CORRESPONDENCE

Real-Time Compensation in Flow Cytometry: A Real Need of Time

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Dear Sir,

Compensation is done to prevent spectral overlap and spill-over during data acquisition in fluorescence activated cell sorting (FACS). Spectral overlap occurs because the fluorophores used in flow cytometry emit photons of multiple energies and wavelengths, where emission spectra overlap, fluorescence from more than one fluorochrome may be detected in a channel [1–3]. With newer advanced machines with more lasers and many more detectors or “channels”, the importance of compensation cannot be undermined. The conventional single Antibody (Ab) stained cell tube run before standardization of a protocol experiment, and getting a desired compensation settings is not sufficient in this age of multicolor and multi-tube experiments, real time compensation takes care of other problems apart from compensation like steric hinderance and tube specific “spill overs”. In our experience, even if an experimental protocol is standardized beforehand with single tube run experiments, a “real time” compensation setting contributes significantly to the results. Hence we recommend after standardization of protocol by single Ab experiments for compensation, a tube specific compensation should be applied and voltage and gain settings

adjusted during acquisition to simulate real time data collection. This procedure of real time compensation has better results as compared to single tube runs in standardized protocols as explained in Fig. 1. To elaborate this point we acquired data from two sets of experiments one which was run on standardized settings (single Ab tube experiment) of protocol and the other when compensation was done in real time while acquisition (Figs. 1 and 3). The difference in voltages in different channels was also compared (Fig. 2).

The compensation algorithm needs to be performed both on positive population and a negative population (Fig. 1). For best compensation, selection of controls is important—either internal control with similar fluorescence as that of the test cells or external beads can be used, when added to the same tube which have surface test markers acting as controls. Internal control should have adequate adherence to fluorochromes and the individual samples should have the same carrier particles for the fluorochromes, [3–5] this can be done by using known positive controls and comparing their fluorescence [4].

In our experiment, the voltage and compensation settings of single Ab tube (Fig. 2) were saved in the general

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Fig. 1 Fluorescence compensation for standardization of protocol. **a** Shows overlapping in different channels. **b** Shows after compensating

protocol. During routine test runs, practically all samples required some change in settings, inspite of running them on standardized protocol (Fig. 1). This can also be seen in the compensation matrix of the general protocol and the real time acquisition protocol which was modification of the general protocol (Fig. 3). Thus in our experience although recommended, single antibody standardization allows compensation which cannot be generalized to all the samples, attributed to steric hinderance, inadequate antibody density, instrumentation problems etc. This problem is not encountered in newer FACS instruments and acquisition softwares which allow recording primary data

simultaneously with the visualization of compensated data and compensation and change of setting after data acquisition also. These softwares are in any setup superior and more user friendly when compared to actual compensation experiment, but in settings where these softwares are unavailable, or while working softwares like-CXP for Beckman Coulter machines, or systems that do-not support a free software installation, the above experiment may offer some advantages. Real time compensation can only be done in adequate samples and not samples with low counts.

	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FL2	0.00		9.00	1.68	0.00	0.00	0.00	0.00	0.00	0.00
FL3	0.00	51.26		15.00	0.00	0.00	0.00	0.00	0.00	0.00
FL4	0.00	9.48	31.68		0.00	0.00	0.00	0.00	0.00	0.00
FL5	0.00	0.00	6.00	36.00		0.00	0.00	0.00	0.00	0.00
FL6	0.00	0.00	0.00	0.00	0.00		4.18	16.99	0.00	0.00
FL7	0.00	0.00	0.00	0.00	0.00	96.06		20.00	0.00	0.00
FL8	0.00	0.00	0.00	0.00	0.00	5.42	9.36		0.00	0.00
FL9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		20.67
FL10	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	8.00	

A

	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		1.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FL2	10.80		9.00	2.40	1.00	0.00	0.00	0.00	0.00	0.00
FL3	0.00	39.00		2.20	0.00	0.00	0.00	0.00	0.00	0.00
FL4	0.10	4.90	11.00		0.00	0.00	0.10	0.00	0.00	0.00
FL5	0.00	0.50	1.40	29.68		0.00	0.10	0.00	0.00	0.00
FL6	0.00	0.00	0.00	0.00	0.00		0.20	4.20	0.00	0.00
FL7	0.00	0.00	0.00	0.00	0.00	40.47		9.00	0.00	0.00
FL8	0.00	0.00	0.00	0.00	16.00	12.00	23.27		0.00	0.00
FL9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		22.60
FL10	2.80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.80	

B**Fig. 2** Compensation matrix for **a** standardization protocol, **b** real time protocol at time of acquiring process samples

Fig. 3 Sample run on standardized protocol (a) more compensation was done real time for removing overlapping (b)

With many laboratories using the still older method for compensation, real time compensation may be supplemented by single tube standardization experiments for better and accurate results.

Author Contribution AM and SS wrote the paper, AM, SC and PT made the concept and experimentation setup, SR and SS did the experiment and instrumentation, PT, SR and SS reviewed the paper.

Compliance with Ethical Standards

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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