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Measuring cell viability with membrane impermeable zinc fluorescent indicator

Christian J. Stork, Yang V. Li *

Department of Biomedical Sciences, Molecular and Cellular Biology Program, Ohio University, 346 Irvine Hall, Athens, OH 45701, USA

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Abstract

Recent findings suggest that the accumulation of cytoplasmic zinc $[Zn^{2+}]_i$ is a ubiquitous component in the cell death cascade. Zn^{2+} can be liberated from intracellular stores following oxidative stress and contribute to cell death processes. Here we show that the membrane/cell impermeable Zn^{2+} fluorescent indicator Newport Green (NG), which is non-toxic and impermeable to the membranes of healthy cells, can label unhealthy cells in tissue slices in a manner comparable to the traditional viability indicator propidium iodide (PI). Using confocal microscopy, we detected PI labeled nuclei colocalized with NG fluorescence. Our results indicate that cells which absorbed PI into their nuclei also allowed cell-impermeable Zn^{2+} dye to penetrate their plasma membranes, subsequently exhibiting cytosolic and nuclear fluorescence. As in PI staining, we observed marked increases in NG fluorescence in damaged/dead cells of tissue slices. Two other cell impermeable fluorescent Zn^{2+} dyes, Fluozin-3 and Zinpyr-4, also stained cytosolic Zn^{2+} in PI labeled cells. Our data indicates that the application of a Zn^{2+} fluorescent indicator is a fast, simple, non-toxic and reliable method for visualizing cell viability within in vitro tissue preparations. Accordingly, we demonstrate that intracellular accumulation of Zn^{2+} correlates with neuronal death.

Keywords: Cell viability; Propidium iodide; Newport Green; Zinc; Fluozin-3; Zn^{2+}; Apoptosis; Rat

1. Introduction

Taking notice of recent findings that accumulation of cytosolic zinc $[Zn^{2+}]_i$ is a ubiquitous component in the cell death cascade, and that a cell loses membrane integrity as it dies, we verified that a cell membrane impermeable fluorescent Zn^{2+} indicator could stain damaged or dead cells. Reliable measurement of cell death is critical in the biological analysis of cytotoxicity. Current methods of detecting cell viability in tissue preparations exploit the properties of healthy cell membrane's ability to exclude dye, or alternatively, are designed to utilize a healthy cell's metabolic activity to promote a dye's conversion to a visible state (Haugland, 2001; Ying et al., 2003). These types of assays are used in the investigation of apoptosis, signal transduction, traumatic tissue damage, and identifying new cytotoxic agents for anti-tumor therapy or high-throughput screening. An extensively employed cell viability indicator propidium iodide (PI) capitalizes on the exclusion principle to visually differentiate dead cells in a tissue preparation. PI, a charged molecule, is normally excluded from a cell's lipid membrane, yet passes freely through the permeabilized membranes of dead and dying cells. Once inside a cell, PI intercalates within DNA and forms a bright fluorescent complex. When analyzed using a fluorescent detection system, in this case a confocal microscope, cells that have lost membrane integrity and labeled with PI, will emit lower frequency fluorescence throughout the nuclei.

Zinc is an essential micronutrient and a vital component of enzymes and other proteins (Vallee and Falchuk, 1993). Zn^{2+} homeostasis is meticulously regulated so that free Zn^{2+} is essentially absent in the cytosolic fluid with estimations of concentration being below the picomolar range (~1 pM) (Colvin et al., 2003; Frederickson and Bush, 2001). Current evidence supports the involvement of Zn^{2+} in the cellular signaling of apoptosis and mechanisms of cellular necrosis (Frederickson et al., 2005; Hamatake et al., 2000; Lobner et al., 2000). Recent reports describe the accumulation of intracellular Zn^{2+} ($[Zn^{2+}]_i$) as a important component of the cell death cascade (Bossy-Wetzel et al., 2004; Land and Aizenman, 2005). The accumulation of $[Zn^{2+}]_i$ precedes other morphological and molecular changes following neuronal damage (Land and Aizenman, 2005). Other

* Corresponding author. Tel.: +1 740 593 2389.
E-mail address: Liy1@ohio.edu (Y.V. Li).

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evidence indicates that endogenous zinc may play a key role in neuronal death after acute brain insults such as ischemia, seizures, and trauma (Choi and Koh, 1998; Frederickson, 1989; Koh et al., 1996; Tonder et al., 1990; Wei et al., 2004). In these brain injury paradigms, intracellular accumulation of zinc correlates with neuronal death at the single cell level and supports the theory of detecting $[Zn^{2+}]_i$ increase as a measurable component of cellular damage.

Like other divalent ions such as Ca^{2+} and Mg^{2+}, cytosolic Zn^{2+} can be detected with fluorescent Zn^{2+} indicators. However, unlike Ca^{2+} and Mg^{2+}, cytosolic free Zn^{2+} concentration in normal cells is very low (~1 pM) (Colvin et al., 2003; Frederickson and Bush, 2001). There are two forms of Zn^{2+} indicators, designed to respond to changes of $[Zn^{2+}]$ in either the intracellular space or the extracellular space (Haugland, 2001). The membrane permeable diacetate form of the indicator (a non-polar ester) can pass through cell membranes. Once inside the cell, intracellular esterase activity cleaves the molecule and traps the active ion indicator inside the cell. Opposite in solubility, the cell impermeable form of Zn^{2+} indicator is designed so as to be excluded from the contiguous membrane of healthy cells. The dipotassium salt form of the dye has a large size and an ionic charge which collectively dictate the indicator be excluded from cell membranes. Compromised or damaged cell membranes, however, allow the penetration of large and ionic substances without differentiation. In this regard, counting cells after nuclear staining with extracellular dyes such as PI and Trypan blue has been accepted as an appropriate method to measure cell death (Haugland, 2001; Ying et al., 2003).

In this study the cell impermeable Zn^{2+} selective indicator Newport Green (NG) was used to determine if an extracellular Zn^{2+} indicator can fluorescently label non-viable cells as reliably as PI. Similar membrane properties are expected between the two dyes, wherein PI binds to DNA made accessible as a result of cell damage, and NG binds Zn^{2+} ions liberated and made accessible through the same processes. We hypothesize that the same principle of cell exclusion applies to the indications of cell viability analysis using Newport Green dipotassium salt (NG) as with PI, with the dramatic advantage that the entire cell bodies of damaged cells may be visualized, and we believe this is an advantage to labeling the nuclei alone. We employed double staining of cells with NG and PI in which PI labeled nuclei located within the same cells labeled with the Zn^{2+} selective indicator NG.

2. Materials and methods

2.1. Tissue section (slice) preparation

Male Sprague–Dawley rats weighing approximately 200–300 g were used as tissue donors for this experiment. Rats were anesthetized with Ketamine (.1 ml per 100 g). After anesthetization, rats were decapitated, tissue was rapidly harvested, then immersed in ice cold artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2/5% CO_2. Tissue sections (slices) of hippocampus, cerebellum, and cerebral cortex were prepared with a Vibratome™ and cut at 250 μm in thickness. Coronal sections were taken of the hippocampus and cerebral cortex. Cerebellum sections were taken 2 mm para-sagittally. Dissection and slicing were performed in an ice-cold ACSF containing: (in mM) 121 NaCl, 1.75 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 1.25 KH_2PO_4, and 10 glucose; bubbled with 95% O_2/5% CO_2. Tissue sections were immersed in oxygenated ACSF throughout each experiment, excepting simulated ischemia. Following isolation, slices were subsequently stored for at least 30 min in a bath of ACSF (bubbled with 95% O_2/5% CO_2) at room temperature prior to imaging.

2.2. In vitro fluorescence

After 30 min of equilibration post slicing, sections were immersed in ACSF containing 5 mg/ml (w/v) PI for an additional 30 min at room temperature. Sections were then rinsed in ACSF, given 30 more minutes for equilibration, transferred to an immersion chamber on an inverted confocal microscope, and maintained in the chamber using a circular shaped platinum wire with a grid of nylon threads. ACSF was circulated through the immersion chamber (2 ml/min, bubbled with 95% O_2 and 5% CO_2) and contained 10 μM Newport Green DCF (K_D for Zn^{2+} ~1 μM). Sections were equilibrated in the dye/ACSF for 5 min prior to imaging to allow for full dye saturation of the slices. We also measured autofluorescence of hippocampal slices (without loading Newport Green) under the same experimental conditions used in our experiments. With camera (see below) used in our experiments, autofluorescence and photobleaching were negligible, and neither was subtracted from data. Further experiments to confirm the identity of the ions being detected utilized Zinpyr-4 (K_D for Zn^{2+} ~1 nM), and FluoZin-3 (K_D for Zn^{2+} ~15 nM), delivered in the same concentration (10 μM) and using the same parameters.

In some control experiments we utilized Calcein-AM in conjunction with PI. Tissue slices were prepared as described above, then immersed in oxygenated ACSF with 5 mg/ml PI for 30 min. After washing out PI, the sections were incubated in 10 μM Calcein-AM ACSF for 30 min and were then rinsed and equilibrated an additional 30 min prior to microscopic analysis. Calcein-AM (50 μg aliquot) was mixed with 25 μl DMSO to make a 2 mM stock solution, from which 10 μl was drawn and mixed into 2 ml ACSF, then placed into an oxygenated incubation chamber maintained at 32 °C.

2.3. Confocal fluorescence microscopy

Images were obtained utilizing an inverted Zeiss Axiovert LSM 510 Confocal Microscope. The objective lenses used were: ×10 with .3 NA, ×40 with 1.3 NA oil immersion, and ×100 also 1.3 NA oil immersion lens. A differential interference contrast type III objective filter was used in conjunction with the ×40 and ×100 objectives. Multiple magnification settings were utilized so that the anatomy of sections from different tissues could be delineated by cell type composition, as well as to improve visualization of the different spatial organizations between the tissue types. In this way, we can study colocalization in images acquired from regions of interest including

both single and multiple cells. Z-series scans were used to construct 3D images for colocalization analysis. A 488 nm Krypton–Argon laser line was used for excitation of Newport Green with an 505 nm emission filter. A 545 nm Helium–Neon laser line was used for excitation of propidium iodide with a 645 nm emission filter. The 488 nm laser line was also used for excitation of Calcein, and then filtered using a 515 nm emission filter. Colocalization of PI and NG staining was accomplished by doing two channel laser scans, after which Zeiss imaging software produced single channel results and a composite image produced by fluorescent overlay. Colocalization experiments were conducted with minimal laser intensity, and exposure time was minimized, such that photobleaching was insignificant and no corrections were made. Calcein and PI anti-colocalization was constructed exactly the same as colocalization experiments.

2.4. Simulated ischemia

During simulated ischemia experiments, hippocampus slices were bathed with ACSF lacking glucose (NaCl adjusted to 131 mM) and perfused with 95% N_2 and 5% CO_2 for 30 min in the oxygen and glucose deprived (OGD) ACSF, followed by reperfusion in normal ACSF. Slices were then labeled with PI as described above before confocal analysis. PI labeled slices were analyzed after 30 min OGD. Baseline PI images were obtained from slices exposed to normal ACSF for 70 min.

2.5. Chemicals and reagents

Propidium iodide (PI), and all ACSF salts were purchased from Sigma (St. Louis, MO), Zinpyr-4 from Neurobiotex (Galveston, TX). Newport Green DCF dipotassium salt (Cat. No. N-7900), fluozin-3 dipotassium salt (Cat. No. F-24194) and Calcein-AM (Cat. No. C3100MP) were purchased from Molecular Probes (Eugene, OR).

2.6. Statistics

Zeiss analysis software (LSM 5 Image) was used to analyze the statistical correlation of two channel overlap for each experimental paradigm. Pearson's correlation coefficient was employed to evaluate colocalization (values range between 1.0 and −1.0, where 1.0 is a complete colocalization and −1.0 indicates no overlap; Manders et al., 1993).

3. Results

3.1. Cell impermeable fluorescent Zn^{2+} dye newport green staining for cytosolic Zn^{2+}

The visualization of Zn^{2+} accumulation itself has not been explored as a method of evaluating cell viability. To explore this possibility we used Newport Green dipotassium salt (NG), a cell impermeable fluorescent Zn^{2+} dye, in conjunction with the well-recognized cell viability indicator propidium iodide (PI). Cell damage was induced by 5–10 min anoxia. Hippocampus, cerebral cortex, cerebellum were then stained with NG. NG has been used to characterize extracellular Zn^{2+} released from neurons in cerebral cortex such as the hippocampus (Besser et al., 2004; Li et al., 2001b). Another reason for the use of NG in this study is that NG fluorescence is not appreciable in the presence of physiological level Ca^{2+} and Mg^{2+} (Canzoniero et al., 1999; Li et al., 2001a,b). This characteristic is essential to a cell impermeable Zn^{2+} dye not to become saturated by high concentration of Ca^{2+} or Mg^{2+} that are normally present in the extracellular physiological medium (ACSF).

The hippocampus has a unique anatomical structure. The primary neurons (pyramidal and granular cells) are lined up forming a distinct laminar structure throughout the hippocampus. The complex of NG with Zn^{2+} in hippocampal slices yields stable green fluorescence which is most obvious in the laminar structure formed by compact organization of cell bodies. Using confocal microscopy, we observed and differentiated individual neurons (Fig. 1). Cells stained with NG could be visualized in a minute after the initial immersion of the sections in NG (10 μM) and becomes stable in less than 5 min. In these labeled neurons, the whole cell body including the nucleus was stained with NG, with nuclei often demonstrating more intense NG fluorescence.

Other brain regions that we tested included the cerebral cortex (Fig. 2) and cerebellum (Fig. 3). These regions exhibited the same pattern of NG staining as hippocampal sections. The cerebellum is among a few brain regions that does not have releasable Zn^{2+} within the neuronal fibers (Frederickson et al., 2000). Therefore, we chose the cerebellum in this study to demonstrate that Zn^{2+} stained with NG in cerebellar granule cells had not influxed from extracellular sources, but rather was of intracellular origin, coming from the dissociation of Zn^{2+} from zinc binding proteins.

3.2. Colocalization of newport green staining and propidium iodide staining

Propidium iodide (PI) has been accepted and widely used as appropriate method to measure apoptotic cell death. The PI penetrates the damaged cell membrane and fluoresces upon intercalating within DNA. In this study, cell viability was comparatively determined with dual staining of NG and PI in the same tissue sections. When viewed using a fluorescence microscope with a 645 nm filter, cells that have lost membrane integrity showed contrasting red PI staining throughout the nuclei (image B in Figs. 1–3) in the same tissue sections that also stained initially with NG. The dual fluorescent staining revealed colocalization of NG staining and PI staining (image C in Figs. 1–3) with Pearson's correlation coefficients $r = .743$ ($N = 28$), suggesting that cells labeled with NG were damaged or undergoing apoptotic cell death. We ruled out signal overlap accounting for the higher intensity NG nuclear fluorescence by examining slices stained only with PI. Using excitation and emission wavelengths for NG, we did not observe PI staining (data not shown). In control experiments, to show that NG could be used to visualize dying cells, we employed dual staining with the live cell stain Calcein-AM, and with the dead cell stain PI, in hippocampal slices. Fig. 1D–F shows that

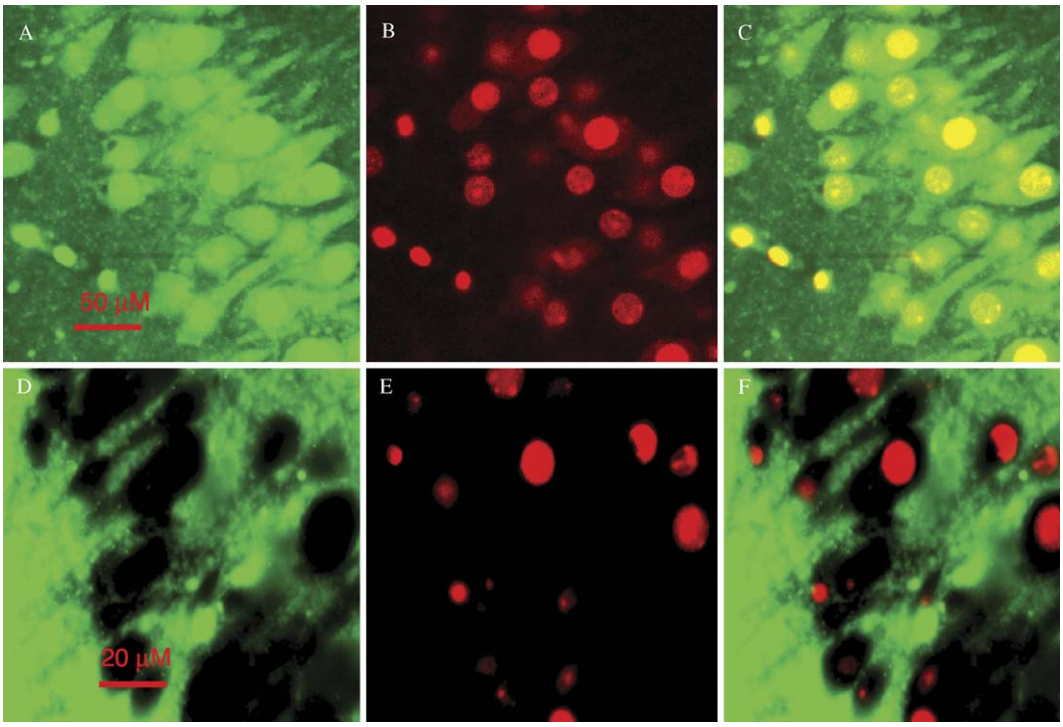

Fig. 1. Microscopic fluorescence images of rat hippocampal sections stained with NG and PI. A–C are images of pyramidal cells of a hippocampal CA1 section viewed at 100×. (A) NG stained cell bodies green with emission at 505 nm; (B) PI stained red nuclei with emission at 645 nm; (C) composite overlay fluorescence images showing colocalization of PI nuclei within cell bodies labeled with NG. D–F are images of pyramidal cells in the same type of preparation, but labeled with the live cell stain Calcein-AM in addition to PI. (D) The image of cells stained with Calcein-AM; (E) the PI stained nuclei of the same area; (F) the composite image showing anti-colocalization of Calcein-AM labeled cells in apposition to PI stained nuclei.

both live and dying cells can be visualized using our method. Unlike NG staining, there was no colocalization of Calcein-AM staining and PI staining (Pearson's correlation coefficients $r = -.286$, $N = 19$), further supporting the use of NG to determine cell viability and to visualize dying cells. Fig. 4 shows that two other cell impermeable fluorescent Zn^{2+} dyes, fluozin-3 and Zinpyr-4, also stained cytosolic Zn^{2+} in PI labeled cells (Pearson's correlation coefficients were as follows: $r = .728$, $N = 19$ for Fluozin-3 and PI; $r = .913$, $N = 10$ for Zinpyr-4 and PI).

3.3. Correlation of cell viability observed with NG and PI

Changes in dead cells were also assessed with NG and PI by simulated ischemia induced by exposure (immersion) of hippocampal sections in oxygen and glucose deprived (OGD)

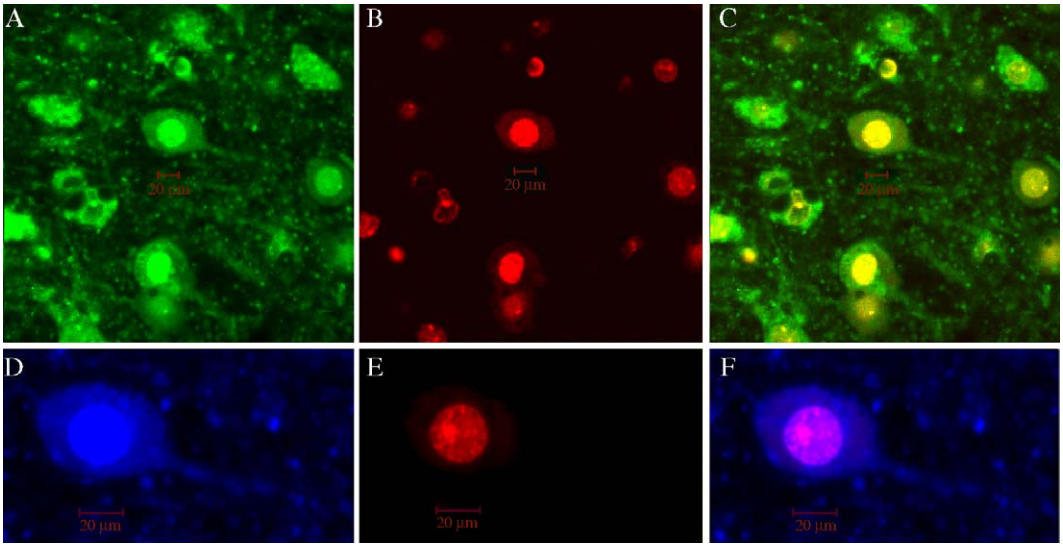

Fig. 2. Confocal acquired fluorescent images of cortical neurons from the rat cerebral cortex stained with NG and PI at 100×. (A) A cortical neuron with NG stained cell body in green, (B) the same neuron stained nucleus in red with PI, and (C) colocalization of PI labeled nucleus within NG labeled cell body and axonal projection.

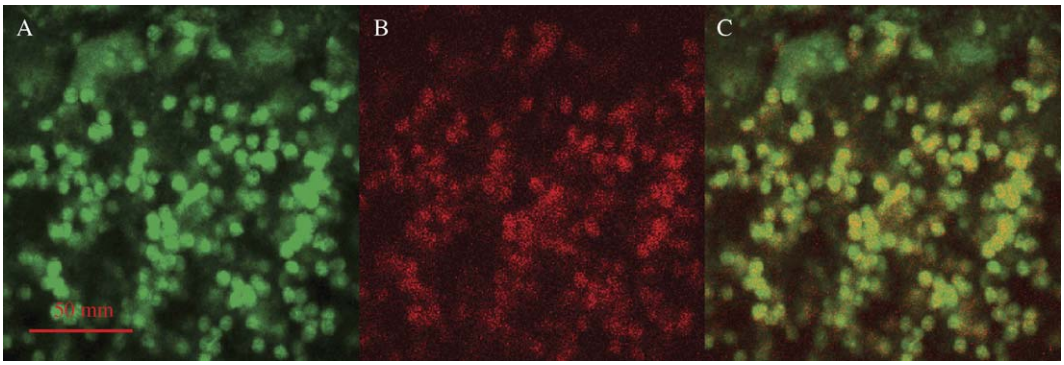

Fig. 3. Microscopic fluorescence images of granule cells of rat cerebellar slice stained with NG and PI at 100×. (A) Granular cells with NG stained cell body green, (B) the same cells stained nuclei in red with PI, and (C) colocalization of PI labeled nuclei within NG labeled cell body and axonal projection.

ACSF continuously bubbled with 95% N_2 and 5% CO_2. After artificially induced ischemia as simulated by 30 min of OGD ACSF, our data shows marked increases in PI staining in hippocampal sections (Fig. 5), indicating increased cell death under this treatment. There were 3 min delay between decapitation and immersion of tissue sections in oxygenated ACSF. The hypoxia induced during this delay accounts for the baseline level of cell damage seen in the both control conditions (Fig. 5A and C). As seen with PI, we observed similar changes in the fluorescence intensity of NG compared to the initial intensity

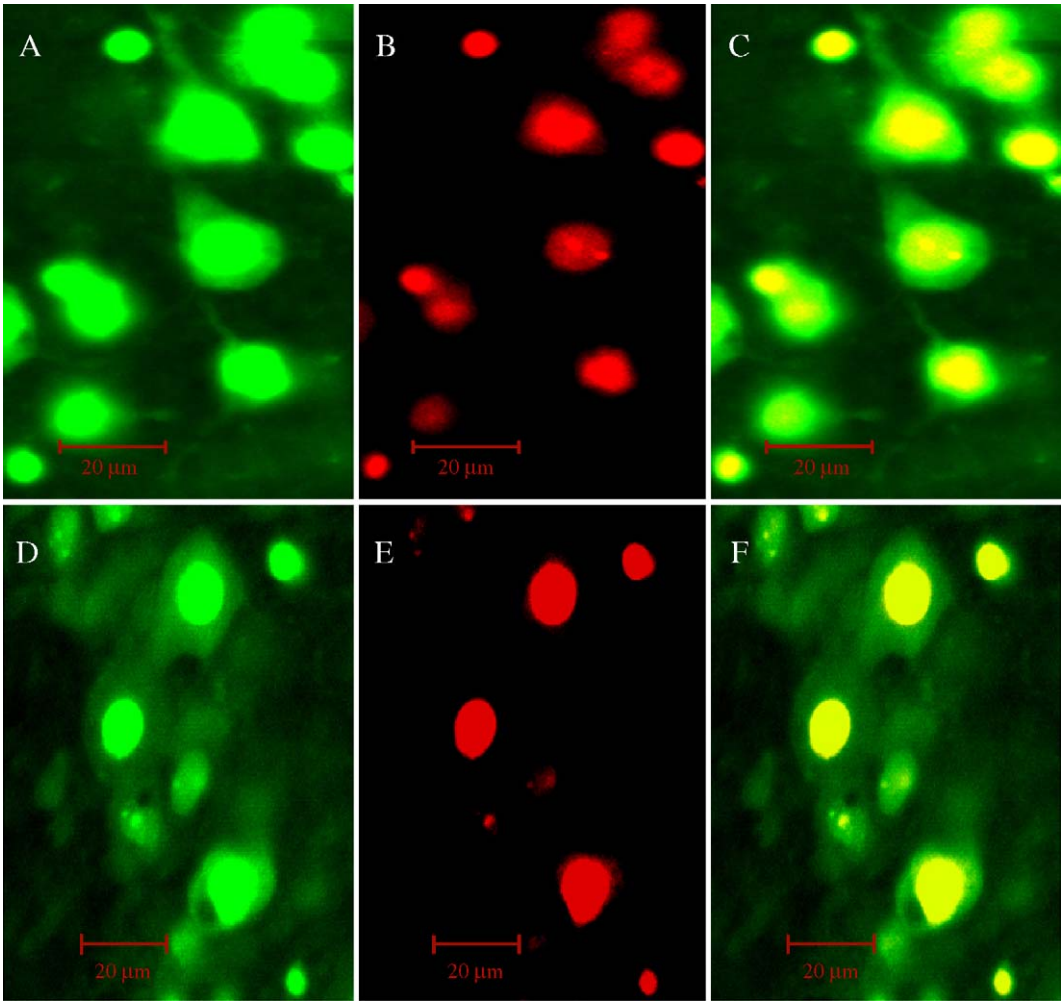

Fig. 4. Microscopic fluorescence images of PI stained nuclei colocalizing with two other cell impermeable zinc indicators. A–C are images of Fluozin-3 and PI dual stained cells within a hippocampus slice. (A) Fluozin-3 labled cells; (B) PI labeled nuclei; (C) PI labeled nuclei colocalizing within Fluozin-3 labeled cells. D–F depict cells within a hippocampal slice dual stained with Zinpyr-4 and PI. (D) Zinpyr-4 labeled cells; (E) PI labeled nuclei; (F) the composite image depicting colocalized PI stained nuclei within Zinpyr-4 labeled cell bodies.

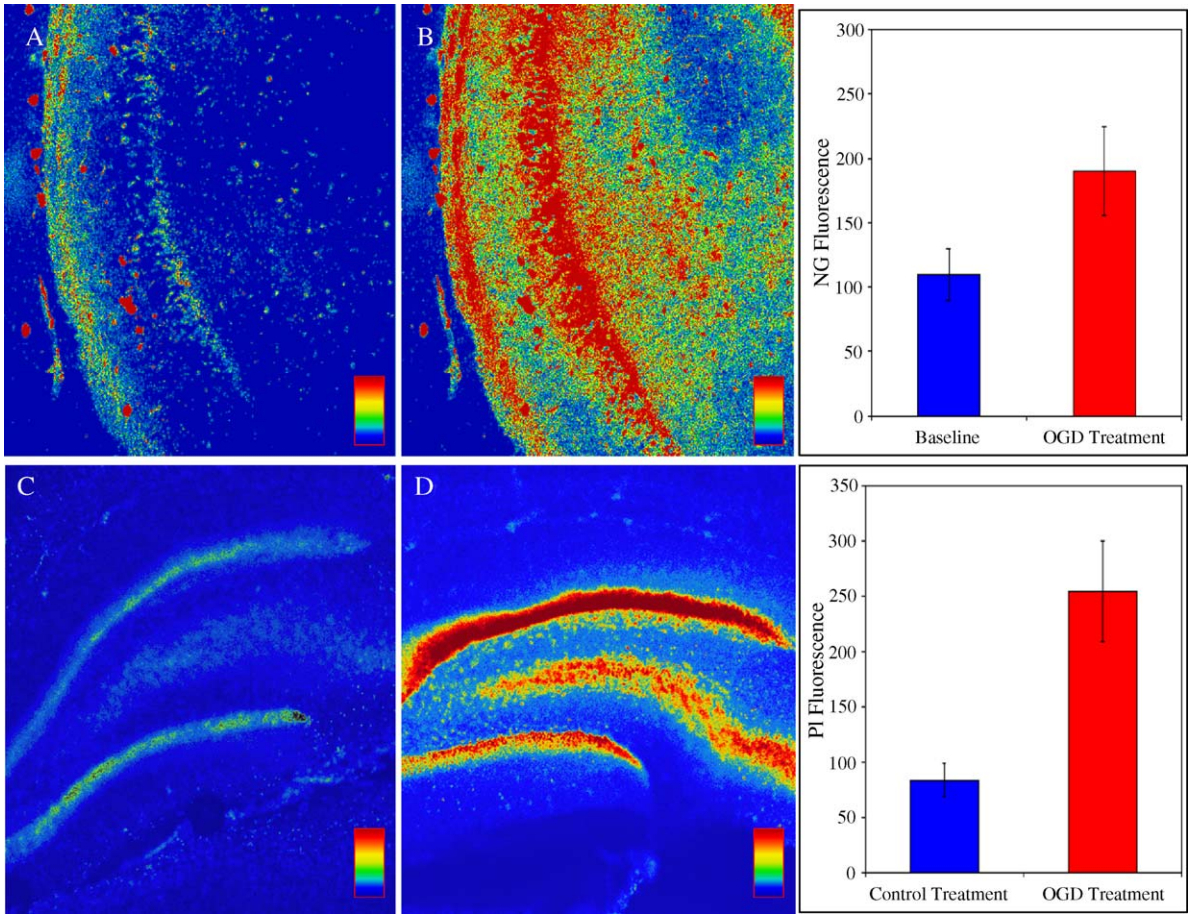

Fig. 5. Detection of cytosolic Zn^{2+} by cell impermeable Zn^{2+} dye demonstrates change of cell viability. (A) Pseudocolor image of a hippocampal section stained with NG (control, scale given at the lower right corner); (B) the increased fluorescence of NG 30 min after OGD treatment in the same hippocampal section in pseudocolor. The bar diagram (mean ± S.D., $N = 8$) summarizes results in A and B shows the increased intensity of NG fluorescence, suggesting increases in damaged and dead cell under distressing condition. The cell viability of the hippocampal section was further confirmed by PI staining in pseudocolor (scale given at the lower right corner) in C–D. (C) Control; (D) the effect of OGD treatment; The bar diagram summarizes data in C and D (mean ± S.D., $N = 8$), showing similar results as the section stained with NG.

of NG before OGD treatment. Therefore, the tissue sections that showed increases in PI staining showed Zn^{2+} accumulation under the same conditions. These results support Zn^{2+} labeling with NG as being capable of revealing a change in cell viability.

4. Discussion

In each of different brain tissue slice preparations, we detected Newport Green (NG) fluorescence labeled nuclei colocalized with propidium iodide (PI). Our results indicate that cells which absorbed PI into their nuclei also allowed cell membrane impermeable Zn^{2+} dye to pass through their plasma membranes and register intracellular fluorescence, suggesting that membrane impermeable Zn^{2+} fluorescent indicators such as NG are effective tools for detecting cell viability. The data supports our hypothesis that the principle of membrane dye exclusion also applies to the Zn^{2+} fluorophore, and its intracellular visualization indicates compromised cellular function. Our observations of fluorescent labeled Zn^{2+} within cells, while using cell impermeable dye, in conjunction with PI fluorescence suggest that cells no longer had intact plasma membranes and that NG penetrated cells by the same mechanism that dictates PI labeling. Our data indicates that the application of extracellular Zn^{2+} dye is a fast, simple, non-toxic, and reliable new method for the determination of cell viability within in vitro tissue preparations.

Our data demonstrates that intracellular accumulation of Zn^{2+} correlates with neuronal death. Others have shown that Zn^{2+} homeostasis is meticulously regulated, such that free Zn^{2+} is essentially absent in the cytosolic fluid with the estimated concentration being below (~1 pM) (Colvin et al., 2003; Frederickson and Bush, 2001). Current evidence suggests that Zn^{2+} is involved in the cellular signaling of apoptosis and pathology of necrosis, which therefore implicates the accumulation of intracellular Zn^{2+} ($[Zn^{2+}]_i$) as a critical component of the cell death cascade (Bossy-Wetzel et al., 2004; Frederickson et al., 2005; Hamatake et al., 2000; Land and Aizenman, 2005; Lobner et al., 2000).

Cell impermeable Zn^{2+} fluorescent indicators are designed so as to be excluded from healthy cells (Haugland, 2001; Ying et al., 2003). The dipotassium salt form of NG has a large size and an ionic charge which dictate exclusion from cell membranes. We have been using cell impermeable NG to study extracellular

Zn^{2+} released from Zn^{2+} containing neuron fibers which are compact in hippocampus (Li et al., 2001a,b). Since the cerebellum contains no Zn^{2+} containing neuron fibers (Frederickson et al., 2000), NG staining of cerebellar granule cells (which form synapses with mossy fibers) indicates that the origin of accumulated intracellular Zn^{2+} was from the cells themselves, and not resultant of influx from extracellular sources. This observation was supported further by data from the extra-nerve system of the kidney (data not shown) where, in separate experiments, we studied kidney tissue sections with dual staining of NG and PI and observed colocalization of NG staining and PI staining.

The use of propidium iodide has long been accepted as an indicator of cell viability. PI staining in conjunction with exposure to NG revealed colocalization of PI labeled nuclei within the cell bodies fluorescing from the Zn^{2+} dye. Visual confirmation of the colocalization and numerical correlation of colocalization indicates that the two dyes were labeling the same cells, and the same number of cells. Like PI, NG can also be used to measure the changes of cell viability in a cytotoxicity assay. This data supports the use of a Zn^{2+} fluorophore as an effective tool for detecting cell viability within in vitro tissue slice preparations.

PI fluoresces in the nucleus of non-viable cells. The use of Zn^{2+} fluorescent indicator as a cell viability tool allows for the rapid visualization of the entire cell body of unhealthy or dead cells. Therefore, the cumulative labeling of cells with NG thereby delineates the anatomical structure of a tissue section. To our knowledge, unlike other dead cell dyes that stain nucleic acid, the use of cell impermeable Zn^{2+} dyes like NG stand out as a novel tool to stain dead cell bodes. In our experiments, the capacity to detect non-viable cells in a tissue slice preparation enhances the experimenter's ability to focus on cells which are still capable of eliciting physiological responses. Another advantage is that NG potassium salt is readily dissolved into physiological solutions, and therefore, is an easy and quick method to label cells. The complex of NG with Zn^{2+} yields stable fluorescence which can be reviewed in less than 5 min. Furthermore, NG is non-toxic to cells (Lukowiak et al., 2001), and thus, can be used in physiological experiments.

In conclusion, a viability/cytotoxicity assay by extracellular Zn^{2+} dye is a fast, simple, non-toxic and reliable new method for the determination of cell viability within in vitro tissue preparations. Other than NG, several new generation of Zn^{2+} fluorophores are available recently such as Zinpyr-4, FluoZin-3, and FuraZin-1 (Burdette et al., 2003; Gee et al., 2002). Fluozin-3 and Zinpyr-4 stained cytosolic Zn^{2+} in PI labeled cells (Fig. 4). Since they all select Zn^{2+} over other divalent ions, we expect that cell impermeable forms of these dyes can be used in measuring cell viability. Furthermore, dual staining with PI confirms the accumulation of $[Zn^{2+}]_i$ in cytotoxicity and neuronal death.

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