‘Range’: A gain and dynamic range independent quantification of spread aiding in panel design

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Abstract

In flow cytometers, ideally each detector receives photons from one specific fluorochrome. However, photons usually end up in different detectors too (fluorescence spillover). 'Compensation' is a process that removes this extra signal from all detectors other than the primary detector dedicated to that fluorochrome. Post 'compensation', the measurement error of spillover signals become evident as spreading of the data. Spillover reduces the ability to resolve single positive from double positive cell populations. For successful multi-color panel design, it is important to know the expected spillover. The Spillover Spread Matrix (SSM) can be used to estimate the spillover spread, but the outcome is heavily influenced by detector sensitivity. In short, the same single stained control sample produces different spillover spread values when detector sensitivity are altered. Many researchers unknowingly use this artifact to "reduce" the spread by decreasing detector sensitivity. This can result in reduced sensitivity and diminished capacity to resolve cell populations. In this article, we introduce 'Range' as an alternative tool that can predict the spillover independent of detector sensitivity. 'Range' is also independent of dynamic range, that allows to compare spread values between different types of instruments, something not possible using SSM.

Key terms: Spread, Spillover, PMT, APD, Dynamic Range

Introduction

Recent developments in Flow Cytometry and fluorochrome technology have made it possible to combine a large number of fluorescent labels to enumerate immune cell subsets and interrogate protein expression from a single biological sample [1]. In conventional flow cytometers, each detector ideally receives photons from one specific fluorochrome as a labeled cell travels through the interrogation point. However, fluorescent light usually ends up in different detectors too (spillover) [2]. Where 'compensation' corrects for spillover signals based on median fluorescent intensities (MFIs), the variance of a spillover signal, as a result of measurement error, is not possible to correct. This error is visible as spreading of the data [2, 3]. A major consequence is a diminished capacity to discriminate double positive cells from single positive cells, where the latter are labeled with the fluorochrome that results in spillover. For this reason, establishing panels for high parameter experiments can be quite troublesome [4]. Therefore, a priori knowledge on the instrument specific spillover characteristics of fluorochromes is important to ensure that the ability to resolve all cell subsets of interest is maintained.

Useful tools/methods have been developed to aid investigators in this process. The most well-known and widely used is the Spillover Spread Matrix (SSM), which provides a metric for signal variance introduced by spillover fluorescence for every fluorochrome-detector combination in a given panel [5]. The SSM can provide valuable insight in which combinations of fluorochromes are expected to work well together in a panel. However, care should be taken when using this method. Each value of the SSM is called Intrinsic Spillover Spread (ISS). These values are highly dependent on detector sensitivity (voltage/gain) or signal amplification of the primary detector. As a result, applying a different voltage over a detector results in a different ISS value for the same sample. More importantly, when different voltage settings are used in the actual experiment, unexpected and undesired outcomes may occur with regard to the ability to discriminate dim cell populations or accurately determine expression levels. Additionally, one may falsely deduce that spread from spillover fluorescence can be minimized by changing detector sensitivity.

Additionally, using the SSM to compare instruments for their capacity to run a given panel requires the tedious process of instrument standardization [6]. This is further complicated by differences in detector characteristics or dynamic range between instruments. Measurement error is strongly dependent on photon number and detector quantum efficiency (QE) for photons of a given wavelength range. In general, this error is larger for long wavelength photons. For example, the widely used photomultiplier tubes (PMTs) are known for their lower QE for long wavelength photons as compared to short wavelength photons. In contrast, avalanche photodiodes (APDs) have a

relatively high QE for long wavelength photons. Thus, a given number of long wavelength photons that strike an APD, results in relatively less measurement error as compared to a PMT [7, 8].

To overcome all these difficulties, we introduce the 'Range' value as a measure of spread that is independent of detector settings and detector type. Instead, the 'Range' value of a fluorochrome is relative to the signal intensity of the fluorochrome that is to be used in the spillover detector. The resulting matrix can be used to aid in panel design just like SSM but easy to compare instruments with regard to data spread for given fluorochrome combinations. As such, 'Range' can be a useful addition to methods that evaluate their respective sensitivity for the individual marker-dye combinations [9]. 'Range' can be used for both conventional and spectral flow Cytometry.

Results

Concept of 'Range'

The 'Range' value is defined as the difference in median fluorescent intensity (MFI) between the 99th percentile and the 50th percentile of the positive population (Fig 1). This value is a measure of total spread including spillover fluorescence in a secondary detector. Some examples of Range can be found in Fig 2. The following abbreviations are used to describe the calculation procedure as explained in Fig 1.

X^{B50} = 50th percentile of compensated positive population of fluorochrome X in dimension B

X^{B99} = 99th percentile of compensated positive population of fluorochrome X in dimension B

Un^{B50} = 50th percentile of compensated unstained population of in dimension B

YSt^{B50} = 50th percentile of compensated stained population of fluorochrome Y in dimension B

NFY^{B} = Normalization factor of fluorochrome Y in dimension B

'Range' value X^{B} = Spread of fluorochrome X in dimension B

(YSt^{B50} - Un^{B50}) = The difference between the positive and negative populations (channel number).

If P (any constant number, in this article we have used P = 9000) is the difference between YSt^{B50} and Un^{B50}, in terms of number of photons received by detector B

Therefore (YSt^{B50} - Un^{B50}) ≡ P

1 channel in dimension B = P/(YSt^{B50} - Un^{B50}) = NFY^{B}

Then 'Range' $X^{B} = (X^{B99} - X^{B50}) * NFY^{B}$

The 'Range' value is made independent from gain settings of the secondary detector, and dynamic range, by applying a normalization factor. The normalization factor is founded on three premises. First, the number of incoming photons remains the same when settings of the secondary detector are changed. Second, differences in signal intensities are linearly proportional to changes in detector settings. Third, there's a linear relationship between signal intensity and signal variance.

The normalization factor allows for the 'Range' value to be determined without prior optimization of detector settings, provided that all obtained data is within the linear range. Because 'Range' is relative to constant value P, it also allows for direct comparison of 'Range' values between instruments with different dynamic range. In the Supplementary Table S1 we have provided the average of three runs from the Fusion 1. 'Range' showed high degree of reproducibility. In the discussion we elaborate on the conditions that must be met. In Supplementary Table S2 we have shown how 'Range' behaves when more than one fluorochrome is associated to the secondary detector, details are in discussion.

Classification of 'Range' values

Since there are no clear characteristics that define different categories of spread [5, 10], we created a different semi-quantitative method to categorize 'Range' outcome as described below:

Percentage of channels occupied by spread = $[(YSt^{B50} - Un^{B50})/$ total channel number$] * 100$

For example, 262,144 is the total channel number for BD machine. 'Range' between 0 to 120, 121 to 220, 221 to 300 and 300+ matches nicely with 0 to 0.5%, 0.5% to 1%, 1% to 1.5% and 1.5%+ of channel occupancy respectively. Comparing percent of channels occupied with 'Range' will provide user a better understanding of spread. We have color coded 'Range' values in 4 categories: 1 – 120 (Green); 121 – 220 (Yellow); 221 – 300 (Orange); and 300+ (Red). While Green and Yellow both provide acceptable discriminative power in most cases, Green should be reserved for markers with low or unknown expression levels. Values with an Orange or Red 'Range' value should be used for markers on distinctly separated cell populations or for markers with exclusive expression on specific cell subsets. For example, if BV421 is combined with Alexa Fluor 647 (AF647), the 'Range' value is 20 (Green). Note that the 'Range' value is not zero due to measurement error of the autofluorescent signal and biological variance. On the other hand, if PE-Cy5 is combined with AF647 the 'Range' value is 611 (Red). At these different levels of spread, 15% and 5% double positive events were identified respectively (Supplementary Fig S1).

Intrinsic Spillover Spread values are dependent on detector gain settings

The ISS value of BV605 changed when the sensitivity of the primary detector changed, because the intensity difference (ΔF) between the positive and negative populations unjustly mimicked a change in photonic input in the secondary detector (Fig 2A). Importantly, the variance as represented by the rSD and 'Range' value remained the same. Additionally, when the voltage of the secondary detector was reduced, the rSD and ISS values were lower too. Conversely, the 'Range' value was unchanged.

'Range' values are independent of detector gain settings

Single stained beads were measured with two different voltage sets. The voltage was manipulated to position the positive population to 110,000 MFI or 50,000 MFI in all detectors. The 'Range' values were the same in both voltage sets, but the ISS values were quite different (Table 1). This observation is possible because 'Range' does not include any component from the primary detector. When the voltage of detector B is decreased, the spread from fluorochrome X in detector B is reduced, but the intensity of fluorochrome Y, which was used to calculate normalization factor, was reduced proportionally, rendering 'Range' values independent of detector voltage. In contrast, when the same data were used to obtain the conventional Spillover Spread Matrices, the lower voltage set showed lower ISS values (Table 1). In Supplementary Figure S2, a series of voltages was applied in

which the voltage of the secondary detector was kept constant, but the voltage of the primary detector was changed gradually. In the second row, the voltage of the primary detector was kept constant and the voltage of the secondary detector was changed. In both cases the 'Range' values remained the same. However, the ISS values changed considerably.

Application of 'Range': Compare instrument performance

While all three Fusions had similar optical configuration and laser power, Fusion 3 performed best with only 6 instances of 300+ range. The performance of The Fusion 1 was considered worst with 12 occurrences of Range values over 300. The findings are summarized in Table 2. The results suggest that Fusion 3, is best suitable for the most sensitive experiments.

Application of 'Range': Compare instruments with different dynamic range

To investigate which instrument is the best for running a given panel in terms of spread, we compared the 'Range' values between a Fusion, Symphony, Quanteon and a Cytoflex (Table 3). The Cytoflex showed the lowest number of 'Range' values above 120. The other instruments showed a higher number of values above 120. The Quanteon and Cytoflex had the lowest number of 'Range' values over 300. The Quanteon had the highest number of 'Range' values between 120 to 199.

Application of 'Range': Investigate the effect of instrument characteristics on spread

'Range' was used to investigate the effect of high and low laser powers in Fusion 2 which showed some interesting findings. At an output power of 140mW, the Red laser resulted in lower spillover spread from APC and APC-R700 compared to lower output power of 100mW. In other cases, higher laser power showed minimal effect on spread. For instance, at 100mW, the 405nm laser showed virtually the same spread in all three violet detectors as compared to measurement at 85mW. This is in concordance with existing knowledge that Brilliant Violet dyes saturate at 80mW. Our data indicates that in our systems, higher laser powers other than for the red laser, doesn't lower the data spread (Table 2).

Discussion

To show how ISS values are dependent on detector sensitivity, we explored two scenarios. First, the voltage of the primary detector was changed (Fig 2A). The spread remained the same as was evident by the unchanged rSD, but the ISS values changed because the value of $\sqrt{\Delta F}$ changed. More specifically, the lower voltage on the primary detector increased the ISS value, because the same $\Delta\sigma_C$ [5] was divided by a smaller number (smaller $\sqrt{\Delta F}$). In the Supplementary Fig S2 we have shown 'Range' remains unchanged if we change primary or secondary voltages. However, the voltage change do effect the ISS values considerably. In the second scenario (Fig 2B – D), voltages of the secondary detectors were also changed. In this example, lower voltages resulted in lower ISS values. In inexperienced hands, this property of the ISS values can lead to adjusting detector settings to 'reduce' the spread, rather than adjusting the panel to fit the spillover characteristics of the fluorochromes at optimal detector settings. This in turn may result in loss of resolution and obscuration of cells that have low expression of markers. Because the 'Range' value is independent of detector sensitivity, bias induced by voltage is prevented. The inherit relation of ISS and detector sensitivity will always make the ISS values subjective. Even when user is not comparing different voltage sets, the influence of detector sensitivity will always be there. 'Range' is currently the only way to remove this subjectivity.

One can expect that the resulting spread due to measurement error of fluorescence is constant and proportional to amplification of the secondary detector [11]. Furthermore, because variance and MFI are proportional to amplification settings in equal fashion, the MFI can be used to correct for the effect of changing secondary detector settings on spread. Additionally, the MFI of the unstained population (Un^{B50}) is subtracted from the MFI of the Stained population (YSt^{B50}) to normalize for differences in autofluorescence between channels. As shown in Table 1, lowering both voltages lowered the ISS values, with same samples. When inexperienced users would lower the voltages to get a 'lower spread' they may risk masking dim populations. This in turn could lead to erroneous conclusions. As 'Range' is independent of voltage, the spread quantitation will remain the same. Ashhurst et al also showed that spread do not change with voltage alteration [11].
In Supplementary Table S2 we presented a special scenario where the 'Range' value of one fluorochrome was calculated for two different secondary fluorochromes. For example, the 'Range' value of APC in detector B695 is different when different secondary fluorochromes (BB700 or PerCP-Cy™5.5) are considered. Because the 'Range' value is relative to the intensity of the secondary fluorochrome, the same spread from APC results in a relatively higher occupancy in channel number when combined with PerCP-Cy™5.5 as compared to BB700. It is helpful in the panel design phase to know that APC with PerCP-Cy™5.5 may be a more problematic combination for the identification of

a double positive population. On the other hand, ISS values remain the same for spread, as these are independent of the fluorochromes associated with the secondary detector.

It is not easy to compare ISS values between systems with different dynamic range, because instruments need to be calibrated first [5]. The ΔF accounts for the dependency of spread on the number of fluorescent photons, the ΔF needs to be numerically similar on both instruments to have a meaningful comparison of ISS values. This calibration is a tedious process. However, because the intensity values in the primary detector don't influence the 'Range' values, this is not necessary in our method. Furthermore, due to the normalization factor, the scaling on every secondary detector is comparable. This makes the 'Range' value excellently suited to compare panel performance on different instruments.
As proof of concept, we compared the 'Range' values for a given set of single stains on different instruments with different dynamic range. Due to higher quantum efficiency for longer wavelength fluorescent light, we anticipated to find less spread in APD and SiPM based instruments in the red emission region as compared to PMT based instruments [8, 12]. Indeed, both APD and SiPM detector based instruments showed lower 'Range' values in detectors for APC and APC-R700 (Table 3).
We matched the other instrument characteristics as best as possible, but these were not exactly the same. For example, laser output powers of the Quanteon and the Symphony were matched, but there was no option to alter the laser power for the Cytoflex. Furthermore, the filter configuration (characteristics and positioning) between the instruments was also not exactly the same (Supplementary Table S3). However, this example shows that it is possible to use 'Range' values for the purpose of comparing instrument performance with regards to spread for a given set of fluorochromes.

'Range' values are empirical and very robust, because it's not a variance-based calculation of spread. For a Gaussian distribution, the 99th percentile can be calculated as 2.325 times the standard deviation (SD) of the mean. However, flow cytometric data follows the Poisson distribution and is known for positively skewed distributions [13]. For this reason, the factor of rSD required to calculate the 99th percentile for each detector is highly variable. The median of 99th percentile was obtained using FlowJo statistics tools. However, since the determination of the 99th percentile is effectively based on just 1% of the data, it could potentially be distorted by outliers. To address this issue, careful singlet selection was performed, the median was used instead of the mean as a measure of central tendency, and a minimum of 35,000 single beads were recorded. This practice has been shown to provide highly robust results with less than 5% variation between tests (Supplementary Table S1).

While the ‘Range’ value is easy to use, there are some important things to keep in mind. First of all, the precision of the ‘Range’ value depends on correct ‘compensation’. For that reason, it is very important that all data is within the linear range of detection. When using beads as carriers, this may cause problems at lower gain settings. Also, a thorough washing of the beads is an important criterion, as any non-specific binding of the antibody to the negative beads will change the photon difference (P), and introduce error in the normalization factor. Lastly, the ‘Range’ value is invalid if the correlation between variance and signal intensity is different at different amplification settings. In other words, detectors and downstream electronics required for further signal processing should function properly.

Another important consideration to ensure reproducibility is the amount of bead bound antibody. This needs to be the same between repeats to ensure the difference in number of photons (P) between the positive and negative populations remain equal between runs. Using antibodies at saturating concentrations is therefore required. At the same time, this will also ensure maximum spread. On most of the commercially available machines manufactures use a set of excitation optics to excite the fluorochromes maximally for practical use. The maximal excitation will ensure that almost the same number of photons will be emitted for both positive and negative populations. It is important to use CD4 kit of same lot number for comparison purposes.

Conclusion

The main take home messages can be summarized as follows:

1. 'Range' values are unaffected by detector gain settings.
2. 'Range' values are independent of detector type or dynamic range. Therefore, 'Range' values can be directly compared between instruments, across different locations.
3. The 'Range' method is a useful tool to aid in panel design.

Nguyen *et al.* already proved that monitoring spread via ISS values is an excellent way to identify poor laser alignment, failing of the laser, other optical problems and to reveal detectors that may need optimization. As such, 'Range' can also be used to track systems over time. However, fluorochrome stability, of tandem dyes specifically, is of major concern, since tandem dye replacement or time dependent degradation will affect the outcome. A set of hard dyed plastic beads, which mimic single stains for all available channels would prove useful for long-term tracking. We strongly believe that this article helps novice users to understand data spread and how to deal with the effects better. Finally, we are certain that the voltage and dynamic range independent nature of 'Range' values will be instrumental in preventing manipulation of detector gain settings to minimize spread, rather than to use settings that provide the best separation and sensitivity.

Materials and Methods

Reagents

1. 5ml Falcon polystyrene round-bottom tubes (Corning Life Sciences, cat. no. 352054)
2. 5ml Falcon polystyrene round-bottom tube, with cell strainer snap cap (Corning Life Sciences, cat. no. 352235)
3. PBS as staining buffer
4. Human PBMC were isolated and stored as mentioned in reference 14.

Instruments

We have used 3 BD FACSAria™ Fusion cell sorters (Fusion) and a FACSSymphony A5 (Symphony) from BD Biosciences, a NovoCyte Quanteon from ACEA (Quanteon), and a CytoFLEX S (CytoFLEX) from Beckman Coulter Life Sciences. Fusion 1,2 and 3 were used with identical optical configuration and laser output power unless stated otherwise. For one of the experiments, data was also generated at higher laser output powers on the Fusion 2 (Fusion 2_High). The output of all lasers of the Symphony and the Quanteon were set to 100mW. A detailed overview of the optical configurations (laser and filters) are given in the Supplementary Table 3.

Antibody-fluorochrome conjugates

All experiments were done using human CD4 evaluation kit (BD Biosciences, cat no 566352). The following fluorochromes were used: BB700, PerCP-Cy™5.5, BV421, BV605, BV711, PE, PE-CF594, PE-Cy5, PE-Cy7, Alexa Fluor 647, APC, APC-R700 and Alexa Fluor 700. Clone matched (SK3) Alexa Fluor 647 conjugated CD4 antibody (cat no 344635), and anti-human Alexa Fluor 647 conjugated CD45RA antibody, clone HI100 (cat no 304153) were obtained from BioLegend. All reagents were stored as recommended by the manufacturer.

Software

BD FACSDiva™ Software (version 8.0.1), NovoExpress, and CytExpert (version 2) were used only for data acquisition. Generation of compensation matrices and other analyses were done using FlowJo version 10.6.1.

The Script

Compensated single stain fcs files are needed for the script to work. 'Range' values are computed using R (R 4.0.3). To do so, the population of stained beads, and unstained beads for fluorochrome X as well as the population of stained beads for fluorochrome Y need to be defined. This is done via

statistical analysis using an analysis of mixture distribution. A first statistical model, using EM algorithm, is performed on the absolute value of the intensities measured by the primary detector corresponding to fluorochrome X. This analysis , performed with the R function normalmixEM from R package mixtools, decomposes the intensities measured on the primary detector as two distinct normal distributions, i.e. the unstained control beads and the stained beads populations. By taking the first and the third quartile of these distributions, low and high boundaries of both unstained and stained populations can be derived, which allows for gating of the data. The same approach is performed to gate the population of the fluorochrome Y stained beads. Once the three different population of beads are defined, the relevant parameters for 'Range' value computations are obtained. The script can be found in the webpage: github.com/RenTissier/RangeComputation.

Data collection

At least 35,000 single beads or 100,000 single cells were recorded. We made sure that the population intensities stays within the linear dynamic range of the detector. Manufacturer recommended quality controls were performed before every experiment.

Sample preparation

Staining of antibody capture beads

1. Antibody capture beads were vortex for ~ 3 seconds.
2. One drop of blank beads and one drop of antibody capture bead were mixed in a tube.
3. The saturating antibody concentration was determined in titration series for every antibody-fluorochrome conjugate used.
4. 0.25µg of antibody was added to all samples (at saturation).
5. The samples were stained at 4°C for 30 minutes in the dark.
6. Beads were washed twice in 3 ml of staining buffer, 5 minute centrifugation at 500 g (acceleration 9, deceleration 5).
7. Re-suspended in 200 µl of PBS.

Staining of PBMC

1. The cells were thawed in a 37°C water bath.
2. Filtered gently.
3. Centrifuged at 500 g for 10 minutes.
4. The supernatant was discarded.
5. 1 X 10^{6} cells were re-suspended in 100µl of staining buffer.

6. The saturating antibody concentration was determined in titration series for every antibody-fluorochrome conjugate used.
7. 0.06 µg of CD4 antibody was added (at saturation).
8. The samples were stained at 4°C for 30 minutes in the dark.
9. Beads were washed twice in 3 ml of staining buffer, 5 minute centrifugation at 500 g (acceleration 9, deceleration 5).
10. Re-suspended in 200 µl of staining buffer.

Intrinsic Spillover Spread values are dependent on detector gain settings

Antibody capture beads were stained as mentioned above. Single stained beads were run only changing the primary PMT voltage from 600V to 400V in steps of 100V. A mixture of these single stains were run using different PMT voltages. ISS values were calculated and compared to the Robust Standard Deviation (rSD).

'Range' values are independent of detector gain settings

1. Saturated single stains of six different fluorochromes (BB700, BV605, BV711, PE-CF594, PE-Cy5 and APC-R700) were prepared as described above.
2. Two sets of PMT voltages were used.
3. Voltages in group one were set to place the positive population at ~ 110,000 MFI.
4. Voltages in the second group were set to place the positive population at ~ 50,000 MFI.
5. Range was calculated for all spillover signals.
6. In a separate experiment single stained beads were run for a series of PMT voltages. In one set only the voltage for the primary detector was changed, and in the second set only the voltage of secondary detector was changed. The ISS and 'Range' values were calculated for both sets.

Application of 'Range' to compare instrument performance

We have three Fusions. The comparison was done using same laser power for all. Only in case of the Fusion 2 another higher laser power setup was used to test the effect of laser power on spread.

1. Single stains were run in all three Fusions.
2. Each experiment in each machine was performed in triplicates.
3. 'Range' value from all the repeat experiments were calculated and compared to check intra assay variation.

'Range', as dynamic range independent measure of spread

1. Fusion, Symphony, Quanteon and Cytoflex were used for this comparison. Fusion and Symphony has a 5 decade scale. Cytoflex and Quanteon has 7 and 7.2 decade scale respectively.
2. For Quanteon and Cytoflex, positive signals were set to approximately 250,000 and 1,000,000 MFI. For the Symphony and Fusion these were set to 110,000 MFI. Spillover from BV711 was not analyzed in the Cytoflex, because it lacked the appropriate detector.
3. Saturated single stain antibody capture beads were run in each system in triplicates.
4. Range was calculated for all spillover signals.

Application of 'Range': Investigate the effect of instrument characteristics on spread

Single stained beads were run at different laser output powers. The 'Range' values were compared (same Fusion) to investigate the effect of excitation intensity on spread.

Usefulness of 'Range':

1. Human PBMCs were used as samples
2. 7 X 10^6 Cells were thawed in a 37°C water bath.
3. The cells were then filtered gently, and centrifuged at 500 g for 10 minutes.
4. The supernatant was discarded, and the cells were re-suspended in 700 µl of staining buffer.
5. 100 µl of the cell suspension was kept aside as complete unstained.
6. 600 µl cells were stained 1st with BV421 or PE-Cy5 or BB700 conjugated CD4 antibody (0.36 µg) for 15 minutes in dark at 4°C.
7. This was divided in 6 equal portions.
8. Five different amounts (0.01, 0.025, 0.05, 0.5 and 1.25 µg) of CD45RA antibody were added.
9. The samples were stained for another 30 minutes in dark at 4°C,
10. Cells were washed twice in 3 ml of staining buffer, 5 minute centrifugation at 500 g (acceleration 9, deceleration 5).
11. Re-suspended in 200 µl of staining buffer.
12. Single stains of BV421, PE-Cy5, BB700 and Alexa Fluor 647 were prepared using BD compensation beads as described previously for automated compensation.

Data availability

All data are available at http://flowrepository.org, under the Repository ID of FR-FCM-Z336 to Z339 and FR-FCM-Z33A to Z33H

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Table Legend

Table 1. PMT voltage independent measure of spread: Two different sets of PMT voltages were applied to set the MFI of the positive population to 110,000 (110K) or 50,000 (50K). Six single stain bead samples were run using these two voltage sets, and the ISS and 'Range' values were calculated. The 'Range' values similar at the different voltages, but the ISS values were different.

Table 2: Comparison of all four Fusion setup: The 'Range' values for 10 fluorochromes run on three different Fusions are shown. For Fusion 2, "2_High" denotes higher laser power as for the other two instruments. The 'Range' values are categorized as follows: 1 – 120 (Green); 121 – 199 (Yellow); 200 – 299 (Orange); and 300+ (Red).

Table 3: Comparison of spread in all four systems: Ten different single stained bead sets were run on four different instruments. 'Range' was calculated for every combination. For BV711 there was no data available for the Cytoflex S. The 'Range' values are categorized as follows: 1 – 120 (Green); 121 – 199 (Yellow); 200 – 299 (Orange); and 300+ (Red).

Figure Legend

Figure 1: Concept of the 'Range': The figure shows the concept of the 'Range' value. Spillover fluorescence from primary fluorochrome X results in spread in detector B, and vice versa. The spread is quantified as the difference of the 99th percentile and 50th percentile in dimension B. Subsequently, this value is multiplied by a normalization factor to make the 'Range' value independent of detector gain settings. The 99th percentile is marked by a Green line.

Figure 2: Effect of PMT voltage on ISS: In A, the primary detector voltage was changed from 600V to 400V in steps of 100V for detector V605. This change did not alter the rSD and 'Range' values. However, the ISS values changed significantly. In B to D, voltages of both detectors were changed simultaneously to 600V, 500V or 400V respectively. The 'Range' values remained the same for both detectors in all cases, but the ISS values decreased considerably.

Table 1.

Detector	Type	B695		R730		V710		V610		Y670		Y610	
Fluorochrome		**110K**	**50K**	**110K**	**50K**	**110K**	**50K**	**110K**	**50K**	**110K**	**50K**	**110K**	**50K**
BB700	Range			577	596	632	632	77	78	39	40	15	17
	ISS			*7,24*	*5,27*	*9,27*	*6,59*	*1,08*	*0,78*	*0,53*	*0,37*	*0,2*	*0,15*
APC-R700	Range	49	52			147	155	21	22	30	32	8	8
	ISS	*0,66*	*0,44*			*2,09*	*1,44*	*0*	*0*	*0,4*	*0,28*	*0*	*0,04*
BV711	Range	89	96	786	788			24	25	15	16	8	8
	ISS	*1,22*	*0,84*	*8,98*	*6,23*			*0,14*	*0,11*	*0,16*	*0,1*	*0,03*	*0*
BV605	Range	106	112	78	80	177	181			85	86	206	212
	ISS	*1,49*	*1,03*	*1,14*	*0,79*	*2,44*	*1,67*			*1,21*	*0,82*	*3,05*	*2,16*
PE-Cy5	Range	721	785	309	298	412	411	38	39			31	29
	ISS	*11,03*	*7,71*	*4,31*	*3,02*	*5,8*	*4,14*	*0,44*	*0,33*			*0,37*	*0,26*
PE-CF594	Range	299	322	63	62	198	195	296	301	179	184		
	ISS	*4,27*	*2,88*	*0,83*	*0,57*	*2,67*	*1,82*	*4,16*	*2,87*	*2,42*	*1,73*		

Table 2.

	Detector	B695	V450	V610	V710	Y582	Y610	Y670	Y780	R670	R730
Fusion	**Fluorochrome**	**Range**	**Range**	**Range**	**Range**	**Range**	**Range**	**Range**	**Range**	**Range**	**Range**
1	**BB700**		29	158	1819	8	17	43	74	329	471
2			28	105	1248	6	14	40	63	266	420
3			50	77	525	7	15	41	60	205	641
2_High			29	107	1280	6	15	40	63	229	375
1	**BV421**	13		44	26	7	10	10	11	23	17
2		16		42	23	6	8	11	13	20	17
3		14		35	20	6	9	10	10	15	13
2_High		15		42	24	7	10	13	15	20	17
1	**BV605**	110	92		220	61	248	87	61	89	107
2		110	77		177	40	194	86	57	79	102
3		101	43		175	42	165	89	54	79	89
2_High		119	53		183	40	198	90	60	83	99
1	**BV711**	75	85	32		7	9	15	62	92	476
2		84	91	27		6	8	16	55	91	586
3		84	55	24		6	8	16	53	63	709
2_High		79	73	28		6	8	17	55	89	538
1	**PE**	125	17	334	101		197	96	55	109	39
2		121	15	400	81		163	99	55	98	46
3		130	15	148	86		172	100	50	92	31
2_High		115	15	396	77		164	104	56	103	46
1	**PE-CF594**	309	19	656	254	67		183	123	208	68
2		245	17	744	148	51		184	116	183	78
3		272	19	239	167	54		188	111	177	55
2_High		241	17	747	141	53		193	118	191	77
1	**PE-Cy5**	736	29	51	629	83	32		232	935	343
2		494	19	35	394	44	26		204	564	321
3		574	26	33	339	42	27		204	548	225
2_High		499	19	35	418	34	26		212	611	308
1	**PE-Cy7**	23	20	30	27	203	23	46		20	36
2		26	18	28	24	159	18	39		23	28
3		22	14	23	22	34	18	16		18	25
2_High		21	17	28	25	57	18	20		23	25
1	**APC**	121	20	29	145	8	12	136	56		160
2		95	17	26	100	6	10	110	51		163
3		116	15	22	119	7	10	118	49		147
2_High		110	18	27	116	6	11	113	51		139
1	**APC-R700**	42	17	26	219	7	10	34	114	570	
2		43	15	24	204	6	8	30	90	224	
3		48	14	21	123	6	8	31	85	105	
2_High		42	15	25	212	6	8	31	90	104	

Table 3.

	Detector	BB700	BV421	BV605	BV711	PE	PE-CF594	PE-Cy5	PE-Cy7	APC	APC-R700
System	Fluorochrome	Range	Range	Range	Range	Range	Range	Range	Range	Range	Range
Symphony	**BB700**		45	68	563	23	18	38	56	523	509
Fusion			29	158	1819	8	17	43	74	329	471
Cytoflex			20	106		5	6	51	38	181	567
Quanteon			54	228	827	30	33	37	55	124	279
Symphony	**BV421**	13		33	27	22	15	14	9	35	20
Fusion		13		44	26	7	10	10	11	23	17
Cytoflex		7		38		4	3	5	5	38	20
Quanteon		33		46	35	29	30	26	25	37	38
Symphony	**BV605**	73	39		177	129	220	81	52	83	77
Fusion		110	92		220	61	248	87	61	89	107
Cytoflex		85	70			44	115	29	29	47	35
Quanteon		95	68		145	103	145	77	46	62	78
Symphony	**BV711**	85	48	23		22	15	17	41	161	604
Fusion		75	85	32		7	9	15	62	92	476
Cytoflex											
Quanteon		97	79	40		29	29	28	54	62	374
Symphony	**PE**	105	16	128	74		260	97	49	105	31
Fusion		125	17	334	101		197	96	55	109	39
Cytoflex		71	20	277			88	32	27	43	43
Quanteon		91	47	182	53		153	71	38	58	44
Symphony	**PE-CF594**	215	18	184	150	156		178	103	198	51
Fusion		309	19	656	254	67		183	123	208	68
Cytoflex		201	19	480		42		49	66	64	103
Quanteon		202	47	334	93	124		134	73	94	70
Symphony	**PE-Cy5**	490	22	28	305	61	41		192	855	319
Fusion		736	29	51	629	83	32		232	935	343
Cytoflex		649	21	39		34	13		156	816	453
Quanteon		435	49	51	370	127	47		141	326	201
Symphony	**PE-Cy7**	31	16	21	29	43	26	18		36	41
Fusion		23	20	30	27	203	23	46		20	36
Cytoflex		22	20	23		306	7	131		41	25
Quanteon		36	47	39	46	74	37	30		38	47
Symphony	**APC**	105	18	20	120	22	18	128	52		280
Fusion		121	20	29	145	8	12	136	56		160
Cytoflex		111	22	24		5	10	145	32		264
Quanteon		121	48	39	127	32	33	129	49		165
Symphony	**APC-R700**	51	16	20	117	22	14	35	85	366	
Fusion		42	17	26	219	7	10	34	114	570	
Cytoflex		105	21	22		5	2	83	60	179	
Quanteon		59	48	36	173	30	29	40	88	267	

Figure 1.

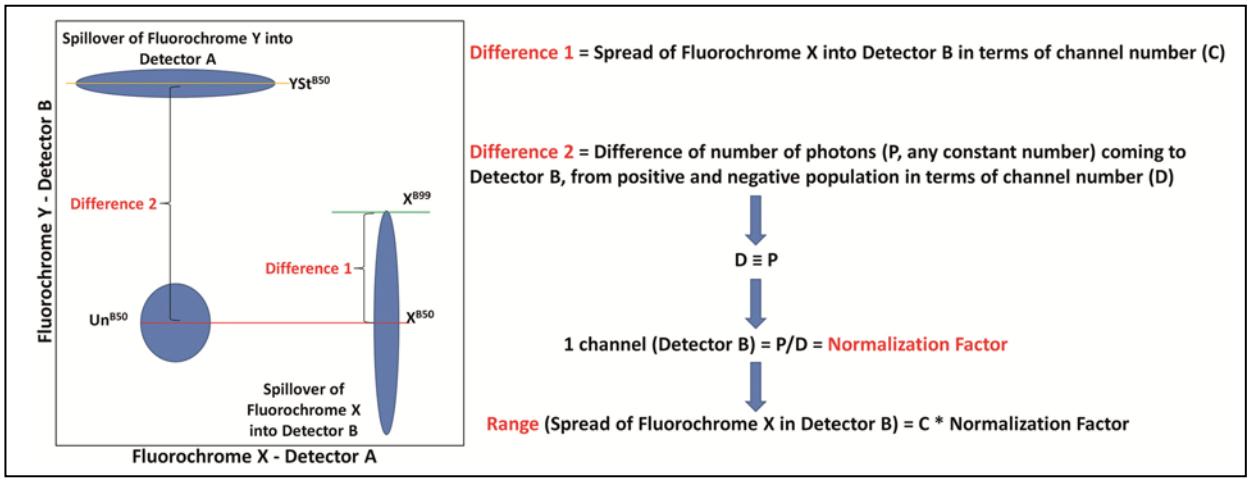
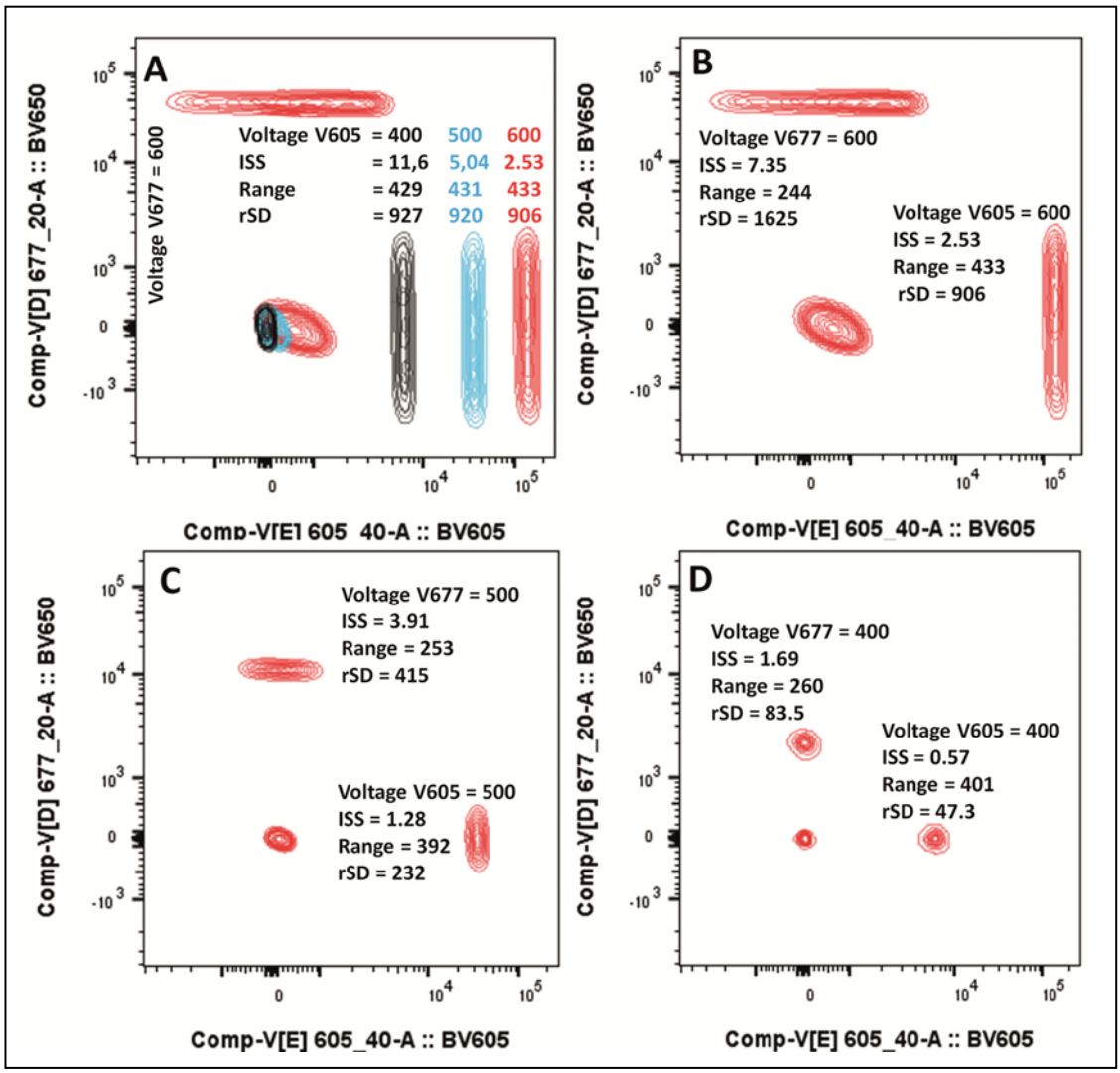

Spillover of Fluorochrome Y into Detector A
YSt^B50
Difference 2
X^B99
Difference 1
Un^B50
X^B50
Spillover of Fluorochrome X into Detector B
Fluorochrome Y - Detector B
Fluorochrome X - Detector A
Difference 1 = Spread of Fluorochrome X into Detector B in terms of channel number (C)
Difference 2 = Difference of number of photons (P, any constant number) coming to Detector B, from positive and negative population in terms of channel number (D)
D ≡ P
1 channel (Detector B) = P/D = Normalization Factor
Range (Spread of Fluorochrome X in Detector B) = C * Normalization Factor

Figure 2.


A
Voltage V677 = 600
Voltage V605 = 400 500 600
ISS = 11,6 5,04 2.53
Range = 429 431 433
rSD = 927 920 906
Comp-V[D] 677_20-A :: BV650
Comp-V[E] 605_40-A :: BV605
B
Voltage V677 = 600
ISS = 7.35
Range = 244
rSD = 1625
Voltage V605 = 600
ISS = 2.53
Range = 433
rSD = 906
Comp-V[D] 677_20-A :: BV650
Comp-V[E] 605_40-A :: BV605
C
Voltage V677 = 500
ISS = 3.91
Range = 253
rSD = 415
Voltage V605 = 500
ISS = 1.28
Range = 392
rSD = 232
Comp-V[D] 677_20-A :: BV650
Comp-V[E] 605_40-A :: BV605
D
Voltage V677 = 400
ISS = 1.69
Range = 260
rSD = 83.5
Voltage V605 = 400
ISS = 0.57
Range = 401
rSD = 47.3
Comp-V[D] 677_20-A :: BV650
Comp-V[E] 605_40-A :: BV605

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Author Contributions Statement: DB conceived, performed and analyzed experiments. MvB helped in critical thinking also generated and analyzed data for Supplementary Figure 2.. DB and MvB wrote the manuscript. FvD actively helped in data collection and played a crucial role in instrument maintenance, which kept the flow cytometers in the best condition. AP helped in sample preparation. RT developed the script.

Additional Information

Competing interest

The author(s) declare no competing interests.

Supplementary Information

‘Range’: A gain and dynamic range independent quantification of spread aiding in panel design

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		B695	V450	V610	V710	Y582	Y610	Y670	Y780	R670	R730
BB700	**Range**		29±2.5%	158±4.7%	1819±4.5%	8±2.5%	17±3.2%	43±2.5%	74±1.8%	224±3%	471±2.3%
	ISS		0.33±2.1%	1.28±0.8%	10.56±3%	0.04±12.2%	0.21±0.3%	0.55±0.8%	1±1.3%	2.4±1.6%	7.18±2.8%
BV421	**Range**	13±0.8%		44±1.1%	26±2%	7±3%	10±2.5%	10±3.1%	11±2%	16±1.9%	17±0.9%
	ISS	0.02±173%		0.44±0.9%	0.19±6.4%	0.01±173%	0.07±0.2%	0.01±173%	NA	NA	0.1±8.3%
BV605	**Range**	110±0.7%	92±2.1%		220±1.7%	61±1.8%	248±2.7%	87±0.8%	61±0.9%	61±2.1%	107±1.7%
	ISS	1.63±0.8%	0.68±0.9%		2.99±1.2%	0.86±0.9%	3.96±1.6%	1.19±0.7%	0.85±0.8%	0.8±1.8%	1.47±0.9%
BV711	**Range**	75±0.7%	85±8.7%	32±1.4%		7±2.9%	9±0.3%	15±5.5%	62±0.9%	63±4.8%	476±1.1%
	ISS	1.04±0.9%	0.89±2.3%	0.16±3.6%		0.01±173%	0.03±1.1%	0.15±2.5%	0.87±0.8%	0.69±1.8%	7.35±0.5%
PE	**Range**	125±1.7%	17±2.8%	334±2.5%	101±1.9%		197±2.8%	96±2%	55±0.8%	74±2.2%	39±2.2%
	ISS	1.76±0.9%	NA	3.34±0.7%	1.37±1%		2.77±1.2%	1.28±1%	0.74±1.7%	0.93±1.2%	0.45±1.8%
PE-CF594	**Range**	309±0.7%	19±1.3%	656±1.7%	254±2.4%	67±2.3%		183±1.1%	123±1.7%	141±1.9%	68±1.9%
	ISS	4.99±3.8%	NA	6.45±1.4%	3.9±2.5%	0.9±0.9%		2.43±0.5%	1.7±0.4%	1.8±0.6%	0.89±2.9%
PE Cy5	**Range**	736±0.5%	29±1.2%	51±0.5%	629±2.4%	83±5.1%	32±2.1%		232±2.6%	637±1.9%	343±3.6%
	ISS	13.1±1.6%	0.39±2.8%	0.62±2%	9.29±1.7%	0.46±1.1%	0.42±1.1%		3.29±1%	8.44±2.8%	5±2.3%
PE Cy7	**Range**	23±7.5%	20±3.2%	30±0.5%	27±1.4%	203±2.5%	23±1.9%	46±2.3%		13±64.6%	36±1.6%
	ISS	0.21±2.7%	NA	0.14±4.2%	0.22±3.9%	0.27±0.8%	0.26±0.1%	0.18±3.9%		0.1±23.2%	0.43±2.5%
Alx647	**Range**	70±2%	21±6%	25±2.1%	47±2%	7±1.6%	9±2.1%	80±0.6%	51±1.3%		225±3.2%
	ISS	1.02±0.9%	NA	NA	0.57±2%	0.02±90%	0.02±86.6%	1.11±1.5%	0.72±0.5%		3.09±0.9%
APC R700	**Range**	42±0.9%	17±2.8%	26±1.8%	219±1.9%	7±5.1%	10±1%	34±3.4%	114±1.2%	388±1.2%	
	ISS	0.57±2%	NA	NA	2.38±0.3%	0.03±0.2%	0.04±19.2%	0.39±2.3%	1.62±1.8%	1.05±0.7%	

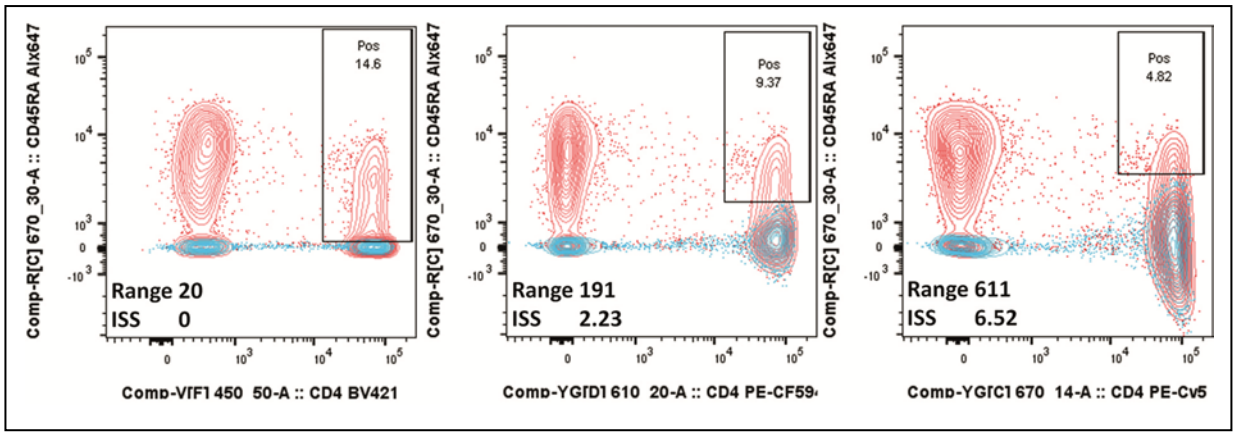
Supplementary Table S1: Reproducibility of 'Range': Three replicative data sets from Fusion 1 were used to calculate 'Range' and ISS values. Each box displays the average of 3 measurements with the relative standard deviation (%).

Detector	B695		V450	V610	V710	Y582	Y610	Y670	Y780	R670		R730	
Fluorochrome	BB700	PerCP-Cy™5.5								Alexa 647	APC	APC R700	Alexa 700
BB700			29	161	1864	8	17	41	72	232	341	483	1695
PerCP-Cy™5.5			20	26	258	8	9	56	63	53	78	117	410
Alexa Fluor 647	69	555	21	25	48	8	10	80	51			223	782
APC	119	954	20	30	147	8	12	134	55			162	568
APC-R700	42	335	17	26	223	7	9	35	116	386	569		
Alexa Fluor 700	21	171	20	25	96	7	9	15	45	23	34		

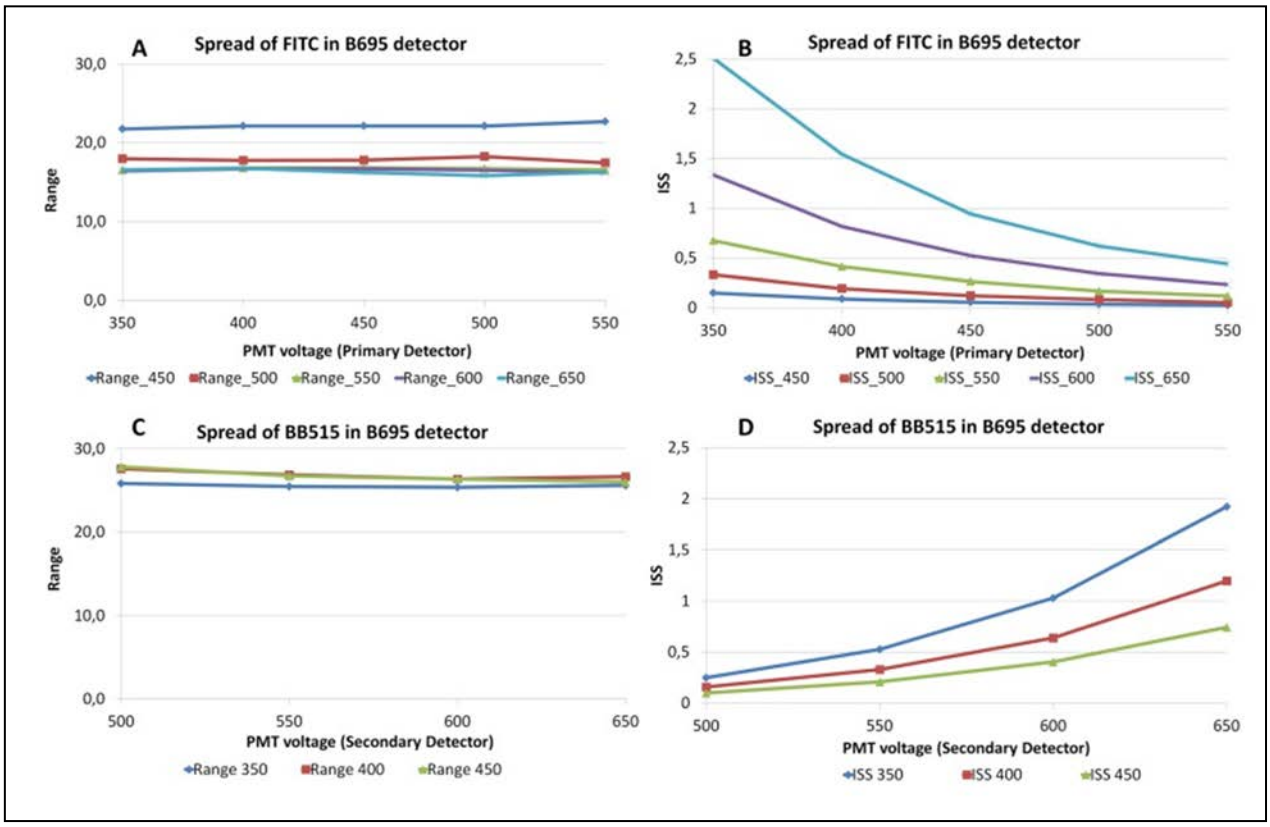
Supplementary Table S2: Effect of multiple fluorochrome in one detector on 'Range': In this table we have presented a special scenario, where B695, R670 and R730 are associated with two fluorochromes. Data indicates same amount of spread (as channel number) translates in bigger 'Range' when a dim fluorochrome is associated with the secondary detector.

Laser	BD FACSAria™ Fusion 1 and 3			BD FACSAria™ Fusion 2			BD FACSSymphony A5			ACEA NovoCyte Quanteon			BC CytoFLEX S	
	Long Pass	Band Pass	Laser Power (mW)	Long Pass	Band Pass	Laser Power (mW)	Long Pass	Band Pass	Laser Power (mW)	Mirror	Band Pass	Laser Power (mW)	Band Pass	Laser Power (mW)
355			NA	770	820/60	50	770	819/44	100			NA		NA
				-	379/28		690	735/50						
							630	670/25						
							600	615/20						
							550	580/20						
							450	515/30						
							-	379/28						
405	750	780/60	85	750	780/60	100	750	780/60	100	757LP	780/60	100	450/45	80
	690	710/50		690	710/50		735	750/30		-	725/40		525/40	
	630	660/20		630	660/20		685	711/25		705LP	695/40		610/20	
	595	610/20		595	610/20		635	661/11		685LP	660/20		660/20	
	505	525/50		505	525/50		595	605/40		598SP	615/20			
	-	450/50		-	450/50		550	586/15		570SP	586/20			
							455	470/20		552LP	530/30			
							-	431/28		495LP	445/45			
488	655	695/40	50	655	695/40	100	750	780/60	100	757LP	780/60	100	690/50	50
	502	530/30		502	530/30		735	750/30		-	725/40		525/40	
	-	488/10		-	488/10		685	710/50		705LP	695/40		488/10	
							635	661/11		685LP	660/20			
							600	610/20		598SP	615/20			
							570	586/15		570SP	586/20			
							505	530/30		552LP	530/30			
							-	488/10						
640	755	780/60	100	755	780/60	140	750	780/60	100	757LP	780/60	100	660/20	50
	690	730/45		690	730/45		690	730/45		-	725/40		712/25	
	-	670/30		-	670/30		-	670/30		705LP	695/40		780/60	
										685LP	660/20		-	
561	735	780/60	50	735	780/60	50	750	780/60	100	757LP	780/60	100	585/42	30
	685	710/50		685	710/50		685	710/50		-	725/40		610/20	
	630	670/14		630	670/14		635	670/30		705LP	695/40		690/50	
	600	610/20		600	610/20		600	610/20		685LP	660/20		780/60	
	-	582/15		-	586/15		-	586/15		598SP	615/20			
										570SP	586/20			
										552LP	561/14			

Supplementary Table S3: Configuration detail: This table lists the optical configurations of the instruments that were used in this study.



Supplementary Figure S1: Usefulness of range: Alexa Fluor 647 conjugated CD45RA was combined with CD4 BV421, PE-CF594 or PE-Cy5 (clone matched), separately. The concentration of the CD45RA antibody was equal in all mixes. The blue contour indicates the control sample , which was stained with a CD4 antibody only. The red contour shows the combined staining. 'Range' and ISS values are displayed. The double positive population is better separated from the single positive population at lower 'Range' values as is exhibited by a higher percentage.



Supplementary Figure S2: Effect of detector settings on 'Range' and ISS: In A and B we have only changed the PMT voltage for the primary detector. 'Range' remains same but ISS dropped with increase of voltage. In C and D, we only changed PMT voltage for secondary detector. 'Range' again remain same but ISS increased with voltage.