System Equalizer: An automated system setup based on detector calibration and reference cell samples.

Ludovic Monheim,[1] Nathanael Schwab,[2] Aaron J Tyznik[2] and Michael Reitsma[2]

[1]BD Biosciences, Erembodegem, Belgium; [2] BD Biosciences, 2350 Qume Drive, San Jose, CA 95131

Abstract

With the rise of full spectrum flow cytometry, instrument setup has become more challenging due to the increased number of detectors and the unavailability of single stains for every channel.

Multiple solutions have been used to overcome this challenge, but they are often time and reagent consuming or introduce subjective user-specific assessments of individual channel performance to define optimal settings. System Equalizer proposes an automated approach that provides an instrument-specific setting that considers baseline definition run and unstained cell reference data.

At a fixed PMT voltage (PMTV) the amplification of the multiple detected signals is linear. However, the response curves for each individual PMT detector have different profiles at variable voltage points.

System Equalizer uses a curve-fitting approach to match the baseline data outside of the electronic noise-sensitive range and extracts the Voltration data run during the baseline to create detector calibration curves.

Unstained cell reference data allows incorporation of the intrinsic particle difference between unstained beads and target cells.

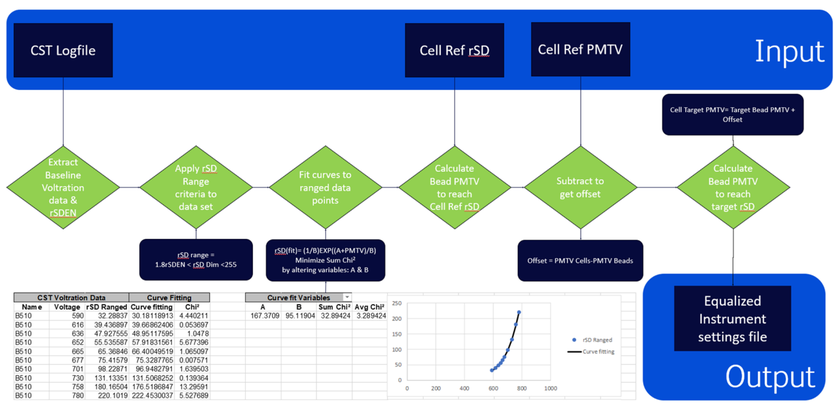
The target PMTVs are set so that cells are detected 1 to 5 times rSDEN and can be directly imported into BD FACSDiva™ Software.

System Equalizer provides an objective way to perform instrument setup based on data (the input logfile and cell reference data) and selected multiplication factor for each PMT detector.

Methods

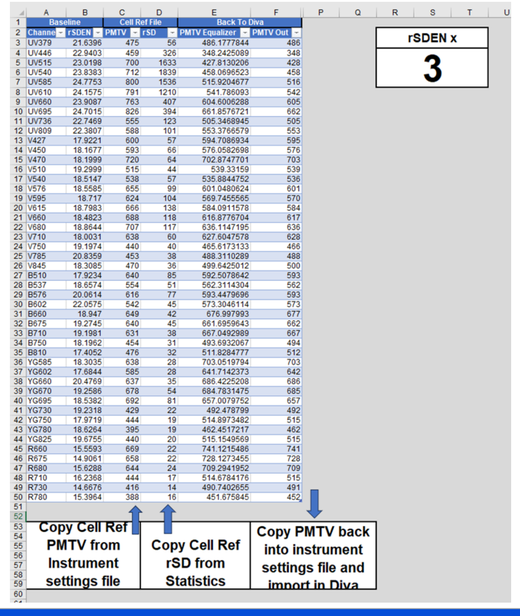
System Equalizer workflow:

Instruments running on FACSDiva™ are calibrated by running a baseline in CS&T™ software. Detector calibration data is extracted from the logfile by file parsing into a csv table to transfer to excel.
Custom calculation columns are added to the data table to get rSD & Stain Index information for every datapoint.
To ensure proper functionality and reduce rSDEN contribution to the curve fit, datapoints are subject to a range criteria evaluation.
Curves are fitted to the datapoints for every detector using the Microsoft Excel add-in Solver to minimize the Sum Chi² value between the fitted curve and the dataset by altering the fitted curve variables A & B.
Unstained Cell reference data is acquired in FACSDiva™. .
Instrument settings (Cell Ref PMTV) and population statistics (Cell Ref rSD) are exported in instrument settings file and .csv files respectively and then added into the Equalizer table.
A delta PMTV between the reference data and the fitted curve is calculated to define the offset between the beads and the reference cells.
A multiplier is selected between 1 & 5 x rSDEN and PMTV are calculated to put the reference cells at that target rSD. Using rSDEN targets a value between 2 & 5 should be used as multiplier, 1x is only to be used for custom defined targets.
The PMTV is exported into an instrument settings file that can be imported back into FACSDiva.
The reference cells are run again to confirm the calculated PMTV are reaching the target rSD.
Using Macro recording and look-up tables all these steps are automated to remove user dependent decisions and allow robust PMTV selection.



CST Voltration Data		Curve Fitting	
Name	Voltage	rSD Ranged	Curve fitting	Chi²
B510	590	32.28837	30.18118913	4.440211
B510	616	39.436897	39.66862406	0.053697
B510	636	47.927555	48.95117595	1.0478
B510	652	55.535587	57.91831561	5.677396
B510	665	65.36846	66.40049519	1.065097
B510	677	75.41579	75.3287765	0.007571
B510	701	98.22871	96.9482791	1.639503
B510	730	131.13351	131.5068252	0.139304
B510	758	180.16504	176.5186847	13.29591
B510	780	220.1019	222.4530037	5.527689

Curve fit Variables			
A	B	Sum Chi²	Avg Chi²
167.3709	95.11904	32.89424	3.289424



Results

1: Voltration curves & target rSD selection
Statistical analysis of multiple Voltration curves show an average of >75% SI for all detectors at 3x rSDEN. This multiplier is proposed as a good balance between sensitivity and negative noise contribution.

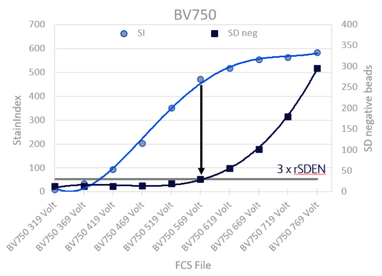


Figure 2: single detector Voltration curve with 3x rSDEN reference.

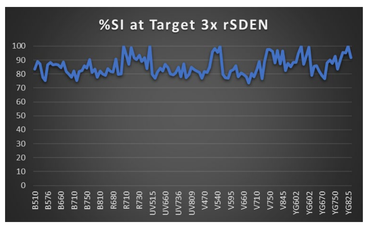


Figure 3: overview of %SI at 3x rSDEN for multiple voltrations across multiple detectors.

2: Dim Bead rSD curves & reference cell rSD curves for offset calculation.
Overlay of bead and reference cell Voltration data demonstrate the offset calculation.

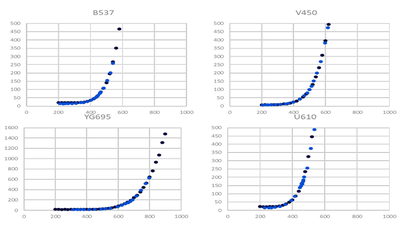


Figure 4: offset included overlay between bead rSD & Cell rSD curves.

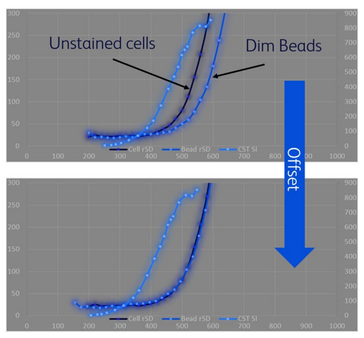


Figure 5: visualization of offset calculation.

3: System equalizer accuracy.
rSD of reference sample is compared before at CST PMTV and after at calculated PMTV to demonstrate accuracy.
For some detectors an iteration can be required for improved accuracy.

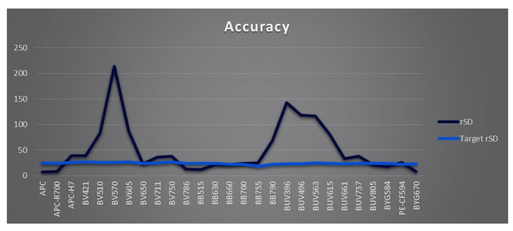


Figure 6:Reference sample rSD at CS&T PMTV and target rSD.

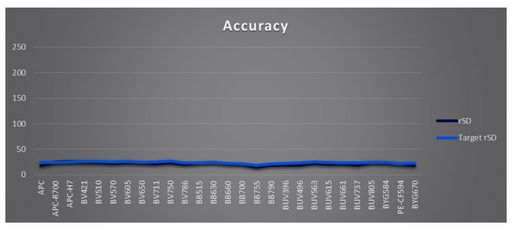


Figure 7:Reference sample rSD at equalized PMTV and target rSD.

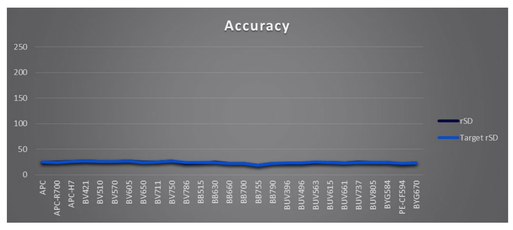


Figure 8:Reference sample rSD at equalized PMTV and target rSD after iteration.

4: Detailed comparison of System Equalizer vs traditional Voltration has been done for a large number of FACSymphony™ A5 SE.
A visualized comparison for data on a single channel is shown below in figure 9.

Channel set-up:
- Select desired channel.
- Calculated offset is shown, enter value to verify calculation match accuracy on the curves.
- Select desired target rSD

Plots:
- Dark Blue: Cell rSD: Unstained cell Voltration curve of rSD vs PMTV.
- Orange: Bead rSD: CST dim bead Voltration curve of rSD vs PMTV.
- Yellow line: rSD Ranged: data points from baseline within acceptance criteria. (criteria adjusted from 10x rSDEN to 255 to include more datapoints on some channels, like V427)
- Bright blue: target rSD: shows x rSDEN.
- CST SI: Shows Bright vs dim bead SI vs PMTV on secondary axis.

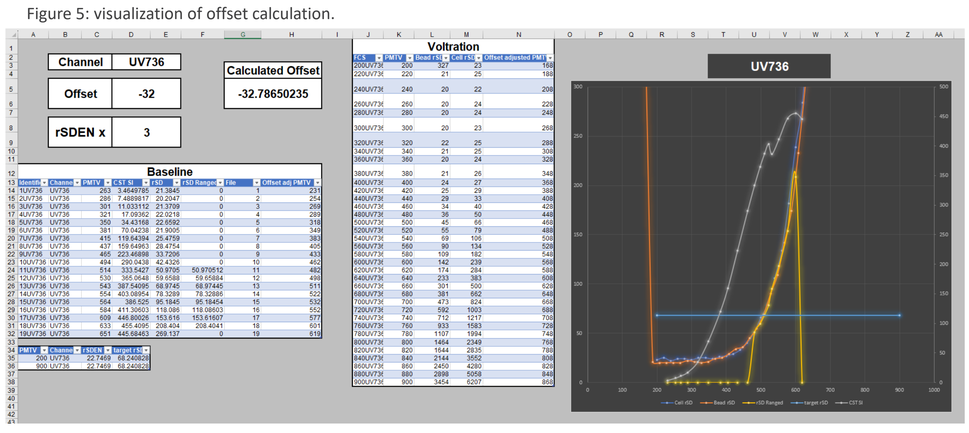


Figure 9: Comparison between traditional Voltration and system equalizer visualized
Data courtesy of Susmita Jasti, Ph.D., Eurofins Clinical Trial Solutions, Vircor BioPharma.

5: System equalizer workflow visualization
Baseline logfile and reference data are loaded into the System equalizer (1,2,3) , target rSD is selected (4) and cells are re-acquired at calculated PMTV to confirm target rSD is reached (5). The time needed to generate the Equalized instrument settings file is less than 2 minutes using this approach.

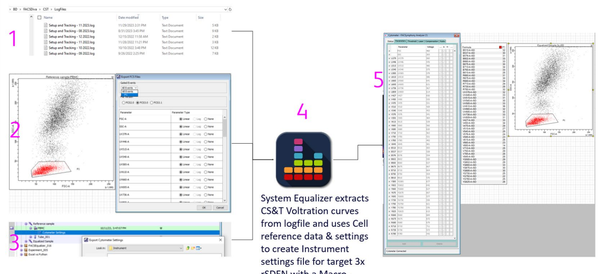


Figure 10: System Equalizer visualized workflow

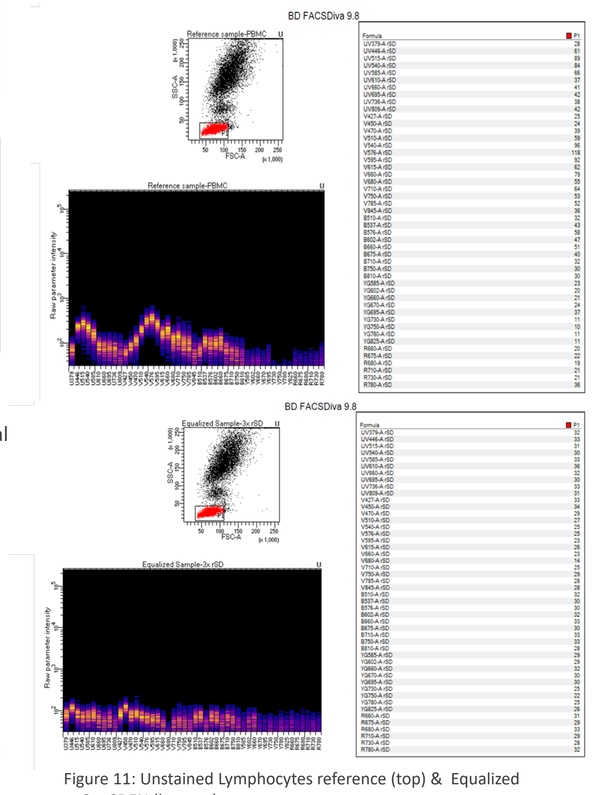


Figure 11: Unstained Lymphocytes reference (top) & Equalized at 3x rSDEN (bottom)

6: System equalizer Excel calculation interface
Baseline logfile is used to build the system equalizer interface table that uses the curve-fit calculation data and Lookup functions to import the cell reference information from a table.
Users only need to select the value for the rSDEN Multiplier. The look-up table has macro enabled fields to extract the data form the cell reference into the system equalizer calculation table and back to FACSDiva™ via an instrument settings file.

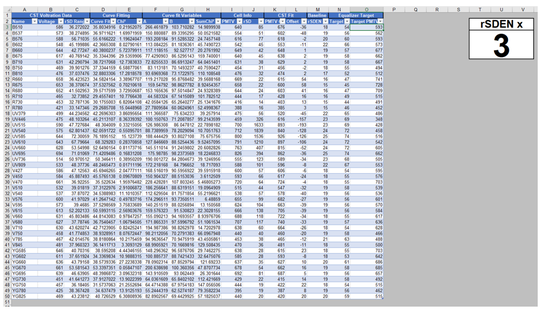


Figure 12: System equalizer calculation table.

Summary and guidance

The System equalizer approach provides a fast and accurate approach to set-up the cytometer at a desired target rSD:

- Using traditional Voltration for PMT setting across multiple systems, it has been determined that we reach >75% SI at a target of 3x rSDEN for unstained cells, which is within the range of selected PMT voltage using inflection point identification
- Adjusting PMT voltages to reach a calculated and defined rSD target provides a more consistent approach to PMT setup compared to traditional visualization of a curves inflection points that is subject to individual bias.
- This new approach to set PMT voltages creates detector-specific calibration curves for all channels from a single reference file recording without the need for multiple file acquisition for each individual channel.
- Setting PMTs to a target rSD can be performed manually, however, this requires the recording of multiple reference files per detector to “zero in” on the target rSD
- The system equalizer using macros and software automation results in an easier workflow for labs to setup assay specific PMTs or set PMTs post service or other system modification.
- The only requirement for setup is reference cells that are commonly available in laboratories.
- Cells with different autofluorescence could benefit from cell-type specific settings. It is possible to create multiple instrument settings with a simple acquisition of each unique cell type i.e. PBMC, Hepatocytes, Skin.