An introduction to Detector Setting Independent (DSI) scaling

TECHNICAL BULLETIN

BD

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Abbreviations

ABD units: Assigned Becton Dickinson units

ADC: Analog-to-digital converter

DSI scaling: Detector setting independent scaling

LED: Light emitting diode

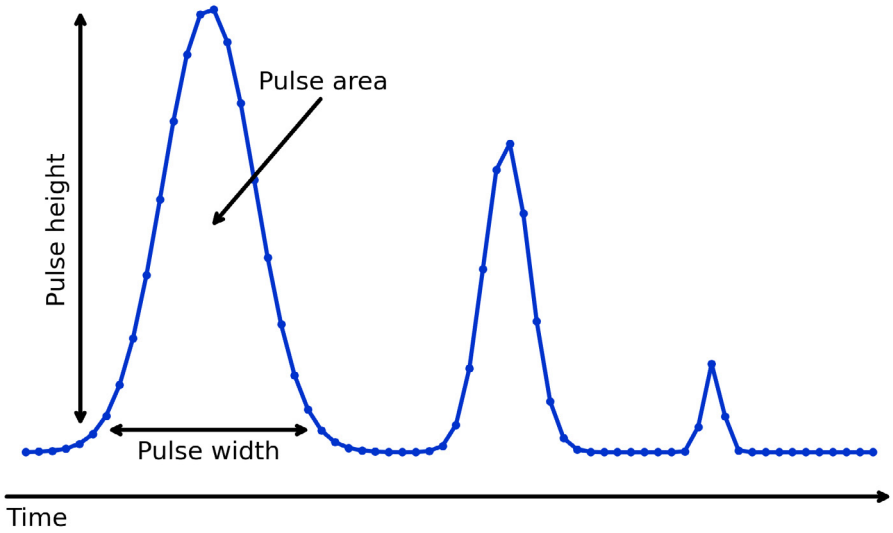
MFI: Median fluorescence intensity

QC: Quality control

Flow cytometry data scaling

The optical detectors of a flow cytometer convert all detected light into electronic signals that are subsequently converted into digital data. A sample pulse is the electronic representation of a signal with measurable height, width, and area values, **Figure 1**. In a digital flow cytometry system, the detector output is continuously digitized, and the threshold defines the signal level at which the system begins to measure a pulse within the continuous data stream. The scaling of an event on the arbitrary unit axis is dictated by these pulse characteristics.
The maximum number of intensity increments on the arbitrary unit scale for the height parameter is defined by the bit-depth of the analog-to-digital converter (ADC) e.g. a 16-bit ADC would have 2^{16} or 65,536 channels.
The number of intensity increments on the arbitrary unit scale for the area parameter is defined by the product of the bit-depth, sampling rate, and pulse duration. This is important to note, as any changes to the detector settings require that the signal amplitude from a given sample remain within this dynamic range to ensure accurate quantification.

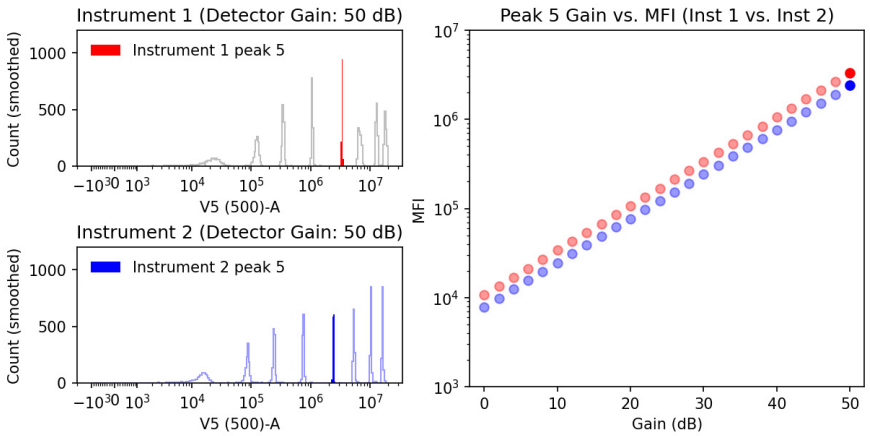
FIGURE 1. Visual representation of a pulse.



Flow cytometry standardization

Data standardization is critical for enabling data to be reliably repeated and reproduced. Since its inception, flow cytometry has scaled data natively in arbitrary units, whereby the signal intensity is dependent on the detector settings, such as voltage or gain. If fluorescent detector settings are increased, the measured fluorescence intensity will also increase, even though the underlying primary fluorescent signal from the particle remained the same, **Figure 2**. However, due to variations in instrument-instrument hardware such as alignment and optical tolerances, the amount of light a detector receives from an identical sample, such as a setup bead, may differ.

FIGURE 2. Comparison of detector gain vs. Median Fluorescent Intensity (MFI) on two instruments.

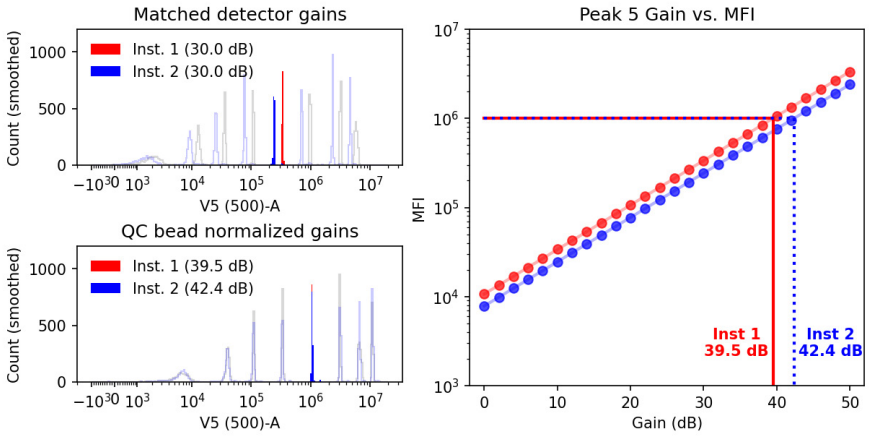


A common way to account for these differences in detected signals, and keep data consistent, has been for Daily QC systems to analyze a multispectral bead. These beads are assigned "target values" in each detector to define where their Median Fluorescent Intensity (MFI) should be. For example, if the Area target value for the V5 (500) detector was $1x10^6$, the detector gain used to put the setup MFI at that value on Instrument 1 would be 39.5 dB, and for instrument 2 it would be 42.4 dB, **Figure 3**. All user-defined settings could then be normalized to this reference value.

For example, if on Day 2 the Instrument 1 Daily QC bead required a gain of 40.5 dB (instead of 39.5 dB on Day 1) to reach $1x10^6$, all user experiment settings would also increase by 1 dB to account for the change in performance.

To date, flow cytometry standardization has utilized detector settings as a mechanism to normalize changes in MFI. The predictable behavior of detector gain and signal intensity, in combination with an instrument bead, has allowed manufacturers to use detector settings as a method of keeping data consistently scaled between instruments. However, because standardized detector settings are required for both intra and inter instrument comparability, considerations of standardization must be incorporated at the outset of a study. Deviating from these settings may introduce discontinuities in the data and compromise downstream analyses.

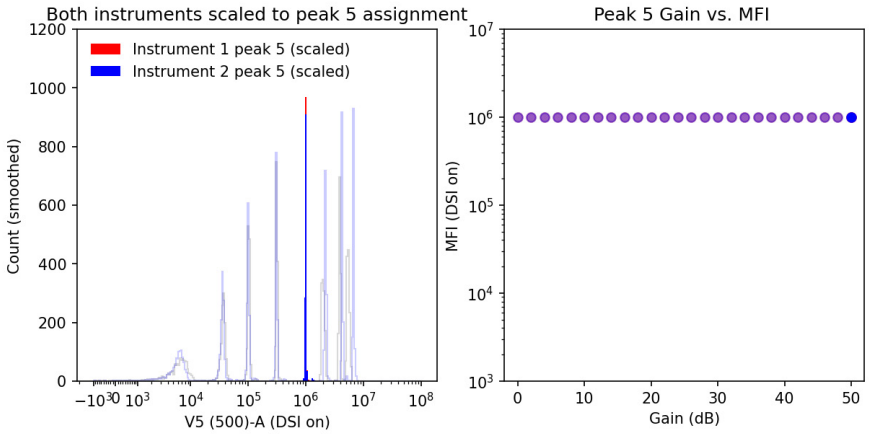
FIGURE 3. Demonstration of beads being used to normalize MFIs using detector gain between two instruments.



Detector setting independent scaling

Detector setting independent (DSI) scaling is a novel approach that aims to decouple the choice of detector settings from reported MFI, allowing consistent data across different detector settings. This is achieved using all the same criteria as previous standardization approaches, with one key distinction. Rather than modifying the detector settings to adjust where we know the reference bead should be on the measurement scale (e.g. target value), we instead calibrate the reported data scale to that reference value, thereby taking account of any differences in performance longitudinally or between instruments without tying users to a given set of settings, **Figure 4**.

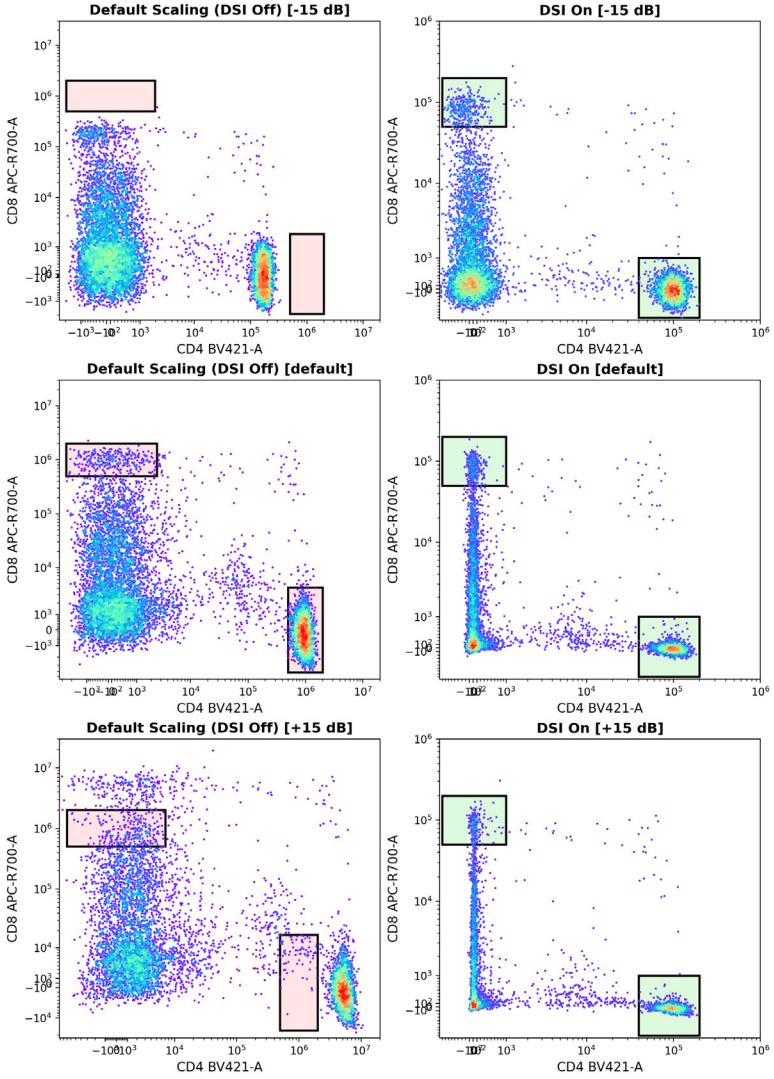
FIGURE 4. Demonstration of normalizing detector scaling based on the setup bead MFI resulting in detector setting independent (DSI) scaling.

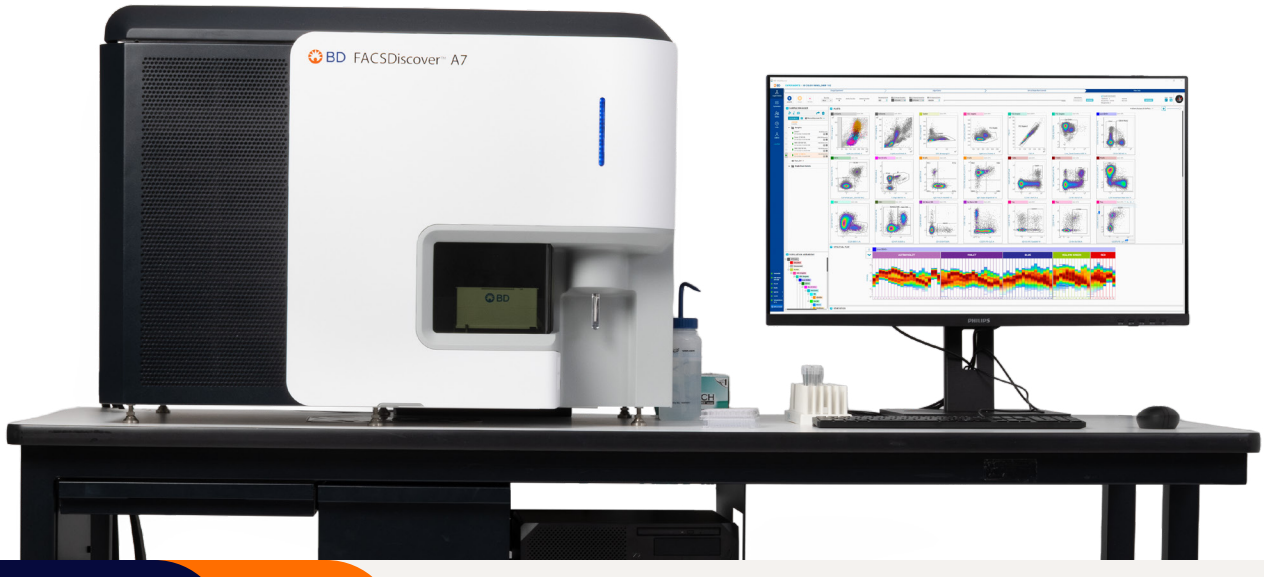


The BD FACSDiscover™ Platform's integrated LED pulser allows for the precise calibration of detector gain day-to-day, which in turn allows for the MFI of a population between detector settings to be precise and predictable, **Figure 2**. This concept of electronic linearity is utilized in transposing the calibration factor obtained at Daily Setup & QC across any of the user's detector settings. Another way of thinking about this is that the detector scale is being calibrated based on the setup bead position on the BD FACSDiscover™ Instruments. The position of the beads at assay setting is determined during setup. When a user changes detector gains to run their samples, because the scaling is calibrated to be linear, we can derive where the BD FACSDiscover™ Instruments Daily Setup & QC bead would appear on the scale at any given detector settings, allowing for a detector scale to be calibrated irrespective of the user's choice in detector settings.

The impact of utilizing DSI scaling is that population MFIs now reflect the underlying signal the detector receives. For example, when we acquire a CD4 and CD8 stained PBMC population and change detector settings, **Figure 5**, the populations only appear within their respective gates at one set of detector settings (default, DSI scaling off) due to the data being dependent not only on the underlying signal but also the instrument settings. With DSI scaling mode enabled, the populations appear in their respective gates at all settings, with only the resolution of the populations being altered by the detector settings.

FIGURE 5. Example of CD4 and CD8 stained PBMCs with default scaling and detector setting independent (DSI) scaling.





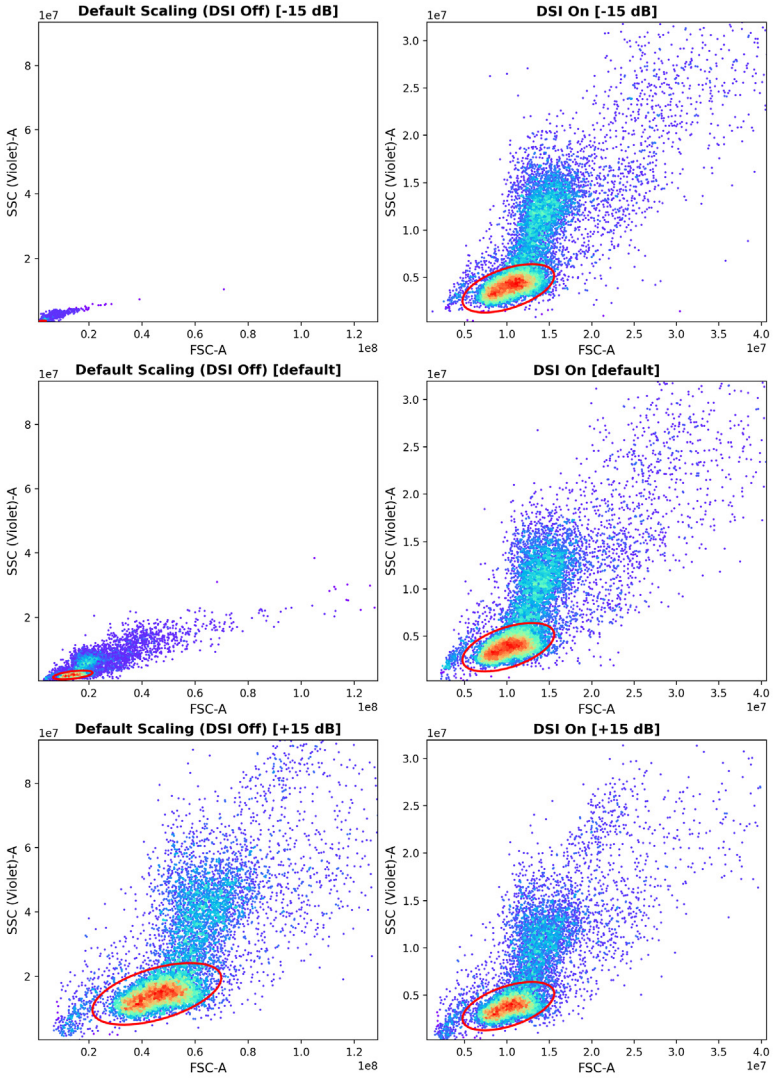
Use cases beyond standardization

While at its core DSI scaling is a standardization feature, a variety of use cases arise where DSI scaling can simplify experiment workflows and open new possibilities for biological comparisons. Below are three representative examples.

Use case 1: Universal gating templates

One of the first interactions a flow cytometry user has when setting up their experiment is the adjustment of light scatter detector settings and trigger threshold in order to identify their cell population of interest. With DSI scaling, users will find their cell populations in the same location irrespective of detector settings used, provided they are on scale for the height parameter and resolved from background, **Figure 6**. This allows users to create universal light scatter gating templates or simply know exactly where to expect and draw a gate for a given cell population, thereby reducing setup time and uncertainty when moving between instruments of the same type.

FIGURE 6. Comparison of light scatter gating when using default scaling and detector setting indepdendent (DSI) scaling.

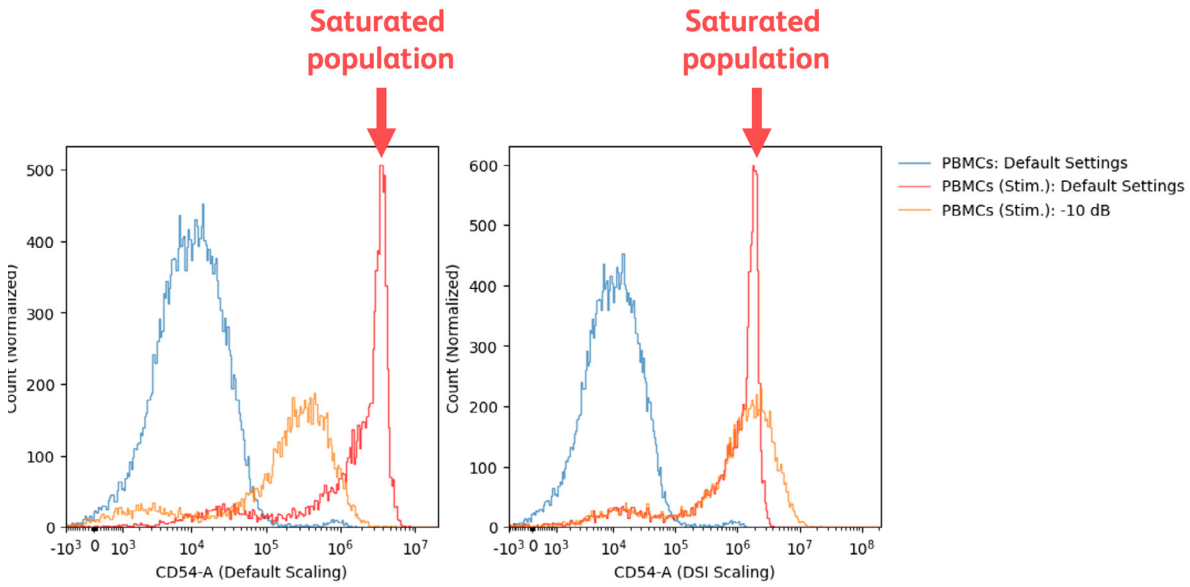


Use case 2: High dynamic range comparisons

In some cases, test samples require different detector settings to remain within scale because the range of fluorescence intensities being measured exceeds the detector's dynamic range.

Examples of this include stimulated and unstimulated cell samples, large and small cell type comparisons, overexpression models in comparison to wildtype or knock-outs, etc. In the example shown, the unstimulated cell population is already positive for CD54, **Figure 7**. When the stimulated cells are acquired at the same detector settings, part of the positive population saturates the detectors, resulting in nonlinear signals and inaccurate data. In order for the stimulated cells to be on scale, we must therefore decrease the detector settings. In this example, we decrease the detector gain by 10 dB. However, we've now lost the true relative signal increase from our unstimulated cell state.

FIGURE 7. Example of using default scaling and Detector Setting Independent (DSI) scaling when wanting to compare sample types that require different settings to be on scale.

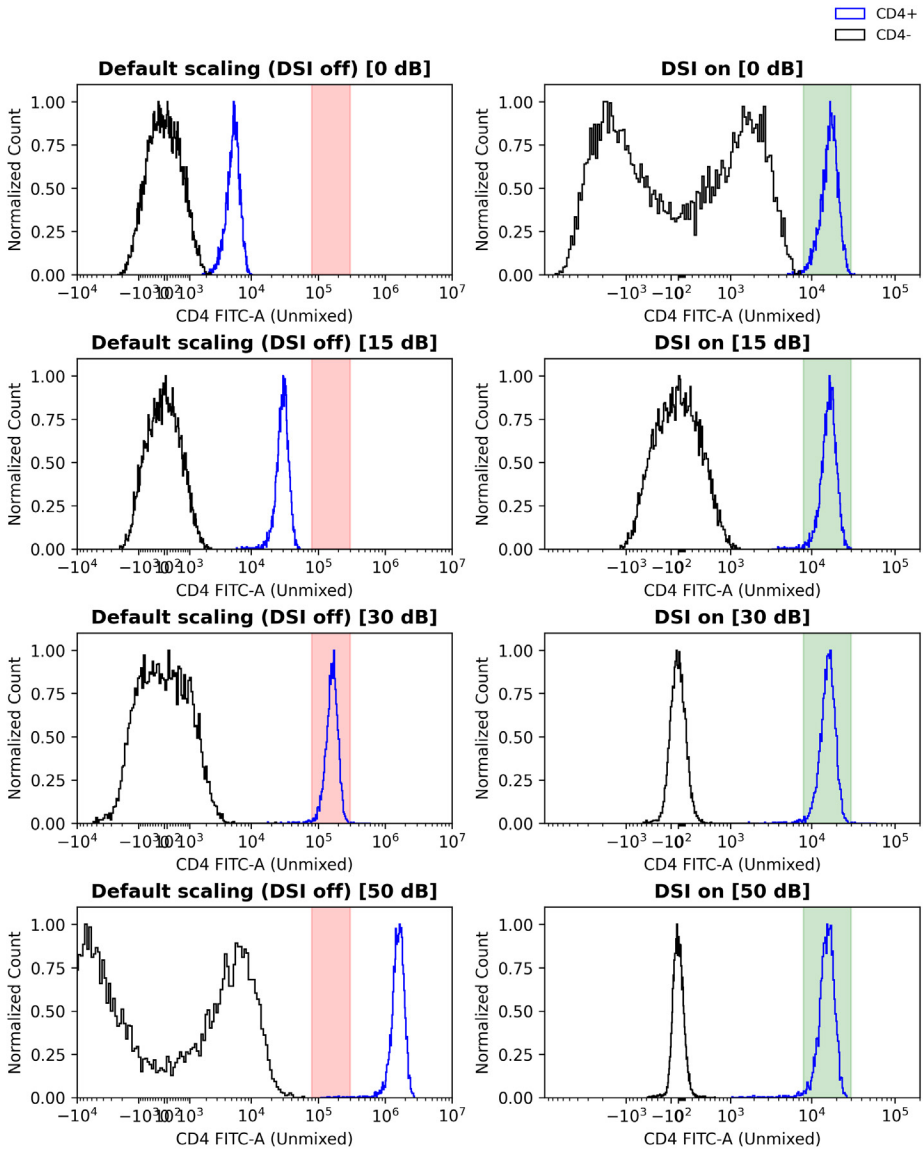


With DSI scaling mode enabled, the stimulated cell population acquired at lower detector gains retains the true underlying magnitude of signal change between the stimulated and unstimulated populations, allowing for MFIs to be quantitatively compared. We can see that DSI scaling still requires all populations to be on scale and resolved in order to be correctly utilized. This is demonstrated in the example with stimulated cells acquired at default settings whose CD54 expression is still impacted by part of the population saturating detectors.

Use case 3: Voltrations

Dedicated workflows known as “voltrations” are often used to find detector settings that maximize resolution (e.g., separation index). These workflows are typically performed as separate analyses because, under conventional scaling, it is difficult to visually compare populations. The position of the positive MFI shifts at different gain settings, but simultaneously the noise of the negative population may expand, making visual assessment of optimal separation difficult, **Figure 8**. In contrast, with the introduction of DSI scaling, optimizing detector settings for resolution can be intuitively identified in real time for most workflows, without requiring dedicated experiments and external analyses. As seen in the CD4-stained lymphocyte example, the positive population location does not change with detector setting changes, only how well it is resolved from the unstained population.

FIGURE 8. Example of performing a voltration with unmixed anti-CD4 FITC labelling on lymphocytes with default scaling and Detector Setting Independent (DSI) scaling. The black populations represent unstained lymphocytes and the blue populations represent CD4 positive lymphocytes.



Frequently asked questions

1. Can DSI scaling be applied to live and recorded data?

DSI scaling is a feature implemented in BD FACSChorus™ Software (v6.5 and higher) which can be turned On and Off whilst acquiring and recording data, as well as to previously recorded data.

2. Can the detector settings be changed when DSI scaling is On?

Yes, users can interact with all instrument settings with DSI scaling On or Off.

3. What happens when populations saturate?

Just as with default scaling, saturated data is not valid and will result in artifacts in data in default scaling or in DSI scaling mode as demonstrated in Figure 7. Users should refer to the spectral plot where the Height parameter data is shown to ensure data is within the detectable dynamic range. This is also the best practice for using default scaling.

4. Does DSI scaling require new setup and QC beads?

No, DSI scaling builds on metrics that already exist as part of the BD FACSDiscover™ Platform next-generation setup and QC today. This is why data recorded data prior to BD FACSChorus™ v6.5 Software can be utilized with DSI scaling mode.

5. What does DSI scaling do to my data?

DSI scaling is applying a single scalar to all data based on the detector gain being used. This scalar is the same value that is used to normalize detector settings between instruments. Rather than normalizing detector settings, this scalar is now being used to normalize the data directly.

6. Can I apply DSI scaling after the fact to older recordings?

Yes, data recorded prior to BD FACSChorus™ v6.5 Software can have DSI scaling enabled when imported or opened within BD FACSChorus™ v6.5 Software.

7. How will it be exported raw, scaled, or both?

FCS file data will export with either DSI scaling On or Off, but not both. Within the FCS file a keyword “DSI scaling” will be set to “TRUE” or “FALSE” to indicate the data type of the file. And a “PnDSI SCALINGFACTOR” keyword will provide the scaling factor used to allow conversion of the data between modes.

8. How will FlowJo™ Software display this so I can compare scaled and raw data?

There are plans for FlowJo™ Software to support dynamic switching between DSI scaling mode On and Off but this will be implemented after the release of BD FACSChorus™ v6.5 Software.

9. How should we guide users in distinguishing which data sets can be reliably compared (i.e., acquired under appropriate conditions) versus those that should not be compared due to suboptimal acquisition conditions?

The scale is calibrated to Assigned Becton Dickinson (ABD) units, which allows data to be scaled irrespective of detector settings and instrument variations. The MFI of populations will be comparable as they are separated from a negative population.

10. How does the system behave when marker MFI values shift, especially when tracking these variations is biologically relevant? Will the system neutralize these shifts, or will it continue to measure and reflect them?

Data is calibrated to ABD units. We are not artificially changing/rescaling data dynamically to make it consistent. This means any observed MFI changes due to the underlying signal will continue to change and be quantitated. Similarly, if a user inconsistently stains their samples or switches fluorophores, the signals will move.

11. Does DSI scaling improve instrument to instrument MFI %CV (for the same experiment template)?

No, DSI scaling is calibrating data based on the BD FACSDiscover™ Instruments setup beads' values. The consistency of the calibration is therefore identical in precision of how well the MFI can be maintained currently using the setup bead, hence no change of the instrument to instrument MFI %CV. The only difference between our current approaches, where we adjust detector settings to maintain MFI, is that with DSI scaling, we calibrate the MFI rather than change settings. Both approaches rely on the same underlying ABD assignments and spectral properties of the beads.

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