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Medium-throughput computer aided micro-island method to assay embryonic dopaminergic neuron cultures *in vitro*

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ABSTRACT

In Parkinson's disease (PD) midbrain dopaminergic (DA) neurons degenerate and die, causing loss of motor function. Currently no therapies exist to ameliorate neurodegeneration or to restore DA neurons, although neurotrophic factors (NTFs) are promising leads. Prior *in vivo* studies the NTFs are routinely assessed *in vitro* by quantifying the survival of DA neurons from embryonic rodent midbrain cultures. Current *in vitro* methods are limited in terms of assay reliability, arduous workflow, low throughput, low statistical power and may obscure detection of molecules with minor yet critically important therapeutic effects.

We have developed a medium-throughput, micro-island culture method. It permits analysis of 10–12 data points from a single embryo – several fold more than any previously published method – and enables comparisons of DA neurons from a single gene knockout (KO) embryo. It is computer-aided, improves statistical power and decreases the number of animals and workload per experiment. This method enhances testing capabilities of NTFs and other factors, and enables small scale screening of chemical drug libraries.

We have validated the method by confirming the known effects of glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN), and demonstrated additive effects via simultaneous addition of GDNF and heparin binding growth associated molecule (HB-GAM). We also show for the first time that DA neurons isolated from GDNF receptor RET-deficient mice are still GDNF responsive, suggesting the presence of an alternative non-RET receptor for GDNF in the DA system. Finally, the method can be adapted for analyses of other low abundance neuronal systems.

1. Introduction

Motor symptoms of Parkinson's disease (PD), i.e. dyskinesia, bradykinesia and tremor are caused by progressive degeneration of dopaminergic (DA) neurons in the substantia nigra (SN). No therapy that could effectively delay disease progression or restore the degenerating DA system exists. Novel therapeutic approaches for PD have mainly focused upon: interference with neurotoxic mechanisms, cellular replacement strategies, and promoting neuronal survival and function (Peterson and Nutt, 2008). In this respect much attention has been placed on therapeutic application of neurotrophic factors (NTFs). To date, glial cell line-derived neurotrophic factor (GDNF) and its close relative neurturin (NRTN) are considered to be the most potent factors for protecting and restoring the DA system both in the *in vitro* primary DA culture paradigm and in animal models of PD (Airaksinen and Saarma, 2002; Andressoo and Saarma, 2008; Peterson and Nutt, 2008). They bind to the specific co-receptors GFRα1 and GFRα2, respectively, and signal via the tyrosine kinase RET receptor. Several other DA neurite outgrowth and cell survival promoting molecules, such as heparin binding growth associated molecule (HB-GAM, also known as pleiotrophin or PTN) have also been discovered. However, due to limitations in available methods, only a fraction of combinations of different growth factors have been assessed, e.g. it has been shown that HB-GAM acts additively with GDNF (Hida et al., 2003; Marchionini et al., 2007). Systematic studies are as yet missing. For the same reason, medium- or high-throughput screening of chemical small molecule libraries on primary DA neurons is very difficult.

As an essential step prior to *in vivo* tests, survival assays of embryonic midbrain cells in culture have been routinely used for testing the efficacy of NTFs and other potentially neurotrophic molecules. In these assays, a potential dopaminotrophic agent is added to dissociate midbrain cultures and the survival of DA cells is quantified and compared to untreated cultures. The most common method for visualizing DA neurons on a culture dish is immunos-

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taining for the dopaminergic marker tyrosine hydroxylase (TH). Virtually every neurotrophic molecule for DA neurons has been initially characterized using this method. However, the method is complicated and arduous due to reasons listed below.

1. *Scarcity of material*. In one E13.5 embryonic mouse midbrain there are about 200,000 midbrain floor cells, of which about 10,000 are dopaminergic. With the traditional setup using 4-well plates, one litter of 10 embryos yields only about ten wells of cells. Thus, experiments require large numbers of laboratory animals, and usage of knockout (KO) animals with embryos of different genotypes is impossible.
2. *Density-dependent variability of the cell cultures*. The midbrain cultures display high internal variability in cell survival, resulting mainly from differences in plating density. Too low a density leads to 100% cell death within days, and too high a density masks potential survival-promoting effects. Well walls create cell density variations, leading to "edge-effects", whereby cells are several fold more dense close to the well edges, or to the "center" effect where cells in the center of the micro-island survive better than those at the edges due to uneven cell plating (Collier et al., 2003; Takeshima et al., 1996). Thus, even plating density is an absolute prerequisite for reliable analysis.
3. *Multiple sources of human error in cell counting*. The large number of cells (usually thousands) and disturbing light reflections from the well walls lead to the necessity of choosing a representative field for counting the DA neurons, which unavoidably creates a source of variation (Collier et al., 2003). Visual cell counting is time-consuming and error-prone. Furthermore, the TH staining does not give a clear "yes" or "no" answer, but illuminates rather a gradient of cells which can either then be counted as dopaminergic or not, dependent upon threshold signal intensity that is arbitrarily assigned by eye, for each cell separately.

Attempts have been made to avoid some of these limitations by plating midbrain cells on glass cover-slips or on micro-islands in the middle of the well (Takeshima et al., 1996). Although this is a substantial improvement, variations between data points stemming from differences in micro-island size and hence cell density (islands lack clearly defined edges), as well as from relying on counting representative fields, still exist. Similarly, subjective assignment of minimum TH intensity also remains.

Here, we set off working towards a protocol that: (i) can be routinely used in every laboratory, (ii) avoids plating density/plating area-induced internal variation, (iii) is sensitive enough to reveal additive effects of neurotrophic molecules, (iv) reduces the amount of animals needed, (v) allows experiments from single embryos, and (vi) releases the investigator from visual–manual cell counting by computer-assisted quantification. We believe that the described method not only reduces the workload of the experimenter to address "classical" NTF associated questions, but also will, especially when automated, enable small-scale screening of chemical drug libraries.

2. Materials and methods

2.1. General overview

Mouse embryonic midbrain floors were isolated at E13.5 and 20,000–30,000 midbrain floor cells containing about 1000–1500 DA cells were plated on each standardized 3 mm^2 micro-island. The dopaminotrophic agent was applied for a chosen time period, after which the cells were immunostained for TH. Approximately 300–600 DA cells survived without NTF treatment until DIV5, thereby providing sufficient numbers for statistical analysis—even with few repeats per NTF. After immunostaining, each micro-island was digitally captured as a single image, followed by computer-based quantification of two complementing parameters: (i) number of TH-positive cells and (ii) total immuno-fluorescence intensity of TH, an indirect measure of TH levels. We also demonstrate that the method is suitable for functional studies, such as dopamine uptake measurements.

2.2. Plate preparation

4-well plates (Nunc, Cat nr 176740) were pre-coated with 300–500 μl of poly-DLL-ornithine (PORN) (Sigma, Cat nr P-8638) (1 mg/ml in 0.15 M borate buffer, pH 8.7) per well for minimum 4 h at RT or overnight at 4 °C. The plates were washed at least 3 times with PBS. PBS was removed holding the plate at an angle to minimize salt accumulation at the bottom of the well. The PORN solution and PBS were filter sterilized to avoid impurities such as dust particles. The washed plates were dried under the laminar flow hood for 1–2 h and kept wrapped in saran at +4 °C until use. The saran wrapped plates can be stored for at least 2 months. In our hands, the use of further coating substrates, such as laminin, enhanced culture variability since (i) different batches vary in quality, (ii) laminin and other integrin family substrates induce substantial cell migration on the micro-island often resulting in DA cell-clusters confounding the computer-aided quantification, and (iii) cells do not attach sufficiently to the plate to enable increase of the medium volume 30 min post-plating (see below). Therefore, other coating substrates were not used.

2.3. Preparation of standard sized micro-islands

2.3.1. Cell density and size of the micro-island

The cells need to be plated with density at which their survival is most responsive to the presence or absence of trophic factors and high enough to minimize the "center" effect (cell density being highest in the center after plating of cells) as much as possible (Collier et al., 2003; Takeshima et al., 1996). When determining the optimal growth area we aimed for a size of a micro-island which can be evenly filled with cells without the amount of material becoming rate-limiting. We also wanted the growth area to be small enough for capturing on a single image for data analysis, but large enough to provide enough statistical power in terms of sufficient number of DA neurons. In our hands the optimal plating density for 5-day experiments is at least 6000 cells/mm^2, and for 8-day or longer culturing periods about 10,000 cells/mm^2. We found the size of the micro-island to be optimal at 3 mm^2. Hence, from the c. 200,000 cells of an E 13.5 midbrain floor one can plate 6–11 micro-islands, each containing 18,000–30,000 cells, of which 900–1500 are DA neurons.

2.3.2. Preparing the micro-island edges

In order to edge and standardize a micro-island area of ~3 mm^2 for plating the neurons, a homemade drill was designed and manufactured. The device was prepared from sharpened metal tube mounted in a plastic stand which exactly matches the 4-well plate well (Fig. 1A i, ii). The inner diameter of the drill was 2.5 mm. However, because the island is etched onto the plate by turning the drill (Fig. 1A iii), the edges of the micro-island are broad and the actual inner diameter of the micro-island is 2 ± 0.2 mm. Guidelines for preparing the drill are presented in Supplementary Fig. 1. A standard sized drill of desired diameter can also be prepared from an inverted 1 ml, 2 ml, etc. plastic pipette. The tip of the pipette may be sharpened with a pencil sharpener and then mounted onto a 15 ml Falcon tube as shown in Supplementary Fig. 2. The plastic drill, however, stays sharp for a shorter time and requires more practice to use.