Journal of Neuroscience Methods 306 (2018) 38–44

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

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

Cell volume changes as revealed by fluorescence microscopy: Global vs local approaches

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ARTICLE INFO

Keywords:
Cell volume changes
Fluorescence microscopy
Morphometry
Calcein

ABSTRACT

Background: Several techniques for cell volume measurement using fluorescence microscopy have been established to date. In this study, we compare the performance of three different approaches which allow for estimations of the cell volume changes in biological samples containing individual fluorescently labeled cells either in culture or in the tissue context. The specific requirements, limitations and advantages of individual approaches are discussed.
New Method: Global morphometric data are quantitatively compared with local information about the overall cell volume, represented by the concentration of a mobile fluorophore accumulated within the monitored cell.
Results: Volume changes induced by variations in the extracellular osmolarity in murine fibroblasts and astrocytes either in the culture or in the acute brain slices were registered by the three- and two-dimensional morphometries and by local fluorescence intensity measurements. The performance of the latter approach was verified using FRAP assessment of the fluorophore mobility. Significantly lower amplitudes of the cortical astrocytes swelling were detected by three-dimensional morphometry, when compared to the other two approaches. Consequently, it failed to detect temperature-induced cell volume changes.
Comparison with Existing Method(s): The three most popular methods of cell volume measurement are compared to each other in this study.
Conclusions: We show that the effectivity of global morphometry-based volumetric approaches drops with the increasing cell shape complexity or in the tissue context. In contrast to this, the performance of local fluorescence intensity monitoring, which is also fully capable of reflecting the instant cell volume variations remains stable, independent of the system used and application.

1. Introduction

The overall cell volume is a carefully controlled parameter in the cellular architecture. Every cell possesses specific mechanisms capable of compensating for rapid volume changes induced by hostile extracellular conditions to a certain extent, such as hypotonic conditions, oxygen, glucose deprivation or ion misbalance. In the central nervous system (CNS), astrocytes respond to any homeostasis disturbance by their swelling, as these cells actively participate in ionic-and neurotransmitter homeostasis maintenance by taking up the excess of K^+ ions/neurotransmitters from extracellular space accompanied by water influx (Cheung et al., 2015; Macaulay and Zeuthen, 2012). They have also developed mechanisms enabling cell volume regulation via the ion/water efflux (Benfenati and Ferroni, 2010; Kimelberg, 2005). Abnormal cell volume regulation represents a serious challenge to the cell adaptability and viability, and stands behind many pathological phenotypes including inflammation, retinal or brain edema, and traumatic or ischemic injury (Kimelberg et al., 1995; Jo et al., 2015; Ryskamp et al., 2014; Pannicke et al., 2006; Papadopoulos and Verkman, 2013). Therefore, measurements of cell volume changes have become a standard and important task for biologists worldwide.

Several techniques for cell volume measurement using fluorescence microscopy have been established in the past two decades. In general, they can be subdivided into two sub-classes: techniques using fluorescence markers only to delimit the cell in the context of the observed culture or tissue, and those deriving relative changes of the cell volume from variations in the intracellular marker concentration.

In the former case, the fluorescent marker is simply present in the cell volume and absent outside the cell. Therefore, fluorescence signal represents a binary indicator of the cell borders here and the cell

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https://doi.org/10.1016/j.jneumeth.2018.05.026
Received 19 April 2018; Received in revised form 31 May 2018; Accepted 31 May 2018
Available online 07 June 2018

volume is measured globally, summing up the volumes of all the labeled pixels. One type of these measurements uses a three dimensional (3D) morphometric analysis of the acquired z-stacks of confocal images, which leads to the direct cell volume assessment (Allansson et al., 1999; Chvátal et al., 2007a; Benesova et al., 2009, 2012; Anderova et al., 2014). Another method detects the changes in the cell volume indirectly, deriving them from the area (S) occupied by the cell in a single two dimensional (2D) image (Dibaj et al., 2007; Hirrlinger et al., 2008; Florence et al., 2012). Under the assumption of isotropic swelling/shrinkage, cell volume is proportional to $S^{3/2}$ (for example, Ryskamp et al., 2014).

The last approach takes advantage of the fact that in general, low concentrations of fluorophores are used for the labeling of biological samples. Under these conditions, the intensity of the excitation beam remains reasonably invariant throughout the observed cell volume. The local fluorescence intensity (F) is thus proportional to the fluorophore concentration and, in the case that a fixed number of freely movable fluorophores is entrapped within a cell, is also inversely proportional to the actual cell volume. Changes of the cell volume can be measured locally as changes in 1/F (Risher et al., 2009). This is of course valid only in the case that fluorescence intensity linearly increases with the dye concentration, i.e. when non-linear processes like the dye self-quenching can be excluded (Hamann et al., 2002).

In this study, we compared the performance of the three aforementioned volumetric methods - 3D morphometry, 2D cell size (area) measurements and fluorescence intensity (concentration) measurements – on a set of cells with progressively increasing shape complexity. Specific requirements, limitations and advantages of the three approaches are discussed.

2. Materials and methods

2.1. Cells and tissues

3T3 murine fibroblasts fluorescently labeled with calcein green AM (www.e-bioscience.com) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) at 37 °C in a 5% CO_2 humidified incubator for 48 h. Cells were harvested using 0.5% Trypsin for 15 min in 37 °C, seeded in full media on the glass plates and cultivated overnight. Prior to observation, cells were incubated with 0.1 μM calcein green AM for 30–45 min at room temperature.

For the visualization of astrocytes GFAP/EGFP transgenic mice (line designation TgN(GFAP-EGFP), FVB background) were used in which the expression of enhanced green fluorescent protein (EGFP) was controlled by the human glial fibrillary acidic protein (GFAP) promoter (Nolte, 2001). Mice were bred in a standard barrier animal house. Both male and female mice were used in the experiments, as no sex-dependent differences in astrocyte swelling had been observed (Benesova et al., 2012). Mice were kept on a 12-hr light/dark cycle with access to food and water *ad libitum*. Altogether 3 pups for astrocyte cultures and 19 mice for preparation of brain slices were used.

Primary astrocyte cultures from the mouse cortex were prepared as follows: after removal of the meninges, cortical tissue of 1-2-day-old GFAP/EGFP mouse pups was triturated and placed on Petri dishes. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of heat-inactivated foetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) at 37 °C in a 5% CO_2 humidified incubator and used between 2 and 4 weeks of growth (all products were obtained from Gibco-Invitrogen). Immunostaining for glial fibrillary acidic protein (GFAP) and the flat, polygonal morphological phenotype of the cultured cells indicated that more than 95% were type-1 cortical astrocytes. After reaching confluence, astrocytes were digested enzymatically by trypsin-EDTA and plated on cover slips at a density of 2–2.5 × 10^4 cells per dish. Morphometric experiments were carried out 3–5 days later.

To prepare the acute brain slices, GFAP/EGFP mice were deeply anesthetized with pentobarbital (PTB; 100 mg/kg, i.p.) and perfused transcardially with a cold (4 °C) isolation solution containing (in mM): 110 NMDG-Cl, 3 KCl, 23 $NaHCO_3$, 1.25 Na_2HPO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 20 glucose, osmolality 305 mOsmol/kg. The animals were decapitated, the brains were quickly dissected out and transversal 200 μm thick slices were cut using a vibrating microtome. The slices were incubated for 30 min at 34 °C in the isolation solution and then transferred to aCSF containing (in mM): 122 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 Na_2HPO_4, 1.5 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose, osmolality 305 mOsmol/kg, where they were kept at room temperature for the duration of the experiments. During the microscopic observation, slices were immobilized in a perfusion chamber (POC-R2, PeCon, GmbH, Germany). Solutions were equilibrated with 95% O_2/5% CO_2 to a final pH of 7.4. Osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Logan, UT). Mice 1–3 months old were used in the volumetric experiments, as no significant age-related differences in cell volume changes were detected in this age range.

All procedures involving the use of laboratory animals were performed in accordance with the European Communities Council Directive, 24 November 1986 (86/609/EEC) and animal care guidelines approved by the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic (Animal Care Committee on April 7, 2011; approval number 018/2011). All efforts were made to minimize both the suffering and the number of animals used.

2.2. Hypotonic and hypertonic treatment/stress

For measurements of cell volume changes induced by osmotic pressure, the cell cultures and/or tissue sections were mounted in the flow chamber with the flow rate of 2 ml/minute and perfused either with artificial cerebrospinal fluid (aCSF; in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose bubbled with 95% O_2, 5% CO_2, pH 7.4, 305 ± 5 mOsmol/kg) or modified aCSF solutions, hypotonic (205 ± 5 mOsmol/kg, containing only 67 mM NaCl) and hypertonic (405 ± 5 mOsmol/kg, containing 177 mM NaCl), to evoke astrocyte swelling or shrinkage, respectively. If not stated otherwise, all experiments were performed at room temperature (23–25 °C).

2.3. Image acquisition

All fluorescence images were acquired on multiphoton laser scanning microscope FV1200MPE (Olympus) with 60x LUMPLFLN water objective (NA = 1.0, WD 2 mm).

In fluorescence recovery after photobleaching (FRAP) experiments the microscope was used in a single-photon confocal mode. GFP fluorescence in cultures of cortical astrocytes of GFAP/EGFP mice was excited by 488 nm line of an Ar laser and detected using a 500–545 nm emission filter. A SIM scanner was used to minimize the time delay between the fluorescence photobleaching in the region of interest (ROI) and the acquisition of the first post-bleach frame. To quantify the initial level of fluorescence, five x-y frames were acquired prior to the bleaching. A circular ROI with a diameter of 2 μm, situated either in soma or primary processes, was bleached with 50 ms pulse of 405 nm laser at maximum intensity. The recovery of fluorescence was monitored at 0.14 s intervals for 30 s. Measured fluorescence intensities were corrected for the scan-induced photobleaching and normalized to the fluorescence intensity measured in a pre-bleach period. The curve fitting was performed in SigmaPlot 12.5 (Systat Software Inc.).

In volumetric measurements, fluorescence of calcein green or GFP was excited in a two-photon absorption mode at 950 nm by a tunable Ti-Sapphire laser system MaiTai DeepSee (Spectra Physics, CA). Fluorescence signal selected by 495–540 nm band-pass emission filter was detected by GaAsP detector. In each acquisition, a cell was