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MODERN SLICE CULTURE FOR DIRECT OBSERVATION OF PRODUCTION AND MIGRATION OF BRAIN NEURONS

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ABSTRACT

For the understanding of histogenetic events in the three-dimensional brain primordia, direct observation of progenitor cells and young neurons is required. Although slice culture, which is one of the tissue or organ culture methods, effectively preserves the *in vivo* microenvironment where normal developmental processes occur, conventional phase-contrast microscopic observation of brain slices fails to provide good visibility of single cells. However, a combination of slice culture with the use of fluorescent dyes and/or the introduction of fluorescent protein genes provides live, three-dimensional information on cytogenetic and histogenetic events at the individual cell level. Dynamic cellular behaviors can then be vividly captured without destroying tissue structures.

Key Words: brain, development, tissue culture, cell migration

INTRODUCTION

Since the pioneering work of Ross Harrison in 1907[1)] in which axonal growth was first observed from fragments of frog neural tube into clotted lymph, “cell culture” and “tissue culture” have become obligatory tools in neurobiological research (Fig. 1).

“Dissociated-cell culture” (or simply “cell culture”) was established when trypsinization techniques were developed,[2,3)] and is still widely used in morphological and electrophysiological analyses. One of its greatest merits is that it allows us to easily observe the behavior of individual cells under a phase-contrast microscope. For example, in the field of neural development, axonal and dendritic formation of a single neuron[4)] as well as division of a single progenitor cell[5,6)] can be monitored live. However, this cellular individuality *in vitro* is obtained at the cost of the cellular complexity which is a normal characteristic of the developing brain.

In contrast, such three-dimensional cytoarchitecture is preserved, at least in part, in “tissue culture” (culture of a whole brain region, slices, or fragments). “Organotypic slice culture”, which was developed in the 1950s,[7-12)] allows slices to retain their basic structural organization. Although in classical slice-culture methods, the occurrence of the morphogenetic events such as

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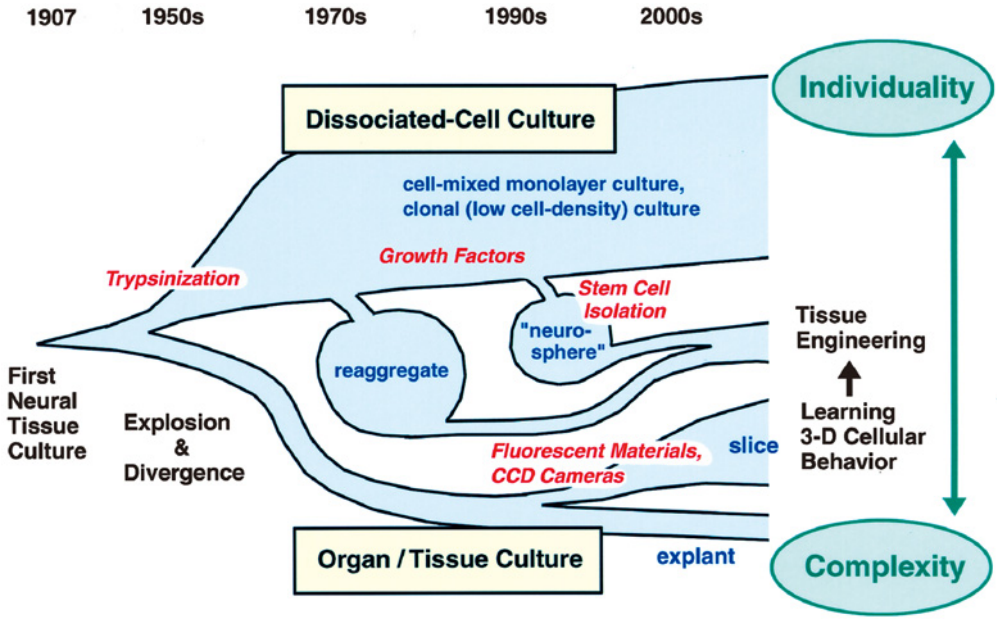

Fig. 1 History and future of the cell and tissue culture in the field of developmental neurobiology. Development of enzymatic digestion techniques (trypsinization) has led to methodological divergence into two major currents, *i.e.,* "dissociated-cell culture" and "organ/tissue culture." In the current of "cell culture," establishment of chemically-defined media supplemented with various kinds of growth factors along with advances in clonal (low cell-density) culture have led to successful isolation or enrichment of neural stem cells in the 1990s. On the other hand, "tissue culture" was greatly advanced in the same decade by the introduction of fluorescent materials and CCD cameras. As described in this article, fluorescent-based slice culture, where cellular individuality and complexity meet, is now assuming a central role in the elucidation of developmental mechanisms. Further methodological convergence that will establish a link between single stem cells and 3-D brain-like structures can be expected in the near future, probably by utilizing the knowledge obtained from slice culture in facilitating singly dissociated stem cells to form tissue-specific 3-D structures.

the production, migration, and maturation of neurons, could be assessed in cultured slices after they were fixed and examined histologically or immunohistochemically,[13] direct monitoring of dynamic cellular behavior was not possible.

In the 1990s, however, a modern slice-culture technique was developed by Susan McConnell and colleagues[14,15] and Hitoshi Komuro.[16] Confocal microscopy of cerebral and cerebellar slices treated beforehand with a solution of lipophilic fluorescent dye, DiI, was effective in monitoring the behavior of some (but not all) cells in the intended field, providing stop-action films that vividly captured the process of cell division and neuronal migration. Fluorescent-based slice-culture techniques, further supported by advances in fluorescent materials and imaging equipment, including CCD cameras and computers, are now providing live, three-dimensional information on cytogenetic and histogenetic events at the level of an individual cell.[17-23]

In this article, the authors will describe the standard protocols of DiI-based slice culture that we are currently using, and show examples of time-lapse observations of neuron birth in the developing mouse neocortex.

MATERIAL AND METHODS

DiI labeling, slicing, and embedding into collagen gel

Brains isolated from the skull and freed from meninges (Fig. 2A) were transferred to DMEM/F12 (1:1) culture medium containing extremely fine crystals of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, DiI C18(3) (Molecular Probes, OR, USA) (Cat.#D-282). This DiI suspension was made by adding 1 ml of a DiI stock solution (10 µg/ml in ethanol) to 10 ml of DMEM/F12, and was kept on ice until use (for 10–20 minutes). The brains were treated with the DiI suspension for 5 minutes on ice and were then washed immediately with fresh DMEM/F12. The labeled brains were transferred with DMEM/F12 onto a silicon rubber-coated (5–10 mm thick) petri dish and sliced (200–300 µm thick) manually using microknives developed for corneal surgery (Fig. 2B,C). Slices were then transferred to 35-mm plastic dishes and embedded in type I collagen solution (Cellmatrix IA; Nitta Gelatin, Osaka, Japan). DMEM/F12 supplemented with insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), sodium selenate (30 nM), putrescine (60 µM), EGF (10 ng/ml), bFGF (10 ng/ml), horse serum (5%), and fetal calf serum (5%) was used for culture.[18,21-23)]

Imaging

The slices were observed using a florescent microscope (IX70; Olympus, Tokyo, Japan) equipped with a 20x objective lens (LCPlanF1; numerical aperture = 0.40, working distance = 6.9 mm). Recordings were carried out manually using a CCD camera (SenSys 0401; Photometrics, Tokyo, Japan) and IPLab 3.5 (Scanalytics Inc., Fairfax, VA, USA) software. Between recordings, the slices were maintained under high oxygen (40%) conditions (Miyata et al., 2002). If the cells to be followed were not oriented parallel to the stage, several images were obtained in different focal planes and subsequently reconstructed using Photoshop 5.0 software (Adobe, CA, USA).[18,21-23)]

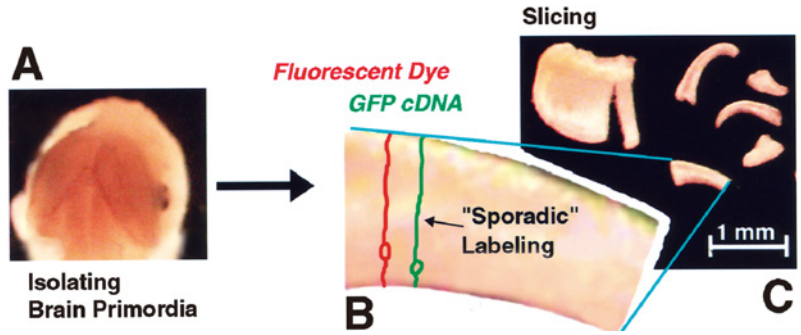

Fig. 2 Flow of fluorescent-based slice culture. Embryonic mouse brains isolated from the skulls of mice at embryonic day 13–15 (A) were treated with a suspension of DiI, lipophilic dye, which leads to random and sporadic labeling of single progenitor cells that span the brain walls (B, bipolar-shaped; see also Fig. 4) from the surface of the brain walls. For visualization of cells with GFPs, either electroporation of cDNAs into the brain walls or infection with GFP-containing viruses are carried out, usually prior to brain isolation. Brain slices (either embedded in collagen gel (C) or grown on membrane filters) are then subjected to fluorescent microscopic observation.

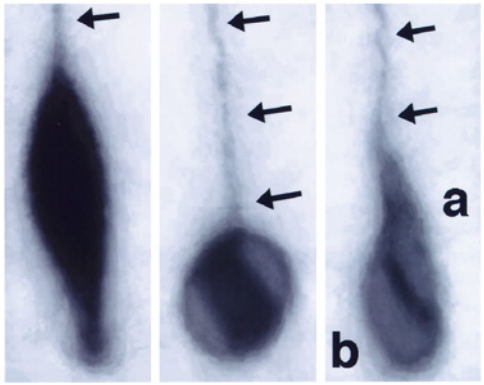

Fig. 3 Division of a singly DiI-labeled progenitor cell in slice culture. Magnified view of a progenitor cell moving downward to the ventricular (inner) surface of the cerebral wall (left), its division (center, 30 minutes later), and its daughter cells (right, at 60 minutes), presented as an inverted version of fluorescent images. A process (arrow) extending from the cell body towards the pial (outer) surface belonged to the right compartment of the anaphase cell body (B), and was then inherited completely by the daughter cell ***a*** (C).

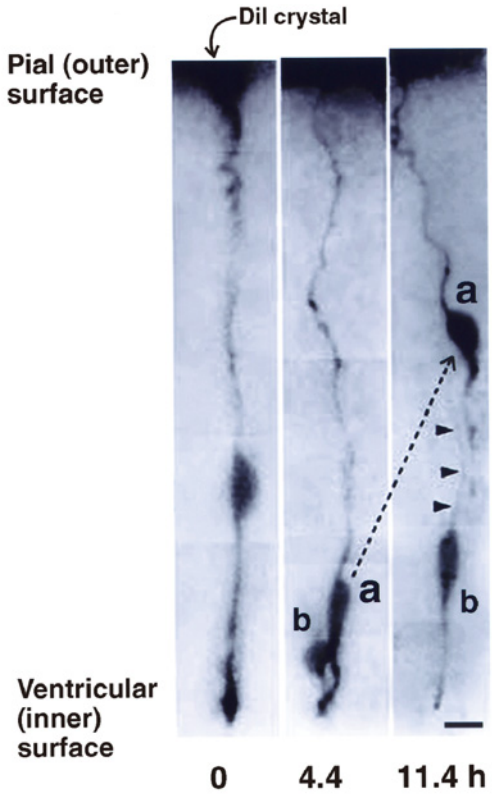

Fig. 4 Inheritance of the radial fiber by a daughter neuron. A bipolar-shaped progenitor cell (0 hour) divided, giving rise to two daughter cells (*a* and *b*, 4.4 hours). One of them (cell *a*) inherited the radial process and used the inherited process for its somal translocation to move towards the outer side, losing its attachment to the ventricular surface, while the other (cell *b* that was born without inheriting the process, and which may be a cycling cell), extended a new radial process (arrowhead). Bar, 10 μm.

IMPACTS

The most significant advantage of DiI-based slice culture is the ability to observe the time-dependent changes in the three-dimensional morphology exhibited by a single cell. Although it had long been believed, based upon previous Golgi and electron microscopic studies,[24,25] that dividing progenitor cells lose their pial processes, our time-lapse observations demonstrated that the process (also referred to as "radial fibers", 100–300 μm long) becomes thin but is neither lost nor divided[18,22] (Fig. 3). Since the retained process is extremely thin (0.5 μm or less) at the end of the M-phase, it seems possible that vigorous glutaraldehyde fixation steps, which generally cause tissue shrinkage, might have led to difficulties in the unequivocal identification of such thin fibers in the previous histological studies.

In divisions that produce a neuron and a cycling daughter (Fig. 4), the neuron can inherit the pial fiber, also grows a thick ventricular process for several hours, and is therefore indistinguishable from its parent cell. The process-inheriting neuron then collapses its ventricular process to take unipolar morphology, leading to the ascent of its cell body using the inherited radial

fiber. Therefore, slice culture has demonstrated that progenitor cells not only produce neurons but also play an important role in the delivery of those neurons through the inheritance of the radial process.

DISCUSSION

The authors discovered the usefulness of three-dimensional culture methods through studies on *reeler*, a mouse mutant that displays abnormalities in the formation of its cerebral and cerebellar cortices. "Reaggregation culture," which is designed to allow cells to reassociate themselves in a test tube,[26] and "explant culture," in which excised brains were grown in collagen gel,[27] were useful in reproducing the phenotype-specific patterns of neuronal arrangement as well as in testing the effect of anti-Reelin antibody on the neuronal assembly[26,27] (Fig. 1). These studies led to the demonstration that Reelin (protein lacking in *reeler*) is essential to the normal positioning of neurons.

Since it is now possible to make an aggregate by allowing isolated neural stem cells to divide continuously ("neurospheres"),[28] one can reasonably expect the formation of artificial brain-like structures from stem cells, including ES cells, in the near future. Lessons learned from fluorescent-based slice culture (in which cells "teach" us how they organize the normal brain structure during development) will be necessary in efforts to engineer tissues of the central nervous system such as the cerebellum, spinal cord, and retina, and may also be necessary for therapeutic modalities intended to reconstruct damaged adult brains using stem cells *in situ*. A convergence of both technique and knowledge will usher in an altogether new era.

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