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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Introduction

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 efficacy 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).

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 Union Biometrica's large particle flow cytometer (COPAS) is a suitable tool for the automated sorting and plating of neurospheres. The comparison of NPC viability, proliferation, migration, differentiation and intracellular oxidative stress for human neurospheres between manually and COPAS sorted and plated human neurospheres is presented. The results show that the automation by the COPAS instrument does not influence the basic performance of neurospheres.

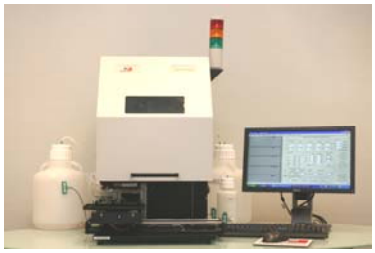
Therefore, we consider the COPAS instrument as a useful tool for higher throughput neurosphere research in toxicology, neuroregeneration, brain development, drug development and brain aging research.

Methods and Materials

Normal human neural progenitor cells used in this study were purchased from Lonza Verviers SPRL (Verviers, Belgium). Human neurospheres were cultured in proliferation medium [DMEM and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin] in a humidified 92.5% air/7.5% CO2 incubator at 37°C in suspension culture. Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin] and plating onto poly-D-lysine (PDL)/laminin–coated chamber slides.

Flow Cytometry using COPAS PLUS HTS for analysis and dispensing of neurospheres

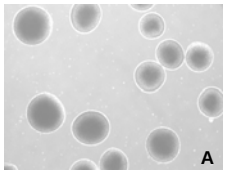
Neurospheres were sorted in two ways: 1) manually under a binocular and plated with a 100µl pipette or 2) using the COPAS PLUS HTS Flow Cytometer (Union Biometrica, Holliston, MA). The COPAS PLUS HTS with Advanced Acquisition Software and Profiler II was used for the acquisition, analysis and sorting of the samples. The COPAS instrument is equipped with a 1000 micron diameter flow cell. The ProfilerII option creates a digital profile for each object

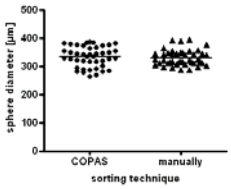


with the 488nm laser. It shows the location and intensity of the optical parameters and allows for extended sorting abilities with user definable sort criteria: peak heights, widths, locations and number for each optical parameter (size, density, 3 channels of fluorescence) in the profile.

Results

Assessment of sorting accuracy. Microscopic images of the human, neurosphere culture before sorting. Scale bar=500 µm (A). 40-50 neurospheres were sorted in a 96-well plate by the COPAS instrument (standard settings) and manually under a microscope .

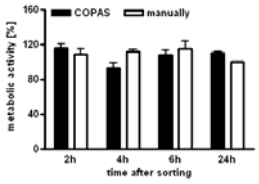




The diameter of the neurospheres was analyzed with the metamorph program (Molecular Devices Corporation). One representative experiment is shown (B).

Results

Viability and Cytotoxicity Assays. Neurospheres of the same size were sorted by the COPAS instrument (black bars) and manually under a microscope (white bars) in a 96 well plate (one sphere/well in 100 µl proliferation medium). Metabolic activity was measured 2, 4, 6 and 24 hours after the sorting using the CellTiter-Blue Assay (Promega) (C). LDH activity was determined as a measure for cytotoxicity by CytoTox One Assay (Promega) (D).



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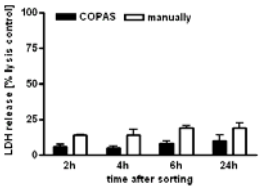
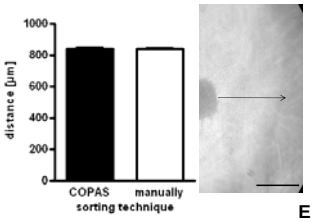


D

Migration and differentiation measurement After 48 hours under differentiation conditions the migration distance of the neural progenitor cells out of the sphere was measured (arrow). Scale bar=500µm (E). After 72h of differentiation the proportion of neurons was quantified by immuncytochemistry using a specific antibody against β(III)tubulin (green) and GFAP (red). Cell nuclei were counterstained with Hoechst (blue). Scale bar=100µm (F).



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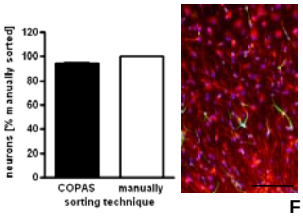
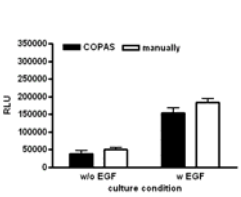


F

Proliferation capacity after sorting. Spheres were dispensed like previously described. After 48 hours cultivation in proliferation medium with and without 20 ng/ml EGF BrdU was added and its incorporation was measured 16 hours later by the luminescence cell proliferation ELISA (Roche) (G). **Assessment of intracellular ROS by DCF assay**. Neurospheres of the same size were sorted by the COPAS instrument (black bars) and manually as described above (white bars). Manually sorted neurospheres treated with 100 µM TCHQ for 2h served as positive control striped bars). DCF fluorescence was determined by FACS analysis of singularized cells (I).



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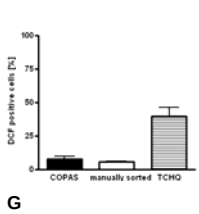

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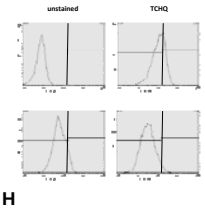

I

Shown are the means ± SEM of 4 independent experiments (20 spheres per condition and experiment) in % DCF positive cells (H). Representative histograms of the DCF-fluorescence within the NPCs after the different treatments (I).

Conclusions

We showed that the COPAS large particle sorter instrument is suitable for the fast sorting and dispensing of neurospheres in 96-well plates. None of the parameters tested in COPAS vs. manually sorted and plated neurospheres was affected. The COPAS sorted same sized spheres as the lab worker picked by hand. The COPAS sorted spheres were as viable as the manually selected ones and none of the functional endpoints – NPC proliferation, migration and differentiation - which build 'The neurosphere assay' was affected. We found no additional ROS generation by COPAS high-throughput sorting. Therefore, our work revealed that the COPAS large particle sorter is, amongst others, a suitable method for high-throughput 3D *in vitro* applications, which contain automated neurosphere sorting and plating.

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