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TITLE:

Atomic Absorbance Spectroscopy to Measure Intracellular Zinc Pools in Mammalian Cells

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KEYWORDS:

Zinc, atomic absorbance spectroscopy, proliferation, differentiation, mammalian cell culture, subcellular fractionation

SUMMARY:

Cultured primary or established cell lines are commonly used to address fundamental biological and mechanistic questions as an initial approach before using animal models. This protocol describes how to prepare whole cell extracts and subcellular fractions for studies of zinc (Zn) and other trace elements with atomic absorbance spectroscopy.

ABSTRACT:

Transition metals are essential micronutrients for organisms but can be toxic to cells at high concentrations by competing with physiological metals in proteins and generating redox stress. Pathological conditions that lead to metal depletion or accumulation are causal agents of different human diseases. Some examples include anemia, acrodermatitis enteropathica, and Wilson's and Menkes' diseases. It is therefore important to be able to measure the levels and transport of transition metals in biological samples with high sensitivity and accuracy in order to facilitate research exploring how these elements contribute to normal physiological functions

and toxicity. Zinc (Zn), for example, is a cofactor in many mammalian proteins, participates in signaling events, and is a secondary messenger in cells. In excess, Zn is toxic and can inhibit absorption of other metals, while in deficit, it can lead to a variety of potentially lethal conditions.

Graphite furnace atomic absorption spectroscopy (GF-AAS) provides a highly sensitive and effective method for determining Zn and other transition metal concentrations in diverse biological samples. Electrothermal atomization via GF-AAS quantifies metals by atomizing small volumes of samples for subsequent selective absorption analysis using wavelength of excitation of the element of interest. Within the limits of linearity of the Beer-Lambert Law, the absorbance of light by the metal is directly proportional to concentration of the analyte. Compared to other methods of determining Zn content, GF-AAS detects both free and complexed Zn in proteins and possibly in small intracellular molecules with high sensitivity in small sample volumes. Moreover, GF-AAS is also more readily accessible than inductively coupled plasma mass spectrometry (ICP-MS) or synchrotron-based X-ray fluorescence. In this method, the systematic sample preparation of different cultured cell lines for analyses in a GF-AAS is described. Variations in this trace element were compared in both whole cell lysates and subcellular fractions of proliferating and differentiated cells as proof of principle.

INTRODUCTION:

Transition and heavy metals, such as Zn, Cu, Mn, and Fe, are found naturally in the environment in both nutrients in food and pollutants. All living organisms require different amounts of these micronutrients; however, exposure to high levels is deleterious to organisms. Metal acquisition is mainly through the diet, but metals can also be inhaled or absorbed through the skin[1-5]. It is important to note that the presence of metals in atmospheric particles is increasing and has been largely associated with health risks. Due to anthropogenic activities, increased levels of heavy metals such as Ag, As, Cd, Cr, Hg, Ni, Fe, and Pb have been detected in atmospheric particulate matter, rainwater, and soil[6,7]. These metals have the potential to compete with essential physiological trace elements, particularly Zn and Fe, and they induce toxic effects by inactivating fundamental enzymes for biological processes.

The trace element Zn is redox neutral and behaves as a Lewis acid in biological reactions, which makes it a fundamental cofactor necessary for protein folding and catalytic activity in over 10% of mammalian proteins[8-10]; consequently, it is essential for diverse physiological functions[8,11]. However, like many trace elements, there is a delicate balance between these metals facilitating normal physiological function and causing toxicity. In mammals, Zn deficiencies lead to anemia, growth retardation, hypogonadism, skin abnormalities, diarrhea, alopecia, taste disorders, chronic inflammation, and impaired immune and neurological functions[11-18]. In excess, Zn is cytotoxic and impairs absorption of other essential metals such as copper[19-21].

Additionally, some metals like Cu and Fe have the potential to participate in harmful reactions. Production of reactive oxygen species (ROS) via Fenton chemistry can interfere with the assembly of iron sulfur cluster proteins and alter lipid metabolism[22-24]. To prevent this damage, cells utilize metal-binding chaperones and transporters to prevent toxic effects. Undoubtedly, metal homeostasis must be tightly controlled to ensure that specific cell types maintain proper levels

of metals. For this reason, there is a significant need to advance techniques for accurate measurement of trace metals in biological samples. In developing and mature organisms there exists a differential biological need for trace elements at the cellular level, at different developmental stages, and in normal and pathological conditions. Therefore, precise determination of tissue and systemic metal levels is necessary to understand organismal metal homeostasis.

Graphite furnace atomic absorption spectrometry (GF-AAS) is a highly sensitive technique used for small sample volumes, making it ideal to measure transition and heavy metals present in biological and environmental samples[25-28]. Moreover, due to high sensitivity of the technique, it has been shown to be appropriate for studying the fine transport properties of Na^+/K^+-ATPase and gastric H^+/K^+-ATPase using *Xenopus* oocytes as a model system[29]. In GF-AAS, the atomized elements within a sample absorb a wavelength of radiation emitted by a light source containing the metal of interest, with the absorbed radiation proportional to the concentration of the element. Elemental electronic excitation takes place upon absorption of ultraviolet or visible radiation in a quantized process unique for each chemical element. In a single electron process, the absorption of a photon involves an electron moving from a lower energy level to higher level within the atom and GF-AAS determines the amount of photons absorbed by the sample, which is proportional to the number of radiation absorbing elements atomized in the graphite tube.

The selectivity of this technique relies on the electronic structure of the atoms, in which each element has a specific absorption/emission spectral line. In the case of Zn, the absorbance wavelength is 213.9 nm and can be precisely distinguished from other metals. Overall, GF-AAS can be used to quantify Zn with adequate limits of detection (LOD) and high sensitivity and selectivity[25]. The changes in absorbed wavelength are integrated and presented as peaks of energy absorption at specific and isolated wavelengths. The concentration of the Zn in a given sample is usually calculated from a standard curve of known concentrations according to the Beer-Lambert law, in which the absorbance is directly proportional to the concentration of Zn in the sample. However, applying the Beer-Lambert equation to GF-AAS analyses also presents some complications. For instance, variations in the atomization and/or non-homogenous concentrations of the samples can affect the metal measurements.

The metal atomization required for GF AAS trace elemental analysis consists of three fundamental steps. The first step is desolvation, where the liquid solvent is evaporated; leaving dry compounds after the furnace reaches a temperature of around 100 °C. Then, the compounds are vaporized by heating them from 800 to 1,400 °C (depending on the element to be analyzed) and become a gas. Finally, the compounds in the gaseous state are atomized with temperatures that range from 1,500 to 2,500 °C. As discussed above, increasing concentrations of a metal of interest will render proportional increases on the absorption detected by the GF-AAS, yet the furnace reduces the dynamic range of analysis, which is the working range of concentrations that can be determined by the instrument. Thus, the technique requires low concentrations and a careful determination of the dynamic range of the method by determining the LOD and limit of linearity (LOL) of the Beer-Lambert law. The LOD is the minimum quantity required for a substance to be detected, defined as three times the standard deviation of Zn in the matrix. The

LOL is the maximum concentration that can be detected using Beer-Lambert law.

In this work, we describe a standard method to analyze the levels of Zn in whole cell extracts, cytoplasmic and nuclear fractions, and in proliferating and differentiating cultured cells (**Figure 1**). We adapted the rapid isolation of nuclei protocol to different cellular systems to prevent metal loss during sample preparation. The cellular models used were primary myoblasts derived from mouse satellite cells, murine neuroblastoma cells (N2A or Neuro2A), murine 3T3 L1 adipocytes, a human non-tumorigenic breast epithelial cell line (MCF10A), and epithelial Madin-Darby canine kidney (MDCK) cells. These cells were established from different lineages and are good models for investigating lineage specific variations of metal levels *in vitro*.

Primary myoblasts derived from mouse satellite cells constitute a well-suited *in vitro* model to investigate skeletal muscle differentiation. Proliferation of these cells is fast when cultured under high serum conditions. Differentiation into the muscular lineage is then induced by low serum conditions[30]. The murine neuroblastoma (N2A) established cell line was derived from the mouse neural crest. These cells present neuronal and amoeboid stem cell morphology. Upon differentiation stimulus, the N2A cells present several properties of neurons, such as neurofilaments. N2A cells are used to investigate Alzheimer's disease, neurite outgrowth, and neurotoxicity[31-33]. The 3T3-L1 murine pre-adipocytes established cell line is commonly used to investigate the metabolic and physiological changes associated with adipogenesis. These cells present a fibroblast-like morphology, but once stimulated for differentiation, they present enzymatic activation associated with lipid synthesis and triglycerides accumulation. This can be observed as morphological changes to produce cytoplasmic lipid droplets[34,35]. MCF10A is a non-tumor mammary epithelial cell line derived from a premenopausal woman with mammary fibrocystic disease[36]. It has been widely used for biochemical, molecular, and cellular studies related to mammary carcinogenesis such as proliferation, cell migration, and invasion. The Madin-Darby canine kidney (MDCK) epithelial cell line has been extensively used to investigate the properties and molecular events associated with the establishment of the epithelial phenotype. Upon reaching confluence, these cells become polarized and establish cell-cell adhesions, characteristics of mammalian epithelial tissues[37].

To test the ability of AAS to measure the levels of Zn in mammalian cells, we analyzed whole and subcellular fractions (cytosol and nucleus) of these five cell lines. AAS measurements showed different concentrations of Zn in these cell types. Concentrations were lower in proliferating and differentiating primary myoblasts (4 to 7 nmol/mg of protein) and higher in the four established cell lines (ranging from 20 to 40 nmol/mg of protein). A small non-significant increase in Zn levels was detected in differentiating primary myoblasts and neuroblastoma cells when compared to proliferating cells. The opposite effect was detected in differentiated adipocytes. However, proliferating 3T3-L1 cells exhibited higher concentrations of the metal compared to differentiated cells. Importantly, in these three cell lines, subcellular fractionation showed that Zn is differentially distributed in the cytosol and nucleus according to the metabolic state of these cells. For instance, in proliferating myoblasts, N2A cells, and 3T3-L1 pre-adipocytes, a majority of the metal is localized to the nucleus. Upon induction of differentiation using specific cell treatments, Zn localized to the cytosol in these three cell types. Interestingly, both epithelial cell

lines showed higher levels of Zn during proliferation compared to when reaching confluence, in which a characteristic tight monolayer was formed. In proliferating epithelial cells, the mammary cell line MCF10A had an equal Zn distribution between the cytosol and nucleus, while in the kidney-derived cell line, most of the metal was located in the nucleus. In these two cell types, when the cells reached confluence, Zn was predominantly located to the cytosol. These results demonstrate that GF-AAS is a highly sensitive and accurate technique for performing elemental analysis in low-yield samples. GF-AAS coupled with subcellular fractionation and can be adapted to investigate the levels of trace metal elements in different cell lines and tissues.

PROTOCOL:

1. Mammalian cell culture

1.1. General considerations

1.1.1. Follow the aseptic techniques for mammalian cell culture previously reviewed[38].

1.1.2. Maintain all cell lines in a humidified 5% CO_2 incubator at 37 °C. Cell culture conditions vary for each cell type. It is important to maintain appropriate culture conditions for each cell line used, as variations of these procedures will lead to aberrant phenotypes and failure of the cell culture.

1.1.3. Ensure acquaintance with the cell line of interest, and follow the protocols provided for each cell type selected for specific experiments (see some examples below).

1.2. General procedure for trypsinization and passing of adherent cells

1.2.1. Remove the cell culture media from the culture plate by aspiration.

1.2.2. Wash the cells gently using PBS without calcium and magnesium (5 mL per 10 cm^2 culture surface area). Add the wash solution to the side of the plate to avoid detaching cell monolayer; swirl the plate to maximize coverage of the solution. For epithelial cells, it is necessary to incubate the cells for 10 to 15 min in the incubator at 37 °C in PBS to facilitate the dissociation of cells in the following steps.

1.2.3. Remove the washing solution by aspiration.

1.2.4. Add enough dissociation reagent (0.25% trypsin) to the plate (approximately 0.7 mL per 10 cm^2). Gently swirl the plate to get complete coverage of the cell monolayer.

1.2.5. Incubate the culture at room temperature for 2 to 5 min. The actual incubation time varies with the cell line used, in the case of epithelial cells is recommended to incubate for 10 to 15 min in the incubator at 37 °C. Observe the cells under the microscope for cell detachment.

1.2.6. When most of the cells have detached, recover the cell suspension in 5 mL of the pre-warmed plating or growth medium (see below for some examples). Mechanically dissociate cell clumps by pipetting over the cell monolayer several times.

1.2.7. Transfer the cell suspension to a 15 mL conical tube and centrifuge them at 1,000 x *g* for 2 to 5 min. The centrifugation speed and time vary based on the cell type. Remove the media by gentle aspiration, being careful not to touch the cell pellet. Resuspend the cell pellet in 5 to 10 mL of the appropriate pre-warmed growth medium and remove a sample for counting using a hemocytometer or an automatic cell counter.

1.2.8. Utilize the appropriate volume of the cell suspension to seed the recommended density for each specific cell line (see below for some examples). Supplement with the adequate volume of culture media according to the size of the culture dish and place the cells back into the incubator.

1.3. Primary myoblasts derived from mouse satellite cells

1.3.1. Before starting, prepare collagen-coated plates for primary myoblast growth. A solution containing 0.02% collagen, and 0.05 M acetic acid in deionized water must be sterilized by filtration with a 0.22 µm sterile filter device. Incubate the culture plates with the collagen solution overnight at 37 °C.

1.3.2. On the next day, aspirate the collagen solution, allow the plates to air dry in the sterile cabinet and store at 4 °C until use.

NOTE: The minimal volumes of collagen are: for 35 mm = 1 mL; 60 mm = 2 mL; 10 cm = 5 mL.

1.3.3. Seed primary myoblasts at 1 x 10^4 cells/cm^2 in 55 cm^2 collagen-coated culture plates. The proliferation media is Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 nutrient mixture culture media containing 20% fetal bovine serum (FBS), 25 ng/mL of recombinant basic fibroblastic growth factor (FGF), and 100 U/mL of penicillin/streptomycin.

NOTE: Proliferating and stock cells should be maintained under 50% of confluency. Cell contact induces commitment of the cells to myogenic differentiation and impairs the growth capabilities of the culture.

1.3.4. For differentiating the primary myoblasts, allow the cells to reach 80% to 100% confluency, which normally occurs after 48 h of plating. Then, change to differentiation media (DMEM, 2% horse serum, 1% insulin, transferrin, selenium A supplement, and 100 U/mL of penicillin/streptomycin). Refresh the media daily until harvest.

1.4. Murine neuroblastoma (N2A)

1.4.1. Plate N2A cells at a density of 2 x 10^4 cells/cm^2 in growth media, containing a mixture of

reduced-serum medium/DMEM containing high glucose and L-glutamine (1:1, v:v), supplement with 10% FBS and 100 U/mL of penicillin/streptomycin. Maintain the stock culture under 50% confluency.

1.4.2. Induce differentiation of N2A cells once confluency reached 25% to 40%. Change to differentiation media containing a mixture of reduced-serum medium/DMEM, 1% FBS, and 20 µM retinoic acid for 7 to 10 days. Refresh the media every other day until cell harvest.

NOTE: As N2A cells differentiate, some cells become round, detach easily, and may undergo apoptosis. Be careful when aspirating and adding the culture media to the plates.

1.5. 3T3 L1 murine pre-adipocytes

1.5.1. Maintain mouse 3T3/L1 cells in DMEM with high glucose, supplemented with 10% FBS and 100 U/mL of penicillin/streptomycin. The adipogenic process is dependent in the confluence of the cells; therefore, do not let the stock culture grow over 50% confluency, as higher confluence will impair the ability of the cells to differentiate.

1.5.2. Plate the cells at 2 x 10^4 cells/cm^2. At this density, the cells will reach confluence in 3 days.

1.5.3. Induce adipogenic differentiation two days after the cells reach 100% confluency. Induction media consists of DMEM containing 10% FBS, 10 µg/mL insulin, 0.5 mM 3-isobutyl-1-methyxanthine, 1 µM dexamethasone, and 10 µM troglitazone.

1.5.4. Replace the induction media after 48 h incubation to differentiation media (DMEM containing 10% FBS, and 5 µg/mL insulin). Refresh the media every other day until harvest. Representative samples of full differentiation are normally collected between 7 and 10 days after adipogenic induction.

NOTE: As adipogenesis progresses, the cells become round and tend to detach easily. Be careful when aspirating and adding the culture media to the plates.

1.6. MCF10A cells

1.6.1. Plate MCF10A cells in DMEM/F12 (1:1, v:v), supplemented with 5% FBS, 100 U/mL penicillin/streptomycin, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, and 20 ng/mL epidermal growth factor (EGF). Recommended seeding density is 1 x 10^5 cells/cm^2. Under these conditions, the cells will reach 100% confluency within 4 days. Refresh the media every 2 to 3 days until harvest.

NOTE: MCF10A cells are difficult to detach. It is recommended to incubate the cells for 10 to 15 min at 37 °C in PBS free of calcium with 0.5 mM EDTA prior to trypsinization.

1.7. Madin-Darby canine kidney cells (MDCK)

1.7.1. Plate MDCK cells in DMEM supplemented with 10% FBS and 100 U/mL of penicillin/streptomycin. Recommended seeding density is 1 x 10^5 cells/cm^2, where the cells will reach 100% confluency within 3 days. Refresh the media every 2 days until harvest.

NOTE: MDCK cells are difficult to detach. It is recommended to incubate the cells for 5 to 10 min at 37 °C in PBS free of calcium and magnesium prior to trypsinization. To prevent cell clumping do not agitate the plates by hitting or shaking the flask while waiting for the cells to detach.

2. Mammalian cell culture sample preparation for AAS: whole cell and subcellular fractionation

2.1. Culture the cells of interest in 55 cm^2 plates. The use of smaller culture plates may yield samples with levels of metals under the detection limits of the GF-AAS.

2.2. Whole cell extract

2.2.1. Aspirate the culture media using a vacuum trap. Be sure to remove all traces of media. Rinse the cells from the desired time points or culture conditions three times with ice-cold PBS free of calcium and magnesium.

2.2.2. Immediately scrape the cells from the plate using a new plastic cell scraper in 1 mL of PBS free of calcium and magnesium. Transfer the sample to a 1.5 mL microcentrifuge tube and centrifuge for 2 min at 2,000 x *g* and aspirate the supernatant. The protocol can be paused here. Store pellet samples at -20 °C or proceed to step 2.4.

2.3. Subcellular fractionation

2.3.1. Aspirate the culture media using a vacuum trap. Be sure to remove all traces of media. Rinse the cells from the desired time points or culture conditions three times with ice-cold PBS free of calcium and magnesium. Scrape the cells off the plate with 1 mL of ice-cold PBS free of calcium and magnesium.

2.3.2. Transfer the samples to a 1.5 mL microcentrifuge tube and keep on ice. Centrifuge for 10 s at 10,000 x *g*. Continue rapid isolation of nuclei if metal analysis of cytoplasmic and nuclear fractions is desired. This procedure will minimize the potential loss of metals due to cell extraction[39,40].

2.3.3. Remove the supernatant by aspiration and resuspend the cell pellet in 400 µL of ice-cold PBS free of calcium and magnesium, containing 0.1% Nonidet P-40 (NP-40), a non-ionic detergent. Transfer 100 µL to a new microcentrifuge tube and consider it as the whole cell sample. This aliquot represents 25% of the sample.

2.3.4. Isolate nuclei by pipetting the cell suspension 5 to 10 times on ice with a P1000 micropipette tip. Nuclei isolation of epithelial cells requires a concentration of 0.5 % NP-40.

Achieve cell dissociation by passing the cell suspension through a 26 ¾ G needle 10 to 15 times, followed by 10 to 15 passages through a 30 ½ G needle.

NOTE: Verify the nuclei integrity by light microscopy using a 40x objective.

2.3.5. Centrifuge the remaining cell lysate suspension (300 µL) for 10 s at 10,000 x *g*. The supernatant will contain the cytosolic fraction. Transfer the 300 µL to a new microcentrifuge tube.

2.3.6. Rinse the pellet containing the nuclear fraction in 500 µL PBS free of calcium and magnesium, containing 0.1% NP-40, then centrifuge for 10 s at 10,000 x *g*. Remove the supernatant and resuspend the pellet containing the nuclei in 100 µL of the same solution.

NOTE: The protocol can be paused here. Samples can be stored at -20 °C.

2.4. Lyse the cells by three sonication cycles (30 s on/30 s off, at 50 kHz, 200 W) for 5 min. Quantify total protein content in the sample by Bradford[41].

2.5. Perform quality control of the purity of the fractions by western blot using antibodies specific for either the nuclear (histones, chromatin remodelers, nuclear matrix proteins) or cytosolic fractions (β-tubulin, β-actin) (**Figure 2**).

NOTE: Do not use sonicators with a metal tip. These can contaminate the sample with metals.

3. Zn content analysis of whole cell and subcellular fractions of mammalian cell cultures by atomic absorbance spectroscopy

3.1. Mineralize the cell culture samples (whole cell or nuclear fractions) by acid digestion using an equal volume of concentrated HNO_3 trace metal grade (CAUTION) for 1 h at 80 °C, then overnight at 20 °C.

3.2. Stop the reaction by adding 30% of the reaction volume of H_2O_2. Bring the sample volume to 500 µL with 18 MΩ purified water.

NOTE: HNO_3 handling should be done wearing chemical safety glasses, a face shield for splash protection, gloves, and an approved vapor respirator if adequate ventilation (fume hood) is not available. The protocol can be paused here. Samples can be stored at room temperature (RT).

3.3. Prepare standard solutions and experimental samples for Zn measurements by AAS equipped with a graphite furnace.

3.3.1. Use 2% (v/v) HNO_3 solution in deionized water, coming from the same batch used for the standard curve as the blank solution for calibration throughout. Use analytical grade standards diluted in 18 MΩ purified water.

3.3.2. Prepare a standard of 1000 ppb of Zn from a commercially available 1000 ppm Zn stock solution with 2% (v/v) HNO_3 solution in deionized water. Prepare working standard solutions from the 100 ppb standard by diluting it to 5, 8, 10, 15, 20, and 25 ppb directly with 2% (v/v) HNO_3 solution in deionized water. Prepare the Zn standards and the blank with a 0.1% (1 g/L) $Mg(NO_3)_2$ matrix modifier.

NOTE: The LOD of Zn is 0.01 ppb (µg/L). By increasing the concentration of the standards, the limit of linearity of Zn was determined to be 20 ppb (**Figure 3**).

3.3.3. Measure standard and samples under the same optimized conditions: flow rate of introduction of 250 mL/min, injection temperature of 20 °C, and GF temperature of 1800 °C.

NOTE: The GF temperature was increased to 2450 °C for cleanout steps, prior to the injection of a new sample or standard.

3.3.4. Optimize the lamp (C-HCL) to a current of 20 A, wavelength of 213.9 nm, and slit of 0.7 nm.

3.3.5. Measure the mineralized samples using the Zn standard curve to determine metal content. As small volumes are measured, the samples can evaporate if the measurements take long periods of time. Therefore, it is recommended to add water to the samples. Dilute the samples with 18 MΩ purified water treated with 0.01% HNO_3 (analytical grade) if the data obtained is beyond the LOD.

NOTE: Typically, samples are diluted to a volume of 500 µL, placed in the automated autosampler of the AAS to be measured right after the standard curve is established. The required volume should be determined by the user and should consider final calculations of concentrations. Finishing the Zn determinations by AAS of a complete experiment in one attempt is recommended. Pausing the measurements may lead to large experimental variations and inconsistencies.

3.4. Convert the ppb measurements to molarity as obtained from Zn measured via AAS. Consider the mass of Zn (65.39 amu). Divide the amount of ppb obtained by AAS by 63,390,000. In cells, the Zn concentrations range from units of pmol to µmol.

3.4.1. Divide the concentration of Zn (pmol or µmol) by the initial mass of protein in the sample determined by Bradford (step 2.4). This calculation will render the amount of metal (pmol or µmol Zn) contained per mg of protein of the sample.

NOTE: It is important to consider the dilution factors utilized during the metal determinations.

REPRESENTATIVE RESULTS:

We tested the ability of the GF-AAS to detect minute levels of Zn in mammalian cells (**Figure 1**).

Thus, we cultured primary myoblasts derived from mouse satellite cells, and the established cell lines N2A (neuroblastoma derived), 3T3 L1 (adipocytes), MCF10A (breast epithelium), and MDCK cells (dog kidney epithelium). First, we isolated whole cell, cytosolic, and nuclear fractions of all these cell types and evaluated the purity of the fractions by western blot (**Figure 2**). Representative immunodetection of the chromatin remodeler Brg1 and tubulin as markers of nuclear and cytosolic fractions, respectively, demonstrated the suitability of the subcellular fractionation protocol described in section 2.3 to perform the elemental analysis (**Figure 2**). Whole cell and subcellular fractions were prepared as described above for GF-AAS analyses, and calibration of the equipment was performed to yield a typical linear standard curve (**Figure 3**).

Figure 4 shows representative light microscopy images of proliferating and differentiated or confluent monolayers of each cell type, and the corresponding Zn levels for these. Importantly, all the cell lines analyzed in this study showed Zn concentrations in the nM range. However, a differential distribution of the metal was detected. Differentiated primary myotubes exhibited higher levels of Zn than proliferating cells (**Figure 4A**). Notably, much of the ion was located to the nuclear fraction in both proliferating and differentiating myoblasts. A similar subcellular distribution of Zn was detected in the neuroblastoma derived cell line N2A (**Figure 4B**). However, measurements showed that N2A cells had Zn levels one order of magnitude higher than primary myoblasts. On the other hand, the established 3T3-L1 cell line exhibited higher levels of Zn when the pre-adipocytes were proliferating than when they were induced to differentiate and form mature adipocytes that accumulate lipids (**Figure 4C**).

We also measured Zn content in two different established epithelial cell lines: the MCF10A derived from human mammary gland (**Figure 4D**) and the dog kidney-derived MDCK (**Figure 4E**) cells. Interestingly, in both cases, higher levels of Zn in proliferating cells than in confluent monolayers were detected. However, the epithelial cells derived from the mammary gland showed metal levels that were 2-fold higher than that of the kidney cells. MCF10A cells showed equal levels of Zn between cytosolic and nuclear fractions in proliferating cells. Once MCF10A cells reach confluence, a 40% decrease in whole cell Zn levels was detected, and the metal was found to be most concentrated in the cytosolic fraction (**Figure 4D**). Conversely, proliferating MDCK cells exhibited higher levels of Zn in the nuclear fraction, compared to the cytosolic fraction, but when the MDCK cells reached confluence, a decrease of Zn in whole cells was detected, with the majority of this transition metal observed in the cytosolic fraction (**Figure 4E**).

FIGURES AND TABLES LEGENDS:

Figure 1: Representative flow chart for elemental (Zn) analyses of mammalian cultured cells.

Figure 2: Validation of the purity of fractions obtained via the rapid isolation of nuclei protocol[39,40]. Representative western blot from differentiating primary myoblasts showing the purity of subcellular fractions. The chromatin remodeler enzyme Brg1 was used to identify the nuclear fraction, and tubulin was used to identify the cytosolic fraction.

Figure 3: Calibration curve of Zn standards. Representative standard curve for Zn determined by

GF-AAS. The GF-AAS was calibrated with the standard Zn solutions diluted at the following concentrations: 1, 3, 5, 7, 10, 15, 20, 25, and 30 ppb.

Figure 4: Zinc levels in different mammalian cultured cells. Representative light micrographs and Zn content in whole cell extracts and cytosolic and nuclear fractions. Data represent the mean of three independent biological replicates ± SEM. (**A**) Murine primary myoblasts proliferating and differentiating for 2 days. (**B**) Murine neuroblastoma N2A cell line before and after induction to differentiate by retinoic acid treatment. (**C**) Murine 3T3-L1 pre-adipocytes and 6 days post differentiation. (**D**) Pre-confluent and confluent monolayer of the human mammary epithelial cell line MCF10A. (**E**) Pre-confluent and confluent monolayer of the kidney epithelial cell line MDCK. Data represent the average of three independent experiments ± SE. Student's *t*-test shows significance, *$p \leq 0.05$.

DISCUSSION:

Atomic absorbance spectroscopy is a highly sensitive method for Zn quantification in small volume/mass biological samples. The described optimization of Zn measurement makes application of this method simple and guarantees ideal analytical conditions. Here, using GF-AAS, we determined the concentration of Zn in whole cells, and in cytosolic and nuclear fractions, from different cell lines. The results show that this technique renders comparable to those obtained by fluorescent probes and ICP-MS. For instance, several fluorescent probes showed labile mitochondrial Zn levels in the range of 0.1 to 300 pM, levels in the Golgi were below the picomolar range, and the endoplasmic reticulum ranged from 800 to 5000 pM[42], which are consistent with levels detected in the cytosolic fractions. Moreover, the Zn-responsive fluorophore FluoZin-3 showed accumulation of labile Zn in small cytosolic vesicles in MCF10A and in C2C12 cells[43,44], which are also consistent with the Zn quantified in the cytoplasm. Moreover, Zn analyses by ICP-MS performed by our group showed that whole cell levels Zn in differentiating primary myotubes[45] are similar to those obtained in this report by GF-AAS. In addition, examples of Zn determinations in C6 rat glioma cells by using AAS and Zinquin-E[46] have also rendered similar ranges to those observed in neuroblastoma N2A cells used here.

To date, diverse techniques have been developed to detect Zn in cells. However, GF-AAS remains an accurate and highly sensitive technique that not only allows detection of free Zn, but it also measures the metal complexed and bound to proteins and potentially to small molecules. Our group recently showed that measurements performed with cell-permeable fluorescent probe (which has a high affinity to bind free Zn^{2+}) directly correlated with the levels of Zn detected by GF-AAS in proliferating and differentiating myoblasts[43]. However, the results obtained by these probes may not be representative of whole cell or protein-bound Zn concentrations. Moreover, since Zn exists as a non-redox active species and as divalent cation, under physiological conditions, its detection in cellular systems remained a challenge. Novel Zn imaging techniques have become available, such as the state-of-the-art synchrotron-based X-ray fluorescence microscopy, radioisotopes, and laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS). Unfortunately, some of these techniques require resources inaccessible to the scientific community[29,42,47-50].

Studies have shown that ICP-MS is a more precise technique than AAS, as relative standard deviation values have been shown to be lower in ICP-MS determinations for metals like Zn. The likely explanation for this effect is that ICP-MS is intrinsically capable of eliminating chemical interferences, as the operating argon temperatures range from 5,000 to 10,000 °C. Some chemical bonds can be maintained at 3,000 °C, which is in the range for AAS operation. These bonds will be completely disrupted at above 6,000 °C. Therefore, these high temperatures reached in the plasma state by ICP-MS can eliminate chemical interferences, leading to better detection limits[51]. In addition, ICP-MS requires larger volume samples compared to GF-AAS, as the latter is capable of operating with volumes under 100 µL, which is significantly less than other spectroscopic methods such as AAS, ICP-OES, or ICP-MS. Given the small volumes involved in the method, GF-AAS is the ideal technique to determine Zn concentration in biological samples, primarily if the analysis involves subcellular fractions, metal detections in purified proteins, or small variations in cellular metal content due to mutations in transporters or metal binding proteins[29,52]. In addition, the sensitivity of the GF-AAS system to determine trace and ultra-trace concentrations (pg/mL to ng/mL) is another advantage.

Important considerations should be taken to ensure reliability of the GF-AAS data. These include adequate calibration, integrity of the graphite tube, and selection of suitable matrix modifier for electrothermal atomization. Matrix modifiers are chemical elements added to the sample, which affect the thermal processes taking place in the atomizer. These modifiers minimize the loss of analyte during pyrolysis and contribute to removal of matrix components. Overall, modifiers may change the sample matrix to evaporate the matrix components at lower temperatures and may work as analyte stabilizers. In addition to these considerations, it is important to perform adequate optimization of the steps for the atomizer temperature program.

Two main considerations should be taken, including 1) establishing the optimum pyrolysis temperature, which refers to the maximum temperature at the atomization step at which no analyte loss occurs, and which allows a maximum absorbance of the analyte with minimal background. One should also consider 2) establishing the optimum atomization temperature, which refers to the minimum temperature at which the analyte is fully evaporated and recorded as a reproducible signal or peak. One of the disadvantages of GF-AAS analyses is element interference, for which a thorough optimization and standardization are essential for accurate measurements. However, to date, GF-AAS represents an important tool in scientific research to detect the metal ions in diverse biological samples. Future applications of Zn (and other metals) detection methods by GF-AAS will include measuring the levels of metals in organs and tissues obtained from animal models or patient biopsies to better understand specific physiological needs for trace elements. In conclusion, GF-AAS is accurate, sensitive, cost-effective, and accessible, and this analytical technique will continue to improve as technological advances move forward for these detection systems.

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The authors have nothing to disclose.

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Figure 1

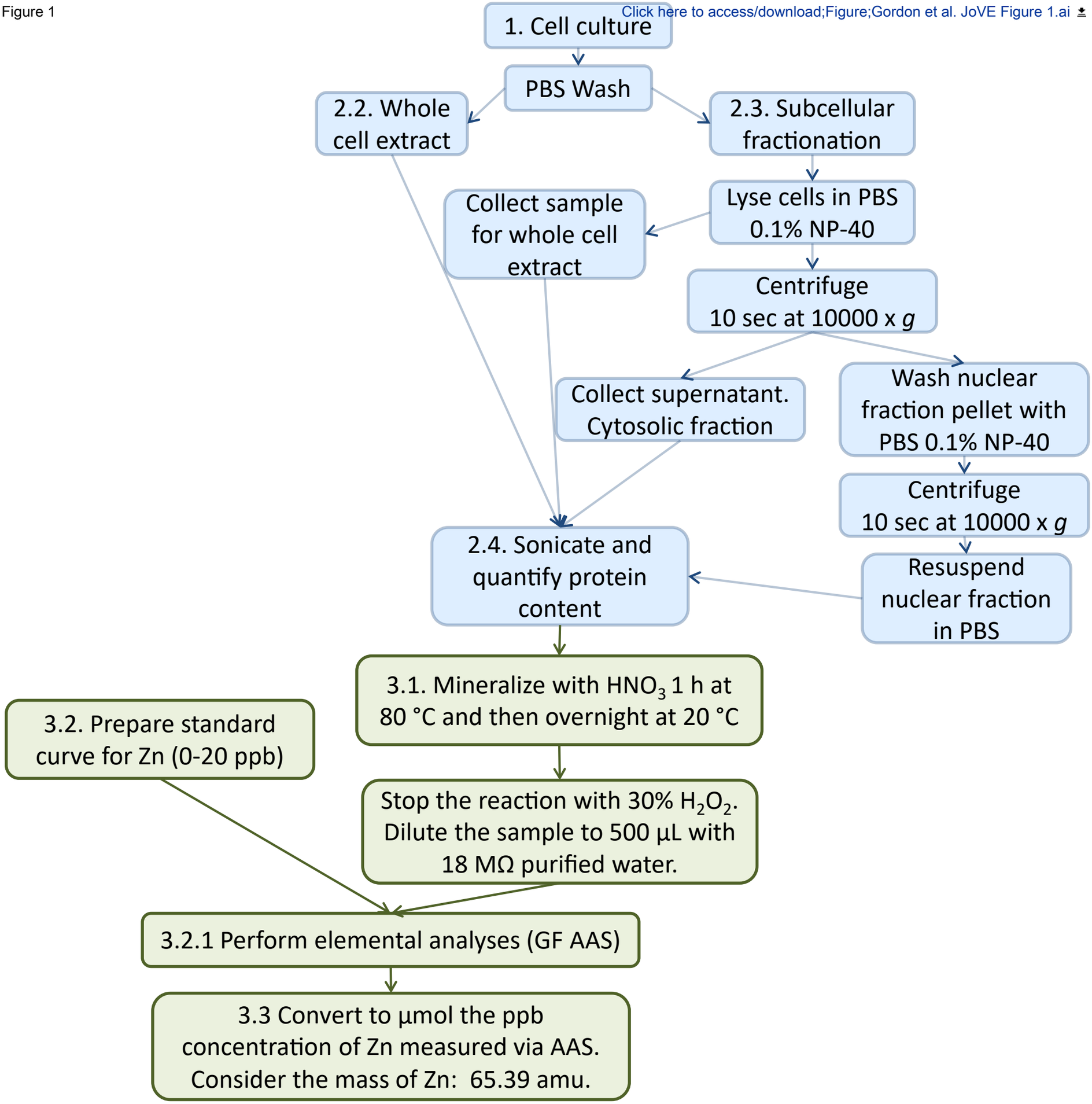


Figure 2

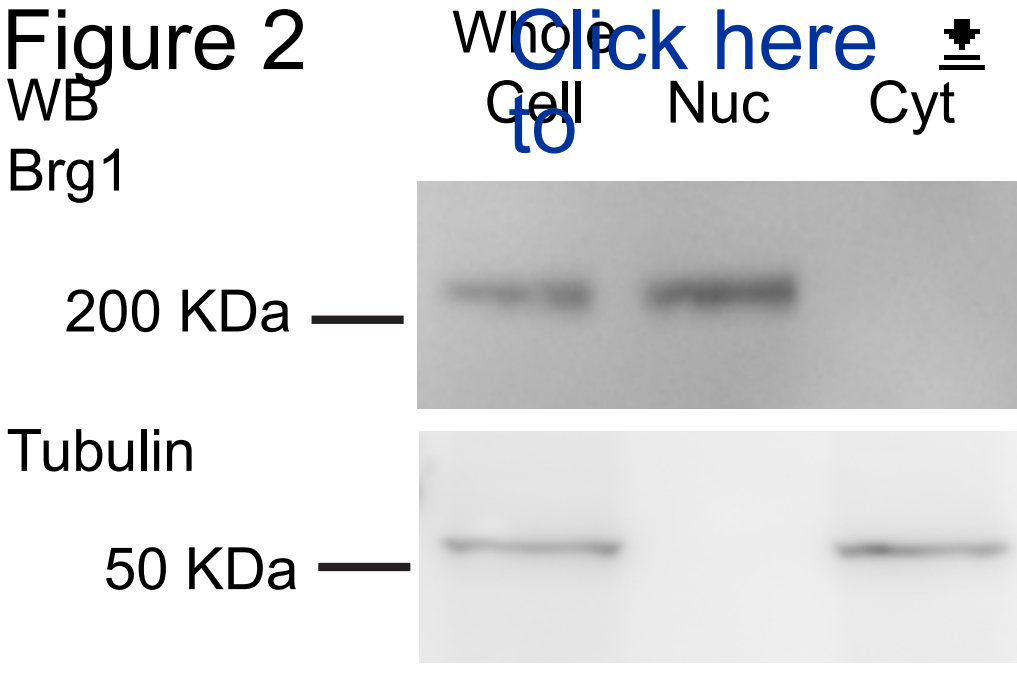
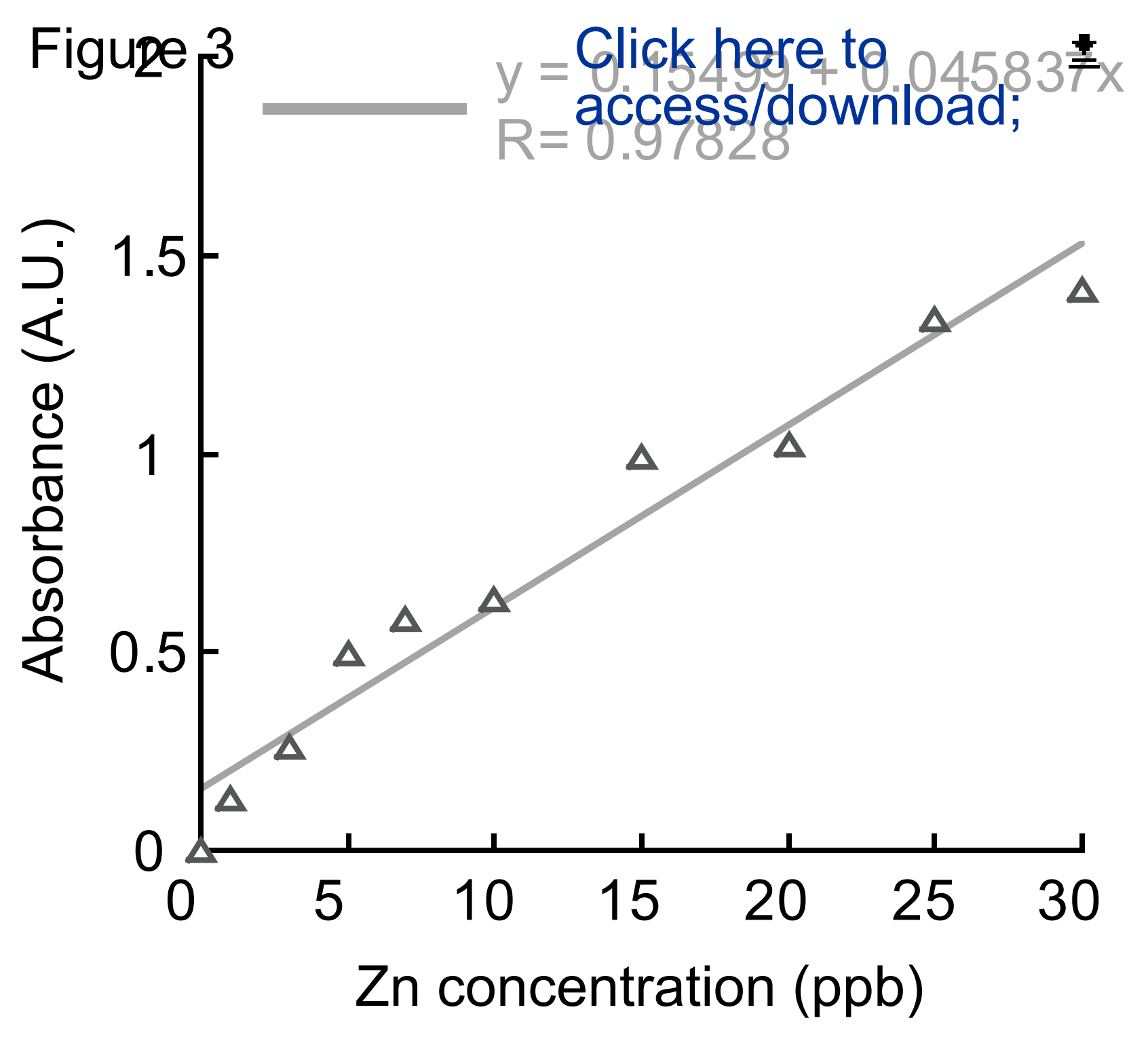


Figure 3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y = 0.15499 + 0.045837x
R= 0.97828
Absorbance (A.U.)
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Zn concentration (ppb)

Figure 4

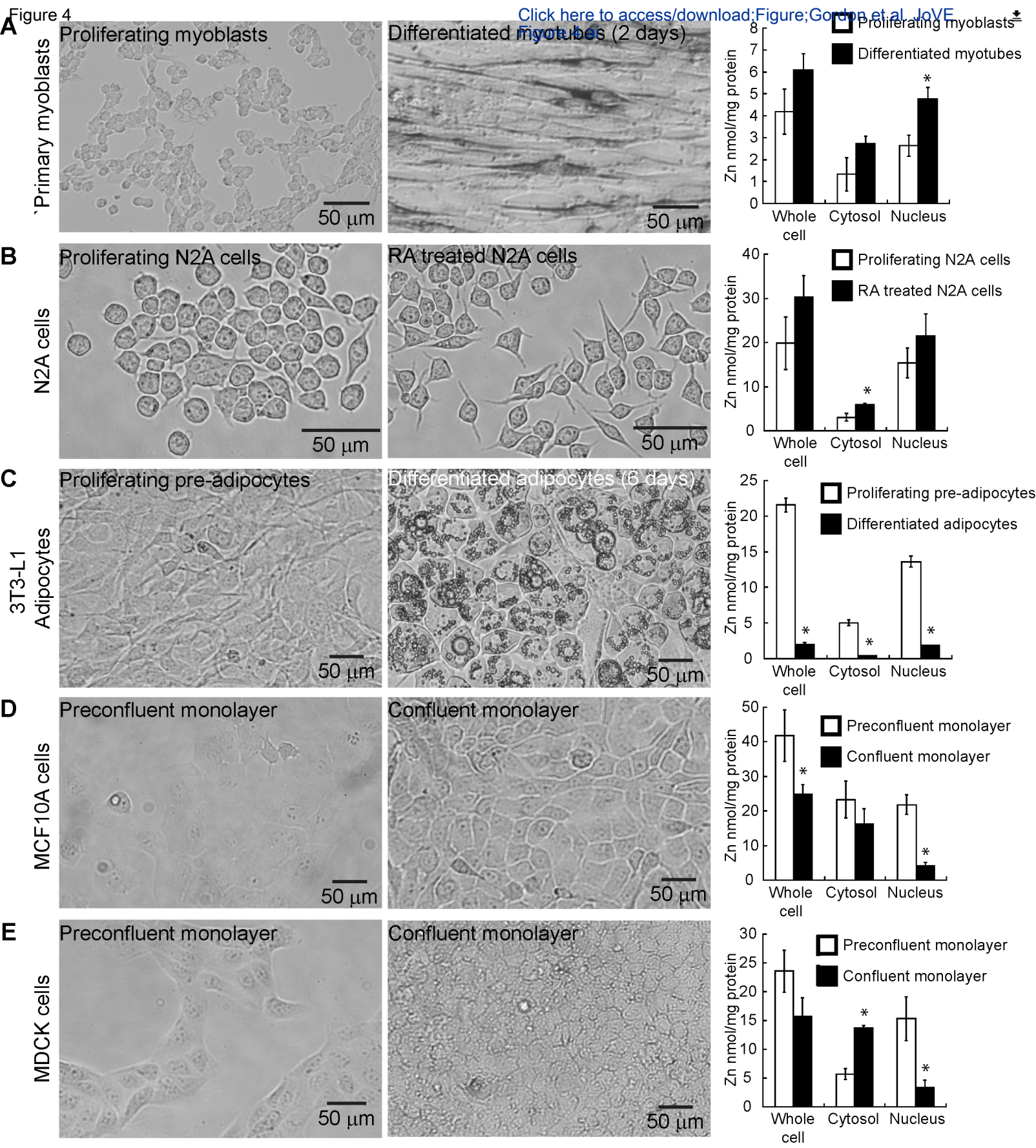

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3-isobutyl-1-methylxanthine	Sigma Aldrich	I5879	
Acetic Acid	Sigma Aldrich	1005706	
Anti Brg1-antibody (G7)	Santa Cruz biotechnologies	sc-17796	
Anti b-tubulin-antibody (BT7R)	Thermo Scientific	MA5-16308	
Bradford	Biorad	5000205	
Dexamethasone	Sigma Aldrich	D4902	
Dulbecco's Modified Eagle's Media (DMEM)	ThermoFischer-Gibco	11965092	
Dulbecco's Modified Eagle's Media/Nutrient Mix (DMEM/F12)	ThermoFischer-Gibco	11320033	
Dulbecco's Phosphate Buffered Saline (DPBS)	ThermoFischer-Gibco	14190144	
Epidemal Growth Factor (EGF)	Sigma Aldrich	E9644	
Fetal Bovine Serum (FBS)	ThermoFischer-Gibco	16000044	
Fibroblastic Growth Factor-Basic (FGF) (AA 10-155)	ThermoFischer-Gibco	PHG0024	
Horse serum	ThermoFischer-Gibc	16050122	
Hydrocortisone	Sigma Aldrich	H0888	
Hydrogen Peroxide (H2O2)	Sigma Aldrich	95321	
Insulin	Sigma Aldrich	91077C	
Insulin-Transferrin-Selenium-A	ThermoFischer	51300044	
Nitric Acid (HNO3)	Sigma Aldrich	438073	
Nonidet P-40 (NP-40)	Thermo Scientific	85125	
OptiMEM (Reduced Serum Media)	ThermoFischer-Gibco	31985070	
Penicillin-Streptomycin	ThermoFischer-Gibco	15140148	
PureCol (Collagen)	Advanced BioMatrix	5005	

Retionic Acid	Sigma Aldrich	PHR1187
Troglitazone	Sigma Aldrich	648469-M
Trypsin-EDTA (0.25%), phenol red	ThermoFischer-Gibco	25200056
Zinc (Zn) Pure Single-Element Standard, 1,000 µg/mL, 2% HNO3	Perkin Elmer	N9300168

Established Cell Lines

3T3-L1	American Type Culture Collection	CL-173
MCDK	American Type Culture Collection	CCL-34
MCF10A	American Type Culture Collection	CRL-10317
N2A	American Type Culture Collection	CCL-131

Equipment

Atomic Absortion spectrophotometer	PerkinElmer	Aanalyst 800
Bioruptor	Diagnode	UCD-200

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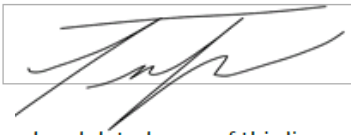
Signature:	 Date: *12/8/18*

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January 28, 2019

Editorial Board

Journal of Visualized Experiments

Dear Editorial Board,

I want to thank you for the opportunity to revise our manuscript entitled *"Atomic absorbance spectroscopy as a tool to measure intracellular Zn pools in mammalian cells"* (JoVE59519) for consideration at the Journal of Visualized Experiments (JoVE), by Shellaina J.V. Gordon, Yao Xiao, Amanda L. Paskavitz, Napoleón Navarro-Tito, Juan G. Navea, and myself.

We have addressed all the editorial comments and concerns of the reviewers. A detailed response to all the comments accompanies this letter.

As I commented in my previous letter, trace elements are critical for mammalian tissue development but are potentially toxic at high levels, meaning their homeostasis must be tightly regulated. Therefore, the development of sensitive, accessible, and accurate techniques is fundamental for understanding imbalances and dysregulation of metal transport and also homeostasis. In this manuscript, we describe the detailed application of atomic absorbance spectroscopy for measuring zinc in whole cell and subcellular fractions of different primary and established mammalian cell lines. Our groups have worked on optimizing these techniques over the past couple of years in order to develop a reliable method for metal quantification in biological samples. It is noteworthy that in this protocol, we focus on zinc measurements, but our procedures can be easily adapted to measure other transition and heavy metals.

We hope you find our revised manuscript suitable for publication at JoVE. Thank you for your consideration.

Sincerely yours,

Teresita Padilla-Benavides, PhD.

Response to editorial and reviewer comments:

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Response: Spelling and grammar issues were corrected by all the authors in the revised version of our manuscript.

2. Please revise lines 212-214, 216-218 to avoid previously published text.
Response: Thank you for identifying these mistakes. Both sentences were changed in the revised version.

3. Keywords: Please provide at least 6 keywords or phrases.
Response: the original version of the manuscript provided 5 keywords (lines 29-30). We have added one more.

4. Please abbreviate liters to L to avoid confusion.
Response: This issue has been corrected.

5. Protocol: Please refrain from using bullets, dashes, or indentations.
Response: This issue has been corrected. However, dashes in the authors' last names must be maintained.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: OptiMEM, Bioruptor, Diagenode, Nonidet, etc.
Response: This issue has been corrected.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: The protocol text has been corrected for this issue.

8. Please revise the protocol (lines 174-182, etc.) to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
Response: The protocol text has been corrected for this issue.

9. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.
Response: The protocol text has been corrected for this issue.

10. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
Response: The protocol text has been corrected for this issue.

11. 1.1.1.4: Please specify the concentration of trypsin.
Response: The concentration has been added.

12. 2.9: Please provide more details regarding how to measure the samples using atomic absorbance spectroscopy, specifying the parameters used such as pyrolysis and atomization temperature.
We thank the editorial office for this suggestion. We have added details in the protocol in the text and a new Figure 3

13. 2.10: Please describe how to prepare the standard samples and how to measure them.
Response: We thank the editorial office for this suggestion. We have added details in the protocol in the text accompanying which included the measurements of standards and samples.

14. Please note that lines 455-468 describe important considerations that are not reflected in the protocol, please elaborate how to perform these critical steps in the protocol if appropriate.
Response: Thank you, the text has been added on how to perform the calculations.

15. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: This issue has been corrected.

16. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.
Response: This issue has been corrected.

17. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: Essential steps have been highlighted in green.

18. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
Response: Essential steps have been highlighted in green.

19. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Response: Essential steps have been highlighted in green.

20. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.
Response: The table of materials has been formatted accordingly.

21. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.
Response: References have been properly formatted.

22. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Response: References have been properly formatted.

Reviewers' comments:

Reviewer #1:
The authors have contributed a nice article in regards to using GF-AAS to quantify zinc levels in various mammalian cell types. A few points should be considered prior to moving forward with this submission.
Response: We thank the reviewer for the positive comments on our manuscript.

1. A previous JoVE article showed that cation levels could be quantified using AAS (Duerr et al. 72, e50201, doi: 10.3791/50201 2013). Adding this manuscript and a description of it in the background section would be a nice addition for this manuscript.
Response: We thank the reviewer for the suggestion. The reference has been added to the introduction and discussion sections.

2. To better understand the method, a flow chart of the procedure would be highly beneficial.
Response: We thank the reviewer for this suggestion. A flow chart has been added as Figure 1.

3. A number of times in the protocol, an "appropriate" amount is suggested. At these points, it would be beneficial to provide more quantitative details. Otherwise, users will have to look to other papers to find the correct methodology, which seems odd for a methods paper.
Response: We thank the reviewer for pointing this out. We have removed the word appropriate from several points. A few were maintained as they are the prelude to examples in following sections.

4. In the cell fractionation step, how do the authors account from variations between nuclei preparations that result in differing metal concentrations?

Response: We thank the reviewer for this inquiry. We have added explanantory text in the protocol and result section, also a new representative Western blot of the subcellular fractions typically obtained from this protocol (Figure 2) to clarify this point was included.

5. In the representative results section, the authors talk about differences in metal levels within the sole figure. Were these differences significantly different? No indication of significance testing is apparent in the text or figure legend.
Response: We thank the reviewer for pointing out this important omission, and apologize for the mistake. Statistical significance has been added to the figure.

Reviewer #2:
Manuscript Summary:
The manuscript by Gordon et al describes the measurement of zinc concentrations in cell lysates and subcellular fractions of different cell lines, using graphite furnace atomic absorption spectroscopy (GF-AAS). Protocols are well described, the manuscript is well written and it displays how useful this technique can be to determine accurately zinc concentrations. Of course, there are more modern and fancy technologies for this, such as ICP-MS, but not as accessible to the scientific community. This manuscript will enable researchers at small universities or research institutions to take advantage of their GF-AAS equipment to make this kind of metal concentration determinations. Almost any chemistry department has a GF-AAS equipment.
Response: We thank the reviewer for the positive comments on our manuscript.

Major Concerns:
None.

Minor Concerns:
I find the first sentence of the abstract a bit confusing: How do essential transition metal ions compete with non-physiological ones?
Response: We thank the reviewer for noticing this redaction mistake. It has been corrected.

Statistical analysis for the data shown in Figure 1 must be included.
Response: We thank the reviewer for pointing out this important omission. Statistical significance has been added to the figure.

How do the findings of authors regarding changes of zinc levels compare to previously reported data? A discussion on this would enrich the manuscript, especially if previously reported data come from other techniques.
Response: We thank the reviewer for this suggestion. Text has been added in the discussion to address this point.

A comment regarding how AAS compares to ICP-MS in terms of amount of sample needed and detection limit would also be useful.
Response: We thank the reviewer for this suggestion. Text has been added to the discussion to address this issue.