Analysis and sorting of human neurospheres based on size and optical density measurements

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Objective

The purpose of this experiment was to test the feasibility of using the COPAS PLUS instrument (Union Biometrica, Inc.) to analyze and sort human neurospheres based on size and optical density measurements and to determine the influence on viability and proliferation of the neurospheres post-sorting compared to manual sorting.

Introduction

The COPAS PLUS instrument (Union Biometrica, Inc) is a large particle flow cytometer and is able to analyze and sort large objects (~20-700 µm diameter) at a high rate (up to 50 events per second) on the basis of the physical characteristics of size, optical density and fluorescence signals. A 488 nm solid state laser is used to acquire a measure of both the size (TOF) and optical density (EXT) of the sample. Additionally, if the sample contains certain fluorophores that can be excited by the 488 nm light, these emission levels can be detected for each of the objects in the sample.
The COPAS instrument is also a dispenser. A gentle pneumatic sorting mechanism provides a means for analyzing and handling sensitive objects like cell clumps without disrupting the clusters. The result is an instrument that can identify objects with similar features and dispense these to various formats (Petri dishes or multiwell plates) for further use and analysis.

Human Neurospheres as Three-Dimensional Cellular Systems for Developmental Neurotoxicity Testing

Developmental neurotoxicity (DNT) of environmental chemicals is a serious threat to human health. Current DNT testing guidelines propose investigations in rodents and requires large numbers of these animals. With regard to the "3 Rs" (reduction, replacement, & refinement) of animal testing plus the European regulation of chemicals [Registration, Evaluation, & Authorisation of Chemicals (REACH)], alternative testing strategies are needed in order to refine / reduce animal experiments as well as allowing faster / less expensive screening.

Human neural progenitor cells (hNPCs), which grow as neurospheres were used to establish assays suitable for detecting disturbances in basic processes of brain development. Research with neurospheres had so far largely focused on their application for neuroregeneration in disease states of the central nervous system. They were also found useful for basic research on brain development, drug development and brain aging research. Recently, the group around Ellen Fritsche has established neurospheres as a 3D alternative in vitro model for DNT testing (Moors et al. 2009). They looked at a variety of endpoints such as viability, proliferation, migration, differentiation, neurite outgrowth and apoptosis. The data of this group provides support for the usefulness of neurospheres in hazard identification screens for chemicals that may cause developmental neurotoxicity. Increasing the throughput of such assays by, for example, automating the analysis and sorting of the spheres, further enhance the applicability of this approach for *in vitro* toxicology testing.

Materials and Methods

Cryopreserved normal human neural progenitor cells (hNPCs; Lonza Verviers SPRL, Verviers, Belgium) were cultured at 37°C and 5% CO_2 as a suspension culture in proliferation medium consisting of Dulbecco's modified Eagle medium (DMEM) and Hams F12 (3:1) supplemented with B27 (Invitrogen GmbH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), and 20 ng/mL recombinant human fibroblast growth factor (FGF; R&D Systems, Wiesbaden-Nordenstadt, Germany) (Moors et al. 2007). When spheres reached 0.7-1.0 mm in diameter, they were chopped up to passage 5 with a McIlwain tissue chopper.
The viability of the neurospheres after the sorting process was assessed by determining metabolic activity using the CellTiter-Blue® (CTB)Assay (Promega) and the lactate dehydrogenase (LDH) assay (Promega).

Results

Size Measurements:
Human neurospheres were transferred to the sample cup and analyzed. Neurospheres were selected based on their size (TOF) and optical density (EXT) and then sorted into a 96 well plate for microscopic verification. A standard curve for calibrating sizes can be generated by using beads of known sizes and measuring the TOF for these on the COPAS instrument. This allows for the correlation of TOF measurements generated by the COPAS PLUS to actual sizes in µm. Figure 1 shows a screen shot of the software for one of the dispensing experiments. The upper dot plot (upper left side panel) show the TOF vs EXT data for the neurospheres. We selected a polygon region that included the larger neurospheres and excluded the smallest clusters, single cells and debris. Only objects that meet the size and optical density criteria set by this gating window (polygon in upper panel) were dispensed. A second level of selection is defined by the lower panel showing the autofluorescence of the neurospheres in the green and red parts of the visible spectrum. A polygon was drawn to include neurospheres with

intermediate levels of green and red autofluorescence. This panel shows the data plotted on a log scale (Lg(Green) vs Lg(Red)). Only neurospheres that simultaneously meet the chosen criteria for size, optical density and levels of green and red fluorescence were dispensed. If desired, data acquired by COPAS can be exported and retrospectively analyzed in standard commercially available analysis packages.

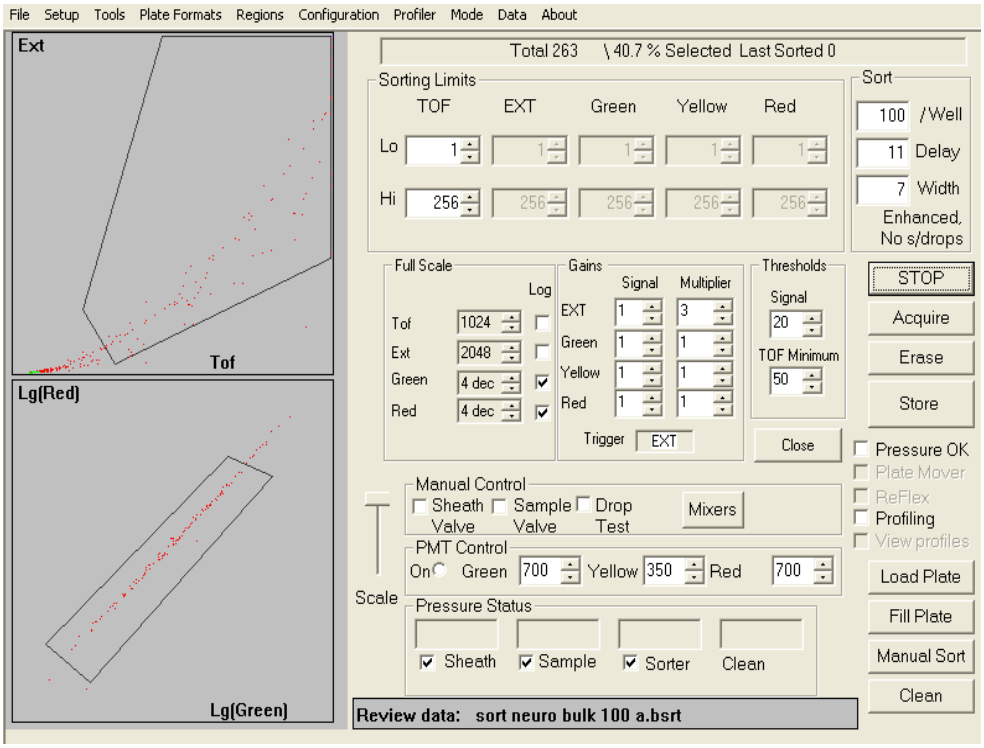

Figure 1. Screenshot of COPAS software with neurosphere data. In the upper left corner a dual parameter dot plot allows for gating for a specific subpopulation of spheres (here TOF vs Ext with TOF ~ size and EXT ~ optical density). This population is then visualized in a dual parameter sorting dot plot (lower left corner). Here autofluorescence signals were plotted against each other (Green vs Red). The right side of the panel allows the user to operate the instrument and change the software settings for data acquisition, visualization of data and various instrument settings.

To determine whether a uniform size of neurospheres could be collected, a selected size was dispensed by the COPAS PLUS and compared with neurospheres collected manually by microscopy. Figure 2 compares ~25 neurospheres of equal size collected by each method. The diameter of the neurospheres was determined using MetaMorph® Software (Molecular Devices).

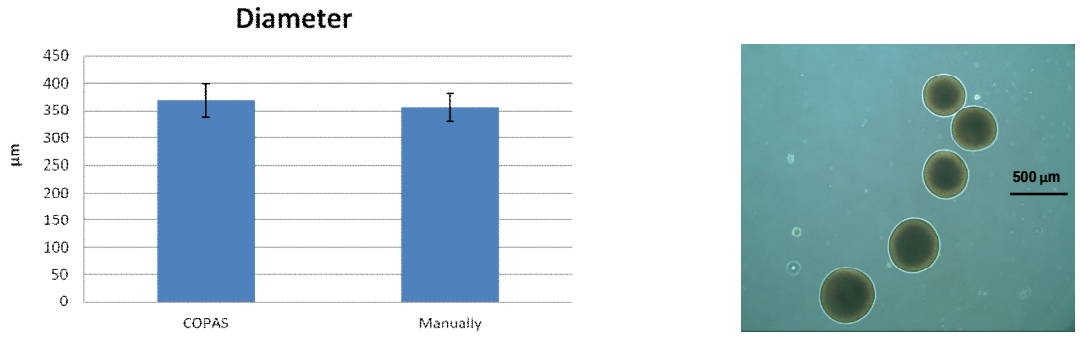

Figure 2. Neurosphere size Comparison of COPAS and manually sorted spheres.(left) A comparison of COPAS sorting and dispensing with manual methods. Approximately 25 neurospheres were sorted into a 96-well plate and the diameter of both groups was determined with the Metamorph program. (right) Microscopic analysis of human neurospheres sorted by COPAS.

Results indicate that the absolute size and the size distribution of the neurospheres analyzed and sorted by the COPAS instrument is similar compared to selection by hand. Microscopic analysis shows that the integrity of the clusters is not affected by the COPAS sorting.

Viability

Viability of neurospheres after the sorting process was assessed by determining metabolic activity using the CellTiter-Blue® (CTB) Assay (Promega) and the lactate dehydrogenase (LDH) assay (Promega).

In the CTB assay neurospheres of identical size were selected by the COPAS instrument and compared to manual selection using a microscope. Neurospheres were dispensed into a 96-well plate (one sphere/well in 100 µl medium). Metabolic activity was measured 2, 4, 6, 24 and 48 hours after the sorting with the CellTiter-Blue® Assay. In Figure 3 the means ± SEM (standard error of the mean) are depicted for 3 independent experiments with 4 spheres per time point as a percent activity of the 48hr manually sorted spheres.

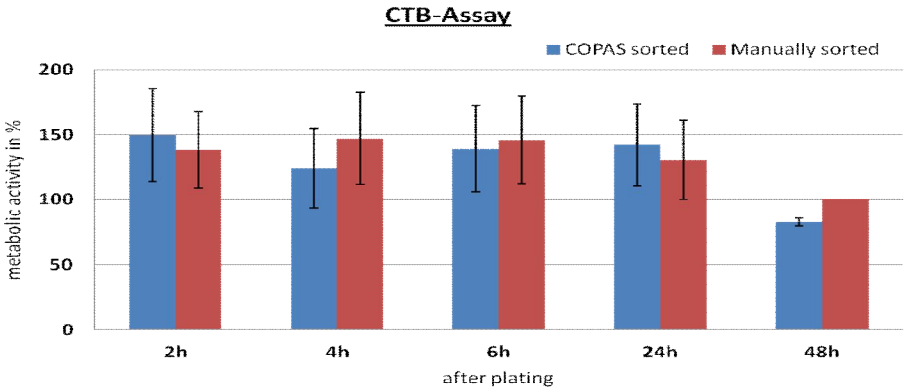

Figure 3. Assessment of cell viability. CellTiter-Blue® Assay comparing COPAS PLUS (blue) and manually (red) sorted spheres.

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The confidence intervals for both methods overlap so it can be concluded that processing the samples with the COPAS PLUS does not induce negative effects on cell viability. It is obvious that viability of neurospheres 48hrs after plating is reduced. However, this occurred for both the COPAS sorted and manually sorted neurospheres. This is probably due to a decrease in growth factor concentration in the media as spheres need a medium change every 48 hours.

In the LDH assay neurospheres of identical size were sorted by the COPAS instrument and compared to manual selection using a microscope. Neurospheres were dispensed in a 96-well plate (one sphere/well in 100 µl medium). Medium was collected 2, 4, 6, 24 and 48 hours after the sorting and the lactate dehydrogenase activity was measured. In Figure 4 the means ± SEM are depicted for 3 independent experiments with 4 spheres per time point and shown as a percentage of the activity of the complete cell lysis with Triton X100.

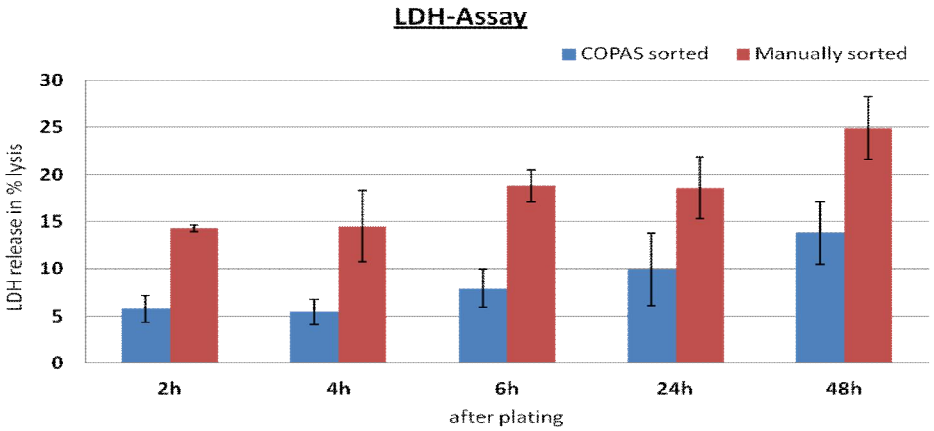

Figure 4. Assessment of cell death. LDH Assay comparing COPAS PLUS (blue) and manually (red) sorted spheres.

The COPAS PLUS sorted spheres give a significantly lower LDH activity which might mean that sorting with the COPAS PLUS gives less stress to the spheres compared to manual sorting. The CTB assay showed a decrease in viability 48 hrs after plating. This effect is mirrored in the cell death assay where LDH activity in the media increases 48 hrs after plating. As noted above, this is probably due to a decrease in growth factors over time.

Proliferation

In the BrdU assay neurospheres of identical size were sorted by the COPAS instrument and compared to manual selection using a microscope. Neurospheres were dispensed into a 96-well plate (one sphere/well in 100 µl medium).
After 48 hours culturing in medium, both with and without 20 ng/ml EGF, BrdU was added. Its incorporation into the DNA was measured after 16 hours using a luminescence cell proliferation ELISA (Roche). In Figure 5 the means ± SEM are depicted for the sum of 4 independent experiments with 6 spheres per exposure in relative luminescence units.

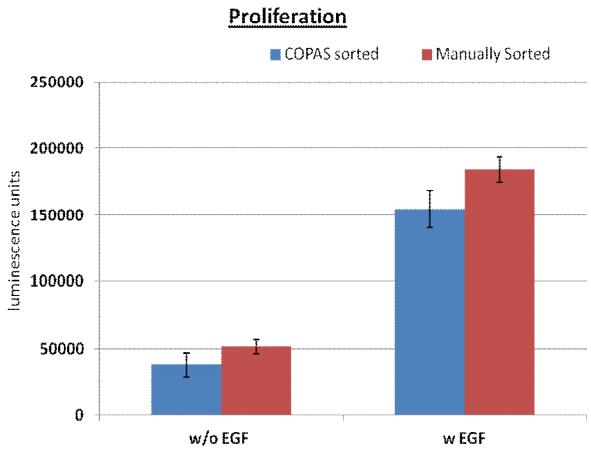

Figure 5. Assessment of cell proliferation. BrdU Assay comparing COPAS PLUS (blue) and manually (red) sorted spheres.

The confidence intervals for both methods overlap so the processing of the samples by the COPAS PLUS does not interfere with neurosphere proliferation as determined by the BrdU-based luminescence cell proliferation ELISA.

Conclusions

These experiments demonstrate that the COPAS PLUS may be used, in the standard configuration using the 488 excitation wavelength, to analyze and sort human neurospheres. The instrument can analyze, accurately select and sort similar cell clusters from a complex mixture of varying sizes. This automated analysis and sorting process is gentle so it does not influence cell viability or cell proliferation compared to manually sorted spheres. The COPAS PLUS provides a level of automation to the process of handling the neurospheres allowing for increased throughput and eliminates any biases that might be introduced by the researcher.

COPAS PLUS large particle analysis instrument brings the method of flow cytometry to the analysis and sorting of neurospheres which are otherwise too large and fragile for analysis on conventional single-cell flow cytometers. Cell clusters can be analyzed while intact, allowing for studies that address questions of cell-cell interaction, tissue development and differentiation. This instrument brings the advantages of flow cytometry – statistically meaningful data, large unbiased data sets, and multi-parametric analysis – to experiments using neurospheres.

References

M. Moors, T. Rockel, J. Abel, J. E. Cline, K. Gassmann, T. Schreiber, J. Schuwald, N. Weinmann, and E. Fritsche. 2009. Human Neurospheres as Three-Dimensional Cellular Systems for Developmental Neurotoxicity Testing. Environ Toxicol Pharmacol 7:1131-1138.