>> CAR T-cell Mediated Cytotoxicity and Cytokine Release in Response to Different Levels of Antigen Expression on Target Cells

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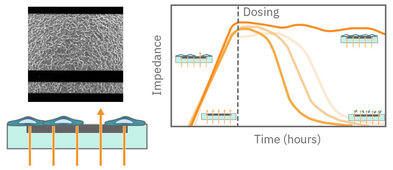
Maestro Z: Dynamic Cell Tracking

Impedance Technology

Cell-based *in vitro* cytotoxicity assays are a valuable tool for screening compounds for toxicity evaluation. Many in vitro cytotoxicity assays rely on dyes, or labels, to measure cell death at a single timepoint after a predetermined exposure time. Assessing the cytotoxicity of a compound label-free, *in vitro*, and at high throughputs is vital for toxicology evaluation.

Axion BioSystems' Maestro Z platform offers impedance-based cell analysis for real-time, label-free monitoring of cell viability, morphology, cytolysis, and signaling. Here, we used the Maestro Z to characterize a cytotoxicity assay for high-throughput screening and dose response analysis.



The impedance is measured from electrodes embedded in the bottom of each well. As cells cover more of the electrode, impedance increases in proportion to the number of viable cells. If a perturbation kills the attached cells, impedance decreases as the cells lyse.

The Maestro Z Product Family

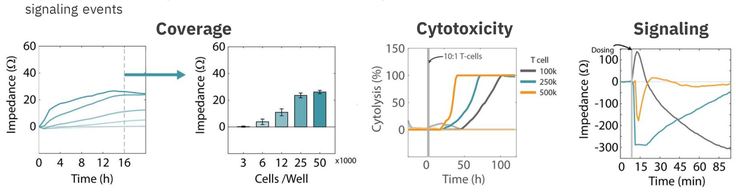
- ***Label-free, non-invasive tracking*** *of cultured cells or spheroids/organoids*
- ***Integrated environmental control*** *provides a stable benchtop environment for short- and long-term toxicity studies*
- ***Automatic and continuous cell monitoring*** *from 96 or 384 wells simultaneously*
- ***"One button setup"*** *automatically docks the plate and adjusts temperature and CO2 levels*
- ***Powerful data analysis*** *to focus on the science, while AxIS Z handles the details with simple setup and automatic experiment tracking*
- ***See your cells*** *with the viewing window included in each well of the CytoView-Z 96-well plate.*
- ***State-of-the-art electrode processing chip (BioCore v4)*** *offers stronger signals, ultra-low frequency content, and enhanced flexibility*

Features	*Maestro Z*	*Maestro TrayZ*	*Maestro ZHT*
Throughput:	96-well	Up to 8 x 96-well	384- and 96-well
Environmental Controls:	Built-in	External	Built-in
GxP Compatible:	✓	✓	✓
Barcode Plate Tracking:	✓	✓	✓
Automation API:	✓	No	✓
Dimensions *(WxDxH)*:	280 x 413 x 225 mm	440 x 450 x 60 mm	280 x 452 x 225 mm

Impedance Assay Measures Diverse Cell Properties

The Maestro Z records impedance at multiple frequencies simultaneously, enabling a thorough characterization of cell behavior, including:

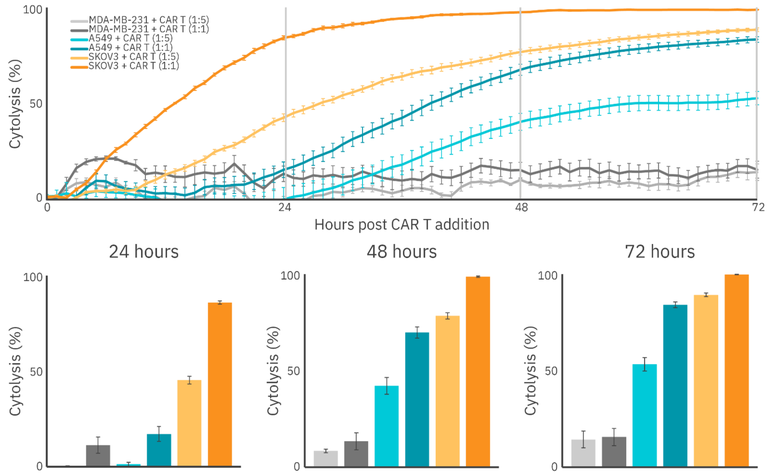
- Coverage/Density – the change in impedance is directly related to the quantity of cells in a 2D and 3D culture covering the electrodes.
- **Cytotoxicity** – dynamic monitoring of cell viability provides measures of the degree and speed of cell death.
- **Morphology** – cell size, shape, and intercellular tight junctions significantly impact the measured impedance.
- **Signaling** – small changes in cell shape or cytoskeleton organization are detected in response to intracellular signaling events



Real-time, Label-free Cytotoxicity Assay

Real-time Tracking Shows Immune Cell Mediated Cytotoxicity is Dependent on Antigen Density

The expression of the CAR within the effector cell population and its affinity for the target antigen are both important when determining the potency of a CAR T-cell. Target cell lines presenting – HER-2 overexpression (SKOV3), HER-2 low expression (A549), and no expression of HER-2 (MDA-MB-231), were treated with HER-2 CAR T- cells, as the Maestro Z recorded continuously for 72 hours. Complete killing was observed in SKOV3 cells at the 1:1 E:T ratio 72-hours post CAR T-cell addition, while A549 cells exhibited only 80% cytolysis and MDA-MB-231 cells showed 20% cytolysis. Similar trends were observed for the 1:5 E:T ratio, with CAR T-cells again exhibiting significant antigen-specific killing.



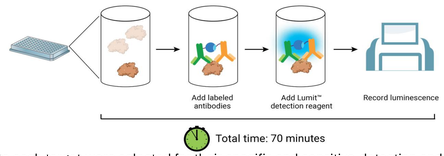
- (Top) Cytolysis time course for SKOV3 (orange), A549 (teal), and MDA-MB-231 (gray) killing by CAR T-cells at E:T ratios of 1:5 or 1:1. (Bottom) Comparison of %cytolysis at 24-, 48-, and 72- hours post CAR T-cell addition.

Promega Lumit® Immunoassays

Fast, No Wash Assay for Cytokine Release Quantification

Lumit® Immunoassays provide a simple and fast alternative to conventional immunoassay methods including sandwich ELISAs and Western blots.

Advantages of Lumit® over conventional immunoassays (ELISA, Western Blot) include:
- Simple add-mix-read protocol with no washing steps
- Direct analyte measurement in the cell culture plate or on medium removed from the cells
- No immobilization to plates, beads or other surfaces required
- Sensitive luminescence detection with wide dynamic range

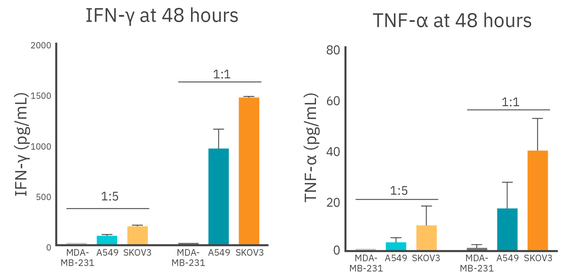


Primary antibodies to each target were selected for their specific and sensitive detection and labeled with the LgBiT and SmBiT subunits of NanoBiT® Luciferase. In the presence of the target, the subunits are brought together to form an active luciferase enzyme. Addition of optimized substrate generates a bright luminescent signal proportional to target abundance.

Antigen Density Effects Cytokine Release

Target Cell Antigen Density Influences Cytokine Release

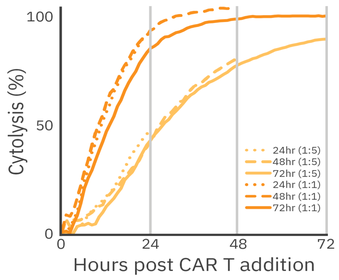
Pro-inflammatory cytokines interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) play a critical role in the immune system. To better understand the impact target antigen density had on cytokine release, we collected supernatant from SKOV3 (high HER-2 expression), A549 (low HER-2 expression), or MDA-MB-231 (no HER-2 expression) cells co-cultured with CAR T-cells. Using Lumit® Immunoassay analysis we observed the highest production of both cytokines at the 1:1 E:T ratio when CAR T-cells were co-cultures with high (SKOV3) levels of HER-2.



- IFN-γ (left) and TNF-α (right) at E:T ratios 1:1 and 1:5 measured in different cancer cell lines at 48 hours.
- CAR T-cells released 41.6% more IFN-γ when co-cultured with SKOV3 in comparison to A549 cells at the 1:1 ratio.
- CAR T-cells demonstrated an 80.5% increase in TNF-α release when co-cultured with SKOV3 in comparison to A549 cells at a 1:1 ratio.

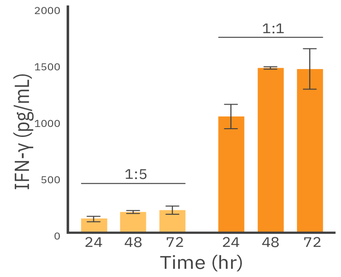
Robust Cytotoxicity and IFN-γ Release from HER-2 CAR T-Cells when Co-Cultured with SKOV3 Cells

HER-2+ SKOV3 cells were used to understand how elevated surface expression of the antigen on target cells impacts CAR T-cell mediated cytotoxicity and cytokine release. Using the Maestro Z and Lumit® IFN-γ (Human) Immunoassay, we observed a parallel increase in both % cytolysis and cytokine release. This suggests an effective activation of the CAR T-cells, highlighting their ability to induce target cell death while concurrently producing cytokines, indicative of a potent immune response.



- (Left) % Cytolysis time course for SKOV3 killing by CAR T-cells at E:T ratio 1:5 or 1:1. (Bottom) IFN-γ cytokine release in SKOV3 cells.
- Each line represents one of three identical plates to signify a 24- (dotted), 48- (dashed), or 72-(solid) hour post CAR T-cell addition timepoint.

- (Right) At the 1:1 E:T ratio, the highest levels of IFN-γ production were measured at 72 hours, 1459 +/- 178.6 pg/mL, when SKOV3 cells reached 100% cytolysis.
- In the 1:5 E:T group, IFN-γ production at 24 and 72 hours were 132.2 +/- 24.2 pg/mL and 209.5 +/- 35.2 pg/mL respectively.



Conclusions

- The Maestro Z platform allows for simple, non-invasive, real-time monitoring of immune cell-mediated killing of target cancer cells and provides a sensitive, quantitative assay for evaluating immune cell potency *in vitro*.
- CAR T potency is dependent on the expression of the CAR within the immune cell population and its affinity for the target antigen.
- Differences in cytokine IFN-γ and TNF-α release were influenced by target cell HER-2 antigen expression on multiple cancer cell lines at different E:T ratios.