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Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D *in vitro* applications

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ABSTRACT

Existing guidelines for testing developmental neurotoxicity (DNT) propose investigations in rodents, which are ethically questionable as well as time and cost intensive. Thus, there is international agreement that predictive *in vitro* methods are needed to increase efficiency of testing and limit the number of animals used. One of a variety of novel approaches for DNT testing utilizes neurospheres, three-dimensional aggregate cultures of primary normal neural progenitor cells (NPCs). Because sorting and plating of single neurospheres is one of the most time-consuming steps within the assay, the aim of this study was to evaluate if the complex object parametric analyzer and sorter (COPAS PLUS™, Union Biometrica Inc.) is a suitable tool for automated sorting and plating of neurospheres. The results of the comparison of NPC viability, proliferation, migration, differentiation and intracellular oxidative stress between manually and COPAS sorted and plated neurospheres of different species show that the automation by the COPAS instrument does not influence the basic performance of neurospheres. Therefore, we consider the COPAS instrument as a useful tool for higher throughput neurosphere research in toxicology, neuroregeneration, brain development, drug development and brain aging research.

1. Introduction

A large number of chemicals are currently in use worldwide for which toxicity data is incomplete and/or lacking. As concern for human health is rising, there is the need for testing methods helping to predict toxicity of such compounds. This is especially true for the potential of chemicals to cause developmental neurotoxicity, as there are only five substances with a scientific basis sufficient to ascribe the potential to disturb human brain development (Grandjean and Landrigan, 2006; US EPA's Office of Pollution Prevention and Toxics, 1998). Guidelines for testing developmental neurotoxicity (DNT) include the US EPA test Guideline 870.6300 and the OECD-guideline 426, which proposes investigations in rodents, mainly rats. Such a DNT *in vivo* testing protocol requires the use of 140 dams and 1000 pups and is therefore ethically questionable and extremely time and cost intensive. Thus, for the need of increasing efficiency of testing (Andersen and Krewski, 2009; Kavlock et al., 2009) and at the same time limiting the number of animals used for such testing (Balls, 2009; Goldberg, 2002) world wide effort is arising to replace DNT animal experiments with predictive *in vitro* methods (Crofton et al., 2011).

To increase predictability, such *in vitro* alternative methods should ideally fulfill certain prerequisites: cells ought to be of human origin (National Research Council, 2007), non-immortalized and not derived from a tumor as this changes the normal cell physiology (Geerts et al., 2003; Moors et al., 2009), and reside in a three dimensional (3D) context (Yamada and Cukierman, 2007).Thereby, Yamada and Cukierman (2007) impressively summarize that 'Three-dimensional (3D) *in vitro* models span the gap between two-dimensional cell cultures and whole-animal systems'. As the key strengths of 3D cultures they indicate that (i) cell morphology and signaling are often more physiological than routine 2D cell culture, (ii) they permit rapid experimental manipulations and testing of hypotheses and (iii) they permit much better real-time and/or fixed imaging by microscopy than in animals. Nevertheless, also

Abbreviations: DNT, developmental neurotoxicity; NPC, neural progenitor cell; COPAS, complex object parametric analyzer and sorter; HTS, high throughput screening; ROS, reactive oxygen species; DCF, dichlorofluorescin; PDL, poly-D-lysin; AU, arbitrary units; TOF, time of flight; LDH, lactate dehydrogenase; TCHQ, tetrachlorohydrochinone; HBSS, Hanks' buffered saline solution; EXT, extinction; RLU, relative luminescence units.

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practicability has to be taken into consideration as for screening purposes cultures also have to be suitable for medium to high throughput screening (HTS) approaches.

During the last years we have established and characterized an innovative cell system for DNT testing consisting of primary normal neural progenitor cells (NPCs). These cells grow as 3D neurospheres in culture and are widely used as model systems for neurogenesis and neural development (rev. in Jensen and Parmar, 2006). For species comparisons neurospheres are also prepared from rodent mouse and rat pups. We showed that these 3D cultures mimic fundamental processes of brain development in culture, like NPC proliferation, migration, differentiation and apoptosis. Moreover, this model allows studying the effects of putative developmental neurotoxins not only on a functional, but also on a molecular level (Fritsche et al., 2005, 2011; Gassmann et al., 2010; Moors et al., 2007, 2009; Schreiber et al., 2010). So far we have mainly focused on characterization of this unique cell system as well as on identification of toxicity pathways in a low throughput for single-picked compounds by employing 'The neurosphere assay' (Fig. 1 in Breier et al., 2010; Fritsche et al., 2011). To be applicable for testing needs, it is crucial to improve the testing capacities to a medium-throughput system. Because sorting and plating of single neurospheres into multi-well plates is one of the most time-consuming steps within the neurosphere assay the aim of this study was to evaluate if the large particle flow cytometer COPAS PLUS (Union Biometrica Inc.) is a suitable tool for automated sorting and plating of neurospheres. Therefore, this technique is not only useful for *in vitro* testing of developmental neurotoxicity but also for other neurosphere applications in the fields of neuroregeneration, brain development, drug development and brain aging research.

For the establishment of such automation it is of highest importance that the automation process itself has no influence on the general cell functions and measured endpoints. Therefore, we compared cell viability, proliferation, migration and differentiation of manually to COPAS sorted and plated neurospheres of different species. Additionally, we determined levels of intracellular reactive oxygen species (ROS) by a dichlorofluorescin (DCF)-assay as a mea-

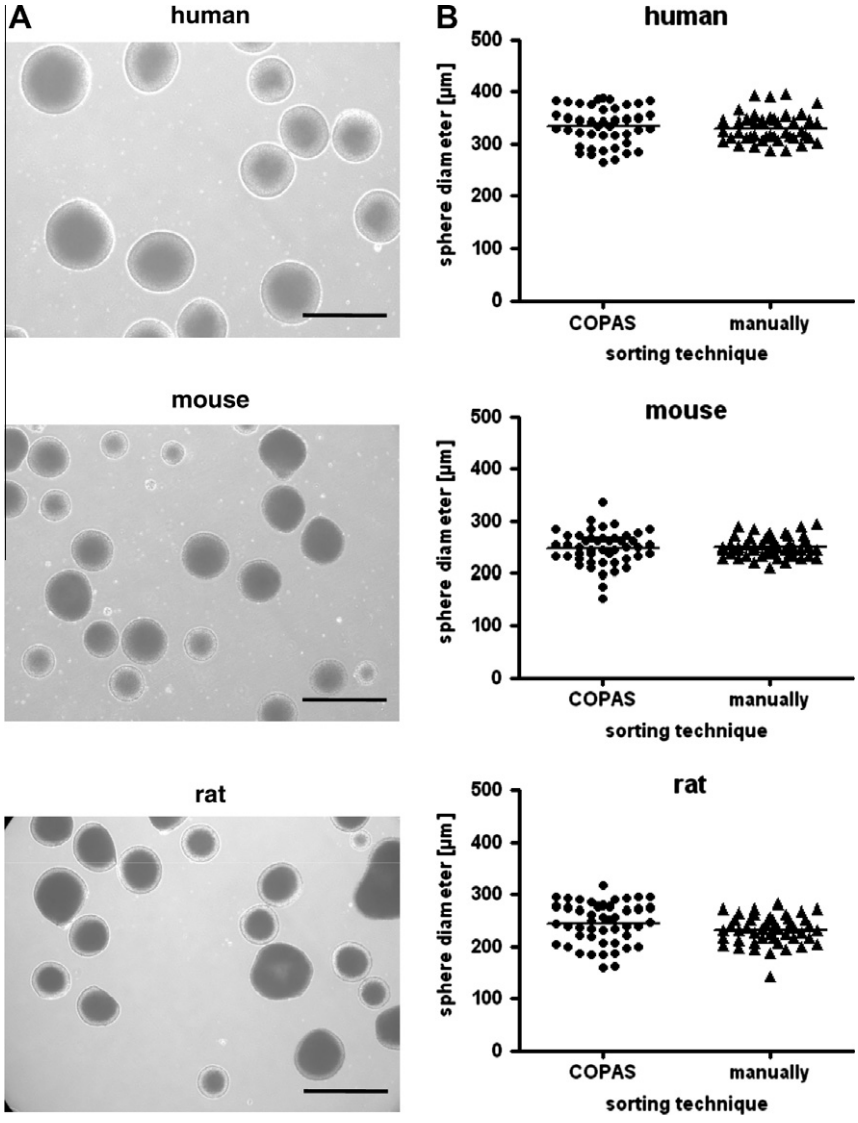

Fig. 1. Assessment of sorting accuracy. Microscopic images of the human, mouse and rat neurosphere culture before sorting. Scale bar = 500 µm (A). Forty to 50 neurospheres were sorted in a 96-well plate by the COPAS instrument (standard settings) and manually under a microscope . The diameter was analyzed with the metamorph program (Molecular Devices Corporation). One representative experiment/species is shown (B).