New Technologies for Sorting QC

Generating new parameters using BD FACS™ Sortware software to understand flow cytometry sample behavior

Joe Trotter
Principal Scientist
BD Fellow, BD Biosciences
Joe_Trotter@bd.com

23-14727-00

BD

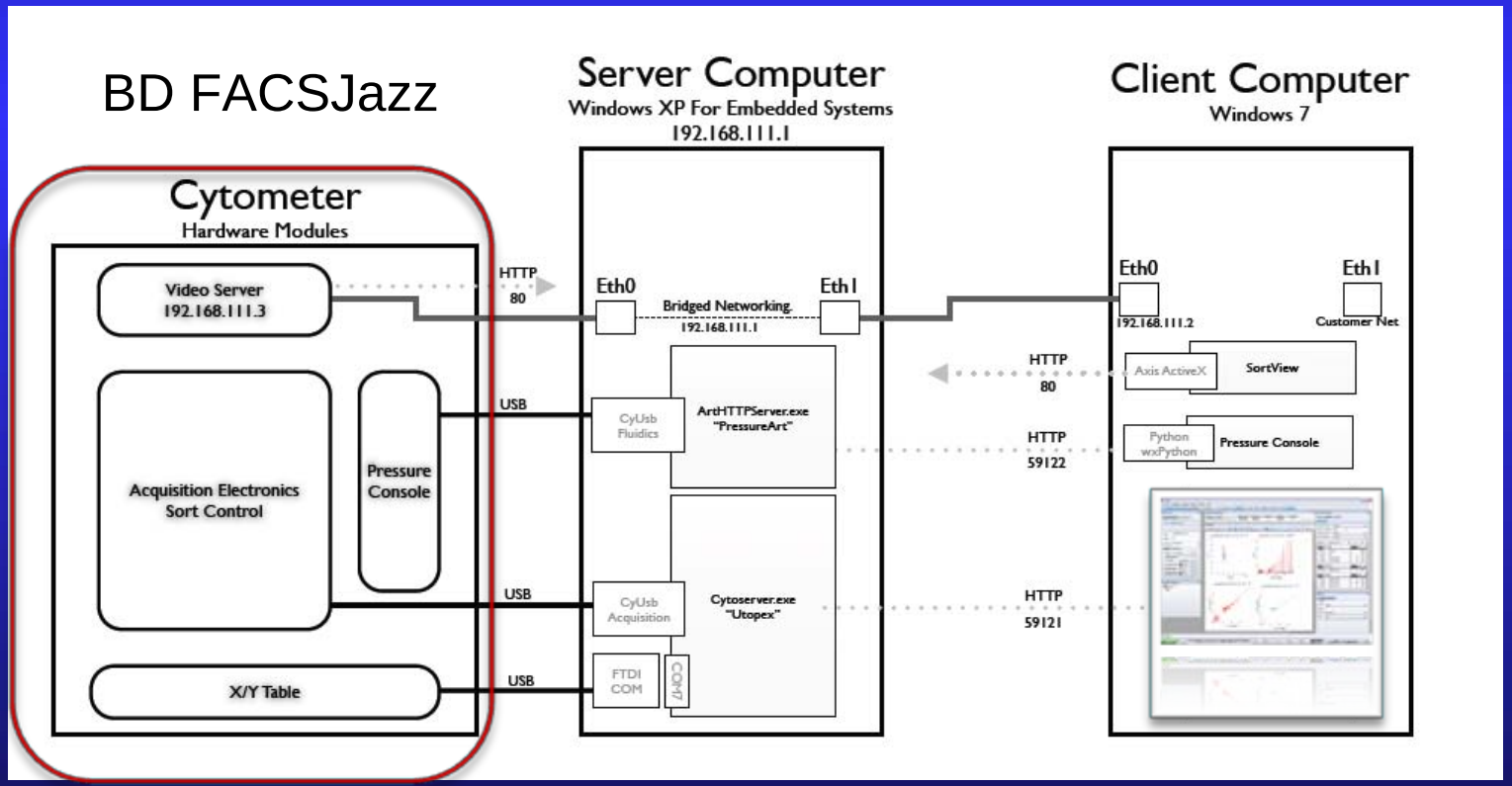
Overview

- Introduction to the systems
  - BD Influx™ and BD FACSJazz™ sorters
- The parameters we normally save in FCS data
- Key information used in firmware but not usually saved
- Time: a new level of precision
- Cell behaviors in flow: impacts for sorting
  - The good
  - The bad
  - The ugly
- Putting it together: some new directions

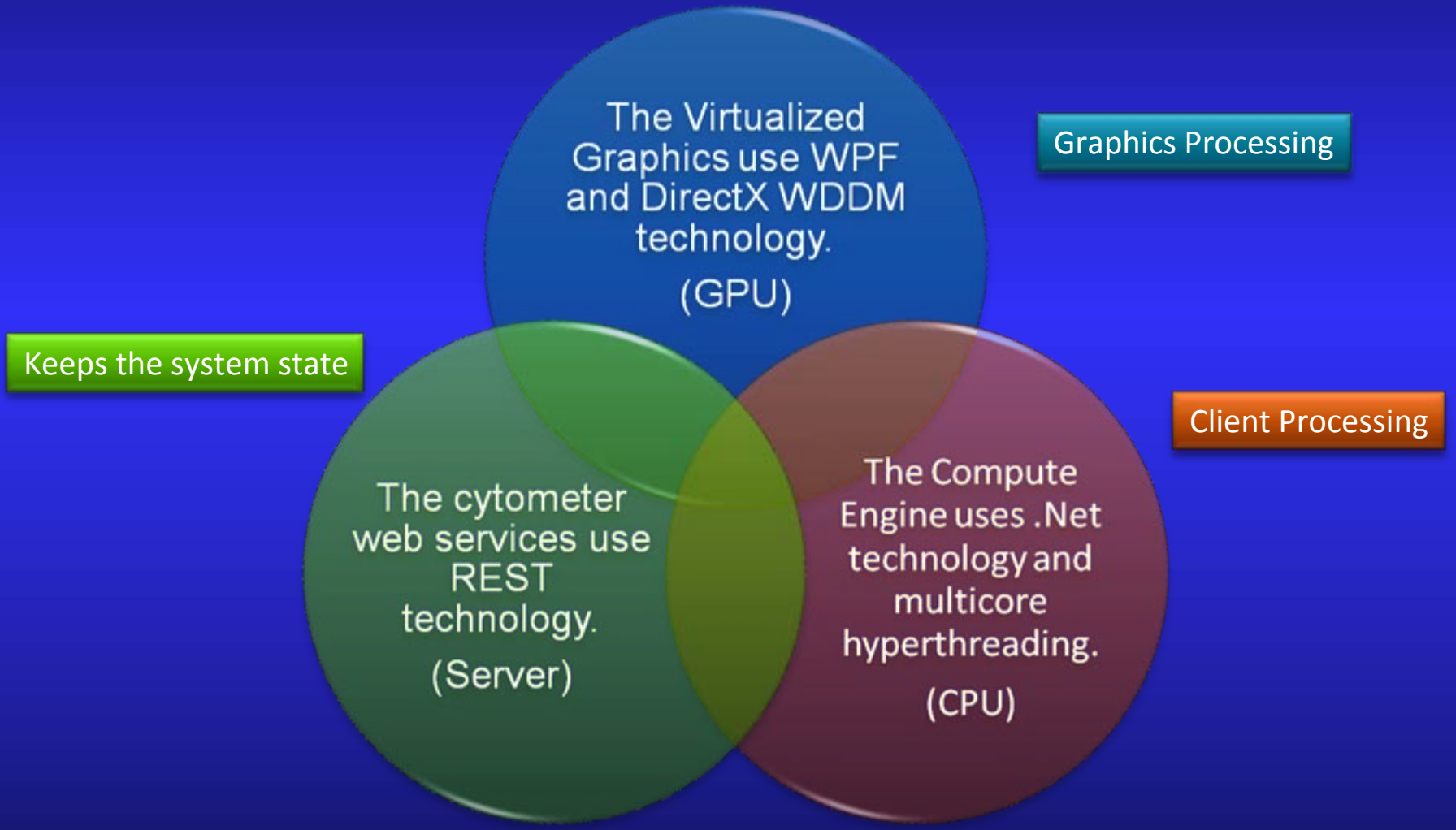
BD

BD FACS™ Sortware Sorter Software

- Sortware is the software suite of components and applications for the BD Influx and BD FACSJazz sorters.



Software Technology Overview



The New Platform

- BD FACSJazz sorter: what it looks like
  - A new sorter with some new parameters

BD FACSJazz Sorter

- 3 lasers, 8 detectors, 6 colors
- Simplified system alignment
  - Compact optical bench
  - Most alignment controls fixed
  - Pinhole camera view
- Preselected sort settings
  - Minimize setup
  - Maximize consistency
  - 100-µm nozzle
  - ~ 40,000 drops per second
  - BD FACS™ Accudrop technology

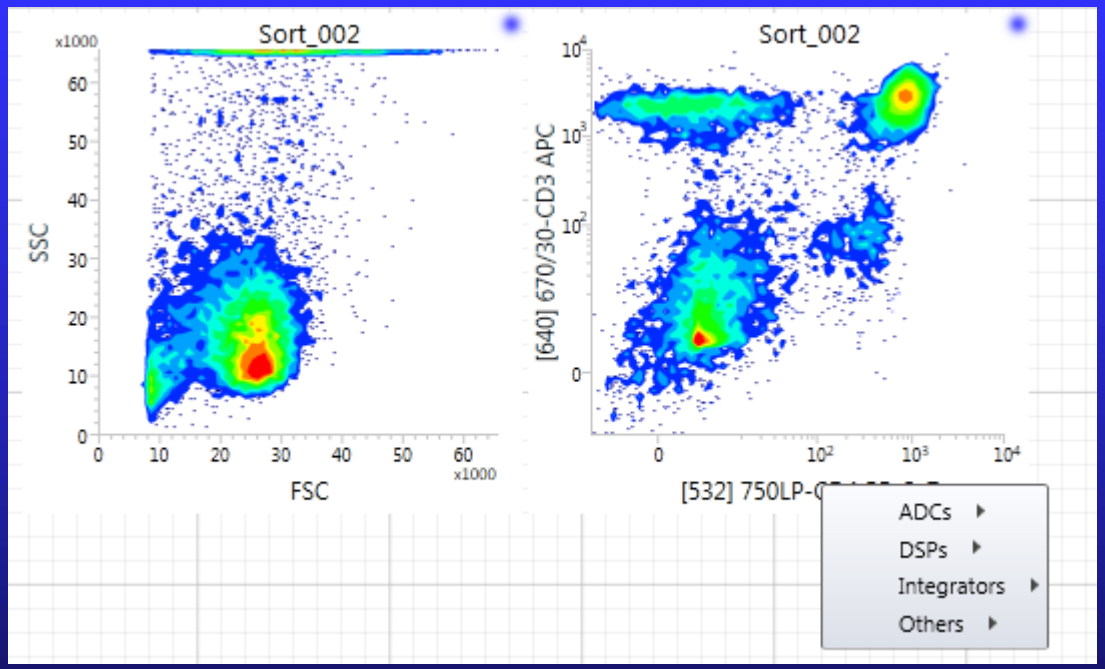
The BD Influx/BD FACSJazz Difference

- The BD Influx and BD FACSJazz cytometers store *all* the event frame information during a sort.
  - Measurement data
    - Scatter data
    - Fluorescence data
    - Time (48-bit timestamp in new firmware)
  - Sort data
    - Complete Sort Setup (drop rate, stream focus, deflection, etc)
    - Classifier information (firmware state for every event)
    - Lookup table (firmware regions of interest [ROI])
    - Drop phase information
  - Index Sorting is built into the system.
  - Sort QC possible: what is happening and why?

BD

What are These New Parameters?

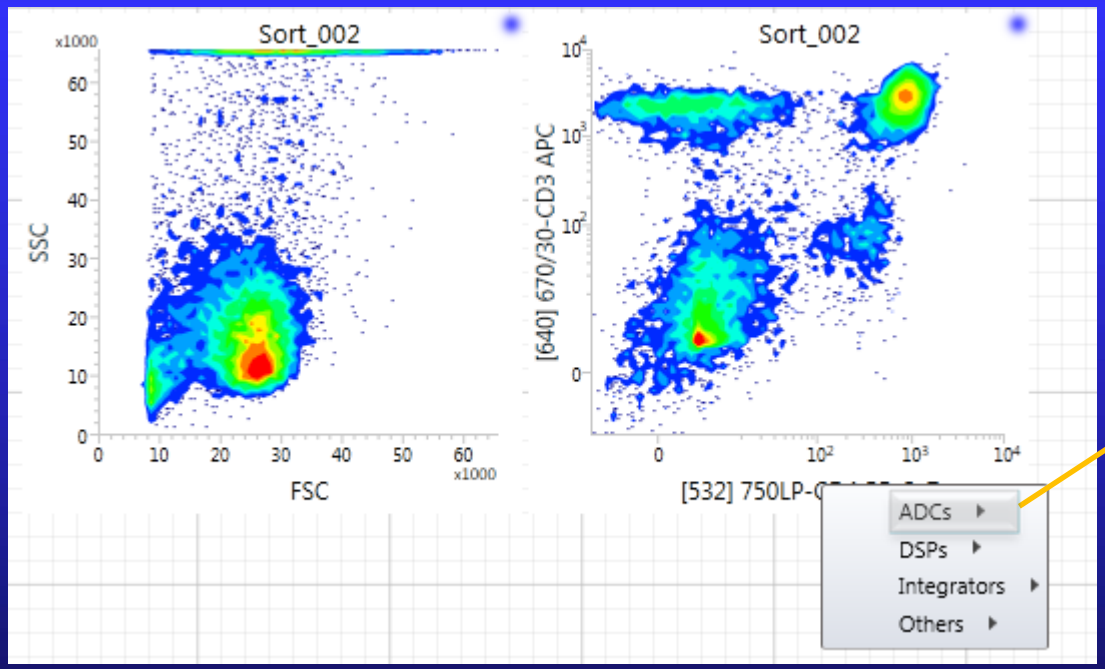
- Many have wondered about the new parameters that show up on the list in Sortware.

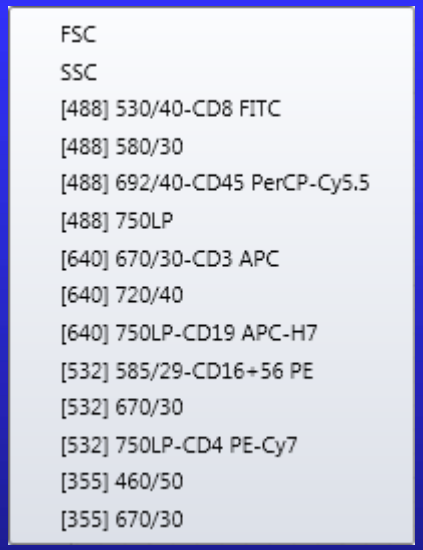


Clicking the plot axis title

What are These New Parameters?

- Many have wondered about the new parameters that show up on the list in Sortware.

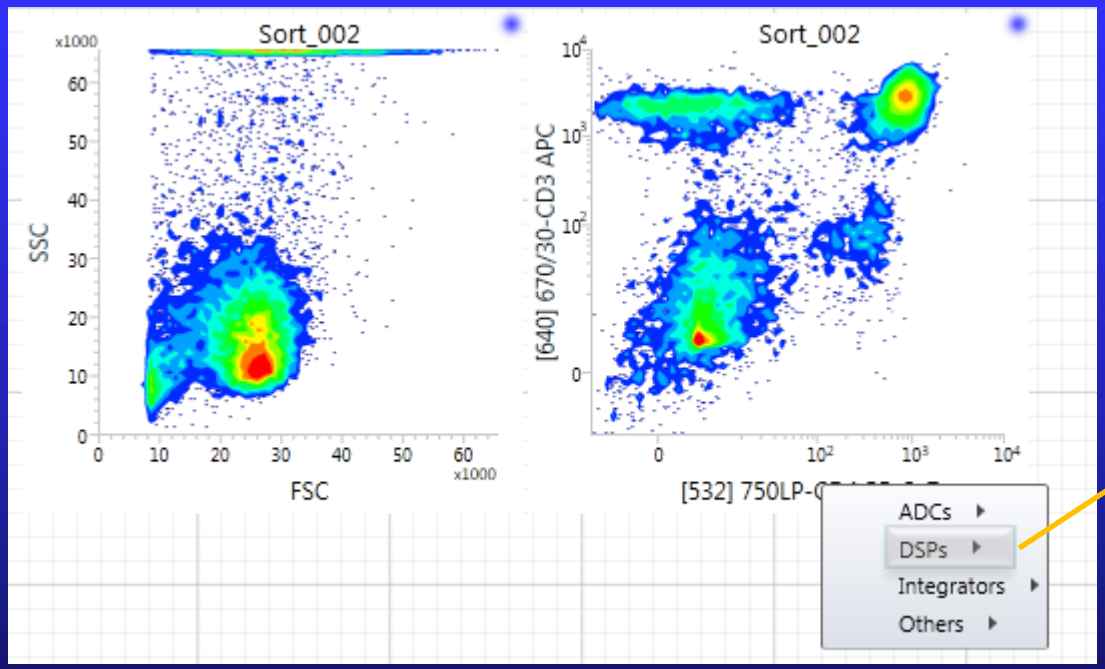




ADCs

What are These New Parameters?

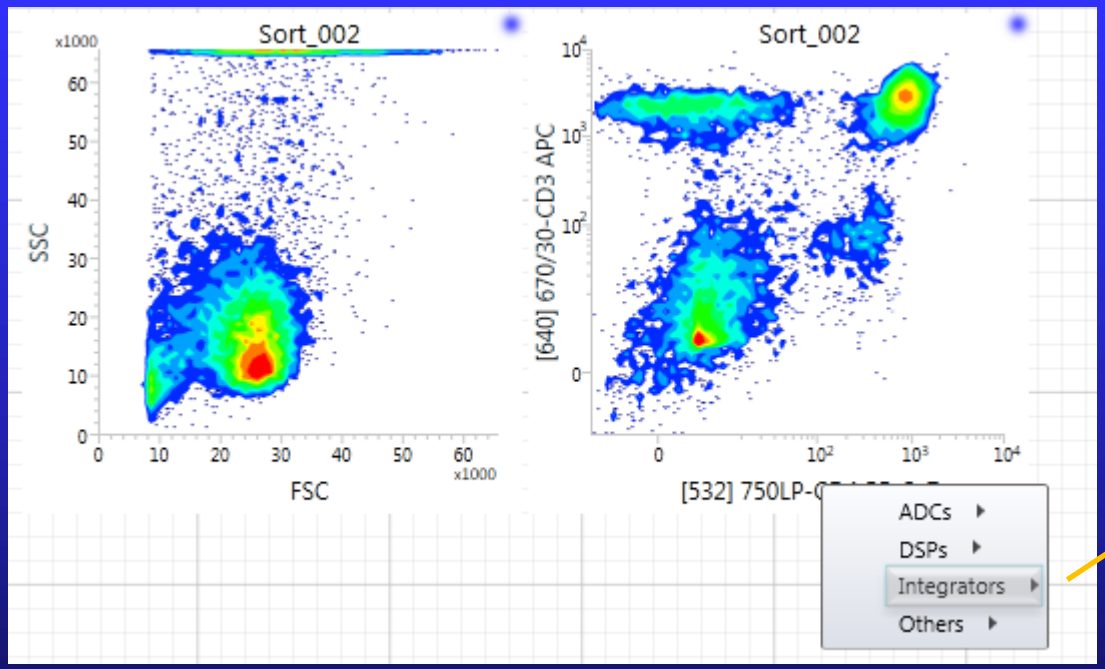
- Many have wondered about the new parameters that show up on the list in Sortware.



DSPs

What are These New Parameters?

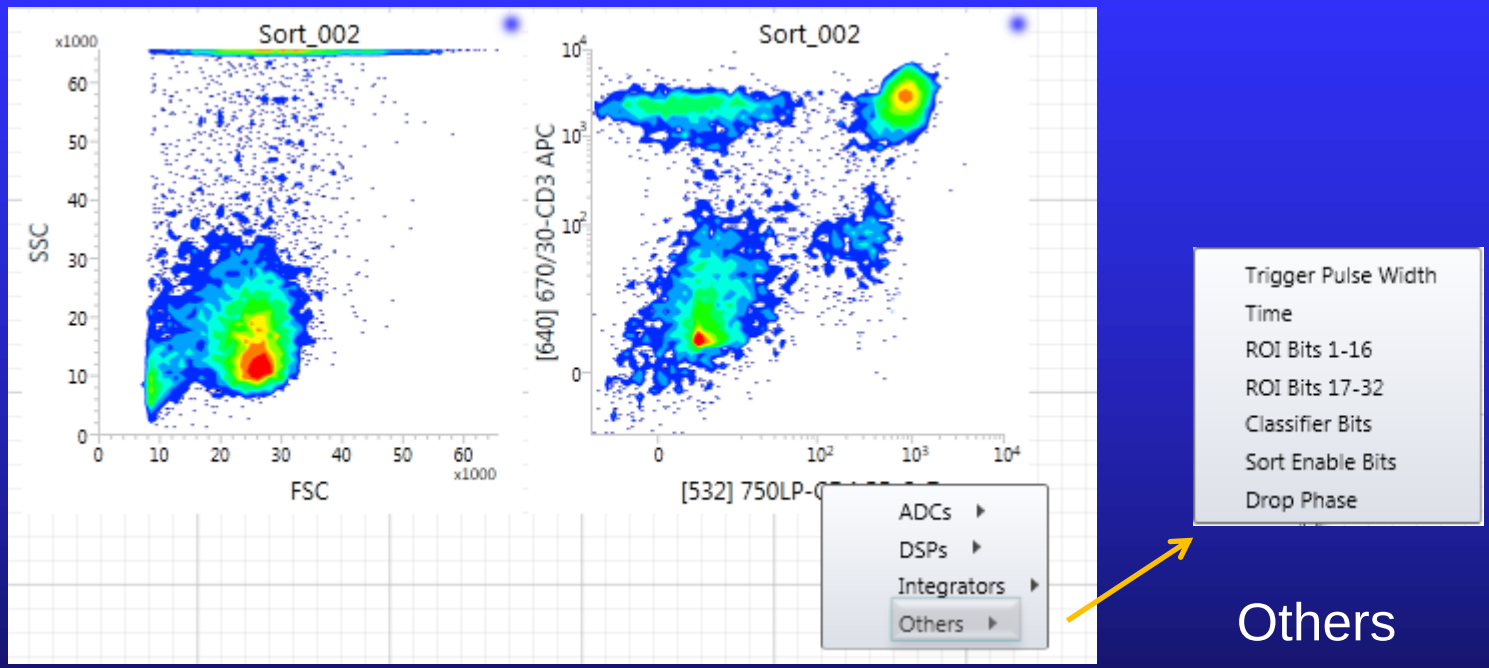
- Many have wondered about the new parameters that show up on the list in Sortware.



Integrators

What are These New Parameters?

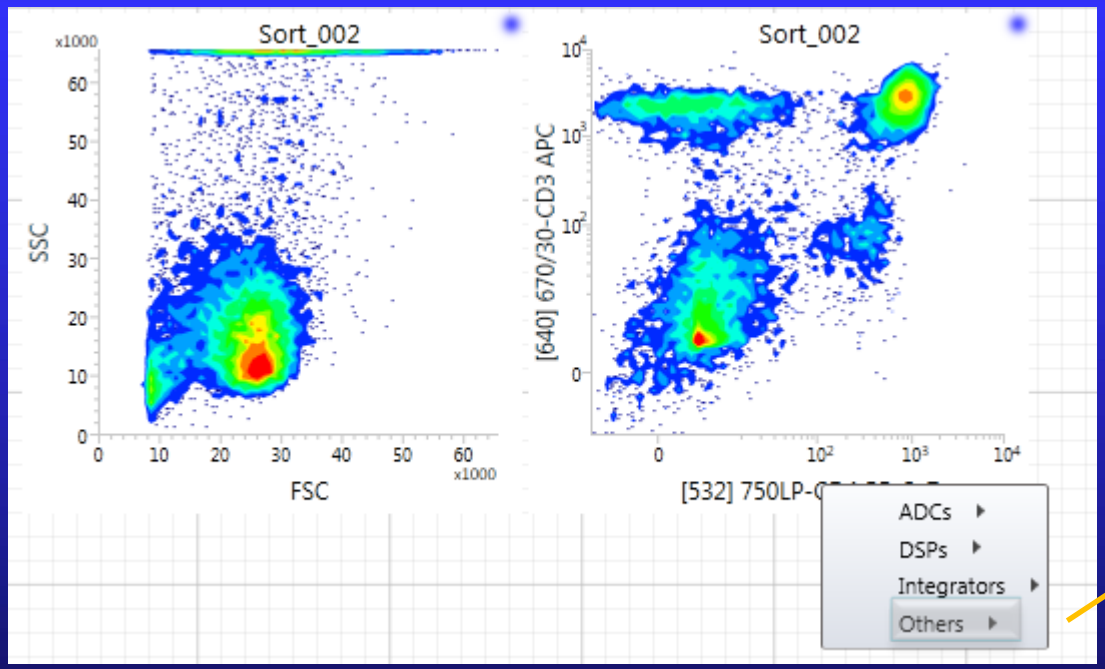
- Many have wondered about the new parameters that show up on the list in Sortware.



Others

What are These New Parameters?

- Many have wondered about the new parameters that show up on the list in Sortware.



Others

The Event Frame

- Key event frame parameters

	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
1	Event Trigger Pulse Width										Event ID					
2	Event Timestamp (Bits 15–0)															
3	Event Timestamp (Bits 31–16)															
4	Event Timestamp (Bits 47–32)															
5	Previous Distance												Drop Phase			

48-Bit Timestamp

- The BD FACSJazz 48-bit timestamp: three words
  - Each clock tick is 17.625 ns.
  - The event stream time rolls over every 57 days.

48-bit time word in firmware

Bits 0–15	Bits 16–31	Bits 32–47

Bits	Word	Channels	Time tick	Span days	Span hours	Span min	Span s	Span ms
0–15	time1	65,536	17.625 ns	1.34E-08	3.21E-07	1.92512E-05	0.001155	1.155072
16–31	time2	65,536	1.155072 ms	0.000876	0.021027	1.261646643	75.6988	75,698.79859
32–47	time3	65,536	1.261646 min	57.41894	1,378.055	8,2683.27	4,960,996	4,960,996,464.5

The Event Frame

- Key event frame parameters

	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
1	Event Trigger Pulse Width										Event ID					
2	Event Timestamp (Bits 15–0)															
3	Event Timestamp (Bits 31–16)															
4	Event Timestamp (Bits 47–32)															
5	Previous Distance												Drop Phase			

A drop phase word is actually drop phase *and* distance.

Drop Phase Word

- Firmware drop phase (7.5.1 – BD FACSJazz)
  - Contains the drop phase enveloping the center of the event (4 bits, 16 possible drop slices)
  - Contains the distance to the previous event in drop slices (12 bits, 4,096 slices, or 256 drops)

Bits 15–4	Bits 3–0

Bits	Component	Range
0–3	Drop Phase	0–15
4–15	Distance to Previous	0–4,096 (256 drops)

The Event Frame

- Key event frame parameters

	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
1	Event Trigger Pulse Width										Event ID					
2	Event Timestamp (Bits 15–0)															
3	Event Timestamp (Bits 31–16)															
4	Event Timestamp (Bits 47–32)															
5	Previous Distance												Drop Phase			
6	ROI Bits 15–0															
7	ROI Bits 31–16															
8	Classifier Word															

Region Tables: up to 32 Regions

- Example: an event is in sort regions 1, 3, and 4 and was sorted left.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
ROI 1–16	**1**	0	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	0
ROI 17–32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Classifier	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

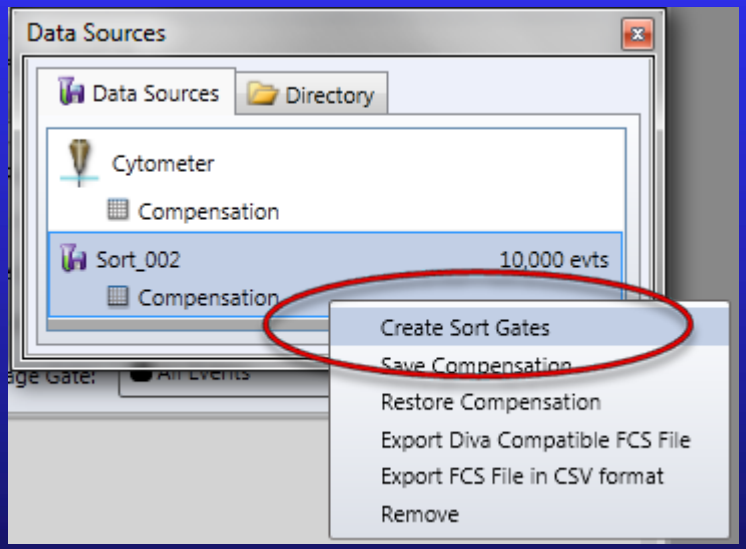
- Example: an event is in sort regions 1, 2, 3, and 6 and was sorted right.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
ROI 1–16	**1**	**1**	**1**	0	0	**1**	0	0	0	0	0	0	0	0	0	0
ROI 17–32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Classifier	0	**1**	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0

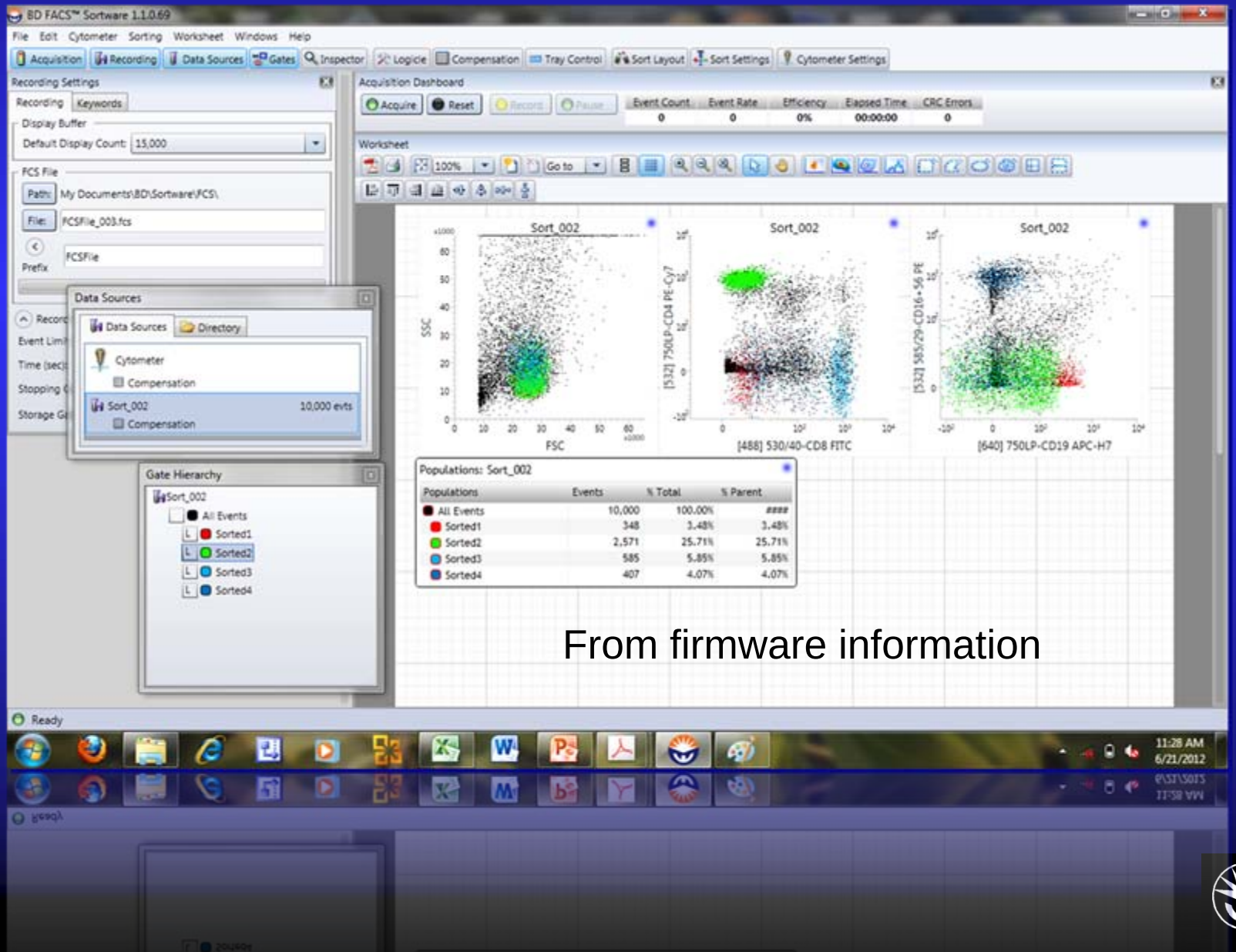
BD

Function in Sortware

- See exactly how each event was classified in the sorting firmware by examining bit states
- Reconstruct sort gates from the firmware event stream

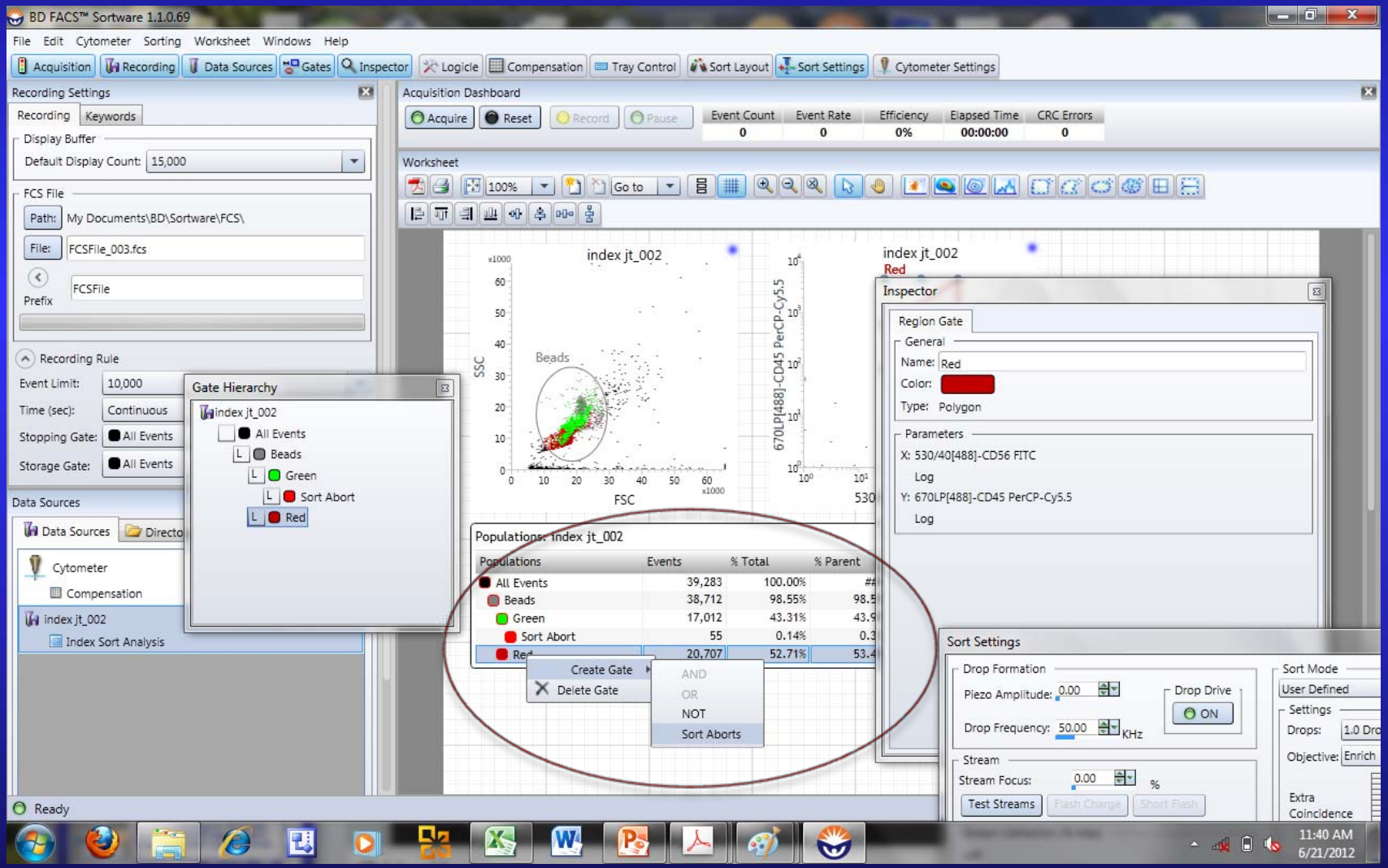


Reconstructed Four-Way Sort



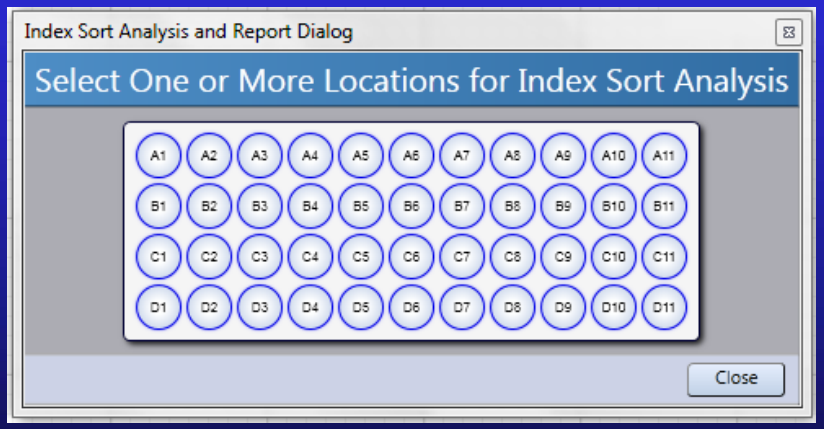
From firmware information

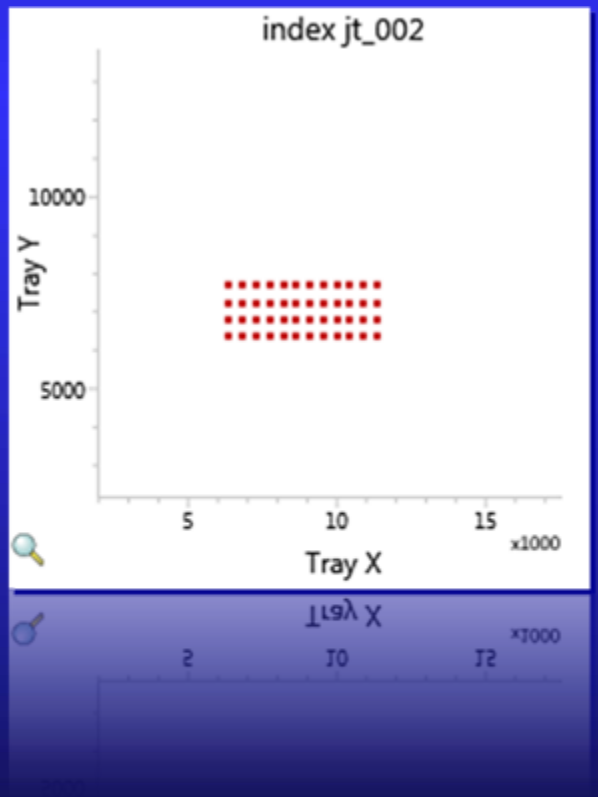
Assess which Events were Aborted



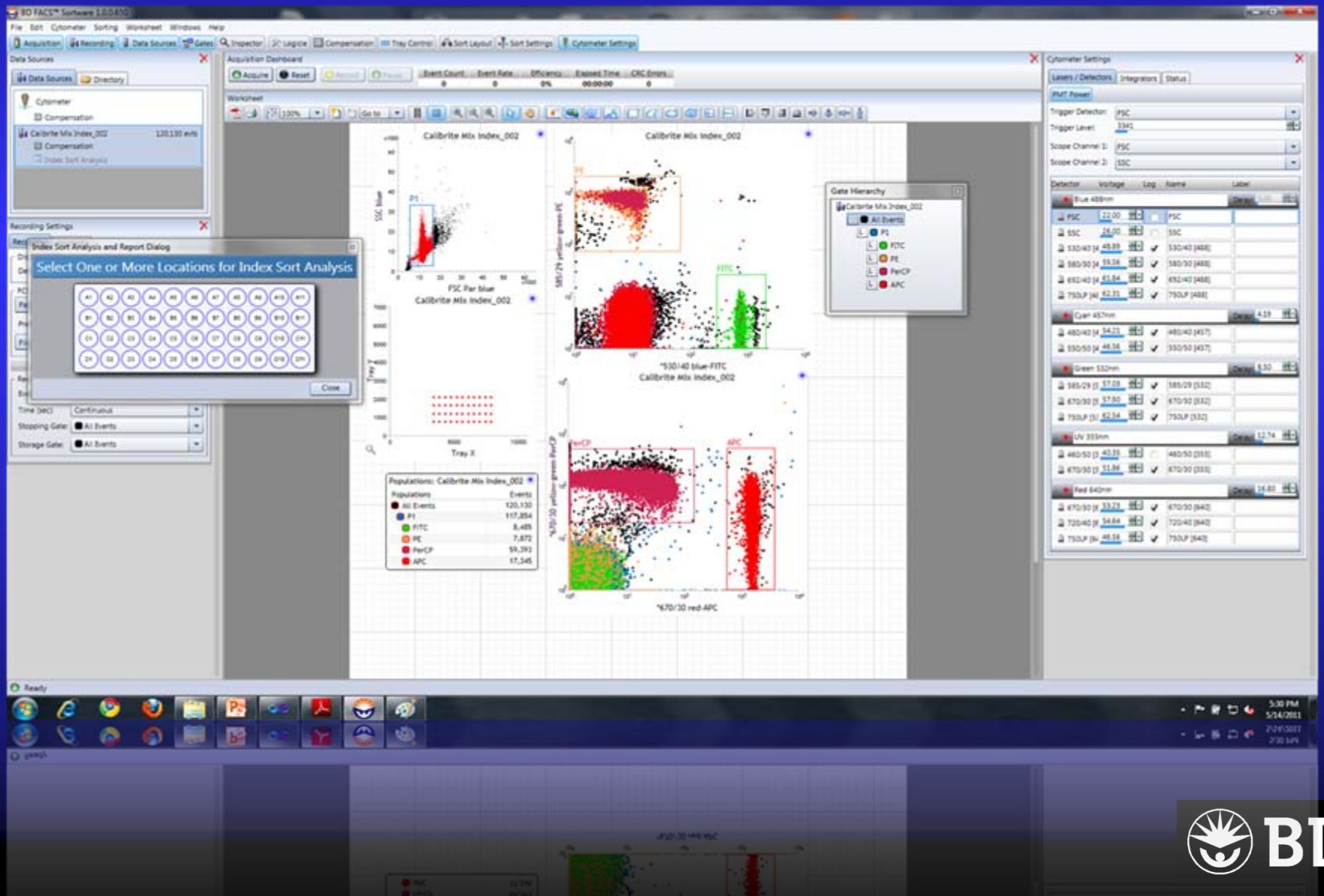
Index Sort Function

- Event Stream contains all the data.
  - Sorted events
  - Aborted events
- Index Sort Mode has:
  - Tray X position (mm x 100)
  - Tray Y position (mm x 100)

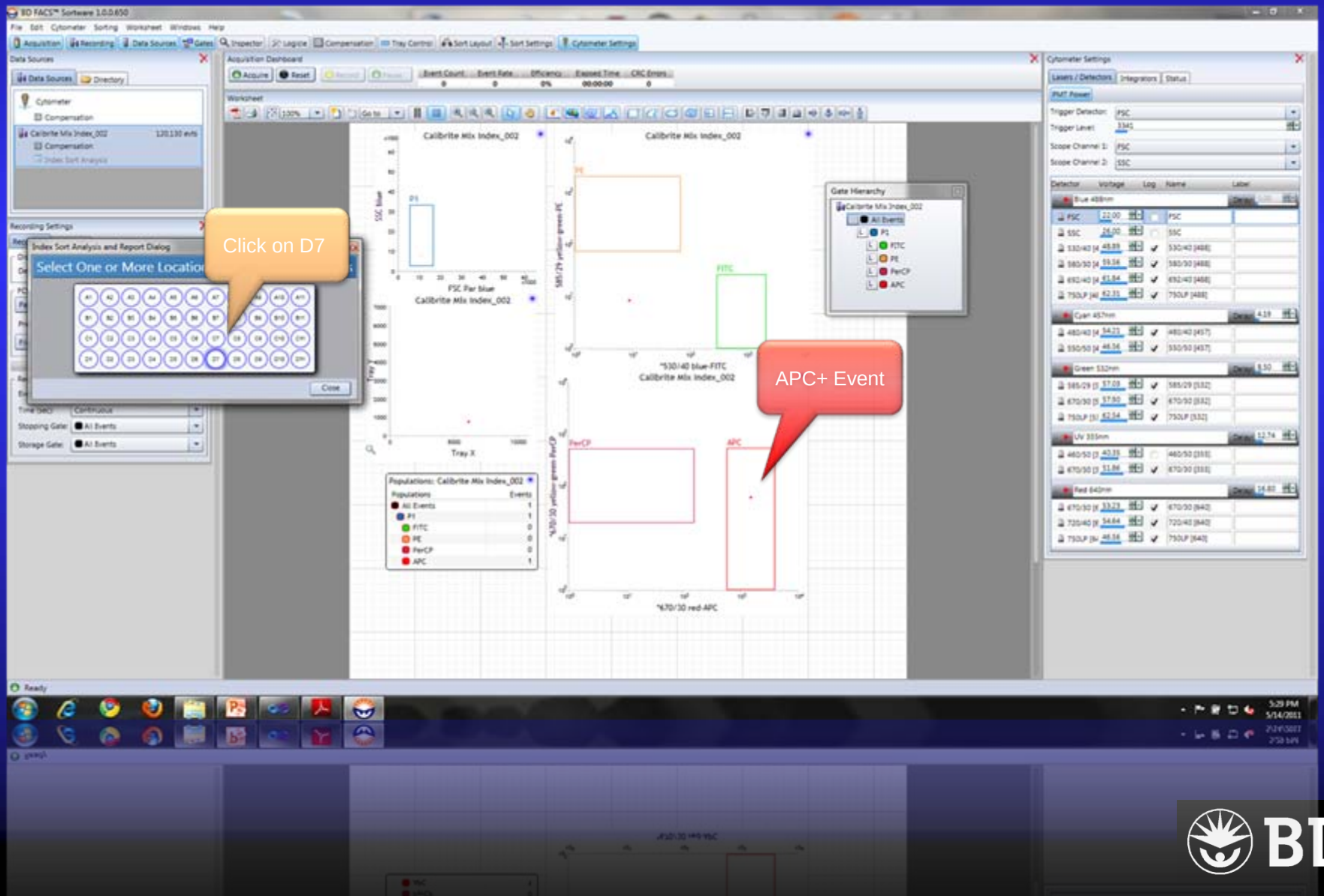




Index Sort Example: Sortware



Index Sort Example



A Look at Some New Approaches

- Created some prototype software to examine Sortware FCS files taken during sorts and assess sample performance.
- The main goals have been to identify the most important features to add moving forward.
- Most of the work has with been done with in-house investigators and Geoff Osborne at the Queensland Brain Institute.
- A need to understand sample behavior…

BD

A Frequent Challenge

- Sorting efficiency is Poisson in an ideal scenario.
  - Efficiency = $e^{-(\text{rate} \times (1 - \text{fraction}) \times d / f)}$
  - d = drop packet
  - f = frequency
- Many samples are not Poisson.
- Cells “entrain,” often in groups or clumps.
- It is common for investigators to “lose” a lot more cells than expected or desired.

BD

Measurements of Dispersion

- The Index of Dispersion: one way to measure how “Poisson” a sample may actually be
  - Variance to Mean Ratio, 1.0 = Poisson

$$D = \frac{\sigma^2}{\mu}$$

  - Fano Factor: a windowed Index of Dispersion

$$F = \frac{{\sigma_W}^2}{\mu_W}$$

The window could be a drop or any other time span.

Using the Event Frame Data

- We can estimate the probability of an event occurring within a defined time widow.
- The Drop Phase and distance information lets us measure spatially and ask questions such as:
  - Is the distribution Poisson?
  - How close were the nearest neighbors when a classified cell is within the “sortable” drop?
  - What is the frequency of neighbors compared to an ideal Poisson sample?

BD

A Simple Metric for Entrainment

- Similar concept to the standard dispersion metrics
  - We can measure the observed frequency of certain events within a given time span W, such as ½- or 1-drop bins.
  - We can calculate the expected frequency for those events based on an ideal Poisson distribution.
  - We can get a sense of dispersion, or cell entrainment, by looking at observed and expected.

$$Entrainment factor = \frac{Observed\ Frequency_W}{Expected\ Poisson\ Frequency_W}$$

  - Poisson samples: Factor = 1.0
  - Clumpy and entrained samples: Factor >1.0

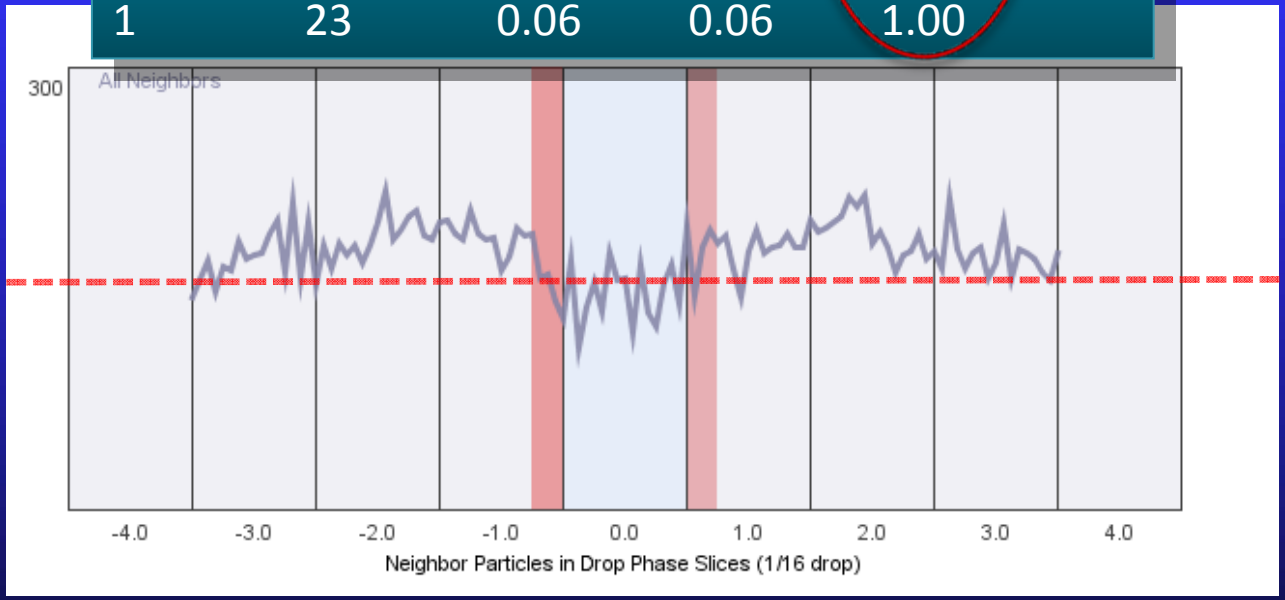
BD

Nearest Neighbor Locations

- Example of a “well behaved” sort
  - Here ... pped based on dista... t particle with...
  - A ra...

Sample Entrainment Factors:

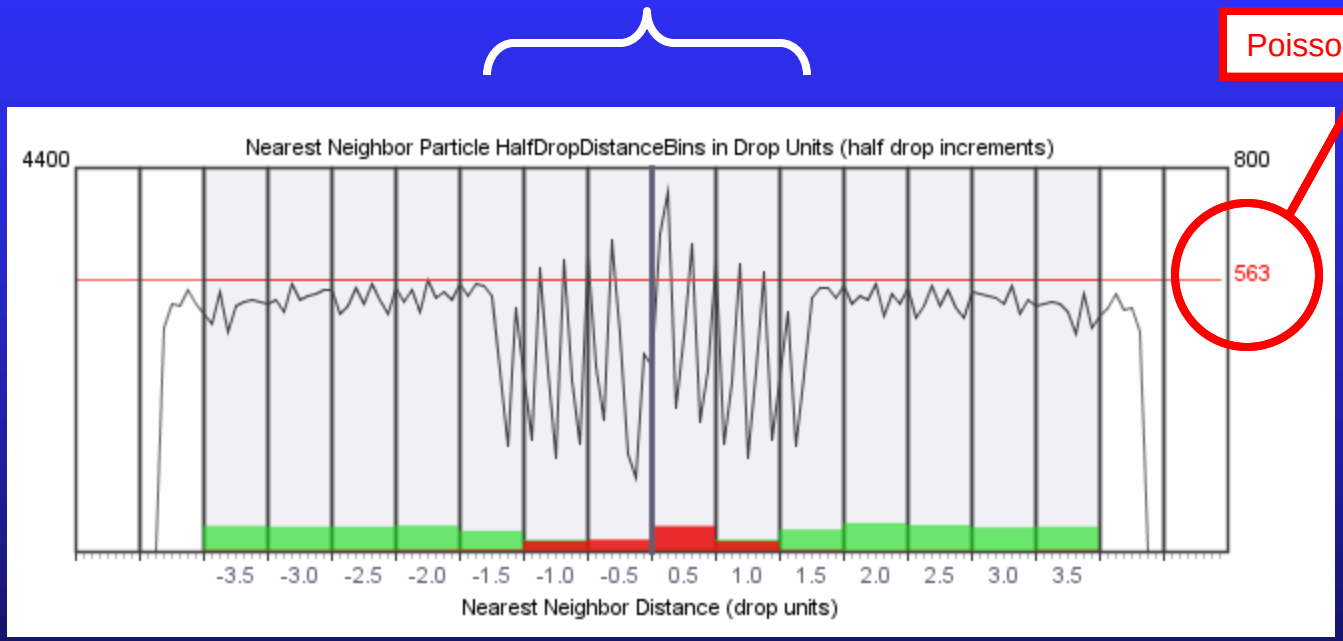
Drops	Events	Ob(%)	Ex (%)	EF
4	459	1.23	1.01	1.22
3	267	0.72	0.58	1.24
2	120	0.32	0.26	1.25
1	23	0.06	0.06	1.00



Comparing Observed to Expected

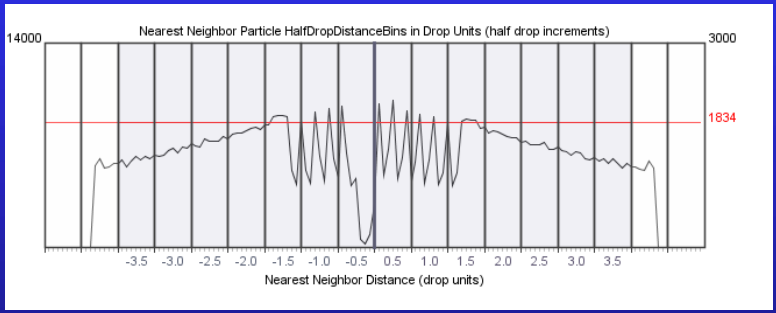
- The firmware event data can be used to look at particle spacing during a sort within 1/16 of a drop, and binned at 0.5 drops.
- Where are the nearest neighbors?

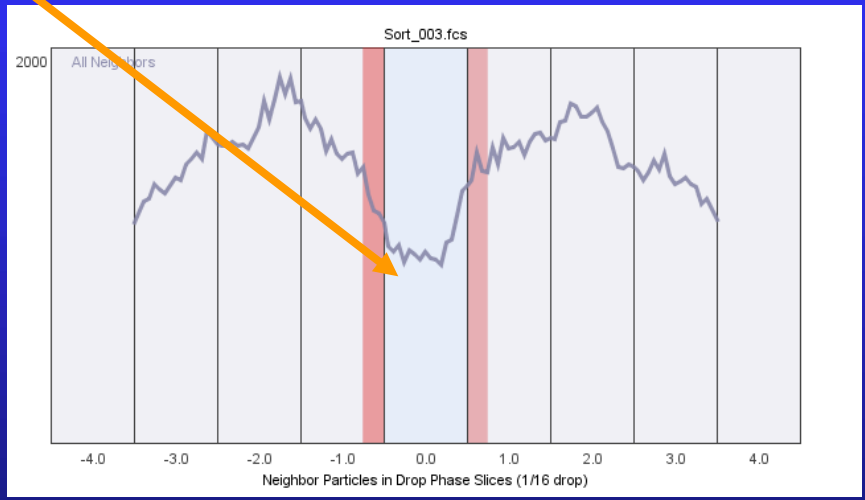
"Saw tooth" effect from electronic aborts (5-µs cycle time)



Fixed PBMC Sort: Why so Efficient?

- Freshly fixed PBMCs 10,000/s
- Event distances are very close to expected
- Distances mapped to phase show that neighbors tend to **not** be in the current drop





Entrainment Factor near 1.0

Previously Frozen PBMC Sort

Analyzed: 28,477 to: 30,607 Timestep: 0.1 elapsed time:213.0 (s) Rate: 4,470 (s)

Analyzed 952,045 contiguous timestamped events, 895,993 events with matched pre/post drop slice halfDropDist.

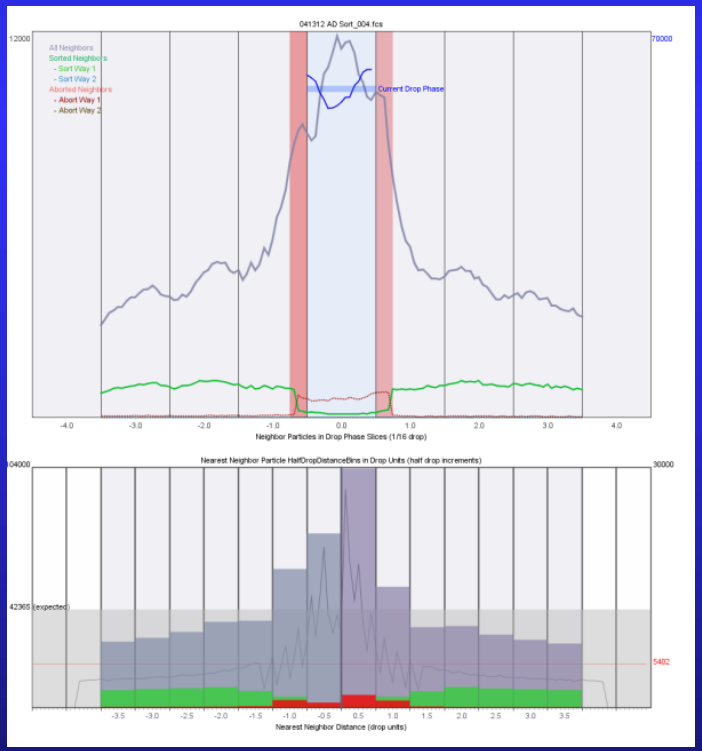
Detected 56,052 probable electronic aborts (efficiency = 94.44%), expected 97.79% based on homogeneous Poisson estimate.

Average is 1 event every 10.98 drops.

Sample Entrainment Factors:

Drops	Events	Freq (%)	Expect (%)	EF
4	163,866	17.21	9.08	1.90
3	134,824	14.16	5.51	2.57
2	108,333	11.38	2.61	4.35
1	75,717	7.95	0.67	11.88

1st 0.5 drop after/Expected 0.5 drop after: 2.44

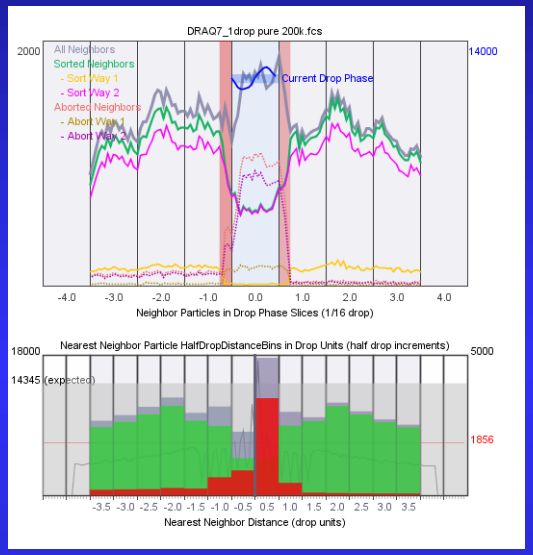


Electronic aborts are high.

Modest rate: one cell every 11 drops.

Entrainment Factor is ~ 12.

Another: A (Good) and B (Problematic)



A 8-Drop Span Distance Analysis (neighbors, 4 drops each direction)

Event	Events	Freq (%)	Expected (%)
Preceding	81,507	43.08	46.26
Following	87,084	46.03	46.26
Both	38,013	20.09	21.40

Sample Entrainment Factor = 0.94 1st half drop/Expected 1.23

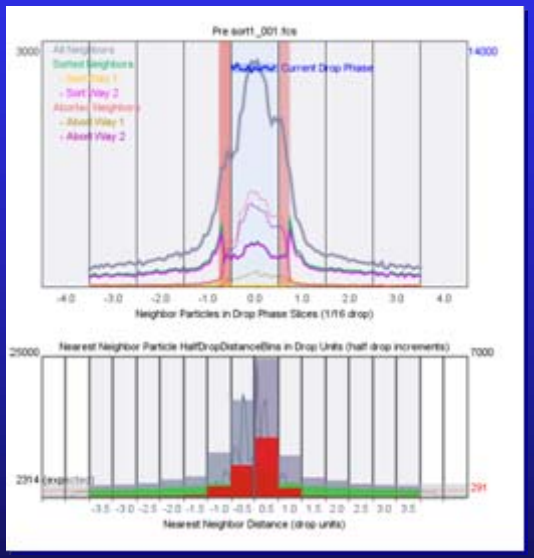
1 event every 6.34 drops.

B 8-Drop Span Distance Analysis (neighbors, 4 drops each direction)

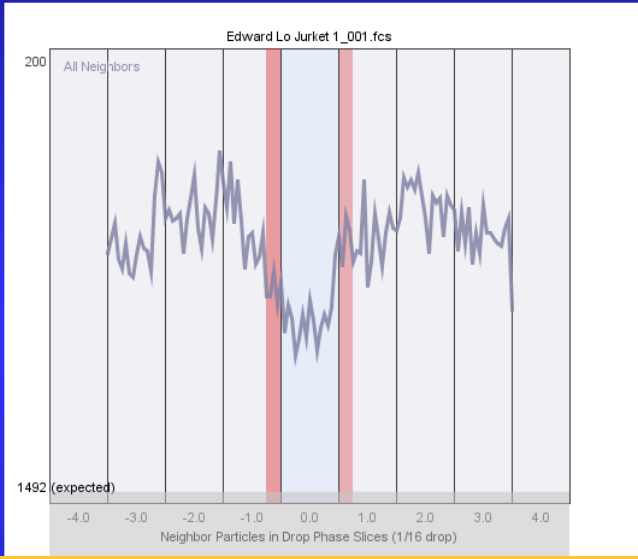
Event	Events	Freq (%)	Expected (%)
Preceding	40,221	20.21	8.80
Following	46,352	23.29	8.80
Both	11,570	5.81	0.77

Sample Entrainment Factor = 7.51 1st half drop/Expected 10.64

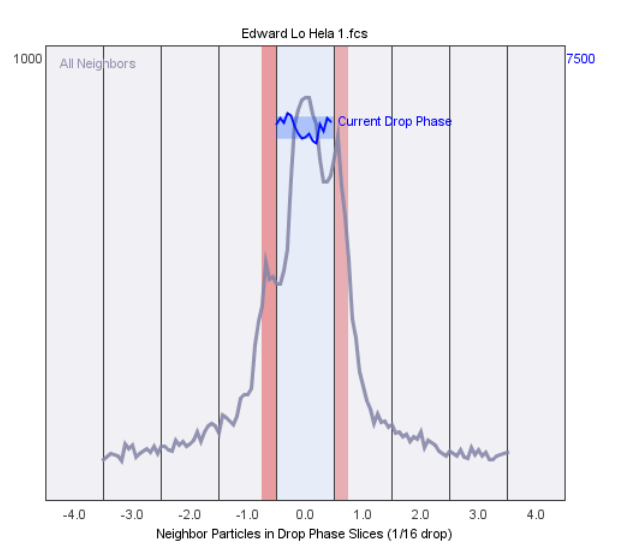
1 event every 42.76 drops.



Jurkat and HeLa: Another Lab



Jurkat cells at 3.96 x 10^6 cells/M



HeLa cells at 9.26 x 10^5 cells/M

- Collected 100,000 events at ~1000 events/s
- Frequency at 58.7 KHz
- Drops: 1.0
- Extra Coincidence: 4
- Jurkat Cells 3 Cell Density Levels:
  - 3.96 x 10^6 cells/M
  - 1.98 x 10^6 cells/M
  - 9.90 x 10^5 cells/M
- HeLa Cells 3 Cell Density Levels:
  - 9.26 x 10^5 cells/M
  - 4.63 x 10^5 cells/M
  - 2.31 x 10^5 cells/M

Cell	Density	1 Drop	2 Drop	3 Drop	4 Drop
Jurkat	3.96 x 10^6 cells/M	0.46	0.45	0.45	0.45
Jurkat	1.98 x 10^6 cells/M	0.64	0.58	0.52	0.51
Jurkat	9.90 x 10^5 cells/M	0.25	0.25	0.31	0.34
HeLa	9.26 x 10^5 cells/M	27.34	10.07	5.36	3.52
HeLa	4.63 x 10^5 cells/M	3.86	1.86	1.29	0.99
HeLa	2.31 x 10^5 cells/M	8.57	3.34	1.98	1.45

BD

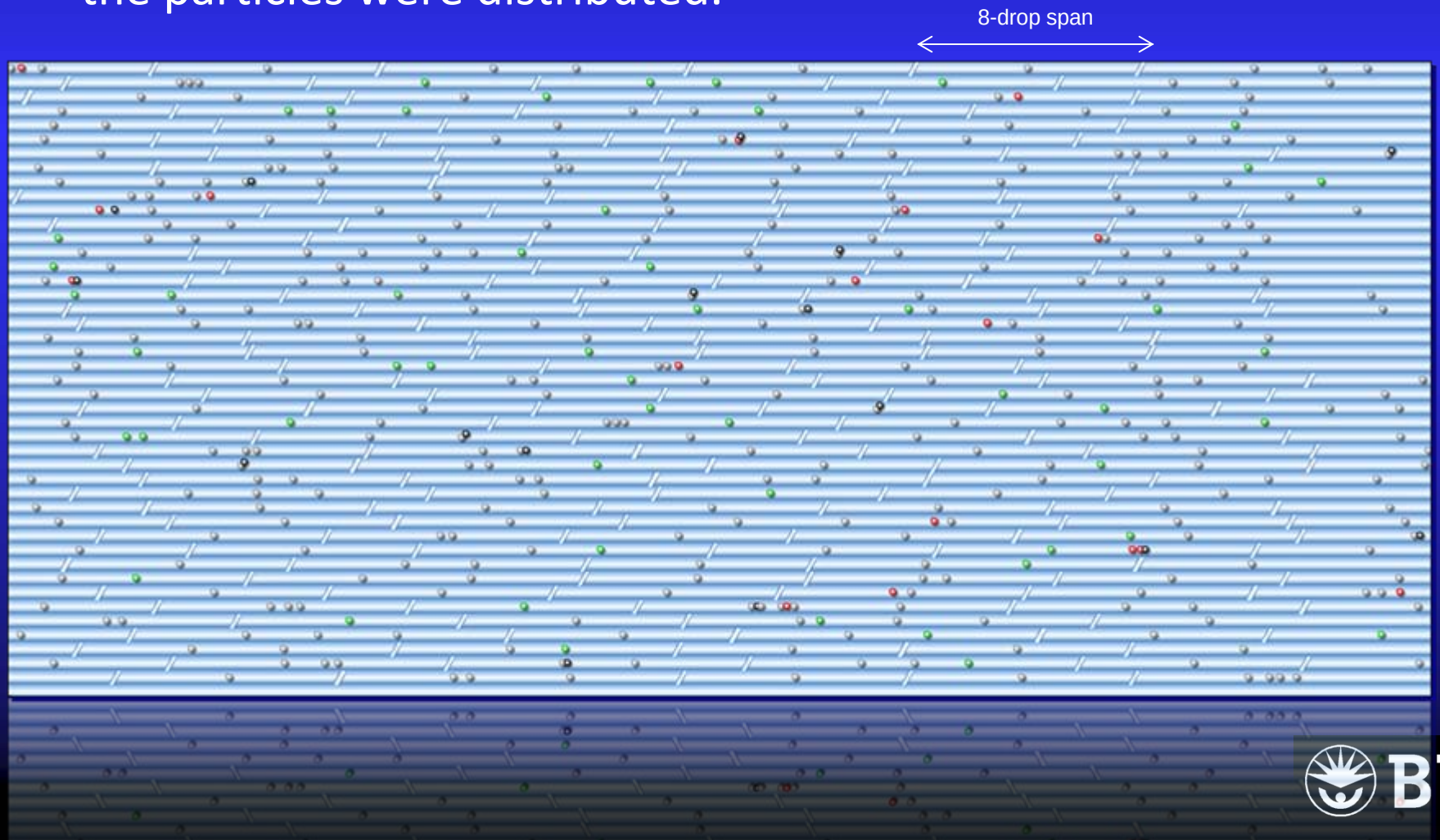
More Work to Do…

- The actual process by which cells leave a cannula and are hydrodynamically focused is complex.
- Many relevant stochastic models to consider before we are done
  - Easier to detect departures from a homogeneous Poisson process than a nonhomogeneous one
    - When is homogeneous Poisson appropriate?
    - When is it a nonhomogeneous Poisson? (spike train)
  - Cox process (doubly stochastic)?
    - Groups within groups, etc…

BD

A Recreation of the Sort Stream

- We can examine a reconstruction of the stream and look at how the particles were distributed.



Acknowledgments

- Ger van den Engh
- Valdis Riekstins
- Barclay Purcell
- Gayathri Parthasarathy
- Chakadhar Dunna
- Geoffrey Osborne*
- Hank Pletcher**
- Brian Warner
- Liping Yu
- Janelle Crane

*Queensland Brain Institute, University of Queensland

**Perelman School of Medicine, University of Pennsylvania