Journal of Neuroscience Methods 280 (2017) 1–10

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

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

Research Paper

An improved method for growing neurons: Comparison with standard protocols

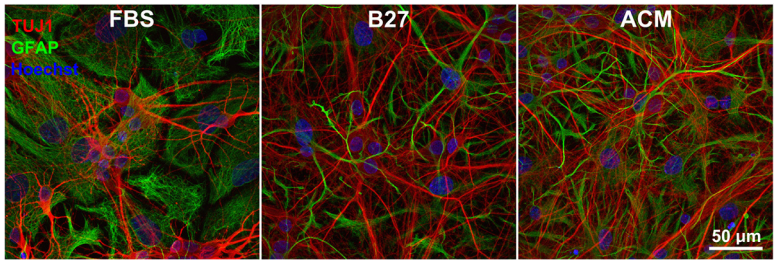
Diletta Pozzi[a,1], Jelena Ban[a,b,1], Federico Iseppon[a], Vincent Torre[a,*]

[a] Neurobiology Sector, International School for Advanced Studies (SISSA), via Bonomea 265, 34136 Trieste, Italy
[b] Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, 51000 Rijeka, Croatia

HIGHLIGHTS

- An improved protocol for primary hippocampal cell cultures is proposed.
- The method relies on serum-free astrocytes conditioned medium (ACM).
- The ACM method is extensively compared with other two commonly used protocols.
- ACM improved morphology and function of both short- and long-term cultures.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 November 2016
Received in revised form 17 January 2017
Accepted 22 January 2017
Available online 27 January 2017

Keywords:
Neuronal culture
Astrocyte
B27 Supplement
Fetal bovine serum
Neuronal branching
Calcium signaling

ABSTRACT

Background: Since different culturing parameters – such as media composition or cell density – lead to different experimental results, it is important to define the protocol used for neuronal cultures. The vital role of astrocytes in maintaining homeostasis of neurons – both *in vivo* and *in vitro* – is well established: the majority of improved culturing conditions for primary dissociated neuronal cultures rely on astrocytes.
New method: Our culturing protocol is based on a novel serum-free preparation of astrocyte – conditioned medium (ACM). We compared the proposed ACM culturing method with other two commonly used methods Neurobasal/B27- and FBS- based media. We performed morphometric characterization by immunocytochemistry and functional analysis by calcium imaging for all three culture methods at 1, 7, 14 and 60 days in vitro (DIV).
Results: ACM-based cultures gave the best results for all tested criteria, *i.e.* growth cone's size and shape, neuronal outgrowth and branching, network activity and synchronization, maturation and long-term survival. The differences were more pronounced when compared with FBS-based medium. Neurobasal/B27 cultures were comparable to ACM for young cultures (DIV1), but not for culturing times longer than DIV7.
Comparison with existing method(s): ACM-based cultures showed more robust neuronal outgrowth at DIV1. At DIV7 and 60, the activity of neuronal network grown in ACM had a more vigorous spontaneous electrical activity and a higher degree of synchronization.
Conclusions: We propose our ACM-based culture protocol as an improved and more suitable method for both short- and long-term neuronal cultures.

1. Introduction

Primary dissociated neuronal cultures, obtained from embryonic or postnatal rodent brain regions such as the hippocampus or the cortex, represent a well-established *in vitro* model for studying neuronal networks, both on a short (network formation) and a long

* Corresponding author.
E-mail address: torre@sissa.it (V. Torre).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jneumeth.2017.01.013
0165-0270/

term (differentiation, maturation) scales (Dotti et al., 1988; da Silva and Dotti, 2002). When cells are dissociated, plated on appropriate substrates and cultured in appropriate media, they grow processes and form *ex-novo* a functional network.

Several media (N2, DMEM/F12) supplemented with serum, most commonly from fetal bovine (FBS) or horse origin, are commonly used for neuronal cultures. However, the chemical composition of the animal serum is not fully defined and includes some factors not present in the brain; moreover, the production of commercial serum is prone to batch to batch variability (Zheng et al., 2006; Arigony et al., 2013). For these reasons, the use of a chemically defined, serum-free medium is recommended for studies in which a complete control of the environment in the culture medium is required (Kivell et al., 2000). Several attempts were made in order to find serum free – media for optimal neuronal growth. Currently, the most widely used is Neurobasal medium with B27 supplement (Brewer et al., 1993; Beaudoin et al., 2012). Moreover, several modifications to Neurobasal/B27 were proposed with the addition/modification of glycoproteins, lipid and hormone molecules (NbActiv4, NS21) (Chen et al., 2008; Roth et al., 2010; Brewer et al., 2008). Most of those protocols were designed to promote long-term neuronal survival in an almost pure neuronal culture: neurons of embryonic (E18) and postnatal (P0) origin were cultured in the presence of <1% and 6–8% of glial cells, respectively (Beaudoin et al., 2012; Cullen et al., 2010).

However, the role of astrocytes for maintaining the homeostasis of neuronal cells both *in vivo* and *in vitro* has been clear for a long time (Araque and Navarrete, 2010; Nedergaard et al., 2003; Barker and Ullian, 2008; Allen and Barres, 2009; Banker, 1980). Different options are now available for a successful hippocampal cell culture, all of them involving astrocytes: I- direct plating of dissociated neurons on a glial feeder layer (Ivenshitz and Segal, 2010), II- suspension of the coverslip with plated neurons above a glial feeder layer (Kaech and Banker, 2006) and III- culturing neurons in astrocyte-conditioned medium (ACM). This third method has an advantage: it does not require a feeder cell's layer while providing all the soluble factors released by astrocytes, such as growth factors, signaling molecules and lipids (Hassanpoor et al., 2013; Ebrahimi et al., 2016; Mauch et al., 2001).

ACM has been demonstrated to increase neuronal survival and stem cell differentiation (Yamashita et al., 1992; Nakayama et al., 2003; Todd et al., 2013). However, the use of ACM in cell culturing protocols is still uncommon and often limited to *in vitro* models of pathologies, where its protective effect on neurons following mechanical injury and hypoxia has been demonstrated (Yan et al., 2013). Standard neuronal culturing methods are mostly based on serum-enriched or serum-free, supplement-enriched media (Beaudoin et al., 2012), both of which rely on commercially available resources. ACM, instead, can be obtained by the researcher from the same animal species used for primary neuronal cell's cultures.

Our goal is to establish an optimal, easy to use and reproducible culturing protocol for obtaining viable, functionally connected and long – term surviving primary hippocampal cultures. For this purpose, we compared two traditional culturing protocols with a new method based on the use of ACM as a growing medium. Our method differs from other previously published protocols (Todd et al., 2013) because the medium is produced in house and it is totally serum – free, unlike the commercial ACM obtained by conditioning of traditional astrocyte medium (supplemented with 10% FBS).

Our comparison is based on a morphometric and functional characterization of neuronal networks grown in the three different media. Above all, we describe a serum – free culturing method for long – term neuronal cultures with preserved functionality and connectivity, reminiscent of that seen in the intact tissue. The three culturing protocols that we compared were: I- FBS based medium (traditionally used in our lab (Ulloa Severino et al., 2016) and by other research groups (Ivenshitz and Segal, 2010; Cohen et al., 2008; Hughes et al., 2010); II- Neurobasal/B27 medium as described in Beaudoin (Beaudoin et al., 2012); III- our serum – free ACM medium. The exact composition for all the three growing media is reported in Section 5 and the conditions are referred to as FBS, B27 and ACM, respectively. The comparison between the three protocols was carried out by maintaining the same experimental parameters (cell density, animal age and functional tests). Therefore, the differences in morphology and electrical activity are attributed to the effect of the different media.

2. Results

2.1. Setting of the plating protocol

We analyzed the morphology, electrical activity and long-term survival of our cell cultures grown in the three different media. All other parameters such as coverslip coating, plating medium and cells density were the same in the three cases. Briefly, after dissociation, the cells were suspended in plating medium (Neuronal medium supplemented with 10% FBS, see Section 5 and Supplementary Table S1 for the complete formulation), seeded at a density of 100,000 cells/sample and incubated at 37 °C for half an hour in order to allow the cell's attachment on the coverslips previously coated with polyornithine (see Section 5). After that, the sample was divided in three groups – in this work referred to as FBS, B27 and ACM – and added to the appropriate growing medium. We used the same plating medium in the three cases because when cells were plated in serum-free Neurobasal/B27 medium, cells death increased and no electrical activity was detectable. Interestingly, when plated in ACM medium, the cells were able to survive for more than one month with consistent electrical activity (Supplementary Video S1).

2.2. Growth cones' morphology

Twenty-four hours after plating, we analyzed the cell morphology by immunocytochemistry. Cells were fixed and stained for filamentous actin (F-actin) and for β-tubulin III, a marker for post-mitotic neurons. At a first inspection, we observed a higher cell growth in Neurobasal/B27-based media, compared to FBS at DIV1. In particular, neuronal growth cones (GCs) were larger, thicker, richer in F-actin and had a more elaborated morphology with many filopodia (Fig. 1**A–C**). To quantify those differences, we measured and compared the surface of GCs among the three conditions. Confocal images were acquired with slice spacing of 0.2 μm, up to 5 μm z-stack thickness, and for each GC a maximum intensity projection of the stacks for F-actin was used. The contours of each GCs were manually traced and the surface was measured using ImageJ/Fiji. At least 20 GCs for each condition were analyzed (see Section 5). The GCs in Neurobasal/B27-based media were significantly larger comparing to FBS. GCs in ACM, in particular, showed the biggest size; however, the difference between ACM and B27 was not statistically significant ($36.27 \pm 1{,}69\ \mu m^2$ in FBS, $86.27 \pm 10.63\ \mu m^2$ in B27 and $102.19 \pm 8.60\ \mu m^2$ in ACM; Fig. 1**D**).

A similar analysis was done by counting the number of filopodia in each GC (Fig. 1**F**): GCs in FBS-based medium had on average 9.48 ± 0.62 filopodia, B27 19.72 ± 1 while ACM 24.24 ± 1.76, matching the most complex morphology observed in B27-based media (examples of elaborated forms – never observed in FBS – are shown in Supplementary Fig. S1).