Cell-Mediated Cytotoxicity Assay by High-Throughput Direct Cell Counting in Microplates using Fluorescence-Based Image Cytometry

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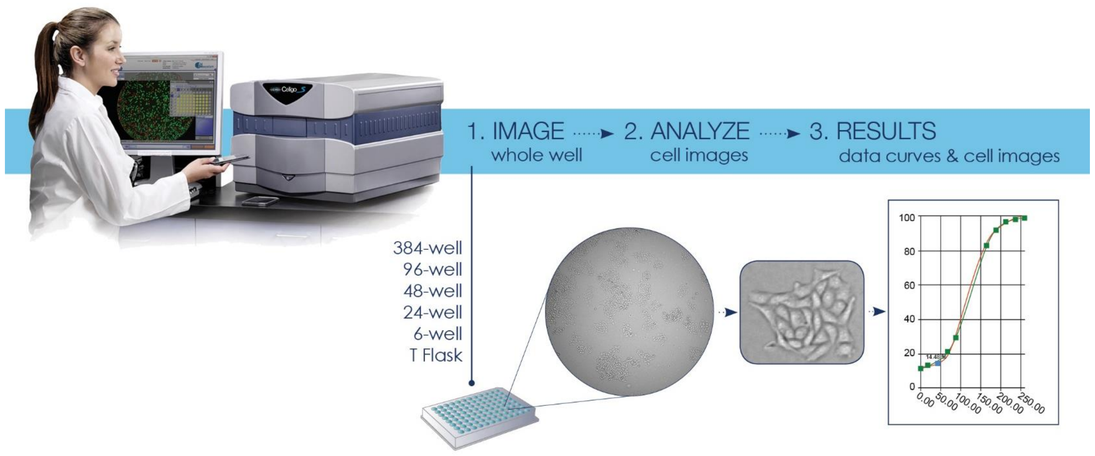
1. ABSTRACT

Cytotoxicity assays play a central role in studying the function of immune effector cells such as cytolytic T lymphocytes (CTL) and natural killer (NK) cells. Traditionally, cytotoxicity assays have been performed using ^{51}Chromium (^{51}Cr) and Calcein release assays. The assays involve labeling tumor cells (target) with radioisotope or fluorescent dyes, when the target cells are subjected to cytolysis by CTLs or NK cells (effector), they releases the entrapped labels into the media upon lysis. The amount of labels in the media is measured to determine the level of cytotoxicity the effectors have induced. These traditional methods may generate inconsistent results due to low sensitivity caused by poor loading efficiency and high spontaneous release of the reagents. In addition, measuring radioactivity or fluorescent labels released in supernatant is an indirect method for analysis. In this work, we demonstrate a novel high-throughput cytotoxicity detection assay using the Celigo imaging cytometry method. Utilizing imaging cytometry, direct cell counting of live fluorescent target cells can be performed, which is a direct method for assessment of cytotoxicity. Human NK cells from one healthy donor were used as effectors, and K562 (suspension) and IMR32 (adherent) were used as the target cells. Both target cells were first stained with Calcein AM, and seeded at 10,000 cells/well in a standard 96-well microplate. The donor NK cells were then added to each well at Effector-to-Target (E:T) ratios 10:1, 5:1, 2.5:1, 1.25:1, 0.625:1, and 0.3125:1. The 96 well plate was then scanned and analyzed using Celigo imaging cytometer at t = 1, 2, 3, and 4 h to measure the % lysis of target cells. The results showed increasing % lysis as incubation time and E:T ratio increased. The propose Celigo imaging cytometry is an accurate and simple method for direct quantification of cytotoxicity, which can be an attractive method for both academic and clinical research.

2. CELIGO IMAGING CYTOMETRY FOR DIRECT CELL COUNTING ADCC ASSAY



1. Celigo Imaging Cytometer is a plate-based 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
LDH Release	Measure release of cytosolic enzyme in the supernatant	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

E:T	1	2	3	4	5	6	7	8	9	10	11	12
A	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1
B	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1
C	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1
D	IMR32						K562					
E												
F	Maximum Release Control						Maximum Release Control					
G												
H	Spontaneous Release Control						Spontaneous Release Control					

- IMR32 (Adherent) and K562 (Suspension) target cells are used to demonstrate the NK Cell-mediated cytotoxicity detection method using Calcein AM staining for direct cell counting
- The Effector-to-Target (E:T) ratio will be 10:1, 5:1, 2.5:1, 1.3:1, 0.6:1, and 0.3:1
- The Maximum Release uses Triton X100 to lyse all cells and release the Calcein AM fluorescent molecules
- Live Target cells are automatically counted at each E:T ratio from t = 1 - 4

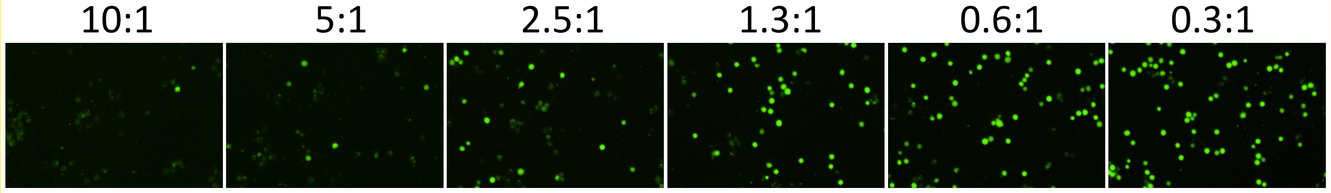
5. CELIGO IMAGING CYTOMETRY EXPERIMENTAL PROTOCOL

Target cells are collected and stained with Calcein AM (10 µM) → Plate the Target cells at 10,000 cells/well → Collect Effector NK cells and add to 96 well plate at different E:T ratios → Image and analyze Calcein AM stained live cells with Celigo at t = 1, 2, 3, 4 h

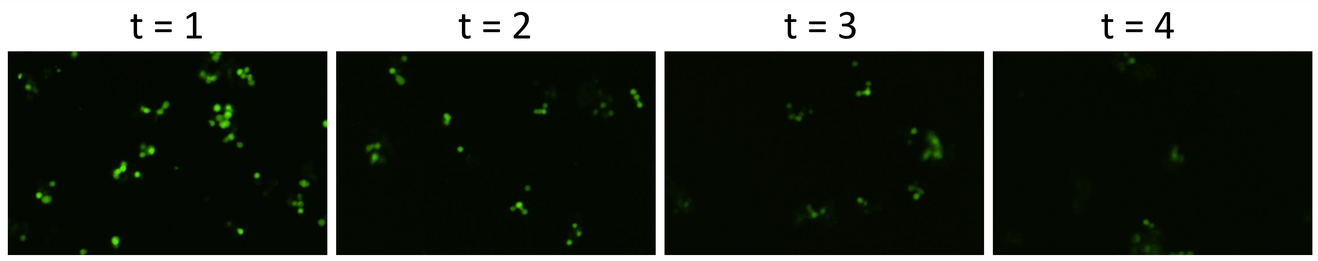
% Lysis Calculation for Cytotoxicity Measurement

- Count # of live Target cells (Calcein AM positive) in wells with Effector cells
- Count # of live Target cells (Calcein AM positive) in wells without Effector cells (control)
- $\%\ Lysis = 1 - \frac{\#\ Calcein\ AM\ Target\ cells\ with\ Effector\ cells}{\#\ Calcein\ AM\ Target\ cells\ without\ Effector\ cells} \times 100$

6. E:T RATIO AND TIME DEPENDENT CYTOTOXICITY FLUORESCENT IMAGES

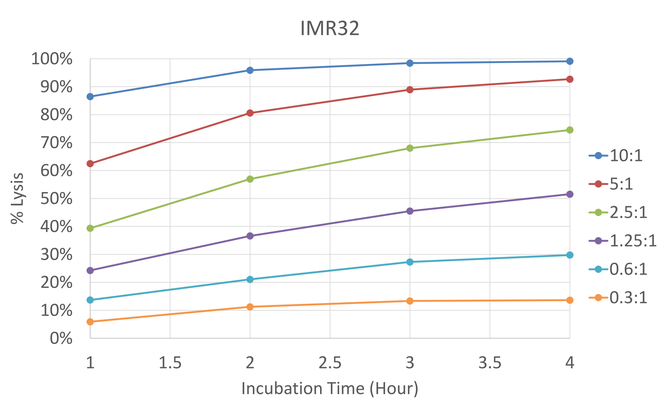


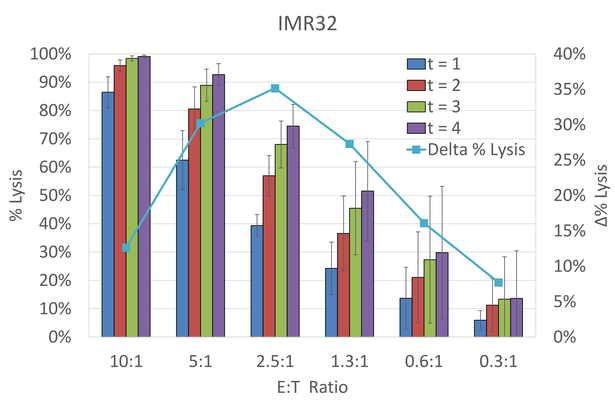
- The example Calcein AM fluorescent images are the K562 Target cells at t = 4 hours, the IMR32 images are similar
- The resulting fluorescent images showed increase in Calcein AM positive Target cells as E:T ratio decreased



- The example Calcein AM fluorescent images are the IMR32 Target cells at E:T = 2.5:1, the K562 images are similar
- The resulting fluorescent images showed decrease in Calcein AM positive Target cells as time increased

7. E:T RATIO AND 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