

# High-throughput Observation and Quantification of ADCC using Imaging Flow Cytometry

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## Abstract

In recent years the development of therapeutic antibodies has experienced significant growth, a trend that is expected to be sustained in the foreseeable future. Many of the new developments in antibody engineering are focused on the improvement of antibody effector functions. Therefore, *in vitro* studies that aid in the prediction of clinical efficacy and in understanding the mode of action of therapeutic antibodies targeting cancer cells are of growing interest. Recent studies have highlighted the importance of Fc effector functions in the therapeutic activity of rituximab (Rituxan®) against lymphoma cells, suggesting that enhanced antibody mediated effector/target cell interactions and their cytotoxic effects on the target cells correlate with better overall response to treatment. To better characterize and quantify these interactions *in vitro*, we directly visualized and evaluated the antibody-dependent cell-mediated cytotoxicity (ADCC) triggered by the therapeutic antibody rituximab when targeting CD20 on Ramos human Burkitt's lymphoma cells and using the human monocytic U937 effector cells. We report the direct high-throughput imaging and quantification of single ADCC events mediated by rituximab in mixed cell populations. U937 cells effector cells labeled with the CMPTX red fluorescent dye incubated in 5:1 ratio with Ramos cells stained with the CFSE green fluorescent dye in the presence or absence of 5 µg/ml of rituximab for 1h, 2h, 4h, and 6h at 37°C in 5%CO<sub>2</sub> were imaged using the ImageStream imaging in flow technology (Amnis Corporation, Seattle, WA). We demonstrate in detail the physical interaction between target and effector cells, their morphological changes, level of apoptosis, and a directional exchange of their cytosolic contents over time. Unlike conventional approaches for the detection of ADCC events, which rely on quantification of the release of traceable compounds from target cells or flow cytometry analysis of population-wide phenomena, the current work combines the statistical power of flow cytometry with the analytical advantages of cell imaging, providing a novel and more comprehensive perspective of effector/target cell interactions during ADCC events. It is expected that similar studies with different cell populations, such as peripheral blood mononuclear cells, would allow the identification of different effector cell populations interacting preferentially with the targeted cancer cells. A direct visualization of the biological activity of antibodies in the context of effector/target cells interactions using imaging methods, such as imaging in flow cytometry, is of interest for the characterization and pre-clinical evaluation of antibody therapeutics at large, as well as for better defining mechanisms involved in effector responses of host antibodies against cancer cells.

## Introduction

In recent years the development of therapeutic antibodies has experienced significant growth, a trend that is expected to remain steady in the foreseeable future, with many new developments in antibody engineering focused on the improvement of antibody effector functions (1). In particular, the mechanism of antibody-dependent cellular cytotoxicity (ADCC) has been shown to be a major effector in the therapeutic activity of trastuzumab (Herceptin®), rituximab (Rituxan®) and other monoclonal antibodies in development, suggesting that enhanced antibody mediated effector/target cell interactions and their cytotoxic effects on the target cells correlate with better overall response to treatment (1). ADCC mediate the elimination of cancer cells through a mechanism that requires the presence of antibody, target cells expressing the antigen on the surface, and effector cells expressing Fc<sub>γ</sub>-receptors such as macrophages, NK cells, monocytes, and neutrophils (2). In some cases, it has been reported that target cells are internalized by phagocytosis in a process known as antibody-dependent cellular phagocytosis (ADCP) into effector cells recruited via the monoclonal antibody (3). Current methods for ADCC determination such as <sup>51</sup>Cr or lactate dehydrogenase release assays rely on the evaluation of the cell membrane integrity by quantification of the release of traceable compounds from target cells or by evaluation of the target cell viability by flow cytometry. In the case of ADCP, effector-target cell interaction status is frequently evaluated by fluorescent microscopy, with the caveat that this analysis can be difficult for rare events and not suited for statistical analysis of the sub-populations.

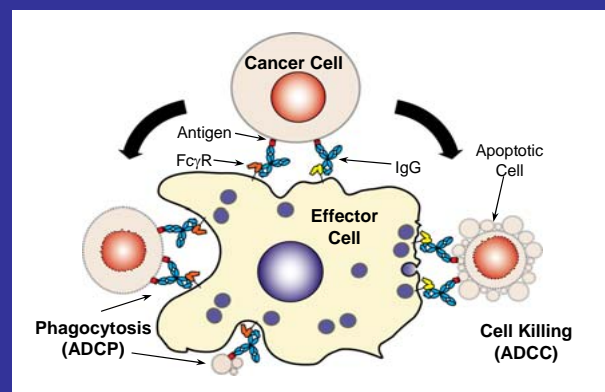
Because of the complexity of the effector/target cellular interactions in the presence of therapeutic antibodies, new technologies are required that allow the monitoring not only of the viability of the target cells, but also of the effector cells as well as their physical interaction. To better characterize and quantify these interactions *in vitro*, we directly visualized and evaluated the interactions between U937 cells (effector) and the CD20 expressing lymphoma cell line Ramos (target) in the presence of the anti-CD20 IgG1 rituximab using imaging flow cytometry. This technique combines the fluorescence sensitivity and high-throughput statistics of flow cytometry with the optical resolution of wide field digital microscopy, allowing the analysis of the status of the population of immune effector cells and target cells and of the interacting cells in the presence of specific antibodies.

## Objective

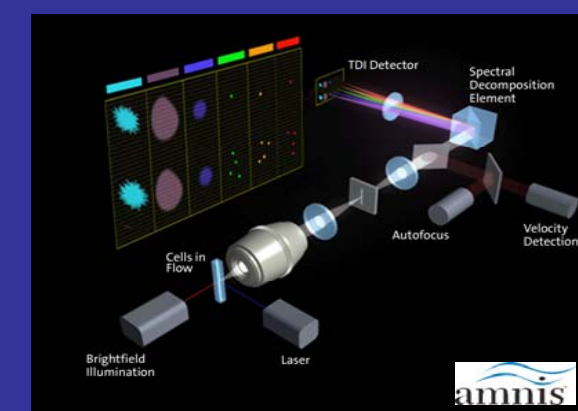
We propose to use imaging flow cytometry to analyze the interactions between immune effector cells and the target cells in the presence of specific antibodies to better evaluate ADCC and ADCP activities *in vitro*.

## References

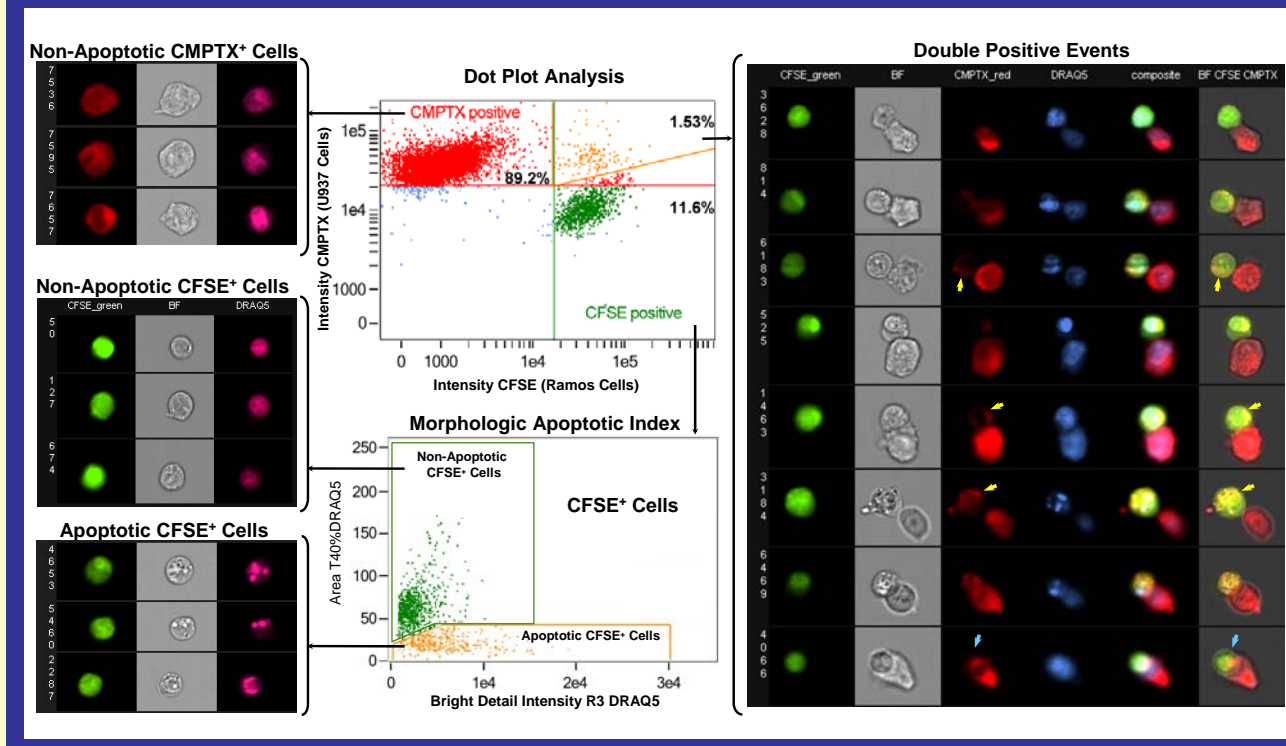
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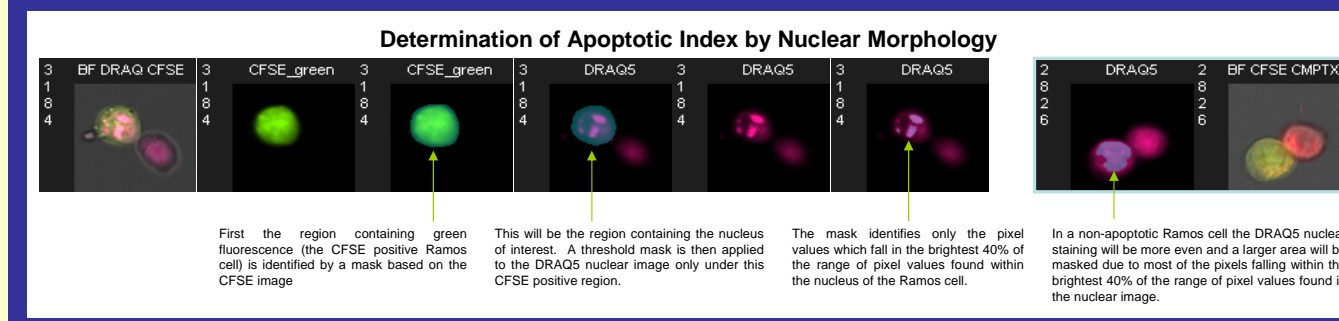
**Figure 1**  
Scheme of the mechanisms of cancer cell killing mediated by immune effector cells together with antibodies. Antibodies bound to tumor associated antigens expressed on the surface of cancer cells and to Fc<sub>γ</sub>R on the surface of effector cells, such as macrophages cells, can induce the death of the cancer cell by either ADCC or ADCP.



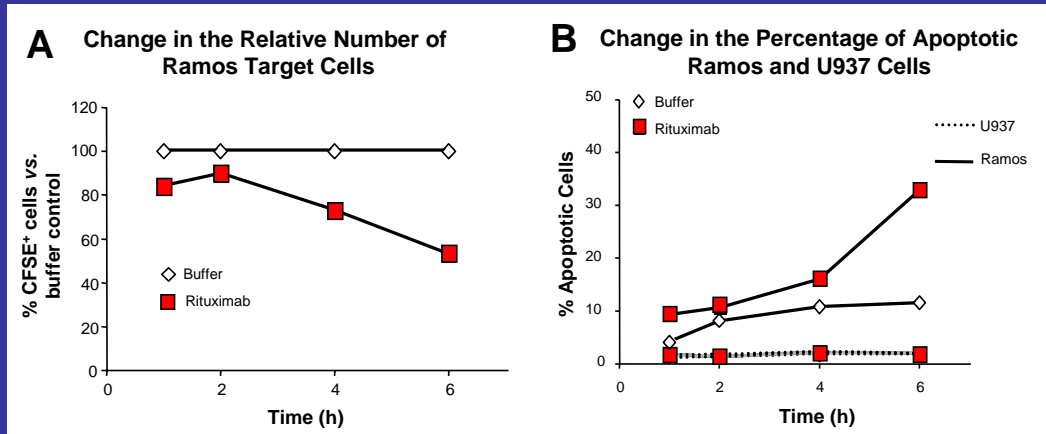
**Figure 2**  
ImageStream Technology. This method allow to image in multiple modes rapidly-moving objects in flow with high sensitivity and high image fidelity. Images are acquired using 488 nm laser excitation containing brightfield, red and green fluorescence channels with a field depth of 2 µm.



**Figure 3**  
Analysis by imaging in flow cytometry. We used an ImageStream multispectral system for imaging in flow cytometry to study the ADCC activity against the Burkitt's B-cell lymphoma Ramos cell line that expresses CD20 by U937 human effector cells in the presence of rituximab. 10<sup>6</sup> U937 cells labeled red with the CMPTX fluorochrome (Molecular Probes, Invitrogen) were co-incubated with 2x10<sup>5</sup> Ramos target cells labeled green with the CFSE fluorochrome (5:1 - E:T ratio) in the presence of 5 µg/ml rituximab. After incubation, cells were fixed and nucleus stained with the DRAQ5 dye. Samples were run in the ImageStream and imagery acquired for 10,000 cells/sample. In the center top we show the dot plot of the treatment. The quadrants in the plot show the events CMPTX<sup>+</sup> (U937, imagery upper left), CFSE<sup>+</sup> (Ramos, imagery left center and bottom) and CMPTX/CFSE<sup>+</sup> (imagery on the right). The plot at the bottom center show the morphologic apoptotic index of CFSE<sup>+</sup> cells. The total number of effector and target cells, as well as their apoptotic index determined using the image data Exploration and Analysis Software (IDEAS®) package. The "yellow arrows" show co-localization of red staining (CMPTX) of the effector cells in the green CFSE area of target cells, suggesting the transfer of cytoplasmic content from effector to target cells, but not in the opposite direction, which is consistent with ADCC. The "blue arrow" shows a very rare event in which an effector cell is engulfing a target cell in ADCP activity.



**Figure 4**  
Measuring apoptotic index. The apoptotic index of the target Ramos cells in contact with a healthy U937 cell was determined based on images of nuclear morphology using the IDEAS software. The Area of the 40% Thresholded DRAQ5 nuclear mask within the Ramos cell can be plotted as a parameter on the Y-axis vs. Bright Detail Intensity R3 (a texture feature in the software package measuring the amount of bright texture with a radius less than 3 pixels) for the DRAQ5 image as well.



**Figure 5**  
ADCC activity and apoptotic status of target and effector cells in the presence of rituximab. Using the ImageStream system imaging flow cytometry we determined the ADCC activity elicited by U937 effector cells (CMPTX) in the presence of rituximab against Ramos (CFSE) target cells in a time course study. The total number of effector and target cells, as well as their apoptotic index was quantified using the IDEAS® software package. In panel A we show that rituximab reduces the relative number of Ramos cells over time. In panel B we show the apoptotic index of both effector and target cells over time. Note the steady increase in the apoptotic index of target Ramos cells over time in the presence of rituximab, but not of effector U937 cells. Although the population of double positive events was significant in all time points, the occurrence of phagocytosis under these conditions was very rare, and in consequence we did not quantify ADCP activity. It is possible that with other effector cells these events will be more frequently observed.

## Conclusions

- We have demonstrated that imaging flow cytometry can be used for the determination of ADCC activity by quantification of total number of target and effector cells, as well as the apoptotic index of both cell populations.
- The imaging analysis allow the observation of cytoplasm transfer from fluorescently labeled effector cell to the target cells, and in some instances the visualization of phagocytosis, although the events of ADCP activity were very rare under our conditions (<5% CFSE<sup>+</sup>/CMPTX<sup>+</sup> events).
- This study demonstrates the feasibility of the application of the ImageStream technology to evaluate simultaneously the ADCC and ADCP activities of therapeutic antibodies against cancer cells *in vitro*.

## Acknowledgements

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