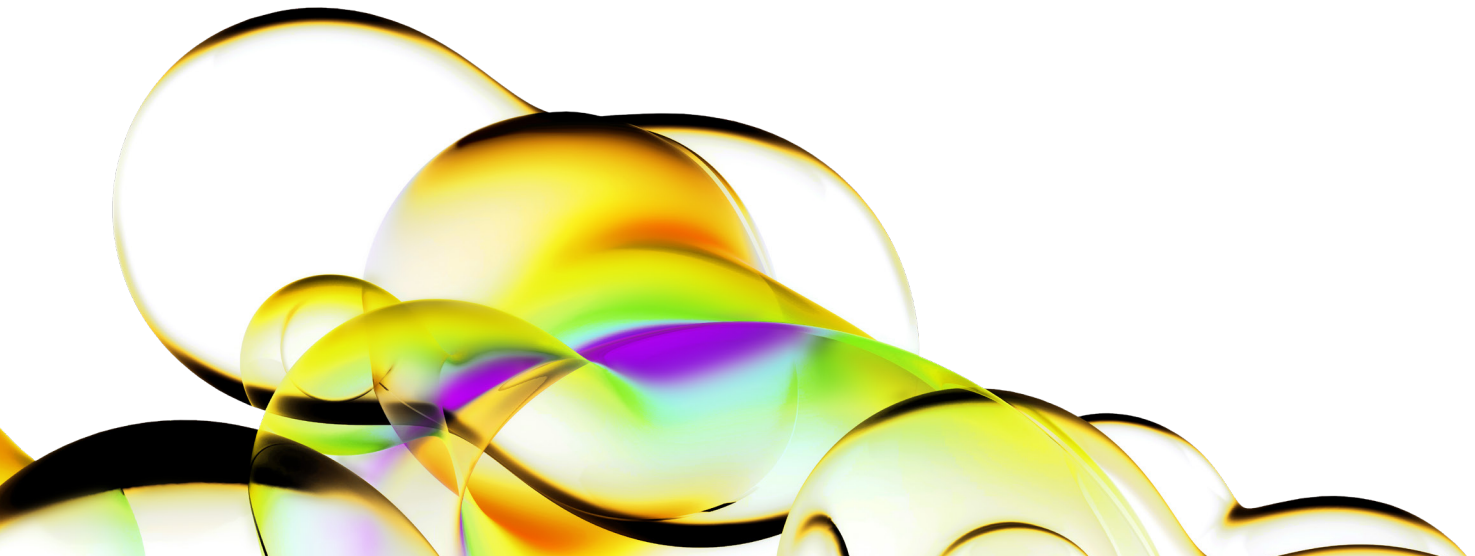
APPLICATION NOTE

Direct cell counting assays for immunotherapy.

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 Chromium-51 (^{51}Cr) and Calcein release assays.

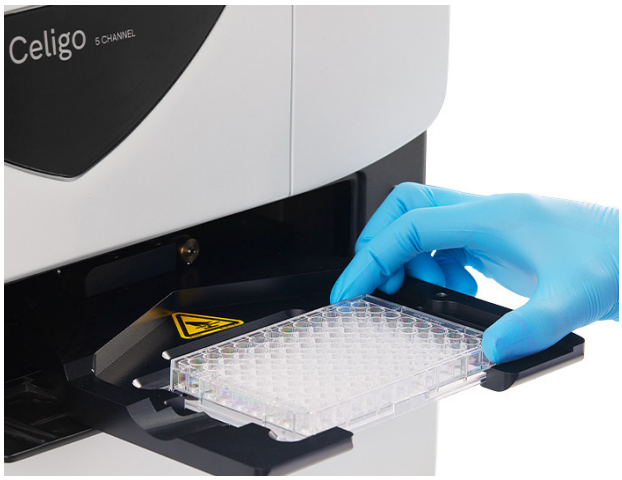
The assays involve labeling tumor cells (target) with radioisotope or fluorescent dyes, when the target cells are subjected to CTLs or NK cells (effector) mediated killing, they release the entrapped labels into the media. The amount of released label in the media is measured to determine the level of cytotoxicity the effectors have induced.

A novel cytotoxicity assay using the Celigo™ image cytometer to directly count live, fluorescently labeled target cells has recently been introduced.



The Celigo image cytometry system for direct cell counting ADCC assay

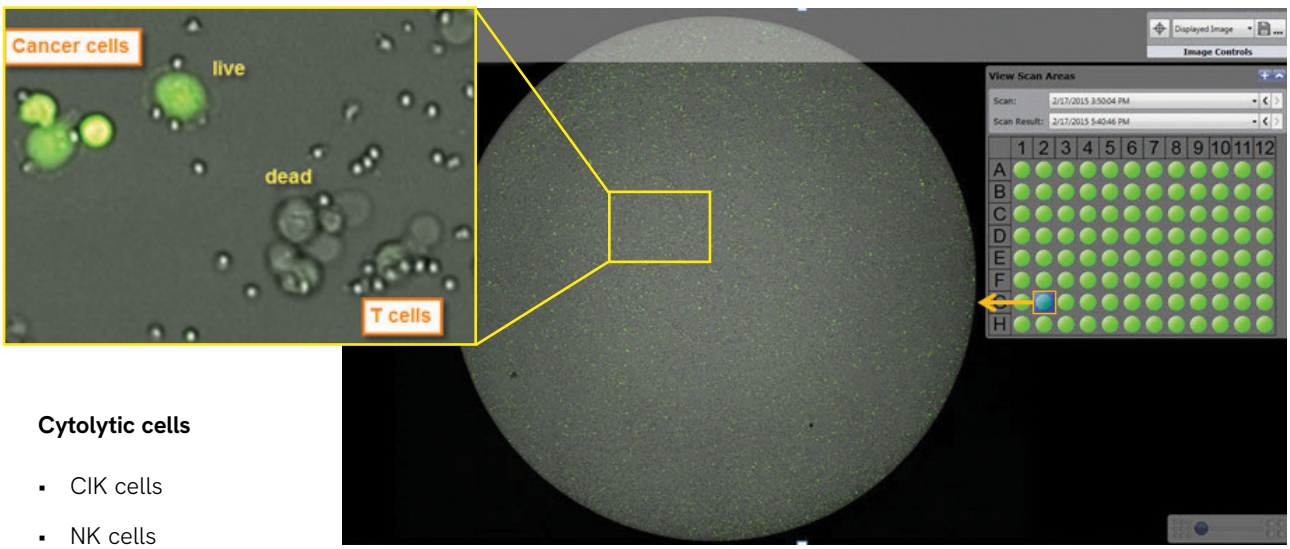
1. The Celigo image cytometer is a plate-based cytometer that can scan the entire well of standard microplates and captures brightfield and fluorescent images.
2. The captured images are analyzed with the Celigo software to measure size, morphology, cell count, confluence, and fluorescent intensity.
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.



Direct cell counting method using Calcein AM

- Use the Celigo to capture and analyze brightfield and green fluorescent images
- Target suspension and adherent cells are stained with Calcein AM and then mixed with the Effector cells
- The number of Target cells (Calcein+) are counted and monitored over time
- Reduction in Target cell number indicates cell-mediated or antibody- dependent cell-mediated cytotoxicity (ADCC)

4. The Celigo image cytometer produces whole well images for 96-, 384-well plates.



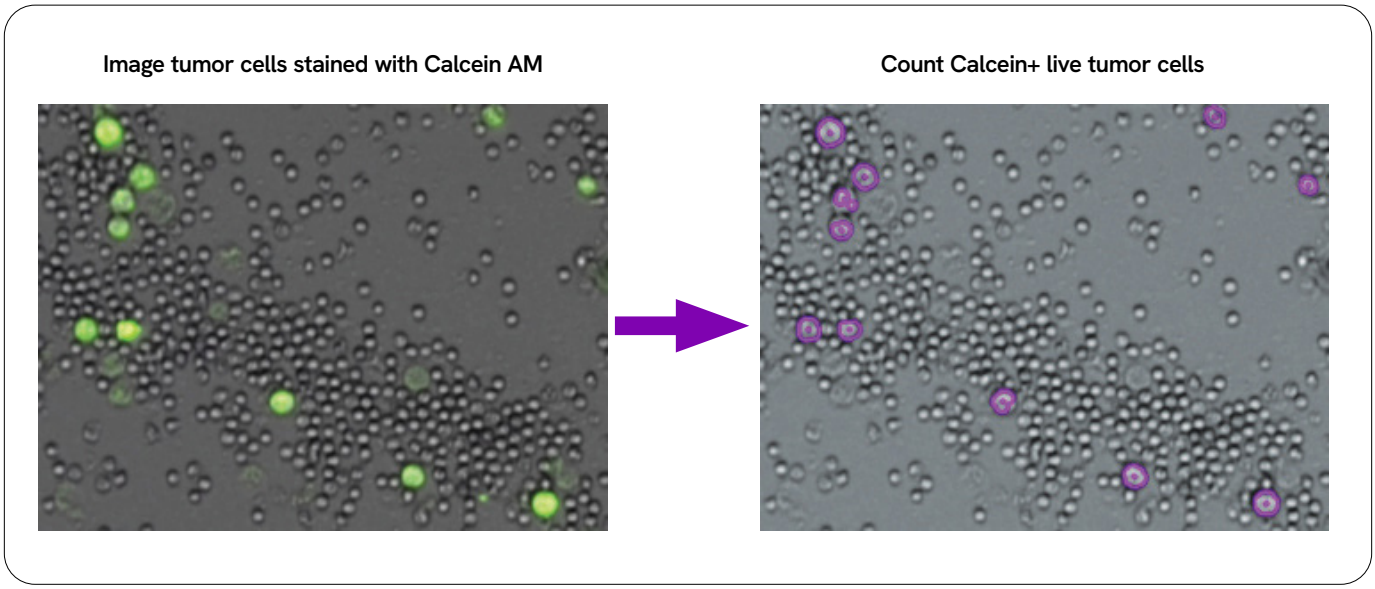
Cytolytic cells

- CIK cells
- NK cells
- Neutrophils
- CAR T cells

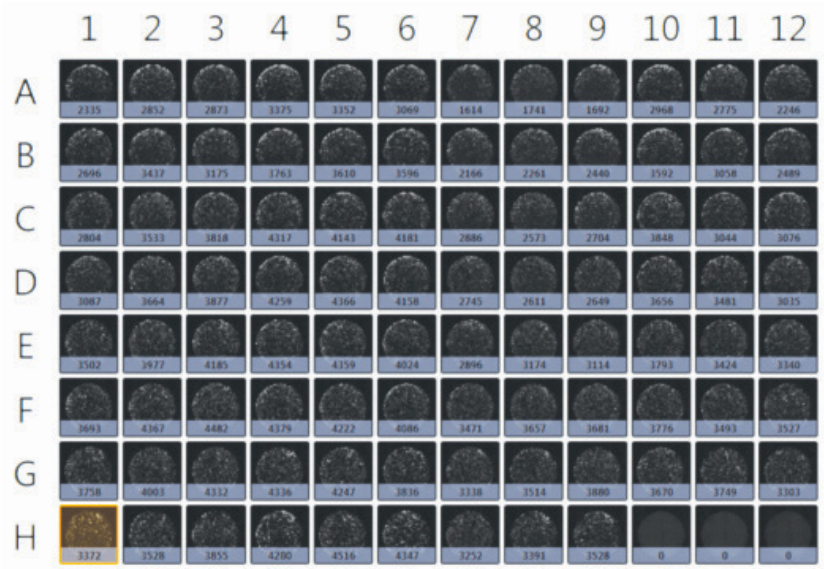
Fluorescent stains

- Fluorescent proteins - GFP, RFP
- Calcein AM
- Cell tracer dyes CFSE
- CellTrace™ dyes
- Viability dyes - PI, DAPI

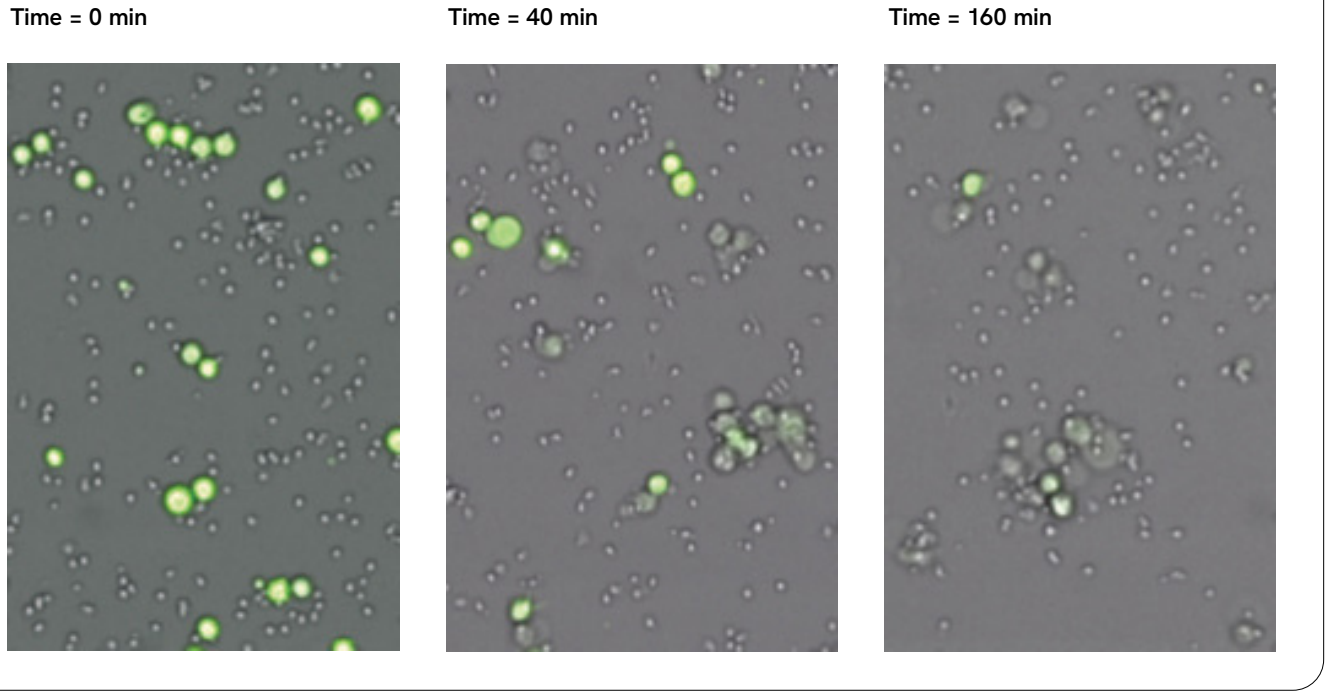
5. Cell images are used by the Celigo software to count live tumor cells for each well.



6. Direct cell counts for each well are reported in the plate map format



7. Each plate can be imaged multiple times during the killing process Time

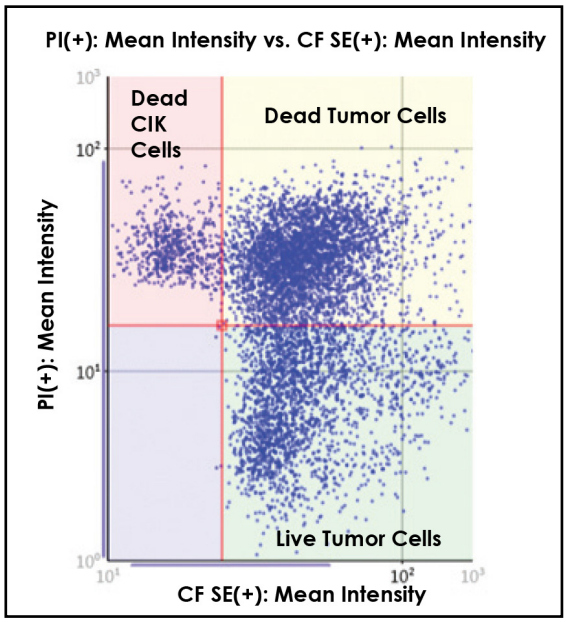


8. Numerical data output into .CSV file with plate level readout.

Live count	1	2	3	4	5	6	7	8	9	10	11	12
A	27	214	883	1454	2094	2895	1234	2040	2702	3400	4158	4856
B	69	548	1405	2535	3197	3342	2097	3223	4608	6457	7123	6857
C	92	431	1297	2272	3303	3729	2173	3418	4764	6273	7158	8091
D												
E												
F	0	0	0	0	0	0	0	1	0	0	0	0
G												
H	3178	3442	4034	4056	3855	4119	9609	9458	9855	9836	9407	9418

9. Total cell count, live cell count, % live cell count and other parameters are generated for each well

General	
Scan area location:	**C2**
% Live:	3.14%
Live count:	1954
Total count:	62203
% Well sampled:	100.00%
AVG live mean intensity:	105.22
SD of live mean intensity:	28.36
AVG live integrated intensity:	15,178.03
SD of live integrated intensity:	11,016.99
AVG total mean intensity:	113.75
SD of total mean intensity:	22.25
AVG total integrated intensity:	3,897.03
SD of total integrated intensity:	3,812.26



Quantification of natural killer cell-mediated cytotoxicity over 4 hours with multiple time point measurement

The Celigo experimental protocol

Target cells arecollected 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

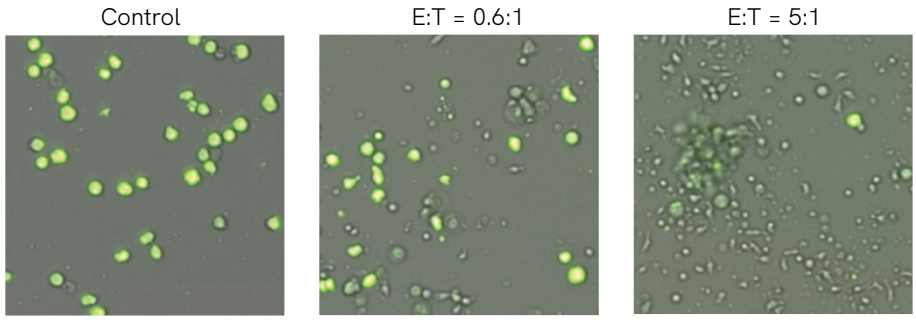
- Count # of live Target cells (Calcein AM positive) in wells with Effector cells
- Count # of live Target cells (Calcein AM positive) in wells without Effector cells (control)

$$\%\ \text{Lysis} = \left[\frac{n_{CAM^+\_t0} - n_{CAM^+\_t}}{n_{CAM^+\_t0}}\right]_{treated} - \left[\frac{n_{CAM^+\_t0} - n_{CAM^+\_t}}{n_{CAM^+\_t0}}\right]_{control}$$

The Celigo experimental protocol

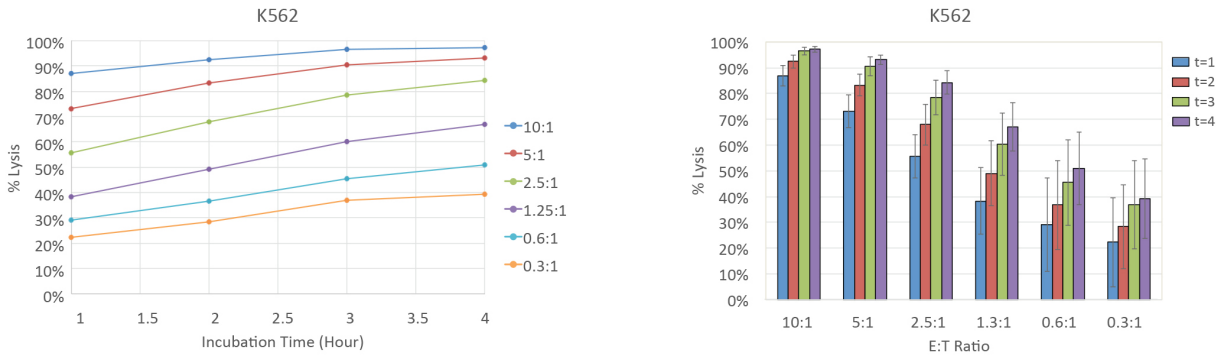
E:T	1	2	3	4	5	6	7	8	9	10	11	12
A	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1
B	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1
C	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1	10:1	5:1	2.5:1	1.3:1	0.6:1	0.3:1
D												
E	IMR32						K562					
F	Maximum Release Control						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 hours
- Cell images at 4 hours for K562 cells

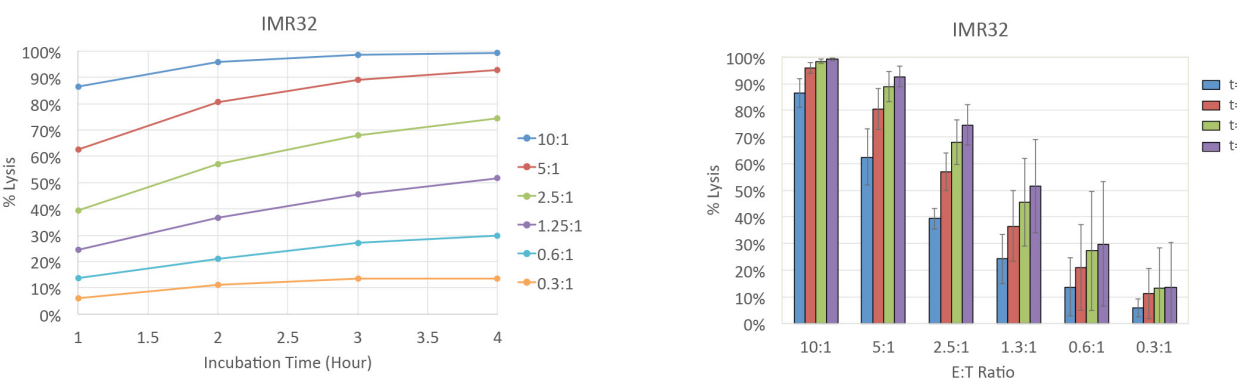


E:T Ratio and time dependant cytotoxicity of K562

- The example Calcein+ 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



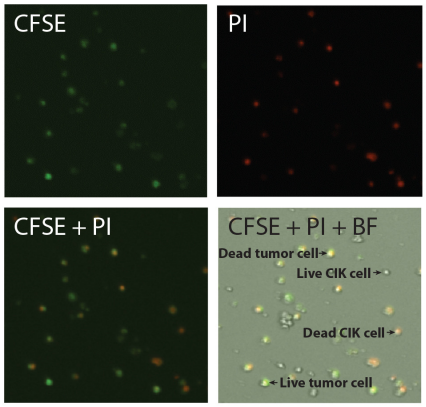
- The live K562 suspension cells were directly measured using the Celigo
- The number of Calcein AM positive cells was counted at each hour and used to calculate the % lysis for each E:T Ratio
- By analyzing the time course data of K562, we can see that the % lysis difference between the E:T Ratios is much smaller in comparison to IMR32

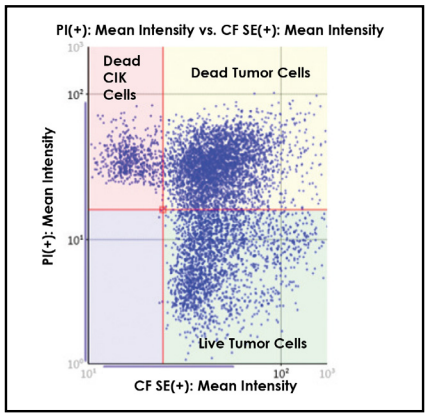


- The live IMR32 adherent cells were directly measured using the Celigo without trypsinization
- The number of Calcein AM positive cells was counted at each hour and used to calculate the % lysis for each E:T Ratio
- By analyzing the time course data of IMR32, we can see that there is a large % lysis difference between the E:T Ratio

Antibody-dependent CIK-mediated cytotoxicity using CFSE and PI

Fluorescent and brightfield cell images and overlay for CFSE and PI

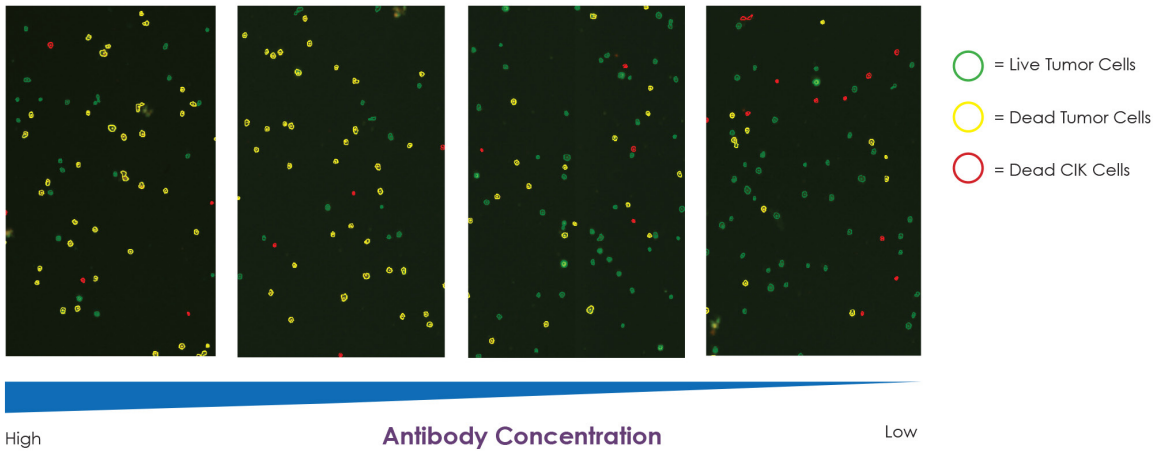




- Live tumor cells (CFSE+PI-)
- Dead tumor cells (CFSE+PI+)
- Live Effector cells (CFSE-PI-)
- Dead Effector cells (CFSE-PI+)

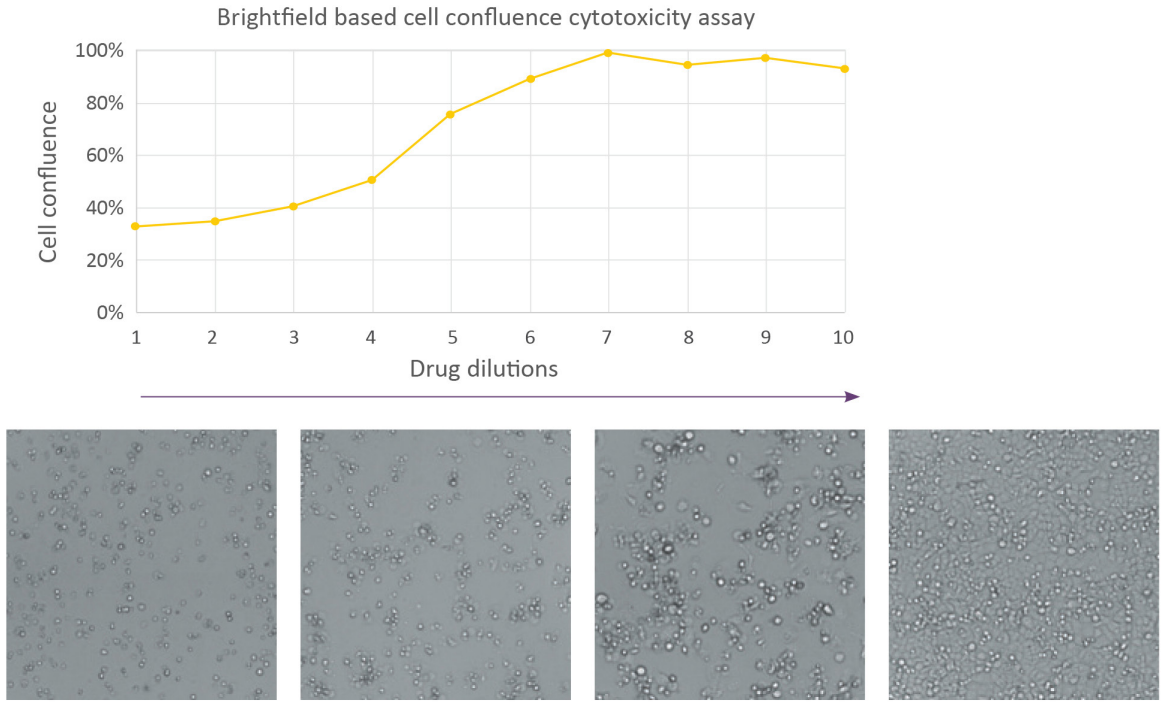
- The fluorescent intensities of CFSE and PI are plotted directly in the Celigo Software
- Quadrant LR indicates the live tumor cells
- Quadrant UR indicates the dead tumor cells
- Quadrant UL indicates the dead CIK effector cells

Antibody concentration dependent cytotoxicity



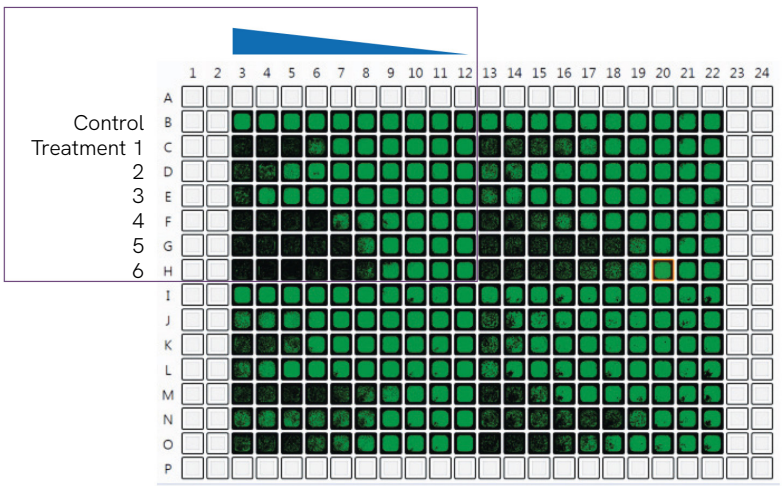
Cytotoxicity assay using brightfield morphological confluence assay

Direct cell killing effect with drug concentration curve



- The captured images show an increase of cell confluence with increased drug dilution.
- The Celigo software produces cell growth curves for each drug treated sample. (not shown here)

Confluence measurements for a multi-drug cytotoxicity assay



Drug treatment, concentration dependent cytotoxicity is represented by well level confluence measurement in a 384-well plate.

Green color indicates the level of confluence per well.

Traditional methods vs. natural killer - cell-mediated cytotoxicity detection methods

Traditional cell-mediated cytotoxicity detection methods

Detection method	Description	Existing issues
Radioactivity Release	Measure release or radiolabels, ^{51}Cr, 101In 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, end point assay only
LDH Release	Measure release of cytosolic enzyme in the supernatant	Indirect measurement of cell death, end point assay only
Luciferase Reporter Assay	Measure luciferase as the cells die	Indirect measurement of cell death
Flow Cytometery	Measure number of viable cells and viability in the sample	Cannot perform in plates, must trypsinize for adherebt cells

The Celigo benefits for direct cell counting ADCC

- 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 is significantly reduced compared to other release assays and Flow Cytometry, from 100,000 to 10,000 target cells.
- The visual observation of Cell-Mediated, ADCC, or CDC effect on tumor cells provides additional information on the functionality of antibodies or complements.

The Celigo assays for immuno-oncology

- **Direct cell counting and confluence measurement using brightfield**
  - Antibody-mediated cell killing (cell proliferation)
- **Direct cell counting method using Calcein AM (or GFP, RFP)**
  - NK cell-mediated cytotoxicity
  - NK ADCC
  - Neutrophil ADCC
  - CDC
- **Direct cell counting method using tracer dye, fluorescent protein, viability dye**
  - CIK ADCC
  - CAR T cell-mediated cytotoxicity