BECKMAN COULTER
Life Sciences

Offline Flow Cytometry Analysis Software:
Importance of Scaling and Data Visualization

Objectives

- Learn about the flow cytometry open platform data structure and how it facilitates for robust offline analysis
- Learn the importance of data scaling and display features in ensuring your data looks consistent with the native environment
- See specific examples comparing the native display to an offline data analysis software across a variety of platforms and manufacturers

Reasons for Considering Offline Analysis

Flow cytometry is a powerful technique and can be applied to answer a variety of questions. The ability to analyze multiple parameters on each cell in a large population gives researchers insight into the heterogeneity of the biological system. The flexibility of the technique has allowed many assays beyond immunophenotyping, to be adapted to the system. Detailed information on cell health and vitality, DNA and RNA analysis and a variety of functional assays yield nuanced understanding when the heterogeneity of the population is factored into the analysis.

Given the diversity of assays possible and the unique application of the technique to research questions, the analysis requirements across different areas varies. The analysis software that is provided with the flow cytometer provides the basic features for the most common situations. Offline analysis packages offer specialized features and capabilities that significantly improve the analysis experience.

For example, analysis needs when visualizing complex multidimensional data can be improved by improved processing capabilities that allow for real time adjustment to the data display allowing the researcher to visually explore the information and identify nuances or unanticipated findings. Multicolor data also places increased demands on algorithms such as fluorescent signal compensation. In contrast, if the aim of the laboratory is to use flow cytometry to monitor some biological event across many samples and over time, the workflow could be improved by batch processing capabilities.

Standardized File Format

The ability of the field of flow cytometry to benefit from third party providers of specialized analysis software comes from the decision to standardize the data acquisition file format. In 1984 a standard format for flow cytometry data was proposed. A Data File Standards Committee of the International Society for Analytical Cytology (ISAC) was formed to maintain the standard, called FCS for flow cytometry standard. FCS3.1 is the current standard and has been adopted by most instrument manufacturers.

The raw data file from a flow cytometer is termed list mode. This refers to the fact that the parameter data collected on each event is stored in the order that the event passed through the interrogation point. The FCS 3.0 standard describes the format that this data is stored into the computer file.

FCS files must have four major segments including HEADER, TEXT, DATA and ANALYSIS. The header section identifies the FCS standard used and outlines the how to find the other sections in the file. The TEXT section contains keywords and keyword values, describing the data set. These include user defined values. The DATA section contains the raw data. An optional ANALYSIS section includes the results of any data processing completed at the time of acquisition.

Offline analysis software takes advantage of this structured file to define the method for loading data regardless of the instrument used for acquisition.

Visualizing Data

Each instrument manufacturer will have settings to scale the data for display during acquisition. Prior to FCS 3.1 data scaling was controlled by the file. With FCS 3.1 data scaling is now controlled by the software doing the analysis. To ensure that the data looks the same in the analysis software as it did during the acquisition, the instrument default preferences need to be applied.

FCS contains a keyword to identify the cytometer used for acquisition. Analysis software can read this keyword, $CYT, and apply preferences that control the display of data and information. When loading the first file from a given instrument, the preferences for that instrument are established. These can then be refined by the user as needed.

The following example shows the instrument default settings that are available for adjustment in Kaluza Analysis Software.

Kaluza Software Options and Defaults

The left list shows the all loaded names of the acquisition instrument. And each one contains its own Cytometer setting information.

The settings are described as below:
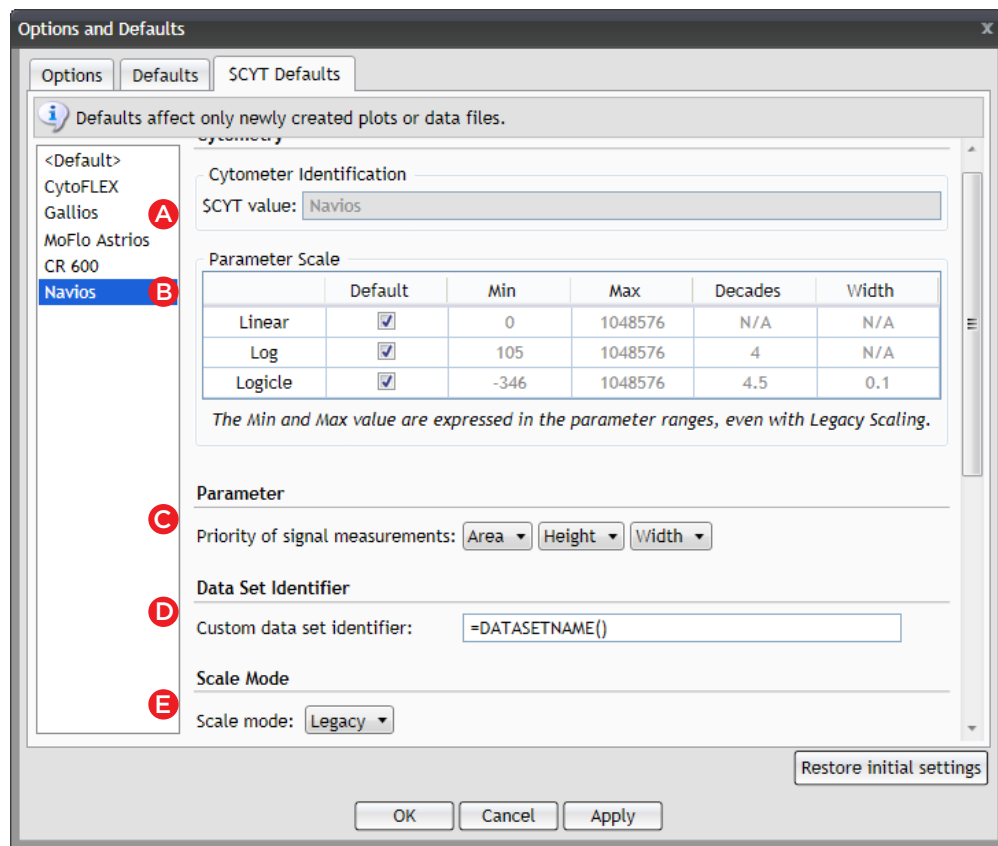
A **$CYT value,** which is the instrument identifier.

B **Parameter Scale** grid, it is used to set the default axis display setting.

There are 3 axis instrument identifier. "Linear" "Log" and "Logicle". The grid has 3 rows to let the user set display setting for each kind of axis. The "Min" column is the displayed minimum vaue for an axis, and "Max" column is the displayed maximum value for an axis. The "Decades" is only used in Log or Logicle axis. The "Width" is the **Negative Percentage** value for Logicle axis. Use the axis setting from FCS file. If the "Default" column check-box is checked, the Kaluza would work the same as Kaluza 1.5a. User could change the settings by unchecking the "Default" check-box, and input the valid expected value in the corresponding cell of grid.

C **Parameter**

The priority of signal measurement (Area, Height, Width). The first signal measurement listed must be the one with the highest resolution on the acquisition instrument. When there are multiple measurements captured for a given PMT, Kaluza uses this order to determine which parameters to use for setting up Compensations and which plots are displayed when Add All Plots is selected.

D **Data Set Identifier**

The default formula used to identify Data Sets when loading new data files(*.lmd, *.fcs). The Data Set Identifier is used in the Data Set column in the Analysis List, as well as anywhere the Data Set name is shown throughout the software.

NOTE The formula may include keywords from the FCS header information, or other custom content.

E **Scale Mode**

Configures the default plot axis scale:
- Full Range scale would make the axis scale to real event data.
- Legacy scale would make the axis scale to maximum 1024, same as Kaluza 1.3 or before.
- Astrios scale changes several Kaluza defaults to provide the means to recreate plots that were created in the Astrios acquisition software.

NOTE Astrios is a Scale Mode option only when Astrios is listed as an available instrument on the left side of this window.
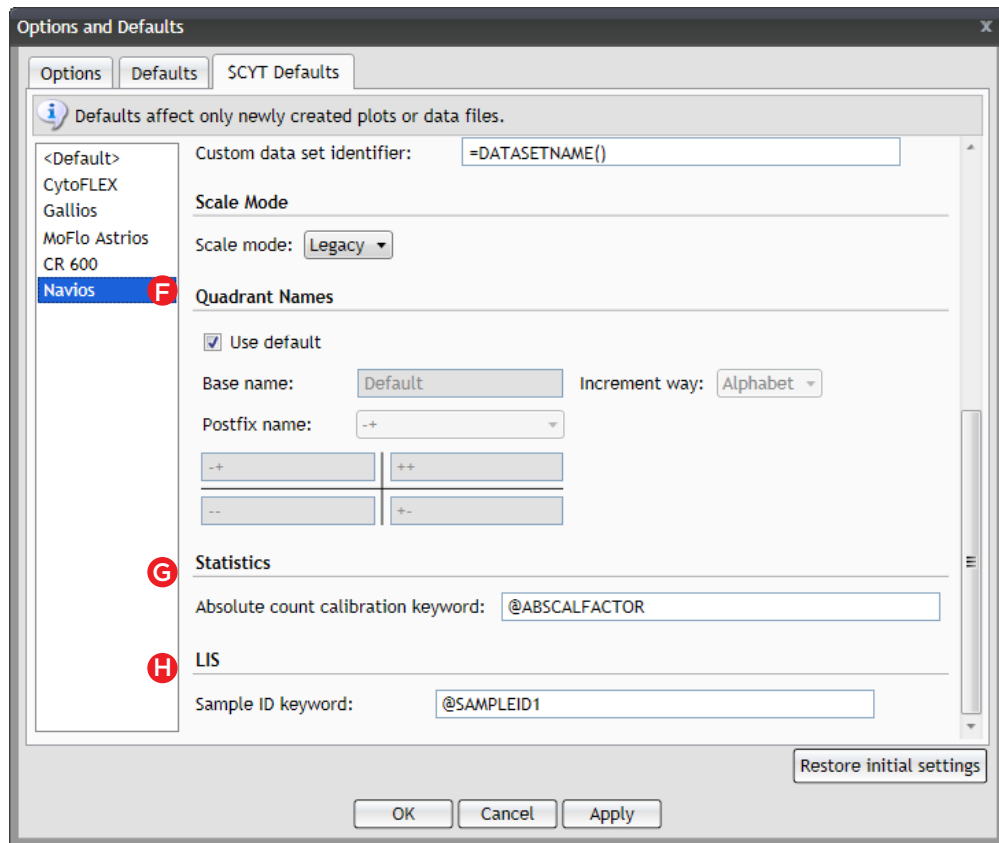
F **Quadrant Names**

The Quadrant Names section allows you to update the naming settings for Quadrant gates. Here, you can choose to use the default gate names or customize the components of Quadrant gate names, including the following:

Base Name: The name of the quadrant begins with the **Base Name.** The **Base Name** is the same in every quadrant.

Increment Way: How names are incremented. Options include: **Alphabet / Number**

G **Statistics**

Allows you to set/change the Absolute Count calibration keyword.

NOTE The Absolute Count keyword defines the calibration factor of the beads used with this sample if Flow-Count or similar absolute calibration particles are used. If the calibration factor is not provided, this keyword contains "NOT SET".

H **LIS**

Allows you to set/change the Laboratory Information System (LIS) **Sample ID Keyword.**

Data Examples

In order to compare data visualization of Kaluza software files from various instruments were collected. We obtained the resulting FCS files from the acquisition, reports documenting the user defined plots, indicating the native scaling, and statistics. The FCS files were then loaded into Kaluza and the same plots and statistics were generated.

Presented plots and values are for illustrative purposes only and are based on customer reported data. Some information has been modified to simplify the presentation. Individual results may vary.

CytoFLEX Flow Cytometer* (Beckman Coulter)

Top row shows CytoFELX data analyzed with CytExpert software. Bottom row shows the same plots generated within Kaluza after importing the FCS file.
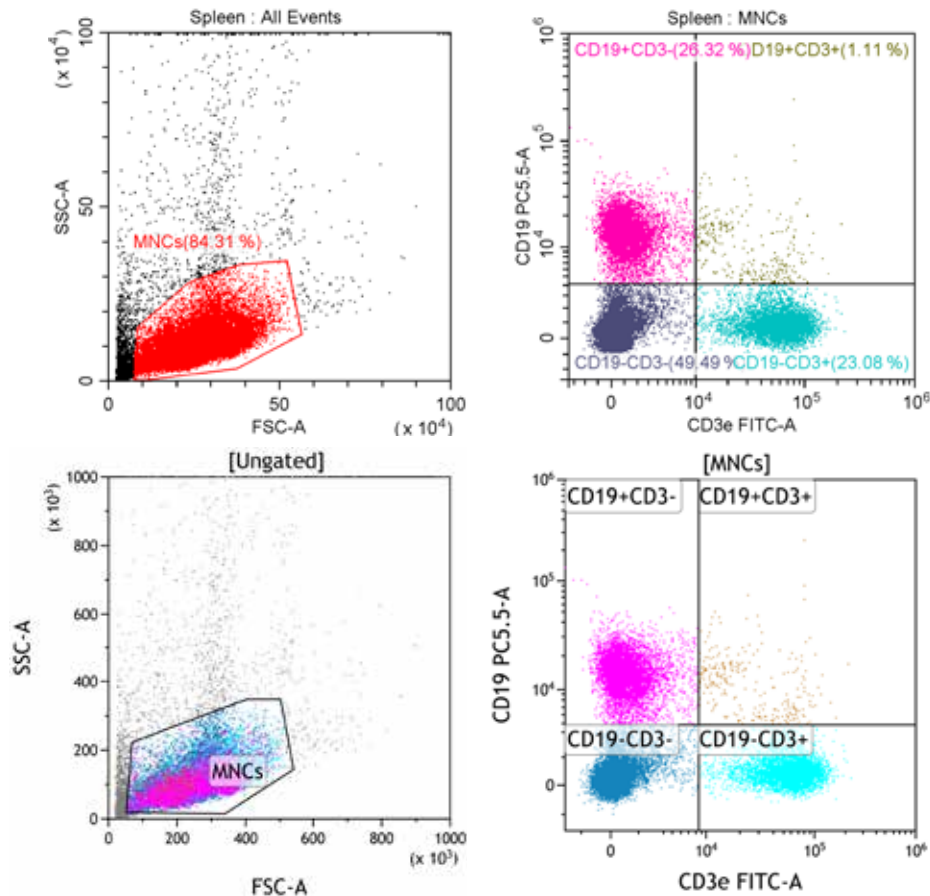
Exported Statistics show consistent numbers and % gated. Variation in the MNC gate is due to the manual placement of that gate.

Table 1: CytExpert Statistics Table.

Tube Name: Spleen
Sample ID:

Population	Events	% Total	% Parent
⬤ All Events	30170	100.00 %	100.00 %
⬤ MNCs	25437	84.31 %	84.31 %
⬤ CD19+CD3+	282	0.93 %	1.11 %
⬤ CD19+CD3-	6696	22.19 %	26.32 %
⬤ CD19-CD3-	12589	41.73 %	49.49 %
⬤ CD19-CD3+	5870	19.46 %	23.08 %

Table 2: Kaluza Statistics Table.

Gate	Number	%Total	%Gated
⬜ All	30,170	100.00	100.00
🟥 MNCs	25,952	86.02	86.02
🟪 CD19+CD3-	6,694	22.19	25.79
🟧 CD19+CD3+	279	0.92	1.08
🟦 CD19-CD3-	13,054	43.27	50.30
⬜ CD19-CD3+	5,925	19.64	22.83

*For research use only. Not for use in diagnostic procedures.

MoFlo XDP Cell Sorter* (Beckman Coulter)

Top row shows MoFlo Astrios data analyzed with Summit software. Bottom row shows the same plots generated within Kaluza after importing the FCS file.
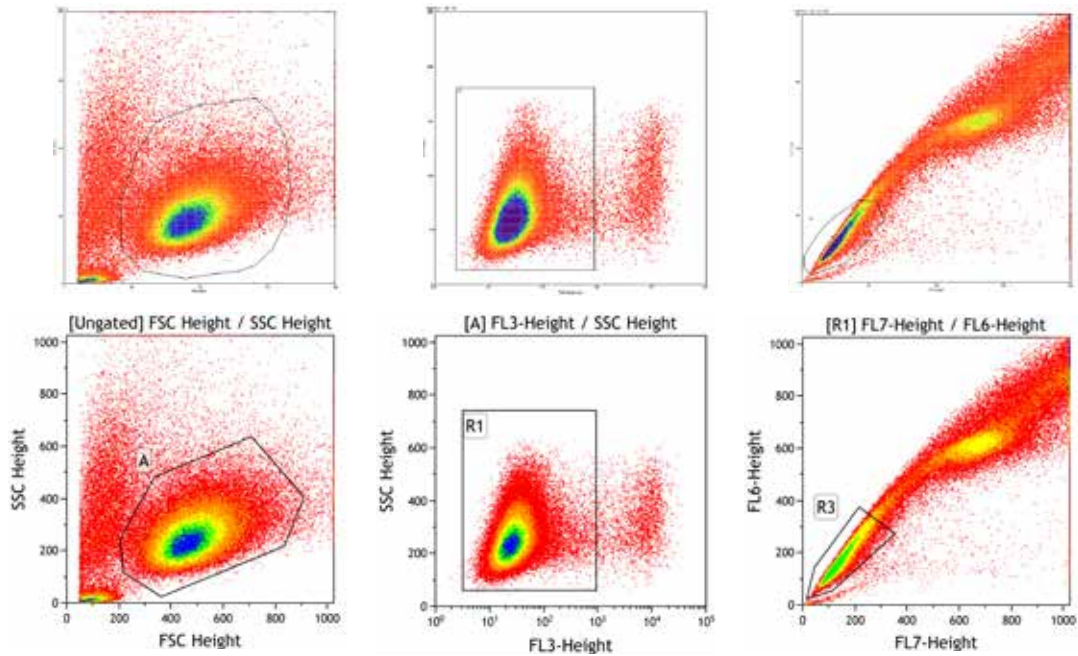
*For research use only. Not for use in diagnostic procedures.

BD FACSVerse™ Flow Cytometer* (BD Bioscience)

Top row shows FACSVerse data analyzed with FACSuite software. Bottom row shows the same plots generated within Kaluza after importing the FCS file.
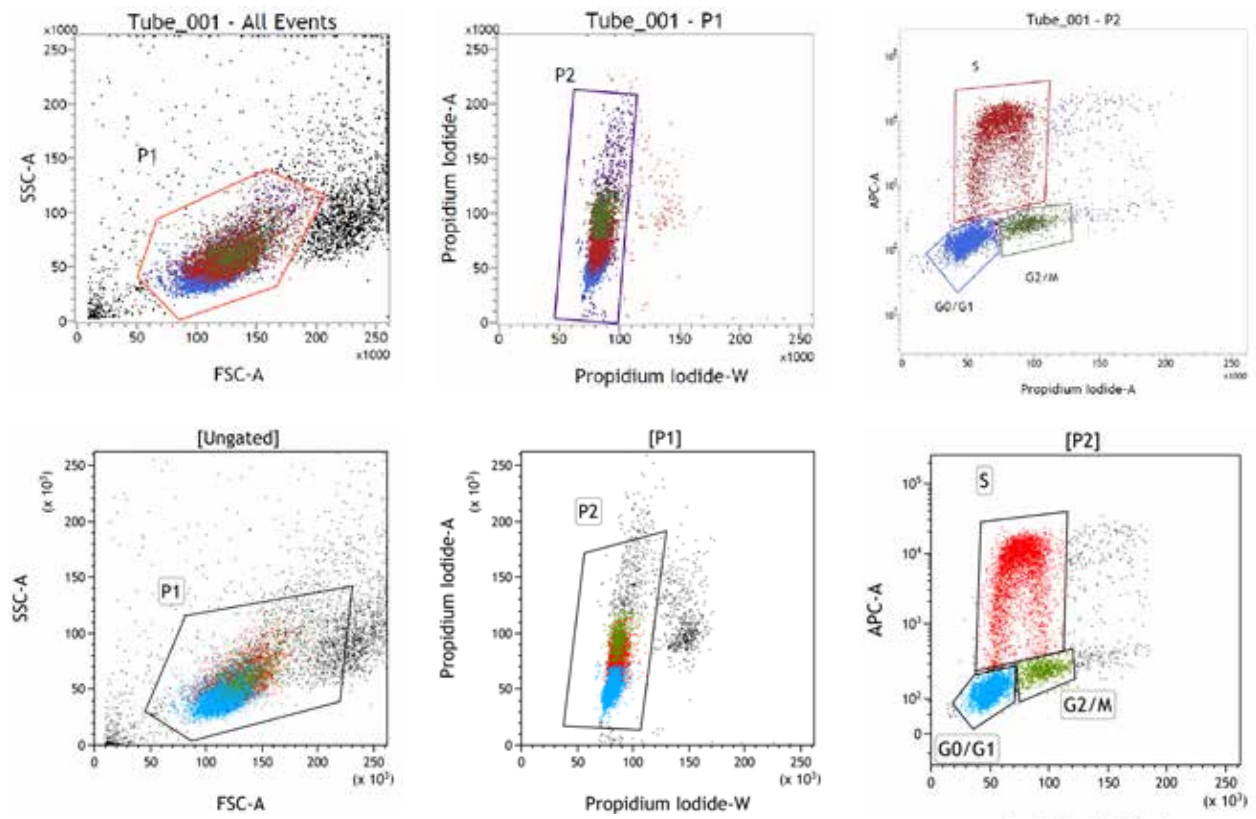
Table 3: FACSuite Statistics

Name	Events	% Parent	% Grandparent	% Total
Tube_001:Tube_001:G0/G1	5,385	52.02	51.31	41.67
Tube_001:Tube_001:G2/M	918	8.87	8.75	7.10
Tube_001:Tube_001:S	3,596	34.74	34.26	27.82

Table 4: Kaluza Statistics

Tube_001

Gate	Number	%Total	%Gated	%GP Gated
G0/G1	5,382	41.64	51.79	48.86
G2/M	898	6.95	8.64	8.15
S	3,720	28.78	35.80	33.77

*For research use only. Not for use in diagnostic procedures.

BD FACSVerse™ Flow Cytometer* (BD Bioscience)

Top row shows FACSCelesta data analyzed with FACSDIVA™ software. Bottom row shows the same plots generated within Kaluza after importing the FCS file.
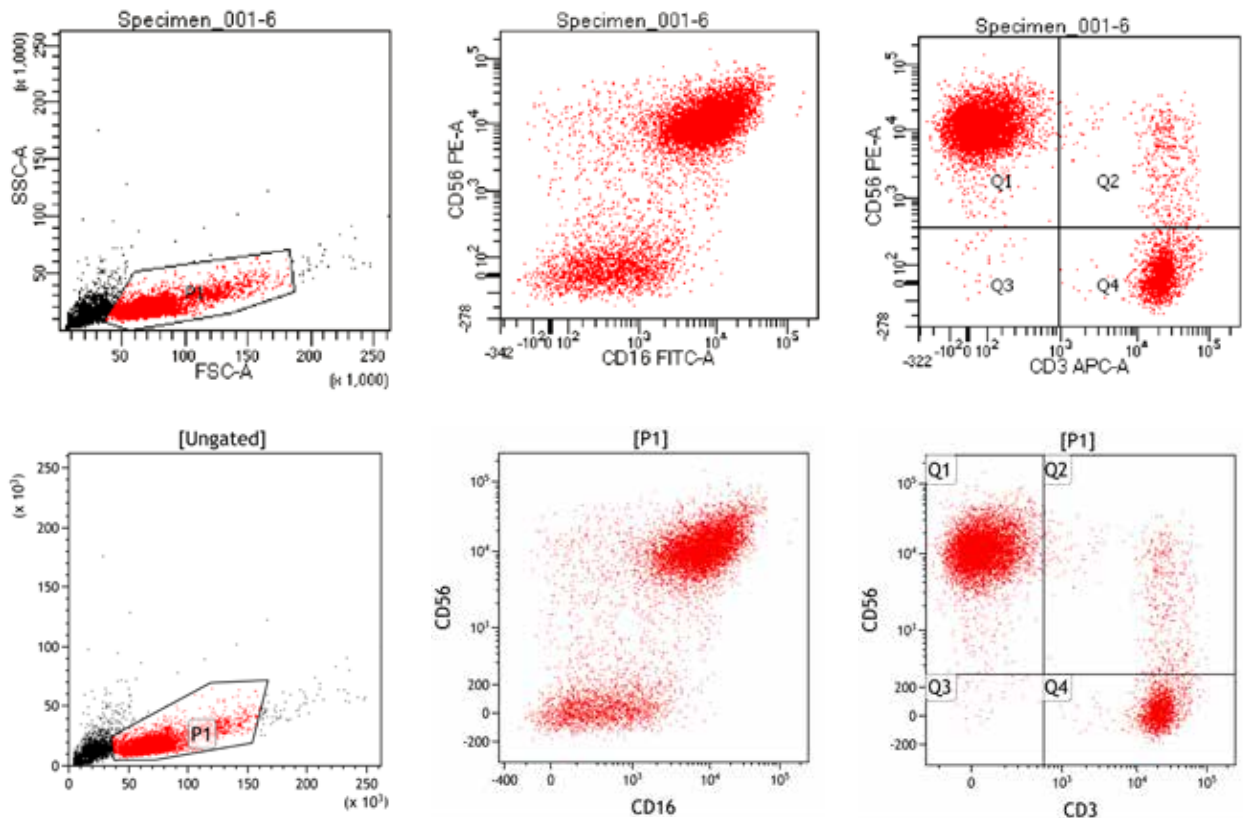
Table 5: BD FACSDIVA Statistics Table.

Experiment Name:		THREE		
Specimen Name:		Specimen_001		
Tube Name:		6		
Record Date:				
SOP:		Administrator		
GUID:		b33cd042-e6e0-4532-8b34-810334899961		

| Population	#Events	%Parent	CD16 FITC-A Mean	CD56 PE-A Mean	CD3 APC-A Mean
All Events	12,991	####	6,729	9,042	4,721
P1	10,150	78.1	7,925	10,296	5,541
Q1	7,768	76.5	9,940	13,082	72
Q2	431	4.2	4,324	6,493	27,563
Q3	33	0.3	4,061	161	74
Q4	1,918	18.9	640	44	22,837

Table 6: Kaluza Statistics Table.

	Gate Number	%Gated	"FITC-A"-AMean-Logicle	"PE-CF594-A"-AMean-Logicle	"APC-A"-AMean-Logicle
All	12,991	100.00	6728.57	16787.86	4720.71
P1	10,186	78.41	7836.62	18930.32	5490.25
Q1	7,766	76.24	9815.62	23990.09	68.69
Q2	509	5.00	4495.37	12160.73	25344.56
Q3	33	0.32	3898.56	616.53	90.81
Q4	1,878	18.44	627.75	163.48	22623.49

Attune NxT Flow Cytometer* (ThermoFisher)

Top row shows Attune NxT data analyzed with system software. Bottom row shows the same plots generated within Kaluza after importing the FCS file.
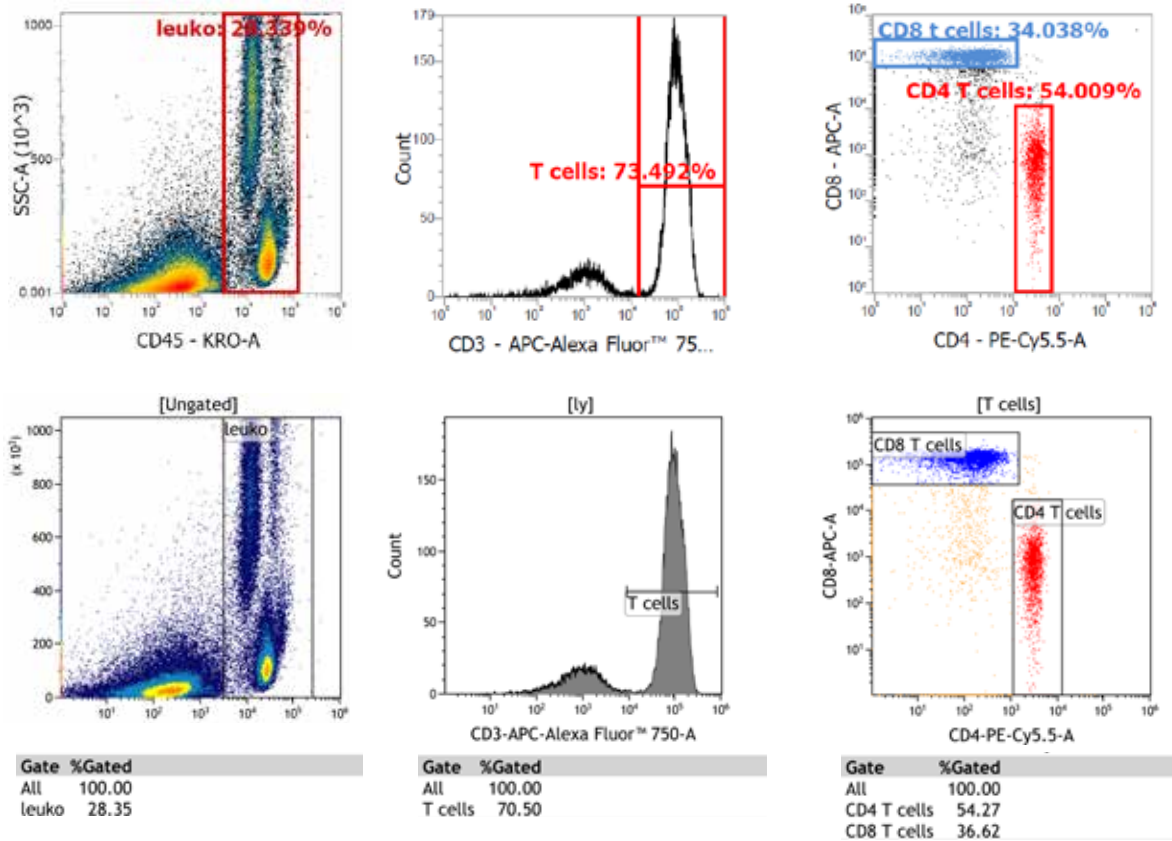
Conclusions

Flow Cytometry Acquisition software, typically provided with the instrument by the manufacturer, provides the basic features for the most common situations, however offline analysis packages offer specialized features and capabilities that significantly improve the analysis experience depending on the needs of the laboratory. Industry standards for flow cytometry data files has facilitated the development of offline packages that are optimized for different types of user needs.In choosinga package users will want to ensure that the data is scaled using the same criteria as specified in the acquisition software. Generated plots will look the same in both the acquisition and analysis software, streamlining the workflow. In this paper, we demonstrated how the Kaluza Analysis offline software package was able to read files from a variety of instruments and suppliers. The data was compared to plots and statistics generated in the native package and provided side-by-side comparisons to compare fidelity.The software will read the parameters from the FCS files and visualize data per the instructions from the manufacturer. An interface with options to adjust these parameters is provided, giving the user exquisite control over their data visualization.