Iterative ultrahigh-content imaging with the MACSima™ Imaging Platform using novel releasable antibody-fluorochrome conjugates based on REAlease® Technology

Jennifer Pankratz, Ali Kinkhabwala, Andreas Bosio, Karolina Schlegel, Claudia Reinhard, Lara Minnerup, Vanessa Schmitz, Sabine Schmachtenberg, Nicole Jansen, Susanne Krauthäuser, Nicole Susan Eckardt, Dmytro A. Yushchenko, Christian Dose, and Jürgen Krieg; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

Technologies allowing for *in situ* high-content imaging of antigens are needed for a deeper understanding of cell biology and particularly for addressing questions related to cell heterogeneity and microenvironment.

Here we present the novel MACSima™ Imaging Platform and tailor-made reagents (REAlease® Conjugates) that enable sensitive ultrahigh-content immunofluorescence imaging of single biological samples. The MACSima Imaging Platform is based on a fully automated imaging system that integrates liquid handling with sensitive multichannel microscopic detection. Iterative imaging with this system consists of three main steps: fluorescent staining, image acquisition, and erasure of the fluorescence signal.

Erasure of the fluorescence signal has traditionally been achieved by photobleaching or chemical bleaching of conventional antibody-fluorochrome conjugates. However, the iterative acquisition of larger samples or multiple samples at a time is limited by the spot-wise photobleaching. Alternative chemical bleaching methods (e.g. peroxide-based), allowing for simultaneous bleaching of the entire sample, are usually harsh and suffer from the adverse effects caused by the used chemical. With the aim to overcome the aforementioned limitations, we developed REAlease Conjugates which enable highly specific multiparameter epitope staining and subsequent efficient release from the cells in a fast and gentle way. Here we show the applicability of the REAlease Conjugates for iterative staining of a single slice of tissue in the MACSima System. We demonstrate that the combination of the MACSima Imaging Platform with REAlease Technology allows for deep proteomic phenotyping of individual cells *in situ* over a potentially unlimited number of parameters.

Methods

1 MACSima™ Imaging Platform

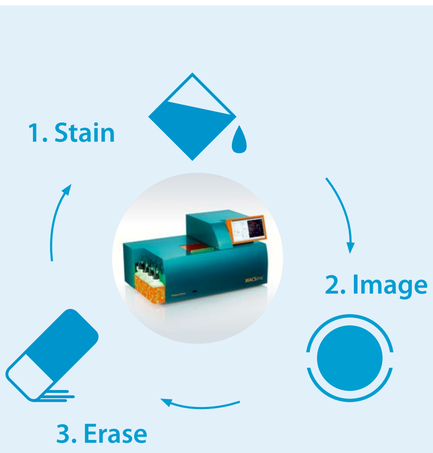

Figure 1

The new MACSima™ Imaging Platform combines liquid handling and microscopy in a completely automated system to enable ultrahigh-content immunofluorescence imaging of single biological samples. The MACSima System facilitates the analysis of hundreds of antigens on biological samples by iterative staining cycles consisting of: fluorescent staining with antibody-fluorochrome conjugates, image acquisition, and erasure of the fluorescence signal (fig. 1). Multiparameter staining and multichannel detection allows for an increase in the parameters detected per cycle. One mechanism utilized by the MACSima System for erasure is based on spot-wise photobleaching obtained through high-intensity light focused onto a region of few millimeters in size on the sample. This enables iterative cycles based on the use of conventional antibody-fluorochrome conjugates. The combination of the new REAlease Technology with the MACSima System provides an alternative fast and gentle signal erasure mechanism.

2 The principle of REAlease® Technology

The novel REAlease® Conjugates for highly specific multiparameter cell labeling rely on recombinantly engineered antibody fragments (fig. 2A). 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 REAlease Conjugates can be rapidly released from the biological sample. Briefly, this release step leads to disruption of the REAlease Conjugate and thereby to a monomerization of the antibody fragments, which spontaneously dissociate from their target epitopes due to their engineered low binding affinity. Therefore, efficient erasure of immunofluorescence signals can be achieved over an entire sample through a fast and gentle release step. REAlease Conjugates serve as an optimal basis for cyclic immunofluorescence microscopy.

Results

1 Multiparameter imaging cycles with REAlease® Conjugates and the MACSima™ Imaging System

Using the novel MACSima™ Imaging Platform, we demonstrate the applicability of REAlease® Conjugates for cyclic immunofluorescence microscopy.

The same thin tissue section (8 µm thick) of a fixed human tonsil, attached to the surface of a glass-bottomed well, was incubated sequentially with REAlease Conjugates and the release reagent (fig. 2). In detail, after 10 min staining with REAlease Conjugates, the well was washed multiple times before imaging. Following imaging, the buffer was exchanged and the section incubated with the release reagent. After 10 min incubation at room temperature, the well was washed and imaged, completing a single cycle. Each image across each row of figure 2B and C is 531 µm × 531 µm in size.

REAlease Conjugates were developed for three fluorochromes, FITC, PE, and APC, and several recombinantly engineered antibodies allowing multiparameter staining and imaging of different antigens in one staining cycle (fig. 2C). The specific multiparameter fluorescent labeling from each staining cycle was released simultaneously over the entire sample and with high efficiency. Only barely visible residual fluorescence signals were detected after incubation with the release reagent.

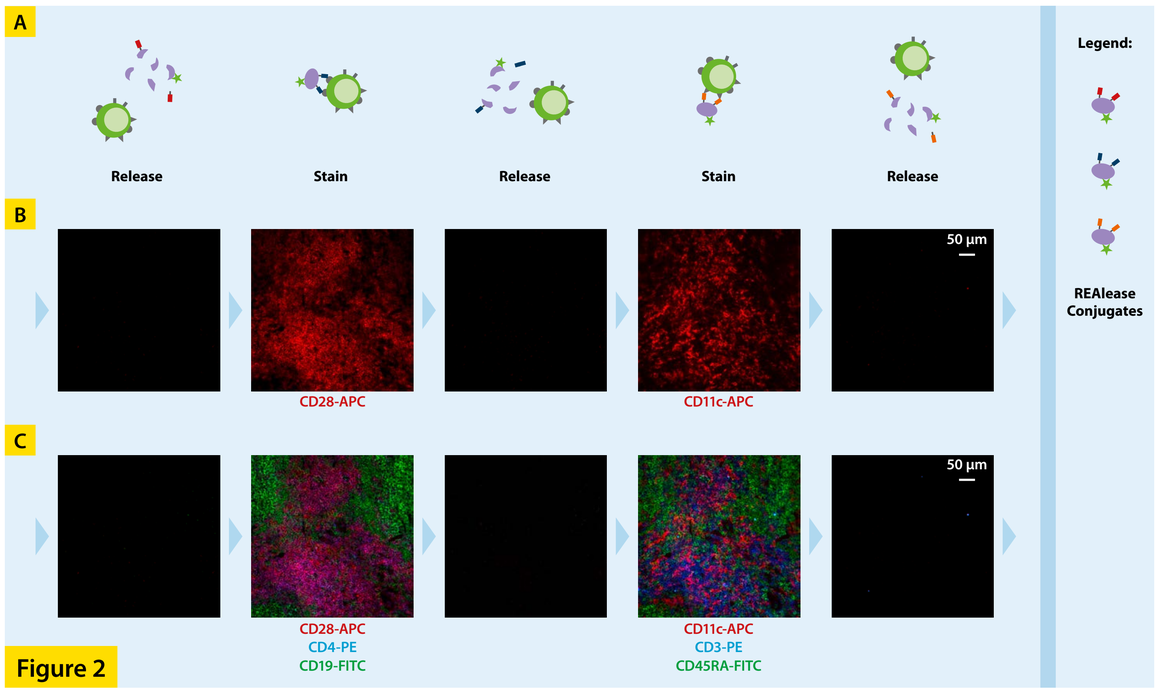

Figure 2

2 Ultrahigh-content imaging of a single biological sample

The option to stain samples and release fluorescent labels in multiple cycles allows for the detailed immunofluorescence microscopy analysis of biological samples with a potentially unlimited number of parameters.

The image sequence in figure 3 displays the results of a cyclic imaging process of an 8-µm thick section of a fixed human tonsil, attached to the surface of a glass-bottomed well. The sample was iteratively stained with two REAlease Conjugates per cycle, one FITC conjugate and one APC conjugate (the first number in each image label indicates the cycle). Acquisition of the nuclear staining images (Hoechst, fig. 3, bottom) in each round helps to reliably register 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 a very low residual fluorescence signal from all prior staining steps, allowing for particularly sensitive and specific immunofluorescence detection.

REAlease Technology is gentle on biological samples compared to other signal 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 achieved due to the high specificity of the release reagent that selectively targets REAlease Conjugates under mild conditions.

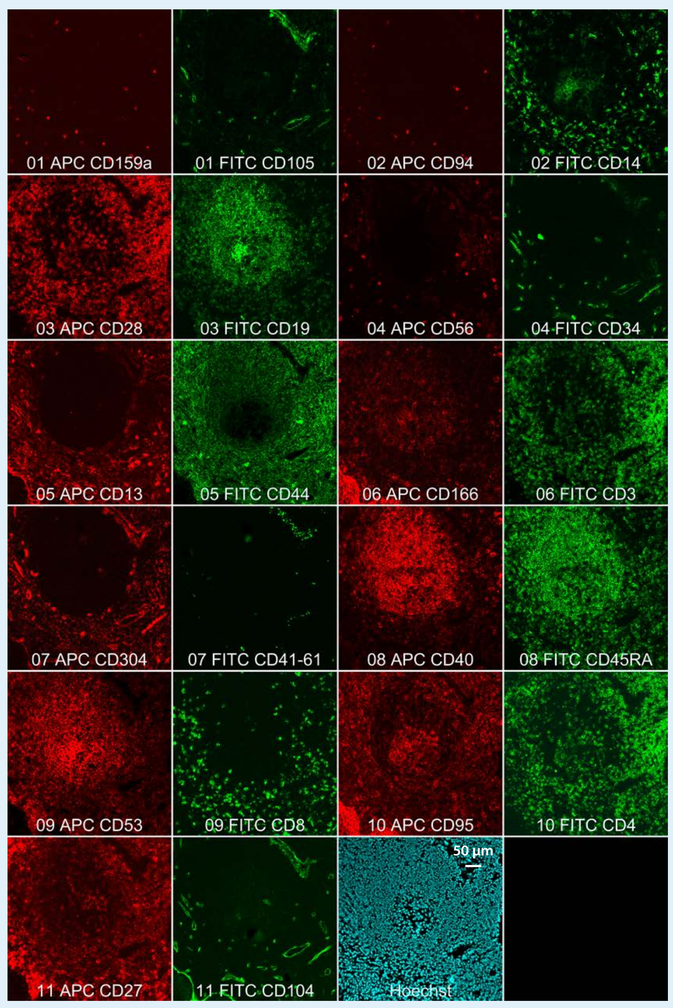

Figure 3

Conclusion and outlook

We introduce here two new technologies, the MACSima Imaging Platform and REAlease Conjugates, which enable ultrahigh-content imaging of a single biological sample. The fully automated MACSima System enables iterative multiparameter stain-image-erase cycles with sensitive multichannel microscopic detection.

REAlease Conjugates allow for highly specific multiparameter fluorescent labeling and efficient removal of all fluorochrome conjugates from the biological sample via a selective and gentle release mechanism.

Iterative imaging with the MACSima System based on REAlease Conjugates improves considerably on existing approaches, permitting rapid analysis of potentially hundreds of antigens and deep insights into biological systems.