

An Overview of COPAS™ Large Particle Flow Cytometry for the Analysis and Sorting of Large Cells and Cell Clusters

Introduction:

Flow cytometry is an established technique for the high throughput multiparametric analysis and sorting of cells. However, many cell types, such as hepatocytes and adipocytes, are too large and delicate for conventional flow cytometry. Likewise, cell clusters, such as pancreatic islets, embryoid bodies, kidney collecting ducts and neurospheres present a challenge for conventional flow cytometry because of their broad size distribution. The alternative – manual microscopic manipulation of large cells and cell clusters – is tedious, slow, and limits the size and scope of experiments. Union Biometrica has developed instrumentation based on the principles of conventional flow cytometry adapted for large, delicate cells and cell clusters.

Technology:

COPAS (Complex Object Parametric Analysis and Sorting) instruments analyze large particles (20-1,500 micron diameter) in a continuously flowing stream at a rate of 10-50 objects/second. Using object size (TOF), optical density (EXT) and intensity of fluorescent markers (FLU) as analytical criteria, particles can be selected and dispensed into Petri-dishes or multi-well plates for further analysis. A gentle pneumatic sorting mechanism located downstream of the flow cell does not harm or change the sensitive objects, making it suitable for fragile and sensitive cells and cell clusters. Samples can be run either live or fixed and analyzed for detection of various stains, dyes, or fluorescent proteins. Multiple fluorescence excitation and emission wavelengths are available for interrogation of the samples. In the following proof-of-principle investigations an instrument with an argon-ion laser (488/514 nm) was used.

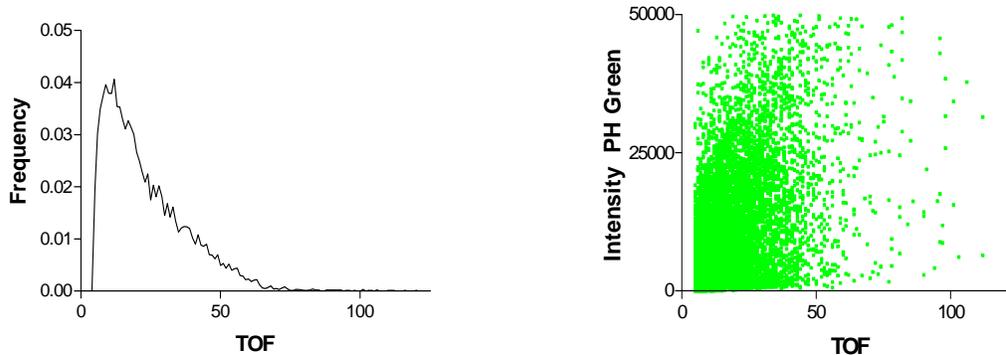
Overview of Application examples:

On the following pages are brief reports on eight different projects which are intended as a quick overview of some of the feasibility experiments that have used COPAS. In some cases only an initial proof-of-principle experiment has been completed so far; in others the methodology is well developed and even published. For more details and to discuss your specific research project, please contact our applications scientists at appsupport@unionbio.com.

- I. **Hepatocytes** frequency distribution and live staining
- II. Analysis of mouse **adipocytes** measuring size and lipid content
- III. Detection and analysis of fluorescence expression of stained live and fixed **adipocyte** samples
- IV. Detection and sorting by size and fluorescence using fluorescently-labeled antibodies to cardiac-specific proteins expressed in **cardiomyocytes**
- V. Mouse **pancreatic duct cell re-aggregates**
- VI. Isolation of mammalian **kidney collecting ducts**
- VII. Automated sorting of adult **stem cells and mouse embryonic bodies**
- VIII. Sorting of **stem cells** cultivated on beads
- IX. Analysis and sorting of **adult cardiomyocytes**
- X. Mammary Particles: **Fibroblast Spheroids and Breast Cancer Histoids**,

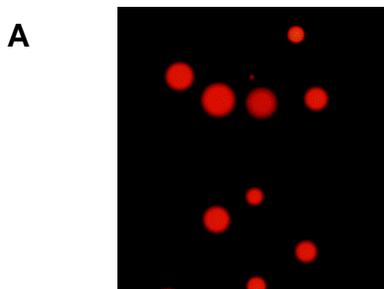
I. Hepatocytes frequency distribution and live staining

A male mouse raised on a normal chow diet was sacrificed and the liver disrupted to single cells for analysis on the COPAS BIOSORT instrument to determine the size distribution of hepatocytes (bottom left panel). COPAS analysis of fluorescence levels of hepatocytes stained with calcein (bottom right panel) is shown.



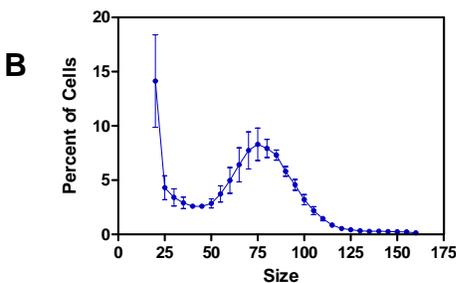
II. Analysis of mouse adipocytes measuring size and lipid content

Lipid staining of adipocytes



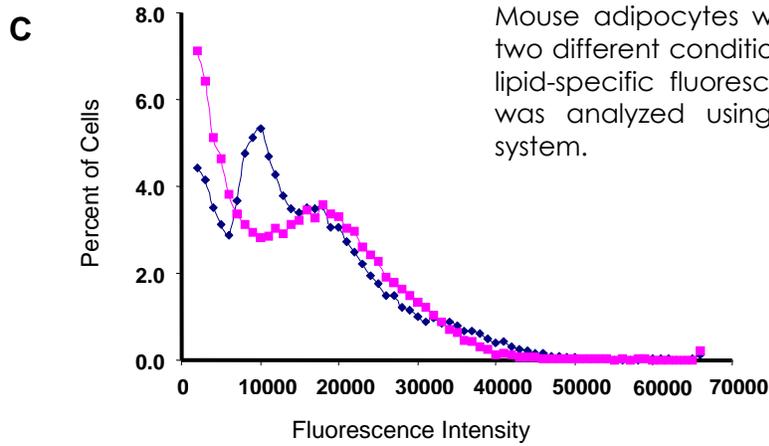
Mouse adipocytes were stained with Nile Red for lipid and visualized under a fluorescence microscope, showing heterogeneous populations of size and lipid content.

Size distribution of adipocytes

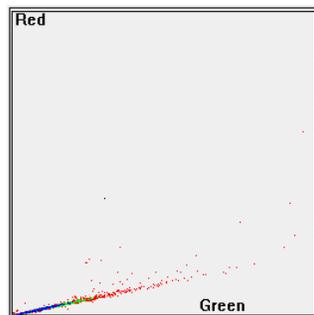


Mouse adipocytes were prepared and their size distribution analyzed using a COPAS BIOSORT flow sorting system.

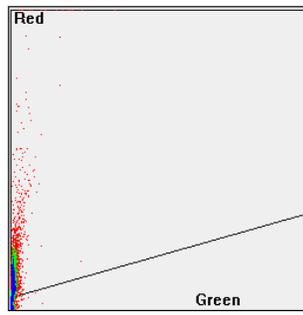
Lipid content distribution of adipocytes



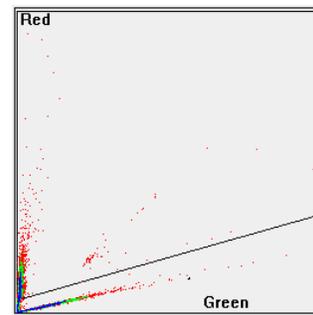
III. Detection and analysis of fluorescence expression of stained live and fixed adipocyte samples



Alexa 488 cells (green)

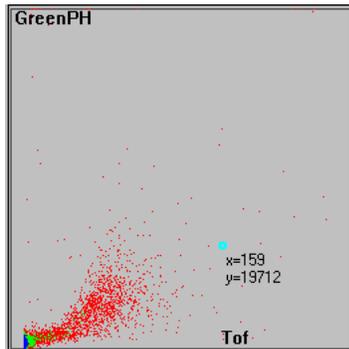


WGA stained cells (red)



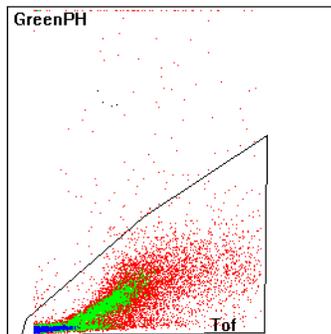
Mix of Alexa 488 and WGA stained cells

IV. Sorting by size and fluorescence using fluorescently-labeled antibodies to cardiac-specific proteins expressed in cardiomyocytes



Cells were fixed and stained with antibodies specific to a protein expressed from a cardiac-specific promoter.

V. Mouse pancreatic duct cell re-aggregates



Pancreatic tissue is from mouse expressing GFP in β -cells. Duct cell re-aggregates can be sorted by setting the sorting window (drawn region) on aggregates that do not contain insulin producing β -cells and are GFP-negative.

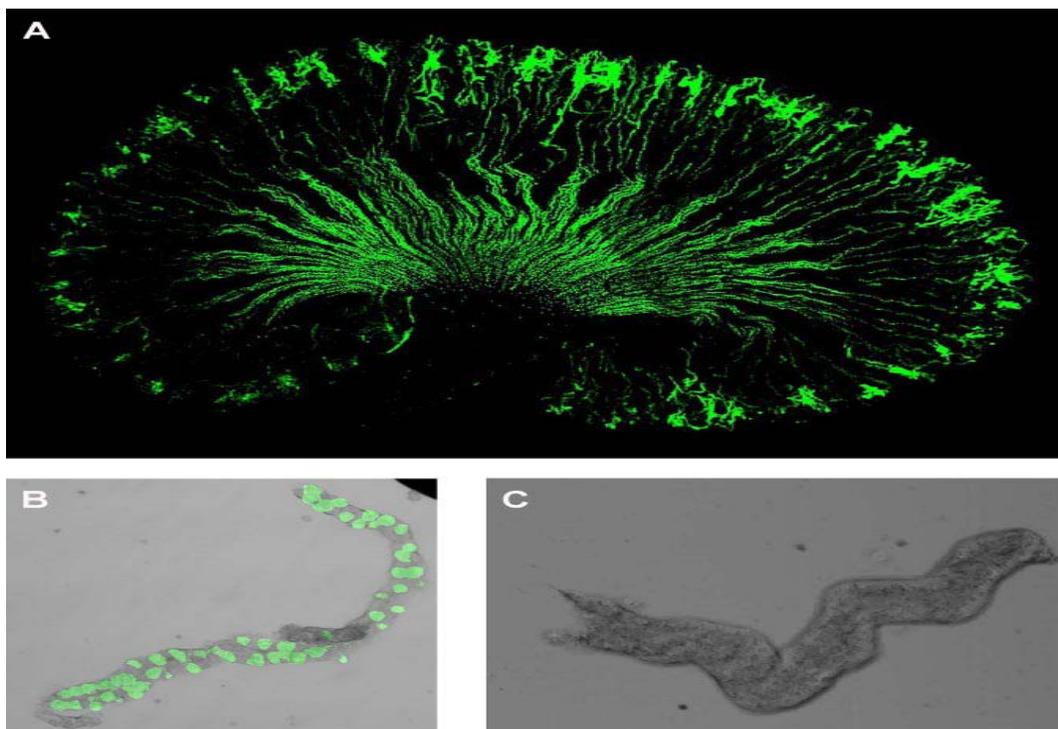
VI. Isolation of mammalian kidney collecting ducts

Automated method for the isolation of collecting ducts

R. Lance Miller,¹ Ping Zhang,¹ Tong Chen,² Andreas Rohrwasser,² and Raoul D. Nelson¹

¹*Division of Nephrology, Department of Pediatrics, and* ²*Department of Human Genetics, School of Medicine, University of Utah, Salt Lake City, Utah*

The mammalian kidney collecting duct has a number of critical roles in maintaining proper salt, pH and water balance. Studies requiring collecting duct isolation are confronted with the challenge that comes with the structural and functional heterogeneity of these structures. Lance Miller, University of Utah, Salt Lake City, UT, has published an automated method for isolating collecting ducts by combining the use of transgenic mice expressing green fluorescent protein (GFP) in the collecting duct with large-particle-based flow cytometry to isolate pure populations. This method allows for isolation of select kidney collecting-duct regions enriched for principal or intercalated cells, based on GFP reporters driven by aquaporin or V-ATPase promoters, respectively. This method yielded sufficient amounts of the different kidney cell types for proteomic analysis. The average length of collecting duct regions was 260 μm , a size too long for conventional flow cytometry.



Kidney from B1-enhanced green fluorescent protein (EGFP) transgenic mouse.

A: 200- μm vibratome section shows the expression of GFP in the connecting tubule (CNT) and collecting duct (CD). B and C: examples of a GFP-positive collecting duct (B) and GFP-negative non-collecting duct tubule.

Union Biometrica

USA: Tel: +1 (508) 893-3115

Europe: Tel: +32-(0) 14-570628

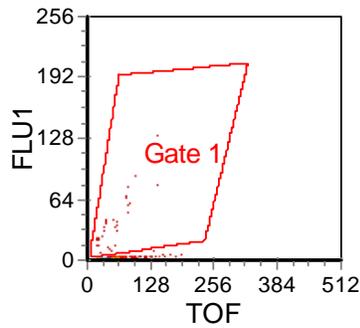
<http://www.unionbio.com>

Fax: +1 (508) 893-8044

Fax: +32- (0) 14-570621

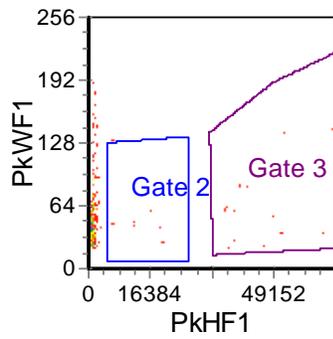
sales@unionbio.com





Gate	# of Events	% of Gated Cells	% of All Cells
None	258	100.0	100.0
Gate 1	26	10.08	10.08
Gate 2	6	2.33	2.33
Gate 3	21	8.14	8.14

COPAS sorting of tubules from a B1-EGFP transgenic mouse kidney. Tubules were sorted based on fluorescence intensity of GFP (FLU1) and time of flight (TOF).



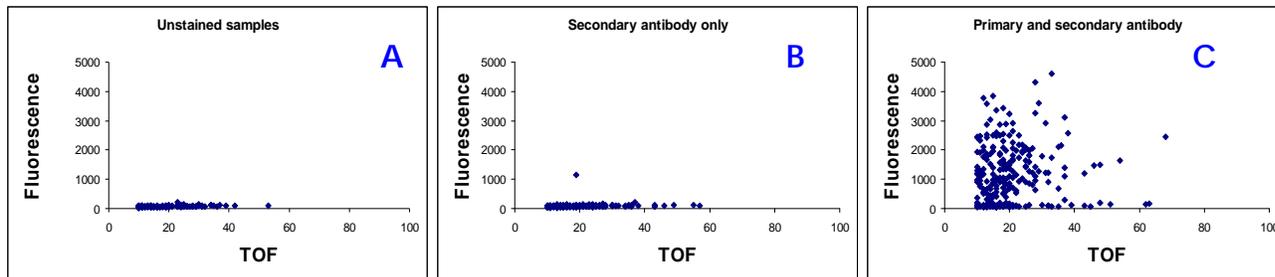
Gate	# of Events	% of Gated Cells	% of All Cells
None	258	100.0	100.0
Gate 1	26	10.08	10.08
Gate 2	6	2.33	2.33
Gate 3	21	8.14	8.14

COPAS sorting based on the GFP expression measurements PEAK HEIGHT and PEAK WIDTH allowed high enrichments of connecting tubules (CNT, gate 3) and collecting duct (CD, gate 2).

VII. Sorting of adult stem cells and mouse embryoid bodies

Detection of specific cell types derived from adult stem cells

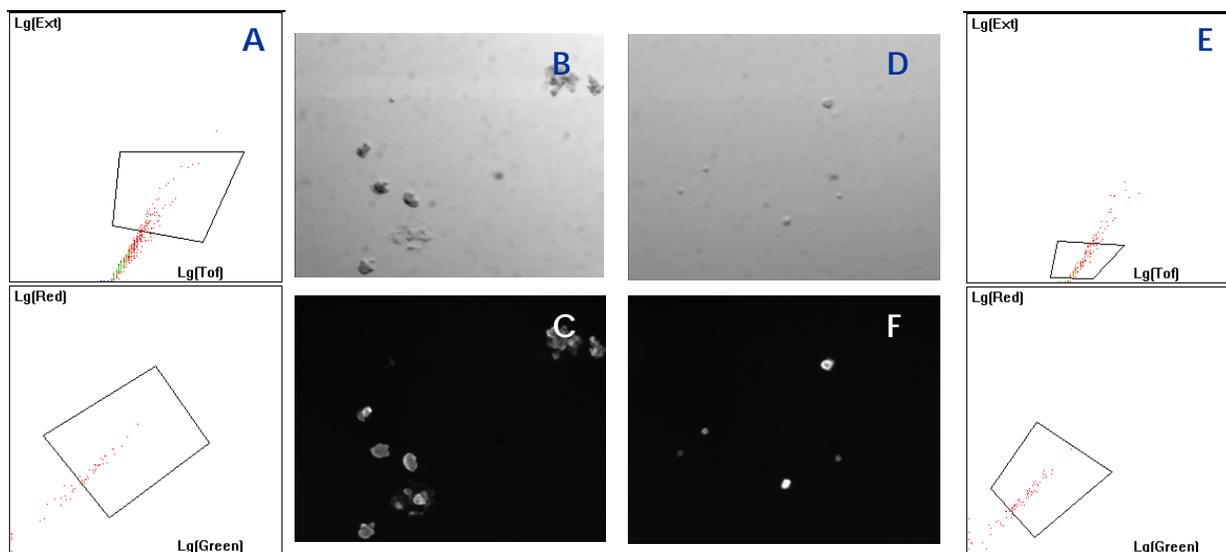
Specific cell types derived from the adult stem cells can be identified by staining with fluorophore conjugated antibodies against the corresponding cell markers. The figure below shows the comparison of the measured fluorescence intensity between an antibody-stained adult stem cell sample and its negative controls. As expected, fluorescence was only detected in the stained sample (seen in graph C below).



Detection of specific cell types derived from adult stem cells based on antibody staining. TOF: Time of Flight, equivalent to length; Fluorescence: Green fluorescence intensity. Fluorophore was conjugated to the secondary antibody. Analysis of raw COPAS data exported into Microsoft® Excel®.

Sorting based on size and fluorescence

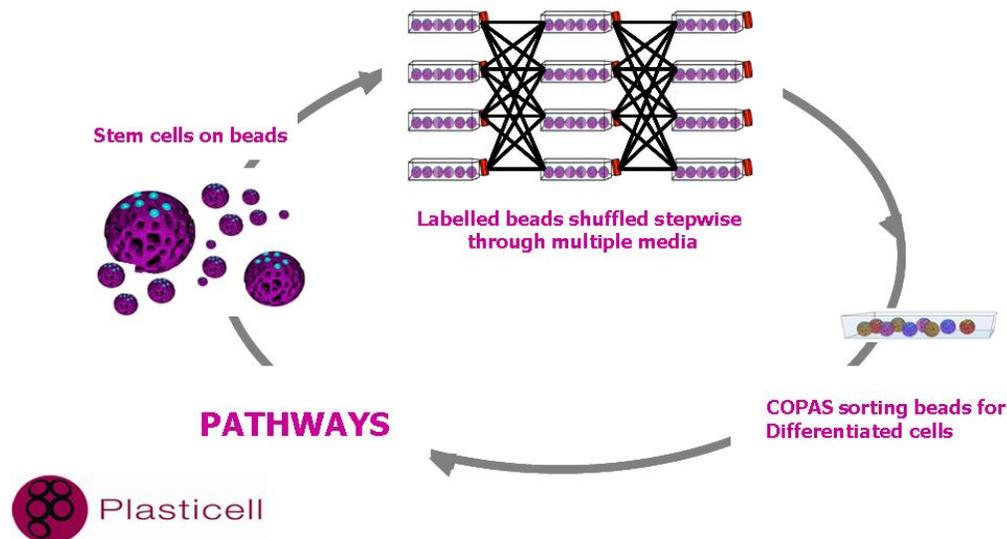
As discussed above, the user can define “gate” regions within a dot-plot to define physical characteristics of objects to include as sorting criteria. In this example, size and fluorescence intensity properties were used. The dot plots below show the gating and sorting regions for collecting fluorescent objects of different sizes from the stained adult stem cell sample. The figure below shows the microscopic images of the sorted objects. By using the log scaling feature within the Advanced Acquisition Package, even small objects are detected with the increased detection sensitivity.



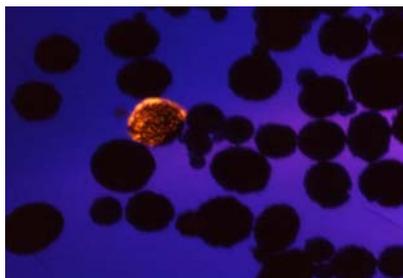
Dispensing large cell/clusters. **A**: Dot plots displaying gate (upper) and sort (lower) regions. **B** (bright field), **C**: (fluorescent) images of objects dispensed from sort of regions in **A**.

Dispensing small cells. **E**: Dot plot displaying gate (upper) and sort (lower) regions. **D** (bright field), **F**: (fluorescent) images of objects dispensed from sort of regions in **E**.

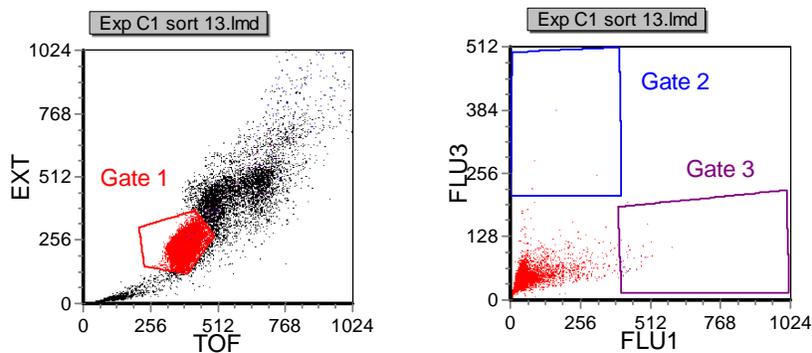
VIII. Sorting of cultivated stem cells on beads



Using Combinatorial Cell Culture™ (images courtesy of Plasticell, London, UK), cells cultured under different conditions on beads are analyzed and sorted using the COPAS PLUS. Cells following the hematopoietic line are stained with pHrodo (Invitrogen). Cells following a neurological pathway express a GFP protein.



Bead-based sample. Black beads do not carry cell clusters. pHrodo stain is visible on macrophages grown on a bead.

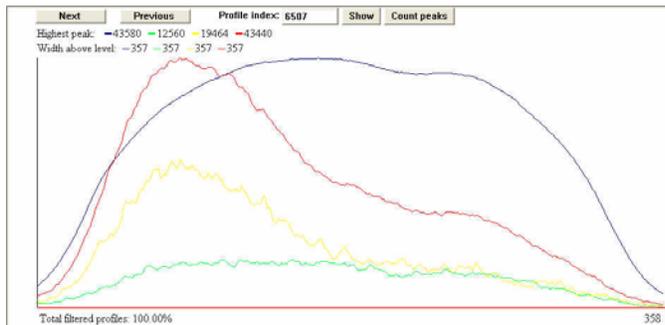


Overlay #	FCS Filename	Gate	# of Events	% of Gated Cells	% of All Cells
1	Exp C1 sort 13.lmd	None	20635	100.0	71.58
1	Exp C1 sort 13.lmd	Gate 1	20635	100.0	71.58
1	Exp C1 sort 13.lmd	Gate 2	4	0.02	0.01
1	Exp C1 sort 13.lmd	Gate 3	30	0.15	0.1

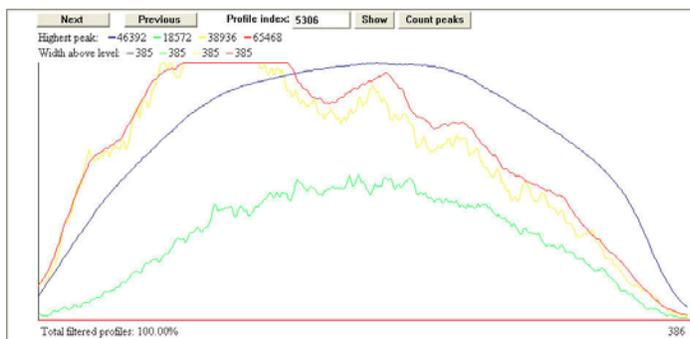
Sort experiment showing statistics for Red cell clusters (FLU1) and Green cell clusters (FLU3).

Profiler II analysis of beads

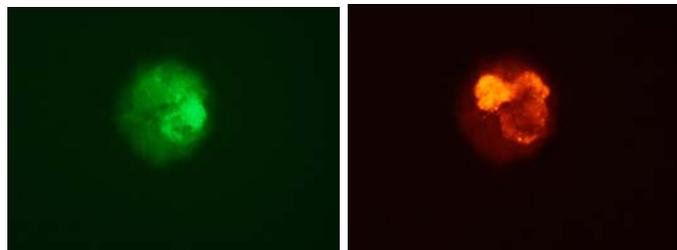
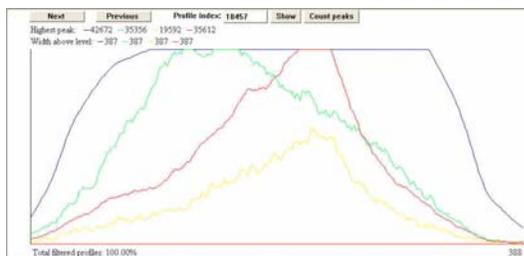
Sorted single beads in a 96-well plate were analyzed using ProfReader software. The blue line represents the optical density of the bead. The red line represents the pHrodo stain pattern of cells on a bead. The yellow line represents the orange emission of the pHrodo. The Green line represents the GFP emission of the cells.



Profile and Image of a pHrodo (red line graph) labeled cell cluster grown on the bead, the group of cells is clearly located on one side of the bead.



Profile and Image of a pHrodo (red line graph) labeled cell cluster grown on the bead, the group of cells is spread all over the bead.



Profile and Images of two cells clusters on one bead, a pHrodo (red) and a GFP (green) labeled cluster.

IX. Analysis and sorting of adult Cardiomyocytes

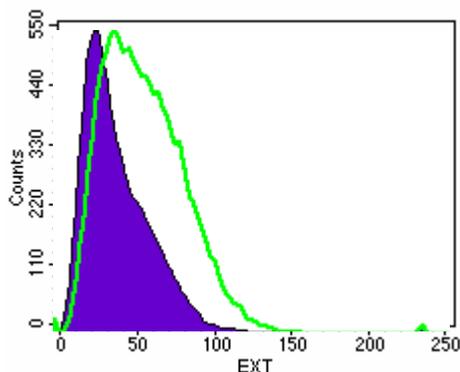
Cardiac growth during development occurs in two phases, initially by myocyte hyperplasia- increase in cell number, then by myocyte hypertrophy- increase in cell size. The second mechanism plays a major role after the early postnatal period in increasing the heart size to maintain cardiac output for the growing organisms. Research on cardiac hypertrophy is critical in understanding the physiological and pathological development in the heart. Size measurement of myocytes therefore is essential to all research of myocytes.

This experiment is the first to test COPAS Size measurement of myocytes. COPAS size measurement revealed the two sets of cells within the myocytes sample.

Materials: Wild type fixed mouse cardiomyocytes. Left Ventricular (LV) and right ventricular (RV) myocytes were separated from the same WT heart.

LV versus RV myocytes

The LV myocytes are larger than RV ones (personal communication). We set out to compare the size of RV myocytes with that of LV ones. More than 30,000 myocytes were measured based on size and extinction. The difference between RV and LV is measurable. In fact, we detect that RV myocytes is 26% smaller than LV ones, consistent with microscopy measurement.



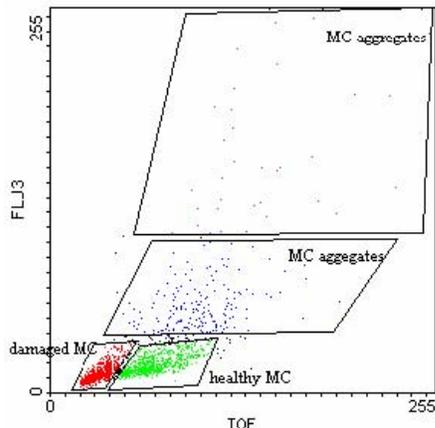
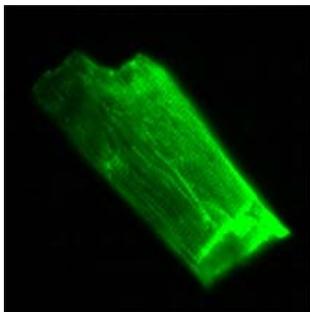
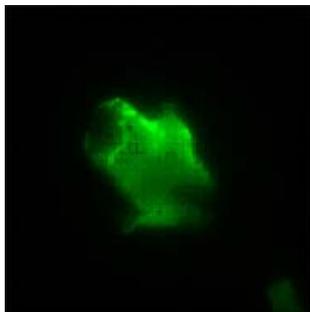
Optical Density	Mean	Median
RV	41.3	36.4
LV	57.6	51.7
RV/LV*100	72%	70%

At left: Histogram displaying the size distribution of myocyte samples: purple-RV, green-LV. Data table displaying average Extinction values of RV and LV samples. (EXT=optical density)

Sorting healthy Cardiomyocytes from damaged myocytes

Healthy cardiomyocytes are rod-shaped and rectangular, whereas the damaged ones become rounded in appearance. In the sample preparation we mixed healthy myocytes and damaged ones in roughly a 1:1 ratio. We analyzed the large myocytes in a blind test to examine whether. COPAS can differentiate healthy myocytes from the damaged ones.

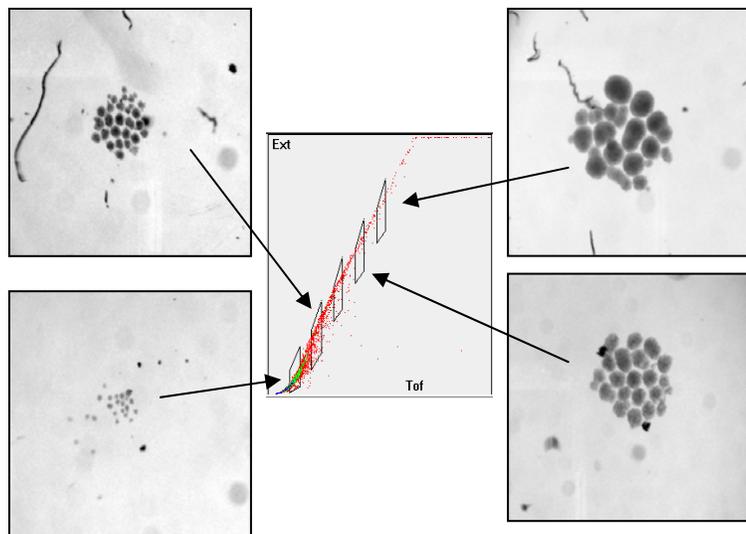
The COPAS system is able to differentiate round shape from rod shaped myocytes.



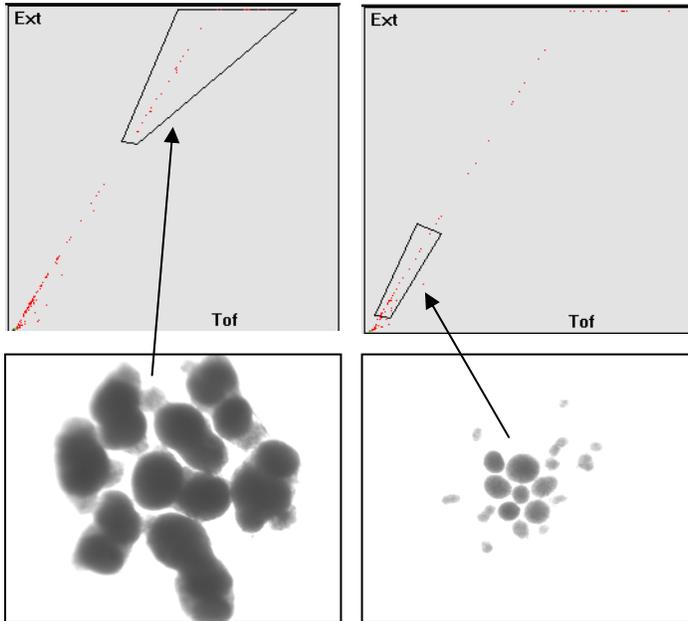
Gate regions identifying populations of healthy/damaged cardiomyocytes and cardiomyocyte aggregates (far right). Images of damaged/rounded MC (far left) and healthy/rod-like MC (middle).

X. Mammary Particles: Fibroblast Spheroids and Breast Cancer Histoids,

Using the COPAS Plus, windows were drawn to dispense spheroids (fixed and not-fixed) according to narrowly defined size regions. As a result, dispensed objects from each of these regions were fairly uniform in size, allowing the user to identify and dispense a particular portion (for instance growth stage) of the entire sample population.



Individual regions redrawn on a single sample dot plot, images of spheroid cells dispensed to individual wells of 96 well plate captured under dissecting microscope



Fixed Histoids dispensed using the COPAS Plus.

Summary:

COPAS large particle analysis instruments bring the methods of flow cytometry to the analysis and sorting of large cells and cell clusters not normally amenable to analysis on conventional single-cell flow cytometers. Large delicate cells such as hepatocytes, adipocytes and cardiomyocytes can be analyzed and sorted. The samples can be live or fixed and stained with conventional dyes and stains developed for optical analysis (microscopy and flow cytometry). Cell clusters can also be analyzed while intact, allowing for studies that address questions of cell-cell interaction, tissue development and differentiation. This instrument platform brings the advantages of flow cytometry - statistically meaningful data, large unbiased datasets, and multiparametrical analysis - to the analysis of large cells and cell clusters.

Profiler II digitizes the object into a succession of peaks and valleys that directly trace the fluorescence intensity of the object as it passes through the flow cell. Profiler II also includes advanced imaging to graphically and numerically display subtle variations in fluorescence and extinction intensity along the length of an object. The result is an optical profile of each object graphically showing the location and intensity of all four optical parameters. Profiler II also enables users to optimize COPAS systems by visualizing data, resulting in better detection of strong versus weak signals.