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General overview of neuronal cell culture

Jennifer Gordon, **Shohreh Amini**, and **Martyn K. White**
Department of Neuroscience, Temple University School of Medicine, Philadelphia, PA 19140

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The ability to produce in vitro cultures of neuronal cells has been fundamental to advancing our understanding of the functioning of the nervous system. The culture of neuronal cells is particularly challenging since mature neurons do not undergo cell division. One way to overcome this is to establish secondary cell lines that are derived from neuronal tumors and have become immortalized. These have the advantage of being able to be grown fairly easily in cell culture to give unlimited cell numbers as well as minimizing variability between cultures. The disadvantage of these cell lines is that they will show many important physiological differences with the cell type from which they were derived. Often such cell lines are induced to display a more neuronal phenotype by manipulations of the culture conditions, e.g., addition of specific growth factors, etc. In Chapter Two, "Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology" by Jane Kovalevich and Dianne Langford, one such cell line is discussed, SH-SY5Y, which has been cultured and used extensively in research on neuronal cells. As described in detail by Kovalevich and Langford, the human SH-SY5Y cell line was derived by subcloning from the parental metastatic bone tumor biopsy cell line SK-N-SH by June Biedler in the 1970's [1]. A modified protocol is presented based on the original culturing conditions described by Biedler for SH-SY5Y cells [2]. The normal culture conditions for SH-SY5Y cells that are routinely used today remain largely unchanged since the 1970s. SH-SY5Y cells can grow continuously as undifferentiated cells that have a neuroblast-like morphology and express immature but not mature neuronal markers [3]. Differentiated SH-SY5Y cells are morphologically similar to primary neurons with long processes and exhibit a decrease in proliferation rate, exit the cell cycle and enter G_0, and show increased expression of neuron-specific markers [3]. As described in the Chapter of Kovalevich and Langford, a number of agents can be used to induce differentiation, e.g., retinoic acid, phorbol esters, dibutyryl cAMP, etc. Markers for differentiation include βIII-tubulin, synaptophysin, microtubule-associated protein-2 (MAP2), neuron-specific enolase, synaptic associated protein-97 (SAP-97) and neuronal specific nuclear protein NeuN. The morphologies of undifferentiated and differentiated SH-SY5Y cells are shown in Chapter 2, Figures 1-3.

Other secondary cell lines have also been used as models to study neuronal cells. In Chapter 3, "Cultured cell line models of neuronal differentiation: NT2, PC12", Nune Darbinian describes the culture of NT2 and PC12 cells. NT2, also called NTera, is a human neuronally committed teratocarcinoma cell line that is able to be induced into neuronal cultures by treatment with retinoic acid and inhibitors of mitosis, whereupon they show expression of

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neuronal markers. Available from the ATCC, NTERA-2 CL.D1 [NT2/D1] (ATCC® CRL-1973™) cells derive from a malignant pluripotent embryonal carcinoma arising in the testis of a 20-year old male [4]. PC12 is a rat cell line that was derived from a pheochromocytoma of the adrenal medulla [5]. PC12 cells cease to proliferate and undergo terminal differentiation into a neuronal phenotype when treated with nerve growth factor [6]. This cell line is also available from the ATCC – PC-12 (ATCC® CRL-1721™). NT2 and PC12 are suitable host cells for DNA transfection and protein transduction as shown in Chapter 3, Figures 4-6. Another type of neuronal cell culture can be derived from mouse teratocarinomas as described in Chapter 4, “Murine teratocarcinoma-derived neuronal cultures” by Prasun Datta. This chapter describes the culture and propagation of the murine embryonic stem cells, F9 and P19 and strategies for the differentiation of these stem cells into neurons. These include protocols which focus on obtaining enriched populations of mature neurons from P19 cells and differentiation of F9 cells into serotonergic or catecholaminergic neurons. These protocols can be employed to dissect pathways such as gliogenesis and neurogenesis that are involved in differentiation of F9 and P19 into glial cells or terminally differentiated neurons.

While neuronal cell lines have been very useful in the study of neuronal cell cultures and continue to be used today, the use of primary cultures is desirable because they are not tumor-derived and hence are more likely to recapitulate the properties of neuronal cells in vivo. However, unlike cell lines that provide unlimited supplies of homogeneous cells, the preparation and culture of primary cells is much more challenging and this is especially true for neuronal cells. Primary cell cultures are not immortal and hence the number of cells available for experiments is much more limited. Furthermore, since animal tissues in vivo are made up of several different types of cell, it is necessary to separate the cell type of interest from other cell types and to determine the purity of the resulting cultures, e.g., by immunocytochemistry with cell lineage-specific markers. In the case of primary neuronal cell cultures, it is necessary to separate them, as much as possible, from astrocytes and oligodendrocytes. Also with primary cultures, there are other important considerations, i.e., obtaining necessary ethical protocol approvals, which in US universities for example, would come from an Institutional Animal Care and Use Committee for animal cells and from an Institutional Review Board for the protection of human subjects for human tissue. Lastly, it should be noted that primary cultures in general are less easy to transfect than cell lines but that specialized transfection protocols or viral transduction can be used to introduce DNA into these cells, as described later. In Chapter 5, “Isolation and propagation of primary human and rodent embryonic neural progenitor cells and cortical neurons”, Armine Darbinyan and her colleagues describe the preparation and culture of primary neuronal cell cultures and also include culturing of neural and oligodendrocyte progenitor cells.

The brain is not the only source from which neurons can be cultured. Yonggang Zhang and Wenhui Hu describe protocols for the isolation and culture of neurons from the gut of the mouse in Chapter 6, “Mouse Enteric Neuronal Cell Culture”. A complex network of neurons embedded within the wall of the gut controls the gastrointestinal tract, which forms the enteric nervous system, also dubbed “the second brain”. The enteric nervous system also contains glial cells and consists of at least two plexuses, the myenteric plexus and the submucosal plexus. The isolation of enteric neurons presents technical difficulties not

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encountered in the isolation of neurons from the brain. There are established protocols for the isolation of enteric neurons from the guinea pig, rat and human but the mouse is particularly attractive because of the availability of inbred and genetically engineered strains and the economic cost. Zhang and Hu describe two methods to obtain enteric neurons from the mouse myenteric plexuses: (i) direct culture of primary neurons as shown in Chapter 6, Figure 1; (ii) induction of neuronal differentiation of enteric neural stem/progenitor cells as shown in Figure 2.

Perhaps no more exciting area in cell biology has emerged in recent years than stem cells. This is particularly the case for neuronal cell culture. As noted above, one problem in culturing of neuronal cells is that mature neurons do not proliferate. This is not the case for neural stem cells and they proliferate and are capable of long-term self-renewal. In Chapter 7, "Preparation of Neural Stem Cells and Progenitors: Neuronal Production and Grafting Applications" by Joseph Bonner, Christopher Haas and Itzhak Fischer, a comprehensive description is given of the techniques involved in the preparation and culture of the stem cells that are involved in the generation of differentiated neurons. This chapter provides in detail the methods required for the isolation, propagation, storage, and differentiation of neural stem cells (NSC) and neural precursor cells (NPC) isolated from rat fetal spinal cords for subsequent *in vitro* or *in vivo* studies. Of particular note, Section 1.2 provides some useful definitions of terms used in the field, e.g., pluripotency, multipotency, progenitor, precursor, etc., which are all clearly defined. Two major protocols are described in Chapter 7: (i) preparation of Neuroepithelial cells (NEPs) from E10.5 rat spinal cord; (ii) preparation of Neuronal Restricted Progenitors (NRPs) and Glial Restricted Progenitor (GRPs) from E13.5 rat spinal cord. NEPs are multipotent cells that can self renew in vivo and can differentiate into neurons, astrocytes, and oligodendrocytes. Thus, NEPs are a true NSC population that represent an intermediate stage between pluripotent immature cells, such as embryonic stem cells and mature differentiated neural cell phenotypes. On the other hand, NRPs/GRPs are a mixed culture of two cell types that together can produce neurons, astrocytes, and oligodendrocytes, but NRPs will only differentiate into neurons while GRPs will only differentiate into astrocytes and oligodendrocytes. Thus, NRP/GRP are not a true NSC population but represent a necessary intermediate step between multipotent NSCs and mature neural or glial phenotypes.

In Chapter 8, "Derivation of neuronal cells from fetal normal human astrocyte (NHA)" by Ausim Azizi and Barbara Krynska, protocols are described for a different approach to the differentiation of cells into the neuronal lineage. The starting point here is commercially available cultures of normal human astrocytes (NHA) that have been isolated from normal fetal human brain tissue. Some of the glial fibrillary acidic protein (GFAP)-positive astrocytes have stem cell properties and the genesis of neuronal lineage cells from NHA in adherent culture can be induced by removal of serum and addition of basic fibroblast growth factor (bFGF). These neuronal precursor cells express doublecortin, nestin and are negative for GFAP and can later mature into neurons after withdrawal of the bFGF. Since this model system of neurogenesis is an in vitro system containing of both neurons and glia, it may be thought of as a "human brain in a dish", which is useful for certain studies requiring assays of the effects of various treatments on developing human neurons.

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Rather than dispersing cells from a tissue and seeding the cells into primary culture, an alternative is to place unseparated sections of tissue into culture, a technique known as *ex vivo* culturing or slice culturing. Kalen Dionne and Kenneth Tyler have authored Chapter 9, "Slice culture modeling of CNS viral infection", which describes this technique applied to the brain. Since the complexity of the central nervous system (CNS) cannot be recapitulated in cultures of disassociated primary cells, the advantage of thin slicing and subsequent culture of CNS tissue is that it allows a valuable means of studying neuronal/glial biology within a physiologically relevant tissue context. Slice culturing facilitates investigator access to both the tissue and the culture medium and these cultures are viable for as long as several weeks after isolation. The protocol described by Dionne and Tyler is based on the membrane-interface method of brain slice culture [7]. Briefly, this procedure involves the placement of explanted rodent brain slices upon a semiporous membrane insert, which sits in a well containing medium such that the slices are suspended at the interface between medium and a humidified atmosphere of 5% CO_2 at 36°C (see Chapter 9, Figure 1). A thin film of medium forms above the slices by capillary action, thus allowing not only hydration and nutrition but also gaseous exchange. In this way, viability can be maintained for several weeks [8].

An important consideration in the isolation of primary cultures is the purity of the culture. There are a number of markers that can be used to label cells for lineage-specific markers to reveal the constituents of the resulting cultures. Classic lineage-specific markers for CNS cells include βIII-tubulin for neuronal cells, GFAP for astrocytic cells, O4 for oligodendrocytic cells and OX-42/CD-11b for microglial cells. In the case of cultures of stem or progenitor cells, lineage-specific markers also exist for the degree of differentiation, e.g., Nestin and SOX2 for neuroprogenitor cells, NG2 for oligodendrocyte precursor cells. The chapters dealing with primary cultures and stem cell cultures contain information about these markers, for example see Chapter 7, Sections 2.3 and 3.3. In addition, Chapter 10, "Neurospheres and glial cell cultures: immunocytochemistry for cell phenotyping" by Amanda Parker Struckhoff and Luis Del Valle, describes a detailed procedure for the immunophenotyping of cultures from the CNS. In particular, Table 1 of Chapter 10 lists specific biomarkers that are available for the different cell types of the CNS. A complementary approach is suggested by Darbinyan et al in Chapter 5, Section 3.4.2 which uses Q-RT-PCR to detect and quantify expression of mRNAs for lineage-specific markers. This has the advantage of providing precise quantitation of marker expression but does not address what percentage of cells in a given culture are positive for a marker and hence it should be used in addition to, and not instead of, immunohistochemistry.

Also included are chapters describing common techniques that are employed with neuronal cultures. For example, it is often of interest experimentally to investigate the effects of ectopic expression of a particular protein in a culture. As noted above, primary cultures in general, and neurons in particular, tend to be refractory to transfection. In Chapter 11, "Transfection of Neuronal Cultures", Ilker Sariyer shares his experience of performing transfection experiments with neuronal cells using a variety of different transfection reagents to define an optimized protocol, which is described in detail. As shown in Chapter 11, Figure 1, transfection of neuronal cells using this protocol with an expression plasmid for

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enhanced green fluorescent protein as an indicator for transfected cells, a high efficiency of transfection is achievable. An alternative approach is to perform transduction of the DNA of interest into cells by cloning into a viral vector, packaging the virus and then infecting the target primary culture. In Chapter 11, "Lentiviral transduction of neuronal cells" Hassen Wollebo and coworkers describe the use of a lentivirus vector system for this purpose.

One of the major defining features of neuronal cells is the polarized transmission of information through axons and dendrites. Two compartment systems have now been developed that allow the investigation of these vectoral functions in neuronal cell cultures. In Chapter 13, "Compartmentalized neuronal cultures", Armine Darbinyan and coworkers describe the AXon Investigation System (AXIS™) device which provides an opportunity to orient neuronal outgrowth and spatially isolate neuronal processes from neuronal bodies. AXIS is a slide-mounted microfluidic system with four wells connected by a channel on each side of the device as shown in Chapter 13, Figure 1. Channels are connected by microgrooves that do not permit passage of the cell body but allow extension of neurites. This facilitates the performance of experiments on the control of the extension of neurites and on the specific constituents of neurites in neuronal cell cultures.

Throughout the chapters on neuronal cell cultures, a number of common factors have emerged that are crucial in the cultivation of these cells. One of these is the substrate on which the cells are seeded. For example, in order for PC12 cells to undergo neuronal differentiation in response to nerve growth factor (NGF), they must be plated on collagen IV-coated dishes (Chapter 3). Poly-D-lysine coated dishes/slides are used in the growth of primary neuronal cultures (Chapter 5 and 13). Enteric neurons will also attach to poly-D-lysine coated plates but better adherence can be achieved with the poly-D-lysine/fibronectin or poly-D-lysine/laminin coating. However, double coating is expensive and needs 1-2 days for the coating process (Chapter 6). Poly-L-lysine and laminin coated dishes are used in the culture of neural precursor cells (Chapter 7). Matrigel is also used as a substrate in many applications (Chapters 6, 7 and 8). The importance of the substrate in promoting or inhibiting neurite outgrowth in *in vitro* cultures of rat dorsal root ganglion (DRG) neurons is explored in the protocols described in Chapter 14 "Quantitative assessment of neurite outgrowth over growth promoting or inhibitory substrates" by George Smith and coworkers. This is important in the study of axonal growth, neurotrophic dependence and the structure and function of growth cones.

Equally as important as the substrate is the composition of the culture medium especially with regard to growth factors and serum. For example, in order for PC12 cells to undergo neuronal differentiation, they are treated with NGF in the absence of serum (Chapter 3). Primary human embryonic neural progenitor cells are cultured in neural stem cell medium, which contains basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and insulin like growth factor-1 (IGF-1) as described in Chapter 5. The protocols for culturing multipotent neuroepithelial cells (NEPs) use culture medium that is supplemented with bFGF (Chapter 7). In Chapter 8, the genesis of neuronal lineage cells from the astrocytes is described by the removal of serum and exposure to bFGF. The serum-free medium and bFGF induces astrocytes to generate neuronal precursors that express doublecortin and nestin but are negative for GFAP.

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Finally, it should be noted that the frequency and cell density is of central importance in neuronal cell culture as is the case for most cells.

In this book, the chapters describe a number of important and up-to-date protocols but it is not intended to be comprehensive. For example, the cell line HEK 293 was generated in the 1970s from normal primary human embryonic kidney (HEK) cells with sheared adenovirus 5 DNA in an early example of the technique of transfection by Graham in the laboratory of van der Eb [9]. Originally thought to be derived by transformation of a fibroblastic, endothelial or epithelial kidney cell, HEK 293 turned out by later analysis to have characteristics of immature neurons, suggesting that the adenovirus transformed a neuronal lineage cell in the original kidney culture [10]. Thus 293 cells express the neurofilament (NF) subunits NF-L, NF-M, NF-H, and many other neuron-specific proteins [10]. Since other independently derived HEK lines also expressed NFs, this suggests that the human adenoviruses preferentially transform human neuronal lineage cells and that HEK 293 is a human neuronal cell line rather than a kidney epithelial cell line [10]. Finally, it should be noted that recent investigations of cellular plasticity led to the recent discovery that induced neuronal (iN) cells can be generated from mouse and human fibroblasts by expression of defined transcription factors such as Ascl1, Brn2, Olig2, Zic1 and Myt1l. These studies have been the subject of an excellent recent review [11].

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