QUICK REFERENCE GUIDE

Cytek® Amnis® ImageStream®X Mk II Flow Cytometer Quick Reference Guide
Collecting Small Particle Data On The ImageStream® System

This guide provides basic instructions for the initial setup of fluorescent small particle data acquisition on the ImageStream® system using the High Gain Mode. Before proceeding, the system should be fully cleaned, samples should be fluorescently stained, and 0.1 µm filtered sheath fluid should be prepared for use. Use this Quick Reference Guide to become familiar with the workflow of small particle data acquisition. For more detailed instrument information, refer to the User's Guide.

SYSTEM REQUIREMENTS

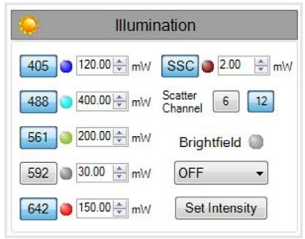
- MultiMag: 60X Objective
- High Gain Mode

RECOMMENDED INSTRUMENT SET UP

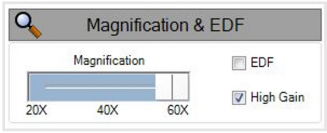
- Autosampler: 96-Well Plate
- High Power Laser:
  488 nm: 400 mW Laser

INSTRUMENT SETUP USING INSPIRE™ SOFTWARE

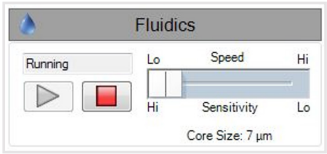
1. Load the small particle INSPIRE™ template or follow the steps below.
2. Set all fluorescence excitation lasers required for your fluorochromes to maximum power.
   NOTE: It is not recommended to use the 592 nm laser as it may cause significant crosstalk.
3. Set the SSC laser power on Ch06 (1-camera system) or Ch12 (2-camera system) to 2-15 mW, sufficient to saturate the Amnis® SpeedBead® reagent.
   NOTE: The 785 nm SSC laser does not detect particles below 150 nm.



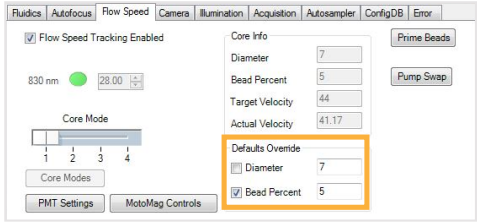
4. Turn off the **Brightfield** channel.
   NOTE: The Brightfield channel increases background and does not detect particles below 300 nm.
5. Adjust the **Magnification** slider to 60X.



6. Turn on **High Gain Mode**.
   NOTE: High Gain Mode is optimized for 60X magnification.
7. Adjust the slider to indicate **Lo Speed** and **Hi Sensitivity**.



8. In the **Advanced Controls** menu, select **Flow Speed**. Under **Defaults Override**, change **Bead Percent** to 5.
   NOTE: This will minimize the number of SpeedBead reagents in the sample.



9. Create a histogram of Raw Max Pixel SSC and draw a region to identify the SpeedBead reagent.
   NOTE: The SpeedBead reagent saturates in SSC and will not exhibit fluorescence. Optionally, create a dot plot of EV fluorescence vs. SSC to eliminate the SpeedBead reagent.
10. **File/Save Template** to use this template for future experiments.
11. Acquire the **All** population by time; a duration of 3–5 minutes is recommended.

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For support and/or questions, contact technicalsupport@cytekbio.com.

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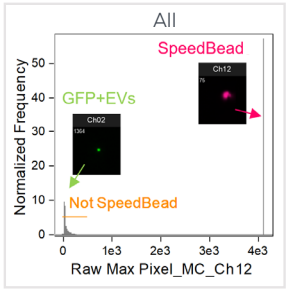
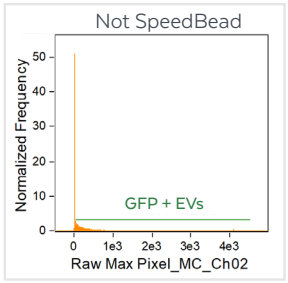
SMALL PARTICLE ANALYSIS IN THE IDEAS® SOFTWARE USING RECOMBINANT HEK293T-DERIVED EXTRACELLULAR VESICLES (EV) EXPRESSING GFP*

1. To gate out the SpeedBead reagent, create a histogram of Raw Max Pixel in Ch12 (or Ch06 on a 1-camera system).



2. Most of the SpeedBead reagent will be saturated (high Raw Max Pixel); draw a gate that captures events with low Raw Max Pixel_Ch12.

3. To find the GFP+ population, create a histogram of the Not SpeedBead population using Raw Max Pixel_Ch02 (Ch02 detects the GFP signal).

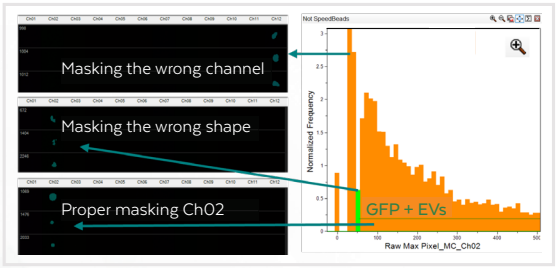


4. Draw a GFP+ EV gate using the image gallery.

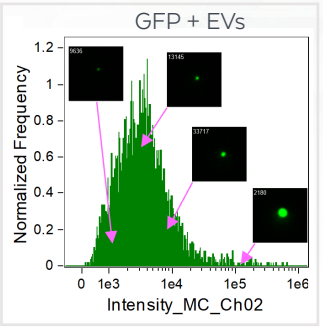
   4a. Zoom in on the bottom left of the histogram.

   4b. Change the view on the gallery to **Population**: **Selected Bin**. Click on the **Mask** icon to show masking.

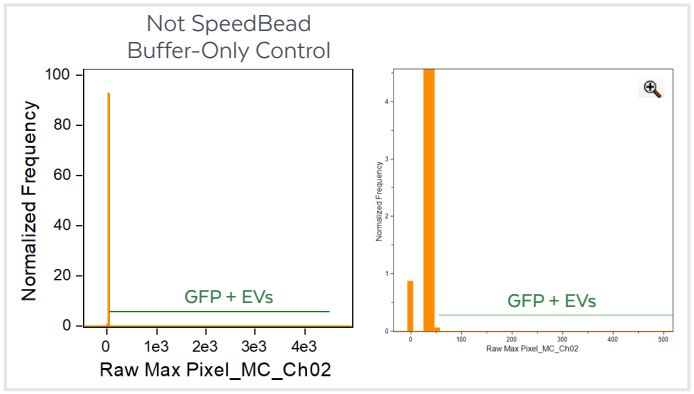
   4c. Determine the gate lower limit on the bin that masks a circle on Ch02, which indicates GFP signal detected above background.



5. Create a histogram of the GFP+ EVs population using Intensity_Ch02. ***NOTE:*** Adjusting the **Image Display Mapping** on the **Image Gallery Display Properties** helps visualize GFP+ EVs.



6. To verify the gates were placed properly, compare this sample analysis to the controls: buffer-only, detergent control, negative staining controls, and stained samples.



7. Continue with additional analysis such as compensation when multiplexing, determining sample concentration, and axis calibration on third-party software.

*MilliporeSigma Catalog Number: SAE0193

405 nm side scatter detection can be performed for EVs using the method described in Woud, WW. Cytometry A. 2024 Oct;105(10) 752-762. PMID39238272.

Perform small particle assay following the MIFlowCyt-EV guidelines in Welsh JA, Van Der Pol E, Arkesteijn GJA, et al. MIFlowCyt-EV: A framework for standardized reporting of extracellular vesicle flow cytometry experiments. J Extracell Vesicles. 2020;9(1):1713526. Published 2020 Feb 3. doi:10.1080/20013078.2020.1713526.

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