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Astrios EQ instrument setup and sample acquisition

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We use this protocol and it's working

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Disclaimer

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

Abstract

In this protocol the authors describe the general steps to follow in order to achieve optimallaser:stream:detectors alignment and sample acquisition on the Astrios EQ flow cytometer (Beckman Coulter). The general concept however can be applied to other jet-in-air instruments.

Materials

Reagents

- 200 nm Fluospheres beads: red (Life Technologies, Catalog number F8794, ex/em 580/605) and yellow-green (Life Technologies, Catalog number F8811, ex/em 505/515)
- $Ca^{2+}Mg^{2+}$-free Dulbecco's Phosphate Buffer Saline (DPBS, Gibco)
- FACS Clean
- FACS Rinse

Hardware

- Astrios EQ
- FACS polystyrene tubes

1 Turn on the instrument at least an hour before running the samples. Let the pressure and stream to stabilize and prime the fluidics system to remove bubbles in the circuit. Turn lasers on and allow them to warm up whilst shuttered.

2 Wash the sample line with FACS Rinse solution for about 20 minutes at a high differential pressure (1 psi over the sheath pressure). Repeat same procedure with clean DPBS for 20 more minutes.

3 Adjust the vertical alignment.

Note

Note: To test whether the stream is vertical or not, raise the nozzle while looking at the relative position of the stream to the pinholes. If the position doesn't change, the stream is vertically well aligned; if it does change, then the verticality must be tweaked. A good vertical alignment maximizes the detection of parameters in all laser paths.

4 Set the triggering threshold to the 561-SSC channel. Adjust the triggering threshold channel and voltage to allow the visualization of the noise population, to approximately 10,000 events per second.

Note

Note: The noise is a random representation of the diffusely scattered photons from the laser beam and stream intercept.Because of the high event rate of these low-level signals, inclusion of these events on some instruments is only feasible in a very limited way due to limitations in their baseline restoration algorithms and other signal processing attributes.Background noise is informative because: i) it serves as a window into the population of EVs that fall under the triggering-threshold; ii) it allows the determination of too much free dye in the interrogation point; and iii) it helps to identify when EV samples are being analyzed at a concentration that is too high and is therefore at risk of coincident detection(Morales-Kastresana et al., 2017; van der Pol, van Gemert, Sturk, Nieuwland, & van Leeuwen, 2012). For these reasons, the authors refer to the noise as "background reference noise".

5 Load a sample containing a mix of 200 nm yellow and red beads at 1×10^{7}beads ml^{-1}(about a 1×10^{6}-fold dilution of original stock) to fine-tune the stream:laser alignment.

Note

Note: Any combination of beads that are excited by different lasers can be used. The goal is to have two populations, whose fluorescence will be collected in different pinholes, in a way that the stream is aligned according to two pinholes. This ensures the correct vertical alignment.

Note

Note: The dimmer the fluorescent beads used, the better the alignment will be for EVs.

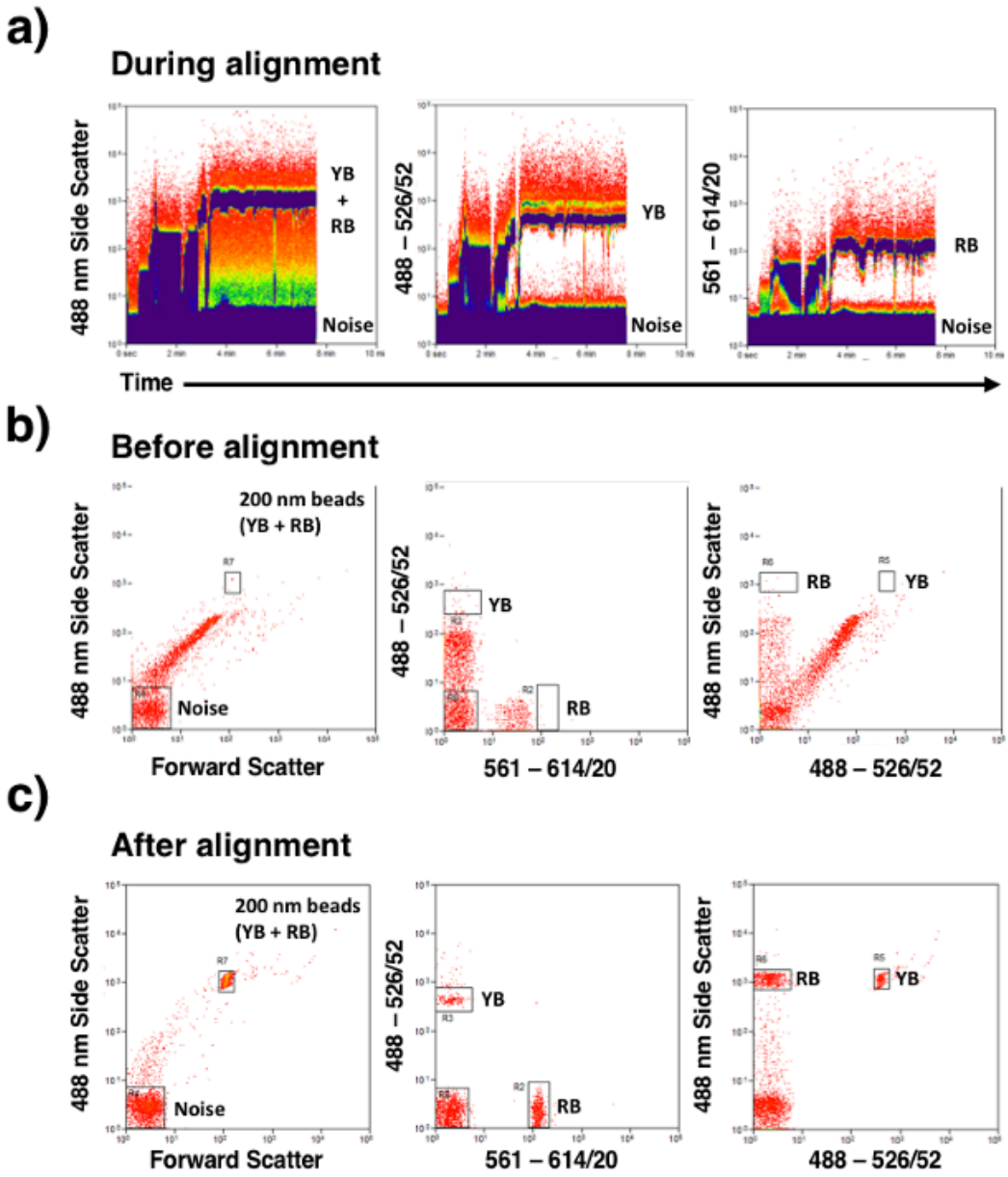
6 Open a dot plot depicting yellow (~515 nm) and red (~605 nm) fluorescent channels in each axis (or correspondent fluorescent axis for the chosen beads). Tweak the alignment until the fluorescence signal is optimized for both bead sets. While doing this, try to keep the total event rate, including the noise, around 10,000-20,000.

Note

Note: Alignment will be optimal when the distance between the noise and bead populations is the biggest in terms of fluorescence, while the bead population remains as tight as possible. Also, the event rate should not increase significantly with respect to DPBS alone, since the contribution of the beads to the overall rate is insignificant.

Note

Note: It is very useful to monitor Time versus any parameter (fluorescence and scatter) to determine how the distance between noise and beads varies with alignment adjustments.



Plots with the time parameter on the X-axis and scatter or fluorescent on the Y-axis during the alignment process to monitor the relative separation between the beads and noise (a). b and c show scatter and fluorescent parameters before (b) and after (c) optimizing the alignment. YB indicates 200 nm yellow fluorescent beads, RB indicates 200 nm red fluorescent beads.

Note

Note: If the bead populations and the reference noise population appear as "split" populations, there is probably drop-drive noise (especially with the 70 µm nozzle), which can be eliminated by dropping the break off point by lowering the amplitude or frequency, to the extent possible while maintaining stable Intelli-Sort settings and a stable breakoff point.

7 Once aligned, acquire and save a representative sample of the beads used for the alignment.

Note

This file will serve as a reference for future alignment, allowing comparisons between the alignment between experiments.

8 Wash the sample line with DPBS for 5 minutes at 1 psi.

Note

Note: As the sample lines clear, monitor the disappearance of the 200 nm beads from the alignment steps over time by referring to the time versus scatter or fluorescence plots. If alignment beads do not disappear completely, increase the pressure up to a differential pressure of 1.4. If beads are still observed, there may be contaminating beads in the sample path (either in the lines, junction points between fittings, or in the nozzle reservoir), and you will need to clear these residual beads before continuing. It can be useful to wash the line with rinse solution and followed by DPBS for 5 minutes each. Once washing is done, change rinse and DPBS tubes for future uses, as these solutions may contain contaminating beads from the sample injection tube.

9 Save an acquisition of 30 seconds of clean DPBS.

10 Load the EV sample, increase pressure up to 1 psi to accelerate the sample loading and then decrease the differential pressure to 0.3 psi.

Note

Note: A 0.3 psi differential pressure is offered here as a reference for what commonly works on AstriosEQ sorters that we have worked with. A differential pressure that permits stable detection of single EVs, avoiding coincidence, should be selected. Authors recommend keeping the differential pressure the same for all samples to be analyzed during an experiment, since event rate can be a valuable source of information.

11 Wait until the event rate is stable (use the time versus scatter plot). Then stop the acquisition in the software (not the machine) and start acquiring again for 30 seconds. Save the acquisition.

Note

Note: Authors recommend saving acquisitions of the same duration, in order to compare event numbers among samples.

12 Wash the sample line with DPBS for 5 minutes at 1 psi.

13 Run samples, save acquisitions and wash with DPBS, as described above.*Keep samples at 4°C and in the dark.*

14 Wash the sample line with FACSClean, FACSRinse and DPBS consecutively, for 10 minutes each at 2 psi. Turn off the lasers and fluidics. Rinse the nozzle and place a FACS tube cap with some clean water under the nozzle tip.

Troubleshooting

15

	ARTIFACT	SOURCE	WHAT TO DO
	False positive signal	Drop drive noise	Reduce amplitude and/or frequency
	Nonspecific binding of antibodies	Add control EVs that do NOT express the target antigen	
	Antibody/dye aggregates	Add control EVs that do NOT express the target antigen	

	Add control antibody/dye alone		
	Background antibody/dye fluorescence	Add control antibody/dye alone	
	Noise fluorescence shifting		
	Low resolution of positive signal over the noise/negative population	Background antibody/dye fluorescence	Wash the excess antibody/dye
	No positive signal detection	Low antigen density	Use a more sensitive instrument, brighter fluorochrome and/or brighter staining method
	Low antibody affinity	Increase concentration and/or incubation time	
	Antibody/fluorochrome conjugate not working properly	Change antibody/clone/fluorochrome/lot	
	Unexpected increase in event rate	Change in differential pressure of sheath tank and sample line	Time vs scatter parameter to identify these peak
	Stuck material in sample line/nozzle	Flush sample line with detergent and PBS with increased sample pressure differential (boost).	
	Contaminating material from previous sample	Run filtered PBS before and after samples, to minimize the presence of contaminating particles.	
	Coincident detection of particles	Sample is too concentrated	Dilute the sample
	Use spike in beads to ensure an operational concentration range		

	Nanobubbles at the interrogation point	Are created due to the high pressure in the nozzle	Run PBS control between samples to have that source of noise identified and controlled

A list of the most frequent artifacts observed by the authors is listed, along with a brief explanation and the suggested controls to consider, either to identify or to avoid such artifacts.