Still Fixing Spectral Unmixing Errors Manually? FlowLogic provides Solution!

Geza Paukovics[1], Vittorio Cordioli[1], Kathryn Friend[2], Ian Slater[1]

[1] Inivai Technologies, Australia [2] Chameleon Science

Aim: An emerging challenge within the Flow community involves an overwhelming amount of data being produced which requires researchers to perform labor-intensive manual corrections to fix common post-compensation and unmixing errors. The aim of the project was to develop AI algorithms to assist with fixing unmixing/compensation errors (automatically or semi-automated) and to simplify the workflow associated with achieving correctly unmixed data for downstream analysis.

Background:

Spectral Flow Cytometry (SFC) and the expansion of commercially available antibody-bound fluorophores have enabled the flow cytometry community to delve into and develop large multicolour immunofluorescence panels (25+ colours). One of the challenges of high-dimensional spectral flow cytometry experiments is finding reference controls resulting in no or limited unmixing errors. To achieve this using the same cell as in the experiment is the ideal reference control, however sometimes this can be difficult if the frequency of cells expressing the target marker is too low, the cells may be valuable or limited. An alternative is to use surrogate particles such as antibody capture beads. However, it has been noted that with polystyrene bead-based controls that seemingly meet all criteria, the fluorescence signature or spectral fingerprint of some dyes can be altered when bound to these beads, resulting in higher frequencies of unmixing errors as compared to cells. (Fig1 and Fig2)

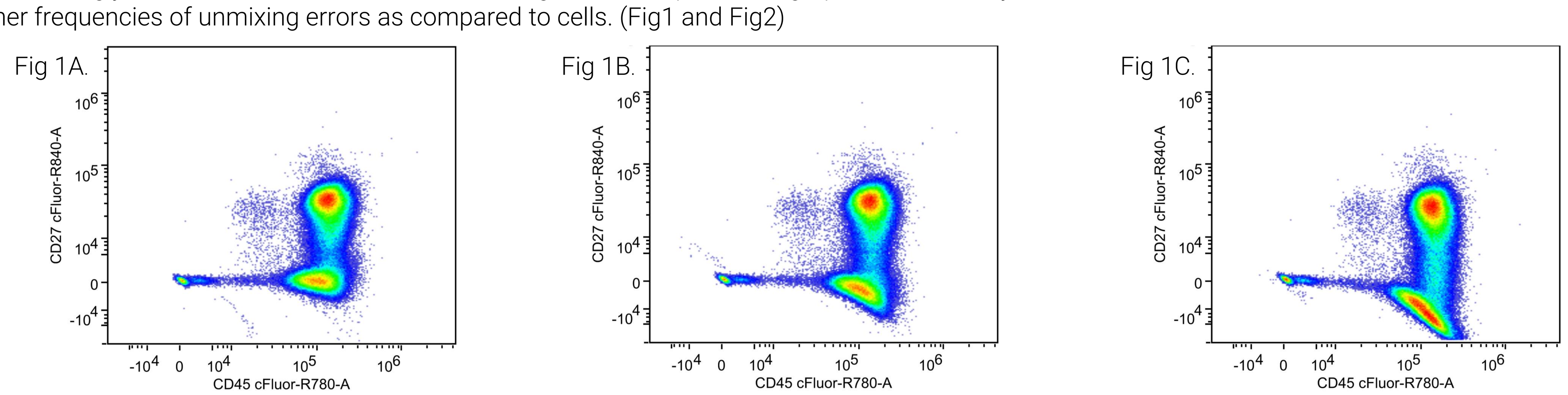
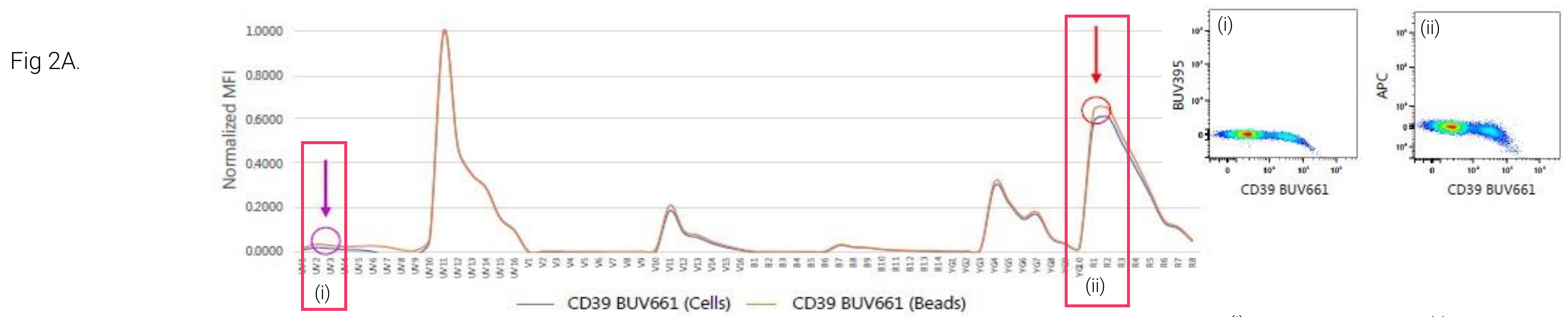


Figure 1: Comparison of Spectral Unmixing Outcomes using three different reference controls for CD45 cFluor-R780. (A) Cells. **(B)** Polystyrene Capture Beads Type 1 **(C)** Polystyrene Capture Beads Type 2 (NB. all plots have been standardized for the same compression and scaling to compare accurately)



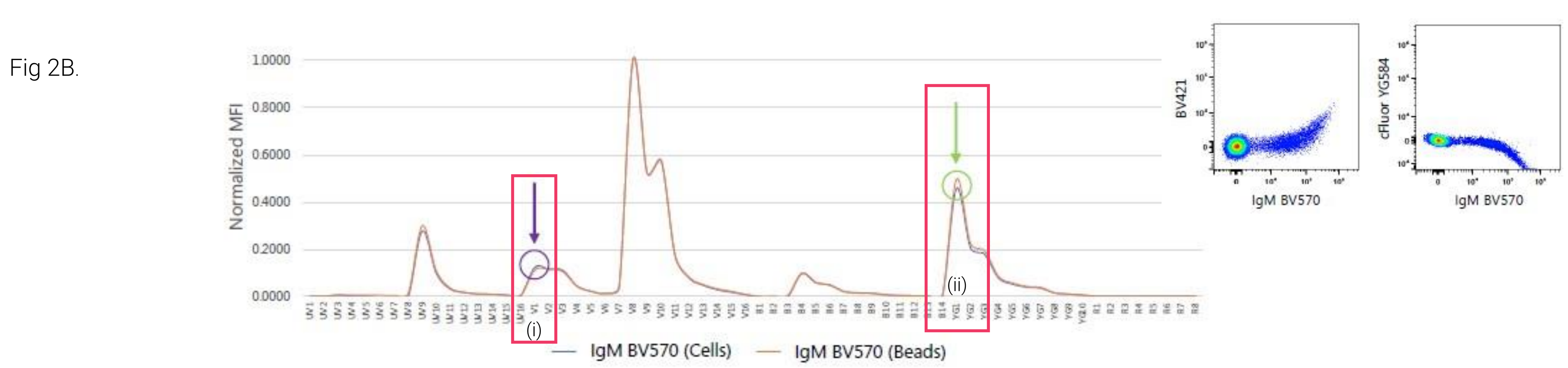


Figure 2: Examples Spectral Unmixing outcomes when antibodies are bound to cells v beads. These unmixing inaccuracies were the result of minor differences in the spectra of fluorochromes when the same antibody was bound to beads vs. cells. **(A)** CD39 BUV661 **(B)** IgM BV570 The outcome of these differences in spectral signatures and their manifestation in the affected channels (bead data only 2A (i)(ii), 2B (i)(ii)
Reference: OMIP69

The Current Approach to Fixing Unmixing Anomalies and FlowLogic Solution approach

Usually, NxN plots are generated and obvious unmixing errors are corrected by "eye" or non statistically and only in a two-dimensional (2D) space still yielding inaccurate results. Why? Manual adjustments or corrections of compensation via 2D dot plots have potential repercussions in all other dimensions/permutations, not just the one being corrected. Manual approaches result in perpetual checking whether one fix caused a potential problem somewhere else resulting in significant time being devoted to prepare the data for downstream analysis especially for complex 25+ colour panels.
The Comp-Check and Fix feature in FlowLogic Solution software assists in correcting any anomalies and simplifies workflows associated with such fixes. A holistic visual overview of the data is provided along with dMFI statistics (similar to Secondary Stain Index (SSI) approach) (reference) with a colour coded reporting table that clearly indicates the consequences of making compensation adjustments for all relevant parameters. There are multiple workflows available while having complete overview and control of the process.
(i) Fully automated AI (ii) Interactive hybrid semi automated (iii) Manual approaches.

Flowlogic™ Flow Cytometry Analysis Software

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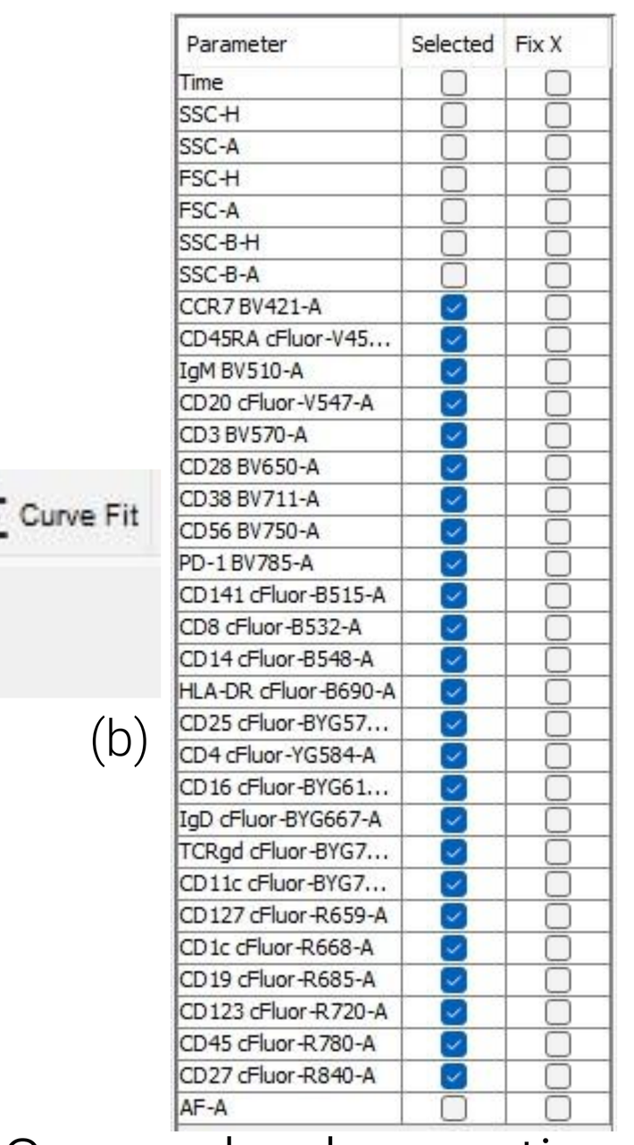
Method:

A Cytek® 25-Color Immunoprofiling Assay, cFluor® Reagent Kit (18C) was used to generate data acquired on the Cytek® Aurora. Single colour compensation controls bound to cells and two different types of polystyrene beads were used to generate the unmixing results, which were then applied to a population of fully stained cells (PBMCs). All criteria for ideal compensation controls were met. Using FlowLogic Solution Software, unmixing anomalies were identified and corrected for downstream data analysis (Fig3a-g).

Process, Workflow and Results:

Fig 3a-g.

Step by step outline of the proposed workflow:



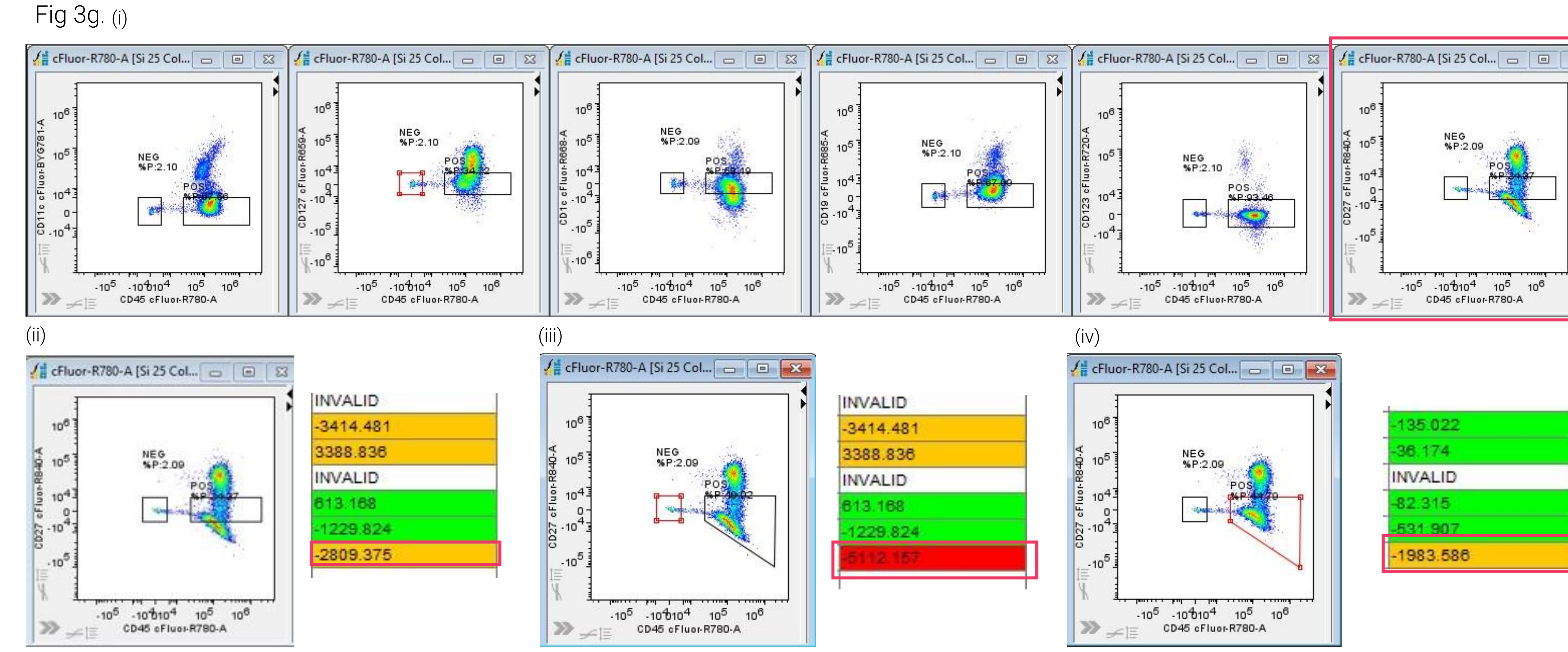
(a) From the advanced function menu – Click on Comp-Fix Tab
(b) Select the relevant parameters
(c) Select the gating hierarchy level from which to start the function from the file navigator
(d) For a faster process especially for files with large number of events – Data Reduction can be applied and is suggested
(e) Click Build All Permutations results in visual displays (dot plots) for all relevant parameter permutations for the data. Cross check negative and positive gate positions (that have been autogated) and adjust if required. Initiate the algorithm by pressing Fix Data. The process takes place in a multidimensional space through multiple rounds of compensation adjustments until the set median dMFI tolerance values are within range and are reported as green.
(f) Median dMFI tolerance values are automatically identified based on the instrument's dynamic range or can be input manually to desired levels. The colour coded SSI type report (post pressing Build All) will identify and flag any anomalies in the dMFI ranges defined.
(g) Amend the minimum number of events required for the algorithm to take into account before taking action (default set to 100 events)

Fig 3e.

	CCR7 BV4...	CD45RA c...	IgM BV510-A	CD20 cFlu...	CD3 BV570-A	CD28 BV65...	CD38 BV71...	CD56 BV75...	PD-1 BV78...	CD141 cFl...	CD8 cFluor...	CD14 cFlu...	HLA-DR cFl...	CD25 cFlu...	CD4 cFluor...	CD16 cFlu...	IgD cFluor-...
CCR7 BV421-A		575.58	-805.554	361.953	-126.314	2386.204	1580.516	43.275	754.583	-398.64	1072.362	-551.083	-1165.025	-775.859	-397.222	-933.463	302.602
CD45RA cFl...	350.869		-2604.032	4333.404	1709.024	850.094	650.298	-21.67	-543.331	886.797	-1327.401	1329.62	332.85	-1795.318	417.934	159.755	-1271.237
IgM BV510-A	INVALID	INVALID		3162.597	1787.042	1269.324	INVALID	-634.796	-447.657	-360.256	-2028.019	1865.538	512.749	-2068.319	1781.02	-9.985	-548.127
CD20 cFluor-...	498.826	INVALID	-3377.883		133.57	2038.308	338.021	-296.688	-1549.066	760.573	-2515.113	2307.414	4922.642	-829.982	860.331	1054.026	1516.892
CD3 BV570-A	-437.385	-285.081	2317.678	-3248.577		899.115	876.836	653.711	2079.719	-805.397	2500.763	-2363.134	-1068.974	1294.915	-2109.854	-1865.726	414.29
CD28 BV650-A	633.572	-1111.451	4175.597	-5027.745	INVALID		600.213	545.39	2059.783	-1828.392	3039.724	-2784.154	-2140.012	172.414	INVALID	-3476.138	1612.592
CD38 BV711-A	2010.08	766.436	-2620.236	2712.898	-1989.498	-325.17		-326.431	-1345.006	-153.445	-713.444	1109.835	-585.513	-747.014	2331.627	2065.222	735.636
CD56 BV750-A	INVALID	2294.14	2744.556	3134.424	309.508	-540.184	221.633		-925.615	296.915	-902.045	1021.754	-682.553	-1637.539	1276.076	-36.391	-854.32
PD-1 BV785-A	INVALID	-830.249	2292.173	-1610.397	INVALID	INVALID	INVALID	INVALID		-515.41	INVALID	-887.914	-706.691	-956.673	INVALID	-1731.685	885.116
CD141 cFluo...	INVALID	INVALID	INVALID	INVALID	INVALID	INVALID	INVALID	INVALID	INVALID		INVALID	INVALID	INVALID	INVALID	INVALID	INVALID	INVALID
CD8 cFluor-B...	145.479	1602.012	-2664.316	4405.163	-977.055	276.328	-426.543	319.685	226.787	3469.688		-817.35	1855.223	2671.337	-2033.401	3391.166	-1424.8
CD14 cFluor-...	2323.885	900.57	-4562.061	4771.664	-3332.649	-358.218	INVALID	-321.425	-1131.405	-32.101	INVALID		5148.717	4021.493	INVALID	3223.608	864.523
HLA-DR cFlu...	231.451	940.053	-2284.7	1763.056	176.566	3198.122	INVALID	316.506	-2063.888	873.695	-1647.946	1373.625		-1653.244	1464.744	175.361	INVALID
CD25 cFluor-...	117.676	-842.882	2830.934	-2795.262	INVALID	INVALID	35.925	98.649	779.297	-1056.474	809.389	-967.045	-674.944		INVALID	-2362.175	892.298
CD4 cFluor-Y...	759.517	-798.305	2506.791	-4077.344	-2396.564	67.389	813.933	43.166	854.334	-2585.444	3126.6	-2767.823	-2149.766	-478.307		-2281.985	1954.692
CD16 cFluor-...	-126.021	1316.311	-1065.024	4621.783	3.714	-556.129	-394.473	15.466	-1043.028	685.637	-503.879	1039.969	5.205	-2008.25	1066.206		-1230.703
IgD cFluor-B...	INVALID	INVALID	INVALID	INVALID	1858.753	2284.546	INVALID	-489.321	-964.059	-288.763	-1443.631	1298.945	3525.958	-2893.453	1674.598	-315.199	
TCRgd cFluo...	INVALID	567.337	889.934	-769.999	INVALID	INVALID	INVALID	561.882	INVALID	1096.725	-2168.714	2388.691	INVALID	-3720.61	2804.13	198.165	395.656
CD11c cFluo...	1215.649	868.801	-3657.911	2876.995	-2714.454	-606.316	INVALID	-367.544	-1529.938	108.557	-829.163	2411.471	3580.726	2241.287	1844.92	2908.806	1544.414
CD127 cFluo...	-157.241	-560.857	3649.143	-3931.166	INVALID	INVALID	727.853	359.592	1884.788	-1764.457	2930.295	-2917.983	-1928.11	1326.202	INVALID	-2927.263	1152.441
CD1c cFluor-...	INVALID	INVALID	INVALID	INVALID	1700.225	INVALID	INVALID	485.808	-1871.049	INVALID	-1726.596	1537.078	INVALID	-2381.722	INVALID	-366.819	INVALID
CD19 cFluor-...	-76.268	INVALID	-1786.748	INVALID	2135.116	1876.889	643.023	-392.924	-997.302	127.212	-2083.072	1979.11	3479.017	-2252.863	1653.215	-374.94	1773.891
CD123 cFluo...	958.723	238.201	-1323.544	1237.239	365.761	-453.494	INVALID	-812.385	-1134.162	1601.77	-2671.407	2349.51	1046.579	-860.249	1370.198	512.71	-1593.015
CD45 cFluor-...	1621.989	762.818	-256.64	992.923	-1779.816	825.306	1908.764	1196.899	1463.34	-2158.511	3829.937	-3377.359	-1453.366	1385.235	-158.71	-646.361	1504.688
CD27 cFluor-...	-162.103	-37.058	-213.025	-379.379	INVALID	3745.289	1217.848	328.61	1452.102	-416.038	2219.19	-2097.04	-854.368	277.329	-1644.951	-1283.522	-14.709

Figure 3e: Typical SSI type report post initiating the Comp-Fix Algorithm. dMFI values are the calculated differences in median negative and positive autogated populations. Green values indicate acceptable range or Low Flag = minimal or no chance of unmixing errors, Orange indicates a Medium Flag = moderate chance of unmixing errors, Red indicates Major Flag = High chance of unmixing errors. Negative values indicate suspected overcompensation. Positive values indicate possible under compensation. Any cells can be selected (by clicking) and the software will display the relevant plot to cross-check. INVALID indicates that the minimal number of events required for the algorithm to evaluate dMFI were not met.

Fig 3g. (i)



(v)

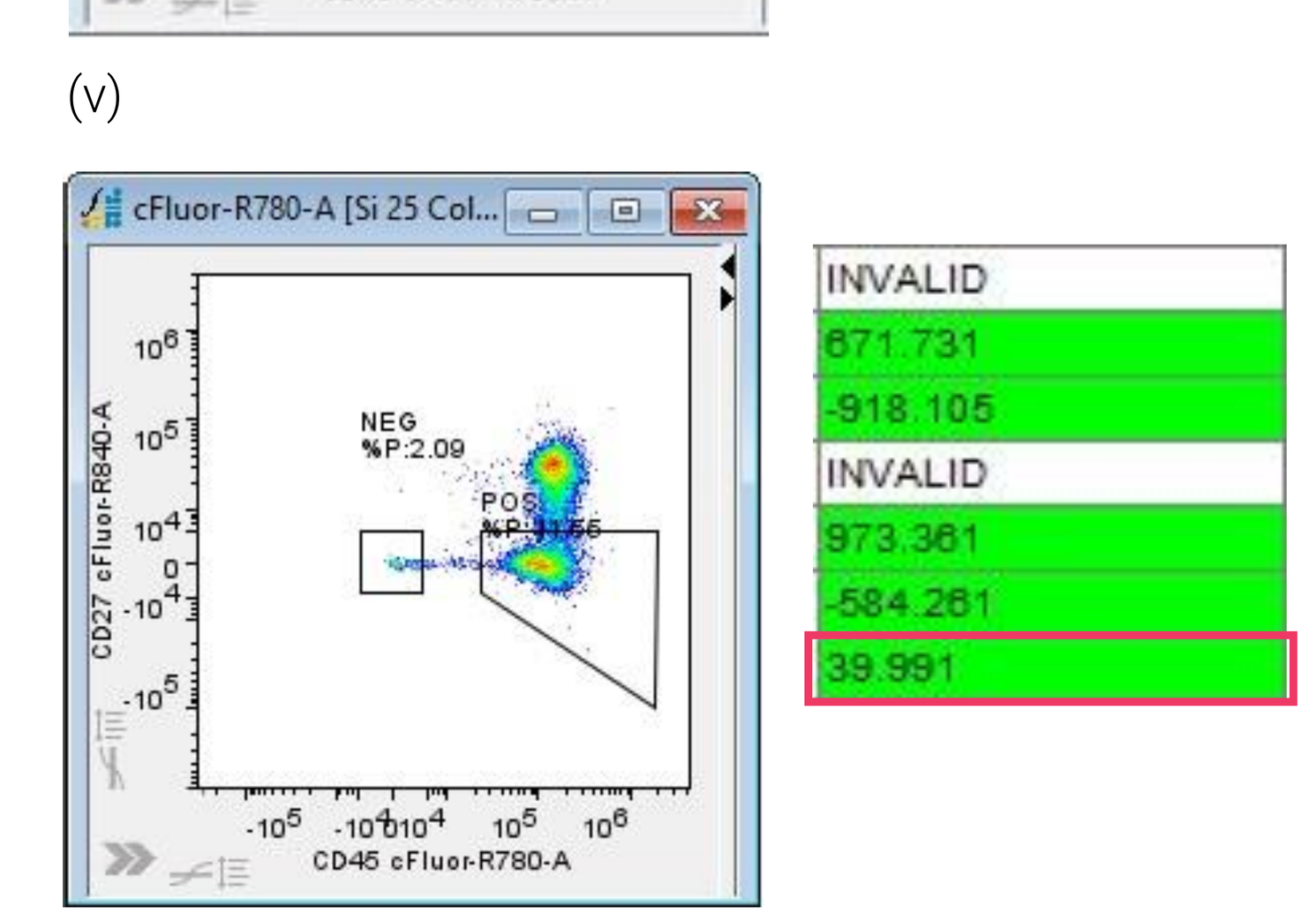


Figure 3g: (i) Post Build All a comprehensive overview is offered to visually inspect the data and statistically investigate the colour coded dMFI report for anomalies. **(ii)** Visual and statistical identification of potential unmixing anomalies. **(iii)** Autogated populations can be modified to more accurately identify problem populations. **(iv)** Results of an automated compensation fix to a tolerance level of under 2000 dMFI, further adjusted to 1000 dMFI with Orange Flag **(v)** Final result as the algorithm achieves dMFI set to a tolerance level of 200.

Conclusion

Here we present an AI assisted Compensation-Fix algorithm that helps in the process of fixing unmixing or post compensation errors. Saving significant time and easing workflow when compared to manual approaches, especially useful for more complex immunofluorescence panels. The use of this algorithm is suited to more advanced users with a strong understanding of the biological processes and phenotypes of target populations.
FlowLogic is user focused and always looking to improve the workflow in your data analysis. If you have any ideas or feedback, please visit us in Booth #12.

Acknowledgement:

For this work we acknowledge Simon Monard from WEHI brighter together for generously sharing and allowing the usage of files he generated using the Cytek® 25-Color Immunoprofiling Assay Panel.