MoFlo™ Astrios* Enhanced Forward Scatter+:
0.2 μm to 30 μm Dynamic FSC Detection

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PURPOSE
Provide researchers with the ability to visualize and sort small (0.2 μm) and large particles (30 μm) simultaneously on two Forward Scatter Parameters.

ABSTRACT
This FSC proof-of-concept poster will focus on the ability to discriminate particles (beads and microparticles) from FSC noise.

Three sections will be discussed: Instrument set-up for small particles, bead resolution, and microparticle sorting (erythrocyte, endothelial and platelet).

Methods: Instrument Set-up: To be able to detect particles smaller than 1 μm, particles residing in the sheath and sample lines were eliminated by using glass 5 mL sample tubes, 0.1 μm inline sheath filter, 0.2 μm air filter, 0.2 μm pre-filtered sample buffer and cleaning both the sheath and sample lines with 10% Sodium hypochlorite (bleach). Bead resolution: Beads were mixed in equivalent concentrations and run using the new FSC optics at speeds of 100 eps. Microparticles: Microparticles for erythrocytes and platelets were harvested from 18 mL human blood, centrifuged 1x at 5K rpm for 15 mins and the supernatant centrifuged in microfuge tubes at the maximum speed (16K rpm) for 20 mins. The pellet was stained with Annexin-V FITC as per standard procedure and stained with additional antibodies for 20 min at RT in the dark. Antibodies: Platelets Mouse Anti-Human CD41-PC5, CD61-PC7, CD42b-PE, Annexin-V FITC, CD45 PB; Erythrocytes: Mouse Anti-Human CD235a-PC7, Annexin-V FITC, CD45-PB Endothelial: CD105-PE, CD146-PC5, Annexin-V FITC, CD45-PB. Flow Cytometry: Bead particles and microparticles were triggered on FSC, SSC and fluorescent parameters. Microparticle PE-tandemns were run both on the 488 nm and a modified 561 nm filter set and sorted at 50K eps.

Results: Instrument Set-up: Utilizing the instrument set-up, the noise particles within the system were reduced to less than 0.2 μm on the FSC-H vs SSC-H histogram plots. Bead Resolution: The 0.2 and 0.3 μm beads were resolvable with a 0.1% overlap between populations and were visible above background noise. Additionally, the enhanced FSC measured beads, sized 0.2 to 30 μm to be resolved concurrently. Microparticles: Erythrocyte and platelet microparticles were visible from FSC noise and sorted on positive markers.

Conclusions:
• Preparing the MoFlo Astrios for small particle analysis is a critical component to successful small particle resolution
• The small particle resolution and sensitivity of the newly re-designed forward scatter allows for a large dynamic range of particles without compromising resolution
• Utilizing both PMTs for the FSC parameters enhances the researcher’s ability to distinguish cellular morphological characteristics thereby, expanding the outcomes of flow cytometry on the MoFlo Astrios.
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. Beam-splitting 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.
INSTRUMENT PREPARATION:

Reduce Instrument Particle Background The FSC Module utilizes the use of both an air filter supplying system air and a 0.04 um sheath filter installed in the system. The air filter is a three-part filtering system which will progressively remove air contaminants of 0.1 um size (93% Stage 1, 99.99% Stage 2, 99.9999% Stage 3). The air filter reduces possible aerosol contaminants and provides the system with filtered air. The 0.04 um in-line sheath filter effectively removes submicron particles that may otherwise be visible in the lower FSC measurement range. It is a drop-in replacement for the current 0.2 um in-line sheath filter.

*Figure 2. In-line sheath filter and air filter provided with the enhanced FSC module.*

The choice of sheath is also critical to small particle detection. The development and testing for the FSC Module to measure 0.2 um particles from noise utilized Beckman Coulter IsoFlow (P/N 8546859). IsoFlow is a non-fluorescent, azide-free balanced electrolyte solution filtered to 0.2 um and provided at a 1x concentration.

Optimize Instrument Threshold Settings

The success of detecting small particles is in part due to measuring the smallest pulses above noise. To ensure that desired signals are captured, the threshold on the Astrios needs to be set properly. On the right, the threshold on the 488-FSC1 trigger is reduced to 0.004% without sample running to visualize the noise. The instrument detects the noise as “events” and gives a high events per second (EPS) of 260,000.

The threshold for the trigger is then increased until the EPS is reduced to 16, giving a resultant 0.016% threshold value (bottom right histogram). The researcher now knows that the signal being measured is not noise, but signal from particles passing through the laser.

*Figure 3. Histograms created from the Astrios Touch Screen panel to demonstrate setting the threshold properly. The top histogram shows a noise population on the FSC1 vs FSC2 Height. As the threshold increases, the population and EPS are reduced.*
Remove Possible Contaminents from Experimental Protocols

Figure 4. Development on the Astrios FSC module included removing particulates from different sample media and testing current media. As seen in the figure, the number of particulates depends on the filtering and expiration date of the sample media. Less particulates indicates a better background for small particle resolution.

The enhanced FSC Module measures small particles, including those in the sheath and sample media. As seen on the left, the different sample media may have background particulates that may give artificial results in a small particle analysis.

REMOVING CONTAMINANTS:

Analyze the Glass and Plastic Components of the Experiment

Figure 5. Preparation for measuring small particles includes analyzing the filtering mechanism and glass/plastic components of the experiment. As seen in the figure above, the 0.04 um DI water in a glass tube gave the least amount of particulates. This media was used to analyze small to larger particles (as seen on the right). The switch from the 5 ml polystyrene to glass tubes reduced the plastic debris.
0.2-30 UM 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 um-30 um beads are measured with an ND=1.

Figure 6. Polystyrene beads purchased from Bangs Laboratories were diluted 1:1000 (0.2-1 um beads) and 1:100 (3-20 um) and analyzed on the Astrios using 100 um tip with drop drive on. The beads were diluted with 0.04 um filtered DI water and ran in a borosilicate glass tube.

Figure 7. 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 um 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 um 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.
DUAL FSC PMT APPLICATIONS:

The dual FSC PMT design of the Astrios enhanced FSC module provides flexibility in triggering and visualizing populations simultaneously. Researchers may use a FSC1 trigger (below left figure) of lysed whole blood, and visualize the entire population on the FSC2 parameter. The populations may also be analyzed on both FSC Log-Height and FSC Height parameters simultaneously (below right).

**Figure 8.** FSC vs SSC Height Log histograms of human lysed blood. Blood was lysed (Beckman Coulter TQ-Prep) and prepared in a glass tube with 0.04 um filtered FACS buffer. The researcher could set the FSC trigger on FSC1 and visualize/sort/analyze the entire population on FSC2.

**Figure 9.** FSC vs SSC (Height-Log and Height) histograms of human lysed blood. A = microparticles, B = red blood cells, C = platelets, D = lymphocytes, E = monocytes, F = granulocytes. Researchers can analyze small particles and large particles simultaneously.

**Figure 10.** Human lysed blood histogram with FSC1 Height Log vs FSC2 Height Log. The different FSC paths may be analyzed for different population attributes (ND filters) as seen in population A and B.

**Figure 11.** FSC1-Height Log vs FSC2-Height Log of the common coccolith, *E. huxleyi*. The dual FSC parameter allows sorting of a non-fluorescent plankton population. The plankton undergoes different life cycles, as seen in the figure.
To confirm the location of human blood cell populations, blood was stained with common cellsurface antigens and run on the Astrios with the enhanced FSC module. The populations were clearly identified in the FSC Height Log histograms gated on fluorescent parameters. The new FSC has distinct separation between microparticles, red blood cells, platelets, lymphocytes, monocytes and granulocytes on a log-log FSC histogram.

**Figure 12. FSC and fluorescent histograms of human blood cell populations. A. Red Blood Cells, B. Platelets, C. Lymphocytes, D. Granulocytes using antibodies as described in the histograms above.**

**FSC IN ACTION: MICROPARTICLES:**

**Introduction**

Microparticles are produced when fragments are shed from the plasma membrane of cells. Nearly all types of cells shed microparticles. (1) Microparticles should be distinguished from exosomes, which are smaller than microparticles (<0.1μm), multivesicular, and produced inside cells. (2) Microparticles have been shown to display both deleterious and beneficial roles in cell maintenance and transcellular information exchange. Shedding of microparticles is caused by activation (2, 3) and stress conditions, including apoptosis. (1) However, microparticles are thought to play a role in hemostasis and can also be shed during cellular remodeling processes (1), as well as function in blood coagulation, inflammation, and cell activation (2).

The new MoFlo Astrios forward scatter design is able to measure particles less than 1 μm in diameter, such as microparticles (3, 4). Using the MoFlo Astrios, erythrocytes microparticles were sorted at high speeds (<50k events per second). Antibodies for endothelial, erythrocyte and platelet microparticles were identified by their specificity, sensitivity, fluorochrome emission characteristics, and availability (2). The MoFlo Astrios could be used as a powerful research device for further characterization of microparticles and other particles smaller than 1μm.
Methods

Sample Preparation: Platelet and Erythrocyte Microparticles

Human blood was collected by venipuncture (8 ml) in a citrated blood collection tube. Six mL of blood was centrifuged in a disposable centrifuge tube at 5000xg for 15 min at 20 degrees C. The supernatant was transferred into 1.5 ml microfuge tubes and frozen at -80 degrees C. The samples were thawed at 37 degrees and centrifuged at the maximum speed on the microfuge (16,000xg) for 20 mins. The supernatant was discarded and re-suspended in 900 ul Annexin V buffer (diluted 1:10 in distilled water) for 4 degrees.

Ten uL of the microparticles/Annexin V solution was removed for single control antibody stains and 20 uL for the multicolor stain and placed in a glass flow cytometry tube. Twenty uL of Annexin-V FITC was added for each single color control and 40 uL added to the multicolor stain. Antibodies were added as described in the table on the left and incubated for 20 min in the dark. Samples were run within 30 min of adding Annexin-V.

**Platelet Microparticles**

<table>
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<tr>
<th>Marker</th>
<th>Fluorochrome</th>
<th>Beckman Coulter Number</th>
<th>Quantity</th>
<th>Specificity</th>
<th>Sensitivity</th>
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<tr>
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<td>IM3546</td>
<td>20uL</td>
<td>Phosphatidyle serine lipopolysaccharides</td>
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<tr>
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<td>Pacific Blue</td>
<td>A74765</td>
<td>10uL</td>
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**Erythrocyte Microparticle Antibodies**

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<th>Beckman Coulter Number</th>
<th>Quantity</th>
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**Endothelial Microparticle Antibodies**

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<th>Quantity</th>
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<td>A74765</td>
<td>10uL</td>
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</tbody>
</table>

**Endothelial Microparticles**

Endothelial microparticles were isolated by a slow spin at 160xg for 10 min at 20 degrees C. Collect the supernatant and centrifuge at 1000xg for 10 min. The resultant pellet was stained as described above.
MICROPARTICLE RESULTS:

Microparticle Controls

The development of this assay required first finding the correct microparticle population through centrifugation and antibody staining. In addition, the samples were spiked with lysed human blood to place the FSC voltages at a location to measure the microparticles, red blood cells, platelets, lymphocytes, monocytes and granulocytes.

Figure 13. Histograms of FSC and SSC Height Log for no sample (above) and lysed blood (bottom).

Endothelial Microparticles

Figure 14. Endothelial microparticles isolated from human blood using human antibodies.

Platelet Microparticles

Figure 16. Platelet microparticles isolated from human blood, positive particles marked by Annexin-V+, CD41+, CD42b+ and CD61+.

Erythrocyte Microparticles

Figure 17. Erythrocyte microparticles isolated from human blood, both whole RBC and RBC microparticle cells are visible in the histogram.

Erythrocyte Microparticles Sort

Figure 18. Sorting out erythrocyte microparticles and red blood cells from human blood. Cells were sorted at >62K eps on both RBC and RBC microparticles. The Astrios was flushed with DI and then a 10 sec acquisition was taken to determine inherent instrument background (see above middle). The sorted samples were then reanalyzed for purity, measuring (with instrument contamination), 86% purity for RBC and 96% purity for RBC microparticles.
CONCLUSIONS:

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REFERENCES:


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