Analysis and sorting of Stem Cell Clusters and Adipocytes,

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Objective:
Establish the proof-of-priciple use of COPAS instrumentation to identify, analyze and dispense cellular samples: Stem cell clusters cultured and adipocytes.

Introduction:
COPAS™ (Complex Object Parametric Analyzer and Sorter) instruments automate the analysis, sorting, and dispensing of "large" objects such as viable small model organisms, seeds, beads, and particles, measuring the object size, optical density, and the intensity of fluorescent markers. Once analyzed, objects are sorted according to user selectable criteria, and then may be dispensed into stationary bulk receptacles or multi-well microtiter plates for high throughput screening. COPAS instruments analyze and sort large objects with a higher speed and precision than present manual techniques. By automating the current, time consuming manual processes, the time required for experiments is dramatically reduced, human error is eliminated, and new experiments that previously could not be considered are now possible.

The COPAS Select instrument features our 500 μm flow cell and is suitable for analyzing and sorting various large cell types and cell clusters.

Materials and Methods:
Two very different cell types were used in this test of the COPAS instrument. These were embryonic stem cell clusters (embryoid body-like) and cultured adipocytes. These two sample types were obtained from collaborators in Cambridge, MA.

COPAS Select (500um flow cell) with a single 488nm solid state laser was used for detection of TOF (time of flight equivalent to length), EXTinction (optical density), and excitation of any fluorophores. COPAS software including Advanced Acquisition Package and Profiler II was utilized in addition to flow cytometry analysis software FCS Express™.

Results:
The COPAS instrument measures five parameters of an object: TOF (Time of Flight, equivalent to the length of the object), EXT (EXTinction, equivalent to the optical density), Green, Yellow, and Red fluorescence signals. The fluorescence is measured as both an integral value (integrated over the length of the object) or peak intensity and peak width (Profiler II options). During sample acquisition, these values are plotted on COPAS software utilizing 2-D dot plots and can be used to identify optical and fluorescent properties that are unique to individual samples.

Sorting stem cell clusters.
Prior to running the stem cell clusters, 100um polystyrene beads were run on the COPAS Select to optimize instrument conditions to acquire and dispense the stem cell clusters of similar 100um size. Once these conditions were set, stem cell clusters were added to the sample cup, a gate region containing clusters of 100um size and larger was drawn to

dispense multiple clusters to each well of a 96 well plate. See **Figure 1** containing COPAS Software screen dot plot of 100um beads and sort window of dispensed stem cell clusters.

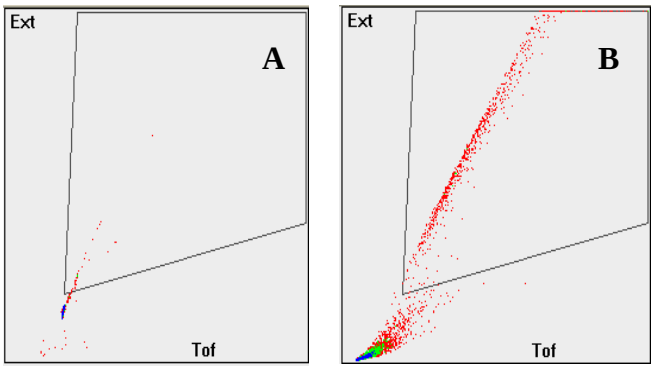

Figure 1. COPAS software dot plot of: **A)** 100um beads, **B)** Dispensed stem cell clusters larger than 100um in diameter. Representative sort region of stem cell clusters drawn on each plot. Dot plot displays Time of Flight and Extinction (max scale = 512)

Profiling feature

Although the profiling feature was not specifically utilized in this experiment, the Profiler II feature measures and plots each parameter (extinction, green, yellow, and red fluorescence values) as the intensity of each parameter changes along the length of the object as it is scanned. The measurement of these changes is displayed as overlaying color graphs plotted along the length (TOF) of the object: blue graph traces the extinction, while green, yellow, and red graphs trace their corresponding fluorescence. (See **Figure 2**) This feature allows for quantification of eight additional criteria (peak height and peak width for EXT, Green, Yellow, and Red parameters) for dot plot representation as well as fluorescent peak count analysis. While these stem cell clusters contained no fluorescence label, the Profiler II feature can be particularly advantageous to identify a small fluorescence signal (small fluorescent peak) representing the auto-fluorescence of the object. Or more simply, Profiler II can be used to illuminate other optical features of the objects within the sample.

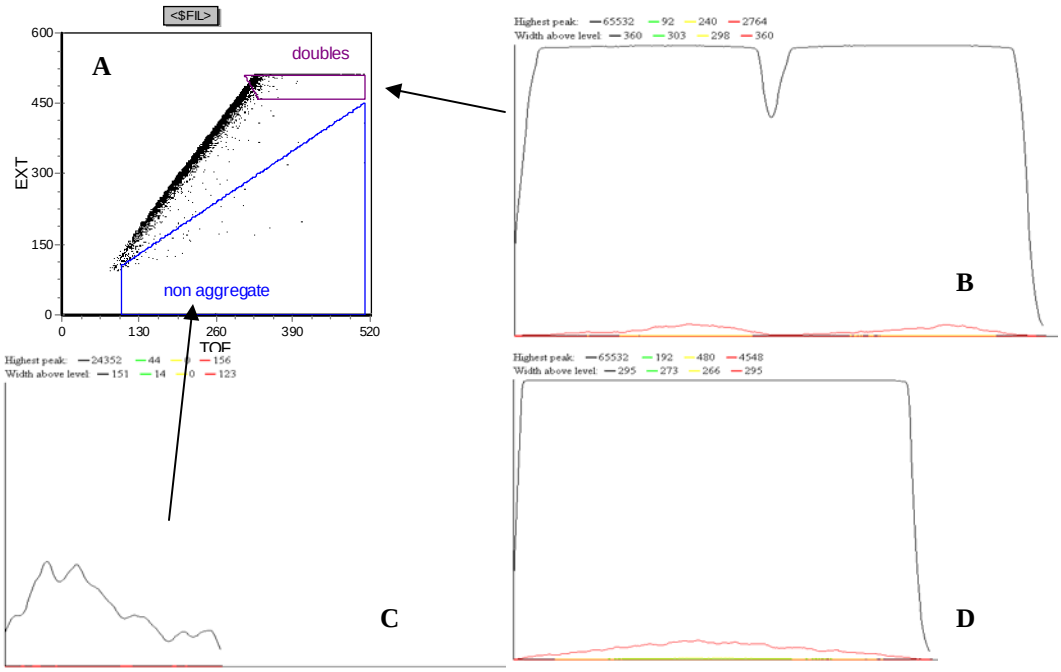

Figure 2. Dot plot and Object profiles of sorted objects. **A)** Dot plot display of objects within sort region, **B)** Object profile of 2 clusters stuck together, **C)** Profile of object that is not a stem cell cluster, **D)** Profile of a single stem cell cluster.
Analysis Dot plot of tof and extinction with representative 'non-aggregate' and 'doubles' regions drawn, profile graphs: X-axis Time of Flight, y-axis extinction and fluorescence intensities.

Figure 2 displays the object profiles from three dispensed objects in the stem cell cluster sample. Notice the profile in **2B** contains two rounded peaks tracing the extinction (blue graph) of two stem cell clusters stuck together (acquired as a single object), while **2D** contains only a single extinction trace corresponding to a single stem cell cluster. **2C**, on the other hand, is an object that does not appear to be a stem cell cluster at all. By visualizing individual object profiles of collected data, we can deduce that some of the objects within the sort region were not single stem cell clusters. This provides an opportunity to define a more selective sort region for a more uniform or 'pure' stem cell cluster collection.

Data analysis

Stored data can be reviewed and statistical analysis applied to identify and quantify differences between individual samples or sub-populations within a single sample. While fluorescence was not a characteristic for analysis in this proof of principle demonstration, it may be useful to review stored data to obtain a total count of stem cell clusters contained in each sample. **Table 1** displays stem cell count analysis of each sample.

Date: sample	# stem Cell Clusters Dispensed	# Objects Fulfilled Sort Criteria	Total Count Single Stem Cell Clusters (analyzed as single)
5.14.09 : 1st sample	5259	7818	9401
5.14.09 : second sample (A)	3962	4687	5903
5.15.09	19505	22496	27074

Table 1. Stem cell count from various samples that were run.
* Total count single stem cell clusters accounts for all analyzed objects approximating population of stem cell 'doubles' and exclusion of 'non-aggregates' in sort region.

Adipocyte sample.
The data for the cultured adipocyte sample was acquired in a slightly different manner. Sensitivity settings were increased for best detection of the very small cells within the sample. After 200 small cells (region **B, Figure 3**) and presumptive adipocytes (region **A, Figure 3**) were dispensed and verified under a microscope, more than 16,000 presumptive adipocytes from region (**A**) were dispensed to a Petri dish. Again Profiler II could be useful to identify small autofluorescence signals and quantitative analysis of the stored data may reveal interesting optical or fluorescent properties of the samples.

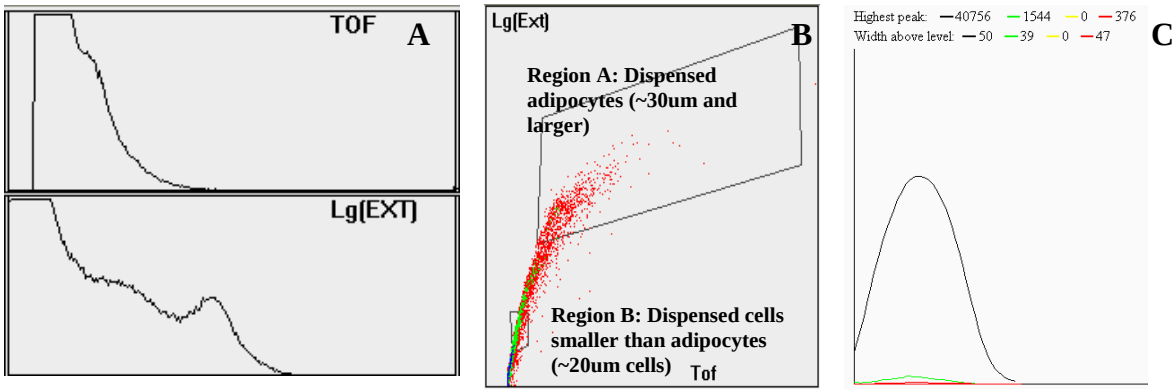

Figure 3. Cultured adipocyte sample. **A)** Histogram representation of cultured adipocyte sample, **B)** Dot plot display with representative sort regions drawn, **C)** Representative profile of an adipocyte dispensed from region A. Histogram and dot plot displays Tof (cell diameter) max scale = 256 and EXTinction (optical density) in log scale 3 decades. Representative sort regions drawn on a single plot. Profile graphs: X-axis Time of Flight, y-axis extinction and fluorescence intensities.

Discussion.
In this report we demonstrate the capability of the COPAS Select (500um flow cell) to identify and dispense different cell populations of stem cell clusters and cultured adipocytes. In just over 2 hours of sample acquisition, over 28,000 stem cell clusters were dispensed in bulk and to various 96 well plates. As a separate test 16,000 adipocytes were dispensed to a Petri dish in less than 45 minutes. Visual inspection revealed that the dispensed stem cell clusters and adipocytes generally remain intact. Additionally, fluorescence detection, gentle sampling, and powerful analysis tools combine to make the COPAS a unique instrument for analyzing and sorting cell and cell cluster types traditional flow cytometers cannot support.