Normalization of Mass Cytometry Data Using EQ Four Element Calibration Beads

Contents

Introduction

IMPORTANT To generate data for comparative studies, it is important to correct signal variations related to instrument performance. In mass cytometry, signal strength reduces over time due to the accretion of cellular material in the instrument during an experiment. Also, manual interventions, such as instrument cleaning and tuning, cause changes in performance. Sample data must be normalized to account for signal drift to minimize measurement variations in the final data to be analyzed. This allows for an accurate interpretation of the biological differences between samples.

This document describes how to normalize sample data using CyTOF® Software 6.7 to correct for instrument signal fluctuations. The method involves spiking cellular samples with metal-containing bead standards—EQ™ Four Element Calibration Beads (PN 201078)— followed by normalizing acquired sample data through algorithmic processing of the bead standards. The output is an FCS file containing normalized single-cell mass cytometry data appropriate for comparative studies.

EQ Four Element Calibration Beads

EQ Four Element Calibration Beads (PN 201078) is a solution of polystyrene bead standards containing known amounts of the metal isotopes cerium ($^{140/142}$Ce), europium ($^{151/153}$Eu), holmium (^{165}Ho), and lutetium ($^{175/176}$Lu). This multi-element solution is used to normalize data in CyTOF Software to a bead-based passport (EQ-P13H2302_ver2) containing global dual count values for bead elements specific to the manufactured bead lot.

CyTOF Data Normalization Method

Data in FCS file format can be normalized for intra-file and inter-file signal drift using the FCS Processing tab in the CyTOF Software 6.7. The Fluidigm data normalization method is a two-step algorithm that first identifies the EQ Four Element Calibration Beads and then applies the dual count values registered by the beads to calculate the normalization factor to be applied to the data.

Step 1: Bead Identification

A bead event is identified in the FCS file if the cumulative signal for the five bead elements is higher than 1662.85 dual counts when the bead-based passport **EQ-P13H2302_ver2** is applied. The median dual count values of the metals in this bead population is then used as the internal standard for normalization.

Step 2: Data Normalization

Data is normalized by multiplying the actual dual counts of each analyzed channel by a normalization factor. A normalization factor for each bead isotope is the ratio of actual median dual counts measured within the specified time interval normalization window (for example, 100 seconds) to the global dual count values stored in the Normalization Passport. After a normalization factor for each bead-specific channel is determined, the normalization factors for all channels between the bead isotopes are linearly interpolated within the time

interval. The normalization factor for cesium is applied to channels below 140 amu, and the normalization factor for lutetium is applied to channels above 176 amu.
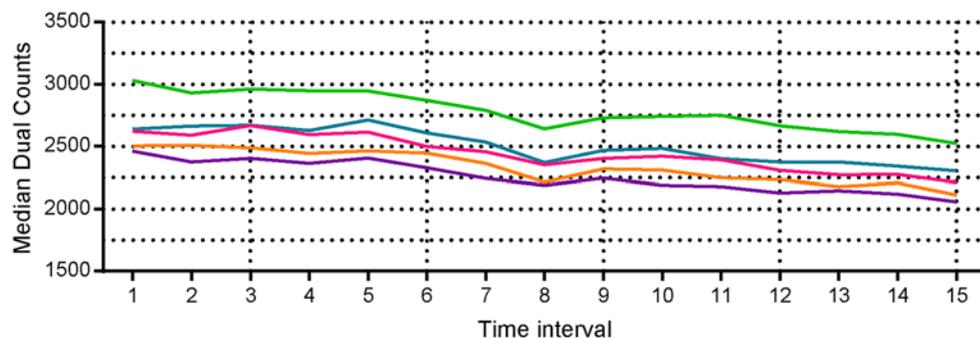

Figure 1. Unnormalized data shows signal drift and time interval segmentation of data over time.

Data Normalization Examples

The following examples demonstrate the effectiveness of the CyTOF data normalization method for correcting signal drift over time (Example 1) and global normalization (Example 2).

Example 1. Data Correction for Signal Drift over Time

Mass cytometry data acquired over an extended period is affected by signal drift. To demonstrate how normalizing the data with CyTOF Software can effectively adjust for signal drift over time, longitudinal data was acquired from cells stained with metal-conjugated antibodies on a Helios™ instrument for a total of 7,200 seconds (2 hours).

Method

1   10 million Jurkat cells (human acute T cell lymphoma cell line) and 10 million Ramos cells (human Burkitt's lymphoma cell line) were stained with Cell-ID™ Cisplatin (Fluidigm, PN 201064), surface marker antibodies (Fluidigm, various PN), and Cell-ID Intercalator-Ir (Fluidigm, PN 201192A) according to the Maxpar® Cell Surface Staining with Fresh Fix Protocol (Fluidigm, PN 400276 A3).

2   Cells were then centrifuged and washed once with Maxpar Cell Staining Buffer (Fluidigm, PN 201068) and twice with Maxpar Cell Acquisition Solution (Fluidigm, PN 201240).

3   Cells were centrifuged, re-suspended to 1×10^6 cells per mL in 0.1X EQ Four Element Calibration Beads (Fluidigm, PN 201078) in Cell Acquisition Solution (Fluidigm PN 201240), filtered through 35 μm mesh, and divided into two aliquots (2 mL each).

4   Data for each aliquot was acquired on a Helios mass cytometer using the WB Injector for 3,600 seconds.

Analysis

All FCS files were normalized using the default data normalization settings in CyTOF Software 6.7.

An unprocessed version and normalized version of each data file was uploaded to Cytobank and analyzed to examine the median dual count values for surface antigen expression across samples acquired over time.

Data files were manually gated to remove debris and non-singlet events. As shown in Figure 2, DNA+ events were gated on DNA+Bead– (^{191}Ir+ vs ^{140}Ce–), then singlet events were gated using Event Length (^{191}Ir+ vs Event_length).
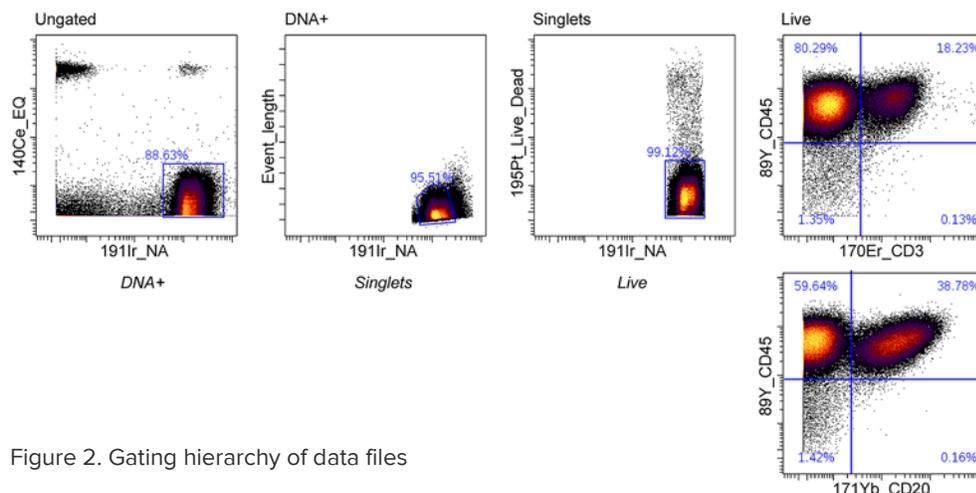
Figure 2. Gating hierarchy of data files

Three cell surface markers—^{89}Y-CD45, ^{154}Sm-CD3, and ^{171}Yb-CD20—were analyzed to assess the correction for signal drift over time. To obtain this data, cells were subsequently gated on bivariate plots of CD20 vs. CD3 to discriminate CD45$^+$CD20$^+$ events (Ramos cells) and CD45$^+$CD3$^+$ events (Jurkat cells). Median dual count values for the relevant channels in each population were obtained every 30 minutes and plotted in Figure 3.
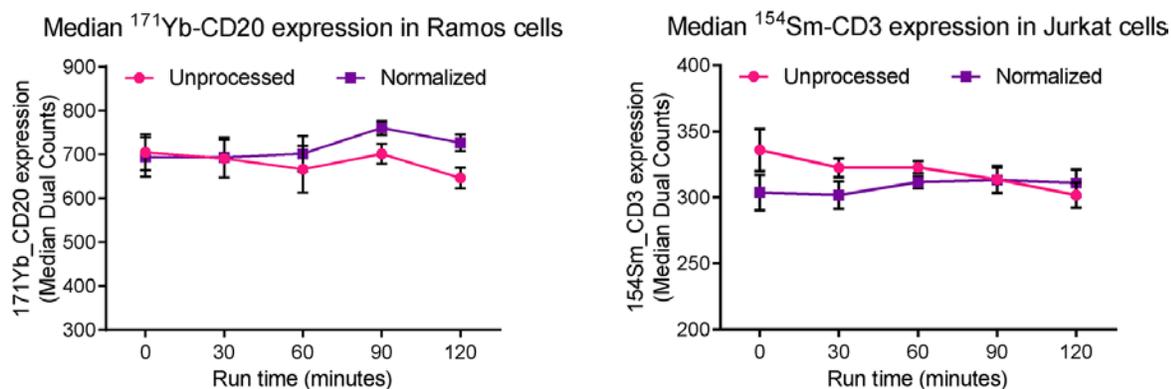

Figure 3. Median cell marker (left graph, CD20 in Ramos cells; right graph, CD3 in Jurkat cells) dual count values of the same sample acquired over time. Data plotted demonstrate the difference between unprocessed (before processing) and normalized (after processing) data.

Expression for cell surface markers is effectively normalized across a longitudinal sample acquisition (2 hours) on the same instrument when the default data normalization settings are applied.

Example 2. Data Correction for Global Normalization

Mass cytometry samples, when stored properly, can be run on different days on the same instrument. Consequently, the data can be acquired across a variety of tuning conditions. To demonstrate how data normalization adjusts for differences in signal intensities due to changes in tuning parameters, human peripheral blood mononuclear cells (PBMC) were stained with metal-conjugated antibodies and data was acquired with a Helios instrument at different times and with different tuning settings.

Method

5   PBMC stained with surface marker antibodies (Fluidigm, various PN) and iridium intercalator (Fluidigm, PN 201192A) were washed and stored as a pellet.

6   Cells were re-suspended to 1 x 10^6 cells per mL in 1 mL of 0.1X EQ Four Element Calibration Beads and filtered through 35 µm mesh.

7   The sample was split into 3 tubes for 3 separate 1,000-second acquisitions. Tuning was performed between each acquisition to achieve different instrument response results. Details of the sample acquisition settings are outlined in Table 1.

Table 1. Instrument response settings for each sample

Sample	^{159}Tb Mean Dual Counts	^{169}Tm Mean Dual Counts	^{140}Ce Median Dual Counts	^{153}Eu Median Dual Counts
Sample 1	1,455,262	1,429,967	2,941	3,389
Sample 2	1,062,919	1,024,060	1,960	2,505
Sample 3	784,510	755,328	1,547	2,014

Analysis

All FCS files were normalized using the default data normalization settings in CyTOF Software 6.7.

An unprocessed version and a normalized version of each data file were uploaded to Cytobank and analyzed to examine the median dual count values for surface antigen expression across samples acquired on the same instrument with different tuning settings.

Data files were cleaned up to remove debris, non-singlet events and dead cells. DNA+ events were gated on DNA+Bead– (^{191}Ir+ vs ^{140}Ce–), then singlet events were gated using Event Length (^{191}Ir+ vs Event_length) as in Figure 2.

Two cell surface markers—^{154}Sm-CD3 and ^{171}Yb-CD20—were analyzed to assess the correction for differences in instrument tuning. Median dual count values for each marker in positive populations were obtained for each file and plotted in Figure 4.
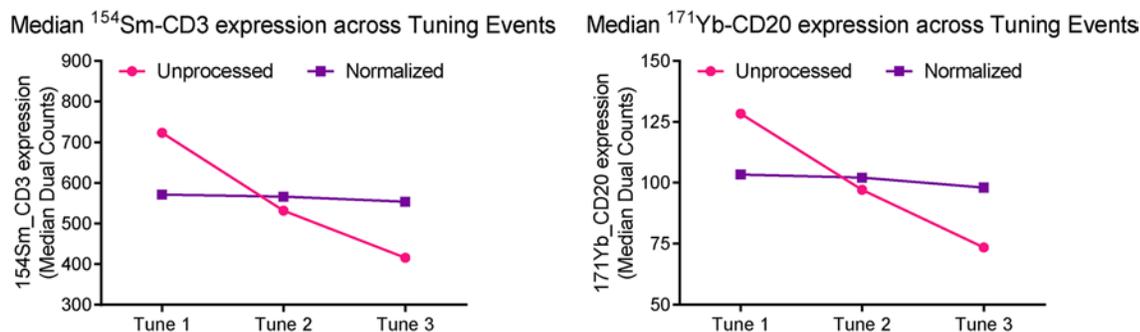
Median ^{154}Sm-CD3 expression across Tuning Events

Median ^{171}Yb-CD20 expression across Tuning Events

Figure 1. Median cell marker (left graph, CD3; right graph, CD20) dual count values acquired from the same sample with three different tuning settings. The data plotted demonstrate the difference between unprocessed (before processing) and normalized (after processing) data.

Expression for both cell surface markers is effectively normalized across multiple tuning events on the same Helios instrument when the default data normalization settings are applied.

Data Normalization Procedure

CyTOF Software Method

Before You Begin

- **Minimum EQ bead requirement.** For effective and statistically relevant data normalization, it is critical that a minimum of 50 single bead events are detected per time interval (the default time interval is 100 seconds).

- **File concatenation.** Normalize the data first, and then, if required, concatenate files.

   NOTE FCS files created by third-party party software cannot be normalized.

To Normalize Sample Data

1   Start CyTOF Software 6.7.

    Open FCS Processing: click **Process** > **FCS Processing**.
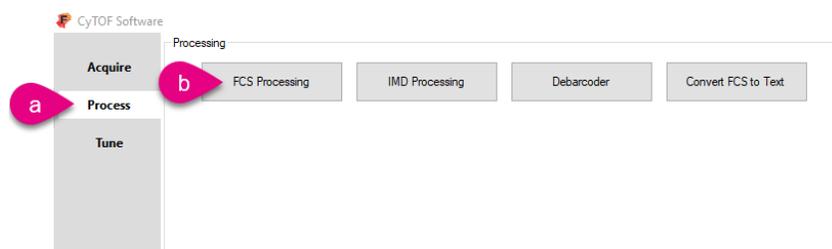
2   Under **Files**, confirm that **Original data (if available)** and **Concatenate** are **not** selected, and then click [...] to browse for FCS file or files for processing.

    NOTE To select more than one file for processing, hold Ctrl and click additional files.
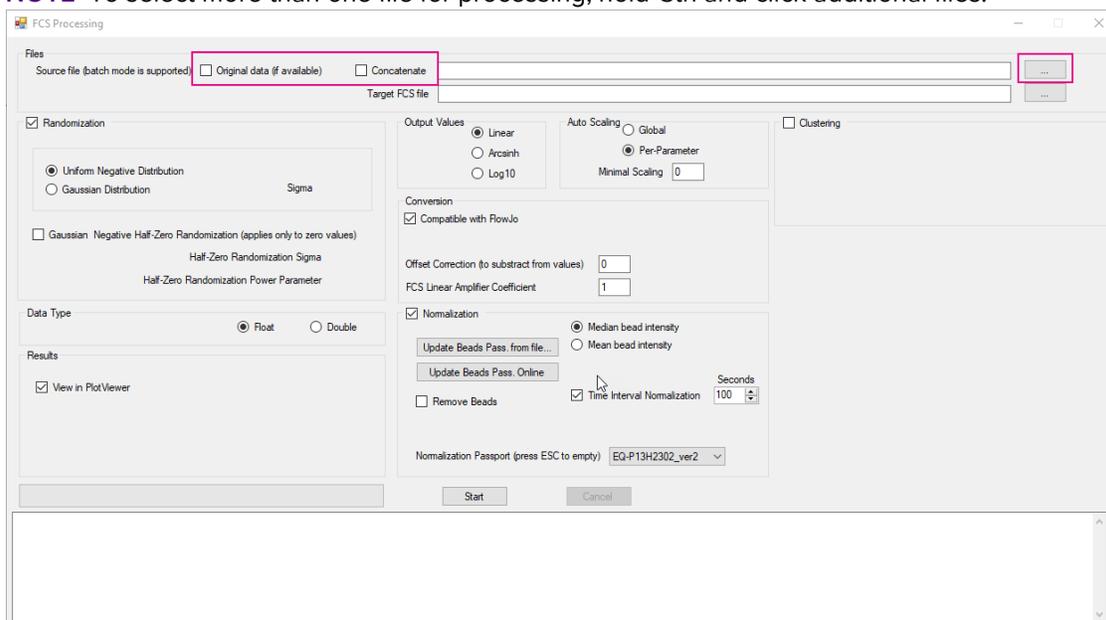
3 Confirm that **Normalization** is selected, and then confirm the
following default options:

- **Median bead intensity** is selected.

- **Time Interval Normalization** is selected and **Seconds** is set to 100.

- Remove Beads is **not** selected.

- **Normalization Passport** is set to EQ-P13H2302_ver2.

    IMPORTANT It is strongly recommended that you use the default median signal
    intensity setting from filtered EQ Four Element Calibration Beads events to reduce the
    influence of non-singlet bead events on the data normalization results.

    NOTE The Normalization Passport EQ-P13H2302_ver2 and is applicable to all
    commercially available lots of EQ Four Element Calibration Beads.
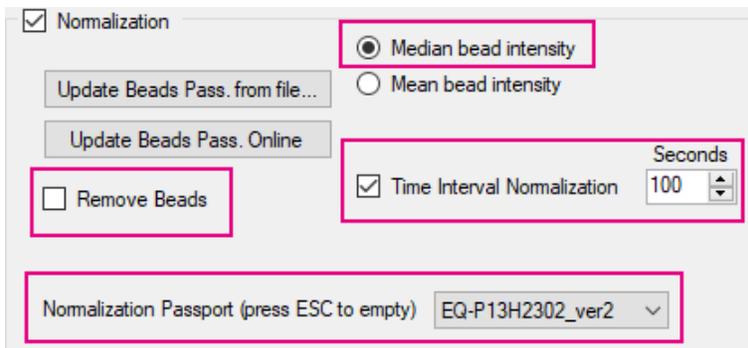
4 To begin the normalization process, click **Start**.
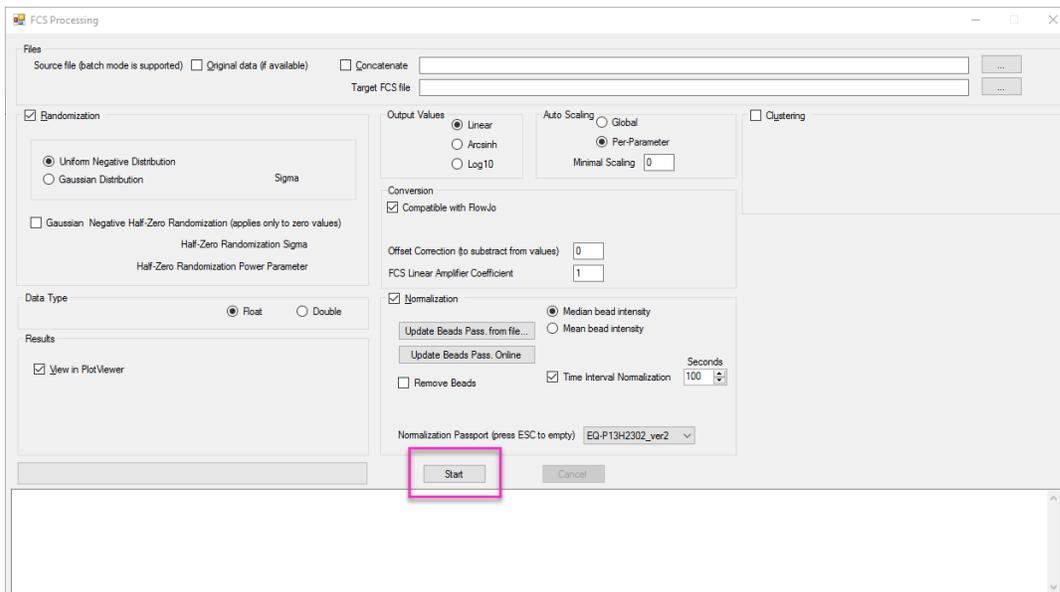
After the normalization process starts, the window at the bottom of the FCS Processing window displays time-stamped status updates until the normalization process is complete. Successful completion of data normalization is indicated by the message **processing completed successfully**.
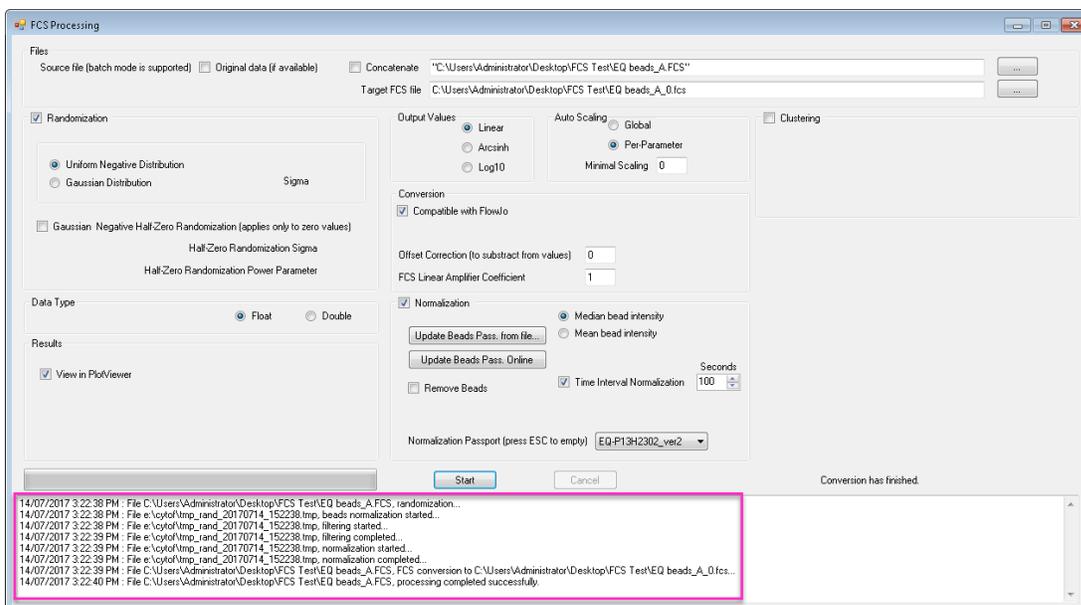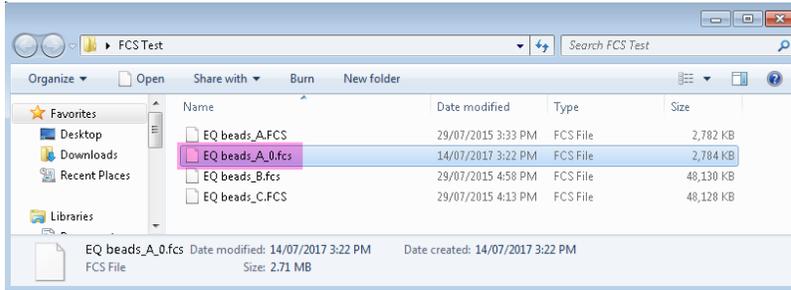

When normalization of a single FCS file is complete, Plotviewer displays automatically.
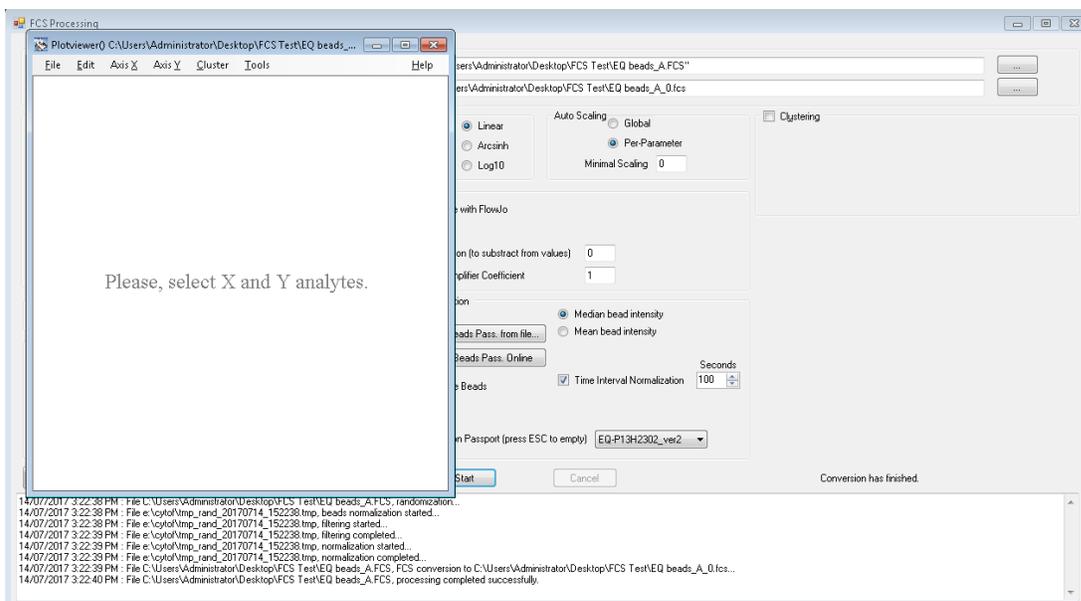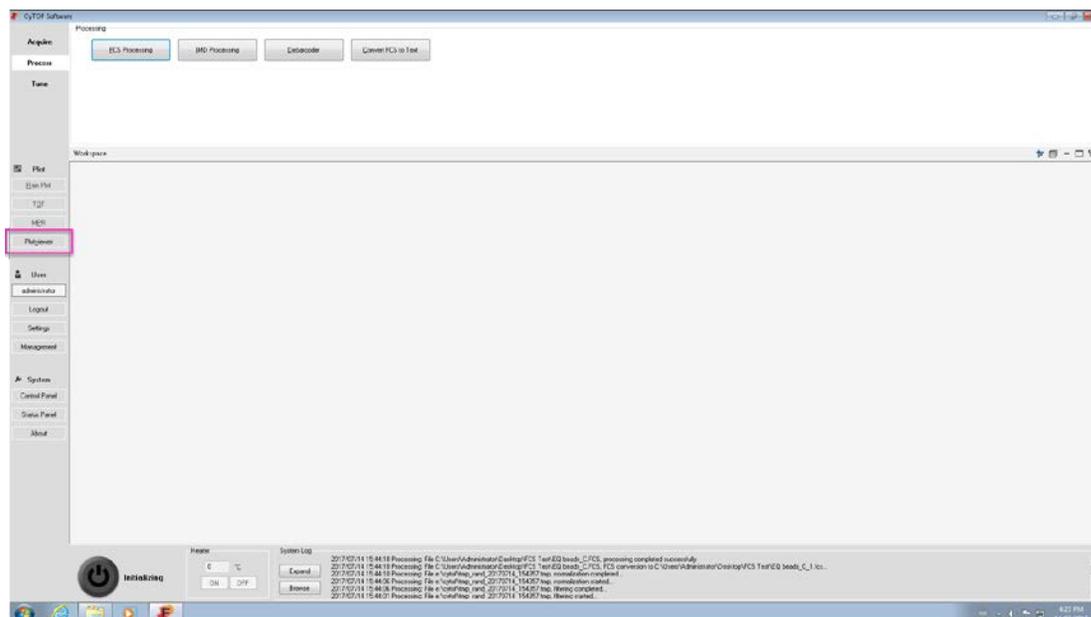
The normalized file is saved in the same folder as the source file and appended with the suffix "_0" for individual files.

If multiple files are selected for processing, Plotviewer is not displayed after processing is complete. A normalized file for each input file is saved to the same location as the source files appended with the suffix "_1".
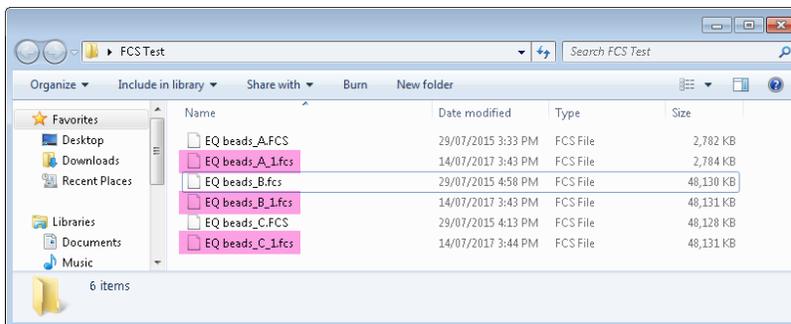
To view these files in Plotviewer, click Plotviewer from the main CyTOF Software menu.

Troubleshooting

Observation	Probable cause	Recommendation

The following message is displayed after normalization completes:
 	
One or more time intervals within the following files did not contain enough beads for normalization. Therefore, data in those intervals is not recorded in the following output normalized files: [list of affected files]
	One or more time intervals in the original FCS file did not contain the minimum 50 beads required for normalization. Time intervals with fewer than 50 beads are removed from the normalized FCS file.	Compare the number of events in the FCS file before and after normalization.
		
		If that number is:
		
		• **Unchanged after normalization,** one or more gaps in the data acquisition (where no events were collected) was removed from the normalized FCS file.
		
		• **Slightly lower after normalization,** a time interval (usually the last) did not contain enough beads and the interval was removed from the normalized FCS file.
		Note: Although time intervals may be set to 100 seconds, the file may contain additional milliseconds of acquisition time that exceed the time interval duration (for example, 100.1 seconds) and is not processed.
		
		• **Significantly lower after normalization**, one or more time intervals did not contain enough beads and was removed from the normalized FCS file. If using the default time interval of 100s:
		
		  • Acquisition time is <400s: If the acquisition time is not evenly divisible by the time interval, the last time interval will not be included in the normalized FCS file. Minimally modify the time interval to a factor of the acquisition time resulting in even division of the acquisition time. For example, for a 270s file, change the time interval to 135s.
		
		  • Acquisition time is >400s: Beads may have been inadequately added to the sample. Review Product Insert for EQ Four Element Calibration Beads (PN PRD014).

Normalization does not start and the following error message is displayed: *Sequence contains no elements.*	FCS file was created in third-party software, such as, Cytobank or FlowJo®.	FCS files created by third-party software cannot be normalized.
Files cannot be concatenated. The following error message is displayed: *Input files have different headers.*	The channels analyzed in each FCS file are not exactly the same. For example, one file contains a channel that another file does not.	Verify that both files contain data for the exact same channels.

Appendix A: FCS File Concatenation

File concatenation is necessary when data for a single sample is acquired over more than one file. Create a single FCS file by concatenating the FCS files using CyTOF Software 6.7 and following the instructions below.

Requirements for FCS File Concatenation

- Files for concatenation must have the same acquisition template.

- Any file concatenation should be performed **after** the normalization procedure. Normalize FCS files first to ensure that the normalization process is not affected by a file concatenated in an incorrect time order.

Concatenation Using CyTOF Software 6.7

1 Open CyTOF Software 6.7 and click the **Process** tab.

2 Click **FCS Processing**.

3 To locate the files, click the browse button to the right of the **Source File** text box.

4 Hold down Ctrl and click each previously normalized FCS file to be concatenated.

IMPORTANT To ensure that the data within the concatenated file is correctly ordered, be sure to select the files in the order they were acquired.

5 Select **Concatenate**. Ensure that **Original Data** is not selected.
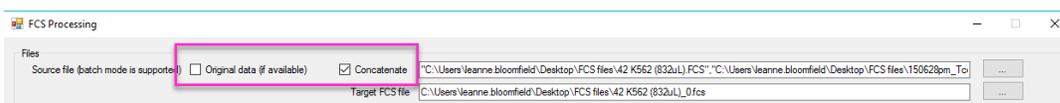
Optional: To select a different path or filename for the concatenated file, click the browse button next to the **Target FCS file** text box or modify the text.
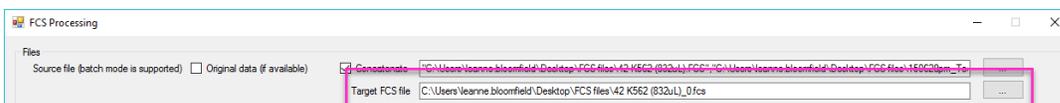
6 Uncheck the **Randomization** and **Normalization** checkboxes.
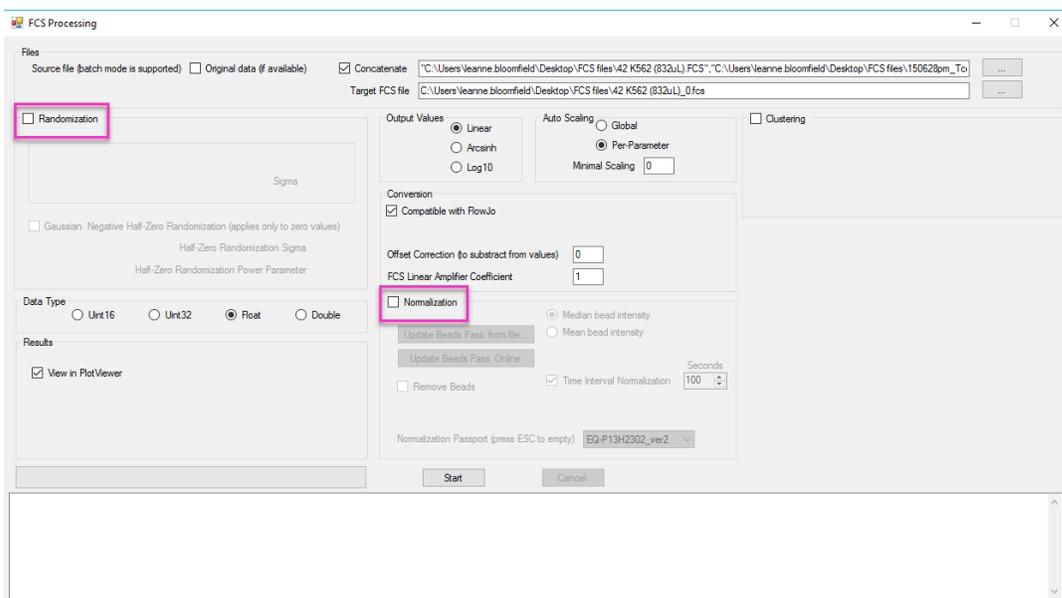
7 Click **Start**.

A concatenated file is generated, saved, and named according to the path and filename entered in the **Target FCS file** field.