REAlease® Technology: Controlled release of antibody-fluorochrome conjugates for maximal flexibility in flow sorting and fluorescence microscopy applications

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Jennifer Pankratz, Sabine Schmachtenberg, Nicole Jansen, Marie Hansen, Susanne Krauthäuser, Ali Kinkhabwala, Karolina Schlegel, Svenja Meiler, Christiane Siewert, Alina Bartholomäus, Nicole Susan Eckardt, Claudia Reinhard, Lara Minnerup, Vanessa Schmitz, Dmytro A. Yushchenko, and Christian Dose
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

Multiparameter immunofluorescent labeling is the method of choice for 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. In particular, the continued presence of the fluorescent antibody labels on the cells blocks specific cellular epitopes and restricts the choice of downstream fluorescence detection channels.
Here we present a new type of antibody-fluorochrome conjugates that enables a highly specific multiparameter cell staining as well as subsequent complete release of the conjugate probes from the sample. These new conjugates rely on i) recombinantly engineered antibody fragments that individually possess low epitope binding affinities and ii) a conjugation chemistry that facilitates cell binding and removal of the conjugates from the cell surface after the flow sorting step. Accordingly, previously blocked epitopes are free for relabeling in downstream applications.
The versatility of this technology is presented based on multiparameter panels enabling clear distinction and discrimination of different cell populations. The applicability of the new REAlease® Conjugates is also demonstrated by flow sorting of human naive regulatory T cells. In addition, we show that the fast and efficient releasability of the new antibody-fluorochrome conjugates can serve as an optimal basis for cyclic immunofluorescence microscopy with the new MACSima™ Imaging Platform, which permits cell analysis with a potentially unlimited number of parameters.

Methods

1 The principle of REAlease® Technology

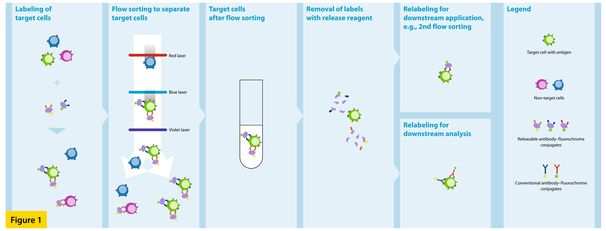


Figure 1

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.
In addition, the option to stain samples and release all fluorescent labels in multiple cycles allows for the detailed immunofluorescence microscopy analysis of biological samples with a potentially unlimited number of parameters (fig. 5).

Results

1 Multiparameter immunofluorescent labeling and release

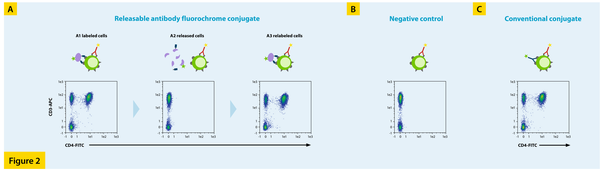


Figure 2

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.

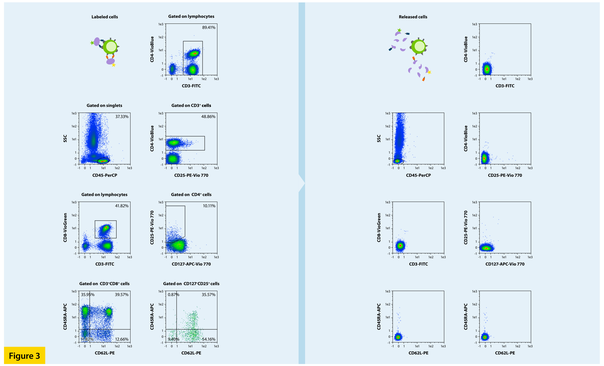


Figure 3

2 Multiparameter flow sorting of naive regulatory T cells

Fluorescence-based cell sorting is an attractive method to obtain highly purified naive regulatory T (Treg) cells. Due to low expression levels of surface antigens used for identification of Treg cells, labeling with bright fluorescent antibody conjugates is required for efficient discrimination and sorting of these cells. Here, human PBMCs were labeled with a panel of four REAlease Conjugates, i.e., CD4-VioGreen, CD25-APC, CD127-PE, and CD45RA-FITC (fig. 4A). Naive Treg cells ($CD4^+CD25^+CD127^-CD45RA^+$) were isolated at high purities using the MACSQuant® Tyto® Cell Sorter (fig. 4B). After sorting, the fluorescent labeling was efficiently released from the target cells (fig. 4C). Removal of the conjugates enabled relabeling of CD4, CD25, and CD45RA epitopes for downstream immunofluorescence microscopy analysis (fig. 4D).

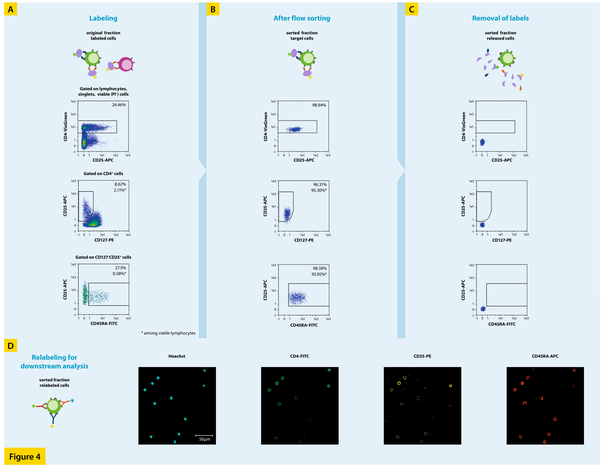


Figure 4

3 Ultrahigh-content imaging of a single biological sample

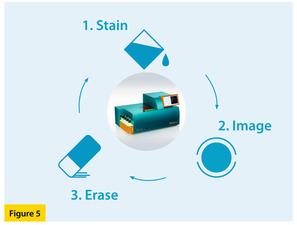


Figure 5

The REAlease Fluorochrome Conjugates can be used as a highly specific and sensitive reagent for potentially unlimited iterative staining of a single biological sample. Iterative staining approaches for imaging consist of three main steps: fluorescent staining, image acquisition, and erasure of the fluorescence signal (fig. 5). With REAlease Technology the fluorescence signal is erased by the addition of the release reagent resulting in a complete removal of the antibody-fluorochrome conjugate from the sample. Accordingly, the utilized fluorescence detection channels become available for fluorescent relabeling in the next cycle.

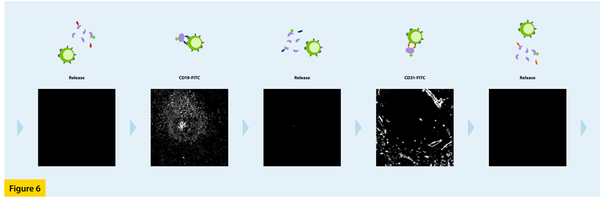


Figure 6

Using the novel MACSima Imaging Platform, developed by Miltenyi Biotec (fig. 5), we demonstrate the applicability of REAlease Conjugates for ultrahigh-content imaging. The same thin tissue section of a fixed human tonsil, 8 µm thick, attached to the surface of a glass-bottomed well, was incubated sequentially with REAlease FITC conjugates and the release reagent (fig. 6). The images are shown at the same contrast level and display a section of 531 µm × 531 µm. The specific fluorescent labeling from each staining step was released with high efficiency, and only a barely visible residual fluorescence signal was detected after incubation with the release reagent.
The image sequence was extracted from a long cyclic run with several REAlease FITC and APC conjugates, the final results of which are displayed in figure 7. Acquisition of the nuclear staining images (Hoechst, fig. 7 bottom right) allows for reliable registration of all stain and release images, enabling accurate subtraction of the prior release image from each subsequent stain image. This image subtraction process removes both persistent autofluorescence signals as well as the very low residual fluorescence signal from all prior staining steps, allowing for particularly sensitive and specific immunofluorescence detection.
In addition, the use of various REAlease Conjugates with different specificities and fluorochromes allows simultaneous multiparameter staining of different antigens, increasing the overall efficiency of each iterative staining cycle. REAlease Technology is gentle on biological samples, especially when compared to other erase approaches utilized for iterative staining cycles, such as fluorophore destruction by photobleaching or oxidation and antibody release by increased temperature or altered pH. Gentle sample processing is due to the high specificity of the release reagent that selectively targets REAlease Conjugates under mild conditions.

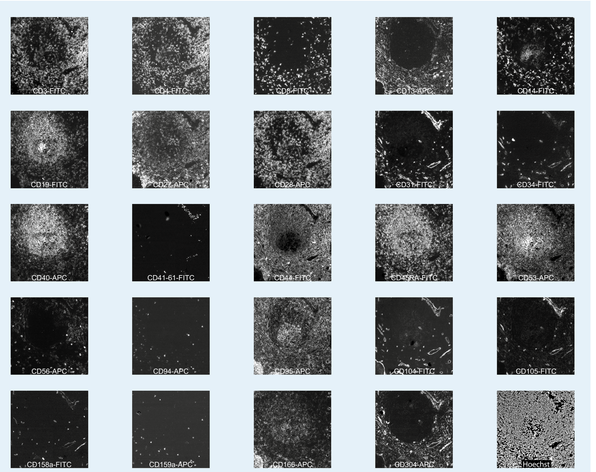


Figure 7

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 and fluorescence microscopy applications;
- facilitate downstream analysis of cells after flow sorting, applying the unmasked epitope or reusing the fluorescence channels;
- serve as an optimal basis for ultrahigh-content imaging of a single biological sample, enabling potentially unlimited iterative multiparameter stain-image-release cycles.

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