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Detecting and minimizing zinc contamination in physiological solutions

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Abstract

Background: To explore the role of zinc (Zn) in cellular physiology it is important to be able to control and quantify the level of Zn contamination in experimental solutions. A technique that relies on a Zn-sensitive fluorimetric probe is introduced for measuring Zn concentrations as low as 100 pM. The method depends on the combination of the Zn-probe FluoZin-3 together with a slow Zn-chelator, Ca-EDTA, that reduces the background Zn levels and allows repeated measurements in the same solution.

Results: The method was used to determine which common labware items could leach Zn into solution. Contamination was predictably found to arise from stainless steel and glass. Perhaps less expectedly it was also introduced by methacrylate cuvettes, plastic tissue culture dishes and other plastic labware. The release of nickel from stainless steel electrodes was also imaged using the fluorescent probe Newport Green.

Conclusion: Zn contamination may arise from rather unexpected sources; it is important that all aspects and components used in the course of an experiment be analyzed for the possibility of introducing contaminants.

Background

There is increasing evidence for the involvement of transition metal ions at all levels of function within cells; as catalysts, structural elements and possibly as second messengers [1]. In particular Zn (this abbreviation will be used to denote Zn^{2+}) a non-redox active metal has come to the fore recently as a candidate cellular messenger.

Investigations of the role of Zn in cellular processes has been facilitated by the development of sensitive fluorimetric probes that have allowed the measurement of Zn both extracellularly and intracellularly [2-4]. In exploring the role of metal ions it is important to ensure that solutions have little metal contamination, as well as being able to control the free metal concentration level with a chelator [5-7].

If the role of a metal ion is to be explored in a cellular process it is often necessary to use solutions that have very low transition metal concentrations while maintaining calcium and magnesium at normal levels (~2 mM). There are essentially two strategies for implementing such a regime. Firstly, physiological solutions can be cleaned by treatment with metal chelating resins and then taking precautions to prevent contamination of the solution [5,8,9]. Secondly, a metal chelator which does not suffer interference from Ca or Mg, can be added to the solution to hold the free transition metal concentrations at very low levels

[10]. For example, EDTA saturated with calcium can be used because EDTA has a far higher affinity for transition metals than it does for Ca and Mg, and will chelate transition metals with the displacement of Ca or Mg [11]. With this strategy it is possible to hold the free metal ion concentration at very low levels, however, the chelator could strip metals from exposed metal binding sites. Moreover, the chelator or the chelator-metal complex may act as agonists or antagonists of receptors [12].

It is certainly possible to reduce the metal contamination in water to levels below ~100 nM with a standard laboratory deionization systems, however as I will show below, metals may be reintroduced into the solution through contact with items that might not seem likely to contaminate the solution.

Metal electrodes since their introduction into experimental neurobiology by Galvani in the eighteenth century have provided an important tool for triggering and recording neuronal activity. There is increasing use of metal electrodes in prostheses for stimulating nerves such as cochlear implants. However, metal ions in solution are often toxic and one has to guard against electrodes serving as a source for ions through leaching or electrolysis.

In this paper I introduce a method for detecting Zn at concentrations as low as 50 pM and use it to determine if common labware items release Zn into solution.

Results

A sensitive method for detecting Zn

FluoZin-3 is a recently developed fluorimetric probe with a high affinity for Zn (K_d 15 nM), yet because it has three carboxyl groups rather than the four of its parent compound fluo-4, has little affinity for Ca or Mg [13]. FluoZin-3 is particularly sensitive to changes in Zn concentration because of the high ratio between the fluorescence of the probe saturated with Zn and that in its absences (140 ± 9, mean ± SEM, n = 3). The free-acid form of FluoZin-3 unlike Zinquin [14] is not membrane permeant so that if it is delivered intracellularly or extracellularly it does not cross compartment boundaries and can be used to detect the release of Zn into the extracellular space [15].

The dynamic range of FluoZin-3 can be improved by the inclusion of a low concentration of Ca-EDTA and excess calcium and magnesium. Because Ca-EDTA has a higher affinity for Zn than FluoZin-3, trace levels of Zn in the solution can be removed, lowering the background fluorescence. When Zn is introduced into the system it binds first to FluoZin-3 and then because of the slow off-rate for calcium and magnesium, EDTA will chelate Zn slowly. This is shown in Fig. 1 where FluoZin-3 has been titrated with Zn in the presence and absence of Ca-EDTA. The ploy of using a Zn fluophore with a kinetic advantage, paired with a strong chelator with a thermodynamic advantage, effectively transforms an irreversible indicator into a reversible one, so that the same solution can be used to determine the concentration of multiple additions of Zn to the same solution. This characteristic also allows the system to be calibrated internally through the addition of a known concentration of Zn to the solution. In a cuvette with 500 nM FluoZin-3 and 25 μM Ca-EDTA it was possible to detect Zn concentration increases as low as 100 pM (Fig. 2).

FluoZin-3 is as sensitive to cadmium as it is to Zn and therefore the assay cannot distinguish between these two metals. If the occurrence of Cd is suspected, its presence can only be confirmed with a physical technique like inductively coupled mass spectrometry (ICPMS) [16]. FluoZin-3 has a very low sensitivity to iron, while copper quenches its fluorescence.

Sources of Zn contamination

The starting levels of Zn contamination in physiological solutions relies on the purity of the deionized water and chemicals. Preventing contamination depends on avoiding contact with items that might introduce metals into the solution. I found that the initial levels of contamination can be minimized by using water dispensed from a high-grade water purification system (Barnstead, Nanopure, in my case), the highest purity reagents and storing the solutions in Teflon bottles. Furthermore, solutions should be brought up to volume in polymethylpentene cylinders (Nalgene) and transferred with metal-free pipettor tips (Fisher Scientific). Chemicals should be transferred for weighing with a Teflon coated spatula (Fisher Scientific). Even the most transient contact with glass or stainless steel can result in contamination. Using these precautions the contaminating Zn levels could be kept below ~100 nM. If lower Zn levels are required the solution should be treated with a metal chelating resin [5].

The combination of FluoZin-3 and Ca-EDTA was used to determine whether some commonly used labware items could introduce Zn into solution. In developing this assay it was found that methacrylate cuvettes (Fisher Scientific) leached Zn into solution. If FluoZin-3 was added to a Hepes buffered saline in a cuvette stirred with a Teflon stir bar (Bel-Art, 9.5 × 8 mm), in the absence of Ca-EDTA, the fluorescence increased almost instantaneously and then grew slowly ($t_{1/2}$ = 47 ± 9 s, mean ± SD, n = 6) to a steady level. The rapid increase corresponds to the probe binding to contaminating Zn in the solution or perhaps introduced with the probe, while the slower increase corresponds to Zn being leached from the cuvette. Zn appears to be introduced into the solution through the abrading

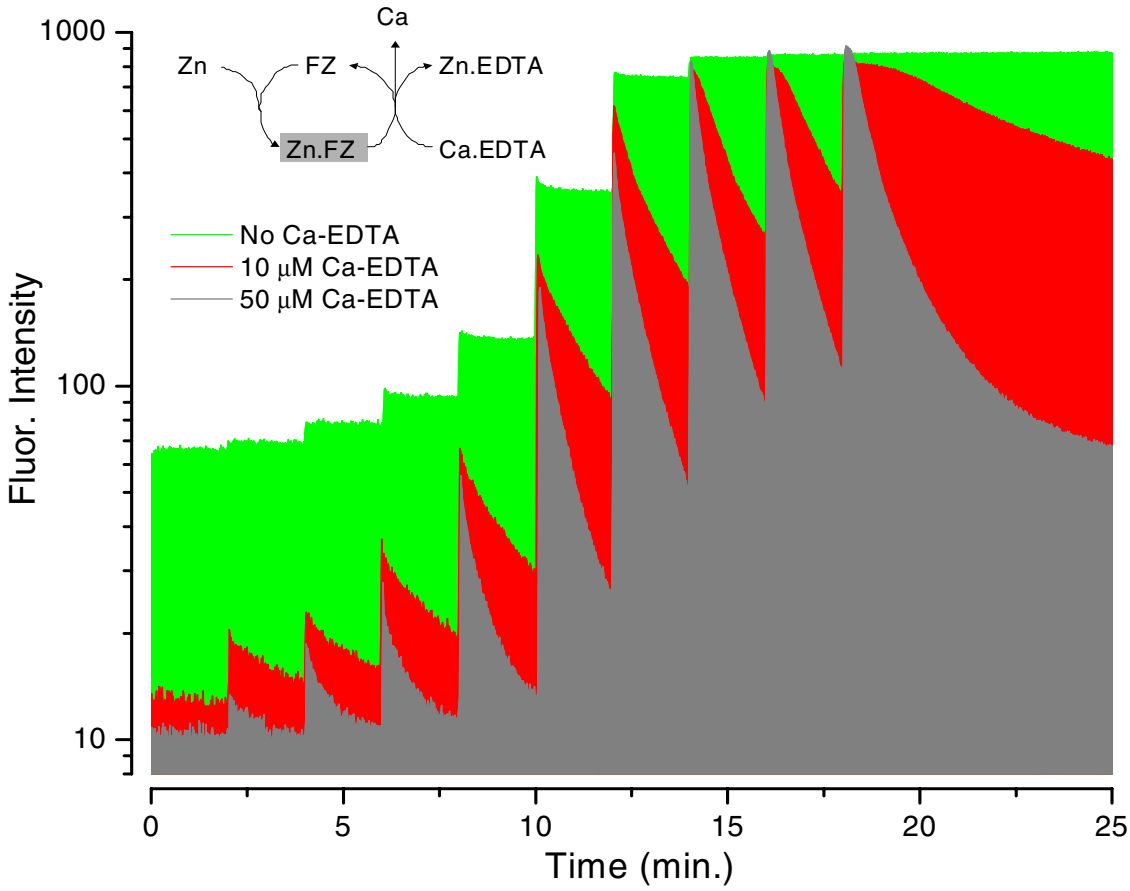

Figure 1
Titration of FluoZin-3 with Zn in the presence and absence of Ca-EDTA. The $ZnSO_4$ additions beginning at 2 minutes and at 2 minute intervals were 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.2 and 10 μM. 500 nM FluoZin-3 in Hepes saline at 26°C.

action of the stir bar on the cuvette. If FluoZin-3 was added to the Hepes buffered saline without using the stir bar but stirred by slowly aspirating the solution five times into a 1 ml pipette tip or by bubbling the solution with nitrogen, the slow increase in the fluorescence was not observed. The slow increase started once stirring commenced (Fig. 3). That it was the grinding of the stirrer rather than the stir bar itself was shown by the fact that rubbing the inside of the cuvette with a metal-free pipette tip led to an increase in fluorescence. Soaking the cuvettes in 2 mM EDTA for at least 24 hrs eliminated the slow release of Zn.

To determine the levels of contamination in water and fluorescent probe, 500 nM of FluoZin-3 was added to a 1 mM Hepes solution that was mixed by slowly aspirating the solution 5 times into a 1 ml pipette tip. Inversion of the cuvette with the open end covered with parafilm led to increases in Zn levels probably through the more vigorous stirring action. The Zn concentration was estimated from the fluorescence using the addition of 1 nM $ZnSO_4$ to calibrate the signal and the linear relationship between fluorescence and Zn in the concentration range of 0.1 to 10 nM (data not shown). Under these conditions the level of contamination corresponded to 7.0 ± 0.4 nM (mean ± SEM, n = 3). Soaking the cuvette in 2 mM EDTA for up to 4 days did not change the level of contamination suggesting that it arises from the water, Hepes and/or the probe. Little metal contamination was found in the probe (personal communication Dr. Kyle Gee, Molecular Probes) and so most of the Zn probably arises from the water and

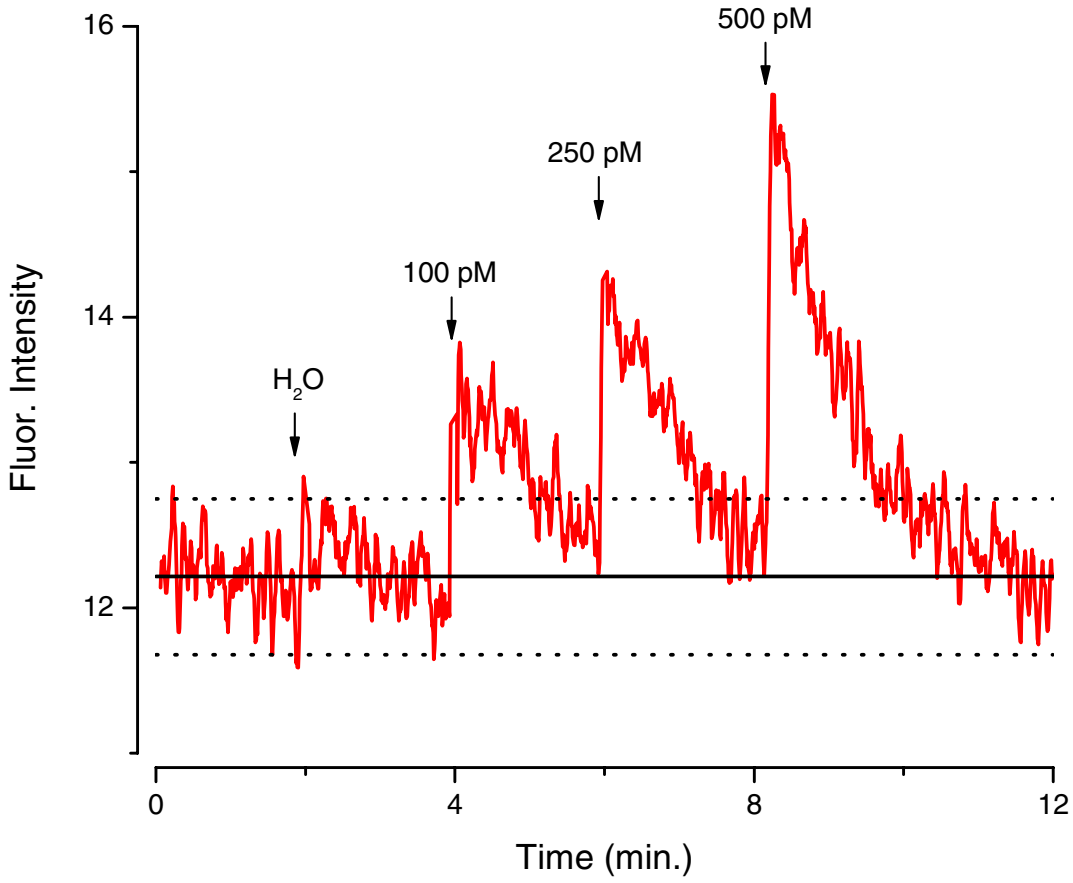

Figure 2
Detecting elevations as low as 100 pM Zn with FluoZin-3. The solution contained 500 nM FluoZin-3 and 10 μM Ca-EDTA in Hepes buffered saline. 'H_2O' indicates the addition of 2 μl water. The trace was subjected to a 7 point Savitzky-Golay smoothing procedure. The solid line is the mean of the data acquired between 0 and 2 min, and the dotted lines the SDs.

Hepes. If the same experiment was performed with the Hepes saline the levels of Zn contamination was found to be 10.8 ± 1.1 nM (mean ± SEM, n = 5).

As a starting point for analyzing how solutions might be contaminated with Zn through contact with various labware items I used the example of an experiment attempting to image Zn release from hippocampal slices, testing all components that came into contact with the physiological saline during the experiment [15,17]. For example, in the course of such experiments I initially used a polyethylene transfer pipette (Fisher Scientific) to move slices from the holding chamber to the experimental apparatus. However, the pipette introduced significant contamination. Indeed, aspirating metal-free buffered solution for only a few seconds was sufficient to introduce detectable levels of Zn into solution.

Passage of a solution through a Hamilton syringe with a metal tip led to the introduction of Zn into the solution even for very short sojourns. Immersing the tip of the syringe into the detection solution for only one to two seconds also introduced contaminating Zn.

The results of testing a number of laboratory items that are likely to come in contact with solutions, for their propensity to leach Zn are presented in Table 1.

Some items, even if they were washed extensively with water, introduced Zn contamination. Only if they were soaked in 2 mM EDTA (1–2 days) could they be cleared of

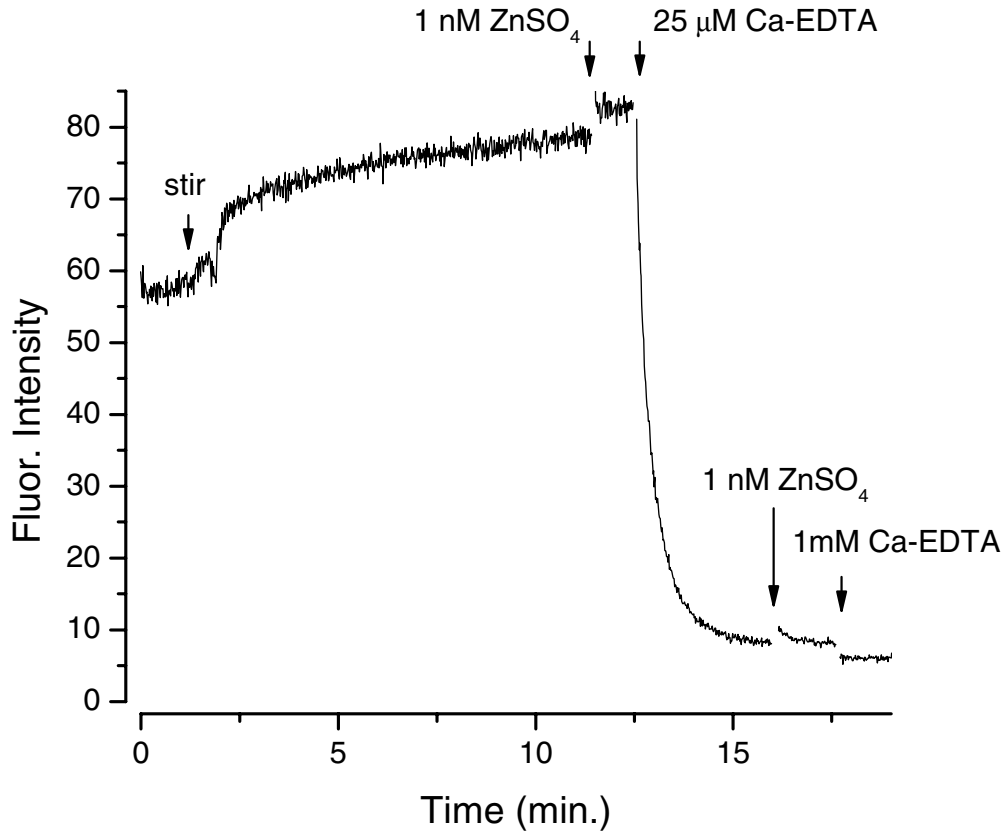

Figure 3
Stirring induces the slow release of zinc from methacrylate cuvettes. The Hepes saline solution contained 500 nM FluoZin-3 in Hepes saline.

Table 1: Zn-leaching by common labware items. Items were tested by immersion in 2 ml of Hepes saline containing 500 nM FluoZin-3 and 25 μM Ca-EDTA in Hepes saline for 5 s and were scored as 'Zn-leaching' if the increase in fluorescence was greater than that induced by the introduction of 0.5 nM $ZnSO_4$.

Zn-leaching	Non-Zn leaching
Glass cover slips	Cell culture dishes (Becton Dickinson, 35 mm)[2]
Hamilton syringes	Cylinders (polymethylpentene, Nalgene)
Latex gloves (Fisher examination gloves powder free)	Metal-free pipette tips (Fisher Scientific)
	Methacrylate cuvettes (Fisher Scientific)[1]
Quartz Cuvettes	MicroFil syringe needle (WPI)
Sintered glass bubblers (Fisher Scientific)	Saran Wrap™ (S.C. Johnson & Son, Inc.).
Tuberculin syringe (Becton Dickinson)	Plastic bubblers (Discard-a-stone, Lee's aquarium and pet products)[1]
	Plastic cover slips (Ted Pella)[2]
	Plexiglass[1]
	Parafilm
	Sylgard (Dow Corning)
	Teflon bottles
	Transfer pipettes (Fisher Scientific)[2]
	Tygon tubing[1]

[1] Soaking in 2 mM EDTA overnight eliminates contamination. [2] Soaking overnight in 10 % nitric acid eliminates contamination.

contamination. This was the case for Tygon tubing and methacrylate cuvettes. Soaking plastic tissue culture dishes (Becton Dickinson, 35 mm) in 10% nitric acid for two days eliminated the metal contamination whereas EDTA was ineffective. While 10% nitric acid was ineffective in treating methacrylate cuvettes. Others items such as Hamilton syringes, glass and tuberculin syringes served to contaminate solutions even after extensive washing in EDTA or 10% nitric acid.

It is worth bearing in mind that most physiological solutions need to be aerated with a gas dispersion tube, which if it is fabricated from glass, will introduce Zn contamination. Disposable plastic fish tank bubblers (Discard-a-stone) presoaked in EDTA can be used to avoid contamination during bubbling.

Zn release from glass

Most glass contains contaminating metals that could leach into solution [18]. To determine the rate and extent of leaching, a glass coverslip was suspended in a stirred cuvette containing 500 nM FluoZin-3 and 25 μM Ca-EDTA. On insertion the fluorescence increased quickly but not immediately suggesting that the Zn is removed from below the surface of the glass rather than from loosely adsorbed metal (Fig. 4). The fluorescence then declined exponentially as the Zn was chelated by Ca-EDTA, declining to a steady-level somewhat above the initial level, suggesting that there is a continuous low-level leaching of Zn into the solution. When the slide was removed the fluorescence declined to baseline levels confirming the last supposition. Soaking glass slides in 2 mM EDTA or 10% nitric acid for 2 days reduced but still did not eliminate the leaching of metal.

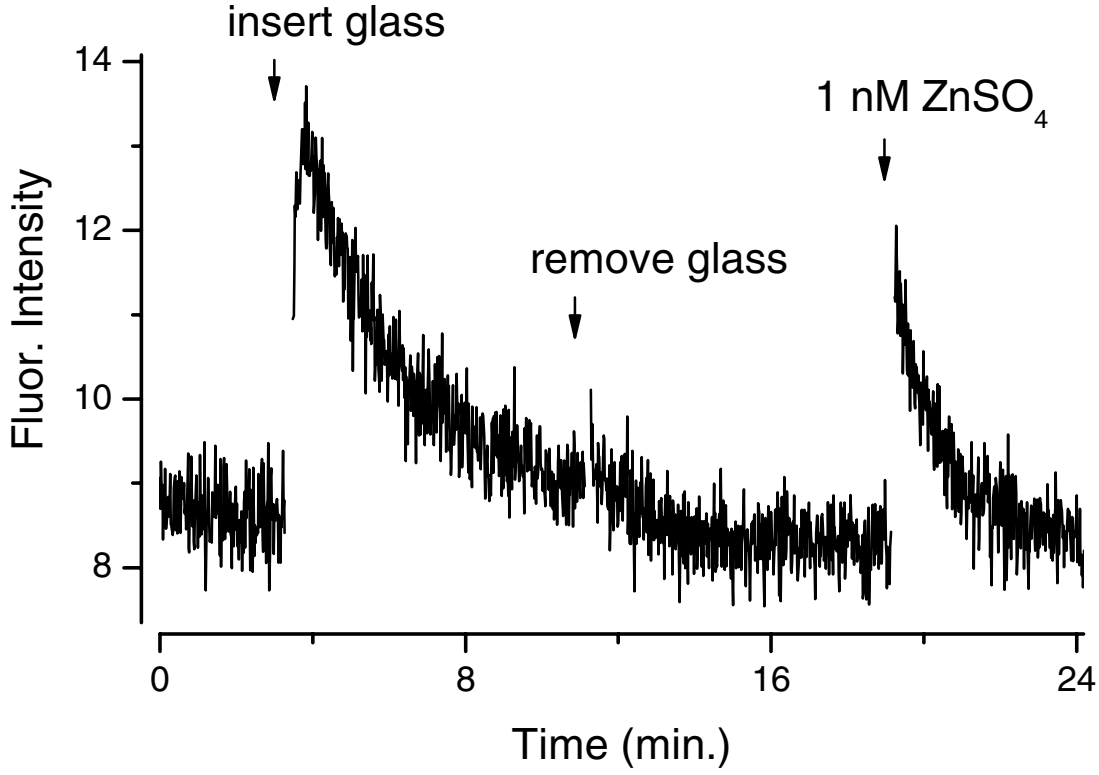

Figure 4
Zinc release from a glass slide. The solution contained 500 nM FluoZin-3 and 25 μM Ca-EDTA. The glass slide (Thomas Scientific, Micro cover glass, 11 × 22 mm) was suspended in the cuvette with about 5 mm immersed in the solution.

A quartz cuvette (Hellma) exhibited an even more dramatic slow increase in fluorescence than the methacrylate cuvettes. Soaking the cuvette for 2–4 days in 2 mM EDTA or 10% nitric acid reduced the slow increase, however, if the treatment was not repeated, the slow increase returned within 2–3 days. This suggests that EDTA or acid removes Zn from the superficial layers that are then repopulated by diffusion of the metal from within the quartz.

In imaging experiments brain slices are often placed in a chamber with a glass bottom, while in some cases water immersion objectives are used, both of which can leach Zn into the solution. This can be prevented by wrapping both the slide and objective with a single layer of Saran Wrap™. Alternatively a plastic cover slip (Ted Pella) can be used or a glass cover slip can be coated with a thin layer of Sylgard™.

Metal release from electrodes

During the passage of current through a metal electrode, ions could be released through electrolysis and in so doing bind to the fluorimetic probe. Stainless steel contains nickel that induces fluorescence in the probe Newport Green (NPG) [19] and to a lesser extent FluoZin-3. To determine if the passage of ions through a stainless steel electrodes might activate fluorescent probes, the metal electrode was placed in a bath containing Hepes buffered saline with NPG and viewed with a water immersion objective. The solution was left unstirred because stirring rapidly dissipates the fluorescence. The fluorescence in the region of the electrodes was monitored while passing current through the electrode. With a stainless steel electrode and 2 μM NPG in the bath, passage of current was marked by an expanding sphere of fluorescence developing around the anode (Fig. 5). Changing the polarity of the electrodes switched the site where fluorescence developed. That the fluorescence was induced by metal release could be shown by the fact that the addition of 10 mM EDTA eliminated the current-dependent fluorescence (data not shown). The fluorescence peaked about 10 s after initiating current flow, the delay may in part reflect the slow rate of Ni binding by NPG, however, it also includes some contribution from diffusion. The fluorescence probably arises from the release of Ni that is 15% of the electrode by weight and is the only component metal capable of inducing fluorescence. NPG is more sensitive to Ni than Zn and iron only weakly affects the fluorescence of NPG. Metal release could also be detected with FluoZin-3 (2 μM) however a smaller signal developed, consistent with the lower sensitivity of the probe to nickel. No fluorescence developed if current was passed through a bipolar tungsten electrode.

Discussion

I have introduced a technique that can detect Zn at very low levels that can be calibrated internally and allows for the reuse of probes that are rather expensive. The method is comparable in its sensitivity to the state-of-the-art physical methods such as ICPMS that require costly dedicated equipment [16].

The sources of metal contamination can be rather unexpected and it is important to analyze all phases of the experiment to determine if contaminating metals might be introduced [20]. My test of labware was of course not comprehensive but the method provides a simple cost effective one for testing any solution or labware.

The method also provides a way of visualizing the release of metals from electrodes such as nickel that can exert a pharmacological action upon the tissue. In addition the technique may prove of value in determining the leakage of metals through breaks in insulation and the leaching of metals from implantable biomedical devices such as stents [21].

The passage of current through a stainless steel electrode can induce the release of metal ions through electrolysis. Therefore, in imaging experiments in biological tissues, where for example Zn release is to be imaged with a fluorimetric probe, artifacts may arise. This can best be guarded against by using tungsten electrodes.

It is perhaps worth mentioning that glass microelectrodes may leach metals into solution and into the adjacent tissue [22-24]. Patch recordings are typically made with an intracellular solution that contains a chelator that can prevent metal diffusing into the cell but may strip metals from intracellular proteins.

Diffusion of metal ions can occur within glass as it is an amorphous solid [18]. Although EDTA or nitric acid can remove metals from the surface layers, diffusion from the depths of glass may replenish the surface metal. This may hold true for other materials, and it is therefore important to test if after EDTA treatment or nitric acid treatment Zn contamination might recur through this mechanism. The method introduced here may be of value in measuring the diffusion of metals within glass.

Conclusions

A simple method is presented for measuring the zinc concentrations above ~50 pM. I have shown that metal contamination may arise from a number of different sources and that rather than acting on faith, it is best to test labware items for their tendency to leach metals into solution. Stainless steel electrodes release nickel, which may lead to artifactual signals in imaging experiments.

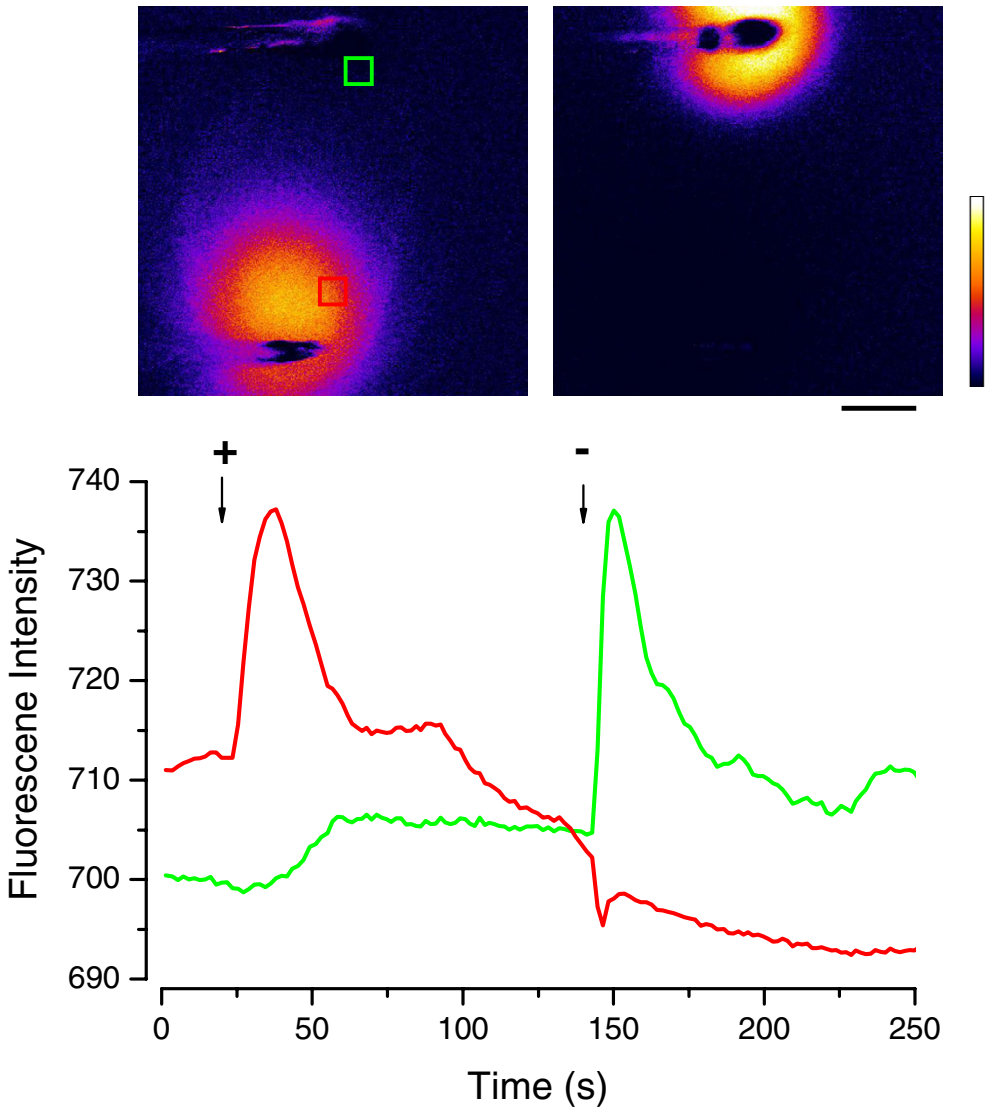

Figure 5
Zinc release from a bipolar stainless steel electrode. '+' indicates the beginning of current passage with the bottom electrode as the anode, while '-' denotes that the polarity was reversed. A 100 Hz square wave with pulse duration of 30 μs was applied for 2.5 s at a current amplitude of 500 μA. The experiment was performed in Hepes saline with 2μM NPG and 50 μM Ca-EDTA. Pseudocolor images are the differences between the fluorescence at the peak of the response and that immediately prior to delivering the stimulus; Left '+' stimulus, right '-' stimulus. Color bar 0–50. Scale bar 100 μm.

Methods

Excitation-emission spectra and fluorescent time-courses were determined on a Hitachi F-4500 spectrofluorimeter using a methacrylate cuvette whose temperature was controlled by a circulating water bath (26°C). Spectra and time-courses were measured in a Hepes buffered saline containing (in mM): 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgSO_4$ and 10 Hepes (pH 7.4).

Electrodes were imaged with a 10 × water immersion objective on an Olympus BX50WI. Illumination was provided by a Till Photonics monochromator at 480 nm, passed through a dichroic (Q495lp, Chroma) and then though a filter (HQ530/60 Chroma) onto the faceplate of a Princeton Instruments cooled CCD camera. Data was acquired by the MetaFluor program (Universal Imaging Corp) and the images were analyzed using the ImageJ (NIH) program.

Current passage through 5 MΩ metal electrodes (stainless steel or tungsten, A-M Systems) was controlled by pClamp software (Axon Instruments) gating a constant current device (AMPI).

Reagents

FluoZin-3 and NPG (Molecular Probes), Hepes (Calbiochem), $MgSO_4$, NaCl, KCl (Sigma), Nitric acid (Fisher Scientific, Optima Grade), EDTA & $CaCl_2$ (Fluka). All Zn solutions were prepared from a standard solution of $ZnSO_4$ purchased as a 0.051 M standardized solution (Aldrich)

Authors' contributions

ARK was responsible for all aspects of this paper including the preparation of solutions and performance of the experiments.

Acknowledgements

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