Compensation and **unmixing** are common methods used to determine the true amount of fluorescence from a fluorochrome when using either traditional or full spectrum Flow Cytometry to carry out multicolor experiments. In these cases, it is necessary to use appropriate single color controls which adhere to all the appropriate rules. If any of these rules are not met compensation or unmixing may not be correct. To **troubleshoot** the acquired panel we need to use a **quantitative approach** to determine if compensation and unmixing are correctly determined.

Secondary **S**tain **I**ndex (**SSI**)

A quantitative metric should be used when determining the success of unmixing or compensation. The **SSI** can be calculated with the MFI of the positive and negative population along with the SD of the negative population, all for the secondary detector(s)/fluorochrome(s).
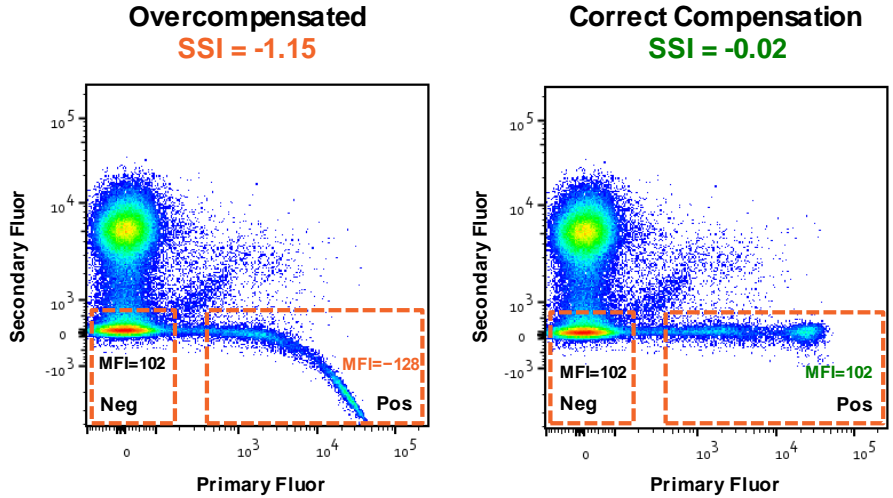
$$SI_{Sec} = \frac{MFI_{pos,sec}^{comp/unmix} - MFI_{neg,sec}^{comp/unmix}}{2 \times SD_{neg,sec}^{comp,unmix}}$$

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Using the SSI allows us to determine if the difference between the MFIs in the secondary channel/fluorochrome of the primary positive and negative populations is within two SDs:

-1 **SSI** +1

If $-1 < SI_{Sec} < 1$
difference is within 2 SDs

💡 Helpful Hints

- Proper controls are necessary for a successful run. Ensure you match your control and experimental fluorochromes exactly, check signal intensity and use the correct negative for your single colors
- Manual compensation or unmixing, or visual tweaking of software derived results **should never** be done
- When testing a panel for the first time, run both single color beads and cells to check which is best for your specific panel

Overcompensated
SSI = -1.15

MFI=102
Neg

MFI=−128
Pos

Correct Compensation
SSI = -0.02

MFI=102
Neg

MFI=102
Pos

SSI Matrix

Use the SSI to systematically check over- or under-compensation/unmixing by building an **SSI matrix**

Primary →

Secondary ↓

	AF700	APC-Cy7	PE	PE-CF594	PE-Cy5	PE-Cy7
AF700		-0.03105	0.083241	-0.00237	-1.66488	-0.01418
APC-Cy7	-0.28516		0.026686	-0.00781	-0.16747	-0.94993
PE	-0.06641	0.09351		0.276773	-0.50025	-0.0976
PE-CF594	-0.12	0.194885	0.027952		2.181774	-0.05772
PE-Cy5	0.282178	-2.769	0.193609	-0.05213		-0.05063
PE-Cy7	-0.11777	0.183122	0.039959	0.003004	1.122928	