# A high-throughput image cytometry-based screening method for the detection of IL2-induced peripheral blood mononuclear cell-mediated cytotoxicity

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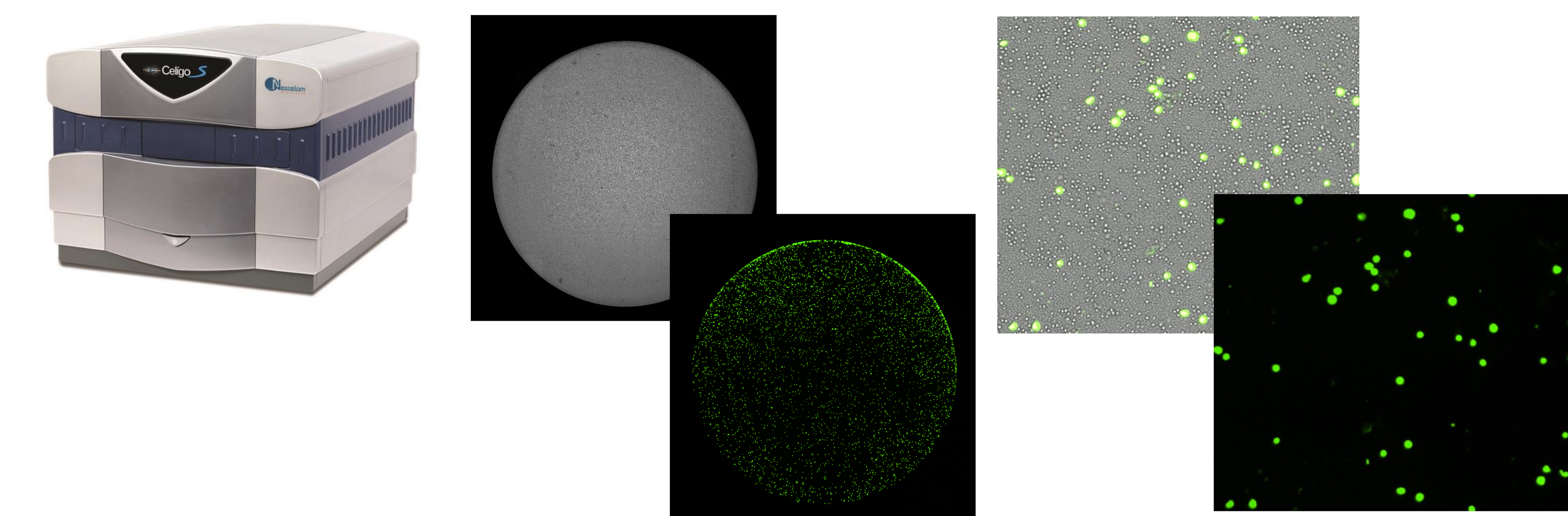
## 1. ABSTRACT

Cell-mediated cytotoxicity assays have been an important functional test for investigating the cytotoxic effect of immune effector on target cancer cells. Cytotoxicity assays have traditionally been performed using the $^{51}$Chromium ($^{51}$Cr) release assay, which involves labeling the tumor cells (target) with radioisotopes. When the target cells are lysed by the immune cells (effector), they release the entrapped radioactive $^{51}$Chromium, which is measured to determine the level cytotoxicity induced by the effector cells. The $^{51}$Chromium release assay is highly hazardous, time-consuming, and can only acquire end point readout. Furthermore, it can generate inconsistent results due to batch variation, ability of the target cell for $^{51}$Cr uptake, and low sensitivity. Above all, this assay is challenging for new investigators due to the difficulty of harvesting equal volume of supernatant from each well without collecting single target cell. In this work, we demonstrate a novel high-throughput cytotoxicity screening assay using the Celigo imaging-cytometry method. First the live K562 target cells were stained with Calcein AM, and were co-cultured with PBMCs from two healthy donors in a flat- bottom 96-well plate in the presence or absence of IL-2. Direct cell counting of Calcein positive cells was implied to calculate the changes in number of live target cells in the same well from T = 0 to T = 4 hours. Thus, accurate cell-mediated killing was determined using the "self-referencing" calculation, which could be a more direct method for assessing cytotoxicity than $^{51}$Cr release. The donor PBMCs were added to each well with Calcein-stained target cells at Effector-to-Target (E:T) ratios of 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. The 96-well microplate was then scanned and analyzed using the Celigo image cytometer at T = 0, 1, 2, 3, and 4 hours to measure the % lysis of target cell. Although it could be argued that release of a dye may not actually represent cytotoxicity by effector cells, nonetheless much like $^{51}$Cr release assay percent cytotoxicity was directly proportionate to:- the duration of the assay, E:T ratio used, and amount of activating cytokine present in the media, all as expected. Further, when PBMCs from both normal donors showed near equal level of cytotoxicity at 4hr; upon calculation of the NK cell lytic unit, it was revealed that one donor possessed much higher level of effector function than the other. The proposed image cytometry method can scan and analyze the entire well area of the entire 96-well plate in 7 min, which can be utilized to perform high-throughput screening of potential antibody- or chemical-based cancer drug, which could be a more efficient method for academic, industry, and clinical research.

## 2. CELIGO IMAGING CYTOMETRY FOR DIRECT CELL COUNTING ADCC ASSAY



1. Plate-based image cytometer that can scan the entire well of standard microplates and captures bright-field and fluorescent images
2. The captured images are analyzed with the Celigo software to measure size, morphology, cell count, confluence, and fluorescent intensity
3. The measured parameters are used to generate cell proliferation kinetic data, GFP/RFP expression, tumor spheroid size change, DNA cell cycle analysis, apoptosis, and ADCC cytotoxicity results

## 3. TRADITIONAL CELL-MEDIATED CYTOTOXICITY DETECTION METHODS

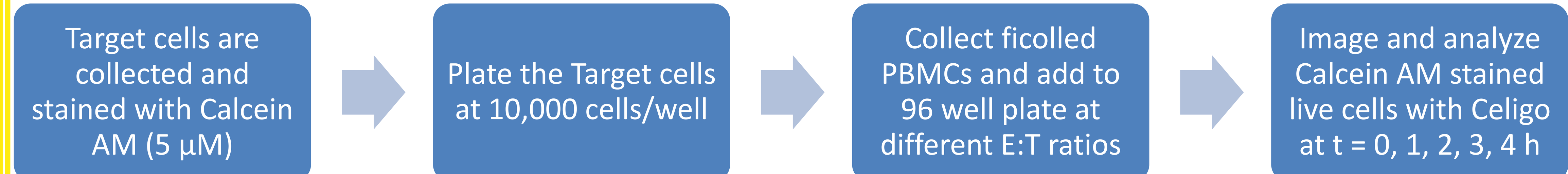
| Detection Method | Description | Existing Issues |
|---|---|---|
| Radioactvitiy Release | Measure release or radiolabels, $^{51}$Cr, $^{101}$In in the supernatant | Handling hazardous material and indirect measurement of cell death |
| Fluorescence Release | Measure release of Calcein AM fluorescent molecules in the supernatant | Indirect measurement of cell death, endpoint assay only |
| LDH Release | Measure release of cytosolic enzyme in the supernatant | Indirect measurement of cell death, endpoint assay only |
| Luciferase Reporter Assay | Measure luciferase as the cells die | Indirect measurement of cell death |
| Flow Cytometry | Measure number of viable cells and viability in the sample | Cannot perform in plates, must trypsinize for adherent cells |

## 4. NATURAL KILLER CELL-MEDIATED CYTOTOXICITY DETECTION METHOD

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 50:1 w/o IL2 | | | | | | 50:1 w/ IL2 | | | | | |
| B | 25:1 w/o IL2 | | | | | | 25:1 w/ IL2 | | | | | |
| C | 12.5:1 w/o IL2 | | | | | | 12.5:1 w/ IL2 | | | | | |
| D | 6:1 w/o IL2 | | | | | | 6:1 w/ IL2 | | | | | |
| E | 3:1 w/o IL2 | | | | | | 3:1 w/ IL2 | | | | | |
| F | Donor 1 | | | Donor 2 | | | Donor 1 | | | Donor 2 | | |
| G | Spontaneous Release | | | | | | Spontaneous Release | | | | | |
| H | Maximum Release | | | | | | Maximum Release | | | | | |

- K562 (Suspension) target cells are used to demonstrate the PBMC-mediated cytotoxicity detection method using Calcein AM staining for direct cell counting
- The Effector-to-Target (E:T) ratios are 50:1, 25:1, 12.5:1, 6:1, and 3:1 in the presence or absence of IL2
- Live Target cells are automatically counted at each E:T ratio from t = 0 – 4 hour
- Cytotoxicity potential of the PBMCs from Donor 1 and 2 are compared

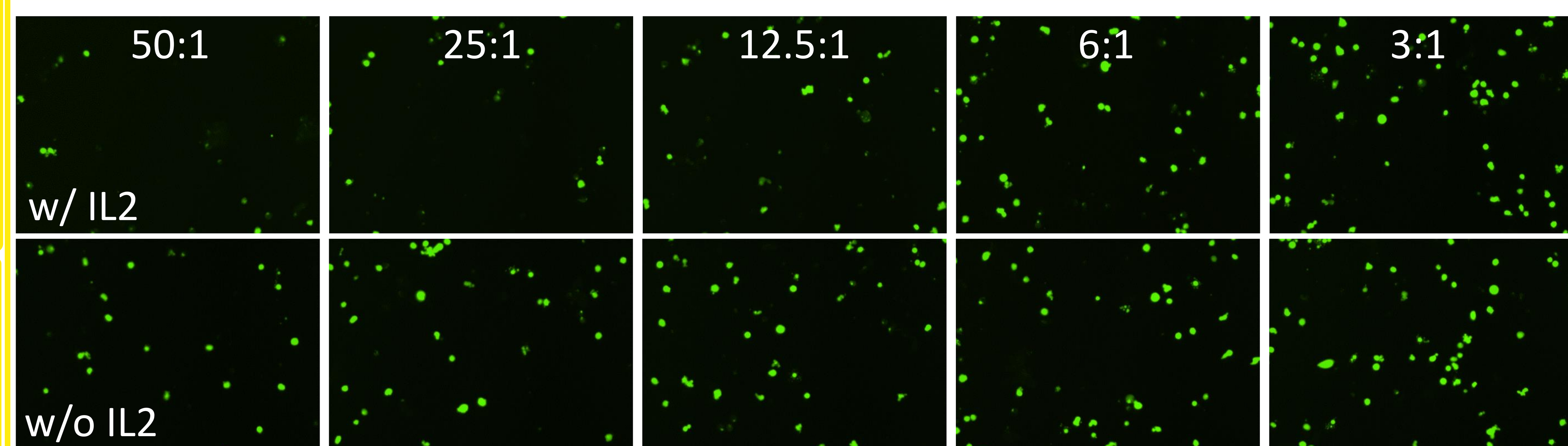
## 5. CELIGO IMAGING CYTOMETRY EXPERIMENTAL PROTOCOL



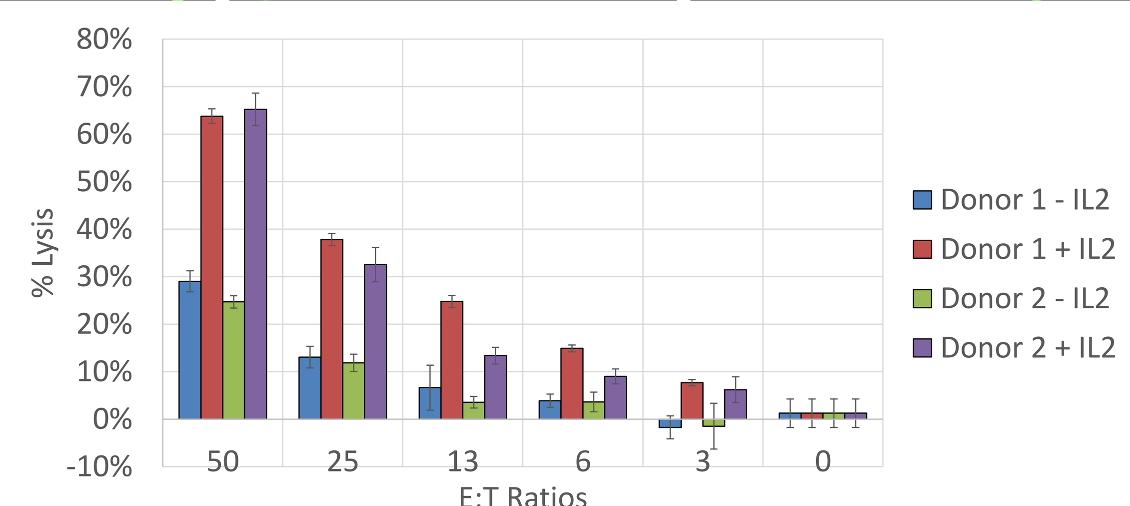

**% Lysis Calculation for Cytotoxicity Measurement**

- Count # of live Target cells (Calcein AM positive) in wells with Effector cells at t = 0, 1, 2, 3, and 4 h
- Count # of live Target cells (Calcein AM positive) in wells without Effector cells (control)
- $\%\ Lysis = 1 - \frac{\#\,Target\,(t=0) - \#\,Target\,(t=x)}{\#\,Target\,(t=0)} - \frac{\#\,Target\,(t=0) - \#\,Target\,(t=x,control)}{\#\,Target\,(t=0)} \times 100$

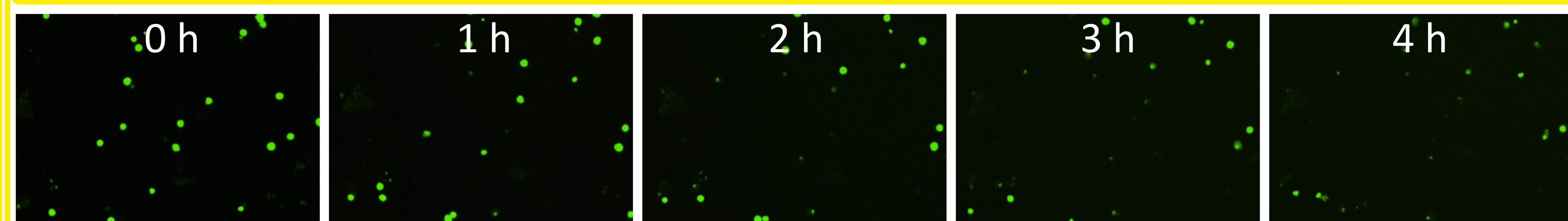
## 6. E:T RATIO DEPENDENT CYTOTOXICITY RESULTS

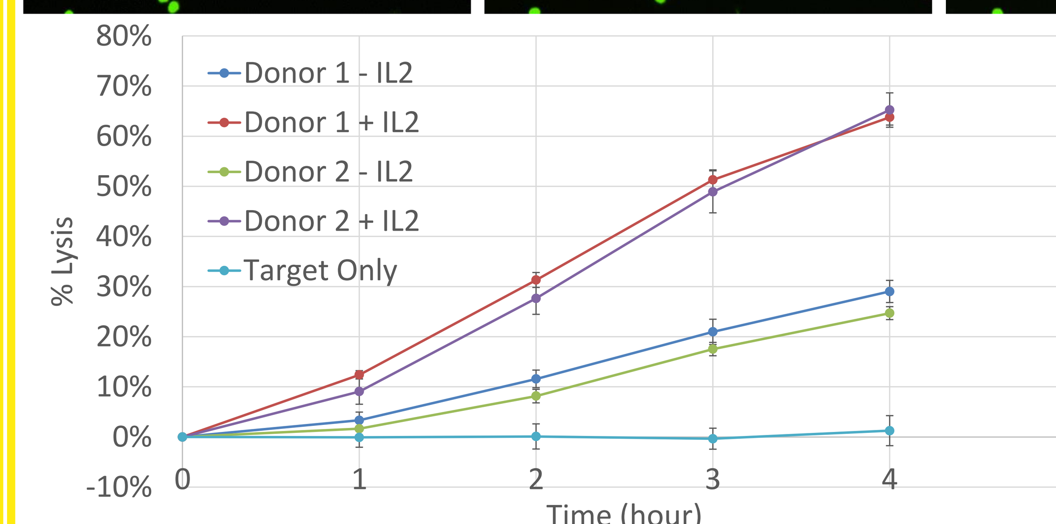



- The example Calcein AM fluorescent images are the K562 Target cells at t = 4 hours
- The resulting fluorescent images showed increase in Calcein AM positive Target cells as E:T ratio decreased
- The addition of IL2 also showed a significant killing of target cells
- It is also shown that Donor 1 has a slightly higher cytolytic potential compared to Donor 2




## 7. TIME DEPENDENT CYTOTOXICITY RESULTS




- Time-course tracking of % lysis can eliminate the need of multiple controls, and the effect of non-uniform cell seeding in the final cytotoxicity calculation
- Adherent cells can be measured and analyzed directly in the plate without trypsinization
- The number of cells used are significantly less than the cells needed for Release assays and Flow Cytometry