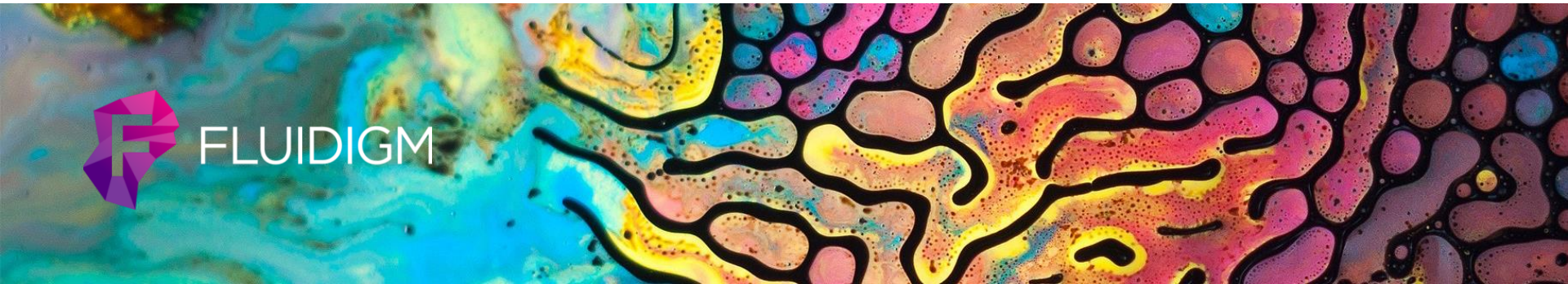


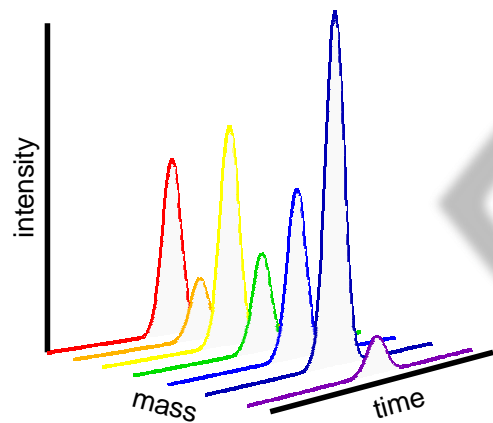
# Data Processing in Helios



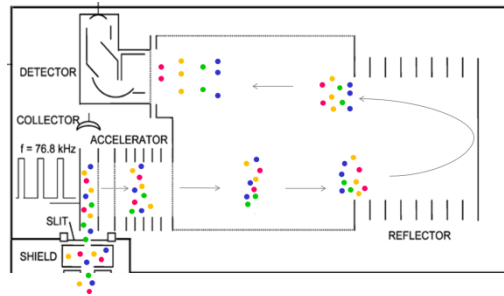
FLUIDIGM



# How Helios Generates Data from Ion Clouds

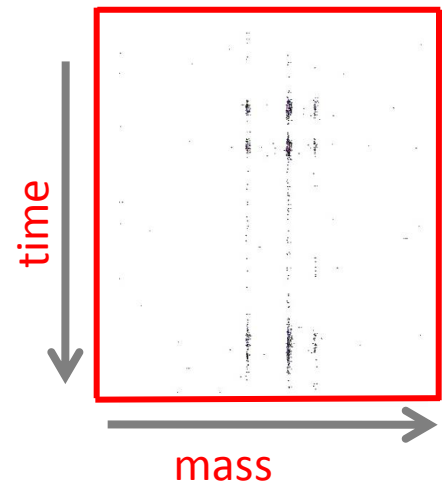
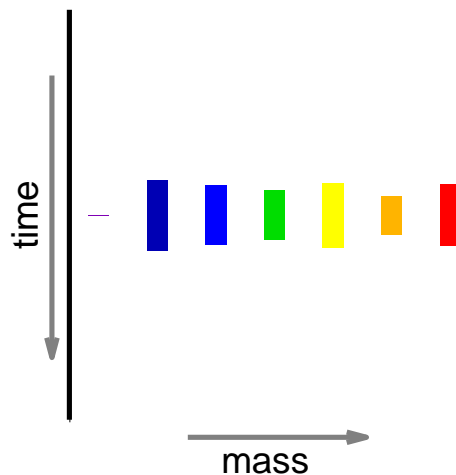
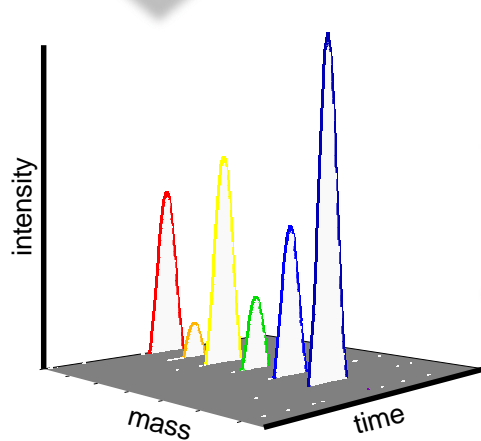


ToF Chamber

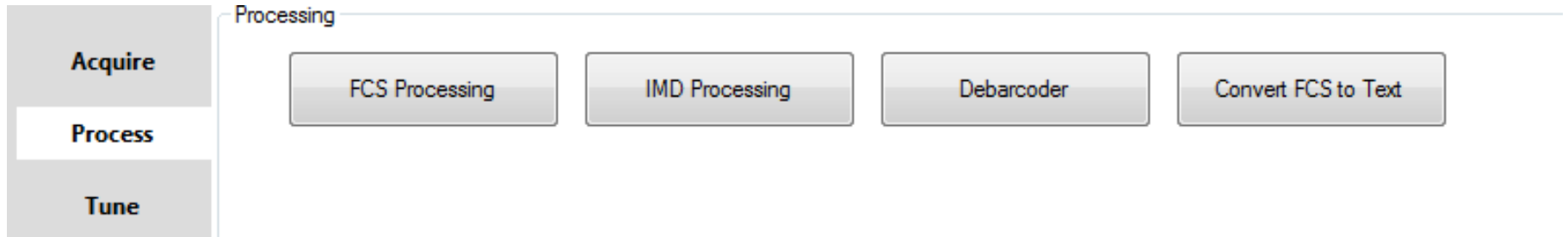


Raw Data  
(pre-FCS file)

Noise Filtering: Lower convolution threshold

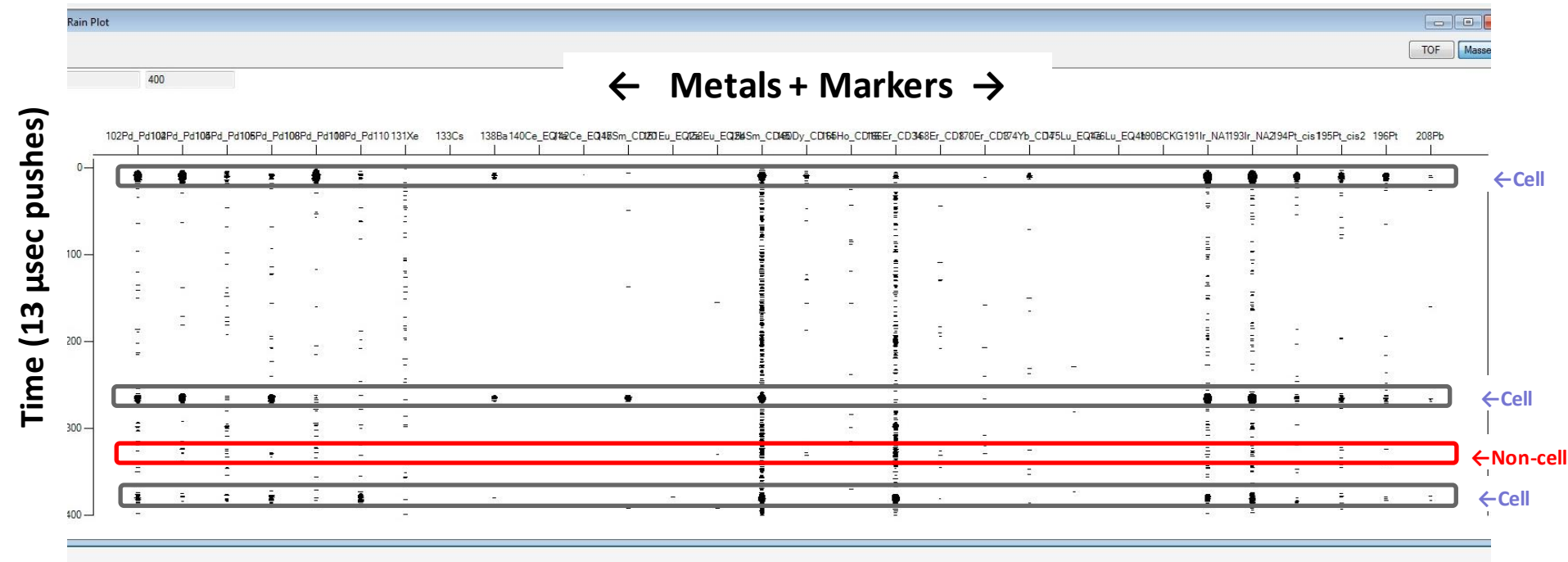


# Helios Software Data Processing



# 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

Collection mode:  Event  Solution

Advanced

Lower Convolution Threshold

Signal Subtraction (per channel)

Min Event Duration

Max Event Duration

Sigma

Dual Count Start

Plot Settings

Pushes to show

Refresh (sec)

Signal to Process

Processing

Noise Reduction

Randomization

Preserve IMD File

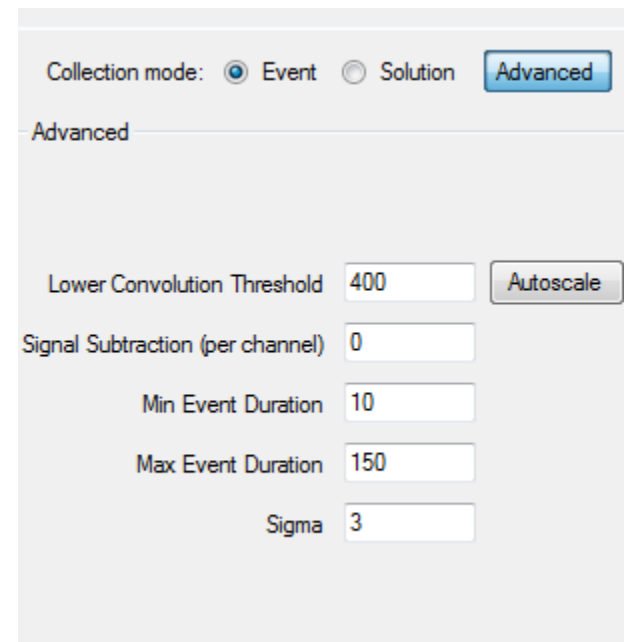
Custom Theshold Filtering

Gaussian Discrimination

# Event Definition

What is an event?

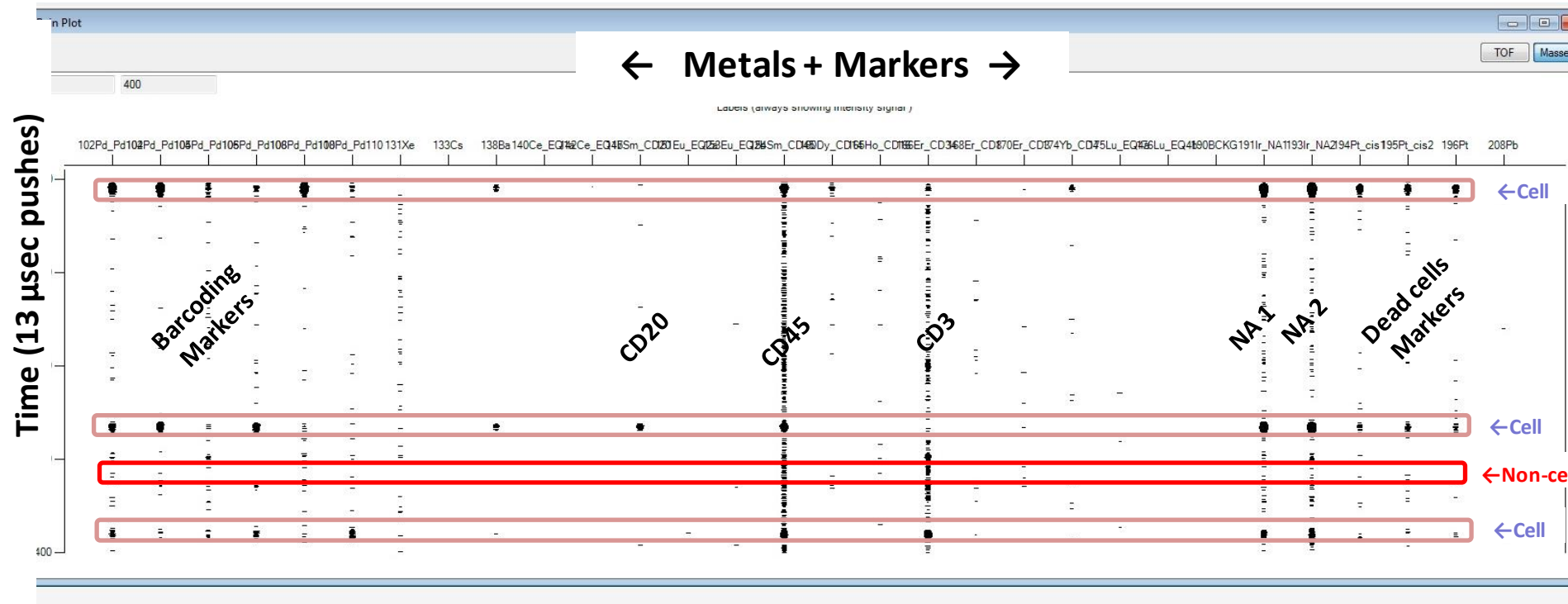
When the total ion current (from all selected channels) exceeds the lower convolution threshold **continuously** for at least 10 pushes (minimum event duration) but no more than 150 pushes (maximum event duration), it will be registered as an event.



The image shows a software interface for defining event parameters. At the top, there is a 'Collection mode' section with two radio buttons: 'Event' (selected) and 'Solution'. To the right of these is an 'Advanced' button. Below this, the 'Advanced' settings are displayed. The 'Lower Convolution Threshold' is set to 400, with an 'Autoscale' button to its right. The 'Signal Subtraction (per channel)' is set to 0. The 'Min Event Duration' is set to 10, and the 'Max Event Duration' is set to 150. The 'Sigma' value is set to 3.

Parameter	Value
Collection mode	Event
Lower Convolution Threshold	400
Signal Subtraction (per channel)	0
Min Event Duration	10
Max Event Duration	150
Sigma	3

# Events



# 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

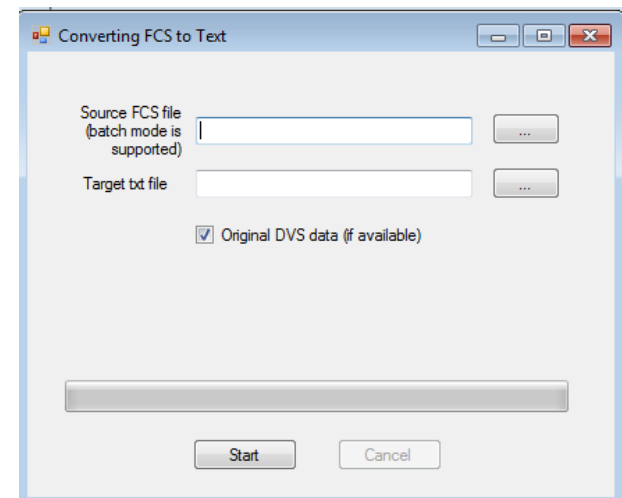
.TXT files contain **non-randomized dual count** data for each successfully resolved event in each channel selected.

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

The screenshot shows the 'Advanced' settings panel in Helios software. At the top, 'Collection mode' is set to 'Event' (selected with a radio button), 'Solution' (unselected), and 'Advanced' (selected in a button). Below this, the 'Advanced' section contains several controls:

- Lower Convolution Threshold:** Input field with value 400 and an 'Autoscale' button.
- Signal Subtraction (per channel):** Input field with value 0.
- Min Event Duration:** Input field with value 10.
- Max Event Duration:** Input field with value 150.
- Sigma:** Input field with value 3.
- Dual Count Start:** Input field with value 1.
- Plot Settings:** A sub-panel containing:
  - Pushes to show:** Input field with value 400.
  - Refresh (sec):** Input field with value 10.
- Signal to Process:** Dropdown menu set to 'Dual'.
- Processing:** Dropdown menu set to 'Realtime'.
- Checkboxes:**
  - Noise Reduction
  - Randomization (highlighted with a red box)
  - Preserve IMD File
  - Custom Theshold Filtering
  - Gaussian Discrimination
- Test Expression:** A large empty text area with a 'Test Expression' button at the bottom right.

# Post acquisition processing: Randomization

Input file format: .FCS or .TXT

The screenshot displays the 'FCS Processing' software window. The 'Files' section at the top includes fields for 'Source file (batch mode is supported)', 'Original data (if available)', 'Concatenate', and 'Target FCS file'. A red box highlights the 'Randomization' checkbox, which is checked. Below this, the 'Uniform Negative Distribution' radio button is selected, with a 'Sigma' input field. The 'Gaussian Negative Half-Zero Randomization' checkbox is unchecked, with 'Half-Zero Randomization Sigma' and 'Half-Zero Randomization Power Parameter' fields. The 'Data Type' section has 'Float' selected. The 'Results' section has 'View in PlotViewer' checked. The 'Output Values' section has 'Linear' selected. The 'Auto Scaling' section has 'Per-Parameter' selected with a 'Minimal Scaling' input field set to 0. The 'Conversion' section has 'Compatible with FlowJo' checked, with 'Offset Correction' and 'FCS Linear Amplifier Coefficient' input fields. The 'Normalization' section has 'Time Interval Normalization' checked with a 'Seconds' spinner set to 100, and 'Remove Beads' checked with a 'Beads' spinner set to 50. The 'Minimum Number of Beads' checkbox is unchecked. A 'Normalization Passport' dropdown is at the bottom. 'Start' and 'Cancel' buttons are at the bottom center.

FCS Processing

Files

Source file (batch mode is supported)  Original data (if available)  Concatenate  Target FCS file

Randomization

Uniform Negative Distribution  Gaussian Distribution  Gaussian Negative Half-Zero Randomization (applies only to zero values)

Sigma

Half-Zero Randomization Sigma

Half-Zero Randomization Power Parameter

Data Type  Float  Double

Results  View in PlotViewer

Output Values  Linear  Arcsinh  Log10

Auto Scaling  Global  Per-Parameter

Minimal Scaling 0

Conversion  Compatible with FlowJo

Offset Correction (to subtract from values) 0

FCS Linear Amplifier Coefficient 1

Normalization  Update Beads Pass. from file...  Update Beads Pass. Online

Time Interval Normalization 100 Seconds

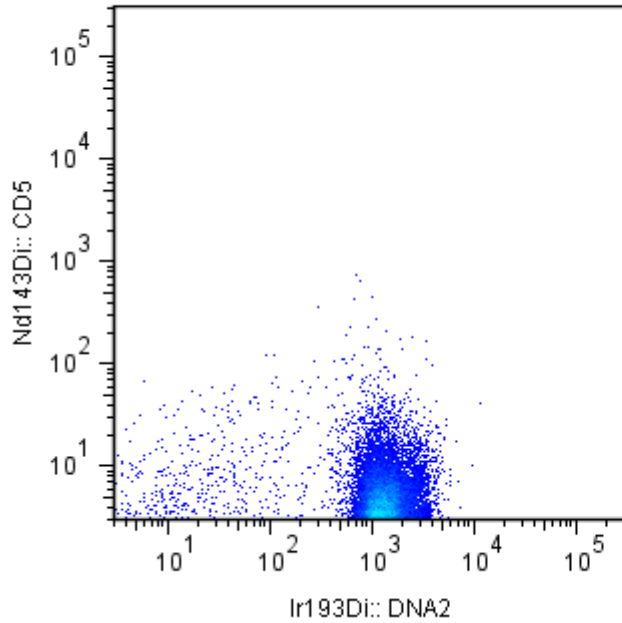
Remove Beads 50 Beads

Minimum Number of Beads 50

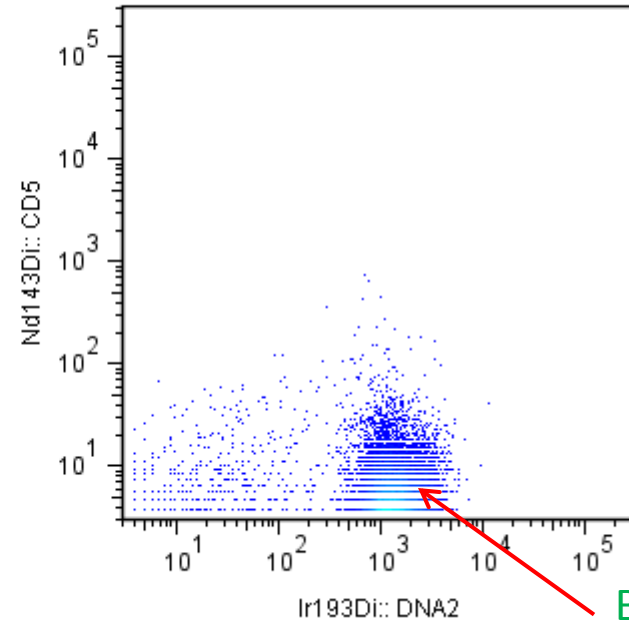
Normalization Passport (press ESC to empty)

Start Cancel

# Resulting files



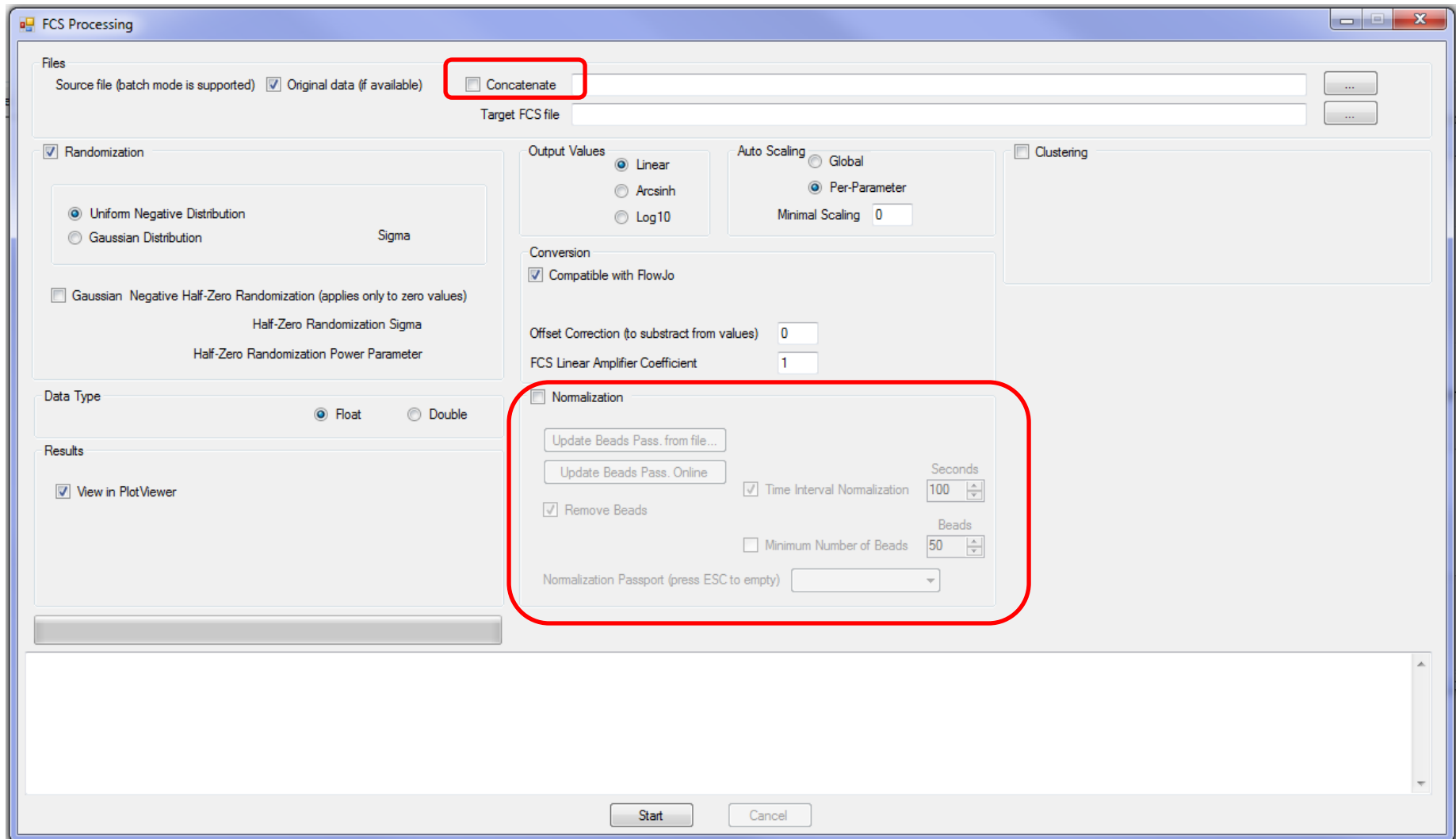
Helios generated data



Processed (non-randomized) data

Binning effect  
"picket fence"

# 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  $D_i$  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  $D_i$  values to Bead population (previously identified) median  $D_i$  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.**

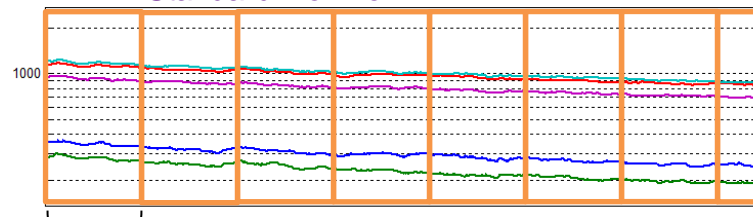
All mass channel values for all events are then multiplied by these normalization factors to obtain the normalized values and data is written to the normalized file.

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

FLDM Standard workflow



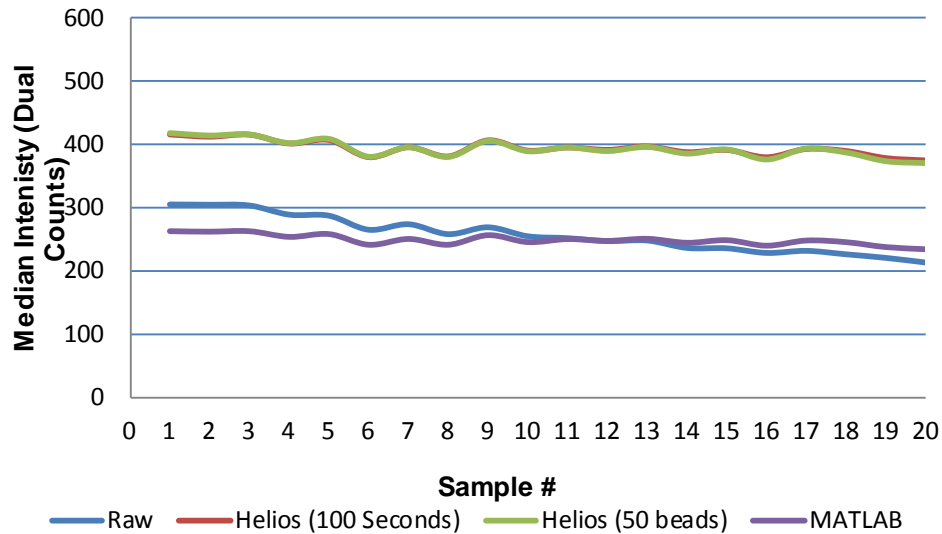
X sec segment with  
X bead min.

# 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

## Drift correction



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

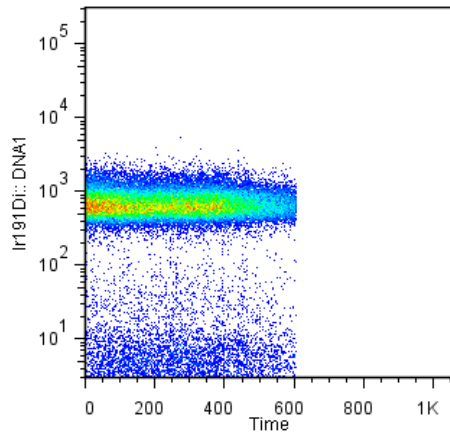
Files are added sequentially by the order of file input

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

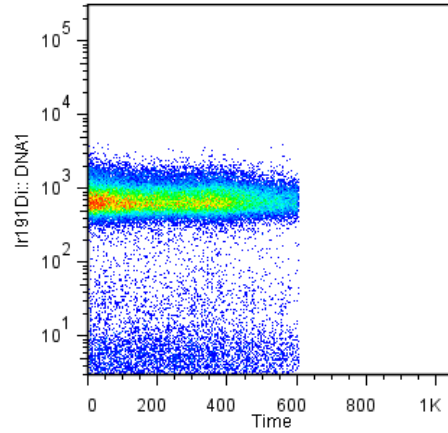
Note: if concatenation and normalization are both required, normalize before concatenating.

# Resulting files

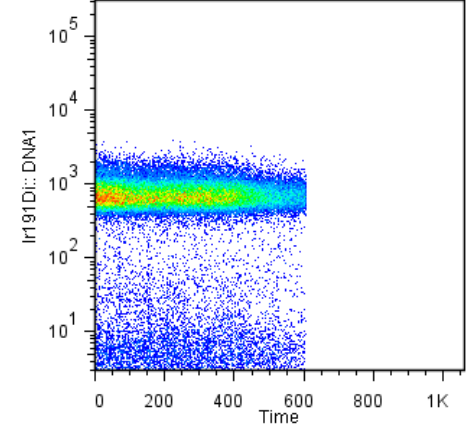
Individual  
FCS files



1

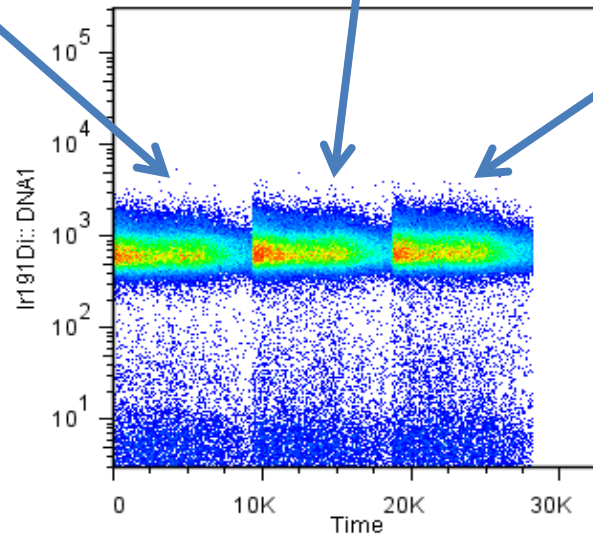


2



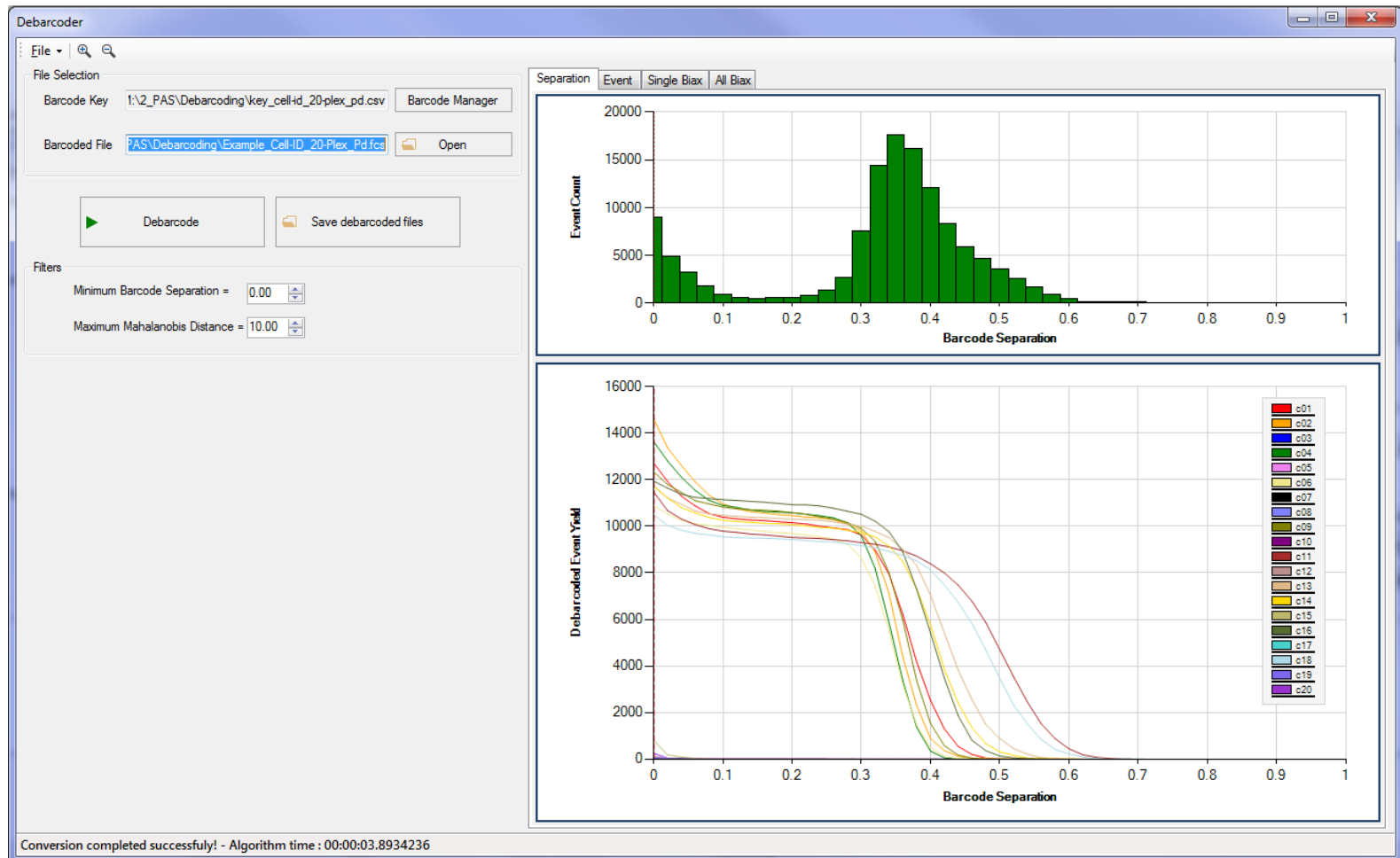
3

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.

