>> Development of an *in vitro* potency assay of immune effector cell-mediated cytotoxicity and kinetics

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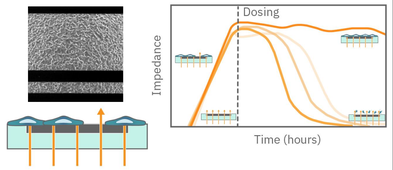
Maestro Z: Dynamic Cell Tracking

Impedance Technology

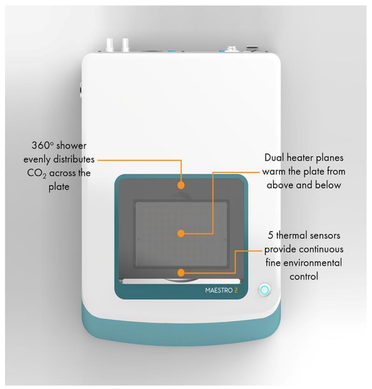
Immune effector T cells are a promising cancer therapy due to their innate cytotoxicity. In addition, engineering chimeric antigen receptors (CAR) to target tumor-associated or neo-antigens can lend high specificity. Assessing the efficacy and potency of such T cell therapies label-free, *in vitro,* and at high throughputs is vital for the preclinical development of these promising therapies.

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 compare the cytolytic potency and kinetics of HER2-specific CAR-T cells against cell lines with varied antigen expression (SKOV3 vs. MCF7) and 2D vs. 3D models.



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.

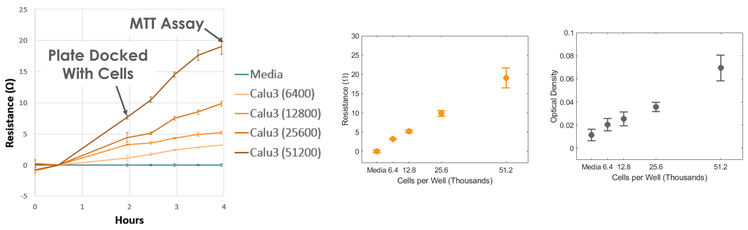
The Maestro Z Platform



- ***Label-free, non-invasive assay*** *for real-time cell analysis.*
- ***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*

Impedance Assay is Directly Correlated with Cell Viability

To validate impedance-based monitoring of cell viability, Calu-3 cells were added to a CytoView-Z plate with varying number of cells per well and monitored for four hours on the Maestro Z platform. The change in resistance was correlated with the number of cells initially seeded, and the resistance continued to increase as the cells adhered and flattened on the surface. At four hours post-seeding, the plate was removed and an MTT assay was performed in the CytoView-Z plate. The resistance measured with the Maestro Z platform was linear with respect to cell number and directly correlated to the MTT assay readings from the same wells.

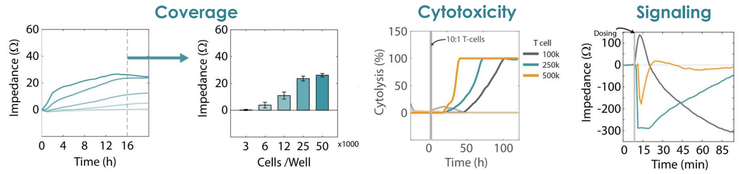


Dynamic Cytotoxicity Assay

Impedance Assay Measures Diverse Cell Properties

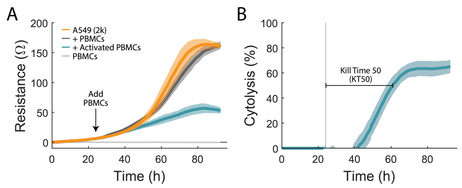
The Maestro Z records impedance at multiple frequencies simultaneously, enabling a thorough characterization of cell behavior, including:

- Coverage – the change in impedance is directly related to the number of cells covering the electrode.
- 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



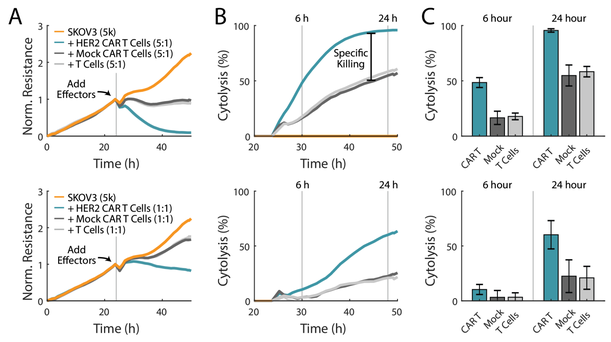
Impedance-Based Assay for Cell-Mediated Cytotoxicity

The impedance measurement is sensitive to the attachment of adherent or tethered target cells, but not the presence of non-adherent immune effector cells. In this way, the assay is naturally sensitive and specific to target cell attachment and cytotoxicity. The attachment and proliferation of the A549 target cells (orange) is measured via the resistance over time. At 24 hours, the PBMCs were added across various conditions. First, the PBMCs were added to some wells alone (light gray), and did not affect the resistance measurement, confirming that the measurement in this assay is specific to the target cells. PBMCs were also added to wells containing the target cells, with (blue) and without (dark gray) anti-CD3 and IL-2 to activate the immune effector cells. The resistance measure was significantly lower when activated PBMCs were added to the target cells, indicating immune cell-mediated cytotoxicity. The dynamics of the cytotoxicity were quantified as the kill time 50 (KT50), defined as the time duration required for 50% cytolysis of the target cells. In this example, the KT50 was 39 ± 3 hours for activated PBMCs added at a 10:1 effector to target ratio.



Antigen-specific Target Cell Killing with HER2-specific CAR T Cells

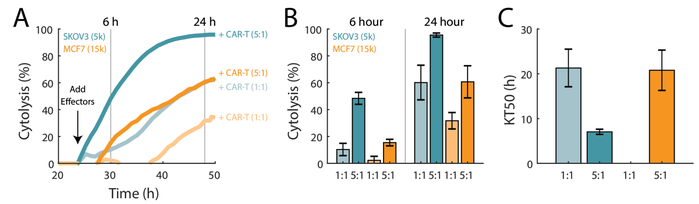
Here, we used HER2-specific CAR T cells (blue) to assess the *in vitro* killing of a HER2 expressing ovarian cancer cell line, SKOV3 (Subik et al, 2010). Changes in resistance as a function of time and different E:T ratios (5:1, top, and 1:1, bottom) were monitored (A). Donor-matched mock CAR T cells (dark gray), which lack the tumor antigen-recognizing domain, and non-transduced T cells (light gray) were used to separate non-specific killing from specific CAR T cell killing. SKOV3 cells treated with CAR T cells showed the greatest decrease in resistance over the time course, with near complete cell lysis in the 5:1 E:T group (A, top). Mock CAR T cells and non-transduced T cells were consistent with each other, and produced less cytolysis than CAR T cells for matched E:T ratios (B), indicating that non-specific killing was limited. Groups treated with CAR T cells showed approximately twice the degree of SKOV3 cytolysis at 6 and 24 hours (C), as compared to Mock CAR T cells.



Immune Cell-Mediated Cytotoxicity

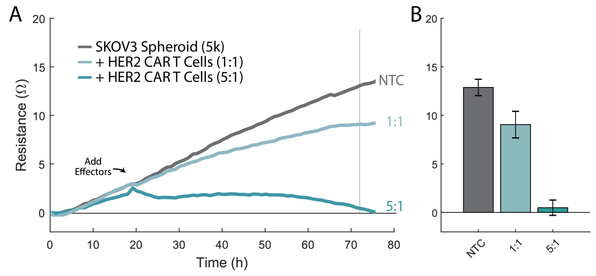
Kinetics and Potency of HER2-specific CAR T Cells across Cell Lines

The kinetics and potency of HER2-specific CAR T cells were compared for SKOV3 and MCF7 cancer cell lines. HER2 is overexpressed for SKOV3 cells, whereas MCF7 cells exhibit a normal level of HER2 expression. CAR T cells showed a greater percentage of cell killing at both E:T ratios for SKOV3 cells when compared to MCF7 cells. Furthermore, KT50 was used to assess the amount of time for 50% of cell death to occur between the two cell lines. Interestingly, SKOV3 cells treated with CAR T cells at 1:1 E:T took approximately the same time to reach KT50 as MCF7 cells treated with CAR T cells at higher E:T ratio of 5:1. Differences in % Cytolysis and KT50 between SKOV3 and MCF7 cell lines may be due to higher expression of HER2 in SKOV3 cells.

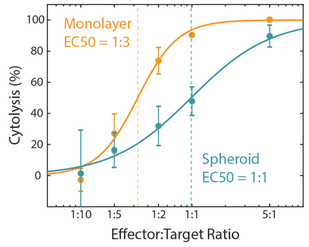


HER2-specific CAR T Exhibit Reduced Potency Against Spheroid Model

Solid tumors exist as complex 3-dimensional structures within the human body. Incorporation of more complex *in vitro* models may better mimic the tumor microenvironment and improve the translational nature of potency assays. Here, SKOV3 cells were cultured in ultra low attachment (ULA) U-bottom plates for 72 hours to generate spheroids and then transferred to the CytoView-Z plate. After 24 hours in the CytoView-Z plate, the spheroids were treated with HER2-specific CAR T cells (A). The dose-dependent decrease in resistance indicates immune cell-mediated cytotoxicity (B).



The potency of HER2-specific CAR T cells was compared for SKOV3 cells cultured as a monolayer and as a spheroid. A cell count assay was performed on a subset of the SKOV3 spheroids at the time of seeding on the CytoView Z plate to ensure consistent total cell number between monolayer and spheroid models. Cytolysis was computed 72 hours after CAR T cell addition. The HER2-specific CAR T cells were more potent against the SKOV3 monolayer model (EC50 = 1:3 E:T ratio) than the SKOV3 spheroid model (EC50 = 1:1 E:T ratio).



Conclusions

- The impedance-based assay for cell-mediated cytotoxicity provides a real-time cytolysis readout, enabling the computation of KT50 to evaluate the degree and speed of target cell death.
- SKOV3 cells, which overexpress HER2, were more sensitive to HER2-specific CAR T cells than MCF7 cells, which exhibit normal HER2 expression.
- HER2-specific CAR T were more potent against SKOV3 monolayer models than SKOV3 spheroid models when controlled for total cell number and E:T ratios.
- Overall, the Maestro Z platform enabled continuous, dynamic, label-free quantification of the potency, efficiency, and kinetics of immune-cell mediated cytolysis.