# AUTOMATED ANALYSIS AND SORTING OF CARDIOMYOCYTE EMBRYOID BODIES

## INTRODUCTION

The COPAS™ SELECT instrument is able to analyze and sort large objects (40-300 micron) on the basis of size, density and fluorescence signals. In this experiment we analyzed and sorted individual embryoid bodies (EBs) from cultured samples. These stem cell aggregates were accurately dispensed into wells of 96- and 24-well plates and visually inspected for viability. The gentle sorting method used by the COPAS SELECT provides a method for analyzing and handling these sensitive tissues with a high level of recovery and a low level of lethality.

## EMBRYONIC STEM CELLS AND EMBRYOID BODIES

Mouse embryonic stem cells (ES cells) are prepared from the inner cell mass of a developing blastocyst. The cells of the inner cell mass normally give rise to the three germ layers from which all tissues and organs of the body develop. The endoderm develops into the organs of the gastro-intestinal tract, the mesoderm into bones, muscles, blood vessels, heart and kidney, and the ectoderm into skin, eyes, glands and central nervous system. The cells of the inner cell mass essentially give rise to all the tissues that comprise the developing embryo. However, these cells can also be grown in culture, as embryonic stem cells, and are used to study the processes of development and differentiation.

One of the main characteristics of ES cells is their ability to proliferate endlessly and to maintain a pluripotent state. This requires special culturing methods and reagents (grown in monolayer culture on fibroblast feeders in the presence of LIF) and allows for the expansion of the total number of ES cells. Under certain conditions, these ES cells growing in culture will form aggregates called embryoid bodies (EB). These aggregates can differentiate into a variety of different tissues including cartilage, bone, smooth and striated muscle, neural tissue and skin. Protocols for the *in vitro* differentiation of ES cells into cardiomyocytes representing all specialized cell types of the heart have been established. As the embryoid bodies differentiate into cardiomyocytes, they closely recapitulate the developmental pattern of early cardiogenesis. Thus, they are an interesting experimental system for understanding the development of the vertebrate heart and potentially provide an avenue of inquiry into the discovery of therapies for heart disease.

There is broad interest in understanding the biology involved in these developmental processes and whether they can be manipulated by external cues to direct and control development along tissue-specific pathways. Furthermore, embryonic stem cells hold enormous potential for use in a number of human therapeutic approaches.

## PREPARATION OF EMBRYONIC STEM CELLS AND EMBRYOID BODIES

### 1. Culture of ES cells on mouse embryonic feeder cells

Mouse ES cells (clone ES-D3, from ATCC, Catalog No. CRL-1934) were cultured on 10cm Petri dishes in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS) and leukemia inhibitory factor (LIF) on a layer of feeder cells (irradiated mouse embryonic fibroblasts). Cells were incubated at 37°C, 7% $CO_2$ and 95% humidity. Cells were split every second day by trypsinising them to single cell suspension and seeding on a fresh dish coated with feeder cells.

### 2. ES cell aggregation

ES cells from one or more Petri dish were trypsinised to obtain a single cell suspension and collected by centrifugation. Cells were resuspended to a density of approximately $2x10^6$cells/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FCS. 4ml per 6cm Petri dish of this suspension were incubated on a rocking table at 50rpm, 37°C, 5% $CO_2$ and 95% humidity for 6h. After this time, the suspension was diluted 1:20 in several T25 tissue culture flasks and incubated for an additional 18h. Under these conditions ES cell aggregates (embryoid bodies) formed, typically around 500 per ml of cell culture suspension.

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### 3. ES cell differentiation

EBs were raised by methods that resulted in increased differentiation to cardiac cells. EBs were collected from the flasks by sedimentation and resuspended in IMDM without phenol red and FCS. EBs were transferred to the sample cup of the COPAS instrument and diluted to an appropriate concentration with IMDM w/o phenol red and FCS (about 200 EBs/ml). The COPAS was run with Hanks' balanced salt solution (HBSS) instead of standard sheath fluid to avoid damage of EBs. Single EBs were collected into each well of a 96-well round-bottom microtiter plate (bacterial grade plastic) filled with 200µl of IMDM 20% FCS. EBs were incubated at 37°C, 5% $CO_2$, 95% humidity for 4 days. On day 5, EBs were transferred to flat-bottom 96-well microtiter plates (tissue culture grade) and incubated under the same conditions for an additional 5 days. After 7-10 days, the first beating cardiomyocytes appeared among the EBs and could be visualized with a microscope.

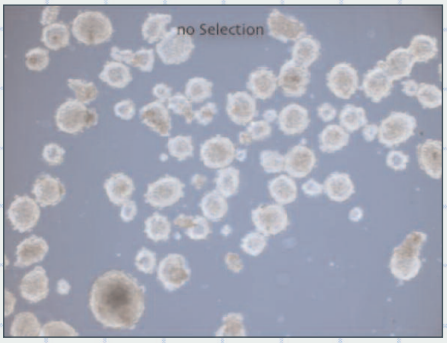


*Figure 1. EBs after overnight aggregation.*

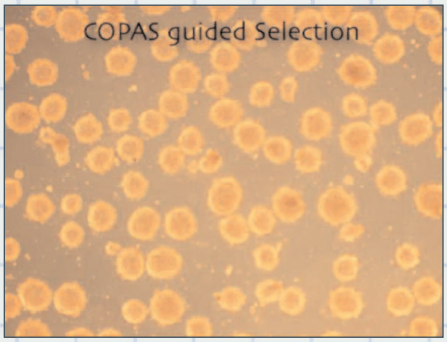


*Figure 2. EBs after COPAS sorting.*

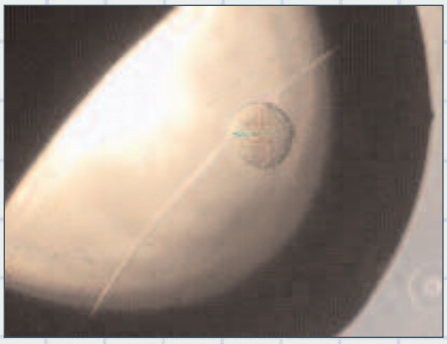

*Figure 3. Single EB sorted into a 96-well plate.*

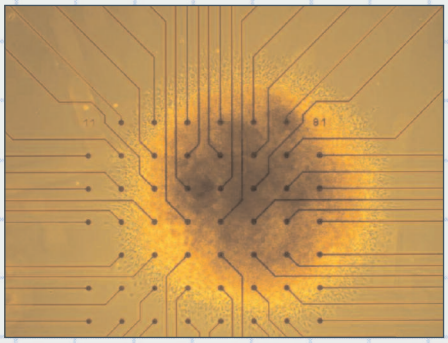

*Figure 4. Single EB plated on multi-electrode chip.*

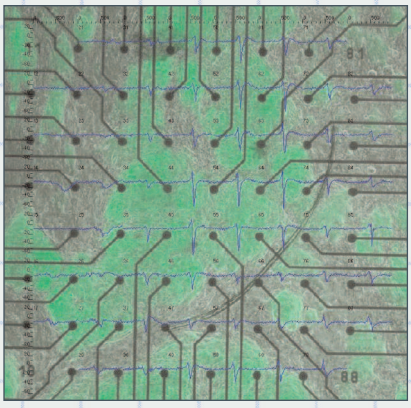

*Figure 5. Functional analysis of single EBs on micro-electrode array*

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## RESULTS

After initial setup of the COPAS SELECT, the samples were analyzed. The embryoid bodies selected for further analysis were the larger aggregates.

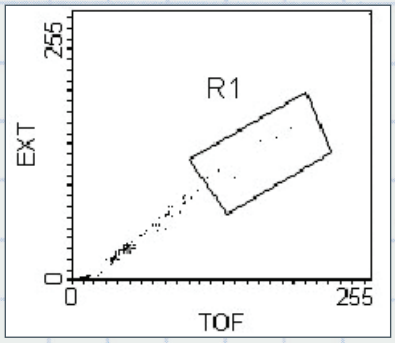


*Figure 7. TOF/EXT for cultured ES cells.*

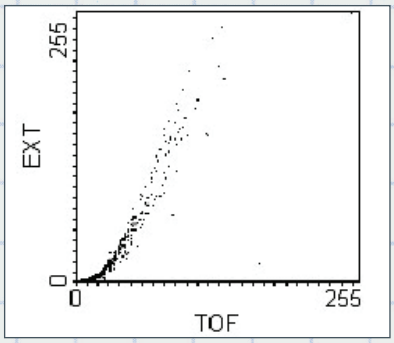


*Figure 8. TOF/EXT for cultured ES cells, lowered instrument threshold.*

Time Of Flight (TOF), the parameter representing the length of the objects and Extinction (EXT), the parameter representing the optical density of the objects were measured and are displayed in the dot plot. A region (R1) was selected for sorting (Figure 7). In this case the largest aggregates were chosen and represent aggregates of around 170 to 230 microns across. These were dispensed into wells of 24- and 96-well plates and allowed to grow before further analysis. The efficiency and accuracy of dispensing to wells was determined by visual inspection and found to be greater than 99%. Visual inspection after the subsequent growth phase also confirmed the viability of the embryoid bodies was greater than 95%. During further experiments in Cologne (at Axiogenesis AG), the application was optimized by lowering the threshold of detection of the system on TOF and EXT in order to avoid contamination of the sorted sample by single cells and debris (Figure 8). While a greater number of smaller aggregates are now detected, the embryoid bodies sorted individually into wells by the COPAS SELECT are more pure. The culture was diluted to get a sample concentration of about 200 events per ml. Depending on the sample preparation, a percentage of 10-30% was selected for sorting.

## CONCLUSION

This early data indicates that the COPAS SELECT instrument is able to analyze and sort embryoid bodies from cultured ES cells. The sorting is accurate to wells of 24- and 96-well plates and the method is gentle, resulting in little or no loss in viability.

## ABOUT UNION BIOMETRICA, INC.

Union Biometrica, Inc. is the pioneer in the development and manufacture of high throughput systems for the screening of "large" particles such as large cells and cell clusters, beads used in combinatorial chemistry libraries, small seeds, and for viable small model organisms including C. elegans, Drosophila, and zebrafish. Union Biometrica's proprietary COPAS™ systems analyze, sort, and dispense objects with a higher precision and speed than any other commercially available technique, thereby dramatically reducing the time required for experiments and even enabling new, previously unfeasible experiments to be performed.

*More information about Union Biometrica, Inc. may be found at http://www.unionbio.com*

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## ABOUT AXIOGENESIS AG

**Axiogenesis AG** is an innovative biotech company with expertise in applied stem cell technology. Using state-of-the-art methods in cellular and molecular biology as well as functional analytics, Axiogenesis develops novel techniques to improve safety and efficiency of new drugs and chemicals.

**Axiogenesis'** first development was the RETox© assay for the identification of toxic and teratogenic compounds. The RETox© assay is based on multiple transgenic mouse stem cell lines and allows for *in vitro* screening for effects of new compounds (e.g. pharmaceuticals, bulk chemicals, pesticides) on the developing embryo and regular differentiated functional tissue. Further improvement of the assay now allows efficient testing of large numbers of new compounds at low cost rates. Moreover, the amount of animal testing in embryotoxicology can be markedly reduced.

**Using Axiogenesis'** CardioBodies©, the MelCor Assay© was developed to search for cardiac side effects in drug development. The MelCor technology platform is based on MultiChannelSystems MEA system and allows electrophysiological screening without the need of time-consuming animal tissue preparation.

*More information about Axiogenesis AG may be found at http://www.axiogenesis.com*