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

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 family of research instrumentation comprises a collection of large particle cytometers utilizing at least 1 excitation laser and up to 8 channels of fluorescence collection. Unique to COPAS instrumentation is the Profiling feature which graphically plots the fluorescence intensity changes along the length of the object as it passes through the laser(s). Large objects up to 1.5mm in diameter can be analyzed for physical and fluorescence characteristics and gently dispensed into a multi-well plate or other collection container for further investigation or reuse. The COPAS Vision also has equipped a camera to take an image of the object inside the flow channel. This image accompanies the cytometry data and can be analyzed using Union Biometrica's FlowPilot software or other image analysis tools.

In this experiment, The COPAS PLUS instrument (Union Biometrica, Inc) equipped with a 1000micorn flow channel, was 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 measurements of both the size (TOF) and optical density (EXT) of the sample object as well as any fluorescence that is excited by 488 nm wavelength of light. After analysis, the instrument can gently dispense the analyzed object in a afluid drop to a collection container such as a multiwell plate. These features make the COPAS PLUS ideal to handle, analyze, and dispense sensitive objects like cell aggregates without disrupting the clusters.

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.

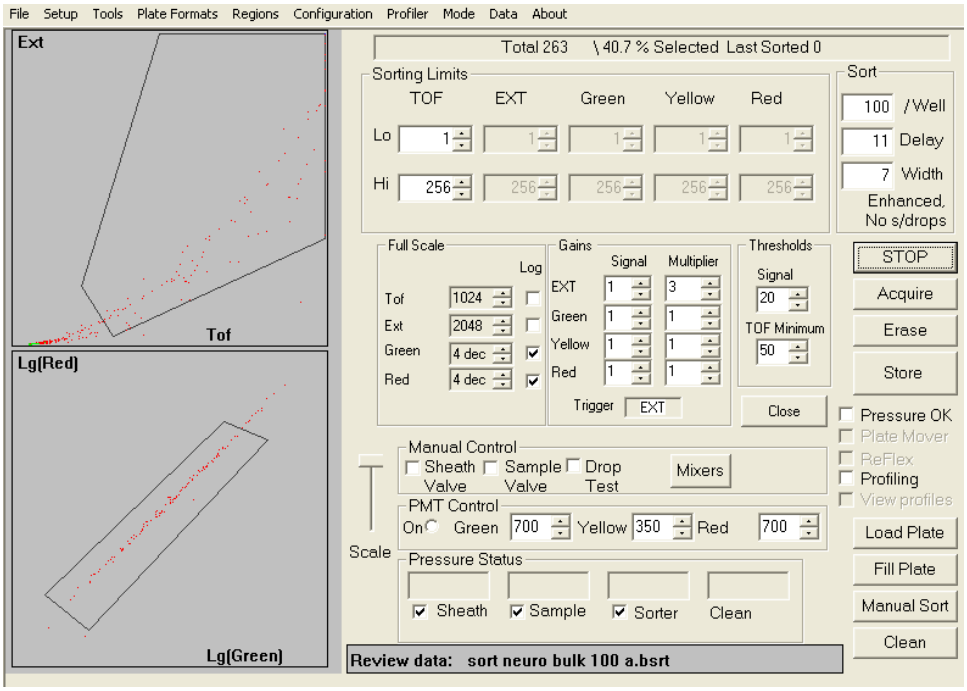

Figure 1. *Screenshot of COPAS software with neurosphere data. In the upper left corner a dual parameter dot plot allows for gating for a specific subpopulation of spheres (here TOF vs Ext with TOF ~ size and EXT ~ optical density). This population is then visualized in a dual parameter sorting dot plot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

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 are analyzed. 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

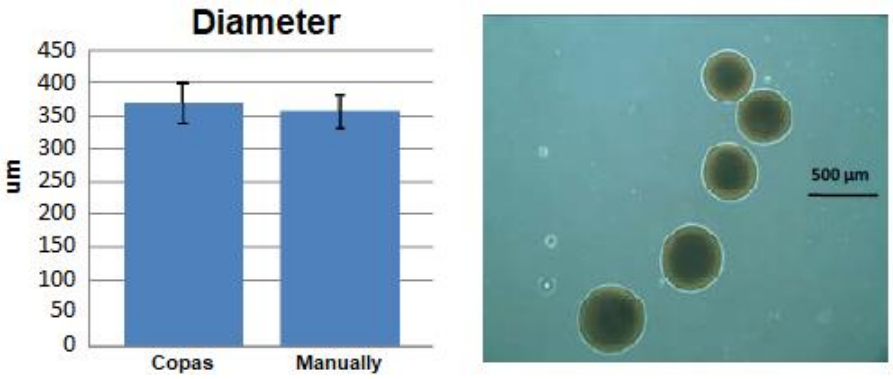

Figure 2 Neurosphere size comparison of COPAS and manually sorted spheres. *(left) A comparison of COPAS sorting and dispensing with manual methods. Appproximately 25 neurospheres were sorted into a 96-well plate and the diameter of both groups was determined with the Metamorph program. (right) Microscopic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

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 auto-fluorescence 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.

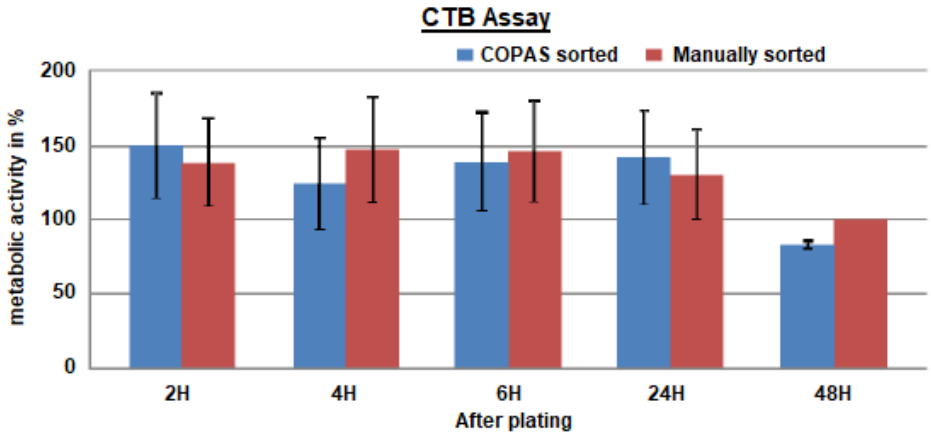

Figure 3. Assesment of cell viability. *CellTiter-Blue Assay comparing COPAS Plus (blue) and manually (red) sorted spheres.*

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).

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

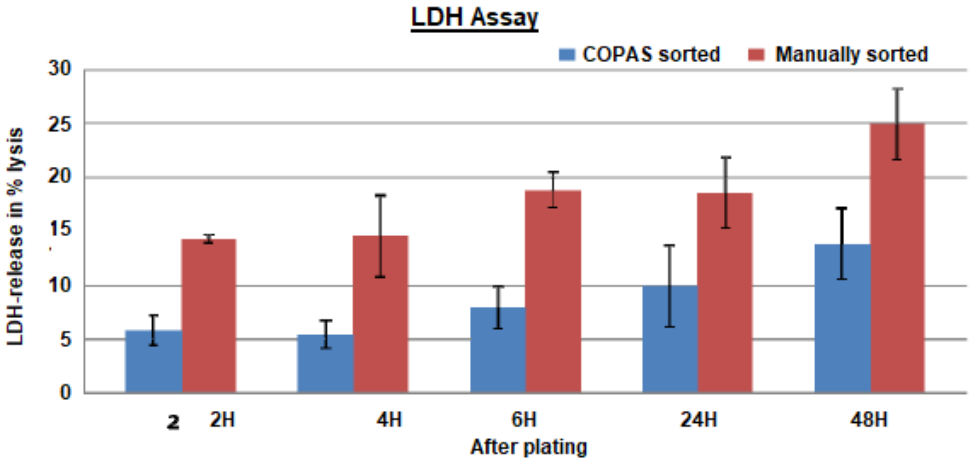

Figure 4. Assesment of cell death. *LDH Assay comparing COPAS Plus (blue) and manually (red) sorted spheres.*

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.

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. 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.

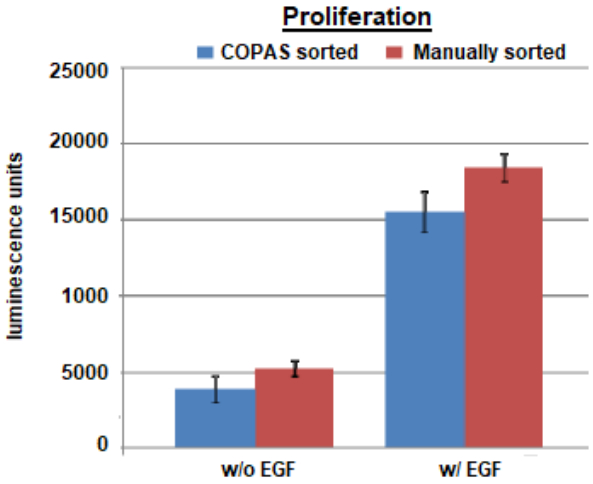

Figure 5. Assessment of cell proliferation. *BrdU Assay comparing COPAS Plus (blue) and manually (red) sorted spheres.*

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 multiparametric 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.

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