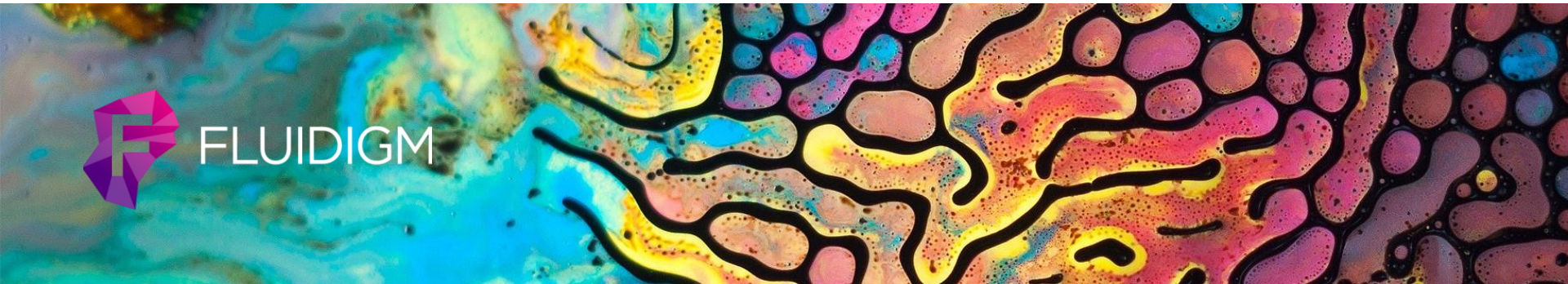
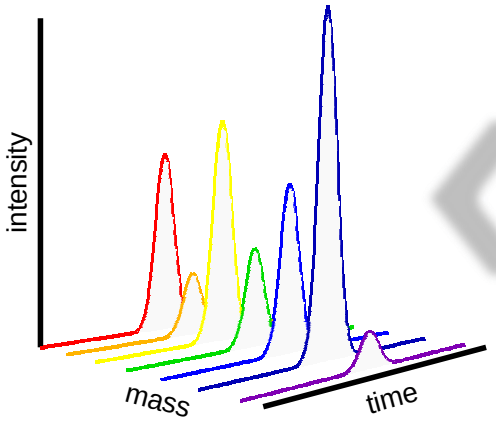
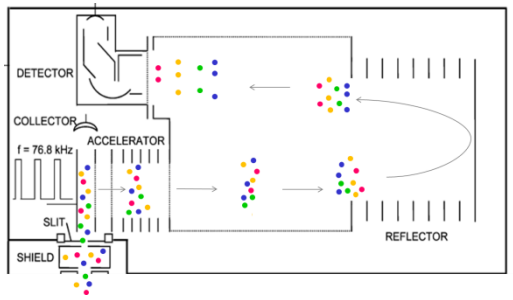
Data Processing in Helios



How Helios Generates Data from Ion Clouds

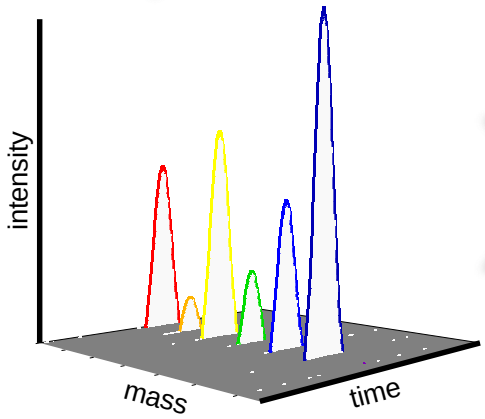


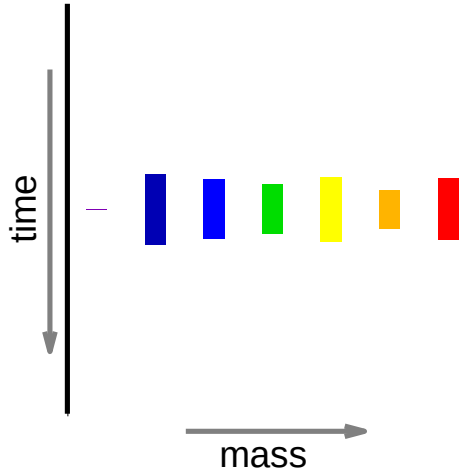
ToF Chamber

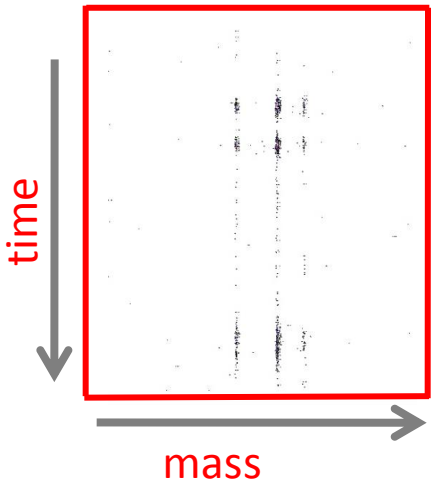


Raw Data
(pre-FCS file)

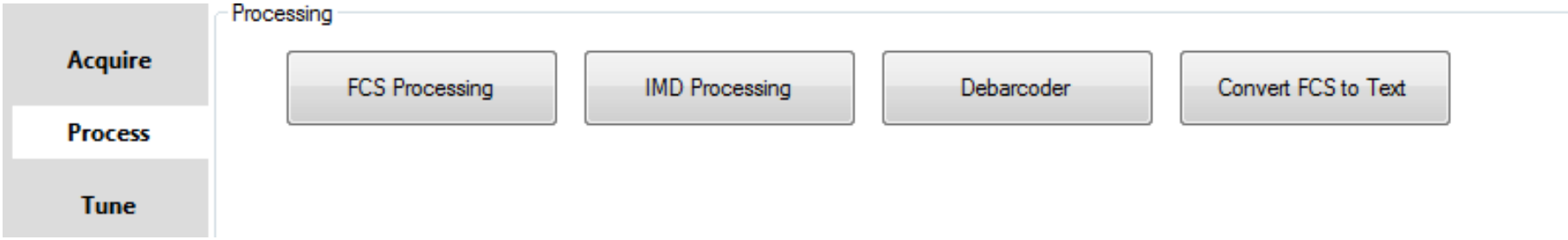
Noise Filtering: Lower convolution threshold





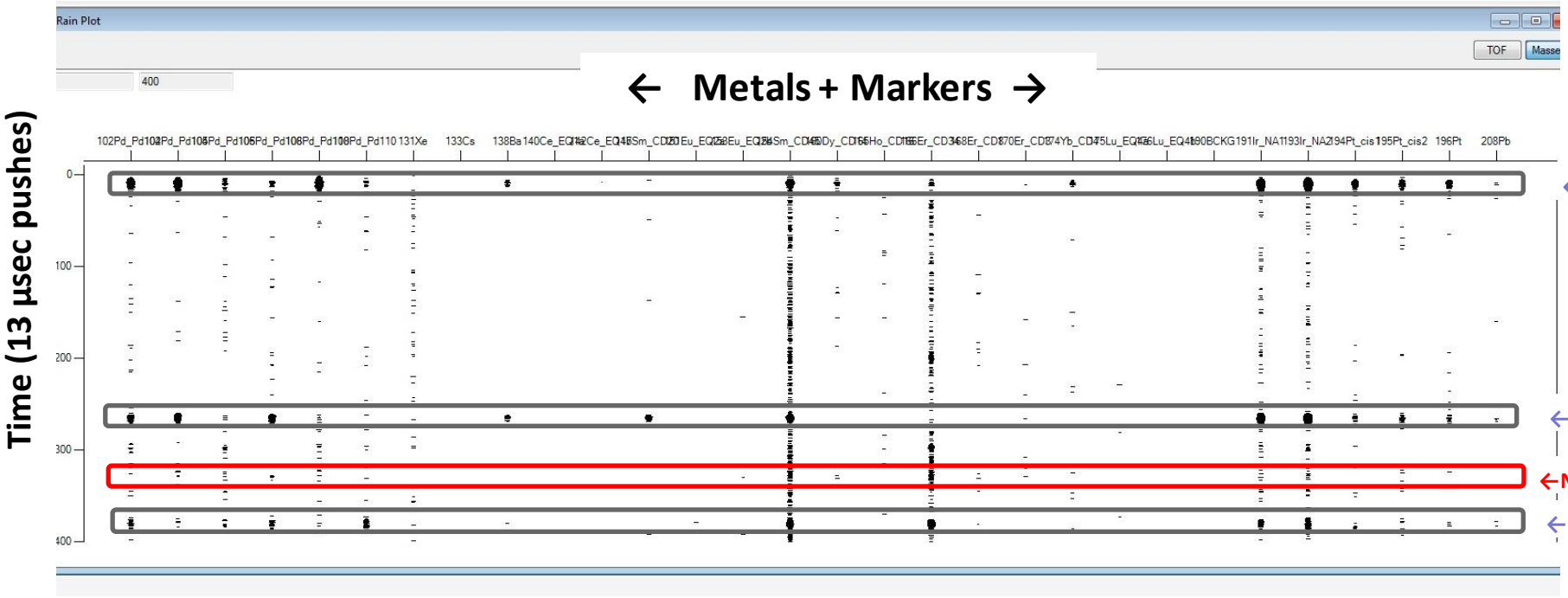


Helios Software Data Processing



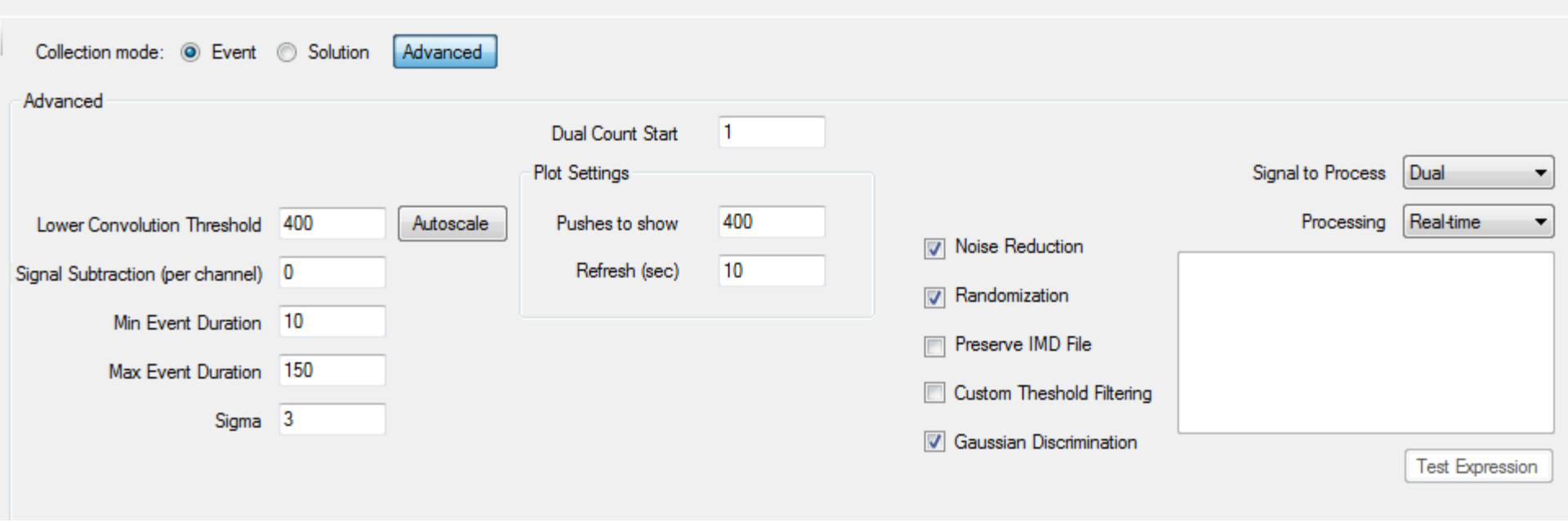
Raw Data: Pre FCS Files

- IMD files
  - Integrated Mass Data = Per Push Data Across all selected channels
  - Includes data from cells and non-cells



IMD to TXT
(behind the scenes)

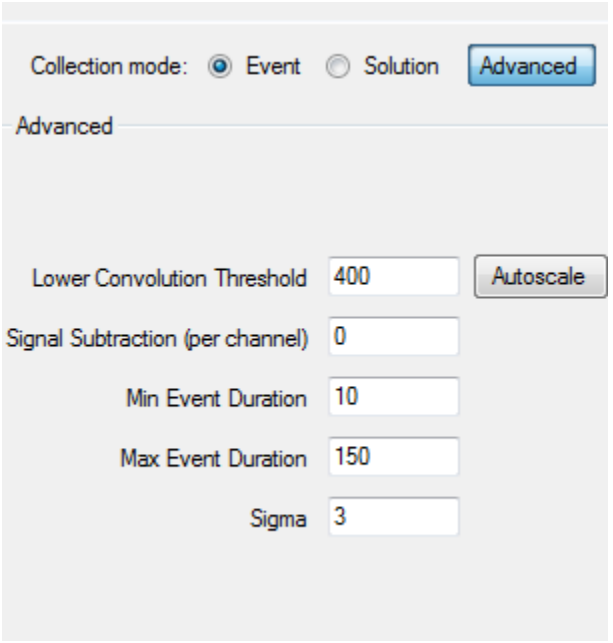
- Only “events” will be recorded into a temporary TXT files which will be embedded in FCS files.
- The parameters necessary to define “events” are in Experiment Manager> Template> Advanced



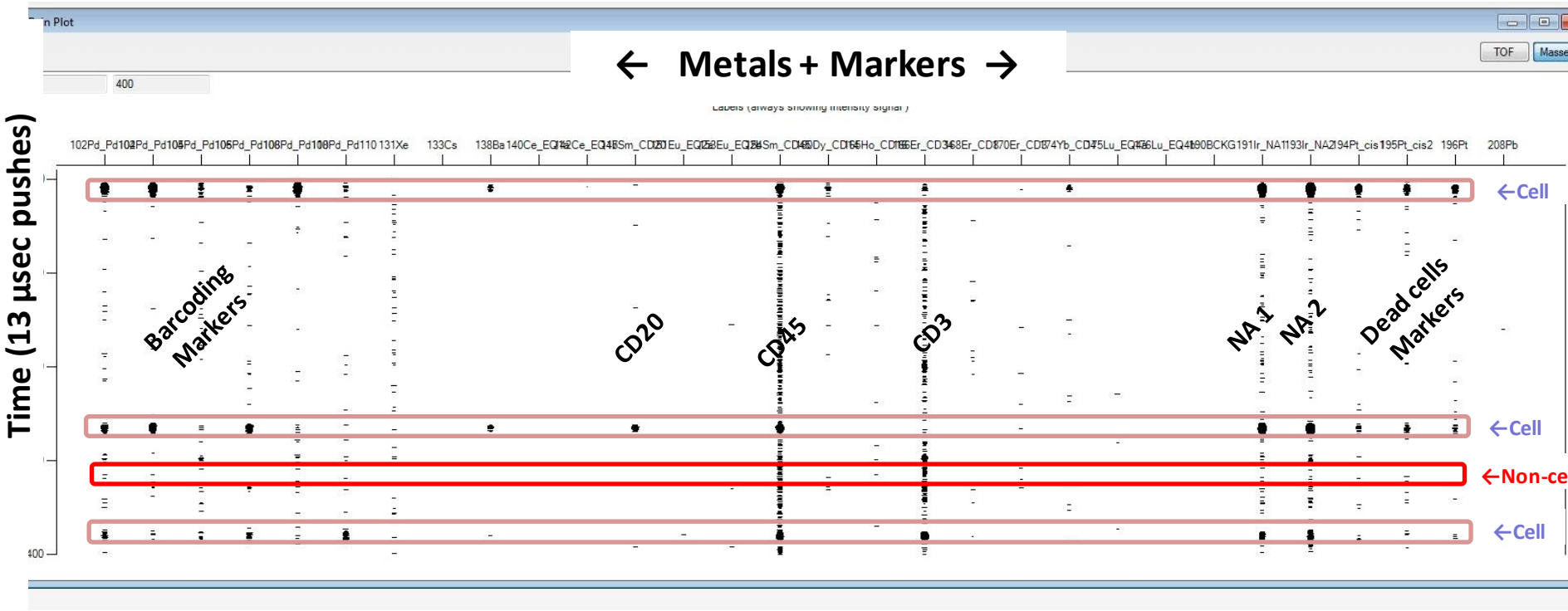
Event Definition

What is an event?

When the total ion current (from all selected channels) exceeds the lower convolution threshold continuously for at least 10 pushes (minimum event duration) but no more than 150 pushes (maximum event duration), it will be registered as an event.



Events


← Metals + Markers →
Time (13 µsec pushes)
Barcoding Markers
CD20
CD45
CD3
NA 1
NA 2
Dead cells Markers
←Cell
←Cell
←Non-ce
←Cell

Events - continued

The software tries to fit each event that comes through into a Gaussian graph (3 sigma)

If the signals are continuous for over 150 pushes software will raise the threshold to try to resolve the Gaussian signal within 150 pushes.

.TXT Files

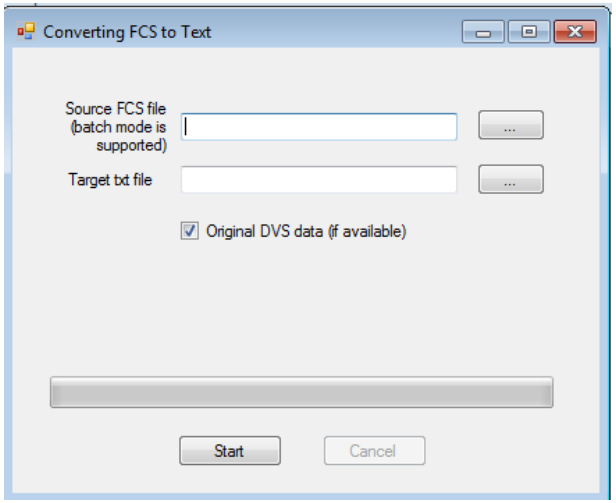
.TXT files contain non-randomized dual count data for each successfully resolved event in each channel selected.

Dual counts are recorded as whole numbers (integers)

In software versions 6.0 and above, the creation of a .txt file is behind the scenes and the final FCS file contains a segment of raw txt data that can be converted to a .txt file

.FCS Files

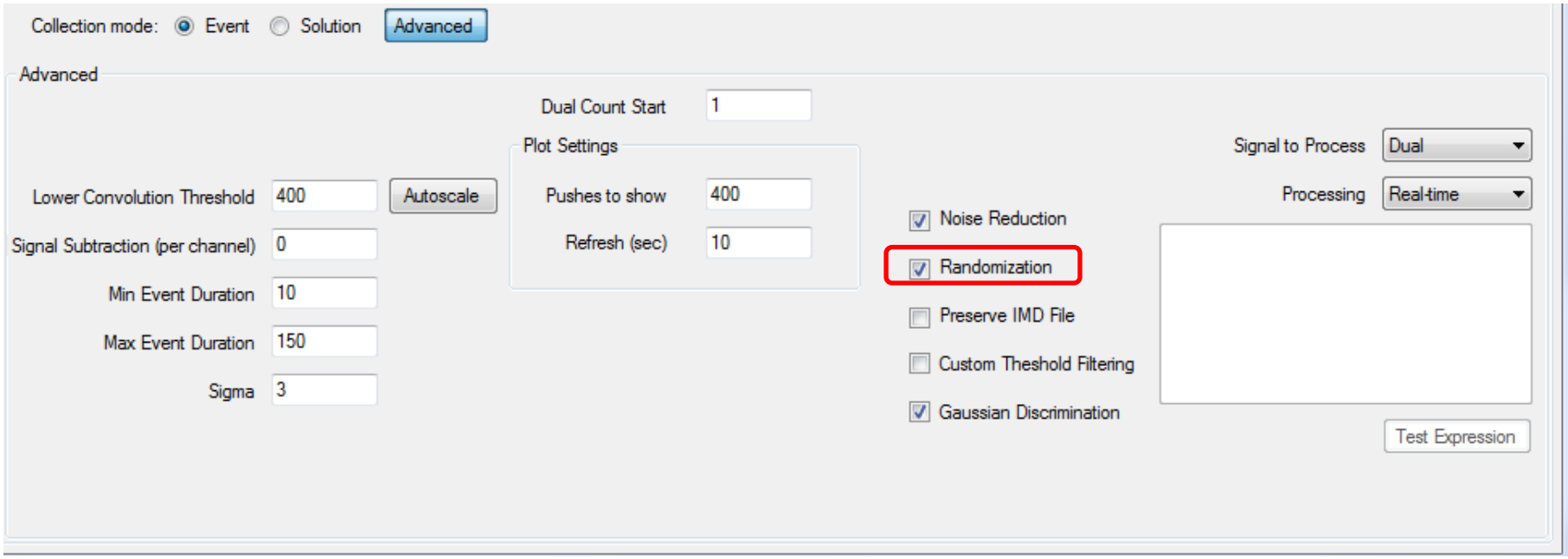
- FCS 3.0 files that are readable by many FCS readers:
  - FlowJo and Cytobank are amongst the most popular tools for cytometrists in single cell studies
- Software (6.0 versions and above) generates a two-data-segment FCS file:
  - Randomized and presented: When the FCS reader opens the .FCS file, the randomized segment of data is displayed by default
  - Non-randomized and hidden: The nonrandomized data segment can be obtained by converting the FCS file into a .TXT file.



Randomization of FCS files

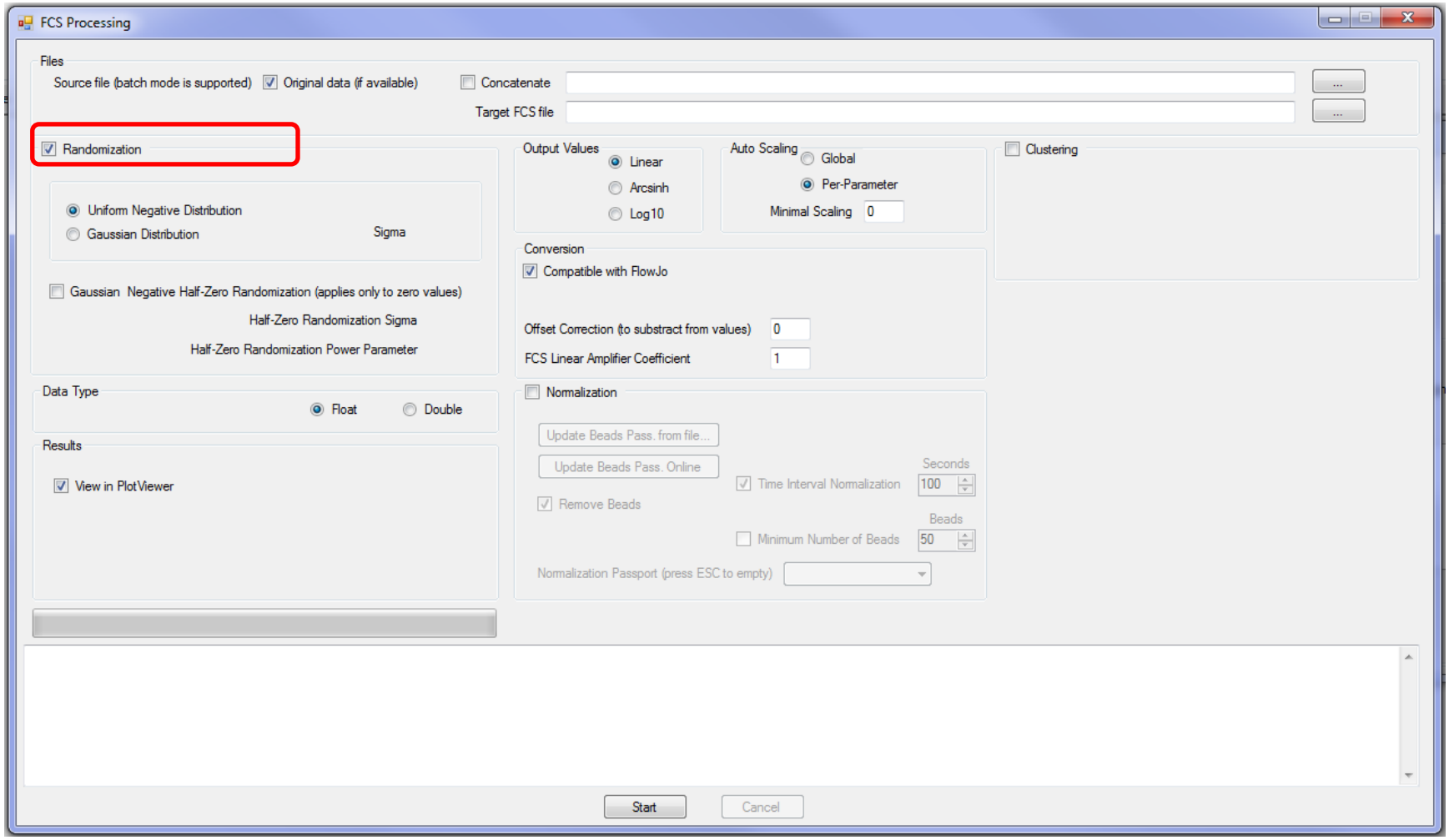
Why is data randomized?

- Helios (and other CyTOF instruments) records “counts” as whole numbers. Display of whole numbers near zero causes a binning effect.
- Whole numbers are therefore randomized to be fractions for better data display in log or bioexponential scales.
- Randomization can be turned on and off in Helios software for templates

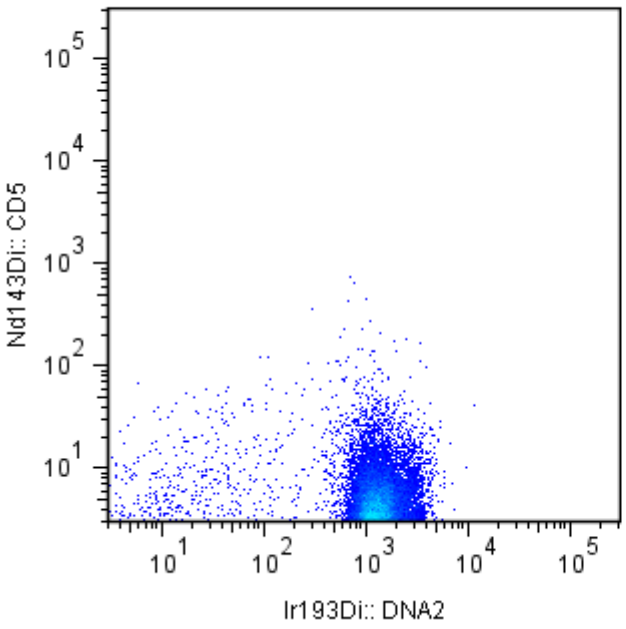


Post acquisition processing: Randomization

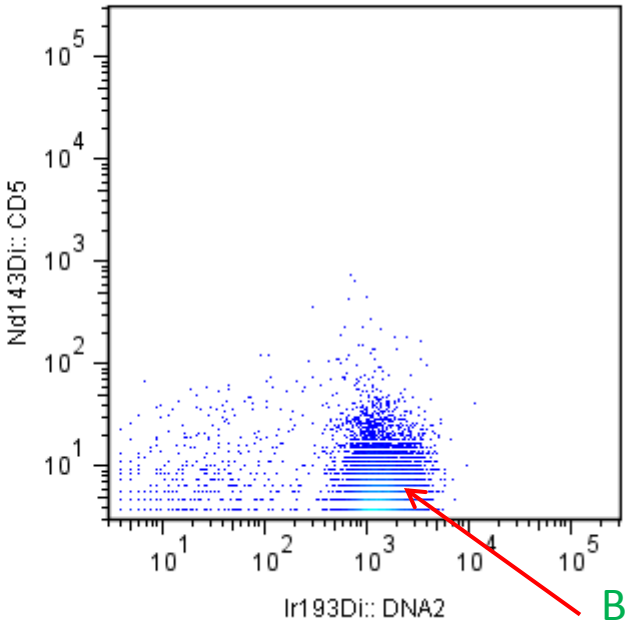
Input file format: .FCS or .TXT



Resulting files



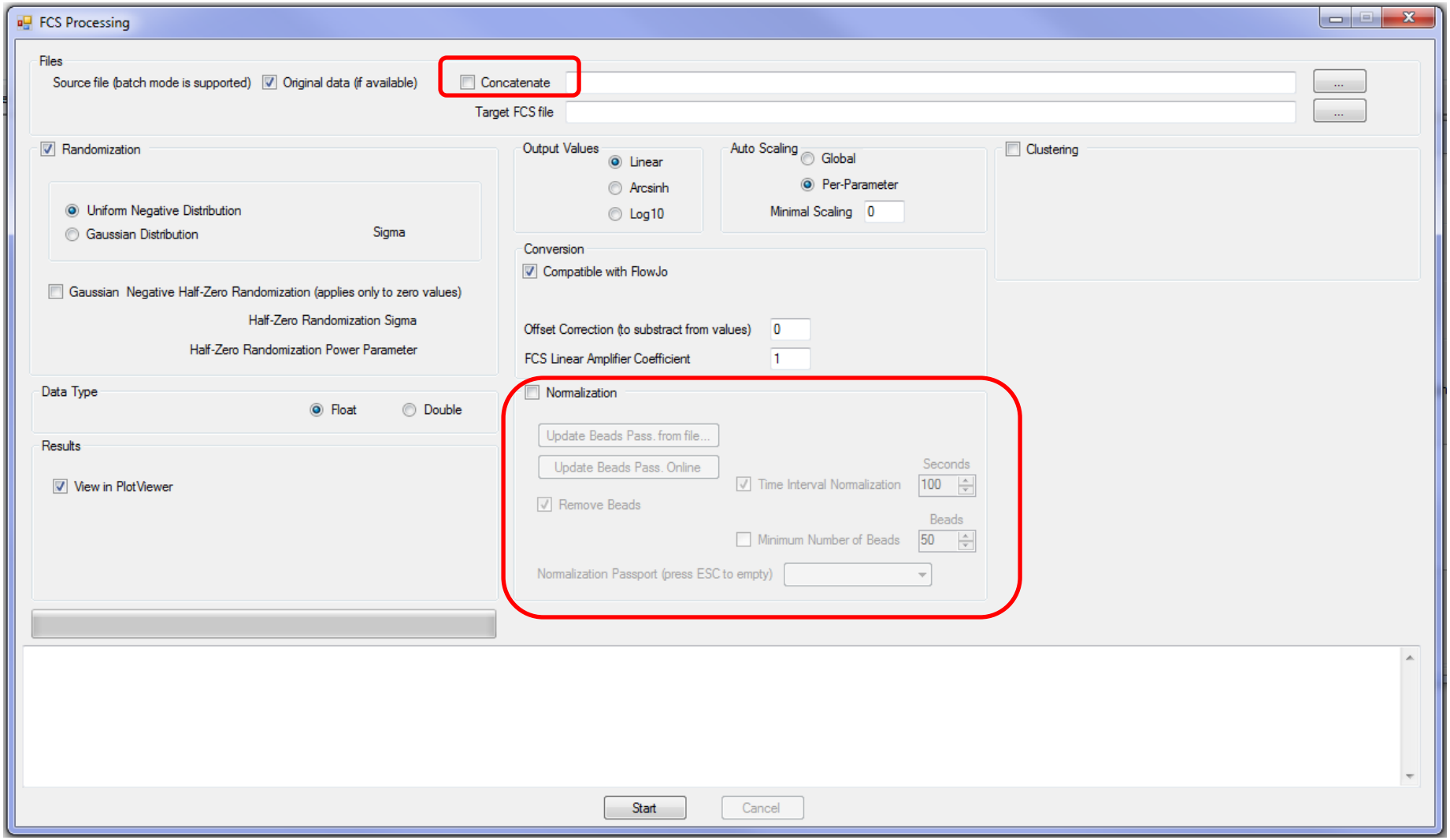
Helios generated data



Binning effect
"picket fence"

Processed (non-randomized) data

Other Post-acquisition FCS processing options



Normalization: Global Standard

- The Helios Normalizer is based on the concept of a **"Bead Passport"**

- The Bead Passport is a global standard generated by the manufacturer for a specific lot of EQ beads.

  - The Passport contains a profile of mean Di counts of all the masses of the particular lot of the beads as determined by multiple measurements during manufacturing

  - This Passport is universal across all instruments of the same type and cannot be changed by individual users.

- Using a global standard allows normalization of data within and across experiments as well as across instruments

- The software is pre-loaded with passports for all the manufactured bead lots

Helios Normalizer is a two-step process:

1. Bead identification

By selecting five of the most abundant isotopes of the elements used in the EQ beads, (Ce140, Eu151, Eu153, Ho165, Lu175), bead events are identified from cell events. The identified bead population is then employed as the internal standard for normalization.

Helios Normalizer is a two-step process:

2. Normalization

The normalization factor is the ratio of Passport median Di values to Bead population (previously identified) median Di values of the encoding isotopes.

Isotopes in the EQ beads cover an extensive portion of the mass range measurable on the Helios instrument. The normalization factors for mass channels between the encoding isotopes are linearly interpolated.

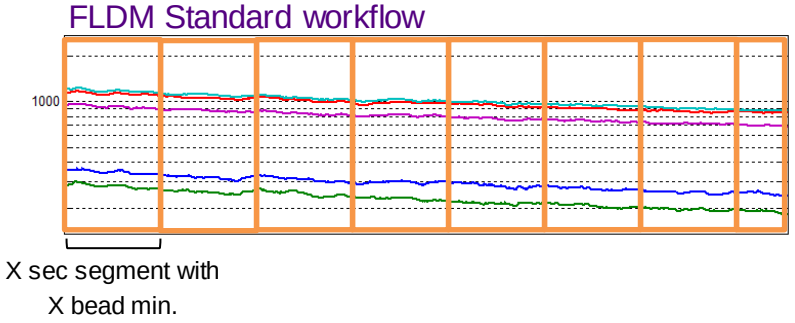
All mass channel values for all events are then multiplied by these normalization factors to obtain the normalized values and data is written to the normalized file.

Note that an external standard (such as a separately run bead sample) cannot be utilized by the algorithm for the normalization procedure.

Time interval Normalization

- The Helios Normalizer can also correct for signal drift over time with **Time interval normalization**.

- File is normalized by the medians of a minimum number of **beads** across **time** segments or the **entire** file
  - Minimum number of beads and time interval are user defined
  - We recommend normalizing every 100 seconds (interval), 50 beads per interval
- Signal adjusted to global standard



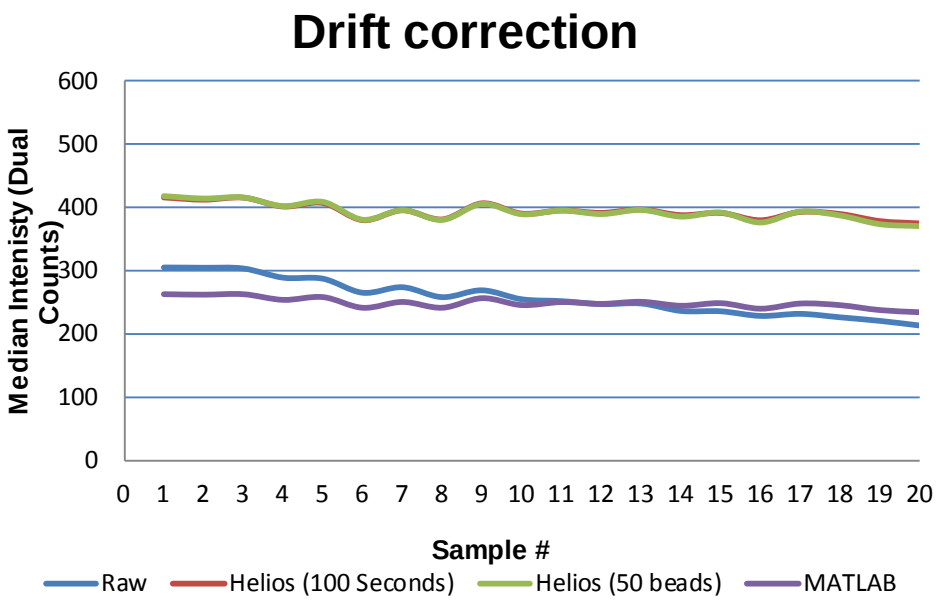
Alternative: the MATLAB ® method

- This method normalizes data using median bead intensity calculated from across the experimental data files instead of a global predetermined standard (Finck et al. 2013. Cytometry A 83:483).

- Note: the MATLAB method (but not the Fluidigm (DVS) method) requires collection of the iridium channel, even if the sample is not stained with iridium.

- Available through freeware offered by Stanford University.

Raw vs Normalized Data



Sample	Raw	Helios (100 Seconds)	Helios (50 beads)	MATLAB
1	305.07	416.06	418.22	262.74
2	304.64	412.55	414.4	261.93
3	303.47	415.79	416.04	262.68
4	288.93	401.73	402.32	253.72
5	287.26	407.28	409.04	258.1
6	264.99	380.09	380.75	241.17
7	273.73	395.78	395.84	250.41
8	258.01	381.25	380.48	241.23
9	268.87	406.79	405.9	256.42
10	254.63	390.49	389.47	245.4
11	251.69	395.43	395.44	250.21
12	246.99	391.61	389.76	247.36
13	247.94	396.85	396.32	250.65
14	236.17	388.03	385.74	244.35
15	235.59	391.51	392.2	248.44
16	228.25	379.9	376.38	239.7
17	231.55	393.11	393.67	247.93
18	226.05	389.78	387.45	245.28
19	220.37	378.61	373.68	237.52
20	213.03	374.92	370.64	234.1
Mean	257.3615	394.378	393.687	248.967
Stdev	28.99514	12.46037	13.98276	8.347561
CV	11.27%	3.16%	3.55%	3.35%

Concatenation

Concatenation is used when it is necessary to combine multiple sample runs into one FCS file

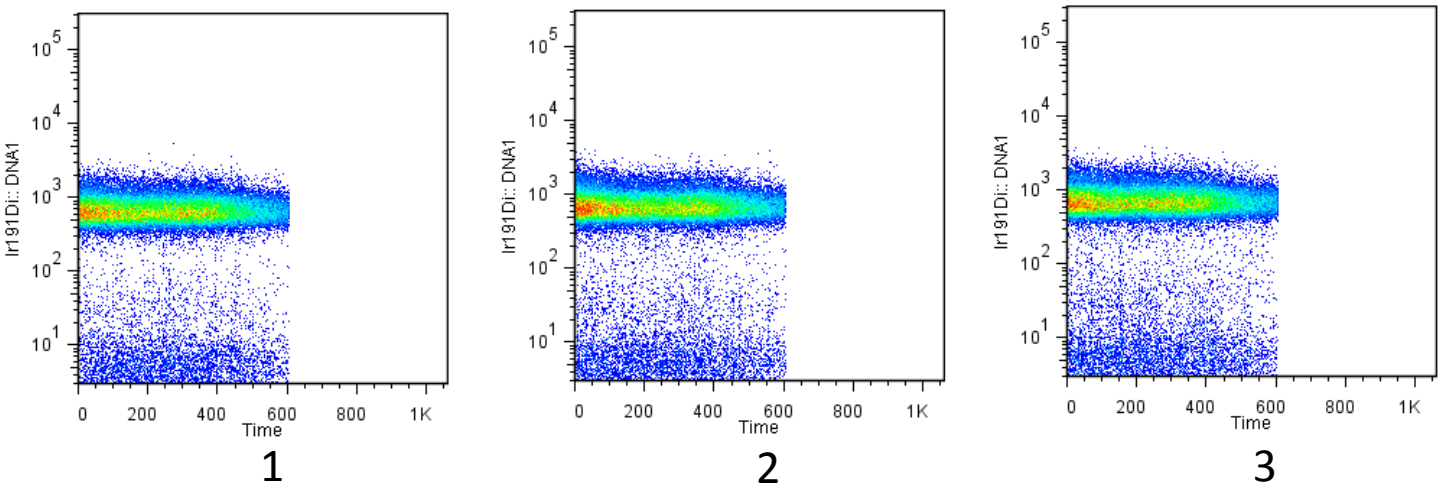
Files are added sequentially by the order of file input

The concatenated FCS file is named according to the first file input and designated as concatenated by a suffix “_0”

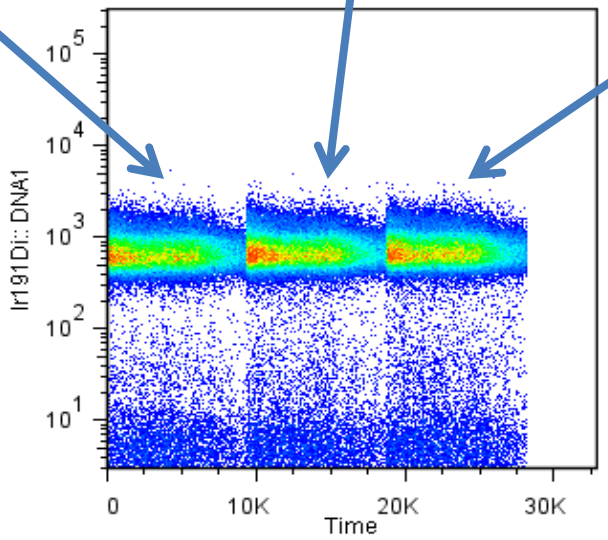
Note: if concatenation and normalization are both required, normalize before concatenating.

Resulting files

Individual FCS files

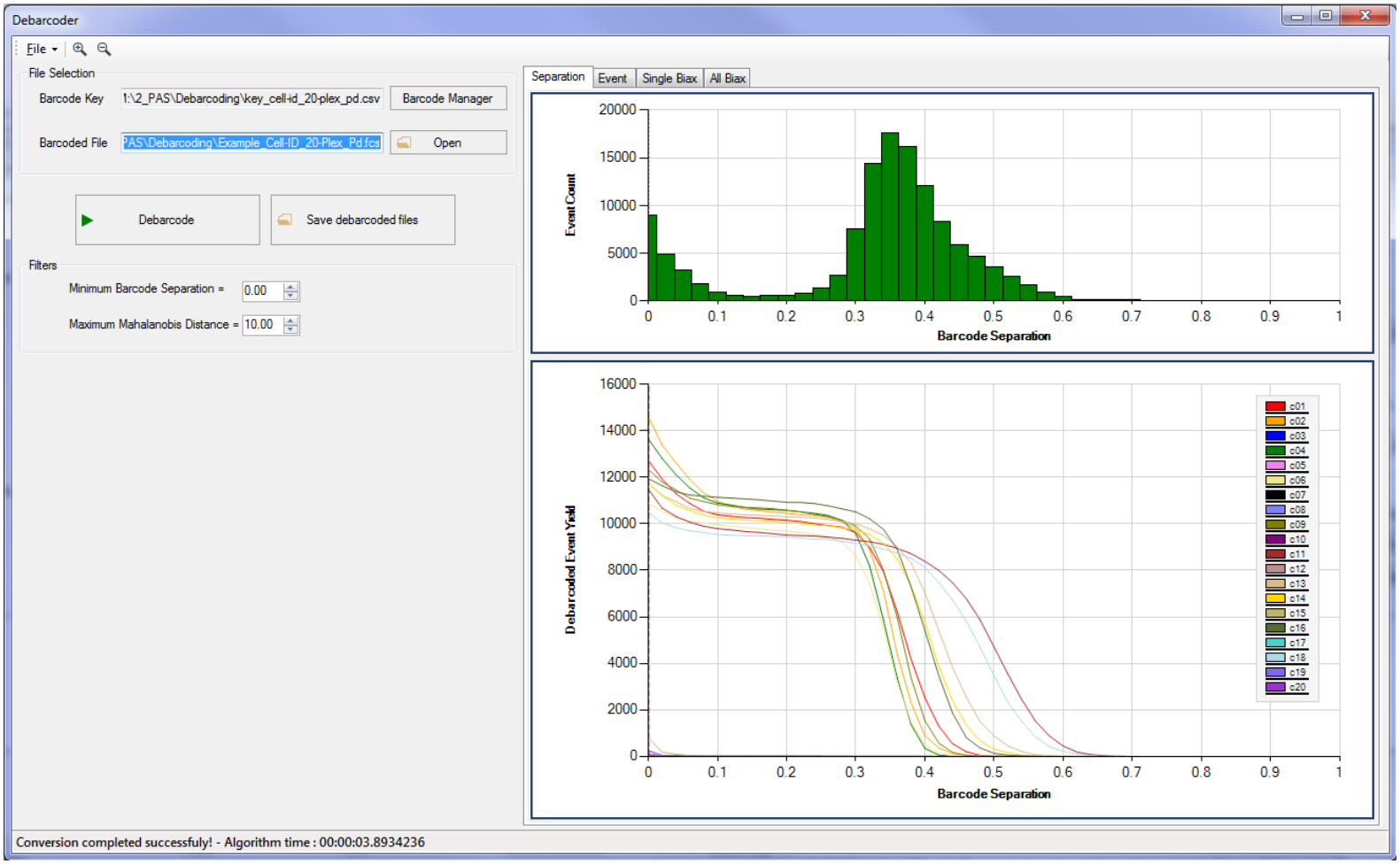


Concatenated FCS file



Debarcoder

For barcoded samples – available for download on the portal.



IMD Processing

- Enabling “Preserve IMD” makes the following reprocessing options available:
  - Reprocess the data based on different event definitions after acquisition
  - Remove channels that have high background (cannot add channels that were not activated during acquisition)
  - Add Gaussian discrimination parameters
  - Post-processing with a custom expression

Simplify the complex quest to understand and apply biology.

FLUIDIGM