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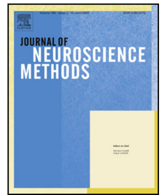

Basic Neuroscience

Long-term primary culture of neurons taken from chick embryo brain: A model to study neural cell biology, synaptogenesis and its dynamic properties

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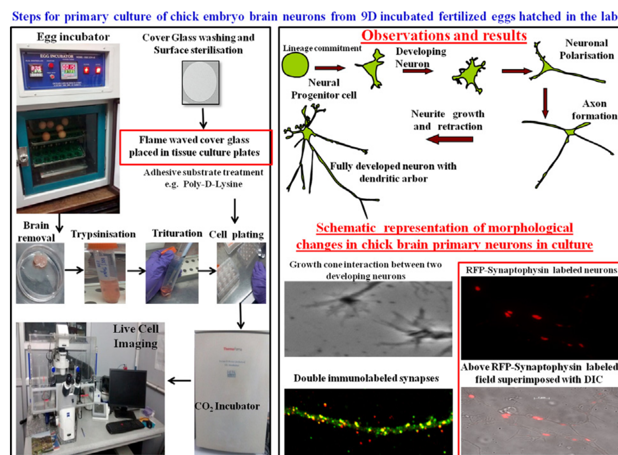
HIGHLIGHTS

- Simple method of glia less primary culture of neurons from chick embryo brain.
- Embryos were hatched in the laboratory from post-fertilized 9 day incubated eggs.
- Cytomorphology and immunostained cultured neurons were studied for at least 35 days.
- Early, late growth events and synaptogenesis were followed under live cell imaging.
- Axonal and SV dynamics were tracked in transfected live neurons and evaluated.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Studying neuronal growth, development and synaptogenesis are among the hot research topics. However, it is faced with various challenges and technical limitations that include but not limited to donor's species and health, threat to life, age of embryo, glial contamination, real-time tracking, and follow-up.

New method: We have successfully standardized a method for long-term primary culture of neurons collected from post-fertilized 9 day incubated chicken embryo brain overcoming the limitations mentioned above. Fertilized eggs were incubated in the laboratory and neurons from the embryonic brain were collected and low-density culture, apparently without glial contamination, was studied at least for 35 days in vitro (DIV).

Results: Neurons were characterized by double immunostaining using stringent neuronal and glial markers. Neuronal differentiation, cytomorphology, neurite and axon formation, development and maturation, spine formation and synaptogenesis were tracked in real-time in a stage and time dependent manner. The neurons were transfected with Synaptophysin-RFP to label synaptic vesicles, which were followed in real-time under live-cell imaging.

Abbreviations: Ab, antibody; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; DIV, days in vitro; DMEM, Dulbecco's modified Eagle's medium; GFAP, glial fibriliary acidic protein; HH, Hamburger and Hamilton; MAP-2, microtubule associated protein-2; PBS, phosphate buffer saline; PSD-95, post synaptic density protein-95; RFP, red fluorescence protein; SV, synaptic vesicle; EtOH, ethanol.

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Comparison with existing methods: Every step was carried out under controlled laboratory conditions. Eggs are easily available, easy to handle, neurons from desired day of incubation could be conveniently studied for long period in apparently glia-free condition. In addition to common factors affecting primary culture, selection of culture media and cover glass coating are other key factors affecting neuronal cultures.

Conclusions: We describe an inexpensive, simpler pure primary neuronal culture method for studying neuronal cell-biology, synaptogenesis, vesicular dynamics and it has potential to grow 3D-multilayered brain in vitro.

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1. Introduction

The human brain is a complex modular organ consisting of billions of neurons and about 1000-fold more synaptic connections among them. During development, early spherical neural progenitor cells give rise to many processes, the neurites; one of these early neurites subsequently transforms into an axon while others develop into dendrites. The growing axons that come in contact with other neurons form terminal presynaptic swellings. These presynaptic swellings possess specific neurotransmitter as well as cognate receptors; they also influence the post-synaptic neurons to express desired receptors. The number of presynaptic swellings, their morphometric characteristics, receptor decoration and other properties may be increased, modified or lost. These individual or collective changes influence somatic and autonomic behaviors including cognition as well as consciousness, sensitivity, new and/or existing learning and memory, or recovery processes following an injury or a disease (Cajal, 1894; Kandel and Spencer, 1968; Mayford et al., 2012). Apparently some of these connections last throughout life while others get modulated, replaced or lost or newly formed. Our understanding of how neurons form such complex, dynamic, spatio-temporally oriented cytomorphologically, neurochemically and anatomically-functional circuitry is still in its infancy.

Studying the neuronal growth and developmental events in vivo in the brain particularly in the animals higher in evolution including humans is desirable; however, in vivo imaging of neurons has various technical and other limitations and concerns. Therefore, it is imperative to select appropriate model(s) to investigate the core and fundamental questions in biology in general and neural as well as brain biology in particular. Experimental studies have been conducted in developing brain as such and/or in primary cultures of neurons derived from the developing brains of various species, e.g. rats, mice, fish, etc. (Darbinyan et al., 2013). Collecting embryos is not very easy, particularly from higher mammals including humans; also it raises various ethical concerns. Surgical removal of embryos from higher animals may pose danger to donor's life and even if one embryo is needed, the remaining embryos in the litter may be wasted or their lives could be at risk. Such complications may be significantly avoided using fertilized chicken eggs.

Genetic manipulation in higher mammals may also lead to embryonic lethality due to complexity as well as complications during course of the procedure in mother's womb (Andermatt and Stoeckli, 2014; Baeriswyl and Stoeckli, 2006). However, external manipulation on chicken embryo (for example) could be relatively easily and precisely targeted with respect to time and space (El-Ghali et al., 2010; Fyfe and MacMillan, 1983). It is important to precisely know the time of fertilization because it has been reported that some genes are on/off or some factors are expressed/repressed only during a specific post-fertilization temporal window during development (Ebendal and Persson, 1988; Godfrey and Shooter, 1986; Hevor et al., 1988). However, it is extremely difficult to know the precise time of fertilization and therefore, it is inherently difficult to temporally correlate and synchronize the post-fertilization developmental events. As a compromise closest we can practically reach is to standardize the incubation period of the fertilized eggs to obtain embryo of desired cell-numbered stage for further study. Accordingly, in this report we present a protocol for primary culture of neurons isolated from chick embryo brains at 9 day incubated post-fertilized eggs. We have successfully standardized the method, such that it provides reproducible and consistent results.

2. Materials and methods

2.1. Equipment

CO_2 incubator (Model No. 300498-4247 Thermo Forma, USA), Egg-incubator (Model No. WZ16, Dayal Poultry Applicant, India, and Model No. OLSC-234-15, Ocean Life Science Corp., India), Haemocytometer (Neubauer improved, Marienfeld, Germany), Laminar flow (Atlantis Cleanair, India), Live cell imaging microscope (Axio-Observer.Z1, Carl-Zeiss, Germany fitted with Camera AxioCam MRm, CO_2 Module S, Temperature Module S, Heating Unit XLS and Incubator XL-S1 fitted with temperature sensor P†100), Sceptor (Handheld automated cell counter, Cat. No. PHCC00000, Millipore, Germany), Spinning disk microscope (Nikon Eclipse Ti, Japan) fitted with spinning disk (CSU-X1, YOKOGAWA) and an EMCCD camera (Andor-iXon3 Model No. DU897, Andor Technology), and Upright microscope (Eclipse TS 100, Nikon, Japan).

2.2. Source of the eggs and their incubation

Kuroiler hatching eggs from white leghorn chicken (*Gallus gallus domesticus*) were procured from Keggfarms Pvt. Ltd., Gurgaon, Haryana, India. The eggs were incubated in the laboratory using an egg incubator Model No. OLSC-234-15, Ocean Life Science Corp., India.

2.3. Reagents and consumables

0.25% trypsin–EDTA (Cat. No. 59428C Sigma–Aldrich, USA), 1,4-diazabicyclo[2.2.2]octane (DABCO) (Cat. No. D2522, Sigma–Aldrich, USA), 12 well tissue culture dish and 35 mm dish (Eppendorf, Germany), disposable syringes (Batch No. 315204JG1, Hindustan Syringe and Medical Devices Limited, India), 100 ml plastic Petri dish (Lot No. 03910501, Corning, USA), Amphotericin B (Cat. No. 15290-018, Invitrogen, USA), B-27 supplement-50× (Cat. No. 17504044, Gibco, USA), Confocal cover glass bottom dish 35 mm (D35-20-1-N, 35 dish, 20 mm Microwell #1 Glass sterile, In Vitro Scientific, USA), Cytosine β-D-arabinofuranoside (Cat. No. C1768, Sigma–Aldrich, USA), 4′,6-diamidino-2-phenylindole (DAPI) (Cat. No. D9542, Sigma–Aldrich, USA), Dulbecco's modified eagle's medium (DMEM) (Cat. No. D6429, Sigma–Aldrich, USA), Ethanol (EtOH) (Reagent grade, Merck, Germany), Ethylene diamine tetra-acetic acid disodium salt (EDTA, Cat. No. 12635, Qualigens Fine Chemicals, Mumbai, India), Fetal bovine serum (Cat. No. 16000044, Gibco, USA), Hank's balanced salt solution 1×, Laminin (5 μg/ml Cat. No. L2020 Sigma–Aldrich, USA), Methanol (Cat. No. SK25620739,

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