Application Note

Topic:
Cancer, Immuno-oncology
Cells:
A549, lung adeno-carcinoma cell line
Tools:
AtlaZ

Immune cell-mediated killing of A549 cancer target cells in real-time

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Summary

Cancer remains one of the leading causes of death, with, according to the World Health Organisation (WHO), around 10 million people dying due to the disease in 2020 (1). Chemo- and radio-therapy are still the dominant treatment types, but advancing therapies such as immuno-therapy have emerged as tools to fight against the disease.

In general, identifying T cells that kill cancer cells *in vivo* is critical to the development of successful cell therapies. The label-free AtlaZ immune cell killing assay can be used to measure rate of killing at Effector : Target (E:T) ratios to predict *in vivo* activity. In order to gain a deeper understanding of cancer cells, real-time and continuous monitoring is necessary to access kinetic and phenotypic information.

The platform used here, AtlaZ, is a quantitative live-cell analysis system and allows for cellular research on cell adhesion and proliferation, cytotoxicity, GPCR, morphology and barrier function, label-free and in real-time. Recordings can be performed in up to six 96-well plates simultaneously or independently. Electrical impedance spectroscopy (2,3) as the methodology behind the AtlaZ system, in combination with the throughput of 6 x 96-wells allows for a so far unmet quantity and richness of information which can be gained from cells.

Here we used the human A549 cell line (Figure 1A) which is a widely utilized epithelial lung adenocarcinoma cell line that was derived from a primary lung tumor. The effector cells co-cultured in our assay were purified human cytotoxic T- lymphocytes expressing CD8 (cluster of differentiation 8), a co-receptor for the T-cell receptor (TCR). Those effector cells exhibit an antigen-induced cell-mediated cytotoxicity by triggering intracellular pathways leading to apoptosis of target A549 cells.

The impedance of planar gold-film electrodes that are used as growth substrate for adherent cells reveals changes in electrode coverage or cell behavior (Figure 1B). Real-time impedance data provide insights in various cell phenotypes, such as cell morphology, proliferation, lateral migration or cytotoxicity even over prolonged periods of time.

A crucial advantage over standard assays, e.g. filter based methods using labels and either an optical or radiometric detection technique, is the continuity of cell monitoring.

Endpoint assays using dyes or labels quantify the potency of effector cells to kill target cells by either measuring target cell viabiliy (i.e. MTT or flow cytometry) or membrane integrity (i.e. LDH). Those assays are labor-intensive and lack kinetic

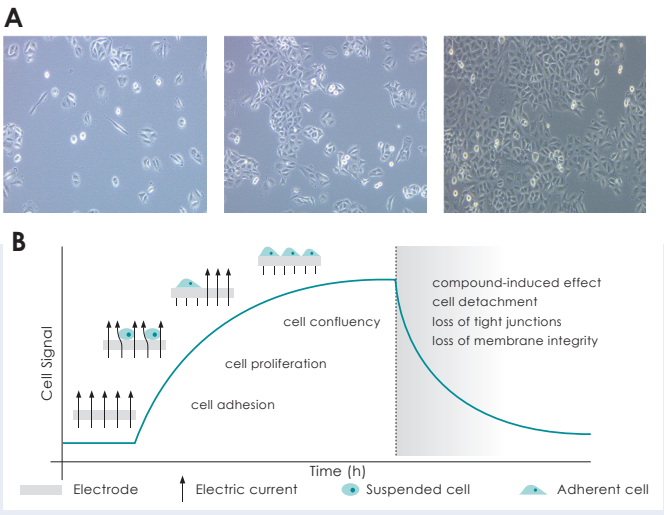


Figure 1: A *Cancer cells A549 one day after plating on Nanion sensor plates (left), after two days (middle) and after four days (right)* **B** *The Cell Signal value offers information on cell adherence, proliferation or cell death.*

information of the recorded effects.

Unique culture plates with integrated electrodes as used in the AtlaZ system enable long-term measurements over several weeks. Thus real-time data on cell adhesion, proliferation and compound effects can be aquired.

Results

Here we used the human A549 cancer cell line derived from a primary lung tumor. Next to the proliferating A549 cells we used effector cells expressing CD8, which is is predominantly present on the surface of cytotoxic T-cells, but can also be found on e.g. natural killer cells.

Figure 1 shows the lung adeno-carcinoma cell line A549 as adhered on the cell culture flasks on days 1, 2 and 4 post-plating.

The A549 cell seeding density was 40 k cells/well, the cells then proliferated and a plateau phase was reached approx. after 14 h. Next, at t = 24 h, T-cells enriched from primary PBMCs (Peripheral Blood Mononuclear Cells) were added at an effector T-cell to target A549 cell ratio of 1:1 (50 k cells), 2:1 and 3:1. The impedance signal of the cell monolayer was recorded every 15 minutes, and is plotted using the unitless parameter *Cell Signal* (Figure 2). The data revealed that increasing E:T (Effector:Target) cell ratios induced a ratio-dependent reduction of the viability of A549 cells seen as a decrease of the *Cell Signal*.

Based on the same data, the AtlaZ Control software also calculates and displays Cytolysis in % (Figure 3A). The data reveal that after approx. t = 27 h the cytolysis of A549 cells gradually increases and reaches a maximum of 37%, 48%, 59% and 57% in the presence of the effector to target cell ratio 1:2, 1:1, 2:1 and 3:1, respectively. Uninterrupted attachment and growth of A549 cells was observed in wells with only A549 cells (Figure 2, grey line).

The Kill Time 50 values were calculated to investigate at what timepoint and which ratios of effector cells killed the cancer cells to 50%. Fig. 3 B shows that 50% of A549 cells were killed after approx. 13 h (E:T = 1:1) or 6 h (E:T = 2:1 and 3:1).

These results demonstrate the capability of CD8 T cells to kill A549 cancer cells.

In summary, AtlaZ allows for label-free and real-time cellular research on cell adhesion, proliferation and cytolysis. Measurements can be performed in up to 6 x 96-well plates simultaneously or independently. The AtlaZ system provides a versatile tool for *in vitro* cell monitoring addressing the demands for versatility, physiological relevance and throughput.

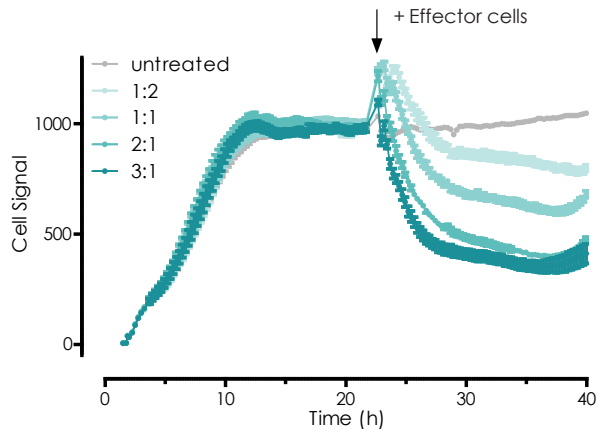


Figure 2: *Effector cells added at t = 24 h after plating of A549 cells. Increasing E:T cell ratios induced a ratio-dependent reduction of the viability of A549 cells, represented as a reducing Cell Signal, data are shown +/- SEM.*

Methods

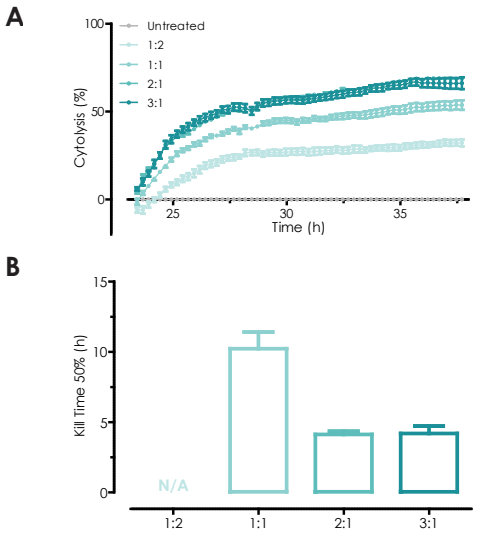
AtlaZ platform

The AtlaZ platform (Nanion Technologies) provides quantitative live-cell analytics by measuring the impedance (Ohm, Ω) of adherent cells as grown on 96-well plates with embedded planar gold-film electrodes (Nanion Technologies).

Real-time impedance data provide insights in various cell phenotypes, such as cell morphology, proliferation, lateral migration or cytotoxicity even over prolonged periods of time. A crucial advantage over standard assays is the continuity of cell monitoring. Continuous measurements reveal the kinetics of cell behavior and allow an in-depth mechanistic understanding without the need for time- and labor-intensive endpoint assays. For example, one of the crucial advantages of data derived from the AtlaZ platform is the possibility to analyse dose-responses at any time during the experiment.

Advanced information content is obtained by using multi-frequency impedance readouts, which is possible with the AtlaZ Control software. With impedance readouts at different frequencies (here a spectrum of 0.1 kHz – 100 kHz) it is possible to further dissect physiological response into deeper levels,

Figure 3: A *Cytolysis in percent (%) as calculated from the data seen in Fig. 2.* **B** *Kill time 50 shows that 50% of A549 cells were killed after approx. 13 h (E:T = 1:1) or 6 hrs (E:T = 2:1 and 3:1). Data are shown +/- SEM.*



and thus zooming in on changes in membrane topography, cell-cell or cell-matrix junctions is achievable. G-protein-coupled receptor (GPCRs) activation is one example here.

Immune-cell mediated killing assay

To investigate PBMC-mediated cytotoxicity, A549 cells were cultured in DMEM medium (high glucose, 1% Pen/Strep, 10% FCS) for 4 days until they reached 80-90% confluency. With a seeding density of 40 k cells/well they were then directly plated on AtlaZ sensor plates without coating. The effector cells were obtained from the blood of healthy donors using a Percoll density gradient (PBMCs). CD8 T-cells were positively selected and purified using magnetic beads. After purification, the cells were incubated in T-cell medium (RPMI 1640, 10% FCS, 1% P/S and 1% glutamine) and stimulated with the cytokine IL-15 (10 ng/ml) for 5 days before being used for the co-culture experiments on AtlaZ. Effector cells were added to the AtlaZ sensor plate at effector to target (E:T) ratios of 1:1, 2:1, and 3:1 24 hours after target cell seeding in a 100 µl volume at the desired final cell concentration. The same volume was added to negative control wells.
The *Cell Signal* values that are displayed were calculated by subtracting the impedance in empty wells with medium, measured at the beginning of the experiment just before cell addition. Data are presented as mean ± SD.

The AtlaZ assay is sensitive to adherent target cells, but not to the presence of non-adherent immune effector cells. This allows to specifically investigate target cell cytotoxicity and is an advantage over other assays where the presence of a second cell type like effector cells could induce background signals.

Workflow of AtlaZ immune cell-mediated killing assay

Target cells are seeded on AtlaZ sensor plates and they adhere on the surface with embedded gold electrodes (Figure 4, 1). Each well of the 96-well plate contains 1 center gold electrode with 0.6 mm in diameter and one reference electrode. After the target cells being adhered, the population starts to proliferate. When the cells reach confluency, non-adherent effector cells (e.g. immune cells) are added (Figure 4, 2).

The cytolysis of the target cells is measured and displayed as *Cell Signal* over time as seen in the figure, additionally the AtlaZ Control software calculates the data as Cytolysis (%) and calculates and displays the data in a KT50 (Kill Time 50) plot (as shown in Figure 3)

Calculation of cytolysis and Kill Time 50

Percent (%) cytolysis was used to quantify cell death and is calculated by AtlaZ Control software as follows:

$$Cytolysis\ (t,\ \%) = \left[\left(\frac{\Delta_{kt}}{\Delta_G}\right)_{\substack{no\\ effector\\ cells}} - \left(\frac{\Delta_{kt}}{\Delta_G}\right)_{\substack{target+\\ effector\\ cells}} \right] \cdot 100$$

where $\Delta_{k,t}=Z_t-Z_0$ and $\Delta_G = Z_0-Z_{min}$. Z_t is the impedance value after the addition of the treatment and Z_0 is the last impedance value before treatment addition. Z_{min} is the smallest impedance value in the region before treatment addition. This means that Cytolysis (t, %) is the effect of the effector cells on the target cells at a certain time t, displayed in percent. $\Delta_{k,t}$ (k=killing; t= time) contains information on the time-dependent cell death based on the impedance change before and after treatment and Δ_G considers the cell proliferation and growth (g = growth) post-seeding.

Next to Cytolysis (%) AtlaZ Control software also displays Kill Time 50 values which are as well determined through the impedance measurements.

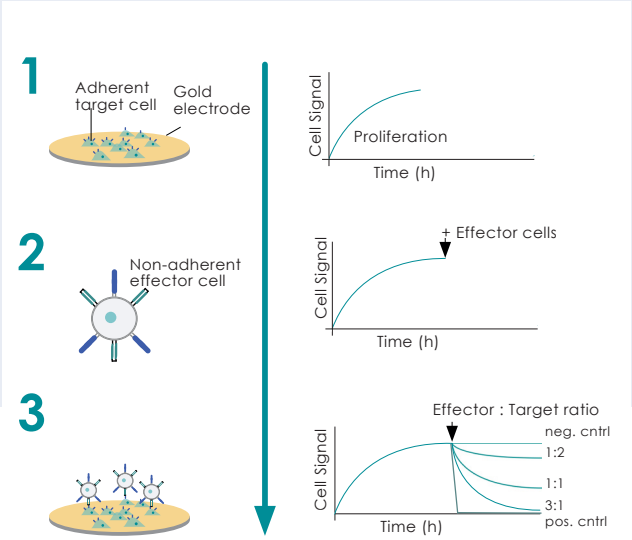


Figure 4: *Workflow of immune cell-mediated killing assay. Target cells are seeded on AtlaZ sensor plates, adhere and proliferate (1), then non-adherent effector cells are added (2). The cytolysis of the target cells is measured and displayed as Cell Signal over time.*

References

1. https://www.who.int/news-room/fact-sheets/detail/
2. Reiß, B. and Wegener, J. (2015) Impedance Analysis of different cell monolayers grown on gold-film electrodes. IEEE Eng. Med. Biol. 7079-7082. org/10.1002/9780470015902.a0025710
3. Lukic, S.; Wegener, J. (2015) Impedimetric monitoring of cell-based assays. Encyclopedia of Life Sciences, Wiley & Co. https://doi.

Key findings

1. Our results demonstrate the capability of CD8 T cells to kill A549 cancer cells.
2. AtlaZ allows for cellular research on cell adhesion, proliferation and cytotoxicity, label-free and in real-time.
3. Recordings can be performed in up to six 96-well plates simultaneously or independently.

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