REAlease® Technology: controlled release of antibody-fluorochrome conjugates for maximal flexibility in flow sorting applications

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Introduction

Multiparameter immunofluorescent labeling is the method of choice for the flow cytometry-based sorting of targeted cell populations from heterogeneous samples. However, existing antibody-fluorochrome conjugates used for flow sorting limit the types of downstream analyses that can be applied to the isolated cells, as their continued presence on the cells blocks specific cellular epitopes and restricts the choice of downstream fluorescence detection channels. Herein we present a new type of antibody-fluorochrome conjugates that facilitates a highly specific multiparameter cell staining for flow sorting as well as subsequent complete release of the conjugate probes from the sample. These new conjugates rely on recombinantly engineered antibody fragments that individually possess low epitope binding affinities. A tailor-made covalent conjugation chemistry allows for their multimerization as well as fluorochrome labeling, generating fluorescent probes comparable to conventional antibody conjugates. Importantly, upon addition of a release reagent, the antibody-fluorochrome conjugates can be rapidly released from the cell surface. Accordingly, previously blocked epitopes are free for relabeling, which provides maximal flexibility for renewed epitope targeting in downstream applications. The versatility of this technology is presented in multiparameter panels enabling clear distinction and discrimination of different cell populations. The applicability of the new REAlease® Conjugates is also demonstrated by flow sorting and downstream analysis of human T cells.

Methods

1 The principle of REAlease® Technology

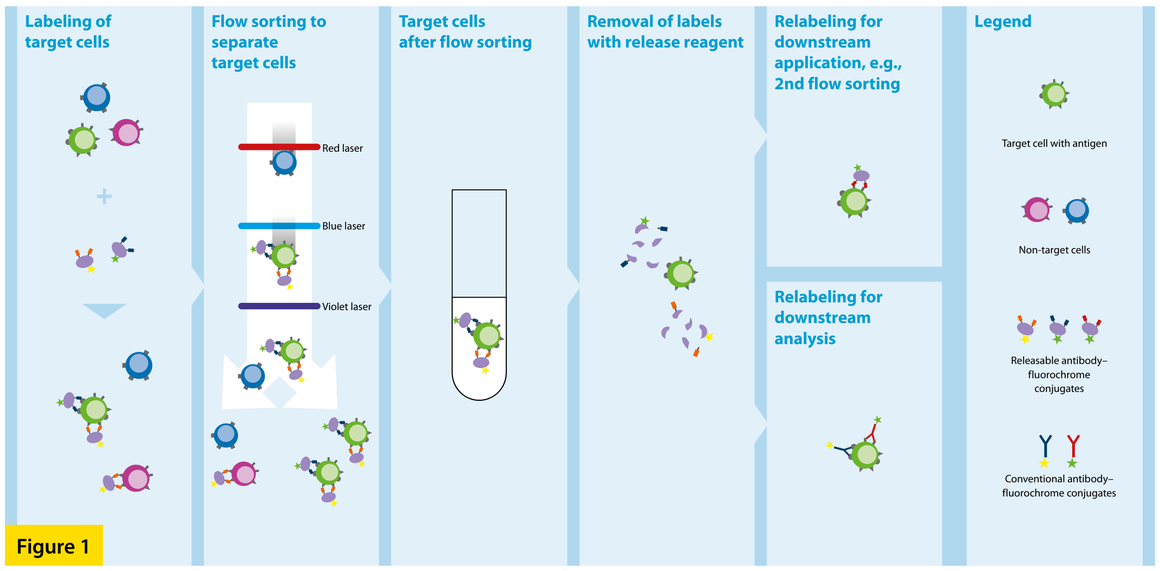

Figure 1

The novel REAlease® Conjugates for highly specific multiparameter cell labeling rely on recombinantly engineered antibody fragments. These antibody fragments are, unlike conventional antibodies, characterized by low epitope binding affinities. A tailor-made covalent conjugation chemistry allows for their multimerization as well as fluorochrome labeling, thereby generating high-avidity fluorescent probes that are comparable to conventional antibody-fluorochrome conjugates. Importantly, upon addition of a release reagent, the antibody-fluorochrome conjugates can be rapidly released from the cell surface. Briefly, this release step leads to disruption of the antibody-fluorochrome conjugate and thereby to a monomerization of the antibody fragments, which spontaneously dissociate from the cell surface due to their engineered low binding affinity. As a result, previously blocked epitopes and utilized fluorescence detection channels become available for renewed epitope targeting or fluorescence relabeling. REAlease Technology is applicable for the multiparameter fluorescence-based sorting of targeted cell populations (fig. 1) providing maximal flexibility for relabeling in any downstream application.

Results

1 Multiparameter immunofluorescent labeling and release

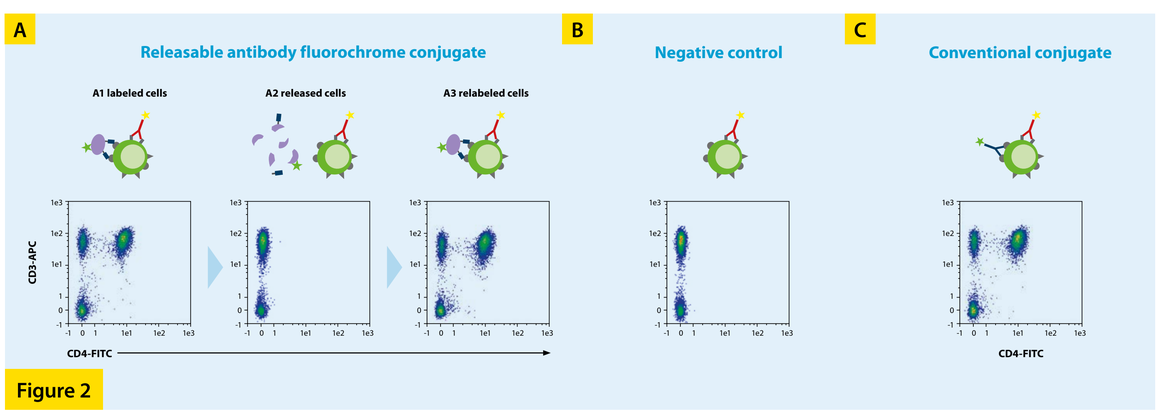

Figure 2

The technological principle and conjugate performance is demonstrated with CD4-FITC conjugates (fig. 2). Human peripheral blood cells (PBMCs) were first incubated with a conventional or REAlease CD4-FITC conjugate and a conventional CD3-APC conjugate for 10 min at 4 °C. Cells were washed and analyzed by flow cytometry using the MACSQuant® X. The staining performance of the REAlease CD4-FITC conjugate was equivalent to the conventional conjugate with comparable brightness and low non-specific binding (fig 2A1 and 2C). Addition of the release reagent initiated fast cleavage of the releasable CD4 conjugate (fig. 2A2). The release was highly efficient and after treatment for 10 min at room temperature no fluorescence signal could be detected (fig. 2A2 and 2B). Release was selective and did not affect labeling with the conventional CD3 fluorochrome conjugate. After blocking of the release reagent, the previously masked epitopes were restained with the same REAlease CD4-FITC conjugate. The labeling efficiency in the relabeling step was equivalent to the initial labeling indicating that epitopes were completely released after removal (fig. 2A1 and 2A3).
Since many targeted cell populations require multiparameter detection of several antigens for their isolation, REAlease Conjugates were developed for eight fluorochromes and several recombinantly engineered antibodies. The versatility of this technology was demonstrated by labeling human PBMCs after erythrocyte lysis with an eight-parameter panel composed of CD4-VioBlue®, CD8-VioGreen™, CD3-FITC, CD62L-PE, CD45-PerCP, CD25-PE-Vio® 770, CD45RA-APC and CD127-APC-Vio 770 conjugates (fig. 3). The panel of REAlease Conjugates allowed for clear distinction and discrimination of different cell populations. After the addition of the release reagent all fluorochrome conjugates were efficiently removed.

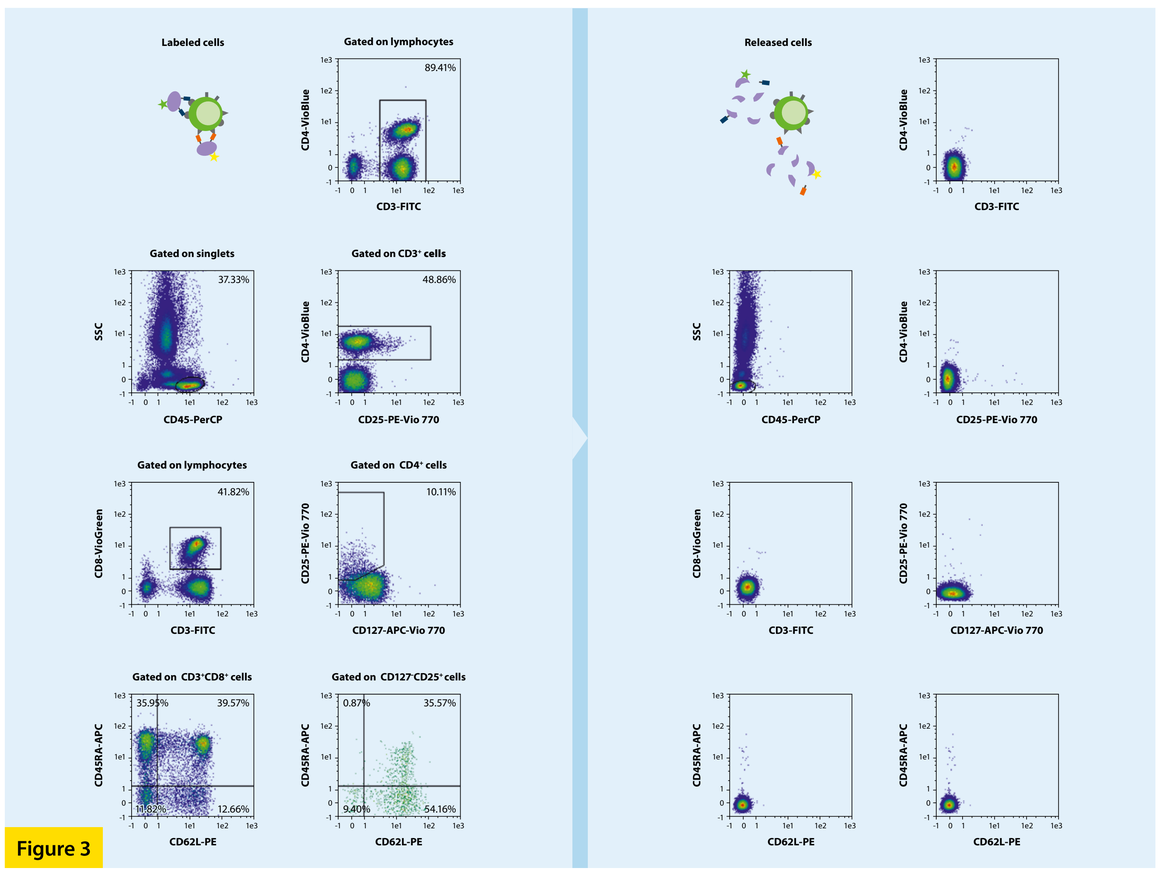

Figure 3

2 Multiparameter flow sorting of naive regulatory T cells

Fluorescence-based cell sorting is an attractive method to obtain highly purified naive T cells. Here, human PBMCs were labeled with a panel of four REAlease Conjugates, i.e., CD3-VioBlue, CD45-FITC, CD62L-APC, and CD45RA-PE (fig. 4A). Naive T cells (CD45+CD3+CD62L+CD45RA+) were isolated with 97% purity among viable lymphocytes using the MACSQuant® Tyto® Cell Sorter (fig. 4B). After sorting, the fluorescent label was efficiently released from the target cells (fig. 4C). Removal of the conjugates enabled relabeling for further immunophenotyping of the cells. Here, the cells were stained with CD197-VioBlue, CD4-VioGreen, CD3-FITC, CD62L-PE, CD45RO-APC, and CD8-APC-Vio 770 (fig. 4D). 7-AAD was used for dead cell exclusion.

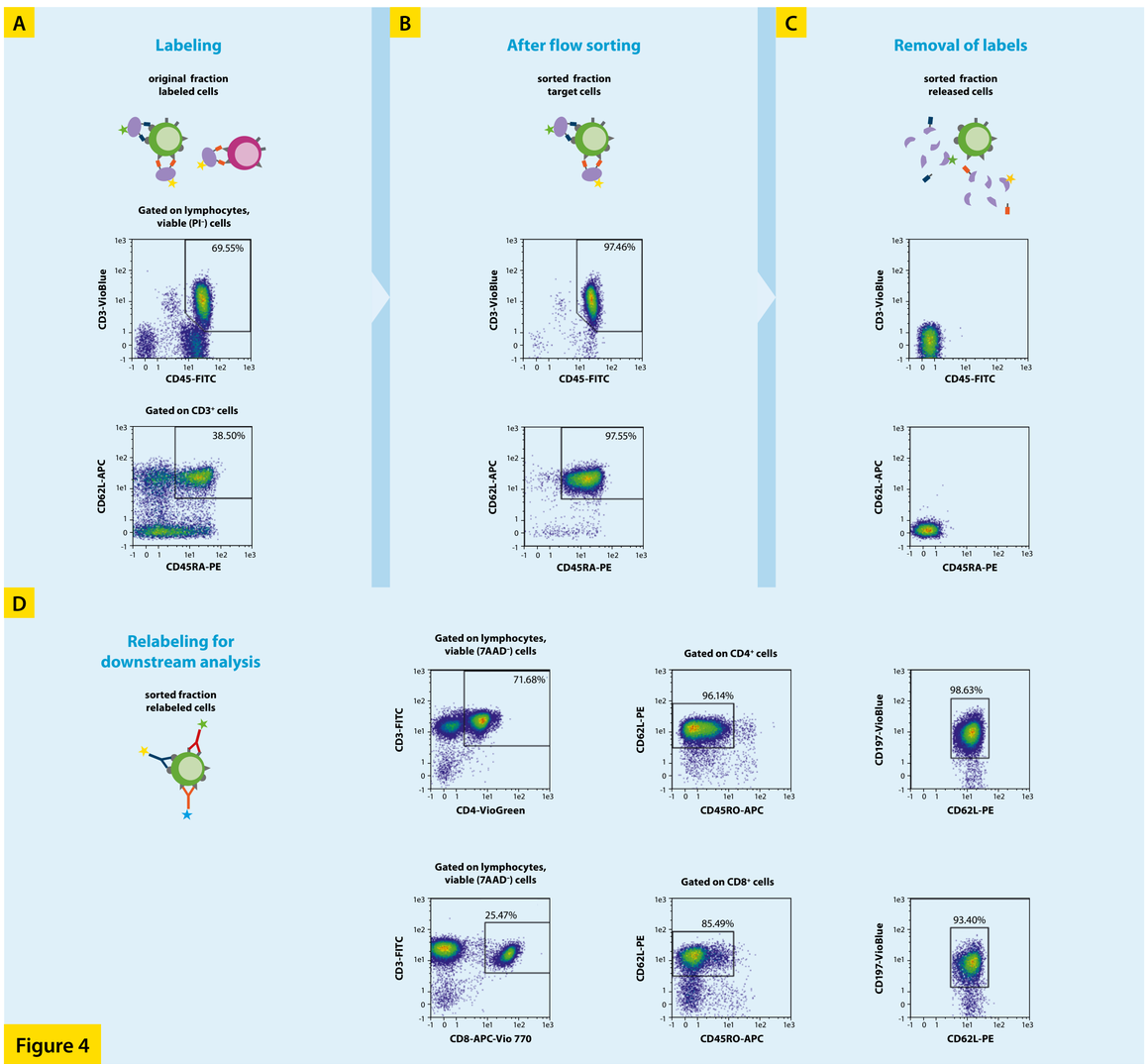

Figure 4

Conclusion and outlook

We developed a new technology allowing for highly specific multiparameter fluorescent cell labeling followed by efficient removal of all fluorochrome conjugates from the cell surface. The technology is based on REAlease Conjugates, which:

- show brightness and specificity similar to conventional antibody-fluorochrome conjugates;
- can be rapidly and completely released from cells in a controlled manner, with high selectivity and under gentle conditions;
- permit multiparameter cell staining for flow sorting experiments;
- facilitate downstream analysis of cells after flow sorting, applying the unmasked epitope or reusing the fluorescence channels.