

Enhanced Forward Scatter Module+ on the MoFlo™ Astrios*: Small Particle Applications

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Enhanced Forward Scatter Module+ on the MoFlo™ Astrios*: Small Particle Applications

PURPOSE

Demonstrate the ability of the FSC Module to resolve and sort small (0.2 µm) and large particles (30 µm) simultaneously on two Forward Scatter Parameters.

ABSTRACT

Background: The MoFlo™ Astrios instrument from Beckman Coulter is a high-speed cell sorter capable of 6-way sorting speeds up to 70,000 events per second with 99% purity. Improvements to this high end instrument have been made which allow scientists to identify particles as small as 0.2 µm using Forward Scatter trigger. Specifically, these improvements to forward scatter have enabled scientists to take advantage of the following cell biology applications: *S. cerevisiae*, *E. coli*, murine mitochondria and photosynthetic plankton such as *E. huxleyi*, and *P. marinus*. *S. cerevisiae* and *E. coli* are common organisms used in the field of cell biology. Isolation of mitochondria for use in DNA analysis as well as focused research on a subcellular component is of great interest to researchers. *E. huxleyi* and *P. marinus* are highly-characterized organisms studied in marine biology and have been suggested to play critical roles in the carbon cycle. The improvements to Forward Scatter on the MoFlo™ Astrios instrument now allow scientists to further refine their work in these popular fields of research.

ASTRIOS FSC MODULE

The Forward Scatter Module collects laser light that is scattered into a range of angles within 45° of the direction of propagation of the incident laser beam. The light collecting aperture of the FSC Module fits through a slot in the Illumination Chamber and is located directly across the interrogation point from the laser optics, as seen in Figure 1.

Light scattered from the interrogation point is collected by lenses and split into two paths, each of which directs the light to a separate PMT detector. The main body of the FSC Module is located to the right of the Illumination Chamber. The signal generated by the forward scattered light is a function of characteristics of the particle that was illuminated by the laser, including size, material, and shape.

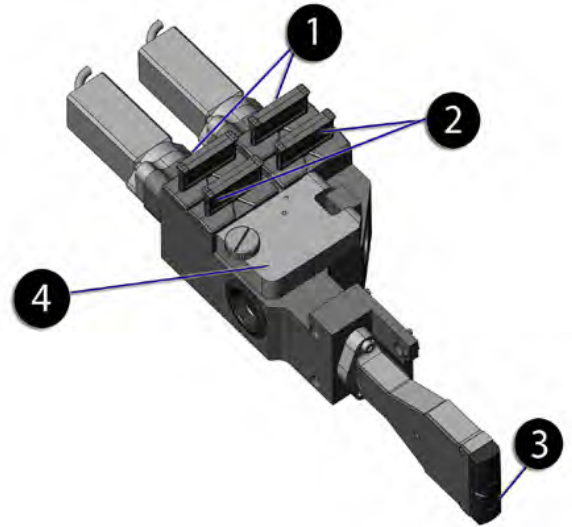
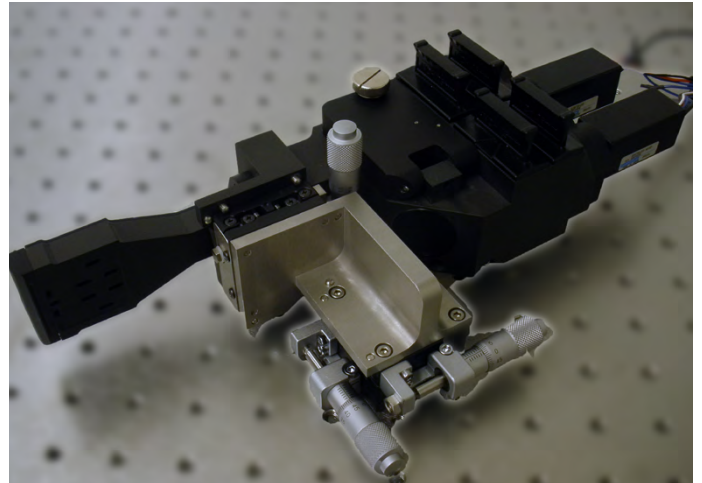


Figure 1. Forward Scatter Module. 1. Neutral density filters in front of the FSC PMTs. 2. Laser blocking filters. 3. Removable FSC cap. 4. Beamsplitting cube.

Figure 1A. The enhanced FSC module that will be released with new MoFlo Astrios systems and optionally upgraded into current systems. The dual PMT FSC module provides flexibility, small particle and large particle detection.



0.2-30 μm FSC DYNAMIC RANGE

The enhanced FSC module contains two separate FSC PMT parameters which allows for different sized particles to be detected simultaneously. The small particles are detected with an ND=0; whereas a larger dynamic range of 0.2 μm -30 μm beads are measured with an ND=1.

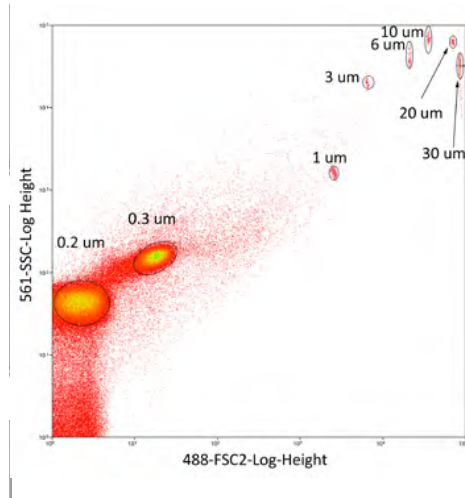
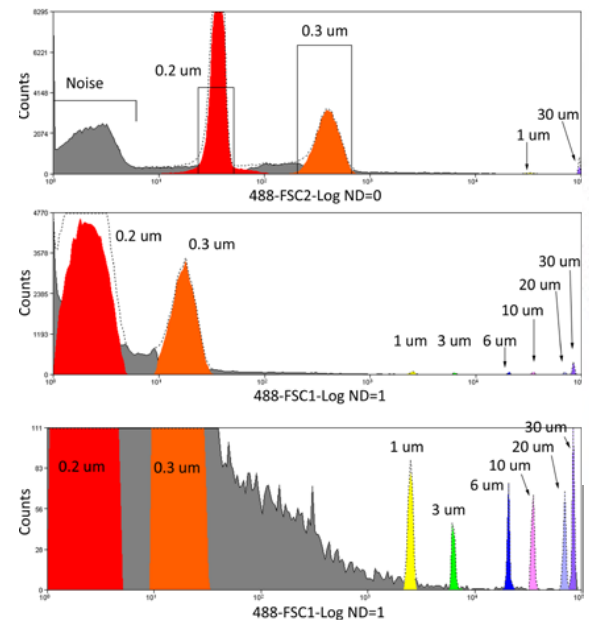


Figure 2. Polystyrene beads purchased from Bangs Laboratories were diluted 1:1000 (0.2-1 μm beads) and 1:100 (3-20 μm) and analyzed on the Astrios using 100 μm tip with drop drive on. The beads were diluted with 0.04 μm filtered DI water and ran in a glass tube.

Figure 3. Three histograms acquired simultaneously to measure the small and large particles. The top histogram was measured on FSC2 with a ND=0, therefore the separation between noise and 0.2 and 0.3 μm beads was large. The second and third histogram are the same histogram, at different y-axis counts. The FSC1 parameter included an ND=1 and therefore the entire dynamic range from 0.2 to 30 μm may be visible, but the separation from noise at the smaller end is not as large.

CONCLUSION

By having the modular dual-PMT FSC, the dynamic range allows researchers to simultaneously visualize both small and large particles. Analyzing and sorting both small and large particles gives researchers another method for cellular analysis.



FSC WITH BACTERIA AND YEAST

Introduction:

Researchers use microbiology for genetic manipulation in studying protein and gene development, protein expression and various other molecular biology techniques. **The ease of separating bacteria and yeast on a flow cytometer/cell sorter gives the researcher the tools to clone and grow microbes for molecular and microbial analysis.**

Bacteria are a rod-shaped organism, 1-5µm in diameter, and may vary in their genetic content. With the simple circular DNA within the cell, researchers commonly use the organism for genetic studies. The genetic splicing of fluorescent proteins adjacent to expressed genes allow for single bacteria to be sorted into 96-well plates (see figure to the right).

Flow cytometry has been used to identify bacterial infections and effects of therapies on bacteria. The use of flow cytometry to identify bacteria is also useful for surface protein screens, gene expression studies (Dako), evaluation of water quality (Dako, Hoefel), detection of yeast and bacteria for rapid wine assessment (Malacchino).

Yeast are part of the Eukaryotic kingdom and over 1,500 different species occur naturally. Most yeast size range from 3-4 µm in size, although some species may get to 40 µm in length.



Figure 4. Artist's interpretation of the *E. coli* with the rod-shaped, 1-5 µm sized particles. The new FSC module clearly separates these organisms from noise. Image taken from 123RF, royalty free stock images.

Researchers use yeast as a model Eukaryote for genetic studies, protein expression and molecular biology analysis.

The industry also uses *S. cerevisiae*'s fermentation process (converting carbohydrates to carbon dioxide and alcohol) for bread and alcohol production (Mattanovich), and genetic studies of yeast (Dako). The ability to sort bacteria and yeast is also useful for viability tests and cultivation of microbial populations

The high speed cell sorter, MoFlo Astrios, with the new FSC Module is able to resolve and sort bacteria (*E. coli*) and yeast (*S. cerevisiae*) from instrument and sheath noise on the dual-FSC PMT parameters.

Figure 5. Scanning electron microscope of yeast. Yeast are irregular in shape when growing and spherical when sporulating. Image taken from <http://www.historyforkids.org/scienceforkids/biology/cells/yeast.htm>



YEAST AND BACTERIA

MATERIALS:

- Zymosan A BioParticles (S. cerevisiae - Yeast), fluorescein conjugate (Invitrogen, Z2841) with an approximate concentration of 2×10^7 / mg lyophilized
- Escherichia coli BioParticles (K-12 strain) (E. coli - bacteria), Texas Red conjugate (Invitrogen, E2863) with an approximate concentration of 3×10^8 / mg lyophilized.
- Tissue culture grade PBS (PBS made within one month of use and sterile filtered to 0.2µm) [add 2mM Sodium Azide to store at 4 degrees for 1-4 weeks]. NaAzide Sigma A8032 lot 106k19.
- Beads for FSC alignment and size: 1 micron

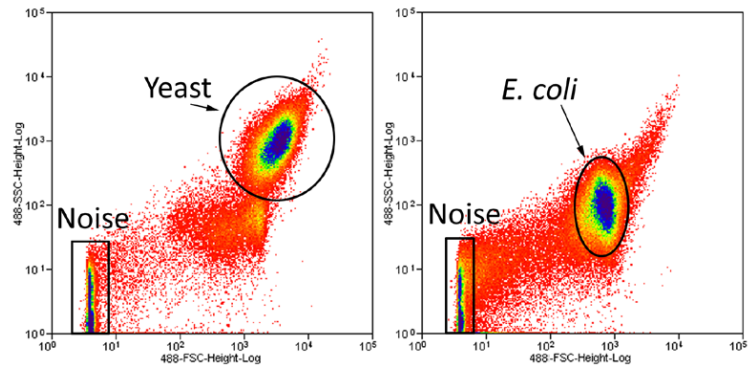
METHODS:

- Add 1 ml PBS to 10mg (entire vial) of lyophilized Zymosan A and E. coli. Resuspend samples as described in the Molecular Probes protocol (stock solution). Add 2 µL Sodium azide stock to each vial. Dilute the Zymosan as described in the table. Mix three tubes, yeast, bacteria and 1 mL of each. Flow cytometry: Set the FSC noise and threshold with no sample running. Use single sample tubes to determine initial populations. Optimize fluorescence (488-513/26 for FITCYeast), (592-620/29 for Texas Red-E.Coli) to move the populations on scale by changing voltage. Measure the overlap of the populations. Run the samples, collecting 10K-15K EPS.

RESULTS:

The enhanced FSC module separates the yeast and bacteria populations from noise with less than 1% overlap measurement. As seen in the figure below, the populations resolve clearly from noise.

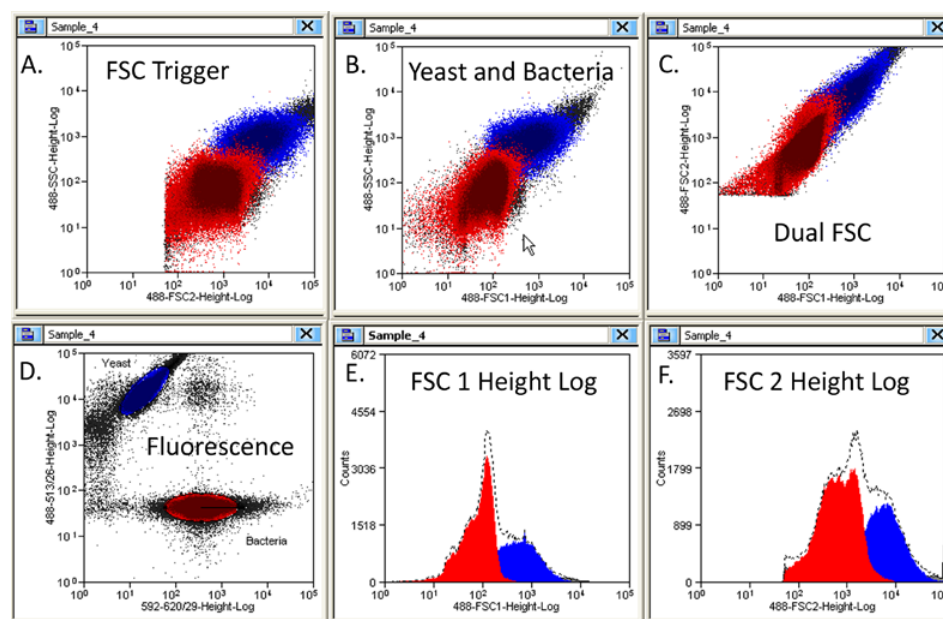
Figure 6. Populations analyzed on the Astrios dual-PMT FSC module with a FSC Trigger. The populations are resolvable from noise on both the FSC Height Log histograms. Researchers are able to analyze and sort E. coli and S. cerevisiae populations, ranging from 1-5 µm in size, on the new enhanced FSC module.



The mixture of the yeast (blue population) and bacteria (red population) ran simultaneously on the Astrios FSC Module were also visible from noise. With the dual parameter FSC, the organisms may be triggered on one FSC and visualized on the other FSC in their entirety (see below left and middle figure). The FSC Height Log parameters may be plotted against each other giving a unique FSC signature for the yeast and bacteria (see right figure). The fluorescence parameters distinguished the yeast and bacteria populations.

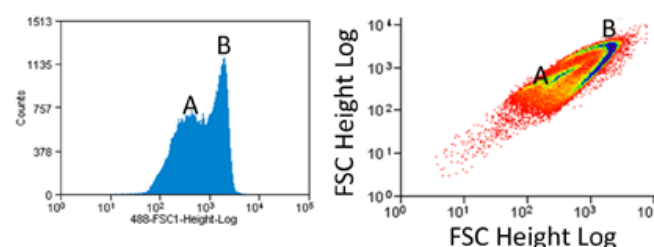
The enhanced FSC module provides flexibility of treating each FSC PMT differently. As seen in the figure below, different ND and band pass filters are applied to the yeast and bacteria mixed sample. The characteristics of each FSC PMT are slightly different giving population variances.

Figure 7. Yeast and bacteria analyzed on the enhanced FSC module simultaneously. A and B are the FSC trigger and FSC Log Height Parameters. C = the dual-PMT FSC showing slight differences in populations depending on the ND and band pass filters in front of the PMTs. D = fluorescence histogram used for coloring only (not trigger or gating). E and F = univariates of the yeast and bacteria ran simultaneously visualized differently on the dual-PMT FSC module.



Different populations of bacteria may be separated via FSC parameters. On the left, two populations of bacteria found in yogurt were overlapping in the univariate FSC histogram (populations A and B), but in the left histogram, with the dual-PMT populations, the two populations were clearly defined and separable.

Figure 8. Separation of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* from yogurt using the enhanced FSC module. The dual-PMT FSC shows two distinct bacterial populations that were overlapping in the univariate.

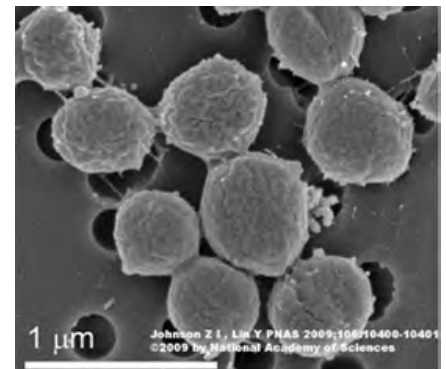


FSC WITH PLANKTON

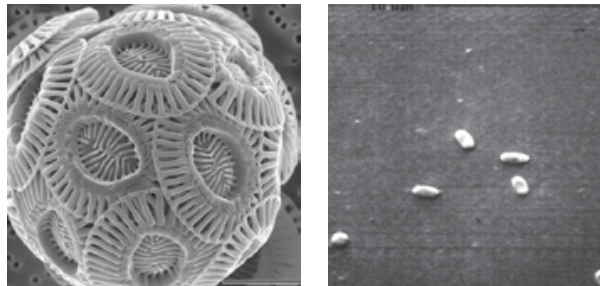
Marine biology, specifically photosynthetic plankton, is another sorting application for the new MoFlo Astrios enhanced Forward Scatter Module. Flow cytometry has previously been used with phytoplankton for rapid and objective evaluation of water quality (Trask), to analyze grazing (Cucci), cell viability (Dorsey), rapid strain identification (Simon), diversity assessment (Marie D), and to characterize plankton populations through molecular (Shi, Marie D) and genetic approaches (Shi). Marine samples, *E. huxleyi*, and *P. marinus*, were used in the tests in this protocol with the Astrios.

The *Prochlorococcus marinus* is a cyanobacteria that inhabits the latitude 40 degrees North to 40 degrees south and is an essential component of the food chain. The bacteria is approximately 0.2 μm and spherical (as seen on the figure at the right). The plankton fluoresces due to the chlorophyll a and b in the dim red spectrum (488 excitation-710/795 emission). Researchers focus on the ecology of these plankton due to the fact they contribute 30-80% of the world's oligotrophic ocean.

Figure 9. Scanning electron micrograph of *P. marinus*. This plankton is one of the smallest and common phototrophs found in the ocean.



The *Emilinia huxleyi* plankton is a coccolithophore plankton found in a broad range of water from the subarctic to the tropics. It serves as a basis for most marine food chains. It is a single-celled phytoplankton covered with calcite disks (coccoliths) (see right image). The phytoplankton has several life cycles where the plankton sheds the coccoliths and is "naked" (far right). In the FSC module testing, the refraction from the coccoliths gives a distinctive scatter pattern in relationship to the "naked" cells and therefore both populations may be distinguished on FSC with the dual PMT FSC parameters. The *E. huxleyi* measures approximately 5-6 μm in the coccolith form.



PLANKTON RESULTS

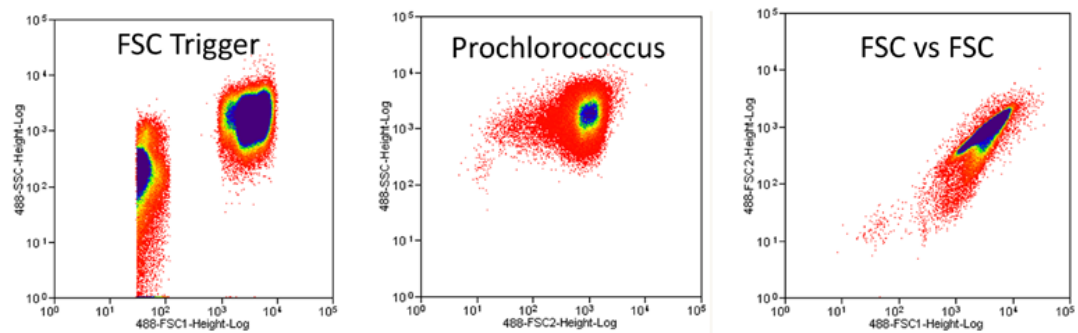
METHODS:

The plankton was grown in sterile 2 L containers supplied with 0.2 μm filtered air in salt water with fertilizer. Instant Ocean, $\frac{1}{2}$ cup per gallon of deionized water, was added to the culture with Microalgae Grow Mass Pack with Silicate. Cells were harvested by gentle centrifugation, roughly 300 x g for 5-10 minutes. The supernatant was decanted/aspirated and the pellet resuspended in a sterile saline solution to achieve 1×10^6 cells/mL.

Results: *P. Marinus*



Figure 10. The *Prochlorococcus marinus*, 0.2 μm , was separable from noise on the enhanced FSC Module as seen in the left two histograms. The first histogram was used as a FSC Trigger with the second FSC to visualize the population. The dual FSC PMT FSC histogram (far right) indicates slight differences in FSC signatures.



Results: *E. huxleyi*

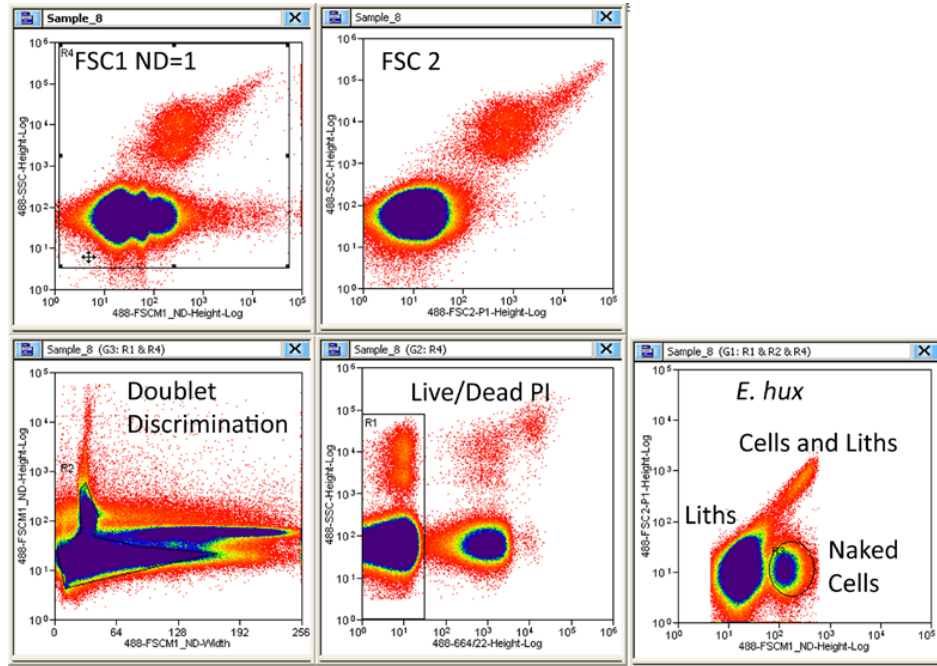


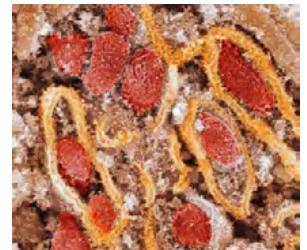
Figure 11. The *E. huxleyi* plankton was analyzed by triggering on SSC-Height Log to be able to distinguish all three populations on the FSC vs FSC dual-PMT parameter. The populations were gated on a doublet discriminator (eliminates aggregates) and also on LIVE/Dead PI. The resultant histogram (as seen below) with the dual-PMT FSC parameter shows three populations as part of the *E. huxleyi* life cycle: Liths, Cells and liths, and naked cells.

The enhanced FSC Module for the MoFlo Astrios distinguishes both *P. marinus* and *E. huxleyi* on the FSC parameters.

FSC WITH MITOCHONDRIA

Mitochondria produce energy in eukaryotic cells and play a significant role in the initiation of cell death. Flow cytometry has been used to quantify transient changes in mitochondrial membrane potential (Warnes) and to analyze molecular pathways important for apoptosis (Campos, Kalbacova). For example, cytochrome c release (Campos CB) and the effects of mitochondrial inhibiting drugs (Kalbacova) have been assessed. Flow cytometry, combined with fluorescent probes, is useful for studying ROS effects on cell death (Mattiasson, Cottet-Rousselle C). Mitochondrial size during stages of the cell cycle has been measured using flow cytometry (Kennady).

Figure 12. Scanning electron micrograph of mitochondria in cellular matrix. Image taken from Science Photo library



Flow cytometry and cell sorting would allow researchers to isolate and analyze mitochondria function and sub-components. With the addition of the enhanced FSC Module to the Astrios, researchers could sort and analyze mitochondria. Below, the FSC is able to distinguish murine mitochondria from noise (size 0.05 μm to 2 μm).

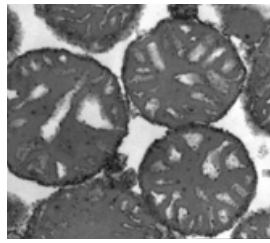


Figure 13. Murine mitochondria isolated from liver are 0.05 to 1 μm in size.

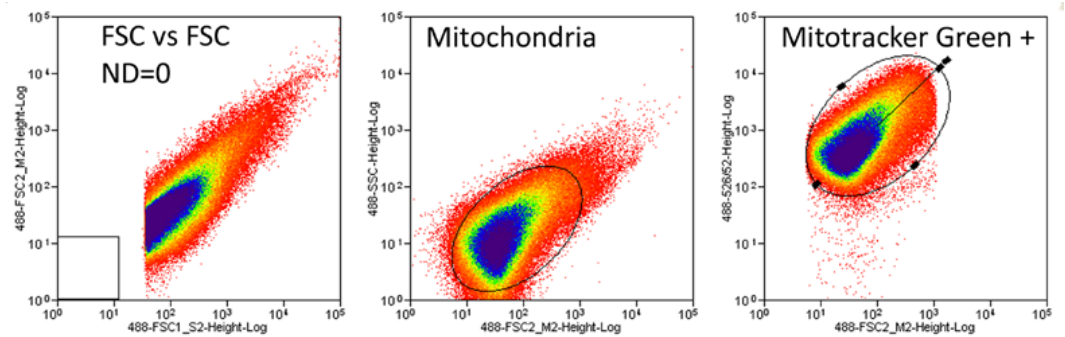
METHODS:

C6-mice were obtained and euthanized via humane methods. The liver was excised from the mouse and placed on ice. The sample was washed once with 1ml 0.9% (w/v) sodium chloride solution. The liver mitochondrial isolation was executed following the Qiagen Qproteome Mitochondria Isolation Handbook and Kit catalog #37612. The isolated liver mitochondria were resuspended in the Mitochondria Storage Buffer. The mitochondria were stained with 500nM MitoTracker Green (Invitrogen M7514) incubated at 37 degrees for 20 minutes. Samples were then placed on ice before analysis.

RESULTS:

The mitochondria stained positively for the Mitotracker green and were analyzed on both of the FSC parameters. The dual-FSC PMT distinguished the entire mitochondria population (middle histogram). The Mitotracker green confirmed the presence of intact mitochondria on the flow histograms.

Figure 14. Flow cytometry histograms visualizing the murine mitochondria on the enhanced FSC Module on the Astrios. The Mitotracker green confirmed the presence of mitochondria in the FSC histograms. The 0.05-1 μm mitochondria were visible and sorted on FSC vs SSC and FSC vs FSC.



CONCLUSIONS:

The enhanced MoFlo FSC module separates small particles (mitochondria) on the FSC trigger.

CONCLUSIONS:

- The seven laser high-speed cell sorter MoFlo™ Astrios was used to differentiate small organisms and particles from noise using the enhanced Forward Scatter
- Improved Forward Scatter functionality on MoFlo™ Astrios enables scientists to expand their set of cell biology applications to organisms and particles such as *E. coli*, mitochondria, and *E. huxleyi*

REFERENCES:

- Trask BJ, et al. Analysis of phytoplankton by flow cytometry. *Cytometry*. 1982 Jan2(4):258-64.
- Cucci TL, et al. Using phytoplankton and flow cytometry to analyze grazing by marine organisms. *Cytometry*. 1989 Sep;10(5):659-69.
- Dorsey L, et al. Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. *Cytometry*. 1989 Sep;10(5):622-8.
- Simon N, et al. Fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes to identify small phytoplankton by flow cytometry. *Appl Environ Microbiol*. 1995 Jul;61(7):2506-13.
- Marie D, et al. Use of flow cytometric sort to better assess the diversity of small photosynthetic eukaryotes in the English Channel. *FEMS Microbiol Ecol*. 2010 May;72(2):165-78.
- Shi XL, et al. Groups without cultured representatives dominate eukaryotic picophytoplankton in the oligotrophic South East Pacific Ocean. *PLoS One*. 2009 Oct29;4(10):e7657.
- Marie D, et al. Eukaryotic picoplankton communities of the Mediterranean Sea in summer assessed by molecular approaches (DGGE, TTGE, QPCR). *FEMS Microbiol Ecol*. 2006 Mar; 55(3):403-15.
- Shi XL, et al. Plastid 16s rRNA gene diversity among eukaryotic picophytoplankton sorted by flow cytometry from the South Pacific Ocean. *PLoS One*. 2011 Apr 28; 6(4):e18979.
- Malacrino Paola, et al. Rapid detection of viable yeasts and bacteria in wine flow by flow cytometry. *J. Microbiological methods*. 2001 Jun; 45(2): 127-134.
- Hoefel Daniel, et al. Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. 2003 Aug; 55(3): 585-597.
- Mattanovich Diethard, et al. Applications of cell sorting in biotechnology. *Microbial Cell Factories*. 2006; 5(12): 1475-5859.
- Flow Cytometry Educational Guide. 2nd Edition. Dako. 2006.
- Nir R, et al. Flow cytometry sorting of viable bacteria and yeasts according to beta-galactosidase activity. *Appl Environ Microbiol*. 1990; 56(12):3861-3866.