Assay Name: NK cell-mediated cytotoxicity using calcein AM

Assay ID: Celigo_01_0001

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Experiment: NK cell-mediated cytotoxicity using calcein AM

Purpose	Measure NK cell-mediated cytotoxicity using calcein AM-stained K562 and IMR32 cells
Current Method(s)	Calcein Release Assay, Flow Cytometry
Target Cell Type	Target: K562, IMR32; Effector: NK cells
Experiment Plan	Scan plate using Bright Field and Green Fluorescent channels
Hypothesis	By measuring the changes in the number of calcein AM-positive cells over time, the % cytotoxicity can be calculated using time 0 and control for normalization

Celigo Setup

Plate Type	Greiner 655090 96-well black wall clear bottom
Scan Channels	Bright field and Green
Resolution	1 µm/pixel
Scan Area	Whole well
Analysis Method	Target 1 + 2
Scan Frequency	Hourly, up to 4 hours
Scan Time	~6 min

Assay Protocol and Plate Setup

Goal

Measure NK cell-mediated cytotoxicity using calcein AM-stained K562 and IMR32 for a duration of 4 hours.

Protocol

Cell preparation

1. K562 and IMR32 were obtained from ATCC and cultured in RPMI 1640 media
2. Donor NK cells were obtained from buffy coat of PBMCs and expanded by co-culturing with irradiated (100Gy) K562 Clone9.mbIL21 and supplemented with 50 IU/ml IL2
3. The K562 and IMR32 Target cells were stained with 10 µM of calcein AM (Nexcelom, Cat# CS1-0119) for 30 min and then washed 3 times with RPMI media
4. The cells were then seeded into a 96-well plate at 10,000 cells/well
5. Next, the Effector NK cells were added following the E:T ratios on the plate map below
    a. The spontaneous release samples were stained Target cells without Effector cells
    b. The maximum release samples were stained Target cells with Triton X-100

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 (Triton X-100)						Maximum Release Control (Triton X-100)					
G												
H	Spontaneous Release Control (Target cells only)						Spontaneous Release Control (Target cells only)					

Data Collection

1. After adding the Target and Effector cells, the plate was centrifuged to settle the cells to the bottom
2. Immediately after, the plate was scanned in Celigo using Target 1 (Green) + 2 (BF) for t = 0 h
3. Repeated the scanning for t = 1, 2, 3, and 4 h

Data Analysis

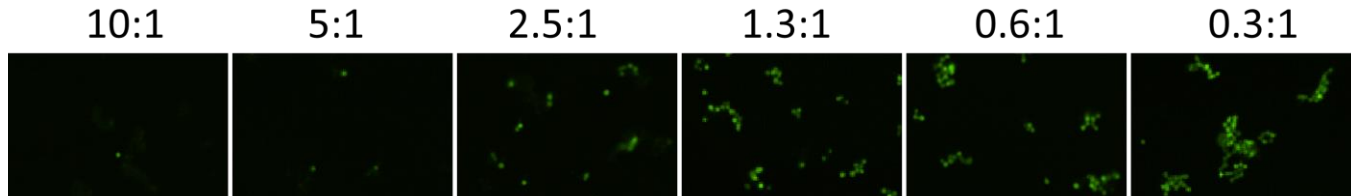
- The images for each time point were analyzed to count total number of calcein AM-positive cells in each well
- The segmentation parameters in Analyze were setup using the calcein AM fluorescent images acquired at the last time point (t = 4 h)
    - The parameters were applied to the other time points for counting calcein AM-positive cells
- Made sure only the bright calcein AM-positive cells were counted
    - The parameters were setup to not count pieces of bright debris
    - The parameters were setup to not count dim cells

Data Calculation

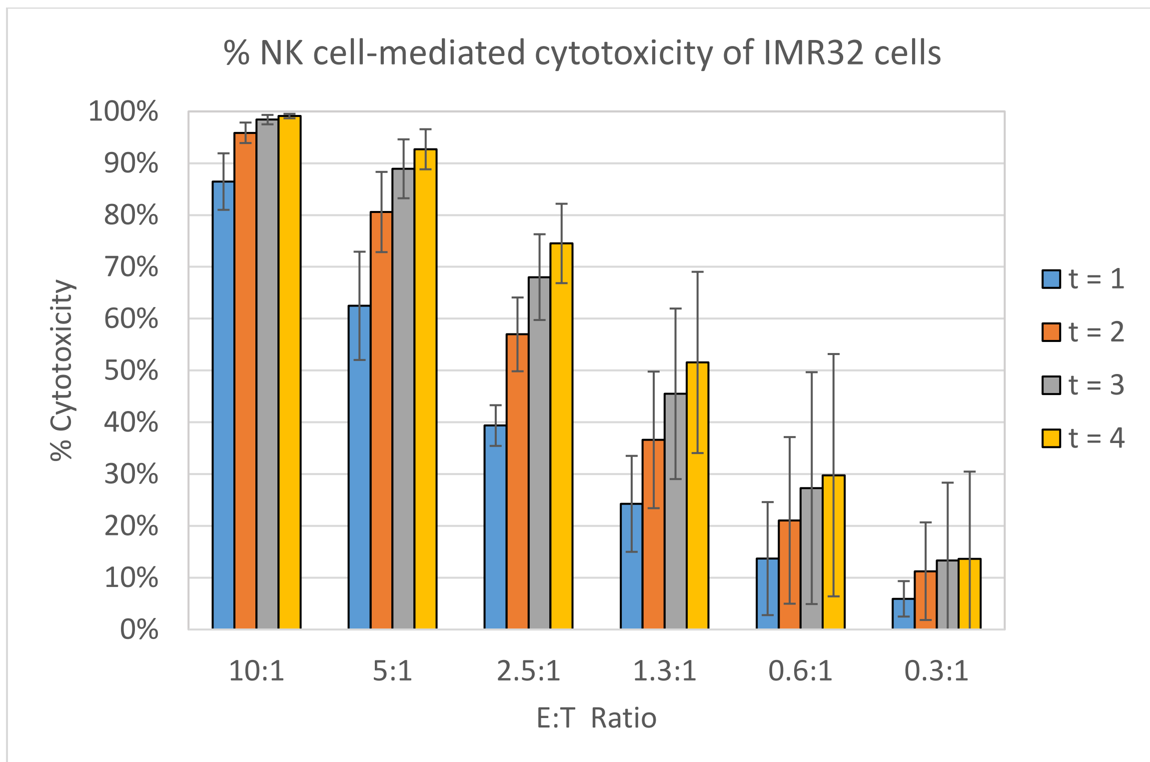
- The calcein AM-positive Target cells co-cultured with Effector cells were counted and recorded
- The calcein AM-positive Target cells only were counted and recorded
- $\%\ Cytotoxicity = 1 - \frac{Calcein\ AM\ count_{treated}}{Calcein\ AM\ count_{control}}$
- The % cytotoxicity was calculated for every well and averaged to generate E:T ratio-dependent response and time-course monitoring

Results

1. Celigo-captured calcein AM fluorescent images at different E:T ratios for IMR32
  - Below are example calcein AM images for IMR32 at different E:T ratios
  - The number of calcein AM-positive cells increased as the E:T ratios decreased

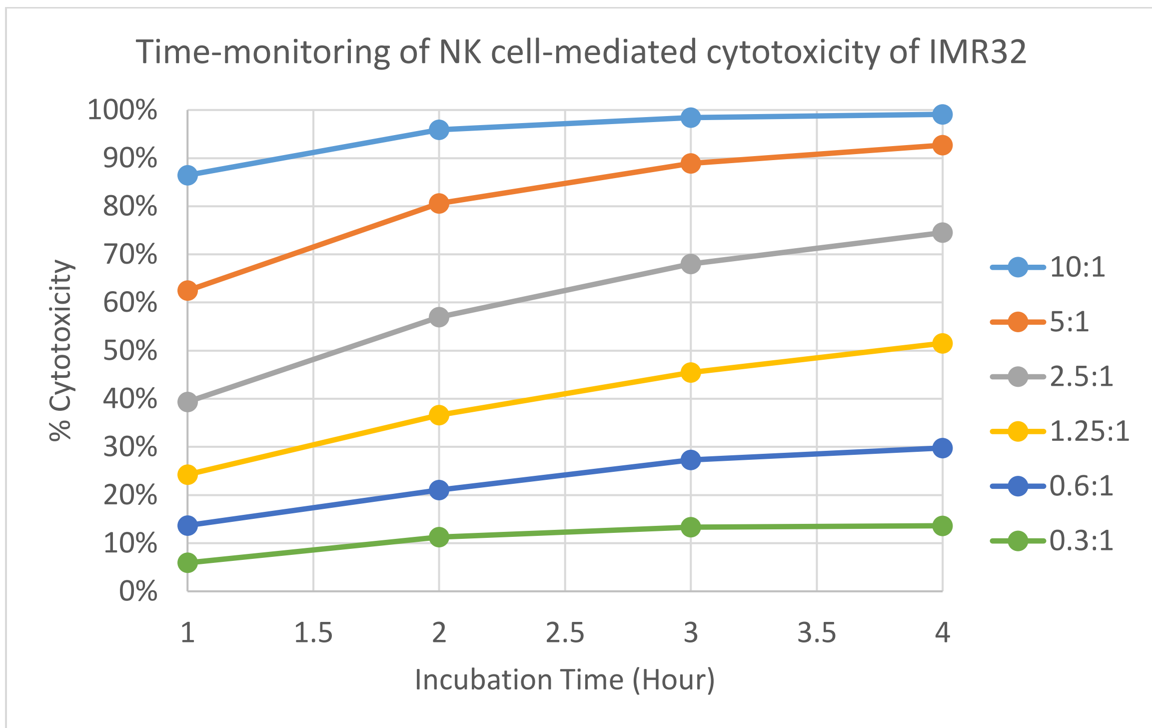


- The bar graph shows the % cytotoxicity at different E:T ratios and different time points for IMR32



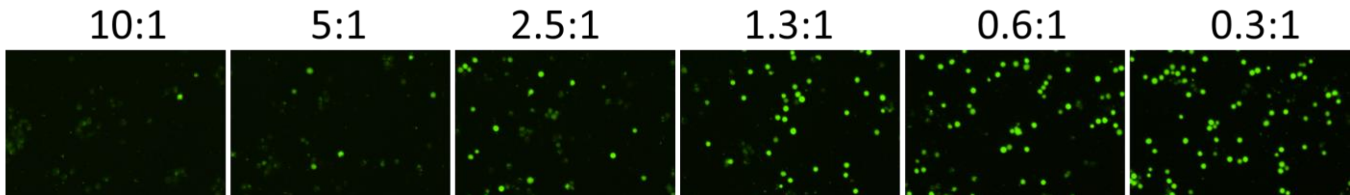
- The % cytotoxicity increased as the E:T ratios increased
- Similarly, the % cytotoxicity increased as the time increased

- The line graph shows the % cytotoxicity at different E:T ratios in respect to incubation time

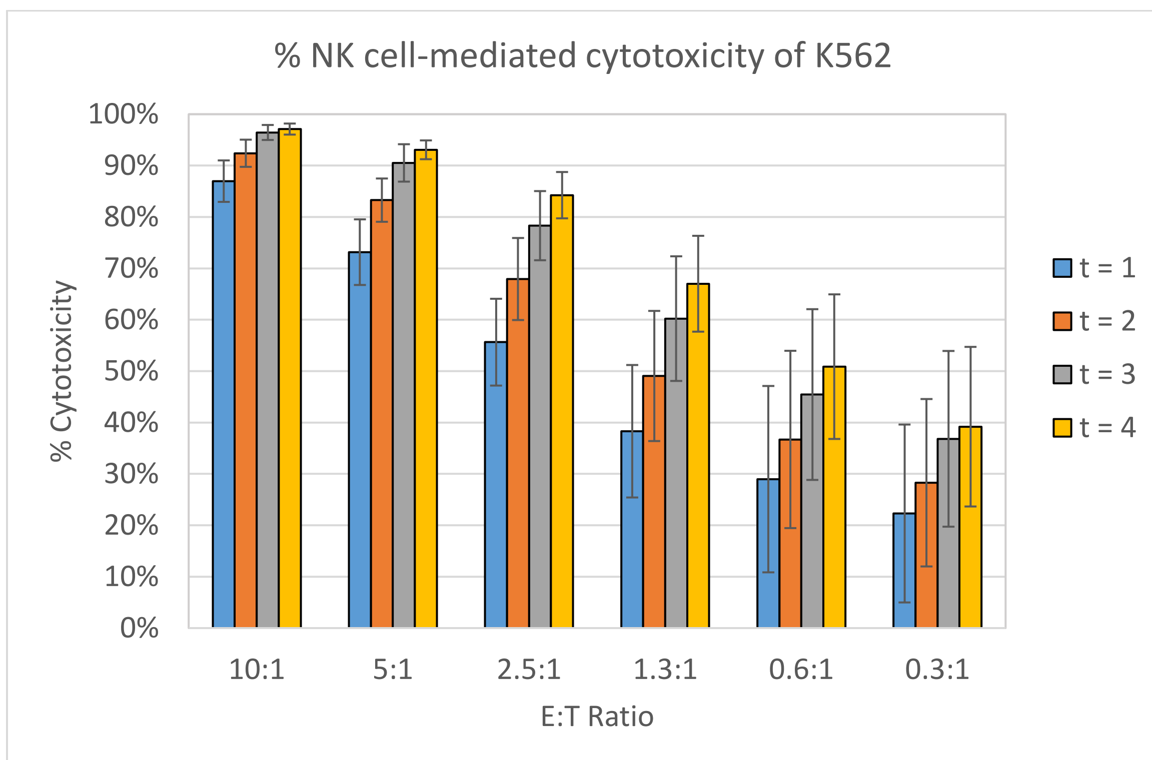


- The % cytotoxicity increased as the time increased for each E:T ratio
- At higher E:T ratios, the % cytotoxicity already reached ~90% at t = 1 h

2. Celigo-captured calcein AM fluorescent images at different E:T ratios for K562
   - Below are example calcein AM images for K562 at different E:T ratios
   - The number of calcein AM-positive cells also decreased as the E:T ratios increased

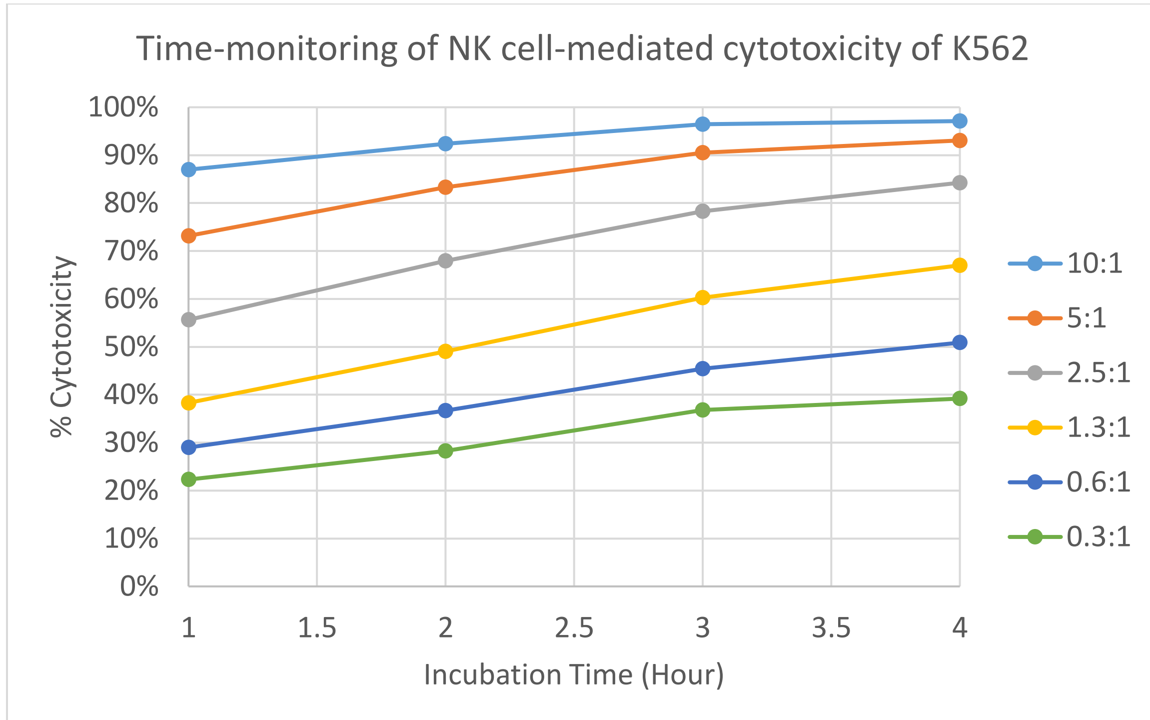


- The bar graph shows the % cytotoxicity at different E:T ratios and different time points for K562



- The % cytotoxicity increased as the E:T ratios increased
- Similarly, the % cytotoxicity increased as the time increased

- The line graph shows the % cytotoxicity at different E:T ratios in respect to incubation time



- The % cytotoxicity increased as the time increased for each E:T ratio
- At higher E:T ratios, the % cytotoxicity already reached ~90% at t = 1 h

Conclusion

- Time-course tracking of NK cell-mediated cytotoxicity can eliminate the need of maximum release control (Triton X-100) used in release assays, as well as normalizing to non-uniform seeding at t = 0 h
- The Celigo was able to count both suspensioin (K562) and adherent (IMR32) live cells
- The number of cells used is significantly less than the cells needed for release assays and flow cytometry assays, which can save time, money and precious primary immune cells
  - Flow cytometry assays and release assays usually require a seeding density of 100,000 Target cells, which translate to 1 million Effector cells at E:T ratio of 10:1
  - Celigo requires less than 10,000 Target cells, which translate to less than 100,000 Effector cells at E:T ratio of 10:1
- The decrease in number of live calcein AM-positive Target cells in the fluorescent images was successfully measured to show the effect of NK cell-mediated cytotoxicity