Journal of Neuroscience Methods 294 (2018) 111–115

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Short Communication

Medium-retaining Petri dish insert to grow and image cultured cells

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HIGHLIGHTS

- For superfusion, coverslips with cultured cells must be removed from Petri dishes.
- This leads to air exposure and unintended $[Ca^{2+}]_i$ elevations.
- A method has been designed to avoid these unintended $[Ca^{2+}]_i$ elevations.
- Cells can be cultured in medium-retaining Petri dish inserts with glass bottom.
- When these inserts are removed from Petri dishes, the cells remain submerged.

ARTICLE INFO

Article history:
Received 11 July 2017
Received in revised form 9 November 2017
Accepted 9 November 2017
Available online 10 November 2017

Keywords:
Fura-2
Glass coverslips
Neurons
Astrocytes
HEK-293 cells
TH::RFP mice
Tyrosine hydroxylase

ABSTRACT

Background: Microscope chambers that accept glass coverslips with cultured cells are often used to monitor intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) during cell superfusion. Unfortunately, the experimental maneuvers associated with the coverslip installation in these chambers (medium removal and re-application) trigger unintended $[Ca^{2+}]_i$ elevations.
New method: To prevent these $[Ca^{2+}]_i$ elevations, a Petri dish insert has been constructed. The insert features a superfusion-optimized well to grow cell cultures. After this insert is removed from the Petri dish, the well retains the medium. This feature allows the inserts to be installed in microscope chambers while keeping the cells submerged at all times.
Results: These inserts were used to test the impact of a transient medium removal from the well (an equivalent of a coverslip removal from the medium) on $[Ca^{2+}]_i$ in primary murine cortical neurons and astrocytes, and in HEK-293 cells. In all of these models, the medium removal/re-application caused a micromolar $[Ca^{2+}]_i$ spike. While in neurons this spike was caused by a Ca^{2+} influx, in astrocytes and HEK-293 cells, it was caused by a Ca^{2+} release from intracellular stores. After the spike, a subpopulation of neurons failed to restore low $[Ca^{2+}]_i$; in 24% of the astrocytes, the spike triggered $[Ca^{2+}]_i$ oscillations. However, prior to the spike, $[Ca^{2+}]_i$ was low and uniform in all these cells.
Comparison with existing method(s): The new method avoids the artificially-induced $[Ca^{2+}]_i$ elevations that take place during the handling of glass coverslips with cultured cells.
Conclusions: The new method allows monitoring $[Ca^{2+}]_i$ without disturbing the basal $[Ca^{2+}]_i$ levels.

1. Introduction

Ca^{2+} is a second messenger and plays important biological roles (Ghosh and Greenberg, 1995). To study effects of drugs on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cells are either loaded with Ca^{2+}-sensitive fluorescent probes or transfected with fluorescent genetically-encoded Ca^{2+} sensors (Carter et al., 2014; Malgaroli et al., 1987; Palmer et al., 2011) and then they are superfused with experimental media. Although Petri dishes with glass bottom are commonly used in cell imaging, the wells in these Petri dishes are not optimized for the laminar flow necessary for effective superfusion. For experiments involving superfusion, cells are typically cultured on glass coverslips that are eventually transferred to special supefusion-optimized microscope chambers. Nonetheless, it takes several seconds to secure the coverslips in these chambers and to re-submerge the cells in medium. During these maneuvers, the cells are transiently exposed to air and, when the

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; GFAP, glial fibrillary acidic protein; K60, 60 mM K^+; RFP, red fluorescent protein; TH, tyrosine hydroxylase; VM, ventral mesencephalon.

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https://doi.org/10.1016/j.jneumeth.2017.11.007
0165-0270/

medium is re-applied, the cells undergo mechanical stress which may acutely destabilize Ca^{2+} homeostasis (Charles et al., 1991; Paoletti and Ascher, 1994). In fact, a subpopulation of neurons with highly elevated basal $[Ca^{2+}]_i$ was observed in earlier experiments (Kiedrowski, 1999; White and Reynolds, 1997). The purpose of this work was to design a method to transfer cultured cells from a Petri dish to a microscope chamber without subjecting the cells to a stress that might destabilize Ca^{2+} homeostasis.

2. Methods

2.1. Cell cultures

Primary cultures of murine cortical neurons and astrocytes were prepared using cryopreserved SPOT kits™ from the University of Illinois at Chicago Research Resources Center (RRC) (http://www.rrc.uic.edu/portal/SPOT_Culture_Kit). Kit production was approved by the Institutional Animal Care and Use Committee. To generate kits with cortical neurons, brain cortex from E16.5 male and female C57BL/6 mice was used. Kits with fluorescently-tagged astrocytes were custom ordered from the RRC. To prepare these kits, P2 TH::RFP mice (a gift from Dr. McMahon, Vanderbilt University) males and females were used. Kits with wild type ventral mesencephalon (VM) neurons were obtained using E13.5 male and female C57BL/6 mice.

To generate neuronal cultures, a SPOT kit™ with cryopreserved suspension of neurons was thawed at 37 °C and diluted 8× dropwise with a warm (37 °C) neurobasal medium supplemented with 2 mM glutamine and 2% B27 (culture medium) and centrifuged at 300 × *g* for 5 min at 20–23 °C. The supernatant was discarded and the cell pellet re-suspended in 200 μl of the culture medium. Five μl of the cell suspension (about 4000 cells) were plated in the middle of poly-D-lysine (PDL)-treated glass coverslip or in the wells of PDL-treated Petri dish inserts from Spot Cells LLC, Chicago, IL (Fig. S1A). The coverslips and the inserts were placed in standard 35 mm Petri dishes. Fifteen minutes after the plating, the Petri dishes were filled with 2 ml of the culture medium. The cells were then cultured in a humidified 37 °C, 5% CO_2 incubator.

To generate neuroglial co-cultures, the neurons were plated in the inserts first, as described above. Then, a SPOT kit™ with astrocytes was processed using the above-described procedure except that the final cell pellet (obtained after 300 x g centrifugation) was suspended in 8 ml of culture medium and 30 μl of the astrocyte suspension (250–500 cells) was plated on top of the neurons (during the preparation of the astrocytes, the Petri dishes containing the inserts with plated neurons were stored in a humidified incubator). Thirty minutes later (after the astrocytes attached), the Petri dishes were filled with culture medium. Two days after plating the neuroglial co-cultures, glial proliferation was curtailed by adding 10 μg/ml of 5-fluoro-2′-deoxyuridine. The latter was not added to the neuronal cultures. The cultures were used for the experiments after 5–7 days *in vitro*.

To prepare Human Embryonic Kidney 293 (HEK-293) cell cultures, a cryopreserved 293FT cell line (Invitrogen) was used. The cells were thawed and processed using the above-described procedure except that Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 1 mM sodium pyruvate, 4 mM glutamine, 10% fetal bovine serum, 100U/ml penicillin, and 100U/ml streptomycin was used as a culture medium. The final cell pellet was suspended in the culture medium to reach a density of about 400,000 cells per milliliter; 30 μl of this suspension were plated in each insert and about 30 min later (after the cells attached), the Petri dishes with the inserts were filled with 3 ml of the culture medium and placed in a humidified incubator (37 °C, 5% CO_2). Experiments were conducted after the cells reached confluence, usually 3 days after plating.

2.2. Experimental media

Standard Locke's buffer contained (in mM) NaCl (157.6), KCl (2.0), $KHCO_3$ (3.6), $MgCl_2$ (1.0), $CaCl_2$ (1.3), glucose (5), and HEPES (10), and pH 7.4 adjusted with NaOH. Ca^{2+}-free Locke's buffer was prepared by omitting $CaCl_2$ and adding 100 μM EGTA. Locke's buffer with 60 mM K^+, was prepared by appropriately increasing the K^+ concentration and decreasing in the Na^+ concentration in the standard buffer.

2.3. Fura-2 fluorescence imaging and superfusion

Fluorescence imaging was performed using a digital fluorescence imaging system controlled by MetaFluor 7.7.8 software (Molecular Devices LLC, Sunnyvale, CA, USA) described in detail in Kiedrowski, 2014. Superfusion was carried out at 37 °C using a MPRE-8 manifold, a bipolar temperature controller, TC2BIP, and 8-channel valve switch, cFlow8 (Cell MicroControls, Norfolk, VA, USA), as described in Kiedrowski, 2011. Medium inflow and outflow during superfusion was controlled using a custom-made fluidic system shown in Fig. S1B and C.

Neurons and astrocytes were loaded for 5–10 min at 37 °C with 0.2 μM fura-2 AM; HEK-293 cells were loaded for 10 min at 37 °C with 4 μM fura-2 AM. In the experiments carried out on cells growing on plain coverslips, after the loading, the extracellular fura-2 AM was removed by a triple manual wash of the cells with Locke's buffer. In experiments carried out on cells growing in the medium-retaining inserts, the extracellular fura-2 AM was removed by a superfusion with Locke's buffer. The images of fluorescence emitted at 510 ± 20 nm after 340 nm (F340) and 380 nm (F380) excitation were saved for off-line analysis; 4 × 4 binning was used in all experiments. The F340/F380 ratio was used as an index of $[Ca^{2+}]_i$. Background fluorescence was measured in cell-free areas and was subtracted before calculating the F340/F380 ratio. Since the HEK-293 cells were confluent, there was no cell-free area to measure the background. Therefore, the background measured in neuroglial co-cultures was applied to the data from HEK-293 cells. The F340/F380 ratio was calibrated *in situ* (Fig. S2), assuming that fura-2 Ca^{2+} K_d is 224 nM and using the approach described in Grynkiewicz et al., 1985.

2.4. Immunocytochemistry

Immunostaining was carried out using cell cultures growing in medium-retaining Petri dish inserts. After fixing the cells with 4% formaldehyde and washing with PBS (Kiedrowski et al., 2002), 50 μl of a blocking solution containing 0.3% Triton X-100 and 10% goat serum in PBS was applied onto the cells in the wells. To detect astrocytes, a primary monoclonal anti-GFAP (1:500, Sigma G3893) and a secondary FITC-labeled anti-mouse IgG (1:500, Sigma F2266) were used. TH was detected using an anti-TH antibody conjugated with Alexa Fluor® 488 (1:50, Abcam, ab192463). All antibodies were applied in the blocking solution for 2 h at room temperature (22–23 °C).

2.5. Statistical analysis

Statistical analysis was performed on uncalibrated fura-2 F340/F380 ratio data using SigmaStat 3.5 software (Systat Software Inc. Richmond, CA, USA). The data were analyzed using ANOVA on ranks followed by Dunn's test. The differences were considered statistically significant if they reached at least $p < 0.05$.