A Closer Look at Compensation Methods in FlowJo

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Cincinnati Children's™

Research Flow Cytometry Facility

Compensation

- 4 different methods of compensation on FlowJo
  - Conventional
  - Autospill
  - “Spectral”
  - “Spectral” Autospill
- Compensation starts with good controls
  - Bright or brighter controls
  - Record plenty of events when acquiring
    - ~30,000 events are best
    - Or at least 100 events in the positive gate

What is Compensation

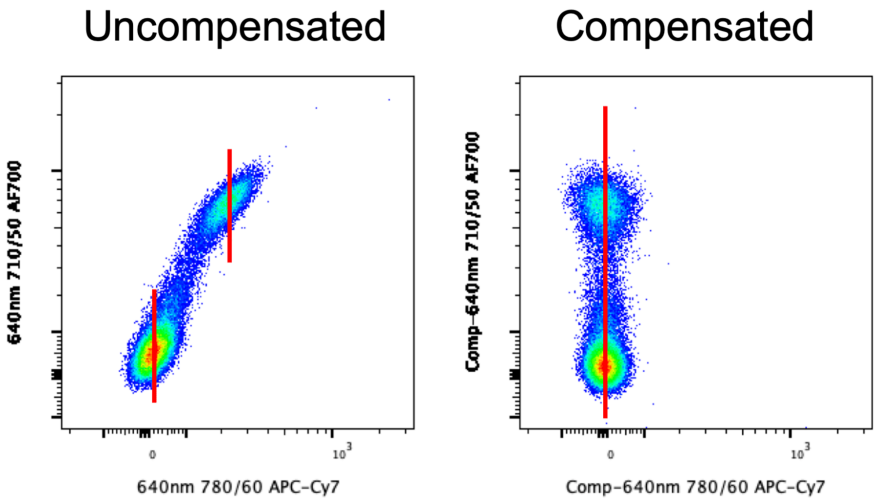


Figure 1. Accurate compensation aligns the MFI of the positive population to the MFI of the negative population.

- A correction of spillover of a fluorochrome into a secondary detector.
- Single Color Compensation controls are acquired and a positive and negative emission is defined

What is Autospill?

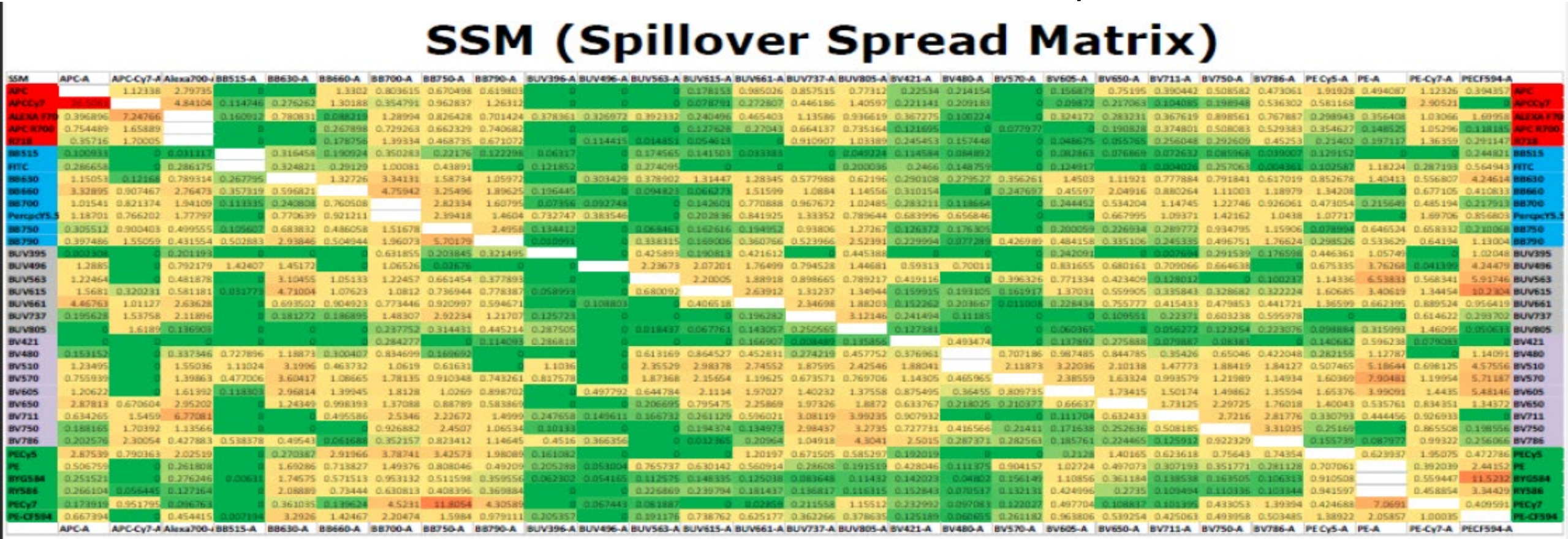
- A Flow Jo compensation algorithm that uses gate-less compensation
- Do not need to define a positive and negative population and do not need an unstained control
- Algorithm will define positive and negative populations
- Can use an unstained control to remove autofluorescence
  - Utilizing an empty detector that has the highest Autofluorescence
- Simple, and more efficient
- Helps remove biased positive and negative gating and human error
- AutoSpill: a method for calculating spillover coefficients in high-parameter flow cytometry. Authors: Carlos P. Roca, Oliver T. Burton, Teresa Prezzemolo, Carly E. Whyte, Richard Halpert, Łukasz Kreft, James Collier, Alexander Botzki, Josef Spidlen, Stéphanie Humblet-Baron, Adrian Liston.

What is Spectral Compensation?

- Need more detectors than fluorescent probes
- Utilizing spectral compensation, signal from non-primary detectors are still used to help unmix the true signal of the primary markers
- Same “process” as traditional compensation
  - i.e. define negative and positive population
- FlowJo spectral algorithm has an optimize weights function to utilize selected comp controls to compare the hypothetical spillover spread matrix (SSM) for different weights

Spillover Spread Matrix (SSM)

- A matrix that reveals how much fluorescence can spillover into each



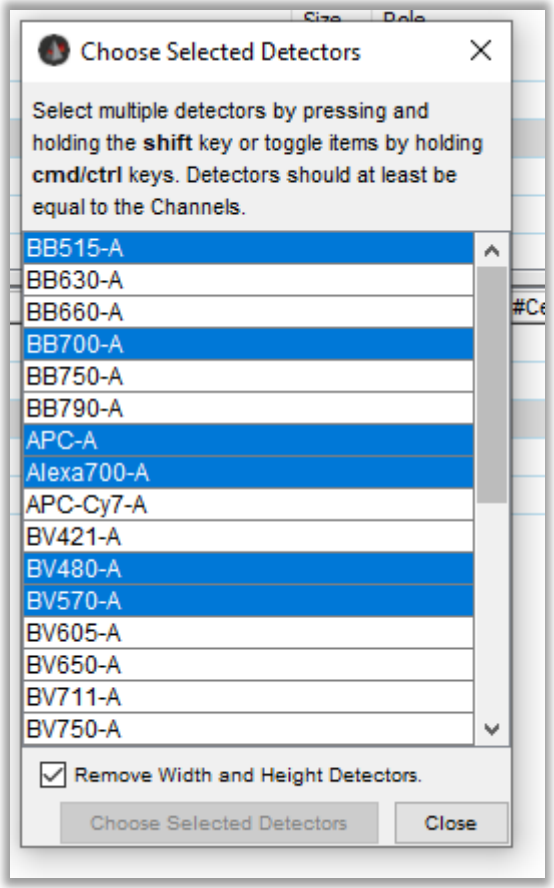
SSM	APC-A	APC-Cy7-A	Alexa700-A	BB515-A	BB630-A	BB660-A	BB700-A	BB750-A	BB790-A	BUV396-A	BUV496-A	BUV563-A	BUV615-A	BUV661-A	BUV737-A	BUV805-A	BV421-A	BV480-A	BV570-A	BV605-A	BV650-A	BV711-A	BV750-A	BV786-A	PE Cy5-A	PE-A	PE-Cy7-A	PECF594-A	
APC		1.12338	2.79735	0	0	1.3302	0.803615	0.670498	0.619803	0	0	0	0.178153	0.985026	0.857515	0.77312	0.22534	0.214154	0	0.156879	0.75195	0.390442	0.508582	0.473061	1.91928	0.494087	1.12326	0.394357	APC
APCCy7	26.5068		4.84104	0.114746	0.276262	1.30188	0.354791	0.962837	1.26312	0	0	0	0.078791	0.272807	0.446186	1.40597	0.221141	0.209183	0	0.09872	0.217063	0.104085	0.198948	0.536302	0.581168	0	2.90521	0	APCCy7
ALEXA F70	0.396896	7.24766		0.160912	0.780831	0.048219	1.28994	0.826428	0.701424	0.378361	0.326973	0.392137	0.240496	0.465403	1.13586	0.936619	0.367275	0.100224	0	0.324172	0.283231	0.367619	0.898561	0.767887	0.298943	0.356408	1.03066	1.69958	ALEXA F70
APC R700	0.754489	1.65889		0	0	0.267898	0.729263	0.662329	0.740682	0	0	0	0.127628	0.27043	0.664137	0.735164	0.121695	0	0.077977	0	0.190828	0.374801	0.508083	0.529383	0.354627	0.148525	1.05296	0.118185	APC R700
R718	0.35716	1.70005		0	0	0.178756	1.39334	0.468735	0.671072	0	0.114415	0.014851	0.054613	0	0.910907	1.03389	0.245453	0.157448	0	0.048675	0.055765	0.256048	0.292609	0.45253	0.21402	0.197117	1.36359	0.291147	R718
BB515	0.100933	0	0.031117		0.316458	0.190924	0.350283	0.22176	0.122298	0.06317	0	0.174565	0.141503	0.033383	0	0.049224	0.114584	0.084892	0	0.082863	0.076869	0.072632	0.085968	0.039007	0.129152	0	0	0.244821	BB515
FITC	0.286658	0	0.286175		0.324821	0.29129	1.00081	0.43891	0	0.121852	0	0.274095	0	0	0	0.200036	0.2466	0.148759	0	0.124917	0	0.004026	0.257063	0.084361	0.102582	1.18224	0.287193	0.564943	FITC
BB630	1.15053	0.12168	0.789314	0.267795		1.32726	3.34131	1.58734	1.05972	0	0.303429	0.378902	1.31447	1.28345	0.577988	0.62196	0.290108	0.279527	0.356261	1.4503	1.11921	0.777884	0.791841	0.617019	0.852678	1.40413	0.556807	4.24614	BB630
BB660	3.32895	0.907467	2.76473	0.357319	0.596821		4.75942	3.25496	1.89625	0.196445	0	0.094823	0.066273	1.51599	1.0884	1.14556	0.310154	0	0.247697	0.45597	2.04916	0.880264	1.11003	1.18979	1.34208	0	0.677105	0.410833	BB660
BB700	1.01541	0.821374	1.94109	0.111335	0.240808	0.760508		2.82334	1.60795	0.07956	0.092748	0	0.142601	0.770888	0.967672	1.02485	0.283211	0.118664	0	0.244452	0.534204	1.14745	1.22746	0.926061	0.473054	0.215649	0.485194	0.217913	BB700
PercpCY5.5	1.18701	0.766202	1.77797	0	0.770639	0.921211		2.39418	1.4604	0.732747	0.383546	0	0.202836	0.841925	1.33352	0.789644	0.683996	0.656846	0	0	0.667995	1.09371	1.42162	1.0438	1.07717	0	1.69706	0.856803	PercpCY5.5
BB750	0.305512	0.900403	0.499555	0.105602	0.683832	0.486058	1.51678		2.4958	0.134412	0	0.068463	0.162616	0.194952	0.93806	1.27267	0.126372	0.176305	0	0.200059	0.226934	0.289772	0.934795	1.15906	0.078994	0.646524	0.658332	0.210068	BB750
BB790	0.397486	1.55059	0.431554	0.502883	2.93846	0.504944	1.96073	5.70179		0.010991	0	0.338315	0.169006	0.360766	0.523966	2.52391	0.229994	0.077289	0.426989	0.484158	0.335106	0.245335	0.496751	1.76624	0.298526	0.533629	0.64194	1.13004	BB790
BUV395	0.003308	0	0.201193	0	0	0	0.631855	0.203845	0.321495		0	0.425893	0.190813	0.421612	0	0.445388	0	0	0	0.242091	0	0.007694	0.291539	0.176598	0.446361	1.05749	0	1.02048	BUV395
BUV496	1.2885	0	0.792179	1.42407	1.45172	0	1.06526	0.02676	0	0		2.23673	2.07201	1.76499	0.794528	1.44681	0.59313	0.70011	0	0.831655	0.680161	0.709066	0.664638	0	0.675335	3.76268	0.041399	4.24479	BUV496
BUV563	1.22464	0	0.481878	0	3.10455	1.05133	1.22457	0.661454	0.377893	0	0		2.20005	1.88918	0.898665	0.789217	0.419116	0	0.396326	0.771334	0.423409	0.128012	0	0.100237	1.14336	6.53833	0.568341	5.91746	BUV563
BUV615	1.5681	0.320231	0.581181	0.031773	4.71004	1.07623	1.0812	0.736944	0.778387	0.058993	0	0.680092		2.63912	1.31237	1.34944	0.159915	0.193105	0.161917	1.37031	0.559905	0.335843	0.328682	0.322224	1.60685	3.40619	1.34454	10.2304	BUV615
BUV661	4.46763	1.01127	2.63628	0	0.693502	0.904923	0.773446	0.920997	0.594671	0	0.108803	0	0.406518		2.34698	1.88203	0.152262	0.203667	0.011004	0.228434	0.755777	0.415433	0.479853	0.441721	1.36599	0.662395	0.889524	0.956419	BUV661
BUV737	0.195628	1.53758	2.31896	0	0.181272	0.186895	1.48307	2.92234	1.21707	0.125723	0	0	0	0.196282		3.12146	0.241494	0.11185	0	0	0.109551	0.22371	0.603238	0.595978	0	0	0.614622	0.293702	BUV737
BUV805	0	1.6189	0.136909	0	0	0	0.237752	0.314431	0.445214	0.287505	0	0.018437	0.067761	0.143057	0.250565		0.127381	0	0	0.060365	0	0.056272	0.123254	0.223876	0.098884	0.315993	1.46095	0.950633	BUV805
BV421	0	0	0	0	0	0	0.284277	0	0.114093	0.286818	0	0	0	0.166907	0.008489	0.135856		0.493474	0	0.137892	0.275888	0.079867	0.08383	0	0.140682	0.596238	0.079083	0	BV421
BV480	0.153152	0	0.337346	0.727896	1.18873	0.300407	0.834699	0.169692	0	0	0	0.613169	0.864527	0.452831	0.274219	0.457752	0.376961		0.707186	0.987485	0.844785	0.35426	0.65046	0.422048	0.282155	1.12787	0	1.14091	BV480
BV510	1.23495	0	1.55036	1.11024	3.1996	0.463732	1.0619	0.61631	0	1.1036	0	2.35529	2.98378	2.74552	1.87595	2.42546	1.88041		2.11873	3.22036	2.10138	1.47773	1.88419	1.84127	0.507465	5.18644	0.698125	4.57556	BV510
BV570	0.755939	0	1.39863	0.477006	3.60417	1.08665	1.78135	0.910348	0.743261	0.817578	0	1.87368	2.15654	1.19625	0.673571	0.769706	1.14305	0.465965		2.38559	1.63324	0.993579	1.21989	1.14934	1.60369	7.90481	1.19954	5.71187	BV570
BV605	1.20622	0	1.61392	0.118303	2.96814	1.39945	1.8128	1.0269	0.898702	0	0.497792	0.644784	2.1114	1.97027	1.40232	1.37558	0.875495	0.36455	0.809735		1.73415	1.50174	1.49862	1.35594	1.65376	3.99091	1.4435	5.48146	BV605
BV650	2.87813	0.670604	2.95202	0	1.24349	0.998393	1.37088	0.88789	0.583869	0	0	0.206695	0.795475	2.25869	1.97326	1.8872	0.633767	0.218025	0.210377	0.66637		1.73125	2.29725	1.76018	1.40043	0.535761	0.834351	1.34372	BV650
BV711	0.634265	1.5459	6.77081	0	0	0.495586	2.5346	2.22672	1.4999	0.247658	0.149611	0.166732	0.261129	0.596021	3.08119	3.99235	0.907932	0	0	0.111704	0.632433		2.7216	2.81776	0.330793	0.444456	0.926933	0	BV711
BV750	0.188165	1.70392	1.13566	0	0	0	0.926882	2.4507	1.06534	0.10133	0	0	0.194374	0.134973	2.98437	3.2735	0.727731	0.416566	0.21411	0.171638	0.252636	0.508185		3.31035	0.25169	0	0.865508	0.198556	BV750
BV786	0.202576	2.30054	0.427883	0.538378	0.49541	0.061688	0.352157	0.823412	1.14645	0.4516	0.366356	0	0.012365	0.20964	1.04918	4.3041	2.5015	0.287371	0.282563	0.185761	0.224465	0.125912	0.922329		0.155739	0.087972	0.99322	0.256066	BV786
PECy5	2.87539	0.790363	2.02519	0	0.270387	2.91966	3.78741	3.42573	1.98089	0.161082	0	0	0	1.20197	0.671505	0.585297	0.192019	0	0	0.2128	1.40165	0.623618	0.75643	0.74354		0.623937	1.95075	0.472786	PECy5
PE	0.506755	0	0.261808	0	1.69286	0.713827	1.49376	0.808046	0.49209	0.205288	0.053004	0.765737	0.630142	0.560914	0.28608	0.191519	0.428046	0.111375	0.904157	1.02724	0.497073	0.307193	0.351771	0.781128	0.707061		0.392039	2.44152	PE
BYG584	0.251521	0	0.276246	0.00631	1.74575	0.571513	0.953132	0.511598	0.359556	0.062302	0.054165	0.112575	0.148335	0.125038	0.083648	0.11432	0.142023	0.04802	0.156149	1.10856	0.361184	0.138538	0.163505	0.106313	0.910508		0.559447	11.5232	BYG584
BYS86	0.266104	0.656445	0.127164	0	2.08889	0.73444	0.630813	0.408396	0.369884	0	0	0.226869	0.239794	0.181437	0.136817	0.116315	0.152843	0.070537	0.132131	0.424996	0.2735	0.109494	0.110936	0.103844	0.941597		0.458854	3.34429	BYS86
PECy7	0.173919	0.951795	0.096763	0	0.361035	0.139624	4.5231	11.3054	4.30589	0	0.067443	0.081887	0	0.02858	0.211558	1.15512	0.232992	0.097083	0.122027	0.497704	0.108837	0.101395	0.433053	1.39394	0.424683	7.0691		0.409591	PECy7
PE-CF594	0.667394	0	0.454415	0.007194	3.2926	1.42467	2.20474	1.5984	0.979111	0.206357	0	0.191176	0.738762	0.625177	0.362266	0.378635	0.125189	0.060655	0.261182	0.963806	0.539254	0.425063	0.493958	0.503485	1.38922	2.05857	1.00035		PE-CF594
	APC-A	APC-Cy7-A	Alexa700-A	BB515-A	BB630-A	BB660-A	BB700-A	BB750-A	BB790-A	BUV396-A	BUV496-A	BUV563-A	BUV615-A	BUV661-A	BUV737-A	BUV805-A	BV421-A	BV480-A	BV570-A	BV605-A	BV650-A	BV711-A	BV750-A	BV786-A	PE Cy5-A	PE-A	PE-Cy7-A	PECF594-A	

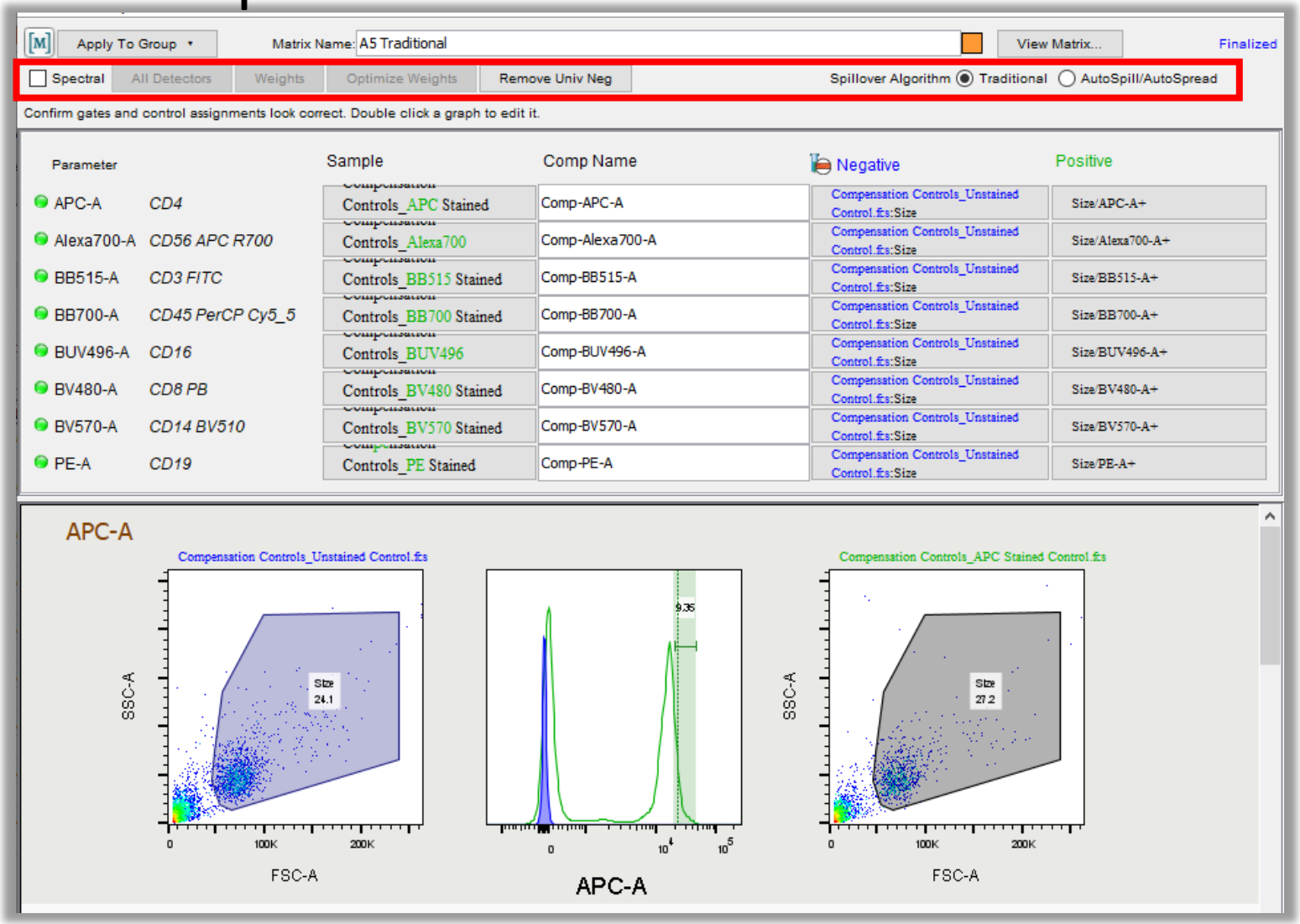
**To read this table: spread of fluor in the row impacts resolution of the fluor in the column. Red means the fluor in that row has significant spread into the dye in the column.

The SSM is independent of MFI (fluorochrome brightness and / or antigen density). MFI is normalized in the SSM formula, in order to assess instrument performance independent of MFI.

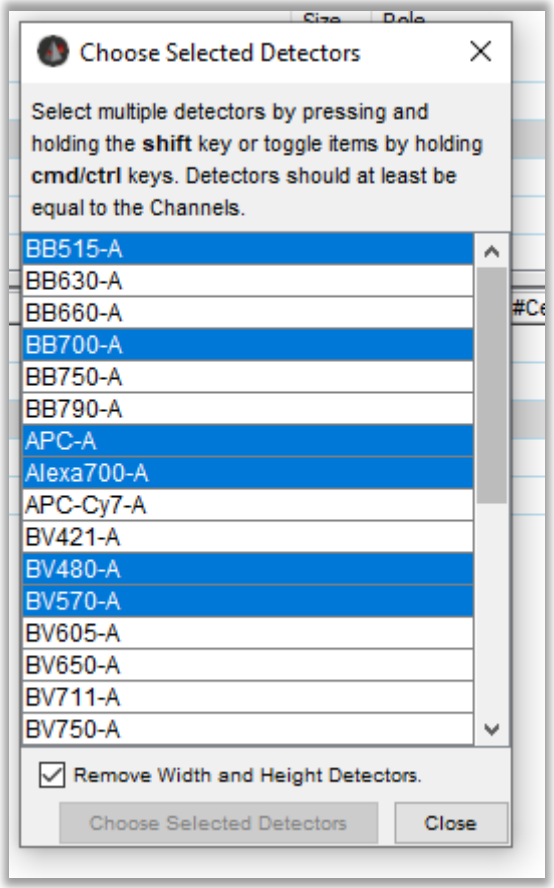
SSM was originally developed to monitor instrument performance (SSM=ΔrSD/√ Δ MFI).

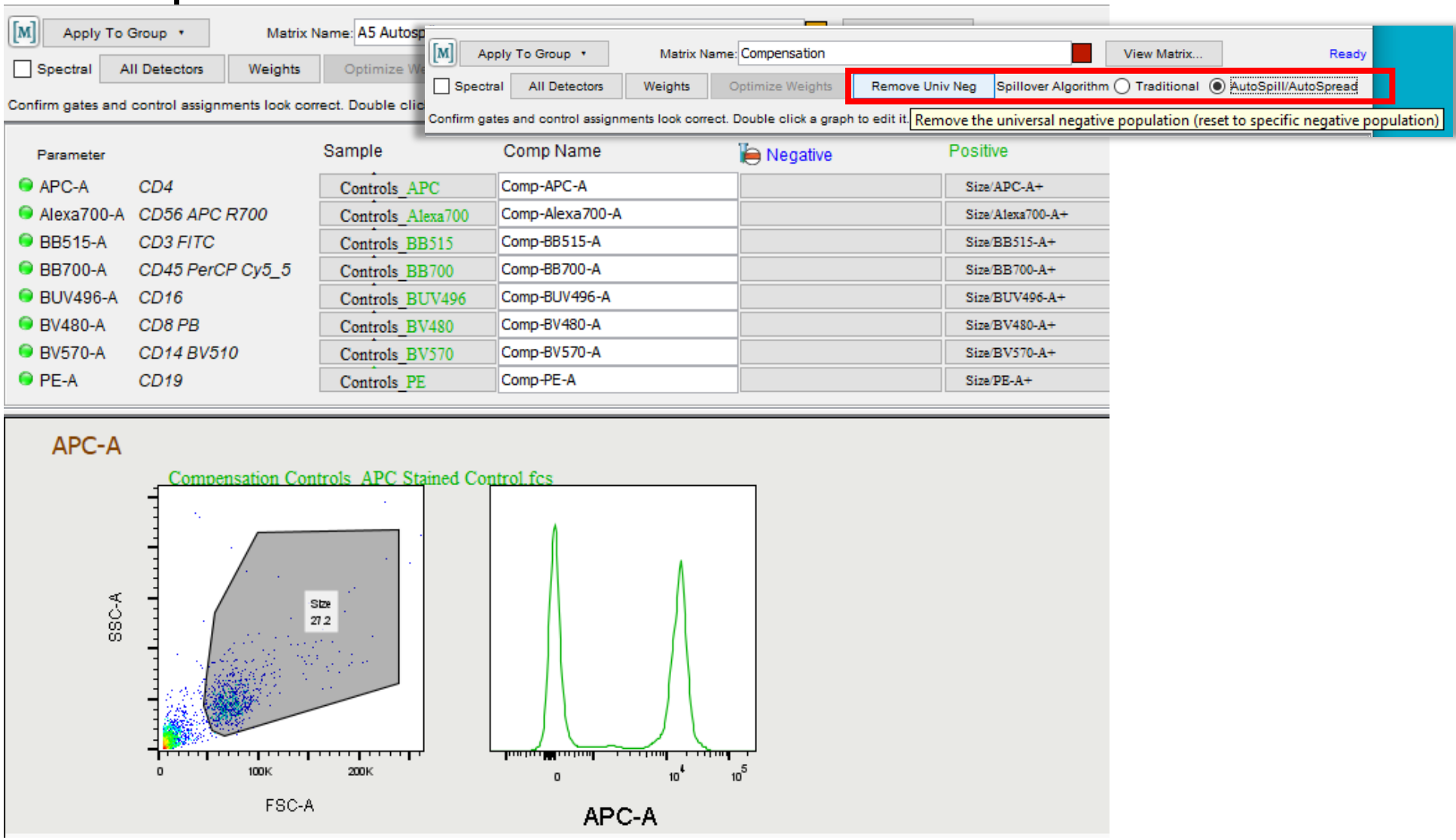
Conventional Compensation workflow



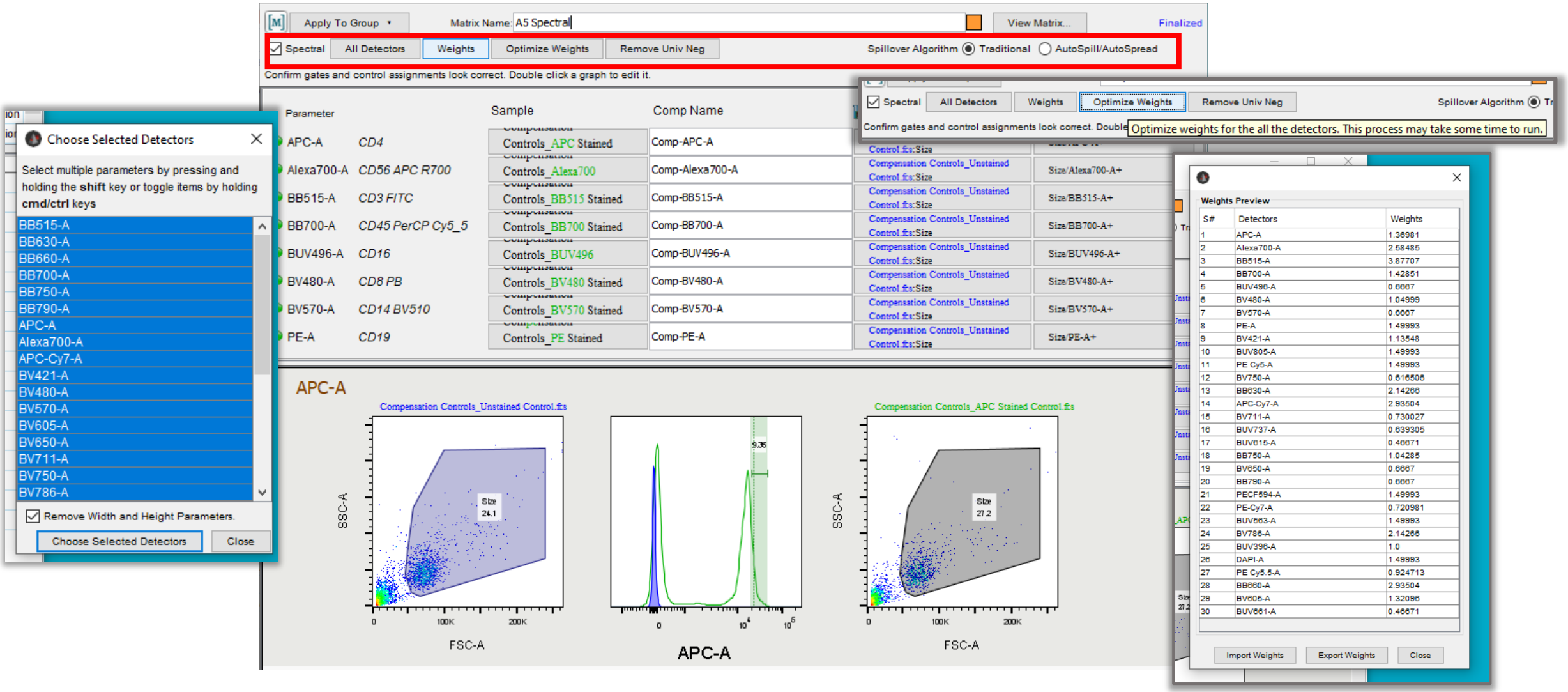


Autospill Compensation Workflow

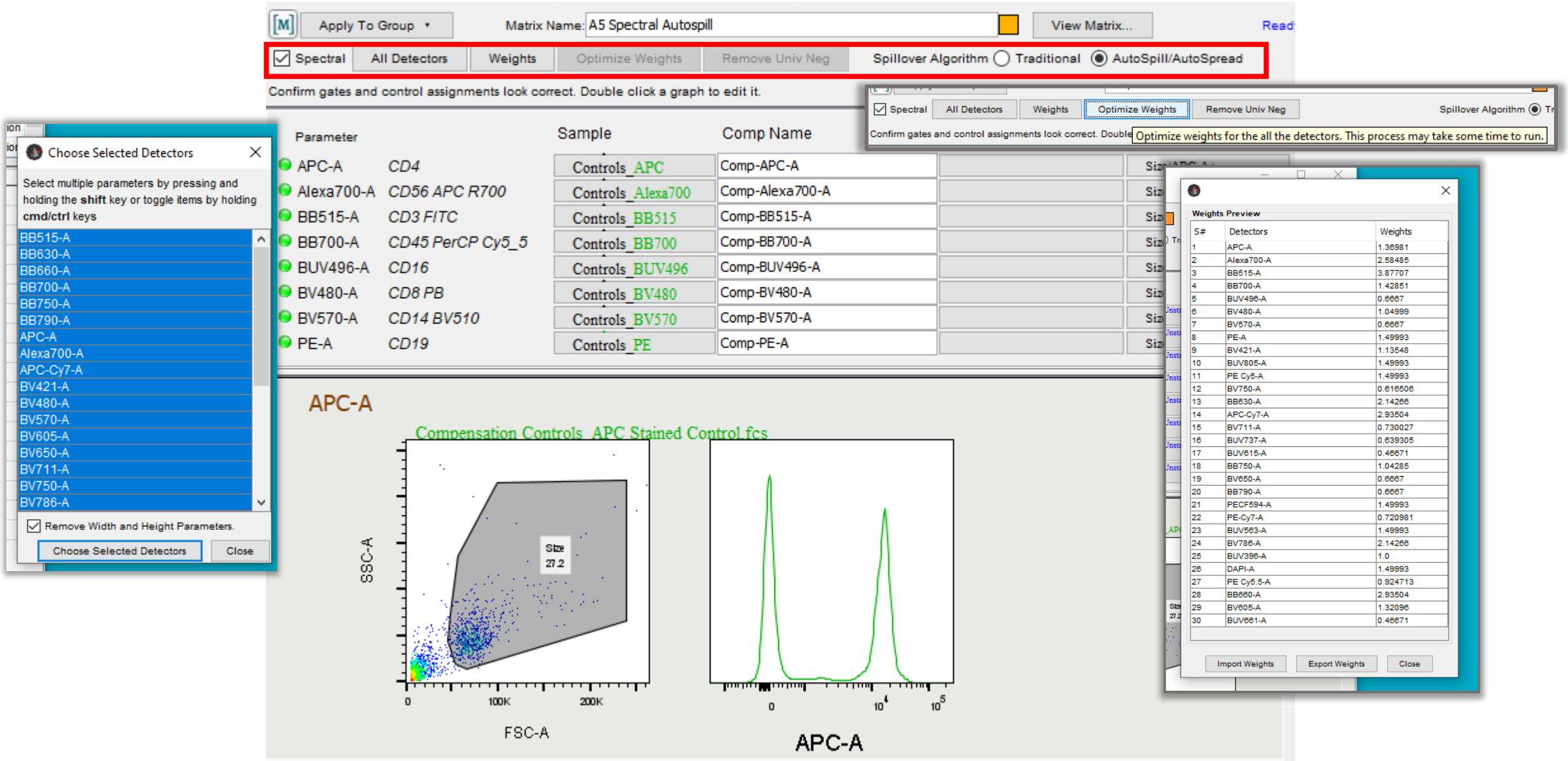




Spectral Compensation Workflow



Spectral Autospill Compensation Workflow

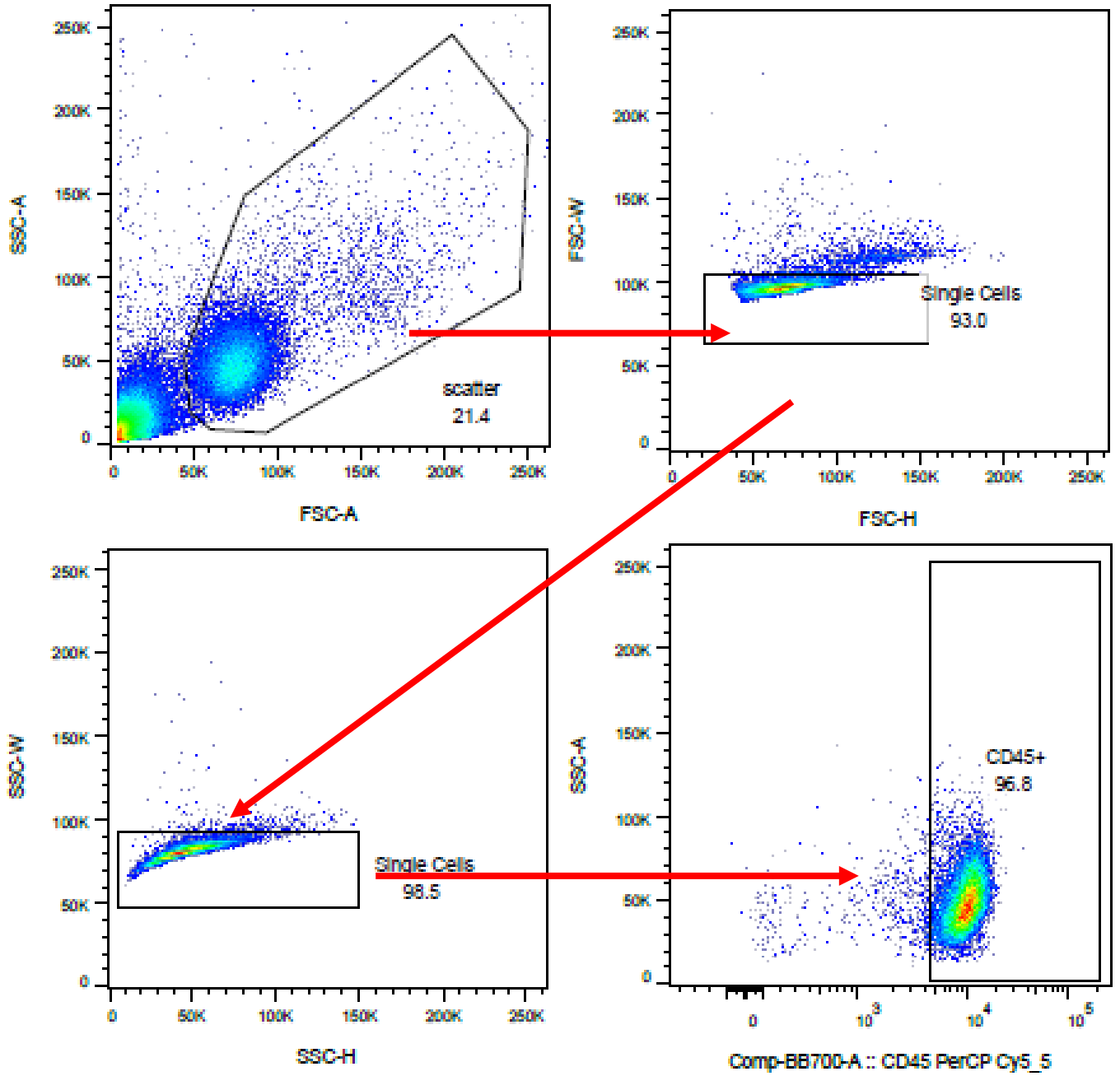


Fluorochrome Markers

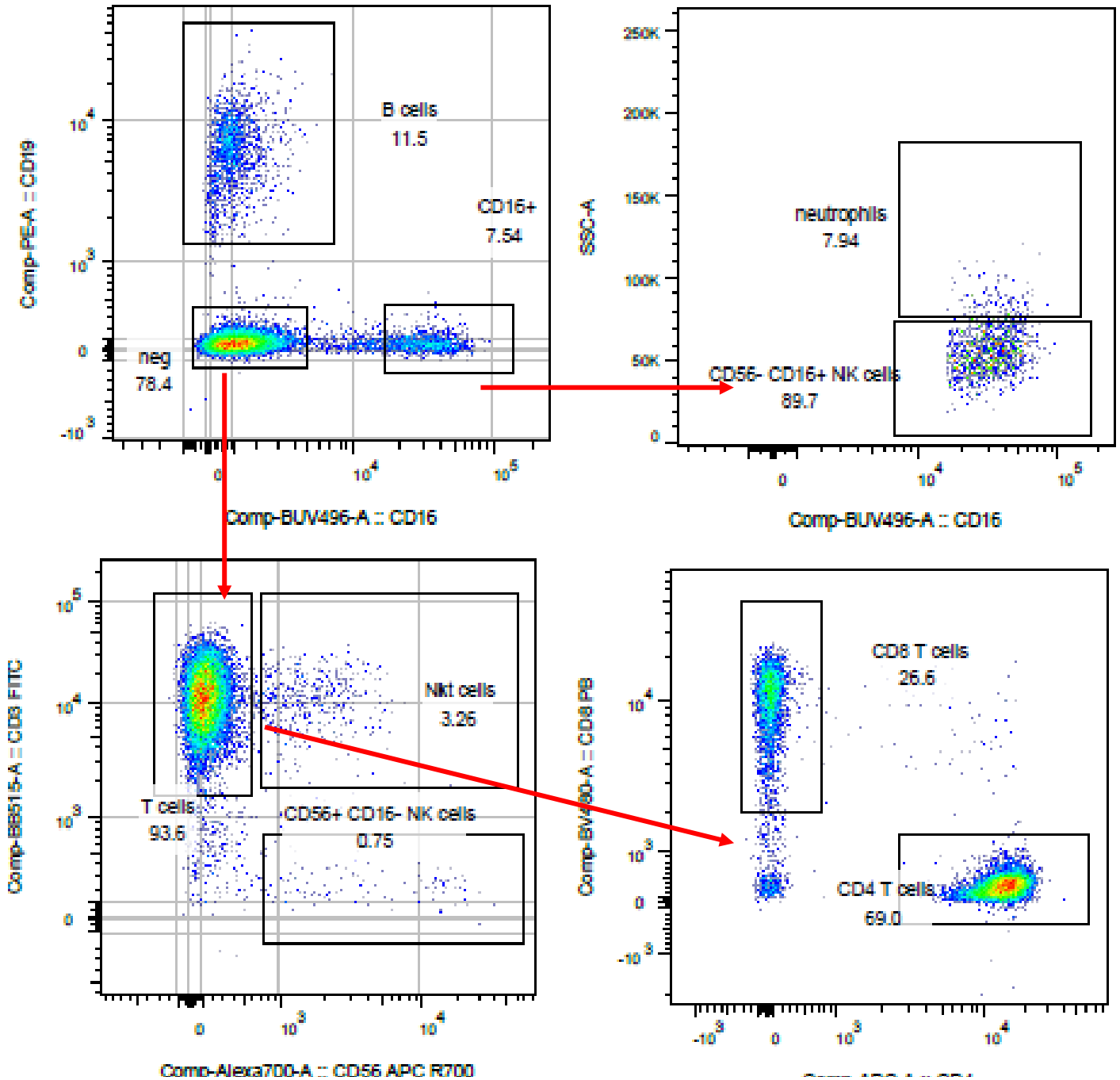
Fluorochrome	Marker
BUV486	CD16
APC R700	CD56
APC	CD4
PacBlue	CD8
PE	CD19
PerCP-Cy5.5	CD45
FITC	CD3
BV510	CD14

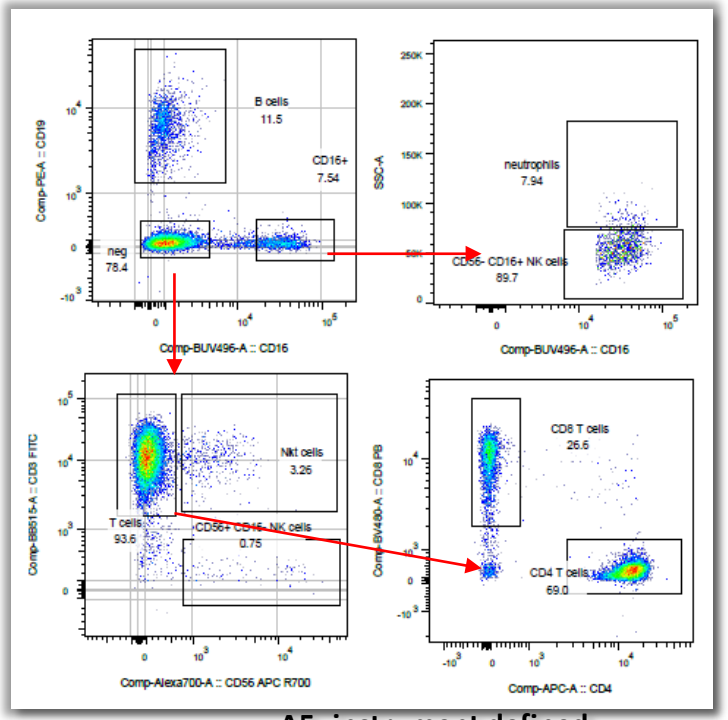
- Stained on lyophilized cells

- Scatter, Doublet and CD45+ gate

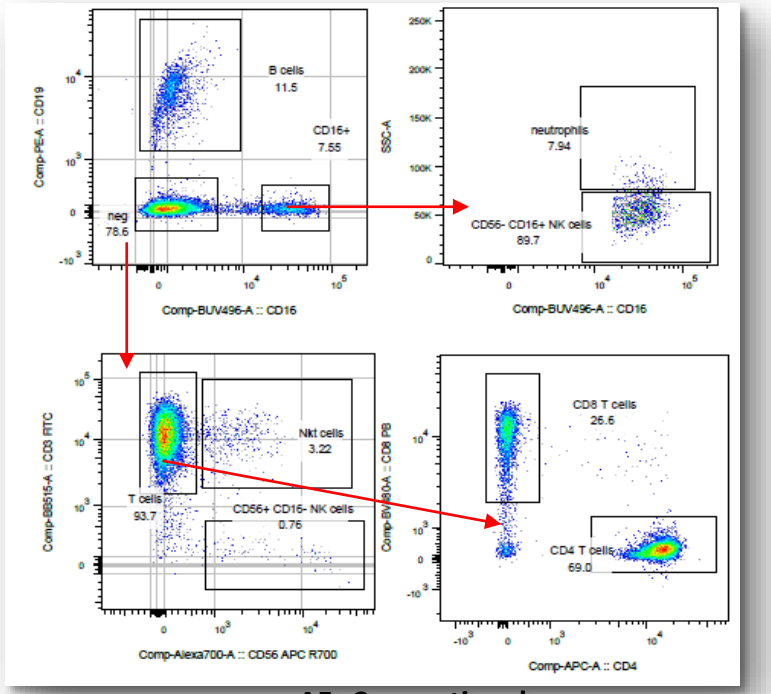


A5 Instrument defined

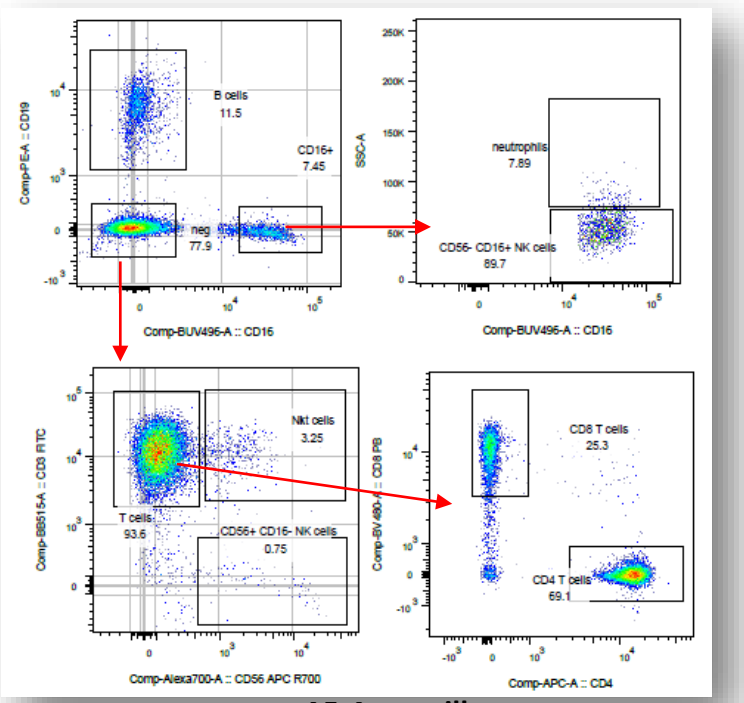




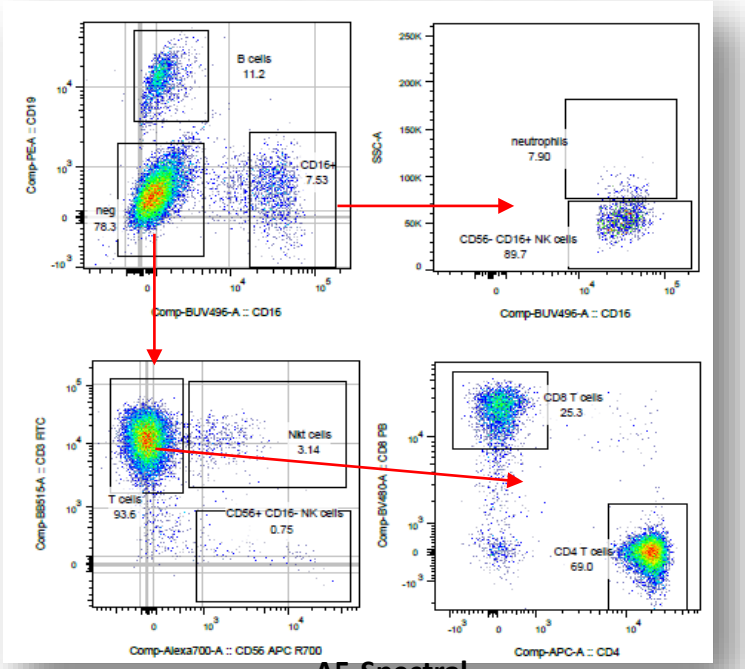
A5- instrument defined



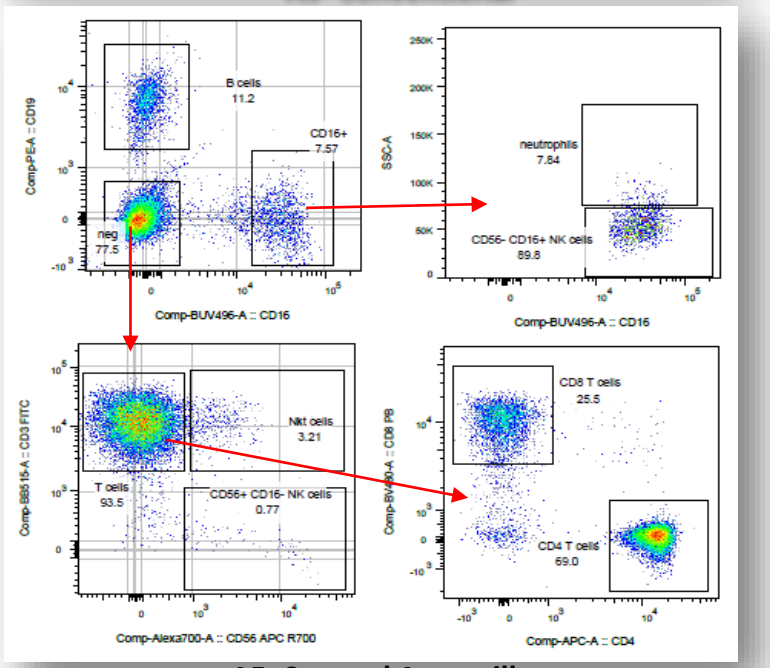
A5- Conventional



A5-Autospill



A5-Spectral



A5- Spectral Autospill

A5 Data

- Identical data set
- Different methods of compensation matrices applied

BD FACSymphony A5 Population Percentages

Parent Population		A5- Acquisition	A5- Traditional	A5-Autospill	A5-Spectral	A5- Spectral autospill	Range
CD45+	CD19+ B cells	11.5%	11.5%	11.5%	11.2%	11.2%	11.2-11.5%
	CD16+	7.54%	7.55%	7.45%	7.53%	7.54%	7.45-7.55%
	Neg	75.4%	75.5%	77.9%	78.3%	77.5%	75.4-78.3%
CD16+	Neutrophils	7.94%	7.94%	7.89%	7.89%	7.80%	7.89-7.94%
	CD56-CD16+ NK Cells	89.7%	89.7%	89.7%	89.7%	89.8%	89.7-89.8%
Neg	T Cells	93.6%	93.7%	93.6%	93.5%	93.8%	93.5-93.8%
	NKT cells	3.24%	3.22%	3.25%	3.13%	3.21%	3.13-3.25%
	CD56+CD16- NK cells	0.75%	0.76%	0.75%	0.77%	0.77%	0.75-0.77%
T cells	CD8 T cells	26.6%	26.6%	25.3%	25.3%	25.5%	25.3-26.6%
	CD4 T cells	69.0%	69.0%	69.2%	69.0%	69.0%	69.0-69.2%

Final thoughts

- All methods of compensation are acceptable
- Conventional and Spectral have a higher chance of bias and human error
- Conventional and Spectral need positive and negative gates defined which could be time consuming
- Autospill could be faster and more efficient for larger panels
- Autospill could take out the bias that conventional has.