Normalization of Mass Cytometry Data using EQ™ Four Element Beads

Introduction

Mass cytometry combines the advantages of single cell high speed analysis common to flow cytometry with the ability to measure over 90 metal-conjugated probes with minimal signal overlap common to atomic mass spectrometry, thereby enabling researchers to generate high resolution phenotypic and functional profiles of cells from normal and diseased states.

Given the depth of the biology revealed, it is critical to account for variations related to instrument performance. In mass cytometry, acquisition of large numbers of cells over the course of an experiment results in signal strength decay caused by the accretion of cellular material in the instrument. Furthermore, manual interventions such as instrument cleaning and calibration trigger shifts in performance. Thus, in order to produce an accurate interpretation of the biological differences between samples, it is imperative that measurement variations are minimized in the final data.

This document describes a method to correct for short-term and long-term instrument sensitivity fluctuations. It involves spiking cellular samples with metal-containing bead standards, followed by normalization of acquired sample data through algorithmic processing against the bead standards. The output is an *.fcs* file containing the normalized single-cell mass cytometry data available for comparative studies with sample data from different individuals, treated under different conditions, at different time points.

Bead Standards

There are two types of beads available from DVS Sciences that can be used for normalization:

- **EQ™ Four Element Calibration Beads** (EQ Beads, Cat# 201078), which contain natural abundance cerium ($^{140/142}$Ce), europium ($^{151/153}$Eu), holmium (^{165}Ho), and lutetium ($^{175/176}$Lu).

- **CyTOF® Calibration Beads** (Eu Beads, Cat# 201073), which contain natural abundance europium ($^{151/153}$Eu).

EQ Beads are recommended for normalization because they have a unique multi-element signature that includes cerium, an element that is not available for mass cytometry panel

design, which enables such beads to be more readily discriminated from sample material. Eu beads can also be used, with two caveats: 1) the cell sample must be labeled with iridium, as this difference enables discrimination of Eu+Ir- bead events from Eu+Ir+ cell events, and 2) only one of the two Eu isotopes (151, 153) are used to label the cells.

Note: This document focuses on normalization using EQ Beads.

Normalization Methods

Two algorithmic processing options are available for normalization of data collected in .fcs file format. Both methods normalize for intra- and inter-file signal drift.

1. **Fluidigm (DVS) method:** Available on CyTOF SW version 6.0.626 and above. This method normalizes data to a global standard determined for each lot of manufactured EQ Beads, and allows normalization of data within and across experiments as well as across instruments The information for each lot of EQ Beads is captured in the form of a "Passport". The software is pre-loaded with passports for all the manufactured bead lots. See Appendix A for a complete description of this normalization method.

   Note: Eu Beads do not have Passport values assigned and data collected using these beads must be normalized using the MATLAB Method, described below.

2. **MATLAB® Method:** Available through freeware offered by Stanford University. This method normalizes data using median bead intensity calculated from across the experimental data files instead of a global predetermined standard (Finck et al, 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.

Sample Preparation and Data Collection

Sample Prep for Manual Loading

1. Remove beads (EQ or Eu) from refrigerator and shake the bottle vigorously to re-suspend.
2. Prepare sufficient volume of 0.1X beads to re-suspend all samples in the experiment by diluting 1 part beads to 9 parts type I ultrapure, 18MΩ deionized water (DIW).

3. After completing the final water wash of the cell sample (see Protocols available at www.DVSSciences.com), but immediately prior to injecting sample for analysis, re-suspend the resultant cell pellet in 0.1X bead solution (~30,000 EQ beads per ml).
4. Filter the cell-bead suspension through a 35 to 45 µm mesh immediately prior to injection into the CyTOF® or CyTOF2® instrument.

Sample Prep for Autosampler Use

1. After the last deionized water wash of the cell sample (see Protocols available at www.DVSSciences.com), re-suspend cells in an appropriate volume of type I ultrapure, 18MΩ deionized water (DIW) and filter each sample through a 35 to 45 µm mesh.

2. Transfer cell samples to a 96 deep-well plate (Whatman 96-well UniPlate Round Bottom Microplates- Whatman # 7701-5200; Fisher Scientific catalog # 09-003-36).

3. Pellet cells, aspirate supernatant and re-suspend pellets vigorously in 50 - 100 µL of DIW.

4. Remove beads (EQ or Eu) from refrigerator and shake the bottle vigorously to re-suspend.

5. Add 50 µL of undiluted EQ or Eu Beads to each sample, pipetting to re-suspend.

6. Program the Autosampler unit to add an appropriate volume of buffer to each well to achieve a cell concentration of approximately 3.0 to 5.0 x 10^5 per mL.

Data Collection

1. When creating the CyTOF or CyTOF2 Template, select the following metals, in addition to those used in cell labeling:
   i. For EQ Beads: select Ce140, Eu151, Eu153, Ho165 and Lu175
   ii. For Eu Beads: select Eu151, Eu153, Ir191, Ir193
2. Set the data acquisition stop value (either volume or time) to ensure that at least 500 beads are acquired per FCS file.

   Note: the bead concentration per sample can often be reduced below 1/10 as the total volume of sample collected per FCS file is increased. A minimum total of 500 bead events per FCS file needs to be collected in order for the normalization algorithm to function properly. For samples where volumes over 100 µL will be collected, reducing the total beads added to each sample will reduce the number of bead-cell coincident events, but care must be taken to ensure that enough beads are collected per unit time to allow normalization.

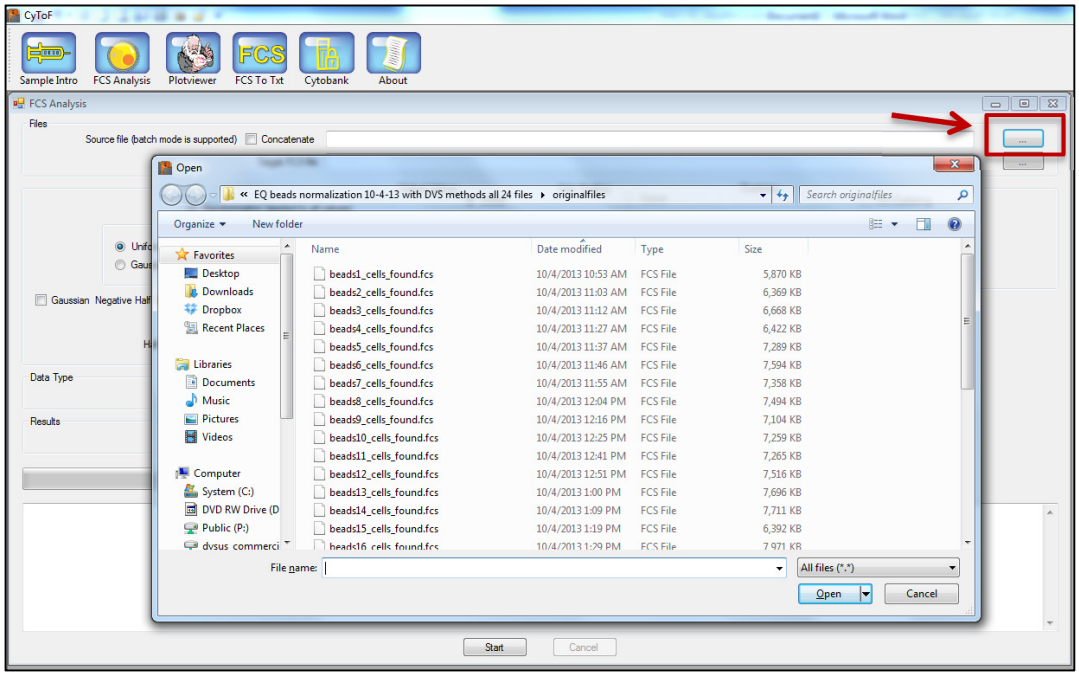
Data Normalization Procedure

Fluidigm (DVS) Method

Note 1: This method works with EQ beads, only, and requires a lot-specific bead passport. See Appendix A for a complete description of this method.

Note 2: If file concatenation is required, it should be performed ***before*** the normalization procedure.

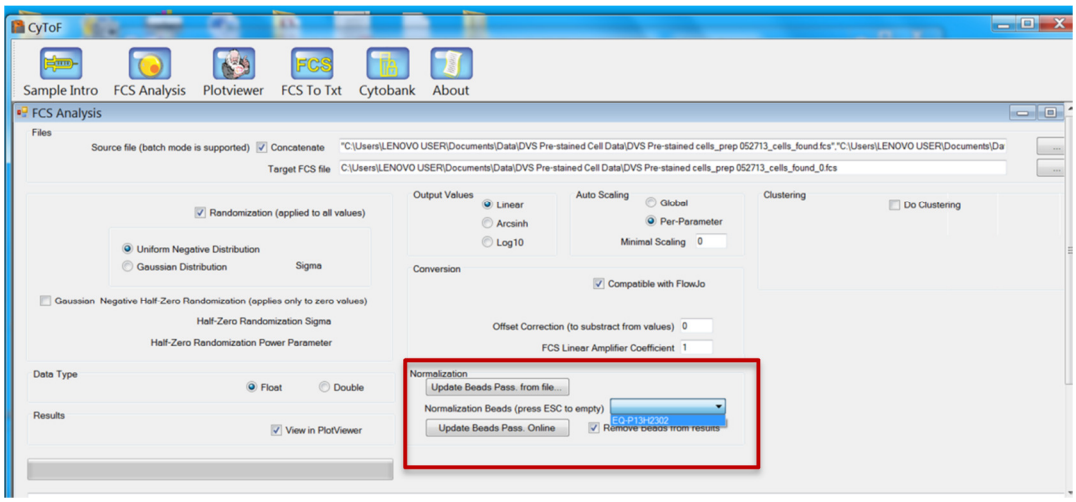
1. If needed, install CyTOF SW v6.0.626 (see Appendix B).
2. Launch the software and click **FCS Analysis** in the menu bar.
3. Load the source .fcs files to be normalized by clicking the button boxed in red below and navigating to the desired folder.
4. Select all the files to be normalized.



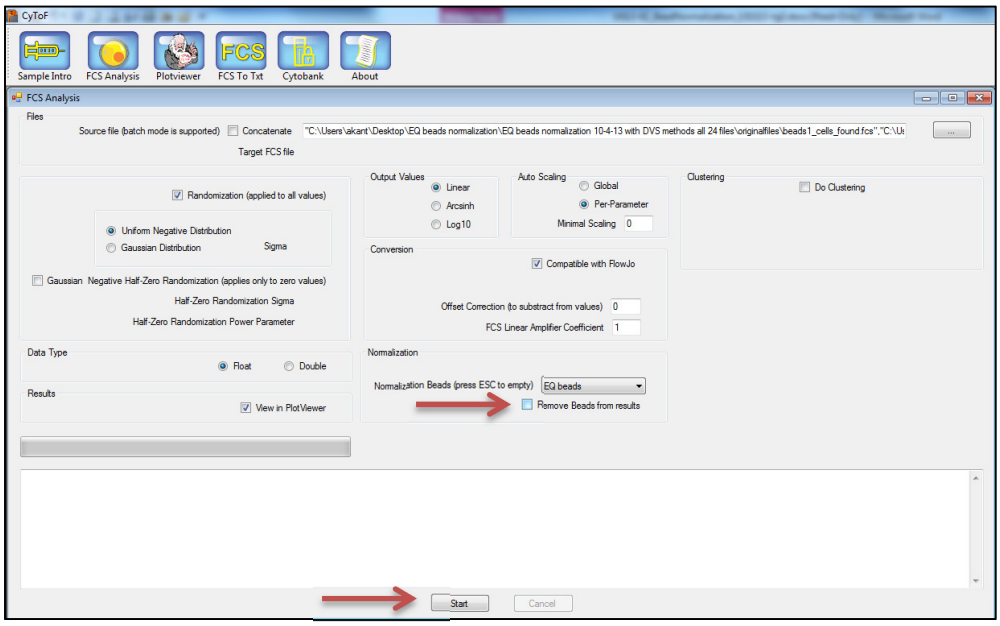
5. Under "Normalization", select the appropriate passport number for the Normalization Beads from the dropdown menu (see red box in figure below).

Note: CyTOF software v6.0.626 comes loaded with passport numbers for released lots of the beads. For each new lot of beads, DVS provides a config file to the

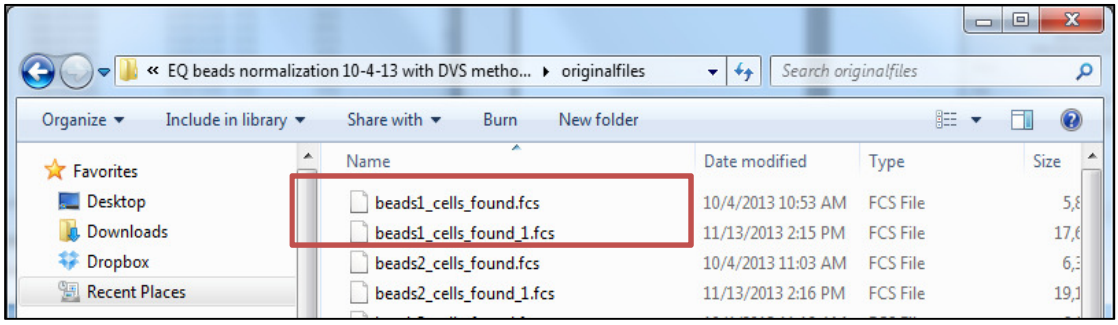
customer along with instructions to add the passport number to the list in the dropdown menu. Please contact technical support at http://www.dvssciences.com/support.php for more information.



6. Uncheck “Remove beads from the result”.
7. Click **Start**.



8. A new file is created for each fcs file that is normalized. The normalized files will be saved in the same folder as the source files, but with a suffix ("_1") appended to the file name.



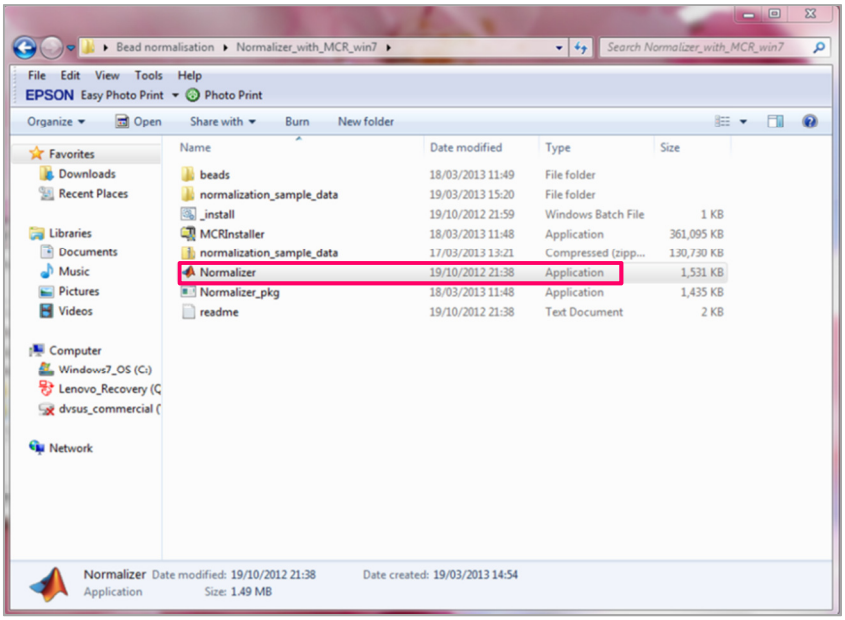
9. The normalized data may now be analyzed using the desired software package.

MATLAB Method

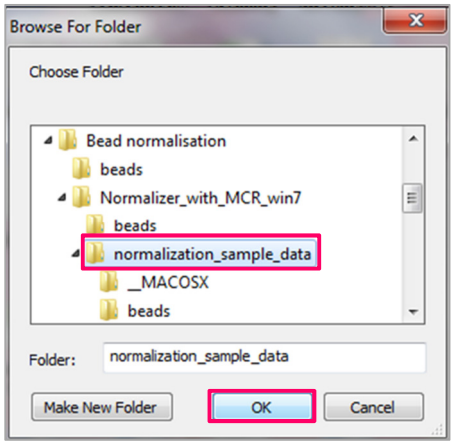
Note 1: This method requires collection of the iridium channel, even if the sample does not contain iridium.

Note 2: If file concatenation is required, perform this ***before*** the normalization procedure.

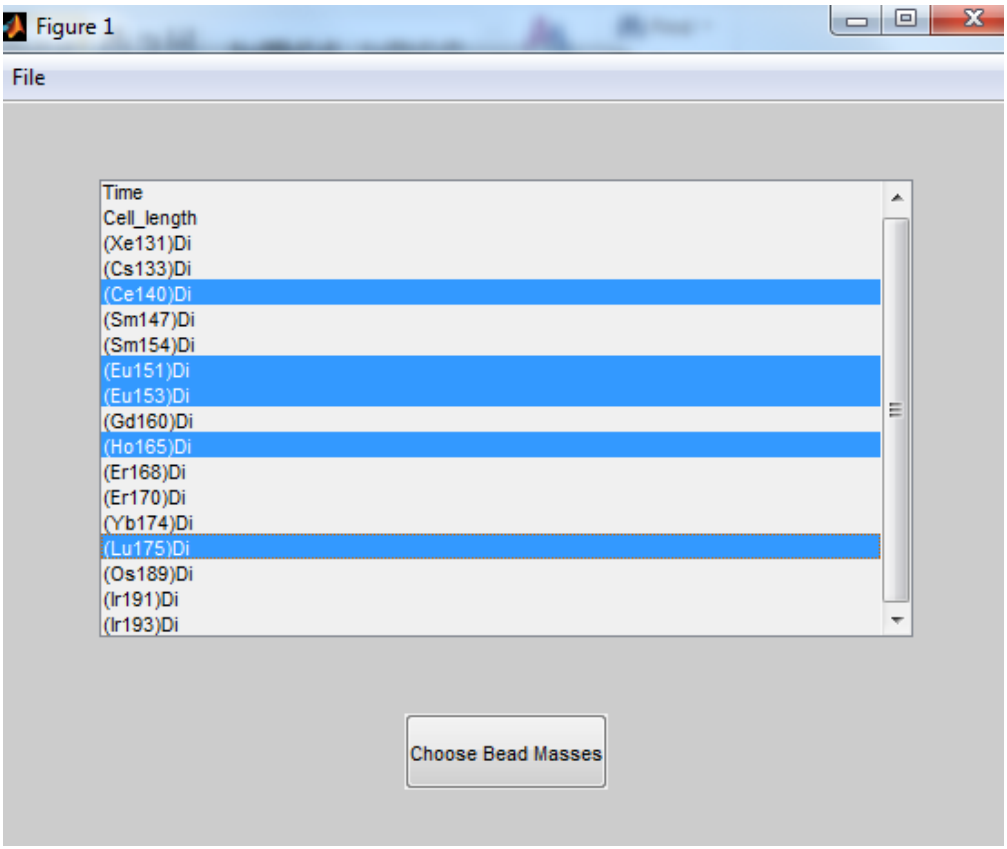
1. Install the MATLAB-based Normalizer Software (see Appendix B).
2. Ensure that the .fcs files to be analyzed are placed in a folder that does not contain any other files. This includes any previously normalized .fcs files which may be located in the sub-folders with the default name of **normed** and **beads**.
3. Open the Normalizer software by right-clicking on the Normalizer application and selecting Open.



4. Select the folder that contains the .fcs files to be analyzed and click **OK**.

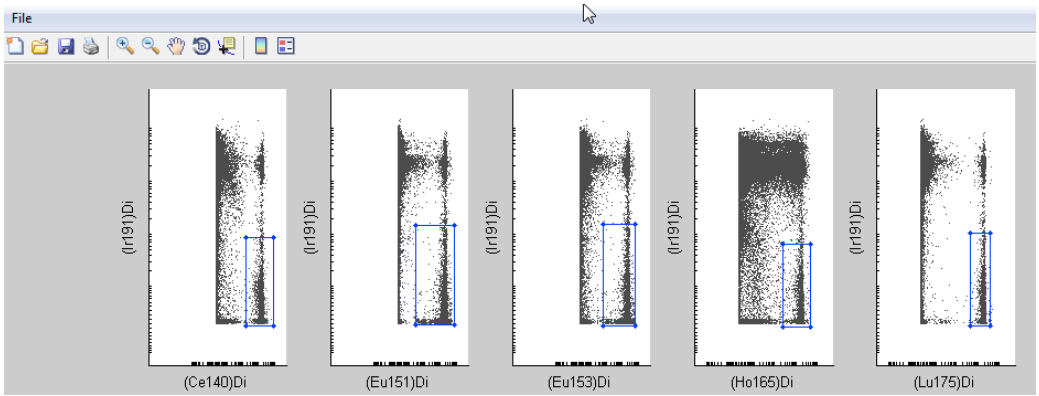


5. The list of acquired CyTOF® parameters will be displayed. From the list, select the masses used for the bead channels and click **Choose Bead Masses**.



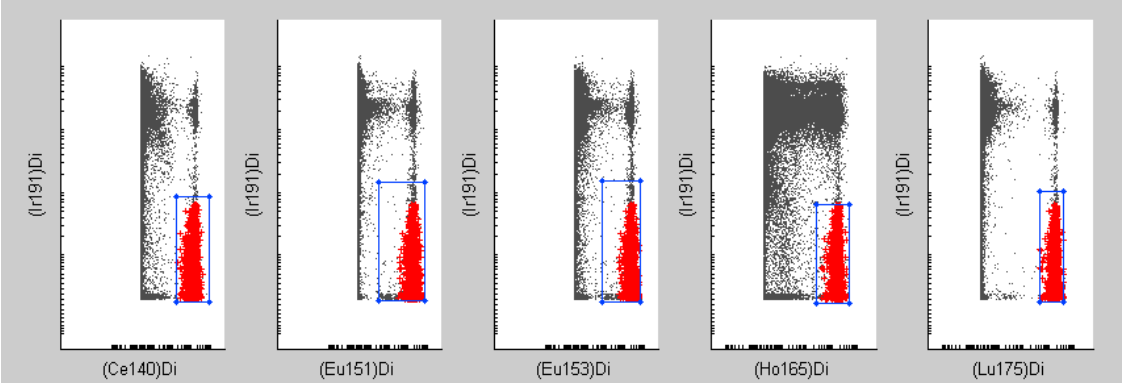
NOTE: the software identifies beads as those events that are positive for all the masses that are selected in this window.

6. The first selected data file will be displayed with each bead mass plotted against ^{193}Ir.

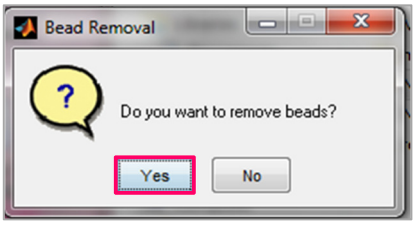


7. Press the space bar to highlight the gated events in red.

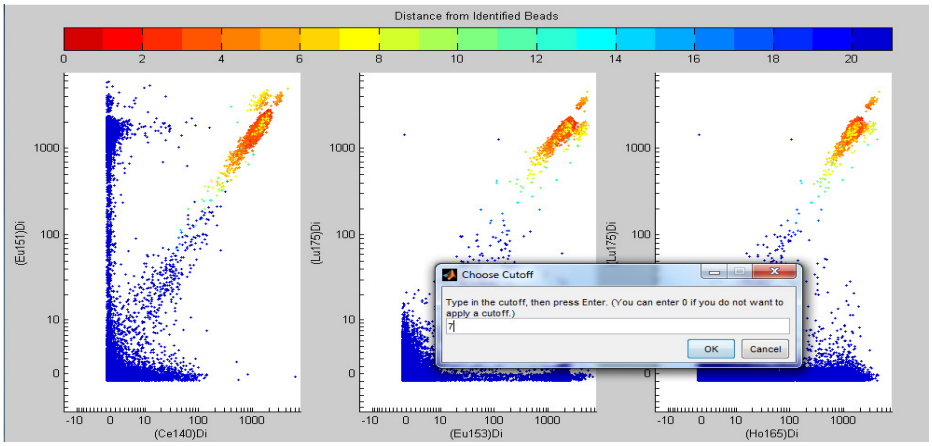
8. Adjust the gate on each plot to include the bead population, defined as high intensity for each bead channel and low intensity for ^{193}Ir. Only those events that are simultaneously present in every gate will be classified by the software as a bead.



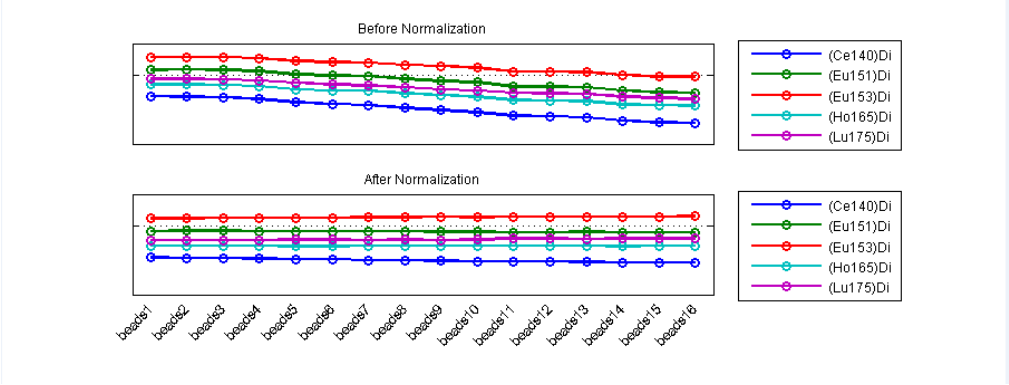
9. After correctly adjusting the gates, press **Enter** to progress to the next file.
10. Repeat this gating procedure for each file to be normalized.
11. The following message will be displayed. To remove the bead events from the normalized data files, click **Yes**.



12. To remove the bead population, select the required cut-off value from the color chart. Red coloration represents the events closest to the gates that were defined in step 6, while blue indicates those events furthest away. A cut-off value of 6-7 is recommended in order to remove both beads and bead-cell doublets. For consistency, it is recommended to input the same cut-off value for each data file.

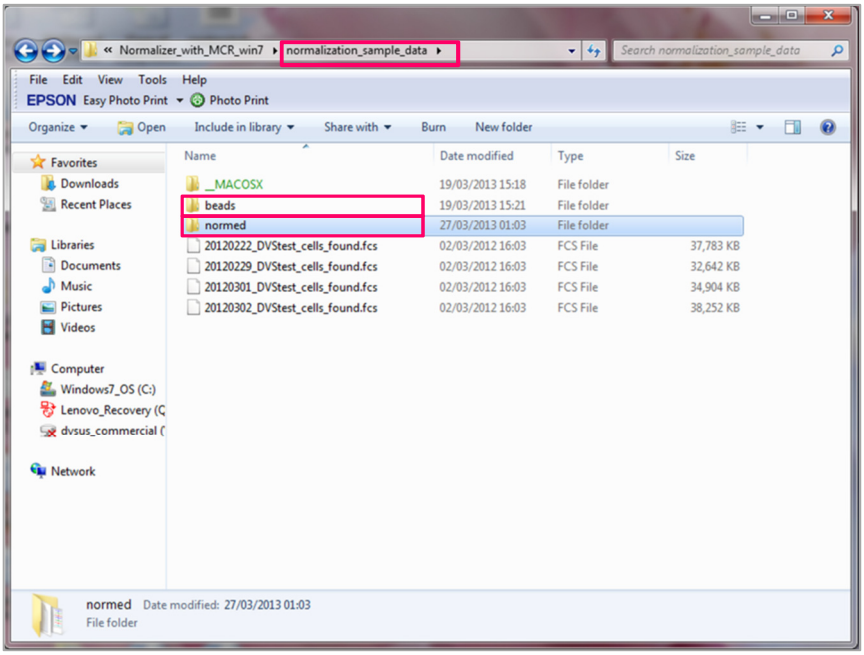


13. Repeat the above step for each individual .fcs file.
14. The normalization is completed and both original and normalized data will now be displayed.

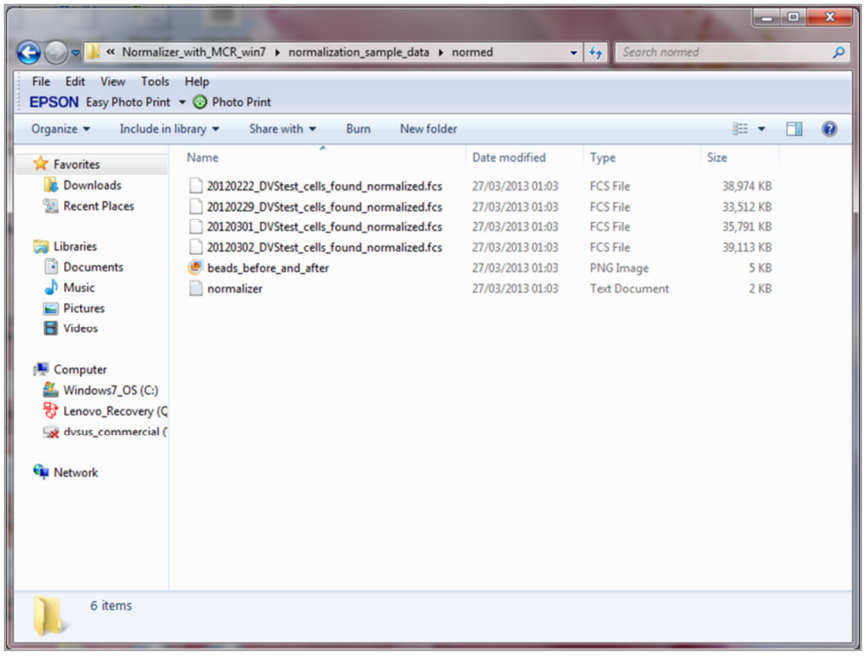


15. Exit the software by closing the above window.

16. Navigate to the folder that was originally selected in Step d, which contains the files that were analyzed. Within this folder are two new sub-folders named **normed** and **beads**.



17. The **normed** folder will contain the normalized .fcs files, along with an image file illustrating the data pre- and post-normalization. The **bead** folder will contain the .fcs files corresponding to the beads.



18. The normalized data may now be analyzed using the desired software package.

Please note that a new folder must be created if you wish to re-process your original .fcs files through the normalizer software. It is recommended to use one folder per normalization experiment.

Example Data

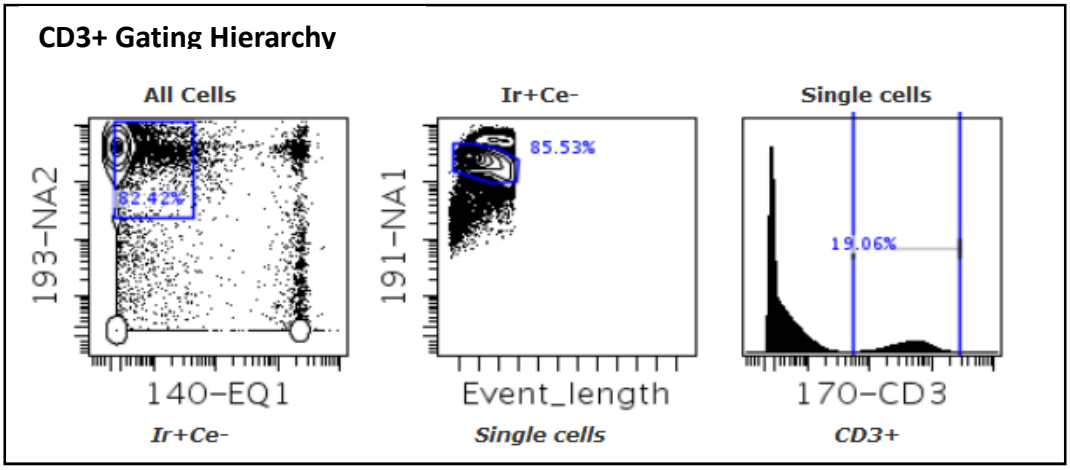
Normalization of mass cytometry data generated from human PBMCs stained with metal conjugated antibodies

1. Four million human PBMC pre-stained with 7 surface markers (DVS, Cat# 201080) were centrifuged and re-suspended in 4 mL of MaxPar® Fix and Perm Buffer containing a 125nM Iridium intercalator (DVS, Cat# 201192A) and incubated at room temperature for one hour.
2. Cells were then centrifuged, washed twice with MaxPar® Cell Staining Buffer (DVS Cat# 201068) and once with MaxPar Water (DVS Cat# 201069).
3. Finally, cells were centrifuged, re-suspended to $3x10^5$ cells per ml in MaxPar water containing EQ Four element beads at 30,000 beads per mL (0.1X dilution of the bead stock).
4. Aliquots of 500 µL each were prepared to acquire 500 second-data files on a CyTOF2 mass cytometer set to collect the channels indicated in Table 1:

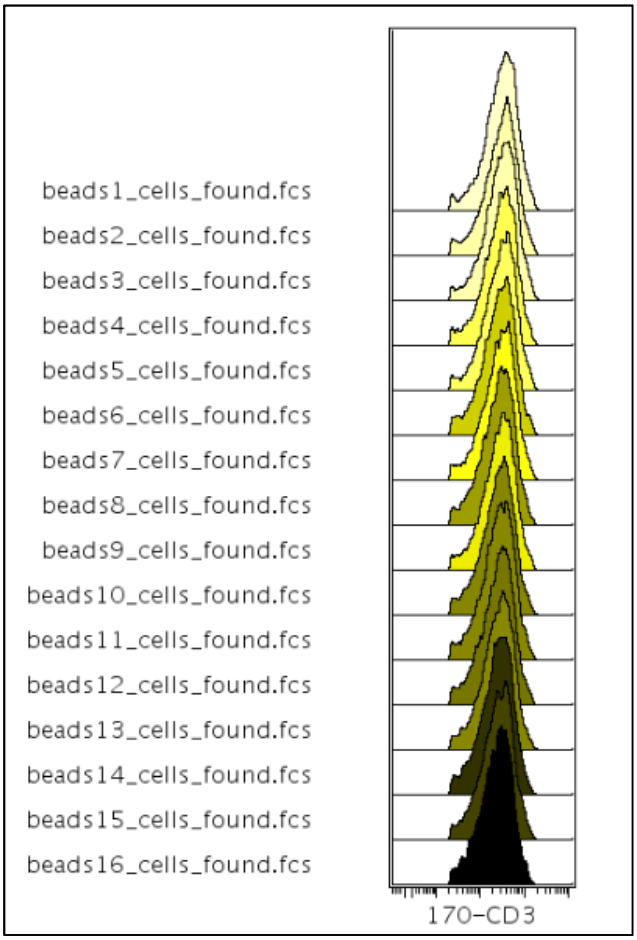
Table 1: Collection channels for EQ Bead + Pre-stained PBMC

Sample	Channel	Target	Note
Pre-stained PBMC	^{147}Sm	CD20	
	^{154}Sm	CD45	
	^{160}Gd	CD14	
	^{165}Ho	CD16	
	^{168}Er	CD8	
	^{170}Er	CD3	
	^{174}Yb	CD4	
	^{189}Os	BKG	
EQ Beads	^{140}Ce	EQ	
	^{151}Eu	EQ	
	^{153}Eu	EQ	
	^{165}Ho	EQ	Common to CD16
	^{175}Lu	EQ	
Intercalator	^{191}Ir	Nucleic Acid	
	^{193}Ir	Nucleic Acid	
Extra Channels	^{131}Xe		
	^{133}Cs		

5. Data was collected and the .fcs files were normalized with respect to EQ beads using the Matlab method.
6. The raw and normalized data files were uploaded to DVS.Cytobank (www.dvs.cyobank.org) and analyzed for comparison in the shift of median Dual Count values for antigens labeled in pre-stained cells.



7. Gating hierarchy for non-normalized raw data files is shown in the figure above. First, cells were gated as Ir+Ce- events to exclude EQ beads from further analysis. Note that the normalized files will not have beads if the normalizer was instructed to remove them in step 2. Single Cells were then gated on iridium dual counts vs. event length plot. Finally, CD3+ cells were gated on a ^{170}Er Dual Count histogram.



8. Median ^{170}Er-CD3 Dual Count values for each file were obtained for raw data and data normalized with Matlab and DVS methods. Results are plotted in the figure below and intensity values are shown in Table 2.

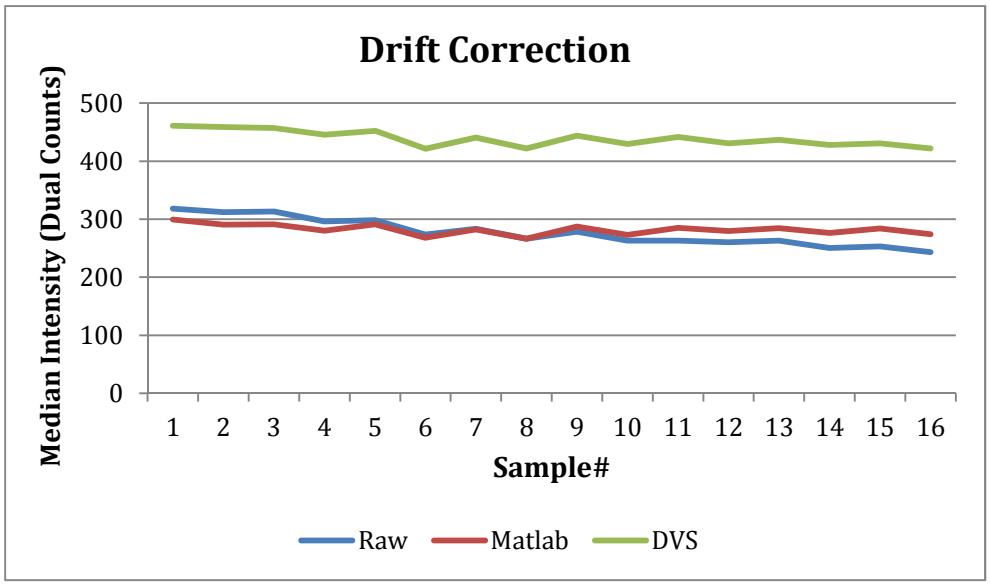


Table 2: Median CD3 Dual Count values prior to (Raw) and after the indicated normalization method

Sample#	Raw	Matlab	DVS
1	318.42	299.60	461.3
2	312.01	290.84	458.86
3	313.31	291.43	457.42
4	296.30	280.27	445.75
5	298.38	291.09	452.41
6	273.64	268.17	421.11
7	283.50	282.42	440.59
8	266.37	266.56	422.09
9	278.27	287.62	443.98
10	262.94	273.15	429.43
11	263.20	284.94	441.67
12	260.59	279.57	430.65
13	263.16	284.35	437.01
14	250.40	276.51	428.16
15	253.07	284.15	430.79
16	243.00	274.36	422.19
%CV	**8.60579454**	**3.18912011**	**3.073086**

Appendix A: Overview of DVS Normalization Method

The DVS Normalization Method is based on the concept of a "Bead Passport", which is a global standard generated by the manufacturer for a specific lot of EQ beads. This Passport is universal across all instruments for the same type and cannot be changed by individual users. 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.

DVS Normalization Method is a two-step process:

1. **Singlet identification**
   Singlet bead events are identified from bead-bead and bead-cell aggregates. This population is employed as the internal standard for normalization.

2. **Normalization**
   The normalization factor is the ratio of Passport median Di values to bead singlet population median Di values of the encoding isotopes. Isotopes in the EQ beads cover an extensive portion of the mass range measurable on CyTOF 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 (a separately run bead sample, for example) cannot be utilized by the algorithm for the normalization procedure.

Appendix B: Software installation instructions

DVS CyTOF Software v6.0.626

Note: This version of software can be installed on a standalone Windows PC computer or on a CyTOF 2 instrument computer, but should NOT be installed on a CyTOF instrument computer. CyTOF users must transfer their acquired fcs data files to a standalone PC to perform normalization.

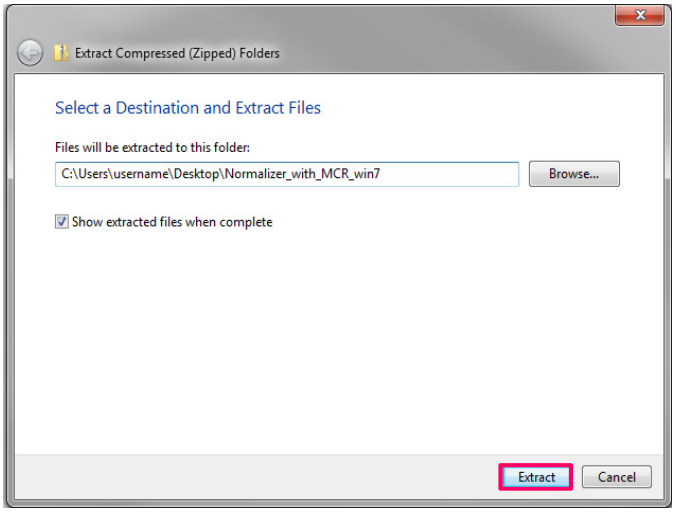
Contact support@dvssciences.com to obtain a copy of CyTOF software version 6.0.626, and directions on installation.

MATLAB-based Normalizer Software

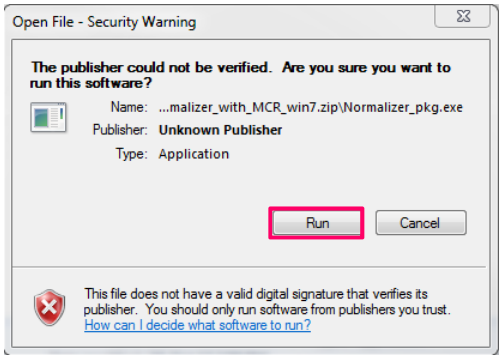
Please note that the current version of the Software is only compatible with the 64-bit version of Windows® 7/8.

Only download files compatible with your computer operating system.

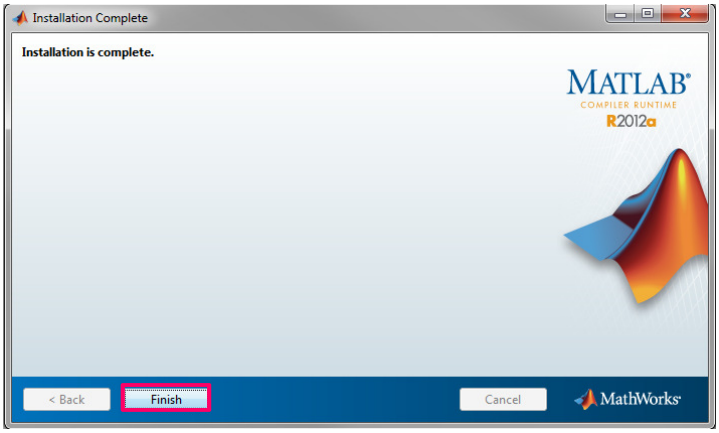
1. Download the MATLAB Compiler Runtime and the Normalizer Software from the following link:
   http://www.stanford.edu/~rfinck/normalization/download_software.html
   The optional sample data can be downloaded from the following link: **https://dl.dropbox.com/u/4437077/normalization_sample_data.zip**
2. Move these downloaded files to your desktop
3. Right-click on the **MCRInstaller** archive on your desktop, select **Extract All…**
4. When prompted to select the destination folder, click on **Extract**.



5. Repeat this procedure for the **Normalization_sample_data.zip** archive as in step 1-2.
6. In the newly created **MCRInstaller** folder, double-click on the **MCRInstaller** executable file.
7. Select **Run** if the security warning window appears.



8. Proceed with MATLAB Compiler Runtime installation onto your computer.
9. Select **Finish** once the installation is complete.



References

Finck R, Simonds EF, Jager A, Krishnaswamy S, Sachs K, Fantl W, Pe'er D, Nolan GP, Bendall SC. Cytometry A. 2013 May;83(5):483-94.