REAlease® Fluorochrome Technology for multiparameter cell sorting

Jennifer Pankratz[1], Sabine Schmachtenberg[1], Anna Bergmann[1], Nicole Jansen[1], Susanne Krauthäuser[1], Marco Vahldieck[1], Andrea Völkel[1], Christiane Siewert[1], Esther Schiminsky[1], Petra Bacher[2], Thordis Hohnstein[2], Svenja Meiler[1], and Christian Dose[1]
[1] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany | [2] Department of Cellular Immunology, Clinic for Rheumatology and Clinical Immunology, Charité - University Medicine Berlin, Berlin, Germany

Introduction

Multiparameter immunofluorescent labeling is the method of choice for sorting of cell populations out of heterogeneous mixtures. However, downstream applications of isolated cells are usually limited since fluorescence channels and epitopes are blocked by the antibody fluorochrome conjugates utilized for the flow sorting experiment. Accordingly, antibody fluorochrome conjugates that would allow for a release of cell epitopes and a reuse of fluorescence detection channels after cell sorting might be of high interest for certain downstream applications.

Herein, we describe for the first time new types of antibody fluorochrome conjugates that enable a highly specific multiparameter cell staining. Unlike conventional fluorochrome conjugated antibodies, the introduced conjugates rely on recombinantly engineered antibody fragments and a conjugation chemistry that facilitates the complete release of the conjugates from the cell surface after the flow sorting step. The resulting cells are free of labeling providing maximal flexibility in downstream applications.

We demonstrate this flexibility in the context of a work-flow for the isolation of highly pure human regulatory T cells (Tregs). These cells were labeled with, for example, CD4-PE-Vio® 770, CD127-FITC and CD25-APC conjugates to be enriched by flow sorting in high purity and cell yields. Final detection of the target cells was achieved by intranuclear labeling with Anti-FoxP3-Vio 667 since a selective removal of the CD25-APC conjugate from the cell surface facilitated the reuse of the APC detection channel and enabled maximum sensitivity for the identification of the targeted Treg cells after flow sorting.

Methods

1 The principle of REAlease® Fluorochrome Technology

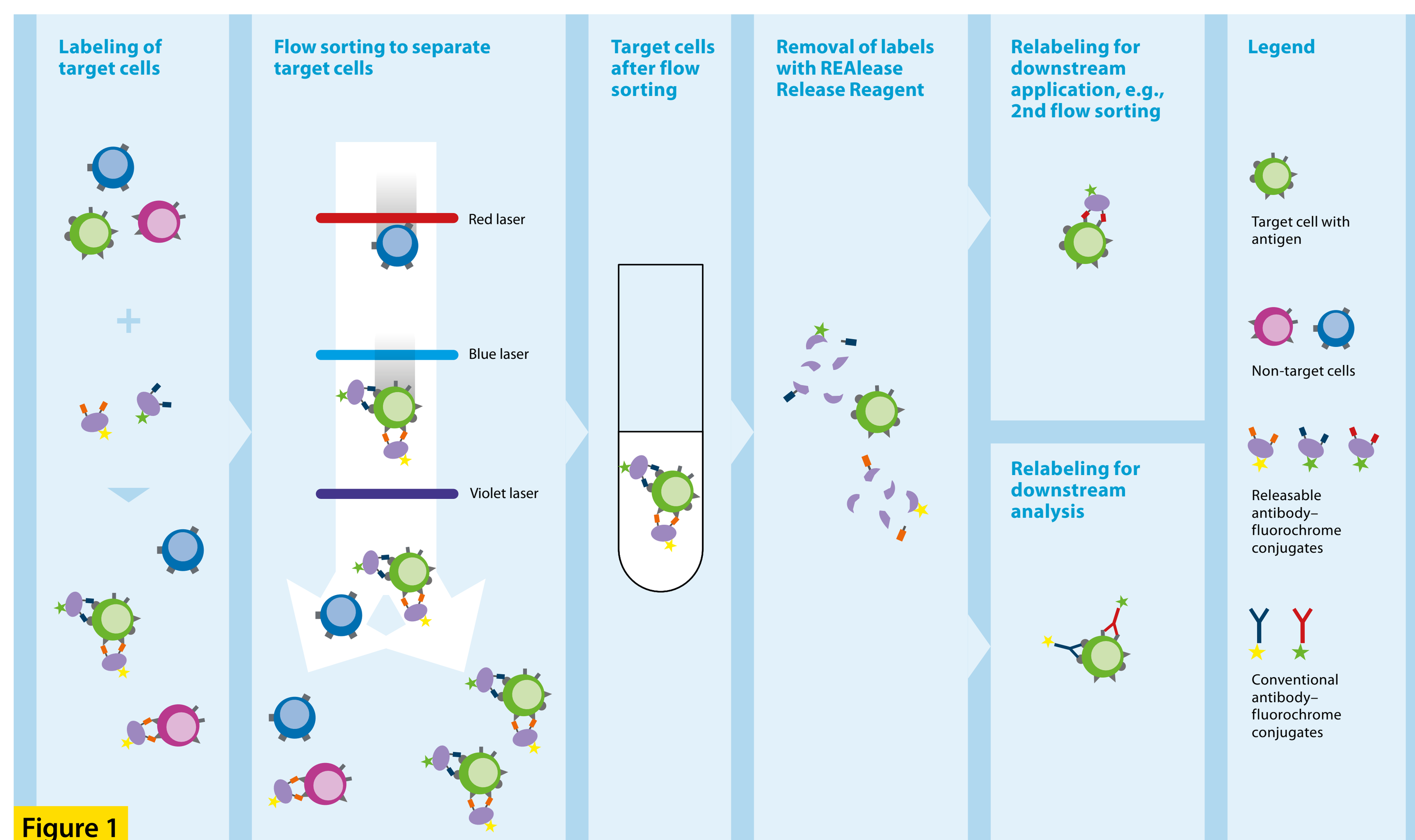

Figure 1

The novel REAlease Fluorochrome Technology relies on recombinantly engineered antibody fragments. Unlike conventional antibodies, these antibody fragments are characterized by low epitope binding affinities. A tailor-made covalent conjugation chemistry allows for a multimerization and fluorescence labeling of these recombinantly engineered antibody fragments (REAlease Fluorochrome Complex) to facilitate high-avidity cell binding comparable to conventional antibody fluorochrome conjugates. This novel and innovative conjugation chemistry allows for a fast removal of the REAlease Fluorochrome Complex from the cell surface by the addition of the REAlease Release Reagent after the flow sorting experiment (fig. 1). The addition of REAlease Release Reagent leads to a disruption of the REAlease Fluorochrome Complex and thereby to a monomerization of the antibody fragments, which spontaneously dissociate from the cell surface due to their engineered low binding affinity. Accordingly, previously blocked epitopes and utilized fluorescence detection channels become available for renewed epitope targeting or fluorescence relabeling, respectively. The result: label-free cells after cell sorting for maximal flexibility.

Results

1 Performance of releasable REAlease® Antibody Fluorochrome Conjugates

Figure 2

The new conjugates that contain recombinant engineered antibody fragments were developed for five fluorochromes VioBlue®, VioGreen®, FITC, PE, and APC. To demonstrate the technological principle and conjugate performance, human peripheral blood cells (PBMCs) were first incubated with a single releasable or conventional CD4 fluorochrome conjugate and the conventional CD3-APC or CD3-PE conjugate for 10 min at 4 °C. Labeled cells were washed and analyzed by utilizing the MACSQuant® X. The staining performance of releasable REAlease CD4 Fluorochrome Conjugates was equivalent to conventional CD4 fluorochrome conjugates revealing comparable brightness and low non-specific binding (fig. 2A2 and 2C). Addition of the REAlease Release Reagent for 10 min at room temperature initiated the fast cleavage of the releasable CD4 conjugate (fig. 2A2). The removal was highly efficient and selective since no remaining fluorescence signal was detected (fig. 2A2 and 2B). The labeling with the conventional CD3 fluorochrome conjugate was not affected. After blocking of the release reagent, the previously masked epitopes were readdressed with the same releasable CD4 conjugate. The labeling efficiency in the relabeling step was equivalent to the first labeling step indicating that epitopes were completely available again (fig. 2A1 and 2A3).

2 Multiparameter immunofluorescent labeling and release

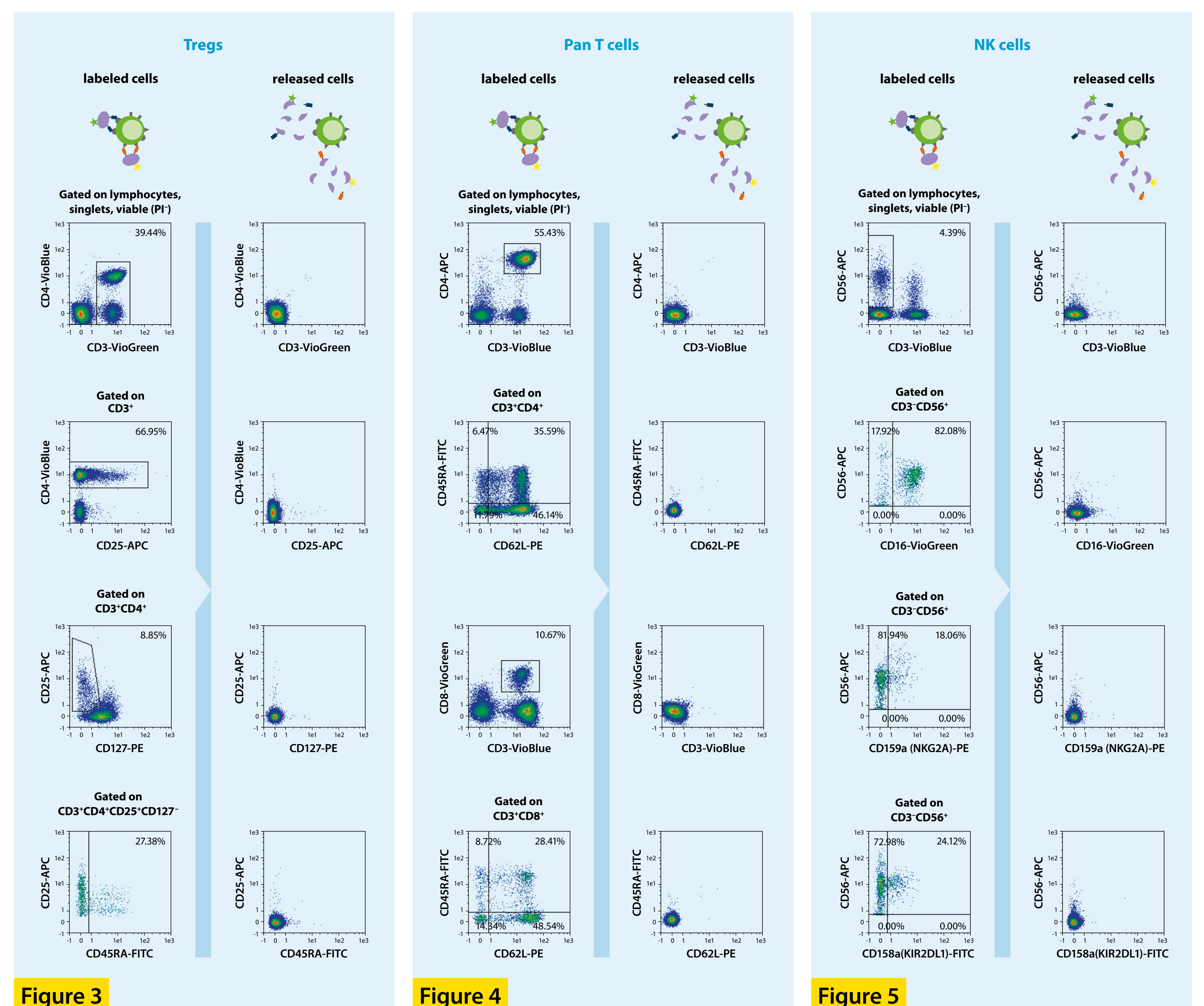

Figure 3 Figure 4 Figure 5

As most target cell populations are defined by the expression of several antigens, an important requirement for their straight isolation by flow sorting is the possibility to label several markers and fluorochromes simultaneously. The new covalent conjugation chemistry of the fluorochrome conjugates allows a reversible multi-parameter cell labeling. To demonstrate the versatility of this technology, we developed REAlease Fluorochrome Conjugates for several cell markers that can be used for multiparameter panel stainings of regulatory T cells, pan T cells or natural killer (NK) cells. As shown in figs. 3 to 5 the five-parameter cell labeling of PBMCs with REAlease Fluorochrome Conjugates allows for clear definition and discrimination of target cell populations:

- naive Tregs: $CD3^+/CD4^+/CD25^+/CD127^-/CD45RA^+$ (fig. 3)
- central memory T cells: $CD3^+/CD4^+/CD45RA^-/CD62L^+$ and $CD3^+/CD8^+/CD45RA^-/CD62L^+$ (fig. 4)
- NK cell subsets: $CD3^-/CD56^+/CD16^+$ or $CD3^-/CD56^+/CD159a(NKG2A)^+$ or $CD3^-/CD56^+/CD158a(KIR2DL1)^+$ (fig. 5)

After the addition of the REAlease Release Reagent all fluorochrome conjugates can efficiently be released.

3 Multiparameter flow sorting of regulatory T cells with relabeling of cell epitopes for cytometric downstream analysis

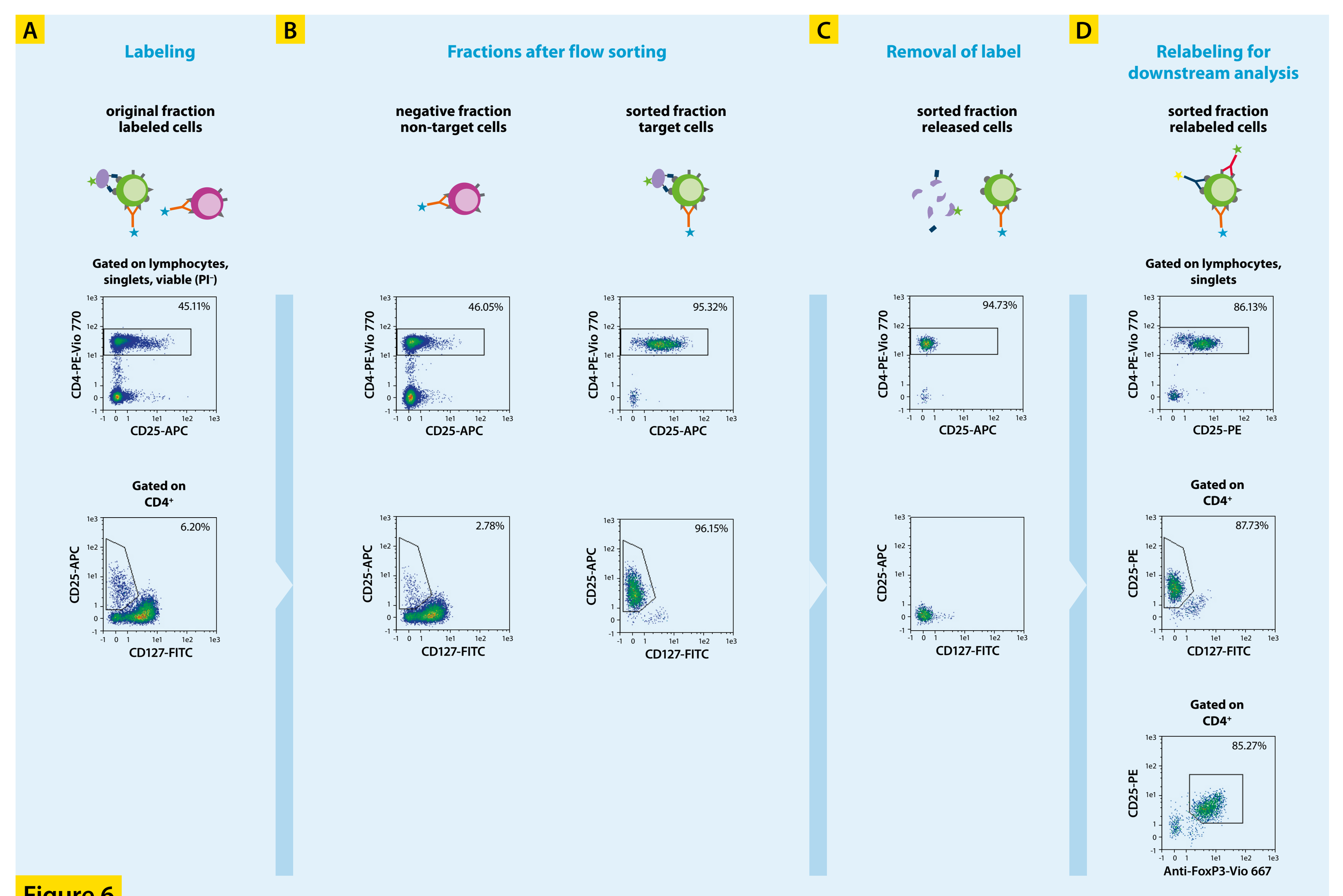

Figure 6

Isolation of Tregs using fluorescence-based cell sorting is a very attractive method to obtain highly purified target cells. Due to low expression of surface antigens used for identification of Tregs, labeling with bright fluorescent antibody conjugates is required for efficient discrimination and sorting of these cells. Here, human PBMCs were labeled with an optimized panel for flow sorting composed of the REAlease CD25-APC Conjugate and conventional CD4-PE-Vio 770 and CD127-FITC conjugates (fig. 6A). Upon isolation of the Tregs ($CD4^+/CD25^+/CD127^-$) using the MACSQuant® Tyto® Cell Sorter, the fluorescent labeling of the target cells with CD25-APC was selectively released (fig. 6B and 6C). The removal facilitated the relabeling of the CD25 epitope with the CD25-PE conjugate and the reuse of the APC detection channel to address the Treg-specific transcription factor FoxP3 with Anti-FoxP3-Vio 667 conjugate. In the cytometric downstream analysis, the identity and purity of the $CD25^+/FoxP3^+$ Tregs in the sorted target cell fraction could be proven with maximum sensitivity (fig. 6D).

Conclusion

In summary, this study represents the development of a new cell labeling technology allowing for a highly specific multiparameter cell labeling followed by an efficient removal of all fluorochrome conjugates from the cell surface.

- The new releasable REAlease Antibody Fluorochrome Conjugates show similar performance regarding brightness and specificity in comparison to conventional antibody fluorochrome conjugates.
- REAlease Antibody Fluorochrome Conjugates support multiparameter cell staining and flow sorting experiments.
- REAlease Antibody Fluorochrome Conjugates can be removed entirely from labeled cells by a fast and gentle procedure, which does not affect cell viability and conventional labels.
- REAlease Antibody Fluorochrome Conjugates facilitate downstream processing of cells by addressing the unmasked epitope or reusing the fluorescence channels for additional analysis.

This technology may pave the way for another generation of antibody fluorochrome conjugates applicable for multiparameter flow sorting experiments in basic research and clinical applications.

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use. MACS, MACSQuant, REAlease, Tyto, Vio, VioBlue, VioGreen, and the MACS logo are registered trademarks or trademarks of Miltenyi Biotec GmbH and/or its affiliates in various countries worldwide. Copyright © 2018 Miltenyi Biotec GmbH and/or its affiliates. All rights reserved.