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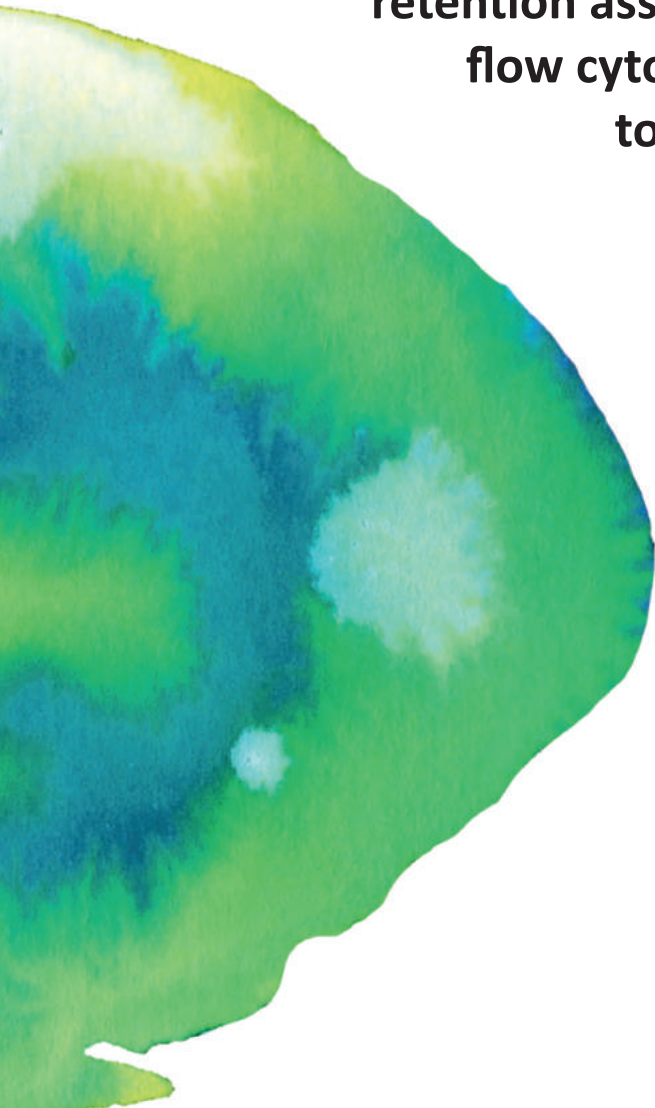
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CHAPTER 4

The modified FACS calcein AM retention assay: a high throughput flow cytometer based method to measure cytotoxicity



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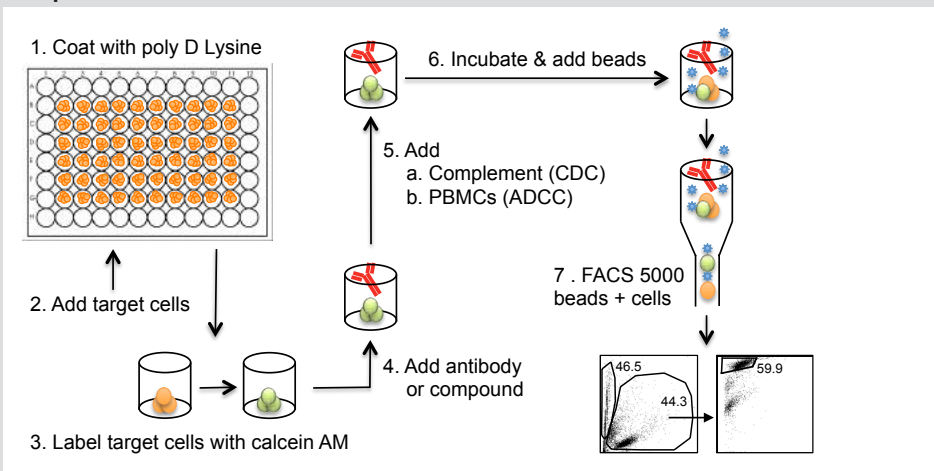
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Abstract

Current methods to determine cellular cytotoxicity *in vitro* are hampered by background signals that are caused by auto-fluorescent target and effector cells and by non-specific cell death. We combined and adjusted existing cell viability assays to develop a method that allows for highly reproducible, accurate, single cell analysis by high throughput FACS, in which non-specific cell death is corrected for. In this assay the number of living, calcein AM labeled cells that are green fluorescent are quantified by adding a fixed number of unlabeled calibration beads to the analysis. Using this modified FACS calcein AM retention method, we found EC₅₀ values to be highly reproducible and considerably lower compared to EC₅₀ values obtained by conventional assays, displaying the high sensitivity of this assay.

Graphical abstract



1. Coat a 96 well flat bottom culture plate with Poly-D-Lysin
2. Seed adherent target cells 3 days prior to the cytotoxicity assay. Seed non-adherent cells after step 3
3. Calcein labeling of target cells on the day of the assay
4. Add test antibodies or compound
5. Add complement for CDC assay (a) or effector cells for ADCC (b) (optional)
6. Incubate at 37°C in the dark for 1 hour (CDC) or 4 hours (ADCC), then add 10,000 FACS beads
7. Acquire 5000 beads by FACS. Process data with Flowjo

Introduction

The goal of anti-cancer therapy is to induce death of malignant cells. Oncolytic agents induce cytotoxicity directly, or indirectly via antibody dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). To determine the cytotoxicity of a compound in vitro, a number of methods can be applied. The classic method is the chromium 51 (^{51}Cr) release assay,^{1,2} in which target cells are labeled with the radioactive compound ^{51}Cr . After incubation with the compound of interest plus or minus effector cells or complement the amount of lysed target cells is determined by the amount of radioactivity in the supernatant. Because the use of radioactive compounds requires skilled personnel, costly equipment and laboratories, non-radioactive cytotoxicity assays have been developed that rely on the release of naturally occurring intracellular enzymes like lactate dehydrogenase (LDH) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from dying cells. The readout in these assays is based on a chemical reaction related to the activity of these enzymes. Both the LDH and the GAPDH assay do not discriminate between cell death by the compound under study or non-specific cell death, including that of effector cells, and results have to be compensated for that.^{3,4} Another alternative to the ^{51}Cr release assay is the calcein release assay.^{5,6} In this assay, target cells are incubated with calcein AM, a non-fluorescent, hydrophobic and cell permeable dye that easily enters living cells. In the cell cytoplasm, AM is cleaved off by esterases via hydrolysis leaving calcein as a hydrophilic, green fluorescent dye that is released into the medium when the labeled cells die. The read-out of this method is the green fluorescence intensity of the medium. Culture media and supplements like fetal bovine serum (FBS) can be auto-fluorescent, causing background signal that can reach up to 40% of the signal observed at maximum cell death.⁵ Other assays include the ATP luminescence assay and the luminescent ADCC Reporter Bioassay. The ATP luminescence assay relies on the quantification of intracellular ATP levels as a read-out for cell viability. As it does not discriminate between target and effector cells the assay is not suitable for ADCC assays.^{6,7} In contrast, the ADCC reporter Bioassay (Promega) relies on the activation of effector cells as a readout, and is therefore applied exclusively for ADCC.⁸

We combined and adjusted existing cell viability assays to develop an assay that allows for single cell analysis by high throughput FACS and that corrects for non-specific cell death. In this assay the number of living calcein AM labeled cells that are green fluorescent are quantified by adding a fixed number of calibration beads. With this modified FACS calcein AM retention assay we developed an improved, accurate, high throughput flow cytometer based method to determine cellular cytotoxicity that can be applied in a variety of assays, including CDC and ADCC of suspension and adherent cells.

Materials and Methods

Cell lines

The cell lines Raji, Ramos (Burkitt lymphoma) and SK-BR-3 (breast cancer) (ATCC) were cultured according to manufacturers instructions. Raji and Ramos cells were grown in suspension at a cell density of 4×10^5 - 2×10^6 or 2×10^5 - 1×10^6 cells/ml in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1x penicillin / streptomycin (PS; Roche), hereafter called complete medium for suspension cells. The adherent breast cancer cell line SK-BR-3 was cultured in McCoy's 5A medium (Sigma) supplemented with 10% heat-inactivated FBS, 1x GlutaMAX™ 150ul/ml (Invitrogen), 1x PS hereafter referred to as complete medium for adherent cells. Adherent cells were cultured to 80% confluency, detached with trypsin- EDTA (Invitrogen) and washed once before use.

Calcein labeling of suspension and adherent cells

Cells in suspension were washed and re-suspended in 10 nM calcein AM (Becton Dickinson) diluted in RPMI 1640 supplemented with 2% heat-inactivated fetal bovine serum at a cell concentration of 1×10^6 cells/ml. Adherent cells were harvested three days prior to the cytotoxicity assay and seeded at a density of 10,000 cells/well in Poly-D-lysine hydrobromide (Sigma) coated 96 well flat bottom culture plate (Corning® Costar® cell culture plates, Corning) and cultured at 37°C with 5% CO₂. On the day of the cytotoxicity experiment culture medium was replaced with McCoy's 5A medium (Sigma) supplemented with 2% heat-inactivated fetal bovine serum, and 1x GlutaMAX™ containing 10 nM calcein AM, in a final concentration of 100 µl/well. Cells were incubated with calcein AM at 37°C for 30 minutes, protected from light, and cells in suspension were intermittently agitated.

Effector cells

PBMC were isolated from buffy coats (Sanquin) by density gradient centrifugation and cryopreserved to secure equal PBMC quality for all assays. PBMC from two donors were used (see Table 2 and 3). The NK cell ratio for each PBMC batch was determined by flow cytometry using mouse anti-human CD56 APC (BioLegend) and mouse anti-human CD16 PE/Cy7 (Beckman Coulter). Thawed PBMC were cultured overnight in complete medium for suspension cells at a density of 1×10^6 cells/ml. For ADCC assays on Ramos cells with rituximab, 500 IU/ml recombinant IL-2 (Aldesleukin) was added during the overnight incubation. The optimal concentration of effector cells was determined at 12.5×10^6 PBMC/ml in complete medium for suspension and adherent cells.

Conventional calcein release assay

Ramos target cells were labeled with 2 μ M calcein AM as described above. For ADCC, target cells were incubated with antibody (see below) and PBMC for 4 hours, with 2% Triton (Sigma) added for the final 5 minutes to ensure maximum calcein release. After centrifugation, cell free supernatant was transferred to a Cellcarrier plate (Perkin Elmer) to measure relative fluorescence units (RFU) using a HTS multilabel reader (Envision; Perkin Elmer). The percentage of specific lysis was calculated as follows: ((RFU value in respective treatment- RFU value in control (spontaneous release) / (RFU value Triton (maximum release)- RFU value in control (spontaneous release)) x 100. The background of the assay was calculated as follows: (RFU value in control (spontaneous release) / RFU value Triton (maximum release)) x 100.

Lactate dehydrogenase (LDH) assay

Unlabeled Ramos cells were incubated with antibody (see below) and PBMC for 4 hours at 37°C in 5% CO₂, with Triton added during the last 5 minutes for maximum LDH release. Cells were incubated with a reaction mixture (Cytotoxicity Detection Kit Plus (Roche)) for 20 minutes at room temperature. The reaction was stopped and optical density was measured at a wavelength of 492 nm on an Envision. The percentage of specific lysis was calculated as described above. PBMC and target cell only conditions were used to measure spontaneous release; for maximum release triton was added. The background of the assay was calculated as described above.

Flow cytometry based calcein retention assay

Calcein AM labeled suspension cells were washed twice, adjusted to a cell concentration of 0.5x10⁶/ml medium and put in U bottom 96-well microtiter plates (Becton Dickinson Falcon™) at a concentration of 1.25x10⁴ cells per well (25 μ l). Calcein AM labeled adherent cells were on the day of the assay also with 1.25x10⁴ cells per well. Antibodies were added at 1:4 dilution (25 μ l) and left for opsonization at room temperature for 15 minutes. The following antibodies were used: trastuzumab (anti HER2/neu, Herceptin®; Roche), rituximab (anti CD20, Mabthera®; Roche), palivizumab (anti RSV-F, Synagis®; Medimmune), and the in-house manufactured monoclonal antibodies AM22 (anti Respiratory Syncytial Virus-F) and AT10-002 (anti influenza virus, hemagglutinin H3).^{9,10} For CDC 50 μ l of 20% rabbit serum complement (Sigma) was added. For ADCC 50 μ l PBMC were added at an effector-to-target (E:T) cell ratio of 50:1. Cells were incubated at 37°C in 5% CO₂ and left for 1 hour for CDC and 4 hours for ADCC. Adherent cells were detached with trypsin- EDTA (50 μ l/well) and transferred to a round bottom 96-well microtiter plate. Samples were measured using a FACS Canto II (Becton Dickinson) or FACS LSR Fortessa X-20 (Becton Dickinson). To assure equal assay volumes, 10,000 Accudrop

Fluorescent Beads (Becton Dickinson) were added to every sample and 5,000 beads acquired per sample. Data analysis was performed using FlowJo software (TreeStar) and the number of live cells was determined by calcein high green fluorescent signal. The percentage of specific lysis was calculated as follows: $100 - ((\text{calcein positive cells in respective treatment} / \text{calcein positive cells in untreated control}) \times 100)$. Experiments were performed in duplo in at least two separate experiments. The background (spontaneously died target cell) of the assay was calculated with the live cell number in the medium control sample and the actual number of target cells that was started with.

EC50

GraphPad Prism Version 6.0f for Mac OS X was used to analyze dose-response data. XY data tables were made with antibody concentration (X) and cell lysis % (Y). Then, X values were transformed logarithmical ($X = \text{Log}X$) and a nonlinear regression (curve fit) analysis and a sigmoidal dose-response curve was created to calculate the half maximal effective concentration (EC50) and the 95% confidence interval (95% CI).

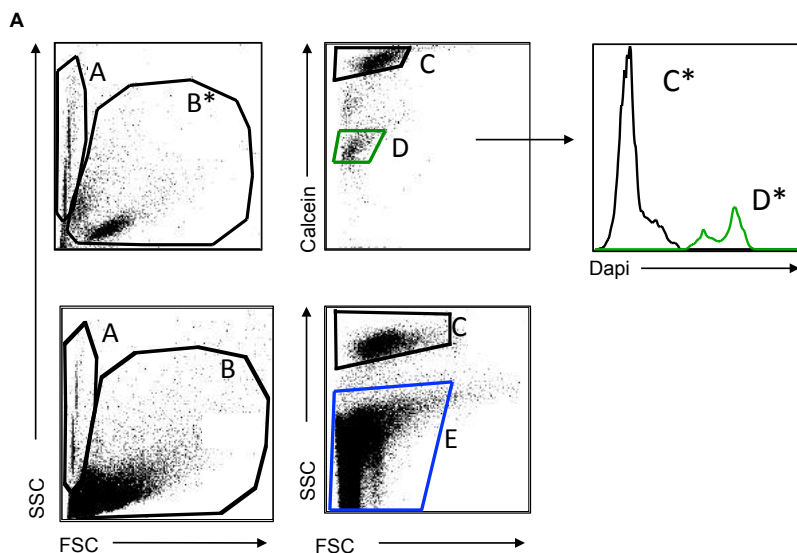
Results

Calcein AM labeling and quantification of live cells

To reliably quantify the capacity of compounds to induce death of target cells, we set up an assay that is based on the green-fluorescent labeling of living cells with calcein. Target cells were labeled with calcein AM that is contained in the cytoplasm of live cells and emits a green fluorescent signal (Emission_{max} 512nm) when excited with a 488nm laser. To be able to measure the same volume for any given sample we added 10,000 Accudrop fluorescent beads (Becton Dickinson) per sample and acquired a fixed number of 5,000 bead events for each sample. In the forward scatter (FSC) versus side scatter (SSC) plot these FACS beads appeared as a narrow band (Figure 1A, gates A) that can be easily separated from the cells in the sample (Figure 1A, gates B). Live cells were gated out as calcein-positive green fluorescent bright cells; these are DAPI negative (Figure 1A, gates C). Dead cells are either calcein-negative cells; these are DAPI positive (Figure 1A, gates D) or have disseminated and are therefore not visible anymore. Effector cells (PBMCs) that were added to the sample can be gated out as calcein-negative cells (Figure 1A, gate E). To calculate the percentage of live cells per sample we used the equation depicted in Figure 1B. The actual number of calcein positive cells was calculated for each cell sample using FlowJo software. To compensate for random cell death induced by cell handling, incubation, complement or PBMCs we normalized cell death to the number of dead cells in the control sample (cells handled similarly to the samples of interest, including incubation with complement or PBMCs, but treated with medium not containing antibodies; 'number of calcein-positive cells in control'). This medium control sample was set as 100% survival.

Complement dependent cytotoxicity assay on suspension cells

We then validated the modified FACS calcein AM retention assay for quantifying cell viability by therapeutic antibodies. Rituximab is a CD20-specific monoclonal antibody that induces CDC and ADCC of B cells, including the B cell lines Ramos and Raji.⁷ Ramos and Raji target cells were labeled with calcein AM and incubated with complement containing rabbit serum to a final concentration of 10% and rituximab or control antibody. One representative example is depicted in Figure 2A (left panels). Dose-response analysis of rituximab concentrations ranging from 0.5 to 3300 ng/ml yielded sigmoidal curves (Figure 2B) and EC50 values of 82 and 24 ng/ml for Ramos and Raji, respectively, with narrow 95% EC50 confidence intervals (Table 1). Complement- containing rabbit serum alone was very well tolerated by the cells resulting less than 5% background cell death. Additional visualization of this process was performed with a high content imager (Operetta, PerkinElmer; 10x objective). Loss of green fluorescence is clearly visible for Rituximab on Ramos cells, whereas no loss of fluorescence is observed for the negative control antibody Palivizumab (Supplementary figure 1)

Figure 1. Calcein AM labeling and analysis of live cells

FACS acquisition of a fixed number of 5000 beads per sample (gates A) and medium-treated cells. Live cells (gates B) are calcein-positive (gates C), dead cells are calcein-negative (gate D). This was confirmed by DAPI staining (histograms). In the lower panels PBMC were added as effector cells (gate E) that can be easily separated from calcein-labeled target cells (gate C).

B

$$\text{Cell death X (\%)} = \left(100 - \frac{\text{Number of calcein positive live cells in X}}{\text{Number of calcein positive cells in control}} \right) \times 100$$

Equation to calculate the percentage cells specifically lysed by the compound under study. 'Number of calcein positive live cells in X' is the amount of cells as depicted in Figure 1, gates C. 'Control' is the average of at least a triplicate measurement of calcein positive target cells in wells with complement or effector cells, but without test compound.

Table 1. EC50 values of rituximab-induced CDC

Target cell	Antibody	Method	EC50 (ng/ml) (95% CI)	Reference
Ramos	Rituximab	ATP luminescence	110	Bornstein ea ⁷
Ramos	Rituximab	Modified calcein retention assay	82 (41.55-105.60)	This paper
Raji	Rituximab	ATP luminescence	180	Bornstein ea ⁷
Raji	Rituximab	Cr release	50	Winiarska ea ¹²
Raji	Rituximab	Modified calcein retention assay	24 (18.41-33.21)	This paper

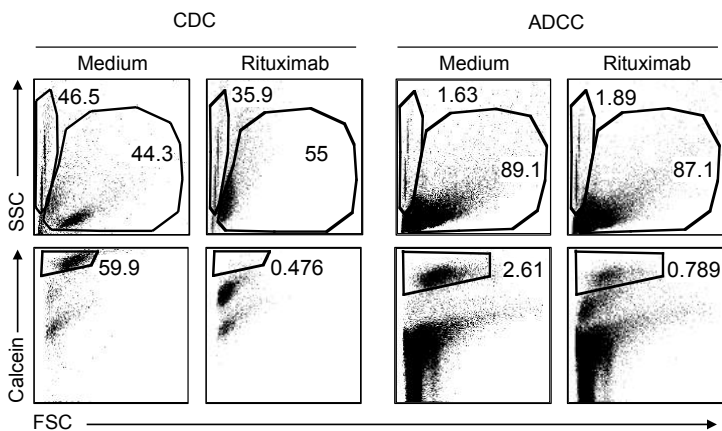
95% CI: 95% confidence interval of the calculated EC50

Antibody dependent cellular cytotoxicity on suspension cells

To measure ADCC, calcein labeled target cells were incubated with human PBMC from healthy donors. A range of effector-to-target (E:T) cell ratios were tested in duplicate. For both batches of PBMC that were used in these assays, the optimal E:T ratio was 50:1. The percentage of CD56+ NK cells per PBMC sample used in our assays was around 10%, resulting in a NK effector:target cell ratio of 5:1. Calcein-labeled Ramos and Raji B lymphocytes were incubated with PBMC and different concentrations of rituximab. In Figure 2A (right panels) a representative example is depicted. Dose-response tests again yielded sigmoidal curves with limited variation between duplicates as can be observed by the narrow 95% confidence interval and EC50 values of 1.2 and 1.02 ng/ml for Ramos and Raji cells, respectively (Figure 2C and Table 2).

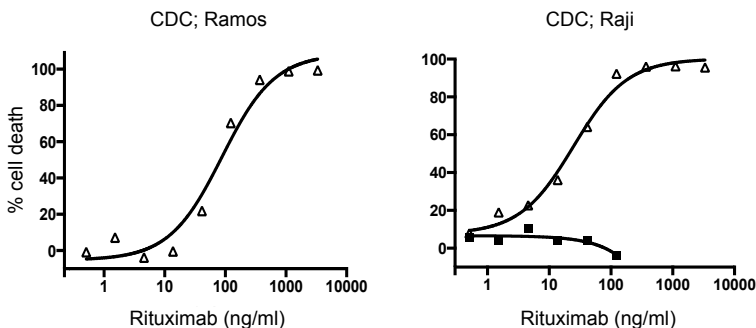
Figure 2. CDC and ADCC of suspension cells

A



Representative examples of CDC and ADCC as measured by the modified calcein AM retention assay. Ramos cells were used as target cells. Rituximab was added at a concentration of 1000 ng/ml.

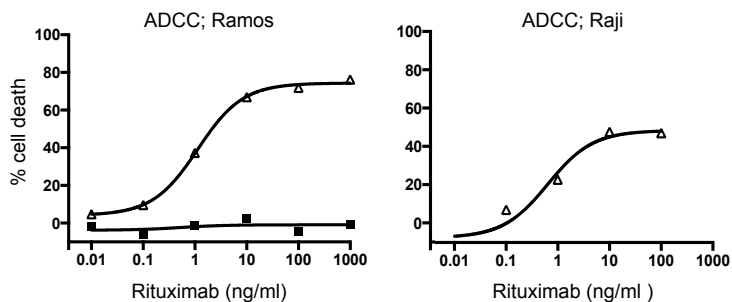
B



CDC of suspension cells with rituximab (open triangles) or control antibody (closed squares) on Ramos cells (left; EC50 82 ng/ml) and Raji cells (right; EC50 24 ng/ml).

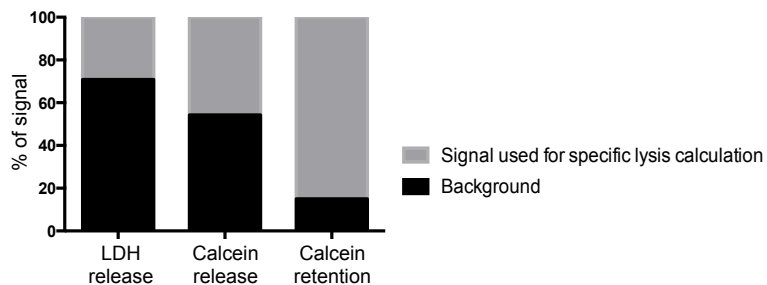
Figure 2. Continued

C



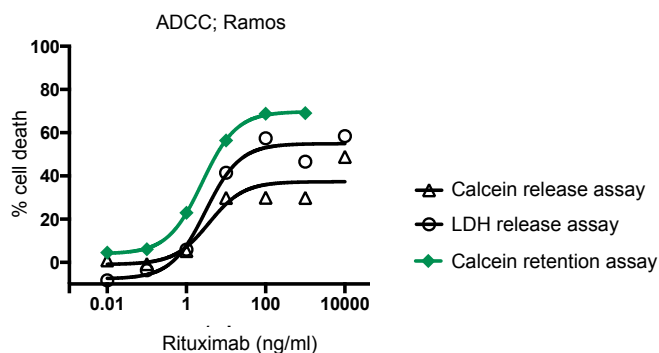
ADCC of suspension cells with rituximab or control antibody on Ramos cells (left; EC50 of 1.2 ng/ml) or Raji cells (right; EC50 of 1.02 ng/ml).

D



Comparison of the LDH release assay, the calcein release assay and the calcein AM retention assay to measure ADCC with Ramos cells as target cells in one experiment, using the same batch of effector cells. Background signals are 70.9%, 54.3% and 15% for the LDH release assay, the calcein release assay and the modified calcein retention assay, respectively.

E



Antibody dose response curves for the three cytotoxicity assays. Rituximab induced ADCC of Ramos cells with the calcein release assay (open triangles, 49% maximum cell death; EC50 3.7 ng/ml), LDH release assay (open circles, 58% maximum cell death; EC50 3.0 ng/ml) and the modified calcein retention assay (green, closed diamonds, 71% maximum cell death; EC50 2.3 ng/ml).

In A-C PBMC donor 4 was used, in D and E PBMC donor 14 was used.

Table 2. EC50 values of rituximab and trastuzumab-induced ADCC

Target cell	Antibody	Method	EC50 in ng/ml (95% CI)	Reference
Ramos	Rituximab	LDH release	16	Breton ea ¹⁴
Ramos	Rituximab	LDH release	10	Heider ea ¹³
Ramos	Rituximab	Not specified	9.6	Bornstein ea ⁷
Ramos	Rituximab	Reporter Bioassay	19	Cheng ea ⁸
Ramos	Rituximab	Modified calcein retention assay	1.1 (0.89-1.28)	This paper
Raji	Rituximab	Not specified	2.8	Bornstein ea ⁷
Raji	Rituximab	LDH release	12	Breton ea ¹⁴
Raji	Rituximab	Reporter Bioassay	19	Cheng ea ⁸
Raji	Rituximab	Cr release	16.2	Le Garff ea ¹⁵
Raji	Rituximab	Modified calcein retention assay	1.02 (0.70-1.54)	This paper
SK-BR-3	Trastuzumab	Reporter Bioassay	10	Murray ea ¹⁶
SK-BR-3	Trastuzumab	Reporter Bioassay	11	Cheng ea ⁸
SK-BR-3	Trastuzumab	Calcein retention	16	Prang ea ¹¹
SK-BR-3	Trastuzumab	Modified calcein retention assay	0.17 (0.13-0.22)	This paper

Frozen PBMC from donor 4 were used as effector cells.

95% CI: 95% confidence interval of the calculated EC50

We then compared the FACS calcein AM retention assay to the calcein release assay and the LDH release assay side by side in one experiment (Table 3). Spontaneous LDH release was high, resulting in a ~70% background signal in the LDH release assay. The calcein release assay had ~54% background signal whereas the calcein retention assay had a background signal of only 15%. The remaining window of 85% of the signal, which can be used for calculating specific lysis, makes the FACS calcein AM retention assay the most sensitive assay (figure 2D). In figure 2E the dose response curves of the three methods to quantify Rituximab induced ADCC of Ramos cells are shown. The FACS calcein AM retention assay yielded the lowest EC50 value, as well as the narrowest 95% EC50 confidence interval (CI) (2.3 ng/ml (CI 1.95-3.2) vs 3.7 (CI 0.84-16.43) and 3.0 (CI 1.45-6.38) ng/ml for the calcein release assay and the LDH release assay, respectively; see also Table 3). The maximum amount of cells killed varied between assays and the ability of Rituximab to induce ADCC was underestimated by 30% when measured with the LDH assay and by ~60% when measured with the calcein release assay.

Table 3. Direct comparison between assays

Target cell	Antibody	Method	EC50 in ng/ml	95% CI
Ramos	Rituximab	Calcein release	3.7	0.84-16.43
Ramos	Rituximab	LDH release	3.0	1.45-6.38
Ramos	Rituximab	Modified calcein retention assay	2.3	1.95-3.20

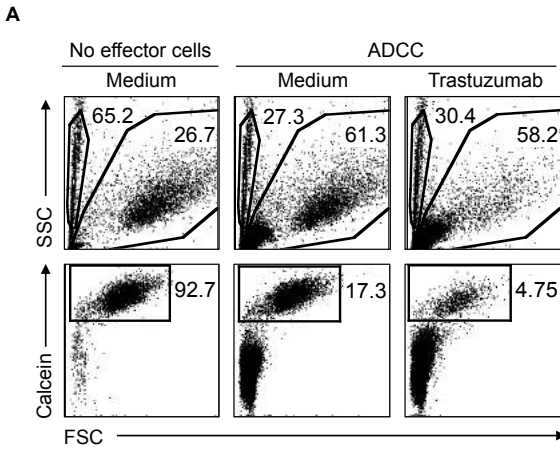
Frozen PBMCs from donor 14 were used as effector cells.

95% CI: 95% confidence interval of the calculated EC50

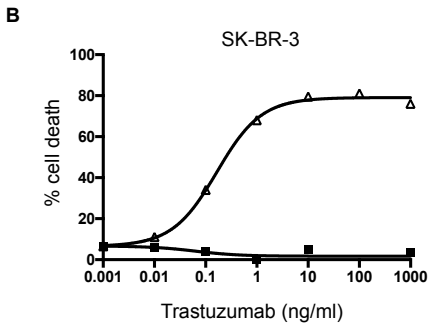
Antibody dependent cellular cytotoxicity on adherent cells

We then applied the modified FACS calcein AM retention assay to adherent cells using the breast cancer cell line SK-BR-3. In conventional cytotoxicity assays adherent cells are detached prior to the assay. To overcome detachment-related effects on cell viability, we plated adherent cells 3 days prior to the cytotoxicity assay on a Poly-D-Lysin coated plate. At the day of the cytotoxicity assay adherent cells were labeled with calcein AM, without detaching them. This approach resulted in the use of viable, calcein labeled cells in the assay (Figure 3A, left panels). Incubation of calcein labeled breast cancer SK-BR-3 cells with PBMC and trastuzumab resulted in death of target cells with a sigmoidal dose-response curve (Figure 3B). The EC50 for trastuzumab on SK-BR-3 cells was 0.17ng/ml (Table 2).

Figure 3. ADCC of adherent cells



Representative example of ADCC of the adherent breast cancer cell line SK-BR-3 by trastuzumab. Viable adherent cells can be labeled with calcein (left panels). Addition of effector cells and trastuzumab (1000 ng/ml) resulted in ADCC (right panels).



Dose-response curve of trastuzumab (open triangles) or control antibody (closed squares).

In all experiments PBMC from donor 4 were used.

Discussion

Established methods to determine the cytotoxicity of any given compound *in vitro* are negatively affected by non-specific signals caused by auto-fluorescence of target and effector cells and random cell death that leads to underestimations of the activity of the compound under study. To overcome this, we developed a novel cytotoxicity assay in which we combined and optimized established cell viability assays. This resulted in an exceptionally sensitive method that allows for single cell analysis by high throughput FACS. As shown in Table 1 (CDC) and Table 2 (ADCC), the EC₅₀ values obtained with this modified FACS calcein AM retention assay are considerably lower compared to published data.^{7,8,11-16} For example, using the chromium 51 (⁵¹Cr) release assay¹⁵ and the reporter-based ADCC bioluminescence assay (Promega)^{8,17} to measure ADCC of Raji cells by rituximab yielded an EC₅₀ of around 17 ng/mg (19 vs 16.2). The LDH release assay generated a somewhat lower EC₅₀ of 12 ng/ml¹⁴. In our hands, the calcein release assay and the LDH release assay yielded an EC₅₀ of 3.7 ng/ml and 3.0 ng/ml, respectively, while depending on the batch of effector cells used the modified FACS calcein AM retention assay produced an EC₅₀ of 1,2 to 2.3 ng/ml (Table 3). The high sensitivity of our assay is the result of a much higher accuracy of compound-specific cell viability analysis as the assay focuses on live cells instead of dead cells. Spontaneous release of enzymes or radioactive isotopes does not interfere with the analysis and background signals are thereby compensated for.

The modified calcein retention assay has other advantages. With its focus on live cells, it allows for less strict timing of analysis as it takes into account all mechanism of cell death (e.g. apoptosis, necroptosis, oncosis). It should be noted that very early apoptotic cells are not detected with any of these methods, as early in the apoptotic pathway the cell membrane is intact and Cr⁵¹, calcein and LDH are contained in the cell. This can be overcome by co-staining with an early apoptotic marker like Annexin V, which stains the flipped phosphatidylserine. Furthermore, for laboratories that have a flow cytometer available the assay is relatively cheap compared to commercially available kits, the read-out is simple, and the assay is safe, as it does not employ toxic or radioactive materials.

EC₅₀ values obtained with our assay were highly reproducible with less than 0.1 ng/ml variation, but only when the same batch of PBMCs was used. The effector cell function of PBMC is to a large extent determined by the percentage of NK cells present and their cytotoxic capacity, which is amongst others dependent on the CD16 FcγRIIIa receptor polymorphism. CD16 Fc receptors with a valine at position 158 have a higher affinity for IgG compared to

CD16 Fc receptors with a phenylalanine at that position.^{18,19} This may result in more efficient effector function of NK cells, however, it is not the only determinant of effector function. Both batches of PBMC that we used had the same FcγRIIIa receptor polymorphism but were isolated at different time-points and showed different effector function. It has been demonstrated that blood processing can impact on NK cell activation and function, and this may add to the variability of NK cell function in ADCC assays.²⁰ Thus, the inter-donor variation for effector cells is large, and PBMC should be tested for their ADCC potency before new compounds are investigated. With the use of CD16 expressing NK cell lines more stable and comparable results may be obtained.²¹

Taken together, we have developed an extremely sensitive, reproducible, high throughput flow cytometer based method to determine cellular cytotoxicity, which can be applied to test the cytotoxicity of compounds *in vitro*.

Acknowledgements

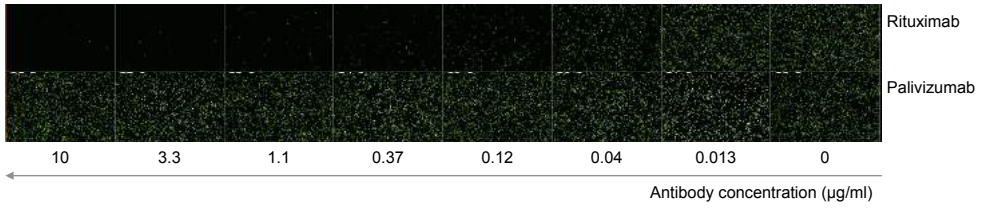
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Supplemental Material

Supplementary Figure 1. Visualization of CDC on Ramos cells



Representative example of CDC with calcein labeled Ramos target cells visualized with a high-content imager (Operetta, PerkinElmer; 10x objective). Rituximab was added at different concentrations as indicated. Loss of green fluorescent cells is indicative for loss of cell viability. Palivizumab was used as negative control.