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TECHNICAL SPOTLIGHT

Thinking of Co^{2+}-staining explant tissue or cultured cells? How to make it reliable and specific

Mark R. P. Aurousseau,[1,2,*] Ingrid K. Osswald[1,3,*] and Derek Bowie[1]
[1]Department of Pharmacology & Therapeutics, McGill University, Montréal, QC, Canada
[2]Graduate Program in Pharmacology, McGill University, Montréal, QC, Canada
[3]Integrated Program in Neuroscience, McGill University, Montréal, QC, Canada

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Abstract

Ca^{2+} and/or Zn^{2+} entry into neurons and glial cells is often a key step driving the processes of neurodevelopment and disease. As a result, a major pre-occupation of many neuroscientists has been in tracking down when and where nervous tissues express ion channels with appreciable divalent ion permeability. The cobalt (Co^{2+})-staining technique is one of the few techniques that allow a snapshot of the entire neuronal circuit, and selectively labels cells expressing divalent-permeable ion channels with a brown–black precipitate. Despite this, its use has been remarkably limited in the past decade. Reluctance to employ this approach has largely been related to an earlier concern with obtaining a reliable and reproducible means of visualizing transported Co^{2+}. Here we show that recent advances have resolved these issues, opening this straightforward and valuable technique to a much larger neuroscience audience.

Introduction

Ion channels involved in the selective transport of Ca^{2+} and/or Zn^{2+} across plasma membranes play key roles in numerous physiological processes as well as being implicated in several neurological diseases (Clapham, 2007; Sensi *et al.*, 2011). Notable examples of ligand-gated ion channels that exhibit appreciable divalent permeability are the ionotropic glutamate receptors (iGluRs) (Shuttleworth & Weiss, 2011; Bowie, 2012), nicotinic acetylcholine receptors (Fucile, 2004), transient receptor potential (TRP) channels (Moran *et al.*, 2011) and purinergic receptors (Burnstock *et al.*, 2011). Although a number of techniques have been developed to study divalent ion transport, such as the use of Ca^{2+}-sensitive metallochromic or fluorescent dyes (Tsien, 1999; Palmer & Tsien, 2006; Albantakis & Lohmann, 2009), few are as useful in providing a snapshot of the entire neuronal circuit as the cobalt staining technique (Pruss *et al.*, 1991; Albuquerque *et al.*, 2001). A particular advantage of this approach is that it offers a relatively quick and simple method to ascertain which cell types express divalent-permeable ion channels without the need for expensive imaging equipment. Furthermore, Co^{2+} staining can be coupled to other staining techniques, such as immunohistochemistry (Albuquerque *et al.*, 2001), making it a valuable tool for studying many problems in neuroscience.

Despite this, the Co^{2+}-staining method has not been adopted widely. Although it was initially developed during early work on TRP channels in sensory neurons (Winter, 1987; Wood *et al.*, 1988), few studies since then have employed it to characterize this ever-diversifying ion-channel superfamily (Wu *et al.*, 2010). Indeed, a survey of the literature reveals that the Co^{2+}-staining method has yet to be applied to the study of other widely distributed divalent-permeable ion-channel families whose importance to central nervous system (CNS) function and disease has only emerged in recent years (Fig. 1). The diminished use of the technique can be traced to problems that hampered early investigators who did not always have robust protocols and, as a result, obtained unreliable staining patterns. However, all of these issues have been resolved in recent years, giving new impetus in using Co^{2+} staining; for example, in the study of Ca^{2+}-permeable AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid)-selective iGluRs (Chung *et al.*, 2005; Santiago *et al.*, 2006; Ma & Lowe, 2007; Osswald *et al.*, 2007; Borbely *et al.*, 2009; Lebrun-Julien *et al.*, 2009; Vilagi *et al.*, 2009). The universality of this approach, however, has yet to be appreciated by a wider neuroscience audience.

Here we provide an overview of the Co^{2+}-staining technique to permit the study of almost any divalent-permeable ion-channel in explant tissue or cultured cell lines. For this, we have provided a simple bench-top protocol that is used successfully in our lab and others on a routine basis. We have also included tips on how to avoid common pitfalls that give unreliable staining patterns as well as straightforward steps that will optimize the outcome. With this information at hand, it will be easy to add the Co^{2+}-staining to all of the other techniques needed to understand the many diverse roles of divalent-permeable ion-channel families in the CNS.

Correspondence: Dr D. Bowie, as above.
E-mail: derek.bowie@mcgill.ca

*These authors contributed equally to this study.

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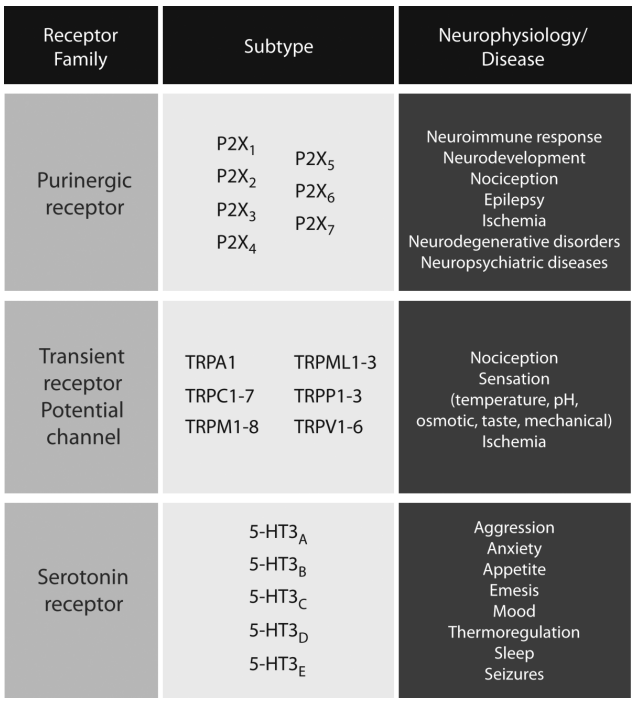

FIG. 1. Chart showing the divalent-permeable receptor families yet to be studied using the Co^{2+}-staining technique. Note, that in each case, the receptor family has a large number of subunits (middle), many of which have been implicated in CNS disorders (right).

Cobalt staining of explant tissue

Basics of cobalt staining

The basis for the Co^{2+}-staining technique stems from the classical use of Co^{2+} solutions as a histochemical tool. Co^{2+} staining was originally described for its capacity to reveal the fine morphological detail of neurons (Pitman *et al.*, 1972). The procedure was remarkably simple: after filling neurons with a Co^{2+} solution, exposure to ammonium sulphide resulted in the formation of a black precipitate specific to the neuron body. This staining could be visualized easily using a transmitted light or electron microscope and was more sensitive compared with fluorescence-based staining techniques of the time. The contrast of the resulting black stain could be further enhanced using a silver intensification step after precipitation (Davis, 1982; Croll, 1986). Importantly, it was later found that specific subpopulations of neurons could take up Co^{2+} ions in response to application of capsaicin (Winter, 1987; Wood *et al.*, 1988) as well as glutamate (Pruss *et al.*, 1991). Not only did this eliminate the need to actively fill neurons with Co^{2+} solutions, it also paved the way for the development of Co^{2+} staining as a functional assay to probe the permeation properties of ion channels expressed at the neuronal surface. Functional Co^{2+} staining takes advantage of the property that several divalent ion species permeate through Ca^{2+}-permeable ion channels. Activation of channels (e.g. via ligand application) on the cellular surface in the presence of extracellular Co^{2+} leads to the intracellular accumulation of Co^{2+} ions. As before, reaction with ammonium sulphide leads to the formation of a dark brown precipitate.

What information can be obtained from the technique?

Several layers of information can be obtained from simply staining a tissue of interest. In the most basic sense, the resulting staining can be used to elucidate the morphology and distribution of stained cells within a given tissue. In this way, populations of neurons expressing divalent-permeable receptors at their surface can easily be screened. In the context of developmental profiling, studies of disease or paradigms of experience-dependent plasticity, such information can be invaluable (e.g. Osswald *et al.*, 2007; Lebrun-Julien *et al.*, 2009). Additionally, simple pharmacological tools can be utilized to identify or characterize receptors expressed at the surface of stained cells. This latter approach is particularly valuable when coupled with a heterologous expression system where Co^{2+} staining can be employed for mutant or drug screening purposes.

How is functional cobalt staining performed?

Functional Co^{2+} staining of explant tissue can be easily completed within just 2 days. Briefly, tissue is prepared and loaded with Co^{2+} ions. Then following Co^{2+} precipitation, specimens are fixed, sectioned if necessary and silver-enhanced. Figure 2 encapsulates a streamlined bench-top protocol and solutions recipe that is intended to complement methodologies provided elsewhere (Engelman *et al.*, 1999; Albuquerque *et al.*, 2001). We emphasize that particular attention should be paid to maintaining tissue health throughout the experiment as this can significantly influence the quality and reproducibility of the resultant staining.

Maintaining tissue quality and staining specificity

Solution composition, temperature and pH can all affect the sustainability of live tissue, as can the thickness of the preparation. For functional Co^{2+} staining, the quality and health of the specimen are absolutely vital for the specificity of staining (Fig. 3). As the assay necessitates a specimen to be exposed to a series of different solutions for a considerable period of time, maintaining tissue quality is even more challenging. To maintain adequate perfusion of nervous tissue, most regions of interest require that vibratome sectioning is thin enough to avoid necrosis occurring in deeper cell layers. Thin nervous tissue, such as the retina, does not require this step. In our experience, cobalt-staining experiments have been successfully performed on acutely isolated brain slices with thickness of up to 250 μm.

In addition to performing tissue dissections quickly and with ice-cold solutions, all Co^{2+} assay solutions containing a bicarbonate buffer should be continuously bubbled with carbogen (5% CO_2/95% O_2) prior to and during the course of the experiment. This maintains normoxic and neutral pH conditions of the immersive solution, resulting in a significant improvement of the final staining quality and specificity. For example, in the rat retina, Co^{2+} staining in the presence of the iGluR agonist L-glutamate (L-Glu) results in the specific staining of inhibitory horizontal cells and AII amacrine cells that is completely lost in the absence of agonist or in the presence of a receptor antagonist (compare Fig. 3B with Fig. 3C) (Osswald *et al.*, 2007). By contrast, when retinae are stained using solutions not bubbled with carbogen, the resulting Co^{2+}-staining pattern is clearly different (Fig. 3D–F), probably due to TRP channel activation (D. Bowie lab, unpublished observation), so much so in fact that staining is observed in the absence of agonist or the presence of a receptor antagonist (Fig. 3D and F). Whatever the mechanism, the L-Glu-specific staining pattern is lost. Another issue is that the bubbling of solutions can damage the specimen. Therefore, we recommend that tissue is placed in mesh-bottom cups positioned in multi-well plates for the duration of the Co^{2+} labelling (Fig. 2D). The carbogen gas mixture can then be delivered through a 27-gauge needle driven

FIG. 2. Co^{2+} staining: streamlined protocol, solutions recipe and set-up guide. A step-by-step bench-top protocol describing the preparation and detailed experimental procedure to perform Co^{2+} staining in both tissue explants (red circles) as well as cultured cells (blue diamonds) (left). The staining procedure can be broken down into three sections: (A) Co^{2+} assay, (B) cryoprotecting and sectioning of tissue explants and (C) silver intensification. Solution recipes including storage recommendations (if required) are shown for all required solutions (right). Photographs of the plate set-ups for tissue explants (D) and cultured cells (E). All chemical product codes are from Sigma-Aldrich (St Louis, MO, USA) unless indicated otherwise.

through the bottom of the mesh. This allows specimens to be submersed in properly oxygenated and buffered solutions without the possibility of being damaged by any turbulence caused by bubbling.

Controlling and calibrating silver enhancement

The success of any Co^{2+}-staining experiment relies largely upon the ability to detect the Co^{2+} precipitate. The need to enhance the precipitate was recognized shortly after the technique was originally described (Tyrer & Bell, 1974). The original enhancement method, known as Timm's silver intensification, is still used to improve the detection of Co^{2+} sulfide precipitate. Unfortunately, Timm's silver intensification method uses solutions that are light-sensitive and require elevated temperatures to achieve proper intensification. This frequently leads to over-enhancement and the need for subsequent de-staining steps (Davis, 1982). Recognizing the problems with Timm's method, Davis developed a light-insensitive intensification method that did not require such high reaction temperatures. Unfortunately, it still involved the inconsistencies of preparing and properly storing various solutions. A simple solution to the aforementioned problems was provided by Nagy *et al.* (1994), who utilized a commercially available silver intensification kit developed for electron microscopy. The IntensEM kit (GE Healthcare, Missisauga, Ontario, RPN-491) provides two light-insensitive solutions (labelled A and B) that

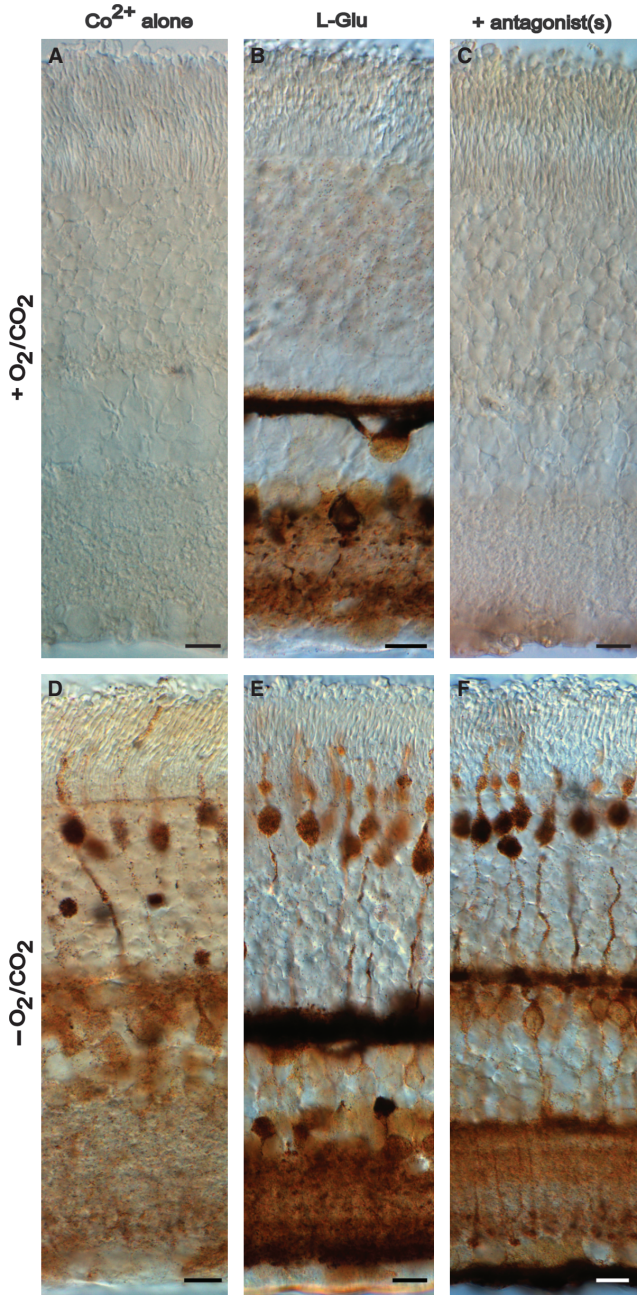

FIG. 3. Co^{2+}-staining specificity relies on maintaining oxygenation and solution pH. (A–C) Photomicrographs showing typical Co^{2+}-staining patterns obtained with rat retinal slices continuously bubbled with carbogen. No staining is observed after incubating the retina with Co^{2+} alone (A), whereas a pattern appears when stimulated with 10 mM L-Glu (B). Staining is absent with the addition of the AMPA receptor antagonist GYKI 52466 (80 μm), indicative of staining specificity (C). (D–F) A different staining pattern emerges when experiments are repeated in solutions lacking carbogen bubbling. Co^{2+} staining is present with (E) or without (D) 10 mM L-Glu stimulation and remains in the presence of both antagonists GYKI 52466 (80 μM) and DNQX (20 μM). Differential interference contrast images were captured with a Zeiss AxioCam HRc coupled to a Zeiss Axioplan 2 upright microscope through a 63× Plan Apochromat (1.40 NA) oil-immersion objective. Scale bars = 10 μm.

enhance precipitated sulphides when equal parts are combined. In our experience, intensification is most consistent if these reagents are mixed and used at room temperature in a dark room. As with other enhancement methods, over- or under-intensification of the Co^{2+} precipitate can lead to resolution or detection problems, respectively (Fig. 4). Therefore, we recommend calibrating the silver enhancement every experimental day to optimize incubation time. Not only does performing such a calibration allow for maximum staining reproducibility between experiments, but it also allows the experimenter to tailor the staining intensity for a given application. For example, in the case where morphological detail is critical, intensification time should be optimized so as to clearly reveal cell bodies as well as finer cellular processes (Fig. 4C and G). Less (Fig. 4B and F) or more enhancement (Fig. 4D and H) results in the loss of fine morphological detail.

Understanding the limitations of the approach

Cobalt staining is essentially a qualitative technique that is most valuable when mapping out where and when divalent-permeable ion channels are expressed. On occasions, it can be used in a more quantitative manner, for example to make comparisons between control and treatment tissue (e.g. Osswald *et al.*, 2007; Lebrun-Julien *et al.*, 2009). To do this, each sample is processed concurrently, especially during cobalt uptake and silver enhancement steps. Reproducibility is a key factor to avoid any fluctuations in the degree of silver enhancement due to tissue thickness or local variations in room temperature. As a rule, the occurrence of staining is taken as proof of the expression of divalent permeable ion channels only if the staining can be eliminated using known pharmacological blockers. The absence of staining need not imply that the cell in question lacks divalent-ion permeability as some ion channels, such as *N*-methyl-D-aspartate receptors (Mayer & Westbrook, 1987) or voltage-gated Ca^{2+} channels (Hagiwara & Byerly, 1981), are impermeable to Co^{2+}. Additionally, the lack of staining may occur due to limited transport of Co^{2+} in cells expressing low numbers of divalent-permeable ion channels.

Cobalt staining of cultured cell lines

With minor modifications (Fig. 2, blue diamond), the functional Co^{2+}-staining method can be applied to adherent cultured cell lines stably or transiently expressing a divalent-permeable ion channel. Cultured cells stained in this way are clearly detectable under bright-field illumination (Fig. 5). One can make use of such a system for the development of a medium-throughput assay for novel drug or mutation screening. More straightforwardly, it can be used to gain insight into the question of how divalent ion permeability relates to receptor composition, as has been shown in some studies of TRP channels (e.g. Woo *et al.*, 2008).

Ensuring cell viability

As with tissue explants, maintaining cellular health throughout the duration of the experiment is crucial. Therefore, the following points should be considered. First, recombinant receptor over-expression can lead to cell death due to overloading with Ca^{2+}. Consequently, it is essential that transfection conditions are optimized to minimize cell death. We have found that this is particularly important when expressing Ca^{2+}-permeable iGluRs, which are readily activated by glutamate released by growing cells. Secondly, it is practical to culture cells on small 12- to 13-mm round glass or plastic coverslips. These conveniently fit in standard 12-well tissue culture plates allowing simple culturing and also manipulation of the coverslip using forceps. Several cell lines do not adhere sufficiently to glass to endure the numerous solution exchanges required. Therefore, we

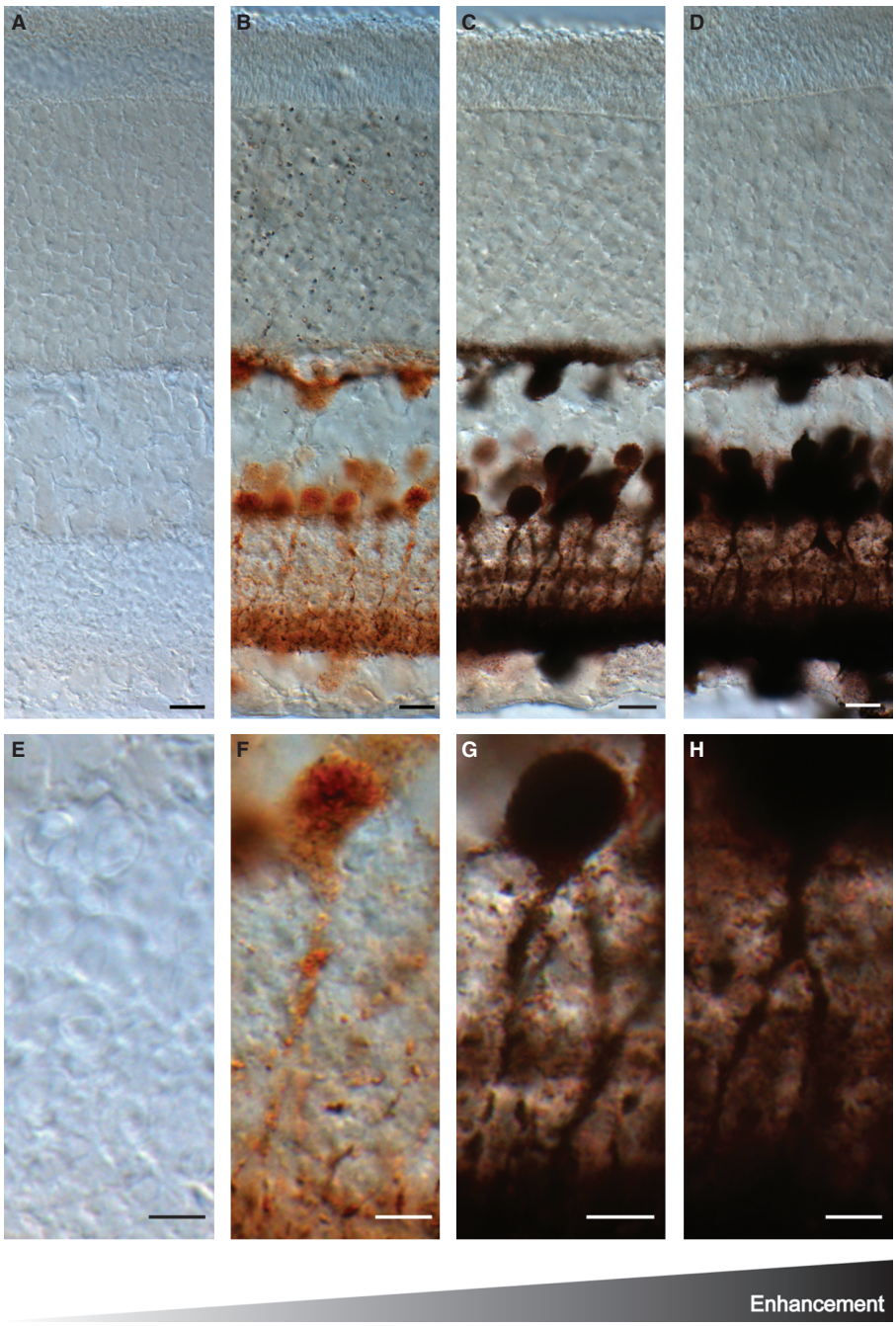

FIG. 4. Ideal Co^{2+} staining intensification is achieved by calibrating the silver enhancement incubation time. (A–D) Photomicrographs taken of the same L-Glu-stimulated (10 mM) retina subjected to increasing incubation times in IntensEM (i.e. silver intensification). (E–H) Higher magnification images to show detail on individual AII amacrine cells. Note that Co^{2+} precipitate is not detectable without enhancement (A and E). Enhancement incubation time can be tailored to the type of experiment performed. Less (B and F) or more enhancement (D and H) incubation times lead to the loss of fine morphological detail. Images acquired as described in Fig. 3. Scale bars = 10 μm (A–D) and 5 μm (E–H).

recommend that coverslips are placed in round mesh-bottom cups positioned in 12-well culture plates (Fig. 2E). In this case, the mesh-bottom cup with the coverslip is ferried between the different Co^{2+}-staining solutions.

As many commonly utilized cell lines do not require supplementary solution oxygenation, two changes are made to the solution recipes used for the Co^{2+}-staining procedure. Foremost, the bicarbonate-based assay buffer is replaced with a HEPES-based buffer (Fig. 2, right column) exempting the need for CO_2 to maintain pH neutrality of the solution. Secondly, to avoid cellular detachment from the coverslip especially during Co^{2+} chelation, cells are washed in 5 mM EDTA (ethylene diaminetetraacetic acid) for 30 s and not 2 mM EDTA for 5 min (Fig. 2) (Albuquerque *et al.*, 2001). Longer incubation times usually result in significant cell detachment and loss.

Quantification

As needed, the quantification of recombinant Co^{2+}-staining experiments can be performed using freeware available online. IMAGEJ (http://rsbweb.nih.gov/ij/) complemented with an automatic cell counting plugin (ITCN (http://rsbweb.nih.gov/ij/plugins/itcn.html), Image-based Tool for Counting Nuclei) can be used. Two images must be acquired from each field: one in bright-field and the other in phase contrast (Fig. 5). Although the bright-field image serves as an easy way to identify Co^{2+}-stained cells, it is not possible to use software to count the total number of cells (stained and unstained) contained in the field. Therefore, the phase contrast image is loaded into the ITCN plugin for automatic counting. The number of stained cells counted from the bright-field image can then be divided by the total number of cells, yielding the fraction of cells that were Co^{2+}-stained.

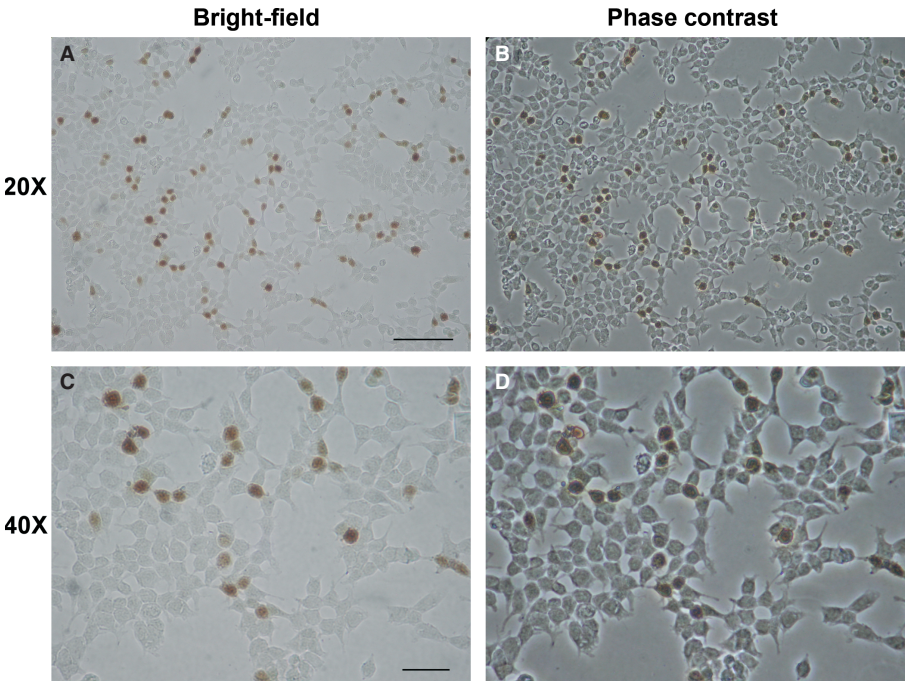

FIG. 5. Cobalt-stained HEK293 cells are easily detected by bright-field microscopy. HEK293T cells transiently transfected with the AMPA receptor GluA1 and stargazin (TARP γ2) subunits exhibit marked cobalt permeability when stimulated with 10 mM L-Glu and 100 μM cyclothiazide. Right and left columns show typical images observed under bright-field and apodized phase contrast microscopy, respectively. Images were captured with a Nikon Coolpix 4500 through an MDC lens coupled to a Nikon TS100F inverted microscope through a 20× or 40× (0.40 NA or 0.55 NA) objective. Scale bars = 100 μm (A and B) and 40 μm (C and D).

Conclusion

With the number and diversity of ion-channel families increasing in recent years, there has been an even greater need to elucidate divalent-ion permeability of native tissues or reconstituted preparations. We propose that the Co^{2+}-staining technique is ideal in this regard for many neuroscientists whether probing expression patterns in developing or adult CNS tissue or using it for medium-scale screening.

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Abbreviations

AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; CNS, central nervous system; EDTA, ethylene diaminetetraacetic acid; iGluR, ionotropic glutamate receptor; L-Glu, L-glutamate; TRP channels, transient receptor potential channels.

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