Multicellular Cytometric Analyses and Sorting on Embryoid Bodies and Embryonic Stem Cell Clusters

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Introduction

Human embryonic stem cells (hESC) provide an appealing source for generating cells or tissues that are of significant clinical potential (e.g. cardiac cells, pancreatic beta cells), or cell populations that have been difficult to grow *in vitro*. One bottleneck in hESC culture is the survival inefficiency of isolated single cells. Consequently, hESC are routinely passaged by a combination of mild enzyme exposure and mechanical disruption resulting in small cell clusters of roughly 100µin diameter. Although, it is desirable to sort hESC for various markers, conventional flow cytometers and sorters dramatically reduce the viability of hESC, likely due to the necessity of breaking them into a single cell suspension before sorting. In this study we examine the capability of COPAS BioSorter™ (Union Biometrica, Inc.) - an air actuated large orifice flow sorter - on ESC clusters as a first endeavor to find a more efficient methodology to analyze and isolate ESC clusters.

Purpose of the Study

To examine the capability of COPAS BioSorter large particle sorter for analyzing and isolating embryonic stem cell clusters or embryoid bodies.

Experimental Plan

Develop protocols for isolating hESC clusters with more than 20 cells per cluster (Figure 2).

1. Examine viability of sorted hESC clusters (Figure 3) and compare with that obtained via conventional FACS.
2. Analyze the isolation and colony formation efficiency of COPAS BioSorter sorted non human primate (rhesus) ESC clusters (Figure 4.
3. Isolate hESC clusters based on GFP expressions (Figure 5).

Materials and Methods

1. Protocols for culturing ESC clusters and alkaline phosphatase assay.
   - H1 hESCs (Wicell, Madison, WI, USA) were cultured on gelatinized tissue culture plates seeded with feeder cells- primary mouse embryonic fibroblasts- that had been exposed to 3000 Rad g-irradiation according to published protocol (reference). The cell layer was washed off the plate and mechanically dispersed and divided into tubes.
   - The hESC clusters were directly sorted into 96-well plates which contain 150 µl of medium (reference) and overnight cultured feeder cells.
   - Colony labeling was by alkaline phosphatase (AP) assay using a kit (Vector Laboratories, Burlingame, CA) following the manufacturer's protocol. The cells were visualized through anti-SSEA-4 (Chemicon International, Temecula, CA) and a biotinylated secondary antibody (Vector Laboratories).
   - hESC were transduced by a lentiviral vector containing GFP driven by a CAG promoter.

2. The COPAS™(Complex Object Parametric Analyzer and Sorter) BioSorter instrument is a large orifice flow cytometer that features a proprietary air-actuated sorting mechanism (Figure 1). The model used in this study is equipped with a 500-micron flow cell, multiline (488/514 nm) Argon laser, 670 nm diode laser and an automatic plate stage for 384/96/48/24-well plates. The fluorescent emission spectrum collected simutaneously are 510 m ± 20 nm(green), 545nm ± 20 nm (yellow) and 610 ± 30 nm (red).

COPAS® BioSorter™

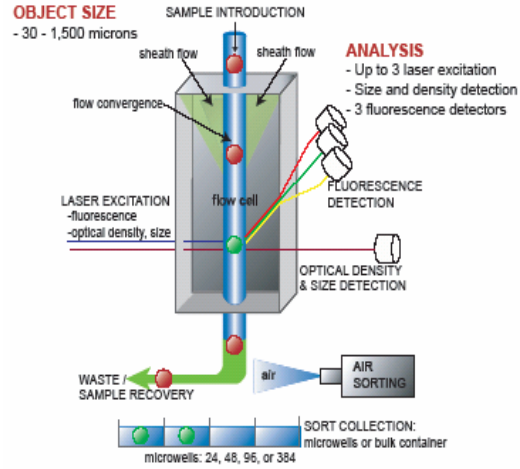


Figure 1. Schematic diagram of fluidics, optics & sorting mechanism.
Key features of COPAS BioSorter include:

- Gentle sorting mechanism that maintains the viability of biological samples
- Large orifice (500 micron) flow cell that accomodates tissues, multi-cellular organisms or cell clusters
- Quantitative and high content analyses on fluorescence, size and optical density in real time

Parameters for Sorting

COPAS Parameters	FACS Counterparts	Measurement
Time of flight	Forward scatter	Size
Extinction	Side scatter	Complexity
Fluorescence	Fluorescence	Fluorescent signals

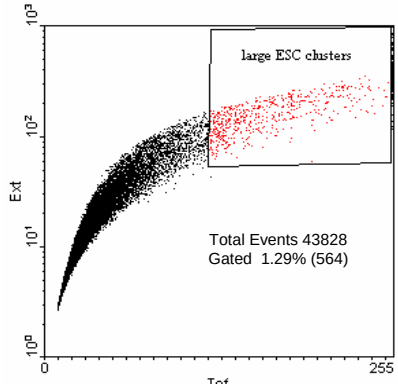
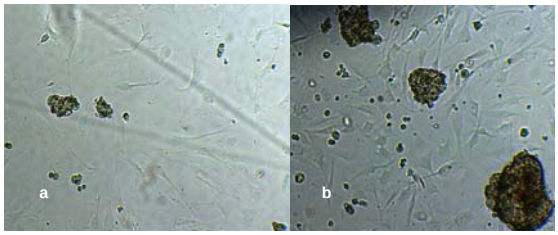
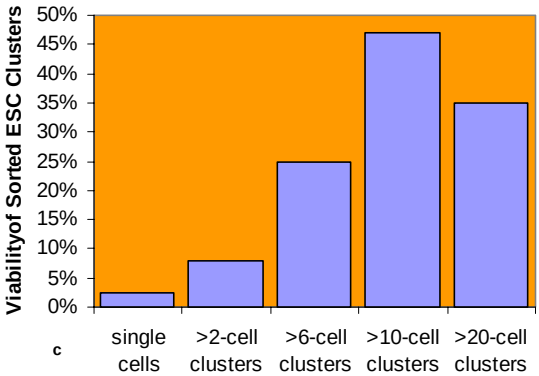


Figure 2. Parameters for isolating hESC clusters.
Time of flight (TOF) is the proxy measurement for the size, reflected by the time that it takes the object to pass through 670nm red laser beam. Single cells and ESC clusters can all trigger the registration of an event; large ESC clusters (>20 cells/cluster) differ from single cells and small clusters in their large TOF and EXT values. We examined the sorted clusters by microscopy to optimize sorting parameters.

Viability of Sorted hESC Clusters





# of sorted	2000	960	480	192	192

Figure 3. Viability result of sorted hESC clusters.
The single cell sorting was performed on a conventional FACS. The ESC cluster sorting was performed on a COPAS BioSorter™ 500. The size of the clusters was examined via microscopy (a and b). The flow rate was ~100 events/second. The viability of the sorted cells or clusters was derived based on colony formation efficiency via alkaline phosphatase assay after 2-day culture as described (reference). It appears that the bigger the hESC clusters, the better the colony formation efficiency (c). The decreased viability in the 20-cell cluster in comparison with 10-cell cluster may be due to sampling error.

Viability of Sorted Rhesus ESC Clusters

# of colonies / well	Plate #1	Plate #2	Plate #3	Total
0	13	40	52	105
1	54	49	28	131
2	17	3	8	28
3	7	3	2	12
4	5	1	6	12
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
Total	96	96	96	288
Viability	64%			

Figure 4. The viability and colony formation efficiency of COPAS sorted rhesus ESC clusters.
Single ESC clusters were sorted into wells in 96-well plate. The size of the clusters were examined via microscopy to be more than 20 cells per cluster. The flow rate was ~50 events/second. The viability of the sorted cells or clusters was derived based on colony formation efficiency via alkaline phosphatase assay after 2-day culture. The wells with 2 or more colonies may most likely be due to the breakage of large cluster after sorting.

Analysis and Sorting on Fluorescent Signals

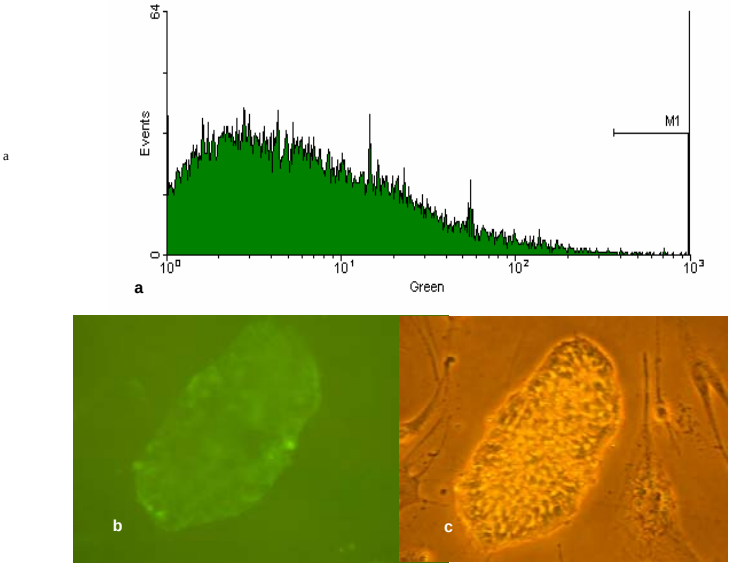


Figure 5. The cytometric analyses on GFP-expressing hESC cell clusters.
The CAG driven GFP is expressed universally in transformed embryonic stem cells. By gating on GFP, we distinguish transformed cells from non-transformed ones. M1 region identifies the gate for sorting GFP-positive hESC clusters (a). The GFP signals of the sorted hESC clusters were verified via microscopy (b). The phase contrast image of the hESC cluster is shown in (c).

Conclusion

1.COPAS BioSorter™ is a superior alternative to conventional FACS for isolating and analyzing ESC clusters. The COPAS sorted ESC clusters maintained far better viability and colony formation efficiency than those sorted by conventional FACS instruments.

2.Quantitative and multimetric analyses and automatic sorting via COPAS provides a high throughput methodology for ESC clusters-based drug and biology studies.

3.Our data show that there is a positive correlation between the sorted hESC clusters and the colony formation efficiency.

Acknowledgments

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Reference

Ware, C. et al 2005, *Biotechniques* Vol. 38, No. 6: pp 879-883.

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