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On the Generation of Efficient Methodologies for Cell-Based Toxicity Screening by Comparative Protocol Analysis and Optimization

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Abstract

In cell biology in general and in high-throughput cell-based screening approaches in particular - e.g. for *in vitro*-based toxicity assessment - various methodologies or protocols are reported in the literature. In the case that a cell-based screening assay, for toxicity evaluation or for assessing another biological question, is to be established for the first time in a research lab, the researcher has to select a number of different protocols from the literature and to create an optimized protocol for the intended use. This is typically required, because optimized protocols, generated following comparative protocol analysis and optimization, are mostly rarely available. In another study, which we refer to as a showcase, we conducted a comparative analysis of three different protocols for neuronal NT2 cell differentiation, available in the literature. From this comparison, we generated an improved and optimized method, allowing for neuronal NT2 differentiation in monolayer cultures with high yield of NT2-N cells, allowing for systematic *in vitro*-based primary screening for developmental toxicants and neuro-toxicants at different stages of maturation. In this commentary, to prevent the same experiments from being repeatedly conducted in different labs around the world and at different times over and over again, we suggest generation of advanced and efficient methods by comparative protocol analysis and optimization, for application in a variety of research fields, related to cell and single cell biology.

Keywords: *In vitro* DNT testing; NT2 human pluripotent stem cells; Protocol optimization; High-throughput screening

Showcase: *In vitro*-Based DNT Testing

Everyday products such as cosmetics, pharmaceuticals, textiles, detergents, plastic or pesticides, contain organic and inorganic chemical substances, that are potentially harmful to humans and the ecosystems. The number and volume of worldwide traded and registered chemicals has dramatically increased in recent years. Simultaneously, the incidence of neurological diseases, including mental retardation and autism, as well as developmental and learning disorders or hyperactivity disorder and attention deficit, has also increased [1-3]. The developing central nervous system (CNS) is particularly susceptible to damage by chemicals and therefore, assessment of the chemicals surrounding us on a daily basis for adverse effects on the maturing CNS – also referred to as developmental neurotoxicity (DNT) - is critical for human health and wealth [4-6]. Currently, as of January 2017, the *CAS Registry (Chemical Abstracts Service)*, the world's largest database of chemical substances, lists more than 126 million unique organic and inorganic chemicals. Of these chemicals, only very few representatives have been evaluated for DNT in recent years [7,8]. The reason for this is possibly because existing guidelines for toxicity evaluation mostly involve animal experiments that are expensive, low in throughput, of poor predictive quality, often not reproducible and, because there is no legal obligation for alternative DNT standardized testing [9-12]. Hence, potentially harmful effects of many of the tens of thousands of chemicals contained in everyday products, still remain unknown.

The Potential of Stem Cells for *In vitro*-Based DNT Testing

In vitro-based DNT testing using e.g. stem cells, such as induced pluripotent stem (iPS) cells or human embryonic stem (ES) cells, as well as human pluripotent teratocarcinoma NTERA-2 (NT-2) stem cells with their almost unlimited proliferative potential, may provide a powerful alternative to animal experimentation [13]. However, the large variety of methodologies and protocols reported in the literature can be challenging when it comes to selecting the appropriate approach for maintenance and application of stem cells for *in vitro*-based toxicity assessment. For example, human pluripotent teratocarcinoma NTERA-2 (NT2) cells are increasingly considered as an appropriate model for such ***in vitro***-based DNT screening approaches [14-19]. NT2 stem cells exhibit many aspects of human embryonic neural stem cells [20]. Upon treatment with retinoic acid (RA), NT2 cells differentiate into post-mitotic cells, showing properties of cells of the central nervous system, also referred to as NT2-N cells [21-26]. These cells are committed towards neurons, astrocytes, and oligodendrocytes, which are the three main cell lineages of the CNS, and have extensively been used for drug *in vitro* screening and for studying human neurogenesis, brain regeneration, and terminal differentiation [20].

Spoiled for Choice - Which Protocol to Choose for a Cell-Based Application?

There are numerous approaches reported in the literature on neuronal differentiation of NT2 cells in aggregate or suspension culture, as well as in monolayer cultures [21,25-32]. These protocols

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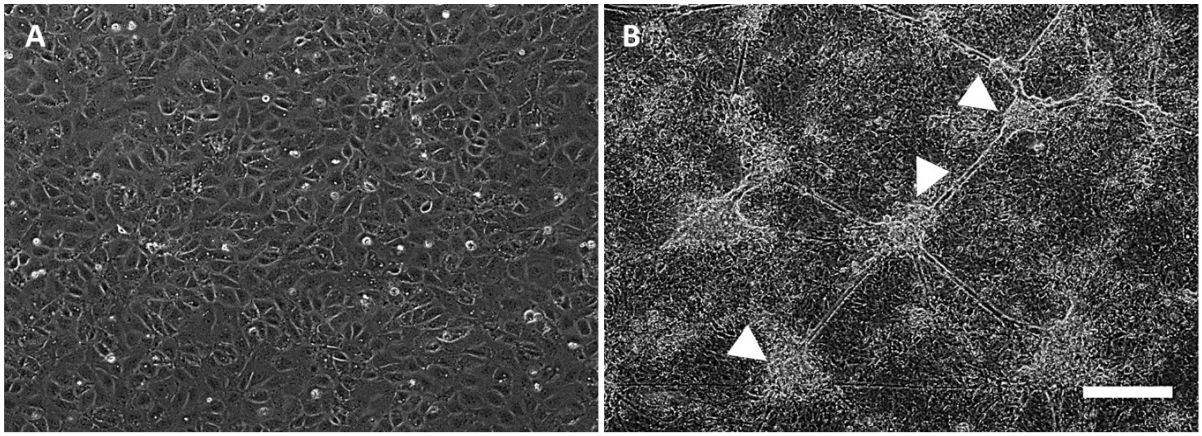

Figure 1: Application of the optimized protocol for neuronal differentiation of NT2-cells in monolayer cultures, generated from comparative protocol analysis. A). Transmission light images of non-differentiated NT2 cells. B). Differentiated NT2-N cells. Formation of interconnected clusters as indicated by white arrows indicates successful differentiation. Scale bar: 200 µm.

mainly differ with respect to the presence or absence and exposure time of a differentiation stimulus (e.g. RA or 22R-hydroxycholesterol), the number and concentration, as well as the incubation time of mitotic inhibitors (cytosine arabinoside, fluorodeoxyuridine and uridine), or the frequency of media exchange, as well as procedures for purification of neuronal cells. The published studies report strongly varying differentiation results including expression of neuronal markers, morphological characteristics of differentiated cells and also efficiency - even from comparable approaches. For example, in the original study published by Andrews in 1984, a large proportion of >50% differentiated cells upon treatment with 10 µM RA, is reported, whereas the studies by Pleasure et al. and Stewart et al. mention proportions of approx. 5% and 20% successfully differentiated cells, respectively [21,24,26].

Protocol Comparison and Optimization

In the case that a cell-based screening assay for toxicity evaluation or for assessing another biological question is to be established for the first time in a research lab, the researcher needs to select a number of different protocols from the literature and apply and compare them, as well as subsequently generate an optimized protocol for the intended use, because data from comparative protocol analysis and optimization are typically rarely available. As this was the case with regard to methods describing differentiation of NT2 cells in monolayer culture at the time we aimed to work with NT2 cells in our lab, we conducted a comparative analysis of three different protocols for neuronal NT2 cell differentiation available in the literature. From our comparative data, we generated an improved and optimized method, allowing for neuronal NT2 differentiation in monolayer cultures with high yield of NT2-N cells, allowing for systematic *in vitro*-based primary screening for developmental toxicants and neuro-toxicants at different stages of maturation [33]. Figure 1 depicts images of non-differentiated NT2 as well as differentiated NT2-N cells obtained from our improved methodology.

The Value of Comparative Protocol Analysis and Negative Results

Despite the fact that there are publications available in the literature on comparative protocol analysis and optimization, not only in the context of stem cell differentiation, but also from other fields of cell biology, such literature is typically only rarely available. This is probably due to the fact that many of the required experiments often generate a large amount of negative results that are typically not published or publishable. However, data from comparative protocol analysis and optimization as well as negative results can be of high value to researchers aiming to establish a specific method in their laboratory, as the provided information may prevent the same experiments from being repeatedly conducted in different labs around the world and at different times over and over again. We thus, suggest generation of advanced and efficient methods by comparative protocol analysis and optimization, not only in the context of cell-based toxicity screening, but also for other research fields related to cell and single cell biology. This can eliminate the need for repetitive experiments, increase efficiency and contribute to furthering the applicability of cell-based screening methodologies.

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