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Standardization in flow cytometry: correct sample handling as a priority

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The Review in which Holden Maecker and colleagues cogently advocate the need for standardization in flow cytometry (Standardizing immunophenotyping for the Human Immunology Project. *Nature Rev. Immunol.* **12**, 191–200 (2012))[1] interested us considerably. As constant users of flow cytometry, we were heartened that the scientific community is able to discuss this sensitive topic.

Maecker *et al*. define the steps needed to standardize flow cytometry-based assays, and posit this standardization as a key requirement for the success of the 'Human Immunology Project', as defined by Mark M. Davies[2]. We agree with Maecker *et al*. on the need to standardize flow cytometry experiments. However, of the myriad obstacles to such standardization, we believe that sample preparation stands out as the main and unavoidable prerequisite for success.

We find that inappropriate sample handling can seriously compromise all subsequent steps in flow cytometry. For example, the purification of leukocyte subsets before flow cytometry-based experiments is a common and hard-to-quit habit that can bias the results. We acknowledge that cell isolation is mandatory for some *in vitro* assays, and it can also be useful for optimizing the interaction of cell substrates with specific flow cytometry probes, as it allows for the removal of dead cells, debris and serum proteins. However, density-gradient separations are highly operator dependent, and they can often lead to the selective enrichment of certain lymphocyte subsets[3]. Moreover, they can directly influence antigen expression and the activation status of isolated leukocytes, and thus can bias the results further[4–9]. Importantly, lymphocyte separation by density gradient leads to the loss of granulocytes, which are the first-line cells of the innate immune system and constitute the largest population of circulating white blood cells. Granulocytes influence most immune functions, including antigen presentation by dendritic cells, T cell differentiation and proliferation, B cell survival and help, and macrophage activation[10,11]. However, the effects of granulocyte removal on immunophenotyping have not been thoroughly studied.

We accordingly recommend, as the first important step towards flow cytometry standardization, that staining be carried out on whole blood as the preferred option in all studies, even in those not related to any clinical trial. Thus, in addition to the well-characterized eight-colour antibody panel proposed by Maecker and colleagues[1], we encourage the use of a mini panel containing probes for granulocyte markers ($CD45^+CD66^+CD14^-$). Furthermore, a permeabilization step that enables the use of extra probes for neutrophil granule markers — such as myeloperoxidase, pentraxin 3 and lactoferrin — could easily increase the sensitivity of blood leukocyte characterization. This flow cytometry approach is relatively straightforward and not labour intensive or time consuming, and it allows researchers to minimize biases that are derived from extensive sample handling. Moreover, it enables the simultaneous characterization of the activation status and functional characteristics of the various leukocyte subsets, as has been achieved in the past in *ex vivo* studies of samples from ~500 human subjects in several clinical settings[12–16].

In conclusion, given that the Human Immunology Project sets a highly demanding goal, we strongly discourage the use of density-gradient purification before flow cytometry analyses, and we endorse the inclusion of neutrophils among the investigated subsets. We firmly believe that these measures will both facilitate the standardization of procedures and allow us to obtain a comprehensive and more realistic picture of all the circulating immune cells in humans.

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Competing interests statement
The authors declare no competing financial interests.

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