CYTOMETRY
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An Eloquent Proof for a Common Challenge

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Sorting cells means manipulating them. This induces biological responses of the cells, resulting in functionalities not representing the previous state of the cells, but indicating effects of sorting procedures. Namely in cases that negative selection is not possible, isolated cells are distinct to their previous characteristics. This is true for bead-based sorting or flow cytometric cell separation and heavily skews functional markers of target cells. Of course, this is a limitation for any following investigation of these cells.

In this issue (page XXX) in the article entitled "An evaluation of T cell functionality after flow cytometry sorting revealed p38 MAPK activation", Immanuel Andrä and colleagues combine flow cytometry and streptamer features to answer a very relevant question: What happens with T cells when undergoing flow cytometric sorting, and how can this activation be described by easily accessible markers?

Sorting cells is a common necessity from research to therapy. From easy technologies such as adhesion to surfaces, physical procedures using density, or protocols making use of active cell migration, up to sorting procedures, the approaches have been refined remarkably and can deliver well-characterized cell populations on demand. Few examples of newer approaches are streptamers (1), single cell sorting methods (2), tracking dye-independent approaches (3), or bead- and column-based technologies (4).

The key technology of the research work presented here was to apply traceless cell sorting by streptamers as a well-established technology (Fig. 1) to sorted cells (1). The idea for this reversible Fab staining originates from antigen-specific multimer staining for T cells and was recently also highlighted in parts by Cossarizza et al. in 2019 (5). As MHC–peptide multimers proved to be detrimental for survival of target cells, reversible antigen specific T cell staining by streptamers was developed by Knabel et al. (6). For this type of staining, the interaction between monomeric MHC and TCR is so weak that the MHC–peptide complex must be multimerized for stable binding. Later the idea was widened not only to make reversible staining available for antigen specific T cells, but also to allow a plethora of surface markers to be targeted by reversible staining reagents. To achieve this goal, antibodies were mutated in their antigen recognition to be still specific to its antigen, but with low affinity enabling the spontaneous dissociation from its antigen if monomeric. For this purpose, Fab monomers had to be generated. After overcoming the technological challenge of mutating and expressing of Fab monomers, the already established MHC-I streptamer system (6) was utilized to multimerize the low-affinity Fab fragments using fluorescence-labeled Streptactin backbones and thereby allow reversible isolation of virtually all cells (1). At low temperatures, the activating/inhibitory stimulus of antibodies and the adverse effects of immune reactions against antibody labeled cells can be minimized. The release of isolation reagents enhances the in vivo functionality of regulatory T cells (7). In line with the development of new reversible staining and cell purification methods, Andrä and colleagues utilized the FAB-TACS®-technology (8) to directly isolate well-defined CD8 positive cell material from freshly drawn unlysed venous blood. These cells were used to evaluate MAPK activation by flow cytometry sorting on a homogenous cell population largely unmodified by preparation effects but highly pure for their readout systems.

Intracellular staining of signal transduction molecules allows today a specific detection of ongoing activation processes. As shown by Bitar et al. (2019) (9), phosphorylation of STAT-5 allows an early, specific, and sensitive detection of ongoing activation and/or proliferation processes. An

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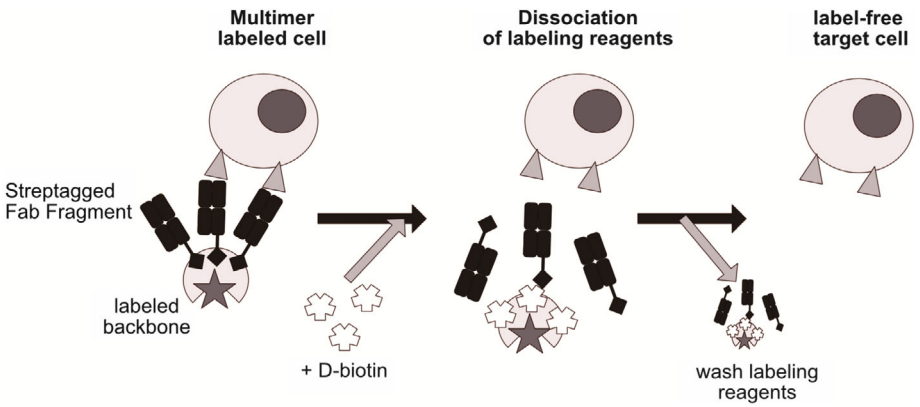

Figure 1. Schematic drawing of the underlying principles for reversible staining. In the drawing, the square represents the Streptag. The cloudlet is for the D-Biotin. The pentamer is a backbone (for flow cytometry analysis). The two black beams are the Fab fragments originating from antibody sequences were point mutated for weaker antigen affinity. Yet by multimerization Fab fragments stably bind to their antigens presented on cell surfaces. Multimer backbones are fluorescence-labeled for flow cytometry analysis. Fab fragments bind to multimers via Strep-Tag® technology. Addition of D-biotin dissolves multimerization and the weak binding of a monomeric Fab fragment to its antigen leads to the dissociation from the cell surface. Disrupted 0-biotin backbone and Fab fragments can be removed by washing steps leaving unlabeled cell material. [Color figure can be viewed at wileyonlinelibrary.com]

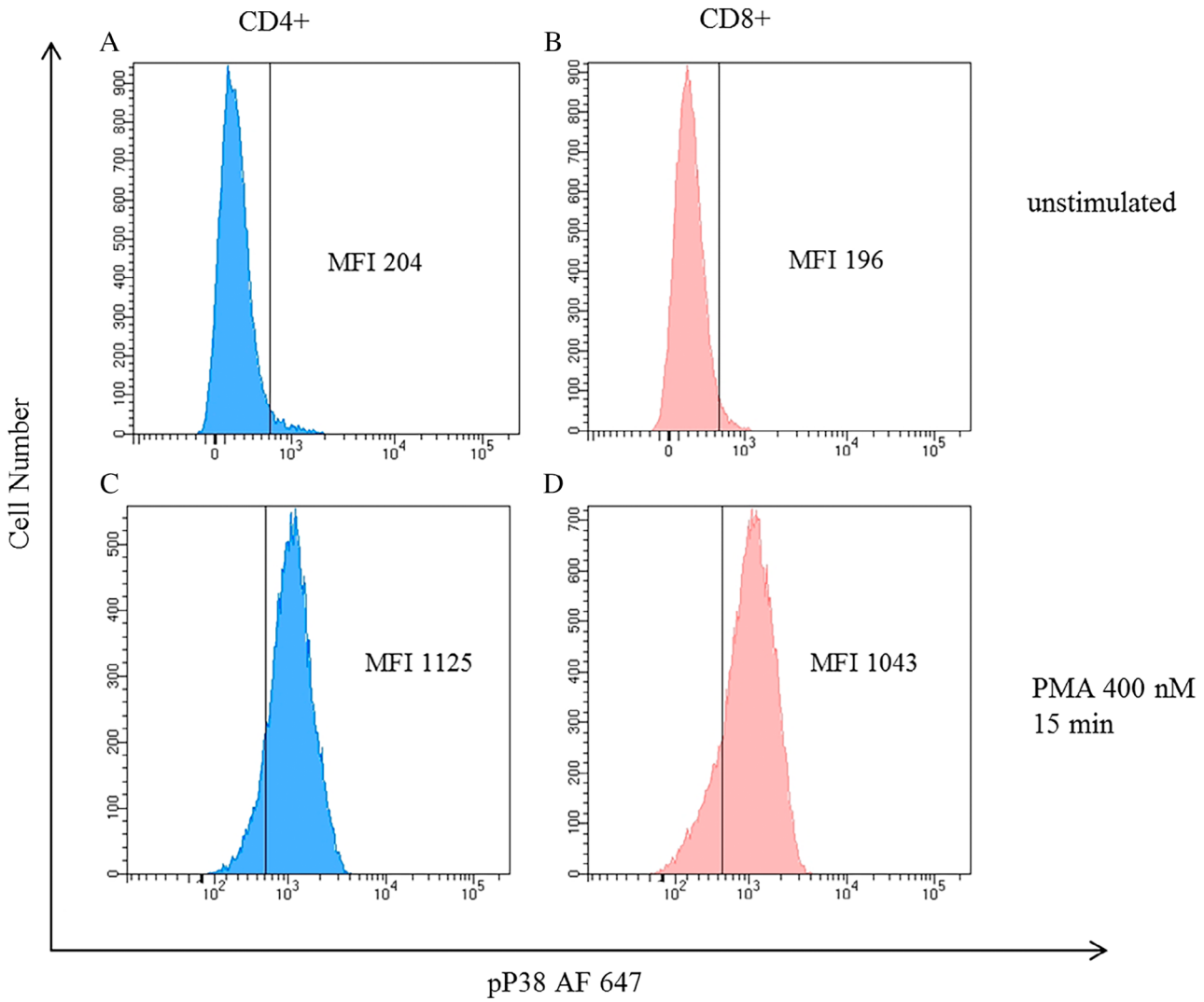

Figure 2. Legend on next page.

increasing number of publications using this intracellular staining method as a surrogate marker indicating ongoing functional changes in cells is huge. As an example, the publication by Crawford et al. (2014) (10) should be cited here. This is also true for specific detection of phosphorylated Map kinase P38, as shown in a typical plot (Fig. 2).

Combining both technologies, Andrä et al. here provide evidence that flow cytometric sorting activates cells in a p38 dependent matter. This finding should sensitize us for reanalyzing countless publications investigating functional characteristics of sorted cells. Additionally, streptamer technology has been proven another time to be superior in this context. Of course, this is not the only paper addressing this challenge. In 2014, Beliakova-Bethell et al. (11) investigated the effect of cell subset isolation methods on gene expression. They expected that flow cytometric sorting may have an impact on the isolated cells and investigated gene expression by microarray analysis. Surprisingly, they found only few alterations in cells sorted by flow cytometry, in contrast to positively selected cells by other methods. They could not find a stress response. Probably, this finding resulted from the microarray technology applied in this approach, merging the cells for analysis and not combining a functional analysis with phenotypic characterization as done by Andrä et al. in this volume. This is also supported by a paper by Weiss et al. (2019) (4) describing activation processes in any kind of antibody- of even Fab-dependent separation processes. This will also be true for other approaches that apply streptamers to positive cell isolation (12). Here, the technology applied was like the streptamer protocol applied by Andrä et al.; remarkably, activation of target cells could be found.

The question what the best technology is seems still to be open. This should be considered in all laboratories sorting cells, independent on the technology applied to. Probably, the 100% untouched cell never will be isolated. But the technology presented here could help understanding this better. In fact, minor changes can result in severe functional alterations, and analyses of the transcriptome may sometimes be not sensitive enough.

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FIGURE 2 Detection of phosphorylated p38 in unstimulated (upper panel) and stimulated (lower panel) CD4+ (blue) and CD8+ (red) subsets by flow cytometry. Peripheral EDTA blood was treated with PMA (400 nM for 15 min) or left unstimulated. Blood samples were lysed and fixed by using "lyse and fix" buffer (BD Biosciences) and incubated at 37°C in a water bath for 12 min. The cells were centrifuged, the supernatant was discarded, and the pellet was washed with 4 ml PBS. The samples were permeabilized by using cold perm buffer III (1 ml) (BD biosciences) and left on ice for 30 min. The pellet was washed three times with a fetal bovine serum stain buffer (FBS) (2 ml) (BD Biosciences) and finally resuspended in 200 µl FBS. For flow cytometric analysis, the T cells were stained with PerCP-Cy TM 5.5 mouse anti-human CD3 (2.5 µl, clone UCHT1, BD Biosciences), BD phosflow TM Alexafluor 488 mouse anti-humanCD8 (2.5 µl, clone RPA-T8 BD Biosciences), BD phosflow TM PE mouse anti-humanCD4 (5 µl, clone RPA-T4, BD Biosciences) and Alexa Fluor 647 mouse anti-human p38 (10 µl, Clone 36/p38, BD Biosciences). After 1 h of incubation in the dark at room temperature, the cells were washed with 2 ml stain buffer, centrifuged, and were suspended in 300 µl of stain buffer. Mean fluorescence intensity (MFI) of cells positive for pP38 are shown. [Color figure can be viewed at wileyonlinelibrary.com]