 well tissue culture plate and the volume of Fura-2 containing extraction volume per well is important in reducing the quantitative influence of endogenous metal ions and maximizing the specificity of Mn detection from the cultured cells. To explore the optimal volume required for extraction and detection of cellular Mn levels, and reduction of endogenous metal ion concentrations in the cell-extracts, different volumes (25 μ L, 50 μ L, 100 μ L, 150 μ L, 200 μ L) of Fura-2 containing extraction buffer could be used to extract cellular Mn from cultured cells following MnCl₂ exposure and compared to the 0 μ M Mn sample for the Mn-Fura-2 standard curve after background subtraction. It is important that the same Fura-2 containing extraction buffer volume be used for both the cellular Mn extraction and generation of the Mn-Fura-2 standard curve. Alternatively, the number of cells plated in the 96 well tissue culture plate could be varied while the Fura-2 containing extraction volume is kept constant. It is critical to ensure that the number of cultured cells used in the experiment is above the minimum number of cells (~ 5,000 cells per well in a 96 tissue culture plate, empirically determined by us) required for Mn detection by CFMEA. The acceptable percent quenching by endogenous metal ions seen in extracted untreated cultured cells should optimally be within ~5% of the RFU for the cell-free Fura-2 extraction buffer itself. Finally, to account for the influence of endogenous metal ions on the measured cellular Mn concentration, the Fura-2 RFU values obtained in each well containing cell-extracts should be normalized to the average RFU value of untreated cultured cells after background subtraction.

Normalization of cellular Mn levels by PicoGreen Assay

The sensitivity and accuracy of commonly used protein detection methods (Bradford and Lowry) can be influenced by the amount of sample or cell number required for minimum detection, and the physical or chemical interference induced by viscosity, salts, detergents, and amino acid concentrations. We observed that cultured cells plated at cell densities below 20,000 cells per 0.32 cm² in a 96 well tissue culture plate had insufficient protein levels to be detected by these and other standard protein assays. Hence, we utilized the PicoGreen assay (described in basic protocol 2) to normalize cellular Mn levels to dsDNA following MnCl₂ exposure. Although the emission spectra of Fura-2 and PicoGreen overlapped, we have observed minimal influence of Fura-2 on PicoGreen fluorescence, as their excitation spectra are distinct (data not shown). Other standard protein assays can be used for normalization of cellular Mn levels if there is sufficient protein to be detected in the cultured cells following MnCl₂ exposure. The PicoGreen reagent is supplied as 1 mL of concentrated dye solution and must be aliquot (~20 µL per 1.5 ml polyethylene microcentrifuge tube) upon receipt and stored at -20°C in the dark for up to 6 months. Given that PicoGreen reagent is susceptible to photodegradation, the working solution must be covered in foil or stored in the dark to protect it from light. On the day of experiment, prepare a sufficient volume of a 1:400 dilution in 1x TE buffer (described in reagents and solutions section) for both the cell-extracts and DNA standard curve before measurement at Ex₄₈₅/Em₅₃₅ nm using a fluorometric plate reader. It is important that the solution is prepared in a plastic container instead of on glass surfaces as this may cause absorption of the PicoGreen reagent. In addition, 96 well tissue culture plates containing cell-extracts and DNA standard curve must be protected from light. When preparing DNA standards for the PicoGreen normalization assay, ensure that the extraction buffer used to prepare the DNA standards and for extraction of cellular Mn from striatal cells are treated the same and contain similar levels of contaminants.

Miscellaneous CFMEA parameters

Although CFMEA was used to measure cellular Mn uptake and transport kinetics in cultured striatal cells and primary astrocyte cells plated in a 96 well tissue culture plate format, the parameters of the assay can be modified to assess cellular Mn transport dynamics using different culture plate formats, cultured cell densities, and cell types. Specifically, cellular Mn uptake and transport dynamics can be assessed in larger cell densities, and cultured cells plated in 6, 12, 24, or 48 well tissue culture plates. To accurately quantify cellular Mn levels in cultured cells plated in a 24 well tissue culture plate and exposed to MnCl₂, similar extraction conditions described in basic protocol 1 can be utilized but with 500 µL Fura-2 containing extraction buffer volume and three washes with 1 mL PBS. This would enable the use of standard protein detection methods to normalize cellular Mn levels to the concentration of protein in the cultured cells. Considerable numbers of cultured cells would yield protein levels that are sufficient for detection by any of the standard protein assays. It is important to ensure that the volume of Fura-2 containing extraction buffer used for cellular Mn extraction from cultured cells completely covers the cells in the wells and is enough to dilute the concentrations of endogenous metal ions. It is necessary to ascertain that the Mn levels being measured are indeed cellular and not plasma membrane bound or extracellular Mn. We have demonstrated that the absence of detergent during cellular Mn extraction does not result in detectable Fura-2 quenching (Kwakye *et al.*, 2010, manuscript under review). Thus, this indicates that the suggested post-exposure PBS washes are sufficient and necessary to remove Fura-2 detectable plasma membrane bound or extracellular Mn. To validate that the Mn detected is in fact intracellular, expose cultured cells to MnCl₂ and thoroughly wash the cells with PBS or the preferred buffer-type. Apply the optimal concentration of Fura-2 containing extraction buffer with or without the solubilizing detergent and measure changes in Fura-2 fluorescence at the Ca²⁺ isosbestic

wavelength at 360 nm. This would indicate the necessity of solubilizing detergents in cellular Mn extraction.

ANTICIPATED RESULTS

The use of ~ 5,000 cells per 0.32 cm² in a 96 well tissue culture plate is sufficient for detection of cellular Mn levels following MnCl₂ exposure. Importantly, the choice of Mn exposure buffer and concentration determines the minimum time required for accurate detection of cellular Mn levels in cultured cells. For example, the minimum exposure time required for precise and accurate measurement of cellular Mn levels and transport dynamics in a striatal cell line (*StHdh*^{Q7/Q7}) (Trettel *et al.*, 2000) following 200 μM MnCl₂ exposure in culture media is about 2 hours. The extended time required for Mn detection could possibly be due to the binding of extracellular Mn to serum proteins (e.g. albumin) present in the culture media. This would reduce the available concentration of extracellular Mn available for cellular uptake. Conversely, accurate cellular Mn measurements could be achieved at least 20 minutes following 20 μM MnCl₂ exposure in either HEPES salt exposure buffer or Krebs Ringers buffer (serum free exposure buffers).

Examination of the ideal Fura-2 concentration for Mn detection

The optimal Fura-2 concentration added to the extraction buffer for detection of maximum cellular Mn levels in cultured cells is dependent on the minimum and maximum concentrations of MnCl₂ used for exposure, duration of MnCl₂ exposure, and most importantly, the approximate expected cellular Mn levels in the cultured cells following MnCl₂ exposure. The sigmoidal nature of the Mn-Fura-2 RFU standard curve is influenced by the concentration of Fura-2 used in the extraction buffer. However, the use of converted %_{MAX} values, rather than RFU to define Mn quantitation by Fura-2 accurately and reproducibly assesses Mn levels with minimal perturbation due to experimental variation in Fura-2 levels or efficiency of fluorescence signal detection by the plate reader.

Modification in the cellular Mn extraction conditions

The cellular Mn extraction conditions described in the basic protocol 1, critical parameters, and troubleshooting sections are the ideal conditions for many cultured mammalian cells. However, if the experimenter chooses to optimize the extraction conditions for the cultured cells used for cellular Mn uptake and transport kinetic studies due to previous experimental evidence of a physical, chemical, or other interferences with the described cellular Mn extraction conditions, it is important to consider the critical micelle concentration of the chosen detergent concentration. For example higher concentrations of Triton X-100 and SDS (>1%) impedes efficient cellular Mn extraction from the cultured cells (Kwakye *et al.*, 2010, manuscript under review).

Interactions between metal ions and Fura-2 at Ex₃₆₀

The Ex₃₆₀/Em₅₃₅ concentration-response curves demonstrated that abundant cellular metals, such as Ca²⁺ and Mg²⁺ had no influence on Mn-induced Fura-2 quenching at concentrations of 10 μM or lower for all tested Mn concentrations. However, Ca²⁺ concentrations above 10 μM Ca²⁺ competitively quench Fura-2 fluorescence (Kwakye *et al.*, 2010, manuscript under review). This may be due to saturation of Fura-2 metal binding sites by Ca²⁺ ions that might possibly induce molecular crowding of the fluorophore and decrease Fura-2 fluorescence. Nevertheless, given the relatively large volume of Fura-2 containing extraction buffer (100 μL or more, depending on the type of tissue culture plate) which dilutes the concentration of endogenous metal ions that might otherwise influence the accuracy of CFMEA, we do not anticipate, nor have we observed, any substantial influence of endogenous cellular metal ions on the quantified cellular Mn levels in cultured cells.

PicoGreen Assay

The presence of detergents, salts, and nuclear contaminants should neither influence the signal intensity of PicoGreen reagent nor alter the linearity of the PicoGreen assay. The sensitivity of the PicoGreen assay enables accurate measurement of dsDNA levels from 1,000 or more cultured cells. Due to the cost per 1 mL volume of the PicoGreen reagent, it would be advisable to normalize quantified cellular Mn levels to protein concentration if the number of cells per 0.32 cm² in a 96 well tissue culture plate exceeds 20,000.

TIME CONSIDERATIONS

The amount of time required to measure cellular Mn uptake and transport dynamics in cultured cells can be divided into five phases: (1) biological preparation, (2) transport incubations, (3) generation of a Mn-Fura-2 standard curve, (4) normalization, and (5) data analysis. The first phase requires significantly more time than the other four phases. The preparation of cultured cells, which includes trypsinization, cell counting, and plating in the 96 well tissue culture plates requires ~ 10 – 15 minutes per 96 well plate. If Mn uptake and transport dynamics are assessed in cells that grow in suspension, this time would vary depending on the method being used to prepare the cells. The second phase typically requires ~ 5 – 10 minutes for the preparation of working MnCl₂ solutions from stocks. Cultured cells exposed to MnCl₂ in HEPES salt exposure buffer or Krebs Ringers buffer require ~ 3 – 5 minutes per 96 well plate to pre-wash two times with 200 µL of either of the indicated serum-free exposure buffers prior to MnCl₂ exposure. In contrast to the duration necessary for the first phase and the preparatory steps of the second phase, the actual Mn transport incubations are conducted in real time. The length of time for transport incubation could be as early as 5 minutes and dependent on the type of cultured cells, experimental design, exposure buffer, and concentration of MnCl₂ used for exposure. Furthermore, generation of a cell-free Mn-Fura-2 standard curve necessitates ~ 5 minutes per experiment. It takes ~ 2 minutes per 96 well plate per assay to measure changes in Fura-2 fluorescence using the fluorometric plate reader. The PicoGreen assay requires ~ 5 minutes to load a 96 well plate with DNA standards and ~ 5 minutes incubation of standards with PicoGreen reagent. In addition, ~ 2 – 4 minutes per 96 well tissue culture plate is required to transfer cell-extracts into a new 96 well tissue culture plate before incubation with PicoGreen reagent for ~ 5 minutes and another ~ 2 minutes per 96 well tissue culture plate to measure PicoGreen fluorescence with a fluorometric plate reader. The duration of the fifth phase is flexible and depends on practice, experimenter's speed of data analysis and familiarity to Microsoft Excel linear regression analysis. It typically takes ~ 5 – 7 minutes per 96 well plate for complete analysis of cellular Mn levels and normalization by DNA. Depending on the experimental set-up, duration and concentration of MnCl₂, and familiarity with Microsoft Excel regression analysis, approximately ten to twenty 96-well plates can be assayed and scored in one day. Finally, CFMEA provides a high-throughput, feasible, accurate, and inexpensive assessment of cellular Mn levels in cultured cells. This assay provides a rapid means to evaluate Mn transport kinetics in cellular toxicity and disease models.

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Fig. 1. Cell-free Mn-fura-2 standard curve

Ten-point Mn-fura-2 standard curve was generated using a cell-free system comprised of 0.5 μM fura-2 in PBS with 0.1% Triton X-100 buffer at $\text{Ex}_{360}/\text{Em}_{535}$. Changes in fura-2 fluorescence induced by MnCl_2 quenching were normalized as percent maximal fluorescence ($\%_{\text{MAX}}$) to the 100% maximal fluorescence value after background subtraction. Data is represented as $\%_{\text{MAX}}$ (x-axis) and $\log_{10}$ scale of MnCl_2 concentration (y-axis). To enable back calculation of Mn concentration from $\%_{\text{MAX}}$, a power curve (solid black trend line) was used for $\%_{\text{MAX}}$ values less than 50% and logarithmic curve (dashed black trend line) for values greater than 50%, on either side of the inflection point of the saturation-binding curve. Mean levels are indicated as $\pm$ standard deviation. $N = 4$ wells/exposure condition. Dashed vertical lines indicate the optimal detection range of CFMEA (10% – 85% maximal fluorescence).

Fig. 2. Inverse relationship between %_{MAX} and total extracted Mn levels (nM)

Wild-type striatal cells exhibit a concentration-dependent decrease in % maximal RFU that inversely correlates with the increase in total extracted cellular Mn levels (nM). Wild-type *STHdh^{Q7/Q7}* striatal cell lines were exposed to different MnCl₂ concentrations for 4 hours in culture media. **(A)** After Mn exposure, changes in %_{MAX} values for each cell-extract were normalized to average RFU of the untreated striatal cells after background subtraction. Data is represented as % maximal RFU normalized to untreated cells (y-axis) and concentration of MnCl₂ exposed to cells (x-axis). **(B)** Total extracted cellular Mn levels (nM) from the striatal cells derived in **(A)** were calculated using the power and logarithmic equations described in Fig. 1 and represented as total extracted Mn levels (y-axis) and concentration of MnCl₂ exposed to cells (x-axis). Dashed line represents the minimal accurate detection limit of CFMEA (100 μM Mn). N=3; 6 wells/exposure condition. Mean levels are indicated as 95% confidence interval.

Fig. 3. PicoGreen standard curve

Seven point PicoGreen standard was generated using a cell-free system comprised of a 1:400 dilution of PicoGreen reagent in 1x TE buffer and varying salmon testes dsDNA concentrations at Ex₄₈₅/Em₅₃₅. The final concentration of salmon testes dsDNA in each well was calculated and plotted as final concentration of DNA (x-axis) and PicoGreen RFU (y-axis). Data points are represented on a linear curve generated by Microsoft Excel. N = 4 wells/dsDNA concentration. Mean levels are indicated as 95% confidence interval. Dashed vertical arrow line indicates the calculated concentration of dsDNA in representative unexposed wild-type striatal cells.

Fig. 4. Normalization of total extracted cellular Mn levels by PicoGreen Assay

Wild-type striatal cells exhibit a concentration-dependent increase in net Mn uptake following MnCl₂ exposure and normalization by PicoGreen assay. The PicoGreen assay described in Fig. 3 was performed on the same wild-type cell-extracts described in Fig. 2. The final DNA concentrations in wild-type striatal cell-extracts were calculated using the PicoGreen standard curve equation generated in Fig. 3. Total extracted cellular Mn levels in each cell-extract were normalized to its respective DNA concentration. Data is represented as femtomoles Mn per μg DNA (y-axis) and concentration of MnCl₂ exposed to cells (x-axis). N=3; 6 wells/exposure condition. Mean levels are indicated as 95% confidence interval.

Table 1

Cell-free Mn-Fura-2 standard DNA standard curve

Number on 96 well plate	MnCl ₂ stock solution (μM)	Vol. (μL) of MnCl ₂ stock solution	Vol. (μL) of extraction buffer	Final [Mn] in 100 μL volume (nM)
A1, B1, C1, D1	1	1	99	10
A2, B2, C2, D2	5	1	99	50
A3, B3, C3, D3	10	1	99	100
A4, B4, C4, D4	50	1	99	500
A5, B5, C5, D5	100	1	99	1,000
A6, B6, C6, D6	500	1	99	5,000
A7, B7, C7, D7	1,000	1	99	10,000
A8, B8, C8, D8	5,000	1	99	50,000
A9, B9, C9, D9	10,000	1	99	100,000
A10, B10, C10, D10	100,000	1	99	1,000,000
A11, B11, C11, D11 (#)	0	0	100	Blank
A12, B12, C12, D12 (*)	0	0	100 uL PBS with 0.1 % Triton-X-100	Blank

(#) Represents wells used for calculating 100% maximal fluorescence value after background subtraction

(*) Represents wells used for Fura-2 background subtraction.

Table 2

PicoGreen DNA standard curve

Number on 96 well plate	Stock [DNA] ($\mu\text{g/mL}$)	Vol. (μL) of DNA standard	Vol. (μL) of working PicoGreen solution	Final [DNA] ($\mu\text{g/mL}$)
A1, B1, C1, D1	0.0781	20	80	0.0156
A2, B2, C2, D2	0.1563	20	80	0.0313
A3, B3, C3, D3	0.3125	20	80	0.0625
A4, B4, C4, D4	0.625	20	80	0.125
A5, B5, C5, D5	1.25	20	80	0.25
A6, B6, C6, D6	2.5	20	80	0.5
A7, B7, C7, D7	5.0	20	80	1.0
A8, B8, C8, D8 (*)	0	20 μL PBS with 0.1 % Triton-X-100	80	blank

(*) Represents wells used for PicoGreen background subtraction.