Flow cytometry for isolation and optical analysis of embryoid bodies from cultured mouse embryonic stem cells.

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Introduction

The study of embryonic stem (ES) cells, as they differentiate in culture to form cell clusters and embryoid bodies, will provide insights into normal cell differentiation, tissue development, and organogenesis. A better understanding of the spatial and temporal cues, as well as, the identity of exogenous growth factors and cytokines involved, can be studied in this simplified model. Traditionally such experiments could only be performed in whole animal studies. Furthermore, with a better understanding of the differentiation of ES cells, they will be useful as tools for drug discovery, toxicology, and the promise of regenerative medicine.

We have developed instrumentation for analysis of large cell clusters and embryoid bodies from cultured ES cells. The technology is based on traditional flow cytometry but modified to accommodate the larger object size and the more delicate handling required to maintain cell viability. We tested this instrumentation using mouse ES cells that formed cardiomyocyte-like cell clusters. Protocols for the *in vitro* differentiation of ES cells into cardiomyocytes representing all specialized cell types of the heart have been established (Kehat et al., 2001). 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 muscle and potentially provide an avenue of inquiry into the discovery of therapies for heart disease. For our experiment, mouse ES cells were prepared for cardiomyocyte differentiation and allowed to grow for brief periods of time. Mouse ES cells can be transformed with plasmids that express GFP protein from a cardiac-specific myosin heavy chain promoter. Various samples were then analyzed on our COPAS flow cytometer instrument. The COPAS instrument is able to analyze and sort large objects (40-300 micron) on the basis of size, density and fluorescence signals. The stem cell aggregates (about 170-230 microns in diameter) were accurately dispensed into wells of 96- and 24-well plates and visually inspected for cardiomyocyte identity and viability. The gentle sorting method used by the COPAS provides a method for analyzing and handling these sensitive tissues with a high level of recovery and a low level of lethality.

Sample preparation

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 (Kehat et al., 2001). 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.

Sample preparation

PREPARATION OF 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 trypsinizing 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 trypsinized 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.

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.

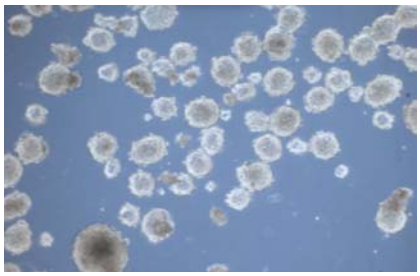

Figure 1. EBs after overnight aggregation.

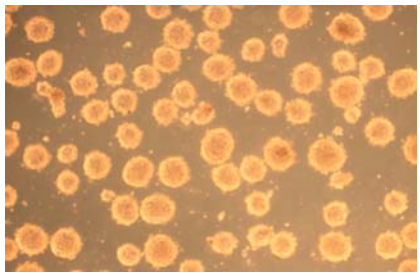

Figure 2. EBs after COPAS sorting.

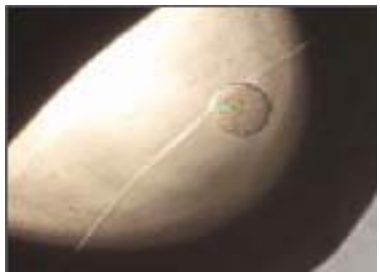

Figure 3. Single EB sorted in a 96-well plate

Figure 4. Single EB at day 6, one day after plating in 96-well flat bottom multiwell plate.

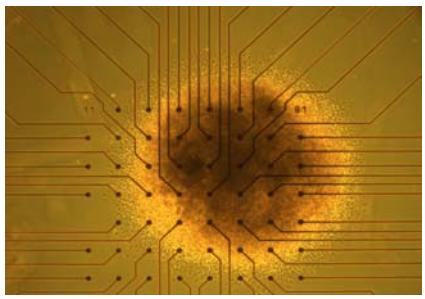

Figure 5. Single EB plated on a Multi Electrode Chip.

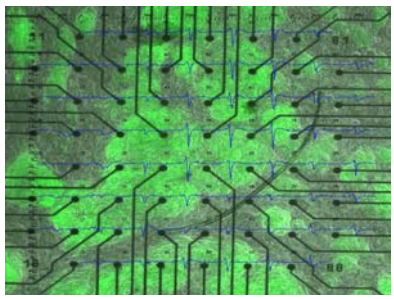

Figure 6. Functional analysis of single EBs on Multi Electrode Chip.

Results

The embryoid bodies are of a range of sizes. We tested whether we could select a particular narrow range for dispensing. 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 an example of the data are shown in the dot plot (Figure 7). Using the software that controls the COPAS instrument, we selected a region for sorting (R1 in Figure 7). The selected region represents the largest objects in the sample. These were collected individually to wells of a multi-well plate and further analyzed.

For the data shown, the embryoid bodies that were collected represent cell aggregates of around 170 to 230 microns across. These were dispensed into wells of 24- and 96-well plates and allowed to grow further. The efficiency and accuracy of dispensing to wells was determined by visual inspection and found to be greater than 99% over several 96-well plates. 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 7). 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. The samples were analyzed at about 20 objects per second (greater than 50,000 per hour). Depending on the sample preparation, a percentage of 10-30% was selected for sorting.

The sorted embryoid bodies were used in a number of different ways including Axiogenesis AG's compound screening for toxicological and teratogenic effects on cardiomyocytes (R.E.Tox Assay) and for Axiogenesis' Multiple Electrode Array System.

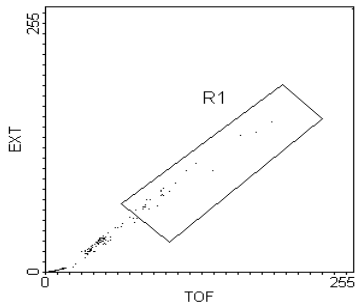

Figure 7. TOF/EXT for cultured ES cells.

Conclusions

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 about 99% for wells of 24- and 96-well plates. The instrument is designed to collect optical measurements for each object sorted, including fluorescence measurements. This allowed us to choose a subset of the sample and sort these accurately. The sorting method is gentle, resulting in little or no loss in viability.

References:

Kehat et al., (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest*, 108:407-14.

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

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