Comparison of Total Fluorescence in Cell Clusters versus Fluorescence of the Individual Cells in the Cluster

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Objective

In the analysis of cell clusters composed of a mixture of fluorescent and non-fluorescent cells it has been questioned how the amount of fluorescent cells in the clusters can be discriminated. The level of fluorescence in a cell cluster is a result of the number of positive cells in the cluster and the intensity of light emitted from the fluorescent marker on/in those cells. In this QTN we compare the analysis of the fluorescence level of an entire cell cluster with the fluorescence measurement of the individual cells of that particular cell cluster. The BioSorter® was used to measure and dispense an individual cell cluster into the well of a multiwall plate. Then, cells of the cluster were separated from each other and analysed by conventional single cell flow cytometry. We compare the fluorescence mean intensity of the cluster as measured on the BioSorter with the total number and mean channel of fluorescent cells within the cluster measured on a conventional single cell flow cytometer.

Introduction

There is an increased interest in studying how cells grow, divide and differentiate in a more natural context, such as a 3D cell cluster, rather than across the surface of a cell culture plate. Many cell types will naturally form cell clusters when given the opportunity, and generate various spheroid and organoid bodies. This approach allows for cell-cell interactions to occur and provides biological insights otherwise missed in flat sheets of cells growing on plastic surfaces or as cells grow in isolation. New methods enable the broad use of transgenically-introduced fluorescent proteins expressed from promoters of interest. Researchers can use these to identify when a cell has transitioned to a different state. Using this approach, cell clusters can be analyzed for these types of changes and transitions, and clusters of different types isolated for further studies.

Here we describe a method for fluorescence analysis of cell clusters that are then dispensed individually into wells of a 96 well plate. After dispensing the clusters, they are dissociated to single cells that are then further analyzed by conventional

single cell flow cytometry. The fluorescence data from the BioSorter large particle flow cytometer is compared with the fluorescence data of the individual cells of each cluster.

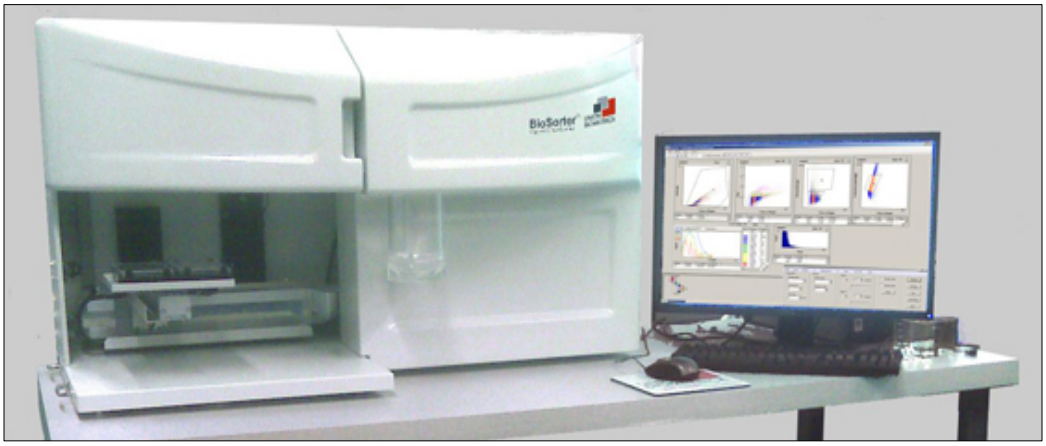
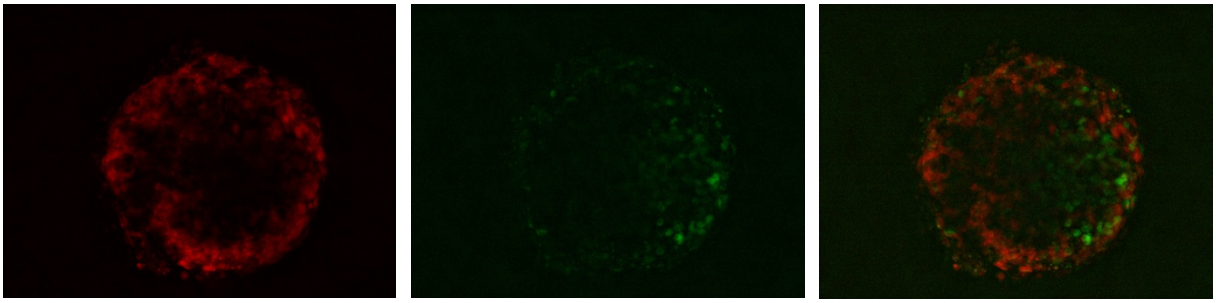


Figure 1. The BioSorter is a large particle flow cytometer that allows high-throughput analysis and sorting of objects in the size ranger 10 µm to 1500 µm.

BioSorter Analysis and Sorting

Embryoid bodies are cell clusters that form from embryonic stem cells grown in liquid culture under certain conditions. Similar cell clusters can form from induced pluripotent stem cells collected and incubated using the "hanging drop" method. We created embryoid body-like cell aggregates starting with 200 cells. The cells were spiked with fluorescent cells so that the final embryoid body could contain between 1 and 500 fluorescent cells each. Green-yellow fluorescent probe for all samples was Venus, the red fluorescent probe was mKO2. The first sample was a negative control (Figure 2). Positive samples were either negative EB's containing a number of fluorescent cells or EB's where the original starting cells where either green or red, spiked with the other color.



Images 1-3: Photomicrograph of EB with red (mKO2) in *left panel*, green-yellow (Venus) in *middle panel*, and mixture of both fluorescent cells in *right panel*.

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A negative control sample was analysed to set PMT levels for the baseline of autofluorescence. Individual embyoid bodies from positive samples were sorted into prefilled wells of a 96 well plate.

BioSorter Analysis Data:

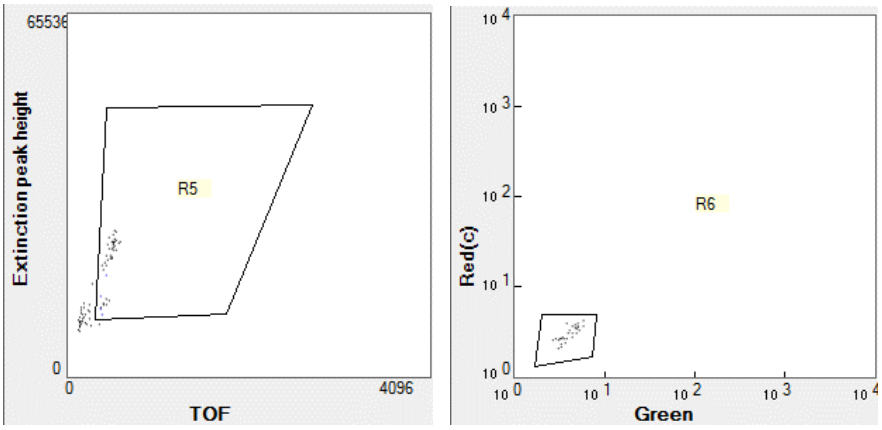

Figure 2: Negative control sample. Plots show the size (TOF) and optical density (EXT) (left panel), total auto-fluorescence (Green versus Red) (right panel) of the clusters.

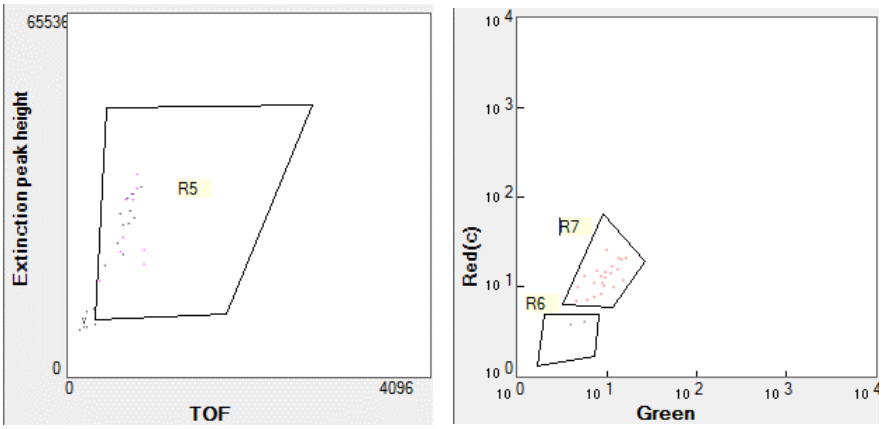

Figure 3: EB sample spiked with mKO2 positive cells. Plots show the size (TOF) and optical density (EXT), total fluorescence (Green versus Red) of the clusters.

Figure 3 shows that the fluorescence levels of the cell aggregates spiked with mKO2 positive cells have more red fluorescence, delineated by gate labelled R7, in comparison with the unspiked aggregates seen in Figure 2. The gate R6 in figure 3 shows the location of the unlabelled cell aggregates from figure 2. Also, the cell clusters in R5 gating region (Fig. 3) indicates the larger sizes of the aggregates than the aggregates of the negative control sample (Fig.2).

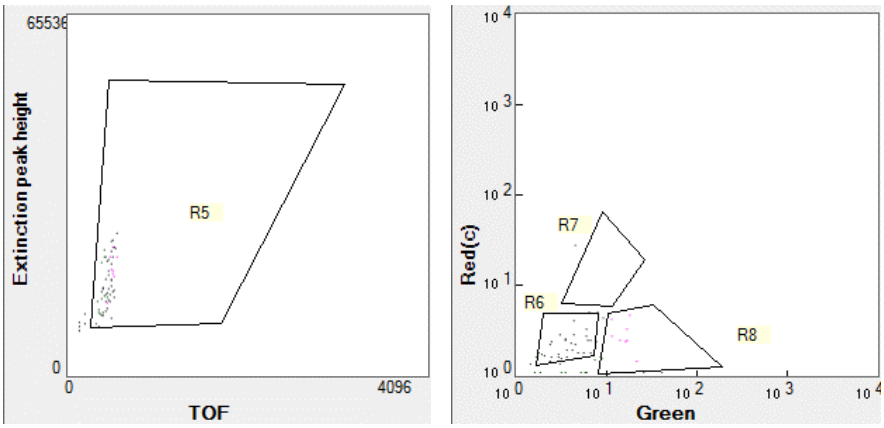

Figure 4: EB sample spiked with Venus positive cells. Plots show the size (TOF) and optical density (EXT), total fluorescence (Green versus Red) of the clusters

Figure 4 shows a shift in the amount of green-yellow fluorescence of the cell aggregates containing some number of Venus positive cells. Many are still in the same region as the negative control (R6) but some have shifted to the R8 region.

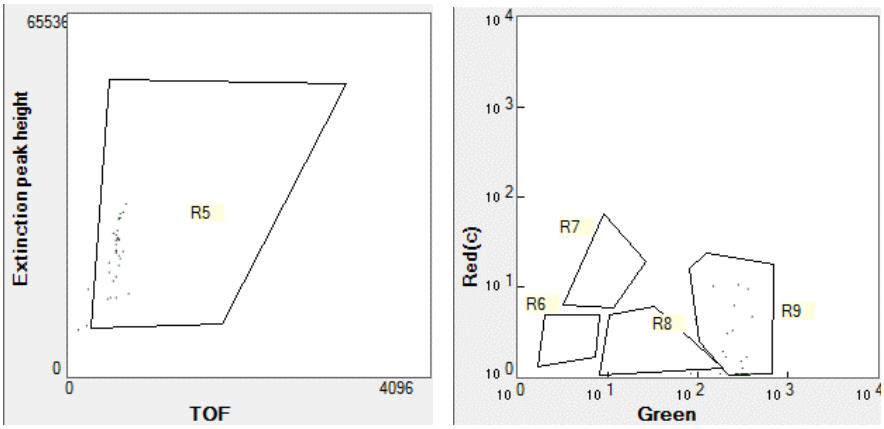

Figure 5: EB Venus positive sample spiked with mKO2 positive cells. Plots show the size (TOF) and optical density (EXT), total fluorescence (Green versus Red) of the clusters.

The figure 5 shows the shift of cell aggregates composed mostly of Venus positive cells and spiked with varying numbers of mKO2 positive cells. These cell clusters have fluorescence values higher along the y-axis indicating greater amounts of red fluorescence than the control sample or the clusters of Venus positive cells alone.

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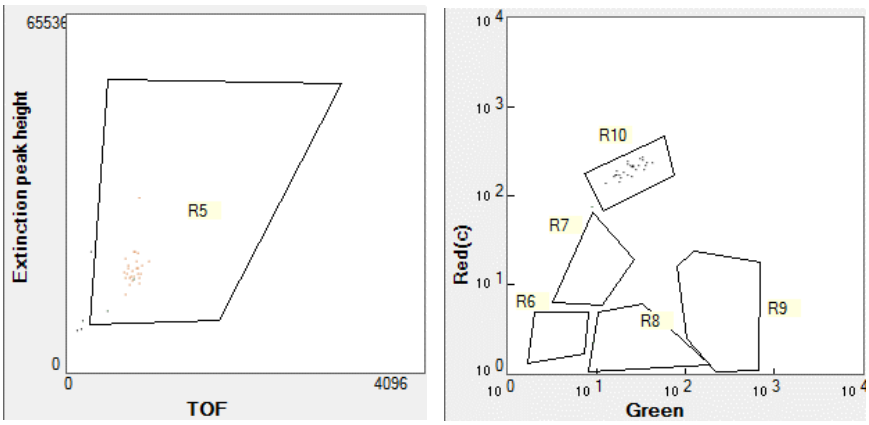


Figure 6: EB mKO2 positive sample spiked with Venus positive cells. Plots show the size (TOF) and optical density (EXT), total fluorescence (Green versus Red) of the clusters.

Figure 6 shows EBs composed mostly of mKO2 cells spiked with varying numbers of Venus positive cells.

FACS Analysis Data

Cell clusters from different samples were dissociated and analysed by FACS. For each EB the percentage of positive cells and mean channel intensity was recorded. Three FACS sample dot plots for 1 EB are shown below.

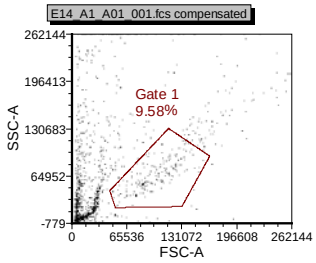
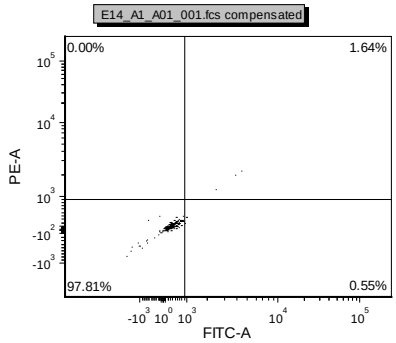


Figure 7: Control sample

BioSorter Values:

Green:	17.3
Green Peak Height:	2224
Red:	62.5
Red Peak Height:	6282



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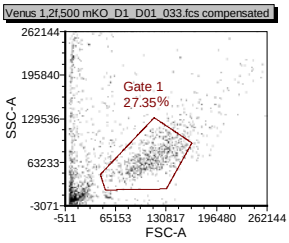


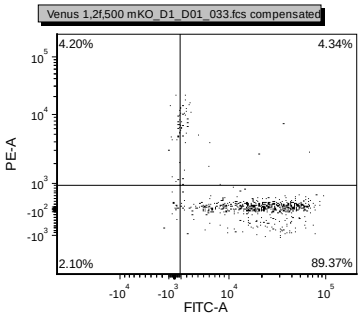
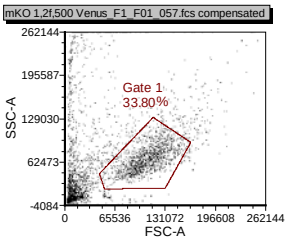


Figure 8: Venus positive sample

BioSorter Values:

Green:	404
Green Peak Height:	65532
Red:	86.5
Red Peak Height:	15459



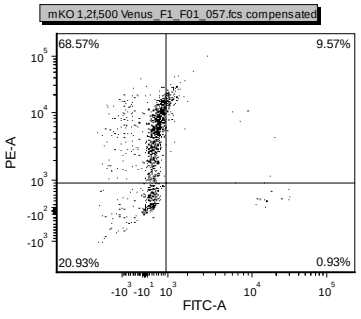


Figure 9: mKO positive sample

BioSorter Values:

Green:	21.4
Green Peak Height:	3292
Red:	226
Red Peak Height:	31332

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Conclusions

The BioSorter large-particle flow cytometer provides instrumentation for analysis and sorting of cell clusters. We show that populations of EBs can be analysed and characterized for their size distribution and relative fluorescence levels. Single EBs were sorted individually into wells of 96-well plates. Criteria for sorting was size and fluorescence but more stringent criteria could be adopted if required by the experimental design. The data from the BioSorter showed that it was able to distinguish cell clusters that contained some fluorescent cells expressing a transgenic protein. In these experiments EBs containing some cells expressing Venus protein (green-yellow), or mKO2 protein (red), or mixtures of these and non-fluorescent-protein expressing cells could be distinguished from each other.

The validity of the data from the BioSorter is supported by analysis of the cell populations by single cell flow cytometry. The individual EBs isolated in a well of a 96-well plate were dissociated into single cells. The contents of each well was then analysed using the FACS Fortessa and the data showed concurrence with the BioSorter data. When the BioSorter measurement of EBs containing Venus-expressing cells showed greater total green-yellow fluorescence than EBs composed of non-fluorescent cells, similar results were observed in the single cell analysis on the Fortessa instrument. Likewise, EBs with the mKO2 cells had higher measurements in the red channel on the BioSorter and higher mean-channel measurement on the Fortessa. Therefore, the fluorescent cells are detected above the background of non-fluorescent cells in the context of the EB cell cluster. Average measurements demonstrate the sensitivity of both systems is comparable regarding fluorescence intensity measurements whether it is measured in the total EB, as with the BioSorter, or by the average fluorescence levels for the individual cells of the populations from dissociated EBs measured by conventional single cell flow cytometry.

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