

Marginal gains in small particle cytometry using BD FACSDiscover™ S8 Cell Sorter and BD CellView™ Image Technology providing improved precision & accuracy.

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Abstract

Small particle cytometry has gained great attention over the past years. Biophysical and immunochemical characterization of extracellular vesicles (EVs) has attracted intense interest due to their emerging diagnostic and therapeutic applications. While the recent pandemic has also increased the attention to study viruses using high-resolution flow cytometry.

Small particle cytometry is a very challenging field of work, as the particles of interest: EV's & viruses are close or below the limit of detection of flow cytometers. Due to several factors: fluidics, electronics & laser intercept, this limit of detection is not a fixed value and traditional flow cytometers have a region of uncertainty where accuracy needs to be considered. The variable abundance of small particles in biological samples require a dilution series to overcome the challenge of swarm detection.

Working on the limits of systems the marginal gains theory can be applied; improving and optimizing performance by a small amount across a number of different areas will lead to significant, noticeable improvements overall.

Here we demonstrate marginal gains in key areas of small particle detection on the BD FACSDiscover™ S8 Cell Sorter platform with the BD CellView™ Image Technology. The combined improvement in these 4 areas: detection sensitivity, noise identification, excitation precision & electronic processing, allows the BD FACSDiscover™ S8 Cell Sorter with the BD CellView™ Image Technology to be used in exploratory experiments for small particle cytometry with higher sensitivity, precision and accuracy vs. traditional BD flow cytometers without small particle option.

The reliability of the platform is demonstrated by comparison of 3 systems on QC standards for EV.

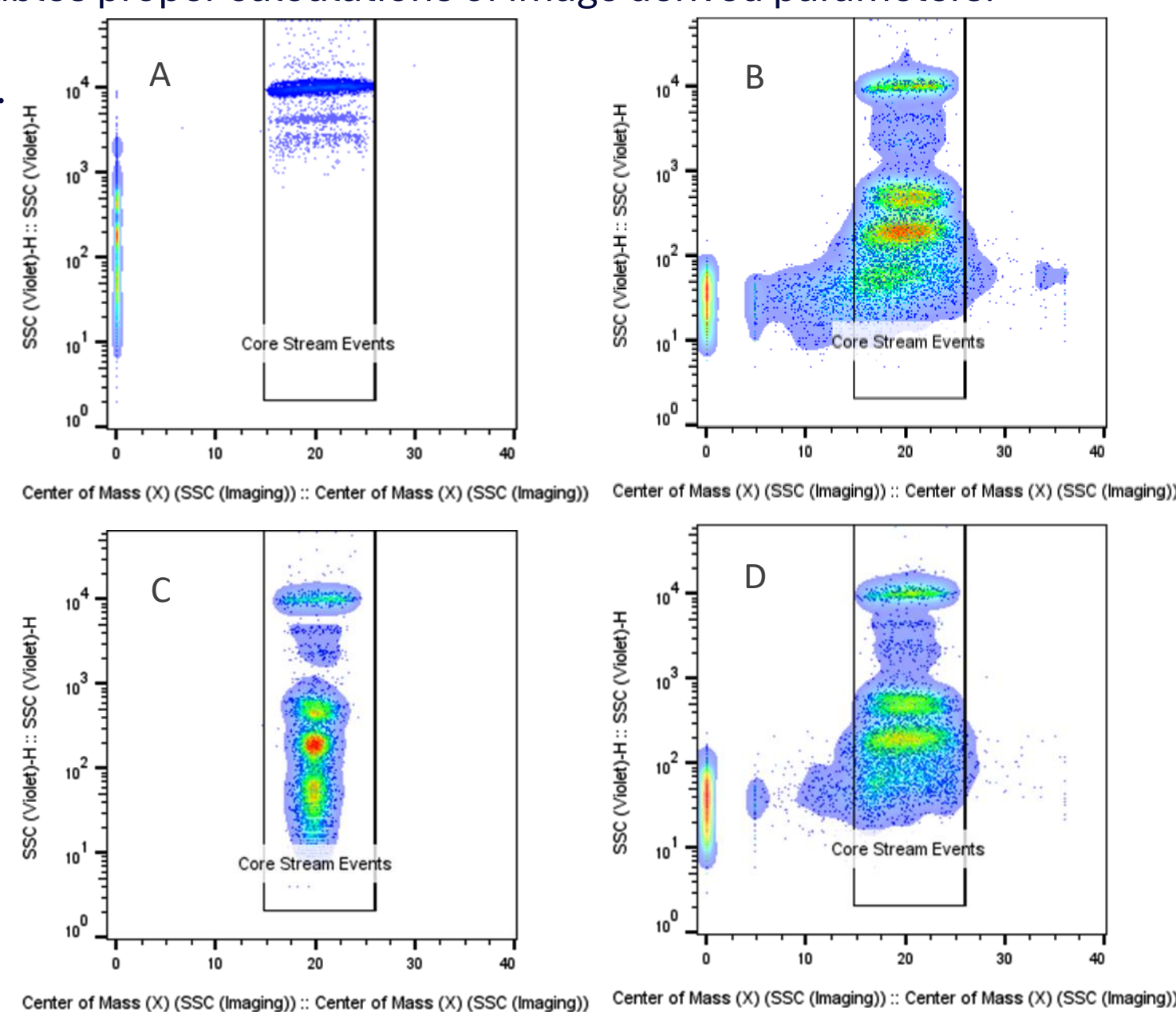
Methods

Instrument setup

Systems have been run using default sheath filter with default settings for the 85µ nozzle. Daily QC with BD FACSDiscover™ Setup Beads and BD CellView™ Calibration Beads and BD FACSTM Accudrop Beads were run. In the experiment SSC Image, SSC Violet and ImgB1 gains were adjusted to include a small amount of electronic noise.

For subpixel detection extra attention is required for the definition of region of analysis. The R.O.A. needs to be defined to the limit of detection without impacting image derived parameters. Here we use the Center of Mass (X) for SSC Imaging as reference guide. Center of Mass (X) is defined as the position of the particle (as defined by the Region of Analysis) in the horizontal direction within an image. R.O.A. adjustment is set using beads and the threshold value will be lowered until the COM X distribution of the smaller events start to increase due to noise inclusion. Bringing the ROA threshold down to 0 will include all pixels in the analysis and disables proper calculations of image derived parameters.

A: R.O.A. too high.
B: R.O.A. too low
C: R.O.A. at 0
D: R.O.A. correct



Reagents:

Megamix-Plus FSC & Megamix-Plus SSC (7802 & 7803, BioCytek): Fluorescent Polystyrene Size estimation beads. size range: 100nm, 160nm, 200nm, 240nm, 300nm, 500nm, 900nm
ApogeeMix (1527, Apogee Flow Systems): Mixture of Fluorescent Polystyrene & Non-Fluorescent Silica Beads. size: (PS) 80nm, 110nm, 500nm, (Silica) 180nm, 240nm, 300nm, 590nm, 880nm, 1300nm

Exosome standards, fluorescent recombinant EV's (SAE0193, Millipore Sigma)
BD FACSDiscover™ Setup Beads (665056, BD)
BD CellView™ Calibration Beads (665055, BD)
BD FACSTM Accudrop Beads (661612, BD)

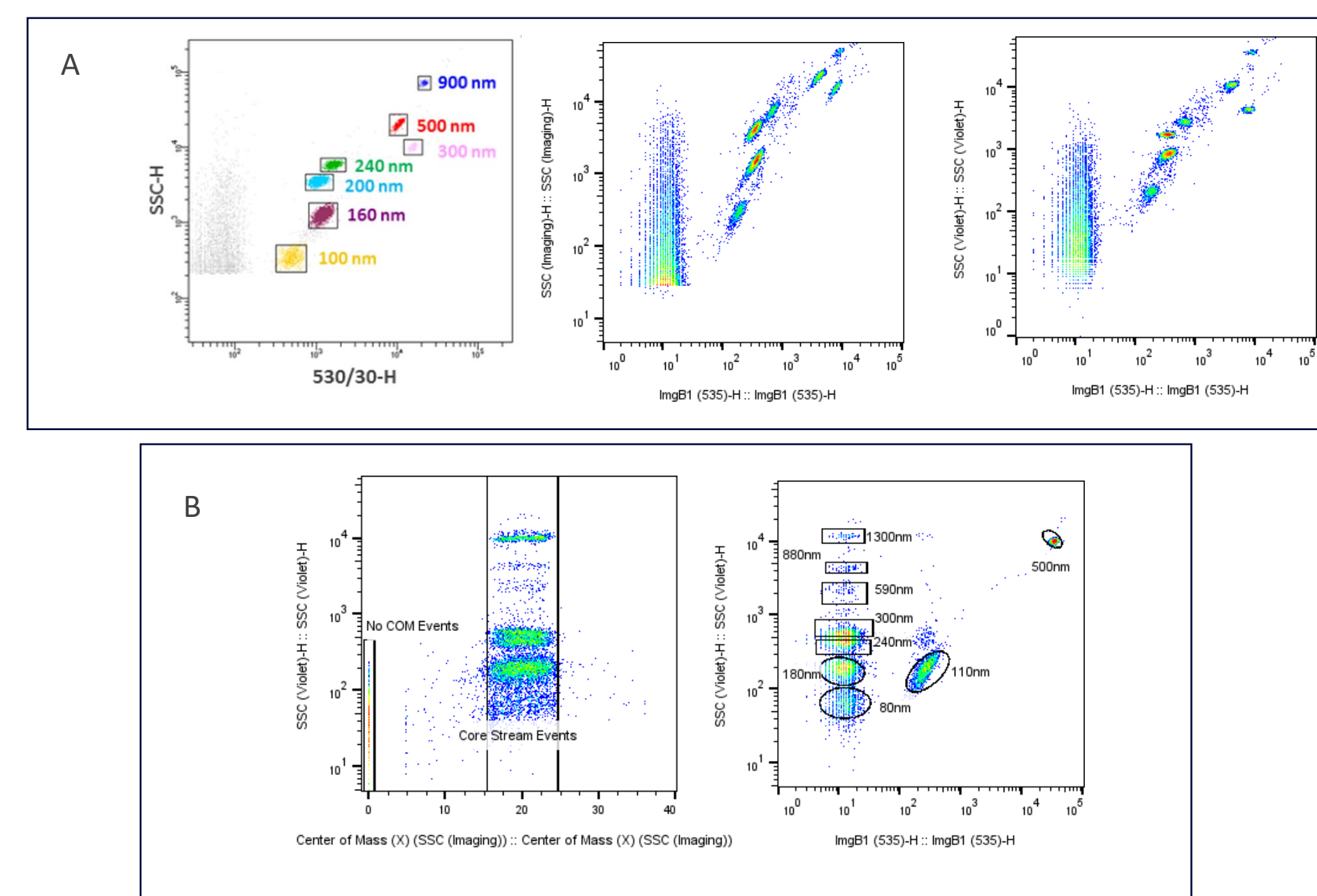
Results (1): Areas of improvement

1A Detection Sensitivity

Latest generation electronics and optimized optical architecture allows a significant improvement of sensitivity using common EV calibration reference standards. Here we compare Megamix-Plus FSC & SSC beads run on a BD FACSAria™ Fusion and a BD FACSDiscover™ S8 Cell Sorter.

(fig.A) Beads are displayed both on SSC Image to show the threshold channel as on Violet SSC to visualize the full detector range. The 100nm population can now be detected a full decade above threshold.

Using the Core Stream gate described below as single gating approach, the ApogeeMix 1592 beads clearly display the 80 nm polystyrene population demonstrating excellent detection sensitivity. (fig.B)



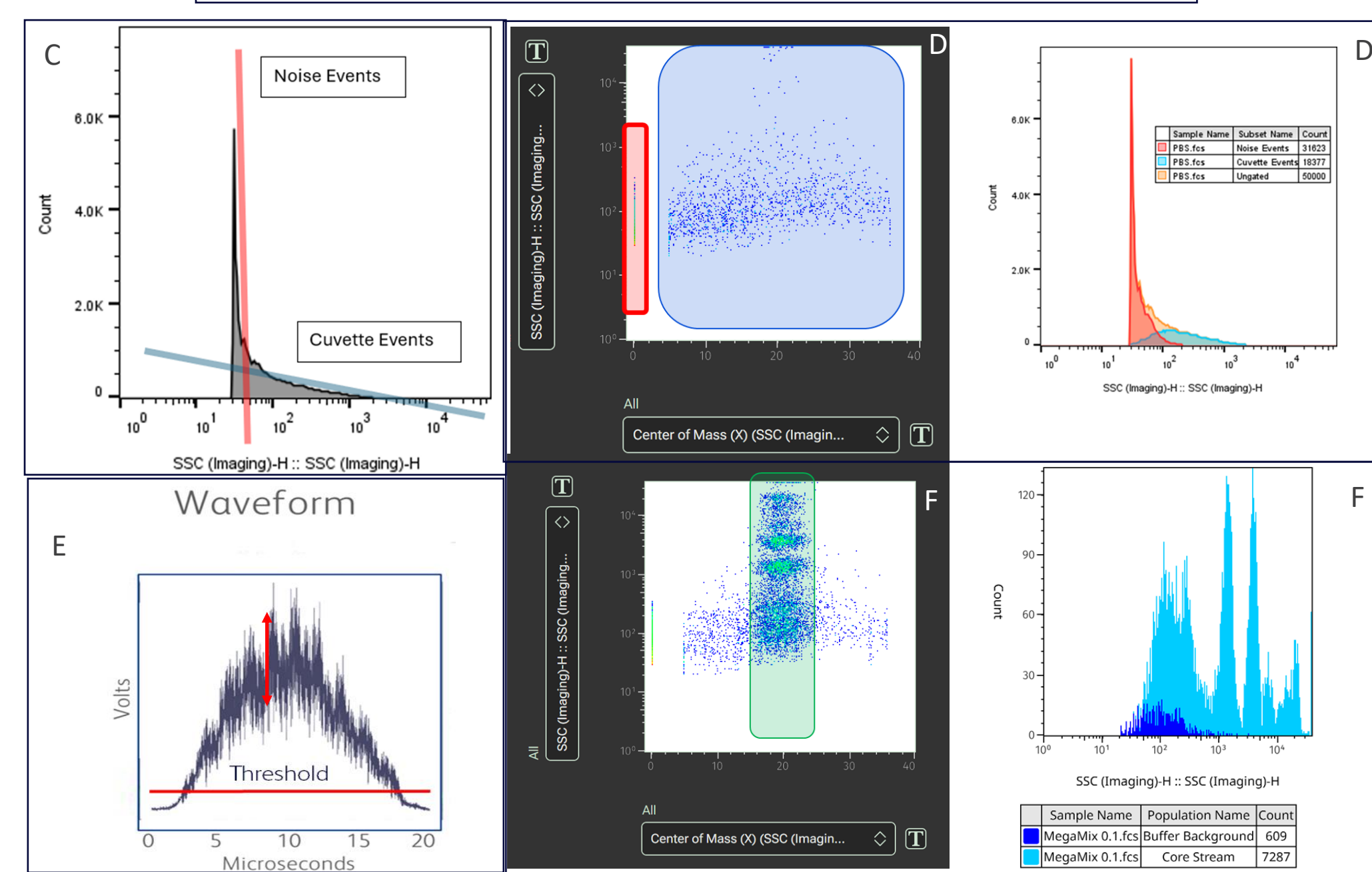
1B Noise identification

To ensure detection at full scale of the cytometer, the threshold & gain needs to be set to a level that includes a small part of true electronic noise.

Fluidic noise coming from events in the cuvette will always be present in a flow system but is hard to quantify. The contribution of events generated in the cuvette summed up with the pure electronic noise events gives a hyperbolic background signal pattern that fits 2 regression curves. (fig.C)

BD CellView™ Image Technology allows to visualize in which segment of the intercept an event is detected using the Center of Mass (X). Applied on SSC imaging this can be used to discriminate the Noise events from the cuvette events. Electronic noise and events too close to the threshold will not contain valid information in their waveform (fig.E). These will show a COM(X) value of 0. (red box) All events with COM (X) values will display where they were detected in the intercept. (blue box) (fig.D)

This allows to visualize the core stream on the dataplot (green box) Buffer background count subtraction approaches to improve accuracy can be designed based on uniform cuvette events distribution of a buffer only sample. (fig.F)

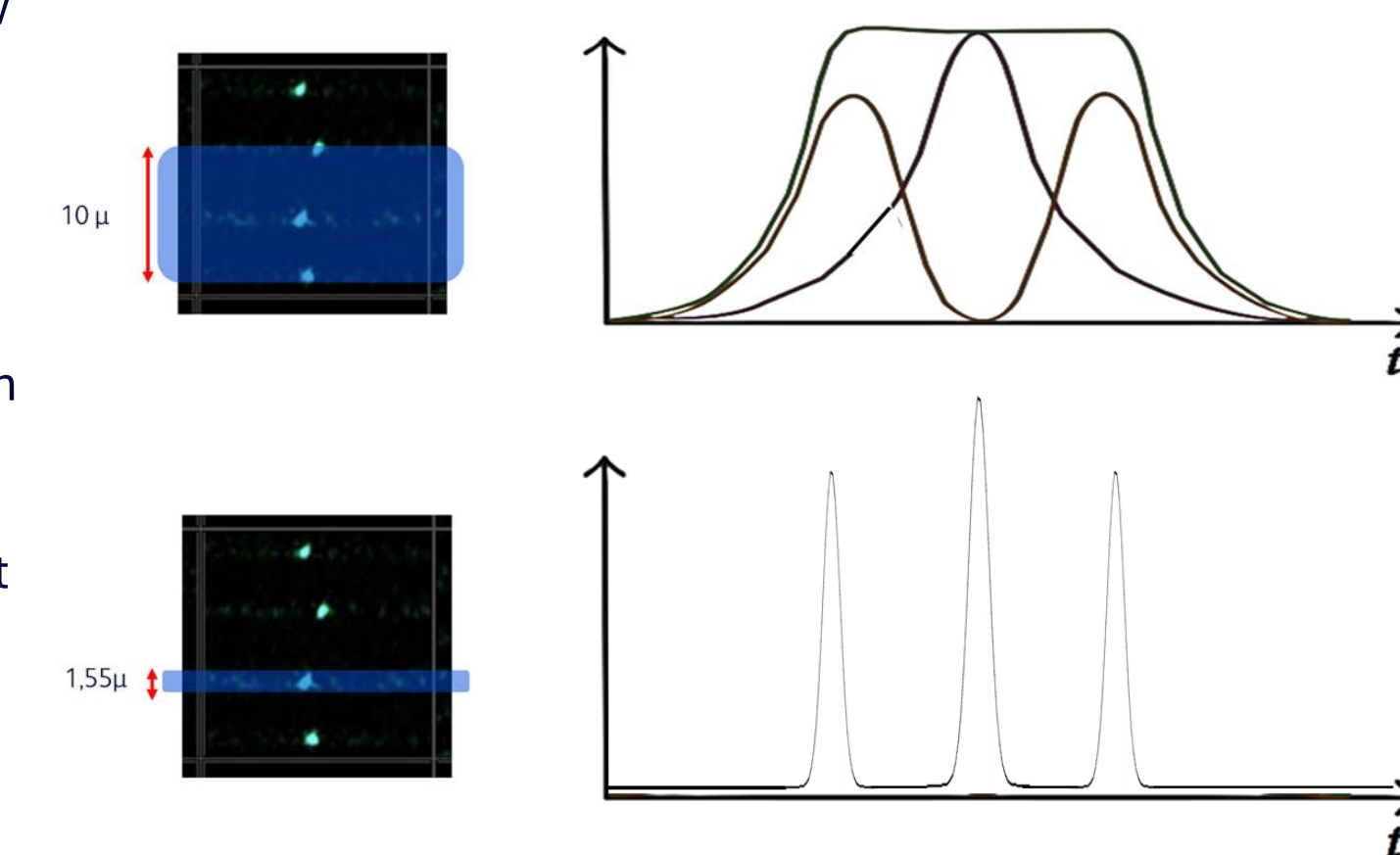


1C Excitation precision

The uniquely engineered BD CellView™ imaging beam has a vertical intercept of only 1.55µ which is much more precise than traditional flow cytometers with specified intercept ranging from 6 to 20µ. This intercept height brings improvement in small particle detection precision as the acquired pulses are more narrow and less likely to be coincident.

The BD FACSDiscover™ S8 Cell Sorter uses a low sheath flow velocity (around 1 m/sec) to allow a sufficient T.O.F. for precise scatter excitation and detection.

Image derived parameters provide additional tools to discriminate multiple particles, where traditional A, H & Width (if available) might not be sufficient to accurately identify doublets & triplets in heterogenous samples like EV. Here we show a visualization of the impact of the intercept height.



1D Electronic processing

On traditional systems, like BD FACSAria™ Cell Sorter, two overlapping pulses are rejected by the electronics as an abort. (fig.G) On the BD FACSDiscover™ S8 Cell Sorter these are merged into a single event to avoid data loss. Imaged derived parameters will allow to gate these appropriately and sort decisions can be made on gating strategy. An example of events that would have been aborted if not merged is shown on the image wall. (fig.H)

The image wall provides a convenient visualization of the samples and can help to identify SWARM effect and allows to reduce the nr of serial dilutions required. An example of swarm detection on SSC of 200nm PS beads, Raw Image wall channel settings not finetuned. (fig.I)

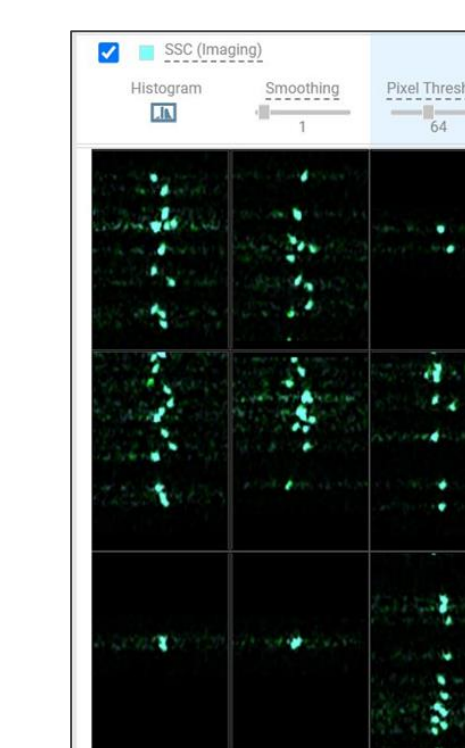
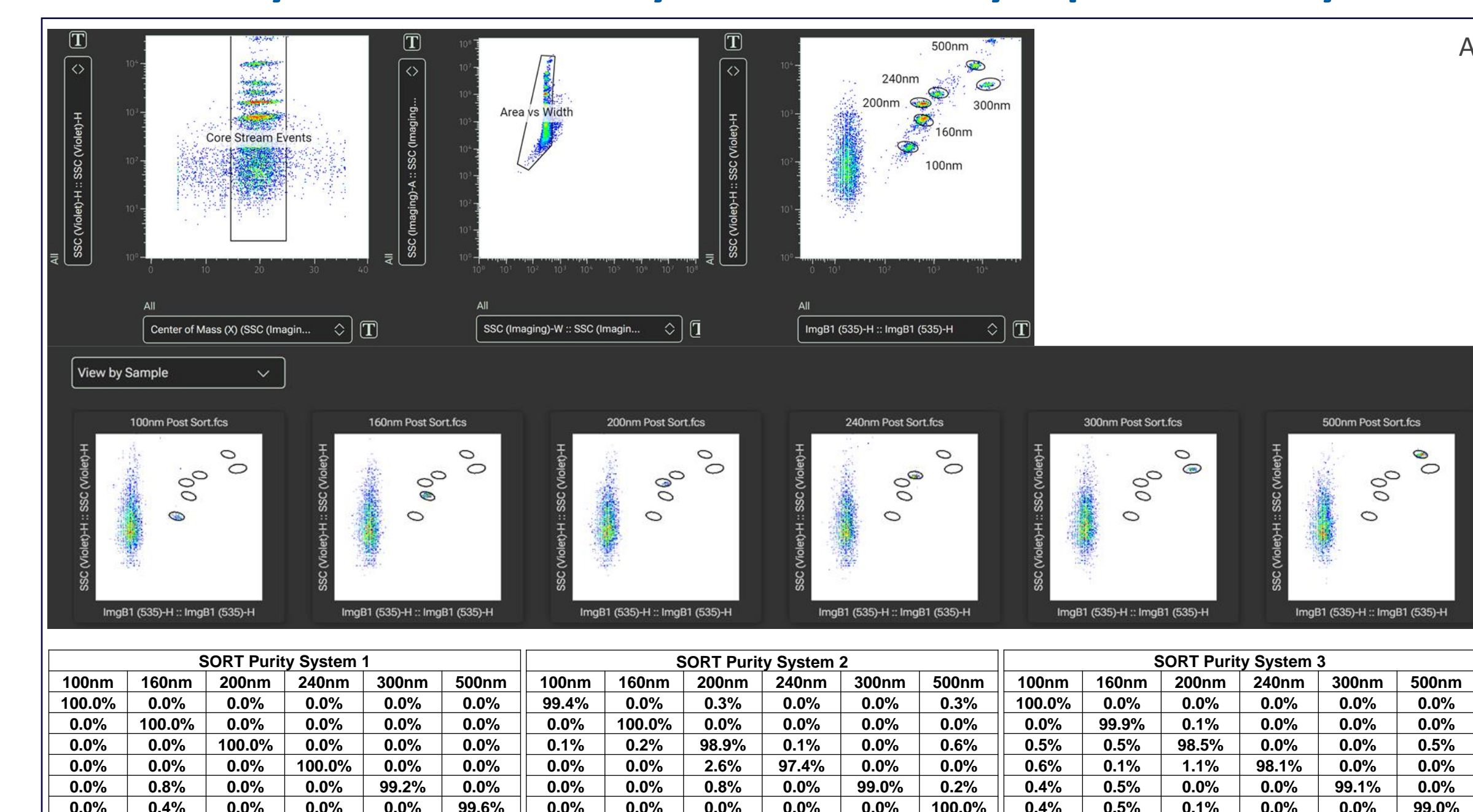


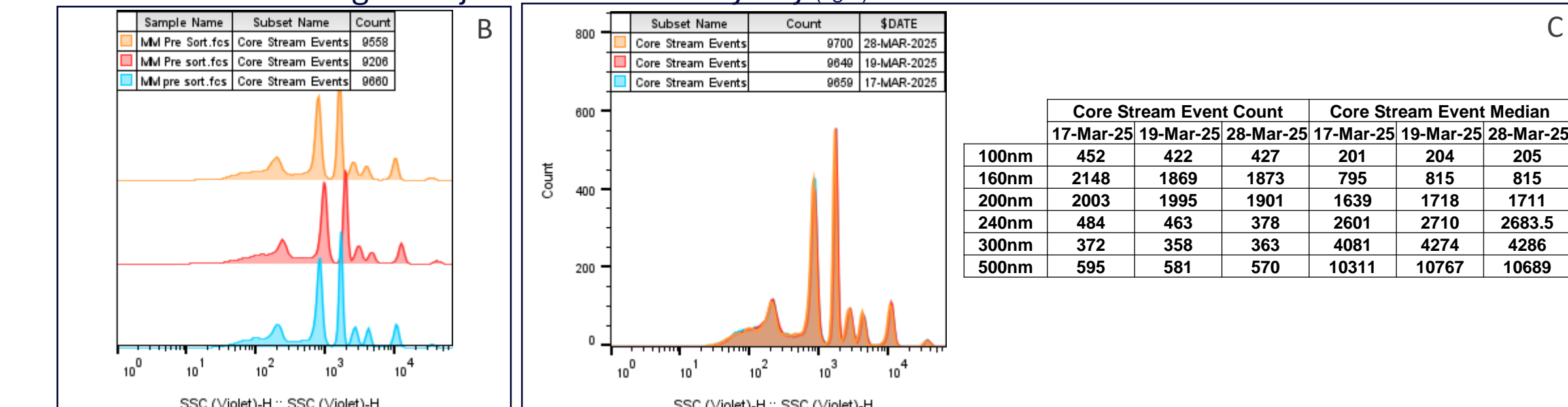
Figure 1G: Pulse with extended window gate. Figure 1H: Abort vs Merge. Figure 1I: Abort vs Merge.

Results (2): System reproducibility for sorting & EV counting

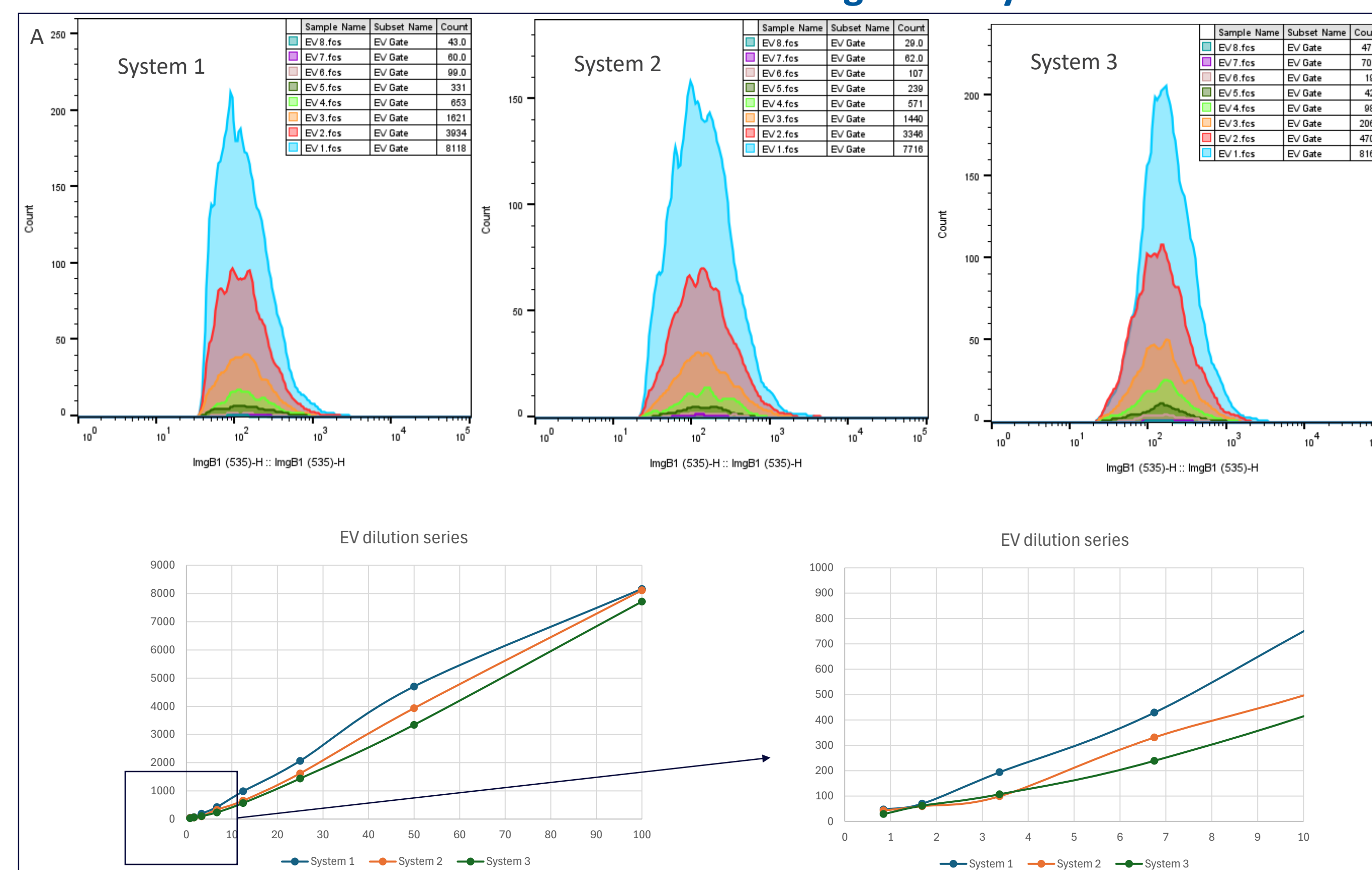
2A: 6-way bead sort on 3 systems and daily reproducibility



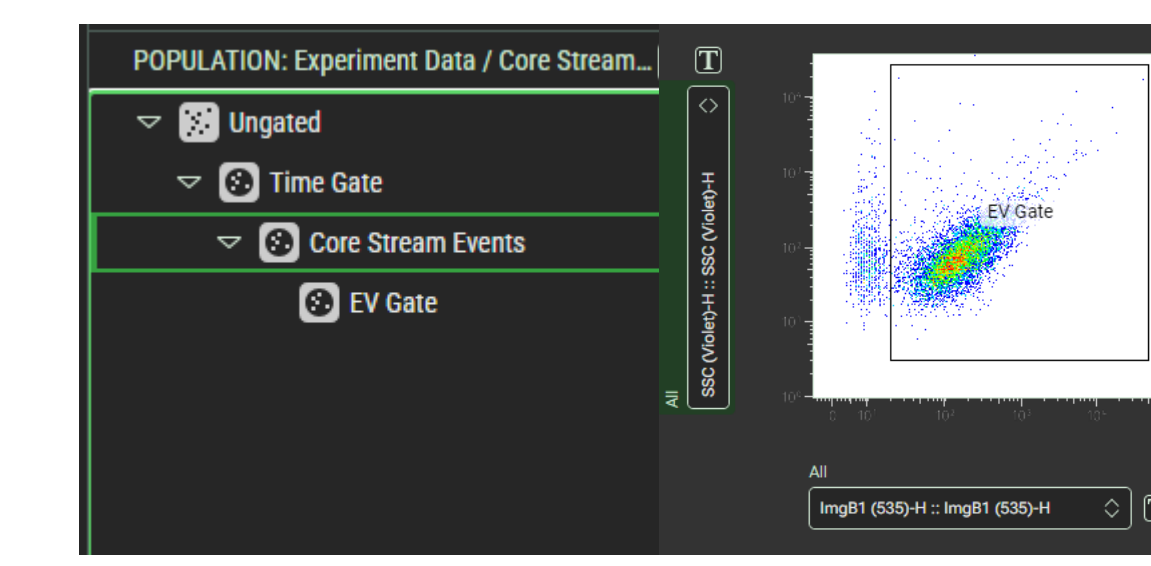
6-Way sorting on 3 different systems showed a 97% and above purity in single cell mode using the sort layout for re-analysis. (fig.A) The experiment was transferred from system 1 to system 2 & 3, minor gain adjustments were required to get populations in their target gates. Overlay of Megamix beads for Core Stream Events of 3 systems shows excellent consistency from 1 system to another. (fig.B) Day to day consistency is demonstrated with an overlay of Megamix using gains defined on day 1, automated QC was run to make the needed gain adjustments for every day. (fig.C)



2B: Serial EV dilution range on 3 systems



Serial dilution of recombinant EV samples shows consistent EV count on all 3 systems. The gating strategy for analysis and a recombinant EV sample example is shown on the right. The threshold was set at lowest on the Imaging SSC.



Conclusions

- The BD FACSDiscover™ S8 Cell Sorter with BD CellView™ Image Technology provides additional tools that allow discrimination of events of interest vs noise. The combined marginal gains in detection sensitivity, Noise identification, higher excitation precision, and electronic processing without aborts, result in an overall improvement of our standard non dedicated solutions for small particle cytometry.
- Noise identification with image derived parameters allow to create a workflow to subtract buffer background within a single fcs file.
- System reproducibility has been demonstrated on 3 separate units using the exported experiment from a single system on QC standard and EV dilution range.
- Day-to-day consistency was also demonstrated for both MFI and population counts.

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