Methods 66 (2014) 325–329

Contents lists available at ScienceDirect

Methods

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

A versatile tool for the analysis of neuronal survival

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ARTICLE INFO

Article history:
Available online 24 August 2013

Keywords:
Cell death
Apoptosis
Transfection
Neurodegeneration
Image cytometry
Fluorescent proteins

ABSTRACT

To understand the principles that govern mechanisms of neuronal survival or death it is necessary to systematically model these processes. Methods involving overexpression or knockdown of a gene of interest using non-viral transfection of primary neurons can easily be adapted to study cell death pathways in primary neurons. However, common biochemical approaches to measure cell death are insufficient to measure neuronal viability in these systems. To investigate the functional role of genes in cultured neurons, we therefore established a cell-based assay using a cotransfection/cocultivation approach in primary cortical neurons from mouse or rat. Using this method, it is possible to use well-established cell culture models of neuronal damage, and to analyze cell survival in genetically different neurons on a single-cell basis following apoptotic stimuli under identical conditions. The duration of the entire protocol is 10 days. Finally, the method may be applicable to a wide range of damage models, primary cells, and cell lines as well as it can be used for high content screening (HCS) studies and downstream image cytometry.

1. Introduction

A complex interplay of intracellular signaling cascades and biochemical pathways as well as intricate cell-to-cell communication are required for normal functioning of the brain [1]. Disruption of one or several of these homeostatic pathways contributes to a variety of disorders of the brain [1,2]. Neurons are particularly vulnerable to disruption of homeostasis and rapidly undergo cell death if energy supplies cease [1], for example after a stroke [2,3]. Systematic modeling of the underlying mechanisms enables insight into the principles that govern neuronal survival or death. Methods for non-viral transfection of primary neurons are well established [4] and can easily be adapted to study cell death pathways in primary neurons through overexpression or knockdown of a gene of interest (GOI). Although electroporation is usually well-tolerated by neurons, the transfection efficiency in long-term culture (i.e. >7 days) is about 20–30% (Mergenthaler, unpublished data). Therefore, common biochemical approaches such as measuring lactate dehydrogenase release to quantify cell death are insufficient to measure neuronal viability after transfection because transfected neurons cannot be distinguished from untransfected neurons.

Here, we therefore describe a versatile cell-based assay using a cotransfection/cocultivation approach in primary cortical neurons from mouse or rat. Using this method, it is possible to use well-established cell culture models of neuronal damage to analyze cell survival or cell death in genetically distinct cells following apoptotic stimuli under identical conditions. The protocol is essentially divided into four steps. It includes the preparation of primary neuronal cells from mouse or rat embryos, the transfection of neurons using Nucleofection™ (i.e. electroporation), and subsequent culture for 9 days, counting of transfected neurons, and using metabolic deprivation (e.g. hypoxia or aglycemia) to injure neurons. Finally, surviving transfected neurons are counted. In total, the protocol takes 10 days to complete. Importantly, other models of acute or chronic neurodegeneration can easily be implemented into the protocol. Likewise, the protocol can easily be adapted to suit a large variety of different types of experimental studies. For example, shorter or longer culture periods depending on the experimental paradigm can be implemented without any further adjustment of the protocol. Simple studies using manual equipment and analyzing only a few conditions ranging to large scale high content screening (HCS) studies can be performed using this protocol. We provide a protocol to perform all essential steps as well as highlight important aspects of image analysis by image cytometry.

2. Description of methods

2.1. Plasmids and cloning

All plasmids are based on pCAG-MCSn1, which allows for constitutive expression of transgenes in primary neurons [5]. Plasmids are available through Addgene (www.addgene.org): pCAG-MCSn1 (Addgene plasmid 45996), pCAG-eGFP (Addgene plasmid 45997), pCAG-mOrange (Addgene plasmid 45998), pCAG-H2b-eGFP (Addgene plasmid 45999), pCAG-H2b-mOrange (Addgene plasmid

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http://dx.doi.org/10.1016/j.ymeth.2013.08.023

46000), pCAG-Bcl_{XL} (Addgene plasmid 46001). Plasmids and cloning are described in detail in Mergenthaler et al., 2012 [5].

2.2. Preparation of primary neurons

We use a protocol based on Brewer et al., 1993 [6] for the preparation of primary neurons from embryonic mouse or rat brain cortex in single-cell suspension [5,7]. Several in-depth protocols outlining the procedure are available for this purpose (e.g. refer to [8,9]). The optimal gestational age is E15 for mouse and E17 for rat. Parent animals should only be mated for a few hours to ensure exact age of the embryos. In order to generate homogeneous neuronal preparations, cortical and subcortical structures should be separated (e.g. remove hippocampus, striatum, olfactory bulb, etc. [8,9]) rather than generating cultures from entire brains [10].

2.3. Transfection of neurons using electroporation

Neurons are transfected on a Nucleofector device (e.g. Nucleofector-I/-II, Amaxa, Lonza) immediately after preparing the cells. Program O-003 for rat or O-005 for mouse, 5 μg DNA per plasmid, and 3×10^6 cells per transfection should be used. Electroporation buffer (EB, 192 mM NaCl, 9.6 mM KCl, 15 mM $MgCl_2$) and standard electroporation cuvettes (2 mm universal fit electroporation cuvettes with long electrodes) can be used instead of commercial kits on a Nucleofector-I/-II device. Neurons should not be left in EB or transfection reagent for more than 10 min. After electroporation, immediately resuspend neurons in DMEM (e.g. Biochrom) containing 3.7 g/l $NaHCO_3$, 4.5 g/l D-glucose, 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine. Plate cells at a final density of 3.1×10^5 cells/cm^2. Replace medium with $^1/_3$ fresh and $^2/_3$ conditioned DMEM (add glutamate to a final concentration of 25 μM) after 4–6 h and replace again with $^1/_3$ fresh and $^2/_3$ conditioned NBM-A (LifeTechnologies, containing B27, 1% penicillin/streptomycin, 0.5 mM L-glutamine, 25 μM glutamate) after 16–20 h. After 5 days, partially replace medium (about $^1/_3$ of total culture volume) with fresh NBM-A (without glutamate).

2.3.1. Note

The desired density of the culture may depend on the particular experimental paradigm. It is affected by the overall viability of the culture (e.g. percentage of dead neurons after preparation). Electroporation itself also influences neuronal survival. A large proportion of cells dies after the procedure, but the remaining cells grow well in culture. It may be desirable to decrease or increase cell density for other applications (e.g. immunostainings).

2.4. Preparation of conditioned medium

The use of conditioned medium may enhance the overall condition of the culture after transfection. Preparation of conditioned medium can be performed in large batches prior to the start of the experiments. Primary neurons should be cultured under standard conditions. Every three days, half the medium (DMEM or NBM-A) should be removed and replaced by fresh medium for a total of 10 days. Pool and store medium at 4 °C. When all medium has been collected, centrifuge medium (1000*g*, 10 min, 4 °C) to remove cellular debris and freeze at −80 °C for long-term storage.

2.5. Analysis of neuronal survival by cotransfection/cocultivation

To differentiate different neuronal populations, one population is transfected with green-fluorescent protein (GFP) and the other one with orange-fluorescent protein (mOrange). See Fig. 1 for a schematic overview. Alternatively, fluorescent proteins targeted to the nucleus (e.g. H2b-GFP and H2b-mOrange) can be used. After transfection, GFP-transfected neurons and mOrange-transfected neurons in suspension are mixed 1:1. By plating out this mix, the two different cell populations are then cocultivated. To investigate the impact of a transgene on the regulation of neuronal fate, a gene of interest is cotransfected with one fluorescent protein (e.g. GFP). We use the potent antiapoptotic and neuroprotective gene Bcl_{XL} as a positive control and single transfections of the fluorescent proteins as negative control in all experiments [5].

After 9 days in culture, immediately before a damaging event (we use hypoxia or aglycemia for metabolic deprivation of neurons; see Section 2.7), and 24 h after the damaging event, we count green- and red-fluorescent cells (or nuclei) in the same area of each well. In all experiments, before damage at least 500–700 cells should be counted per condition per experiment. The ratio of green- and red-fluorescent cells should be calculated (Ratio = "number of green-fluorescent cells"/"number of red-fluorescent cells"). It serves as a measure of the viability of the different cell populations. A ratio of 1 demonstrates that a transgene has no influence on neuronal survival. A ratio >1 (e.g. as in the positive control Bcl_{XL}) indicates a protective effect of a transgene, whereas a ratio <1 indicates a damaging effect. Furthermore, calculating the ratio before and after the damaging event also provides an easy way for normalization of the data for subsequent statistical summary and analysis of different independent experiments.

2.6. Microscopy

Any research-grade inverted fluorescence microscope can be used. Depending on the microscope and the particular experimental paradigm, 20× or 40× objective lenses with high numerical aperture (NA) should be used. We used Leica HCX PL FL L 20×/0.40 or Leica HCX PL FL L 40×/0.60 lenses for our studies. We have used both basic microscopes (Leica DM IL) and fully automated fluorescent microscopes (Leica DMI 6000) with good success. Use specific filters for GFP and mOrange (e.g. Chroma, 490/20×, 505bs, 525/36 m for GFP; Chroma, 555/25×, 575bs, 600/32 m for mOrange). Many research grade microscopes are now fully automated or can be equipped for full automation and can even be implemented into fully automated research environments [11]. Using automated equipment greatly facilitates image acquisition and ensures imaging of the same areas in each well at all time points. In our studies, we use the software tool HCS A by Leica Microsystems for automated image acquisition.

2.7. Metabolic deprivation of neurons

For metabolic deprivation of neurons, oxygen–glucose deprivation (i.e. in vitro ischemia model, OGD), oxygen deprivation (OD), or glucose deprivation (GD) can be performed. For OGD and GD, wash cultures with phosphate-buffered saline (PBS) twice and add BSS_0, for OD add BSS_1. BSS_0: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 10 μM glycine, 1.8 mM $CaCl_2$, 1 mM HEPES; BSS_1 = BSS_0 + 1 mM glucose. OGD and OD can be performed at 0.3% O_2, 5% CO_2, 37 °C for 3 h (OGD) or 6 h (OD) in an In Vivo 2–400 hypoxia chamber (Ruskinn) or at 0% O_2 in a Concept 400 anaerobic chamber (Ruskinn). GD is performed at 21% O_2, 5% CO_2, 37 °C for 6 h.

2.8. Cell counting and automated image analysis

Cell counting can be performed live while sitting at the microscope or after acquisition of microscopy images for all conditions. The latter is the basis for automated image analysis/image cytometry. Many software tools for this application exist and basic operations in image analysis can be performed using the common image processing tool ImageJ. However, when working with larger