**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 labeling and relabeling of cell surface epitopes by 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 workflow for the isolation of highly pure human regulatory T cells (Tregs). These cells were labeled and detected by CD25-APC and CD127-PE conjugates, to be enriched by flow sorting in high purity and cell yield. Final detection of the target cells was accomplished by intracellular labeling with Anti-FoxP3-Vio67 since a selective removal of the CD25-APC conjugate from the cell surface facilitated the reuse of the APC detection channel and enabled maximum flexibility for the identification of the targeted Treg cells after flow sorting.

**Methods**

**1 The principle of REAlease® Fluorochrome Technology**

The new REAlease Fluorochrome Technology relies on recombinantly engineered antibody fragments. Unlike conventional antibodies, these antibody fragments are characterized by highly specific binding affinities. A recombinantly engineered conjugation chemistry allows for a multimodal and fluorescence labeling of these recombinantly engineered antibody fragments (REAlease Fluorochrome Complexes) to facilitate high avidity cell binding comparable to conventional antibody-fluorochrome conjugates. This novel and innovative conjugation chemistry allows for a fast and complete 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 disassociation of the REAlease Fluorochrome Complex and thereby to a reconsideration 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: labeled-free cells after cell sorting for maximal flexibility.

**2 Multiparameter immunofluorescent labeling and release**

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 recombinant 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 staining 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 PMICs with REAlease Fluorochrome Conjugates allows for clear definition and discrimination of target cell populations:

- naïve Tregs CD3+CD4+CD25–CD127+FoxP3–
- central memory T cells CD3+CD4+CD25–CD127–
- CD8+ T cells CD3–CD8+CD25–
- NK cells subtypes CD3–CD56+CD161–/CD56+CD161+

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 cytométric downstream analysis**

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.

**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.

**References**

[1] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

[2] Department of Cellular Immunology, Clinic for Rheumatology and Clinical Immunology, Charité - University Medicine Berlin, Berlin, Germany

[3] The new 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.

**Methods**

**1 The principle of REAlease® Fluorochrome Technology**

The new REAlease Fluorochrome Technology relies on recombinantly engineered antibody fragments. Unlike conventional antibodies, these antibody fragments are characterized by highly specific binding affinities. A recombinantly engineered conjugation chemistry allows for a multimodal and fluorescence labeling of these recombinantly engineered antibody fragments (REAlease Fluorochrome Complexes) to facilitate high avidity cell binding comparable to conventional antibody-fluorochrome conjugates. This novel and innovative conjugation chemistry allows for a fast and complete 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 disassociation of the REAlease Fluorochrome Complex and thereby to a reconsideration 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: labeled-free cells after cell sorting for maximal flexibility.

**2 Multiparameter immunofluorescent labeling and release**

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 recombinant 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 staining 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 PMICs with REAlease Fluorochrome Conjugates allows for clear definition and discrimination of target cell populations:

- naïve Tregs CD3+CD4+CD25–CD127+FoxP3–
- central memory T cells CD3+CD4+CD25–CD127–
- CD8+ T cells CD3–CD8+CD25–
- NK cells subtypes CD3–CD56+CD161–/CD56+CD161+

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 cytométric downstream analysis**

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.

**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.

**References**

[1] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

[2] Department of Cellular Immunology, Clinic for Rheumatology and Clinical Immunology, Charité - University Medicine Berlin, Berlin, Germany