Using xCELLigence® Real-Time Cell Analysis to Monitor Immune Cell-Mediated Killing of B Cells
Introduction

ACEA’s xCELLigence® real-time cell analysis (RTCA) instruments use the principle of cellular impedance to non-invasively monitor adherent cell number, size/shape, and attachment quality in a label-free and real-time manner. Validated in >750 publications over the past decade, this technology has been used extensively in recent years to study immune cell-mediated killing of adherent cancer cells. The protocol included herein expands the application repertoire of xCELLigence RTCA to include monitoring of immune cell-mediated killing of non-adherent cancer cells. Below, a brief description of the xCELLigence RTCA instruments is first provided. This is followed by an overview of traditional immune cell killing assays and their limitations. The xCELLigence RTCA assay principle and work-flow are then described using adherent target cells as an example. After a general overview of how the xCELLigence RTCA assay can be adapted for monitoring non-adherent target cells, a detailed protocol is provided.

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xCELLigence RTCA Instruments

The xCELLigence® Real-Time Cell Analysis (RTCA) instruments enable label-free, real-time monitoring of cell proliferation, morphology, and attachment quality. Though the seven xCELLigence models differ from one another in throughput and functional specificity (such as monitoring cell invasion/migration or cardiomyocyte beating), their setup and workflow are similar. For the xCELLigence RTCA MP model, used in Figure 1 as an example, the instrument contains six cradles that each house a 96-well electronic microtiter plate (E-Plate®). Each of these can be operated simultaneously yet independently of one another, enabling maximal productivity for multiple users. The instrument is placed in a standard CO₂ incubator and is powered and controlled via a cable connected to a work station housed outside the incubator (Figure 1B). The user friendly RTCA software allows for real-time interfacing with the instrument, and includes real-time data display and analysis functions. The specifics of how the xCELLigence instruments monitor cell health and behavior are described in below sections.

Figure 1. The xCELLigence RTCA MP system. (A) Note that the MP instrument differs from the other xCELLigence systems in that it has six separate cradles, each of which can interface with a 96-well electronic microtiter plate (E-Plate®). (B) The instrument is housed in a standard tissue culture incubator and communicates, via a flat ribbon cable, with the analyzer and control units kept outside the incubator.
Traditional Immune Cell Killing Assays

The high specificity and potent cytotoxicity of immune system effector cells make them promising agents for extirpating cancer cells. Though the list of efficacious cell-mediated immunotherapies is growing, realizing the full therapeutic potential of this field will require: (1) continued elucidation of the mechanisms underlying tumor cell recognition and immune cell-mediated killing, and (2) an ability to screen immunotherapy constructs/conditions using patient-derived effector and/or target cells to identify optimal treatment regimens. Fundamental to both of the above is the ability to quantitatively monitor immune cell-mediated killing of target cells under controlled conditions in vitro. Traditional cell killing assays suffer from drawbacks that prevent them from meeting this need efficiently.

Immune cell-mediated killing can be studied by measuring the activation of effector cells or their secretion of cytotoxic molecules (perforin, granzymes, etc.). While these readouts are indeed useful, they don't necessarily correlate with target cell killing efficiency – which is the ultimate measure of therapeutic efficacy. Assays focused on the response of target cells primarily monitor the release of either previously added labels (such as $^{51}$Cr or fluorescent dyes) or endogenous biomolecules (GAPDH, LDH, etc.) upon target cell lysis. Besides the potential artifacts associated with using exogenous labels, the time frame over which such labels are useful is extremely narrow (due to the spontaneous leakage of label out of target cells). Moreover, release assays as a whole suffer from low sensitivity, low efficiency/throughput, and the fact that only endpoint data (mere snapshots in a cell response continuum) is produced.
What is the xCELLigence RTCA assay principle?

ACEA’s xCELLigence® Real-Time Cell Analysis (RTCA) instruments utilize gold microelectrodes embedded in the bottom of microtiter wells to non-invasively monitor cell status including cell number, shape/size and attachment. The major distinguishing features of this technology include enhanced sensitivity, the preclusion of labels and, importantly, kinetic measurement of cell health/behavior.

**Step 1:** Adherent target cells (i.e. tumor cells) are first seeded in the wells of an electronic microtiter plate (E-Plate). Adhesion of cells to the gold microelectrodes impedes the flow of electric current between electrodes. This impedance value, plotted as a unitless parameter called “Cell Index”, increases as cells proliferate and then plateaus as cells approach 100% confluence.

**Step 2:** When added subsequently, non-adherent effector cells (i.e. immune cells) in suspension do not cause impedance changes in and of themselves (due to lack of adherence to the gold microelectrodes).

**Step 3:** If effector cells induce the destruction of the target adherent tumor cells, the corresponding cytolytic activity can be sensitively and precisely detected. The continuous acquisition of impedance data for each well of an E-Plate enables the generation of real-time killing curves for multiple conditions simultaneously.

**Key benefits of using xCELLigence to monitor immune cell-mediated killing:**

1. **Label-Free:** Allowing for more physiological assay conditions; labeling or secondary assays aren’t required.
2. **Real-Time:** Quantitative monitoring of both fast (hours) and slow (days) killing kinetics.
3. **Sensitive:** Capable of evaluating low effector cell to target cell ratios that are physiologically relevant.
4. **Simple Workflow:** Requires only the addition of effector cells to target cells (in the presence or absence of antibodies); homogeneous assay without additional sample handling.
5. **Automatic Data Plotting:** RTCA software enables facile data display and objective analysis, precluding the subjective data vetting that is common to imaging-based assays.
Immune Cell Killing Assay - Adapted for Liquid Tumor Cells

xCELLigence RTCA has successfully been used to monitor killing of diverse adherent tumor cells by a variety of immune effector cells (NK cells, T cells, macrophages, etc.) and in a variety of contexts including the use of chimeric antigen receptors (CARs) and bispecific T cell engagers (BiTEs). In order to apply this impedance-based cell killing assay to liquid tumor target cells ACEA has developed a protocol for immobilizing cells on the bottom of E-Plate wells using antibody (Figure 2A). While this approach is broadly applicable, this protocol focuses specifically on immobilization of B cells. Though they are not naturally adherent, Daudi B cells are effectively immobilized when E-Plate wells are pre-coated with anti-CD40 antibody (Figure 2B). As expected, this immobilization is specific for CD40+ B cells but not for CD40- NK cells or T cells. As seen in Figure 2C, when left untreated the immobilized Daudi cells proliferate on the well bottom – giving rise to a steady increase in the cellular impedance signal. However, upon addition of increasing quantities of T cells (TALL-104) the impedance signal decreases in a dose dependent manner that is consistent with T cell-mediated lysis of the B cells (Figure 2C).

Figure 3. Using xCELLigence RTCA to monitor killing of liquid tumor cells. (A) By coating E-Plate wells with anti-CD40 antibody, B cells can be immobilized. (B) Daudi B cells, non-adherent under normal conditions, proliferate on E-Plate well bottoms that have been pre-coated with anti-CD40. The presence or absence of anti-CD40 coating has no impact on NK-92 cells which are CD40-. The same is true for T cells (not shown here). (C) In a time- and dose-dependent manner T cells (TALL-104) kill immobilized B cells (Daudi).
1. Introduction

The below protocol has specifically been optimized for using the xCELLigence RTCA SP or MP instruments to monitor NK cell (NK-92) and T cell (TALL-104) mediated killing of B cells (Daudi) which have been immobilized on the well bottoms of an E-Plate 96 using anti-CD40 antibody. Assay conditions may require additional optimization if different cell lines or antibodies are used.

2. Reagents & Equipment

Reagents

- **Cells:** Daudi cells (Cat.# CCL-213™), NK-92 cells (Cat.# CRL-2407™), and TALL-104 cells (Cat.# CRL-11386™) were purchased from ATCC.
- **Cell Culture Medium:** RPMI 1640 + 10% FBS + 1% penicillin/streptomycin
- **Interleukin 2 (IL-2):** Peprotech (Cat.# AF-200-02). Working concentration is 100 or 200 units/mL for TALL-104 and NK-92 cells, respectively.
- **Human CD40/TNFRSF5 Antibody:** R&D Systems (Cat.# AF632). Antibody working concentration is 4 µg/mL in PBS.
- **PBS without calcium and magnesium**

Equipment

- **E-Plate 96:** ACEA Biosciences (Cat.# 05232368001 or 05232376001). Because the well size in ACEA’s E-Plate 96 is identical to the well size in the E-Plate 16, the media volumes and cell numbers used in the below protocol are compatible with usage of the lower throughput E-Plate 16. ACEA’s other plate types, the E-Plate L8 and E-Plate 384, are also compatible with this liquid tumor cell killing assay, but cell numbers/media volumes would need to be optimized for the different well dimensions in these plates.
- **xCELLigence RTCA SP or MP instruments:** While all of the xCELLigence RTCA instruments can be used for this liquid tumor cell killing assay, the cell numbers and media volumes listed in this protocol are optimized for the dimensions of the wells in the E-Plate 96 – which is only compatible with the xCELLigence RTCA SP and MP instruments.

For a full description of the seven different xCELLigence instruments please visit: http://www.aceabio.com/products/xcelligence-rtca
3. Protocol

Workflow Summary

Allowing an initial day for target B cells to attach to and proliferate in the E-Plate 96 wells, this assay protocol has been developed for continuous monitoring of cell killing over the course of 4 subsequent days.

- Day 1:
  a. Coat E-Plate 96 with antibody; wash; add medium; equilibrate in 37°C incubator for 1 hour; take background measurement.
  b. Prepare B cells and add to E-Plate 96.
  c. Leave E-Plate 96 at room temperature for at least 30 minutes to let cells settle.
  d. Load E-Plate 96 into xCELLigence RTCA instrument and start data acquisition to monitor B cell attachment and proliferation.

- Day 2:
  e. Prepare T cells.
  f. Pause xCELLigence data acquisition; remove E-Plate 96 from instrument and place inside hood; remove nascent media from wells; add T cells to E-Plate 96.
  g. Load E-Plate 96 back into xCELLigence RTCA instrument and start data acquisition to monitor T cell-mediated killing of immobilized B cells.

- Days 2-5:
  h. Continue data acquisition for as long as desired.
  i. Analyze data.

Detailed Protocol

Coating E-Plate 96 with Anti-CD40 (3 hours)
1. Coat wells of the E-Plate 96 with anti-CD40 antibody by adding 50 µL of anti-CD40 solution (4 µg/mL in PBS).
2. Incubate at room temperature for 3 hours.

» Critical: The extent of antibody adhesion to well bottoms may vary as a function of incubation temperature and duration. To ensure reproducibility between experiments be sure to maintain consistency in the parameters of this coating step.
**Protocol**

**Washing and Background Measurement (5 minutes)**
1. Remove antibody coating solution.
2. Wash wells twice with 200 µL of PBS.
3. To each well add 50 µL of complete medium (or whatever medium will be used during the assay).
4. Place E-Plate 96 back into instrument and initiate background measurement.

**Target Cell Seeding (45 minutes)**
1. Determine Daudi B lymphoblast concentration and seed 80,000 cells in a volume of 100 µL/well. With the medium already present in the wells (from the background measurement step) this gives a final volume of 150 µL/well.
   
   » **Critical:** The number of cells used in this killing assay will ultimately depend on the cell type being used. If using cell types other than those listed here, it is imperative to conduct preliminary experiments to determine optimal cell numbers.

2. Leave E-Plate 96 at room temperature for 30 minutes to facilitate uniform immobilization of B cells on plate bottom.
   
   » **Critical:** Failure to perform this step can result in large well to well variation in the cell seeding density/pattern (which can affect the impedance signal) for the following reason: Immediate warming to 37°C can cause convection currents to form within the well, and these currents can push cells to the well perimeter, resulting in an uneven distribution of cells on the impedance electrodes.

**Monitoring Target Cell Adhesion and Proliferation (24 hours)**
1. Place E-Plate 96 back into xCELLigence instrument located inside incubator and initiate data acquisition. The purpose here is simply to monitor B cell adhesion and proliferation prior to initiating cell killing. Accordingly, measuring impedance every 15 minutes for 24 hours is appropriate.

**Effector Cell Addition (15 minutes)**
1. Determine NK-92 and TALL-104 cell concentrations and prepare serial dilutions in medium containing interleukin 2 (IL-2). The IL-2 concentration in these dilutions should be 150 units/mL for the TALL-104 cells and 300 units/mL for the NK-92 cells.
2. Pause data acquisition, remove E-Plate 96 from instrument and move into hood.
3. Gently aspirate 100 µL of nascent medium from wells. This leaves 50 µL of medium in the wells.
4. Add 100 µL of the NK-92 or TALL-104 dilutions per well. This dilution into the nascent media achieves final IL-2 concentrations of 100 units/mL (TALL-104 cells) or 200 units/mL (NK-92 cells). Effector:target cell ratios will be defined by the density of effector cells in the serial dilutions.

**Monitoring T Cell-Mediated Killing of Immobilized B Cells (assay duration defined by user)**
1. Place E-Plate 96 back into xCELLigence instrument located inside incubator and initiate data acquisition. The purpose here is to monitor T cell-mediated killing of the B cells immobilized on the bottom of the E-Plate 96 wells. Impedance measurements every 15 minutes are sufficient for this purpose. The total duration of data acquisition should be programmed to be longer than deemed necessary; data acquisition can be terminated whenever appropriate.
Protocol

Data Analysis

The full functionality of the RTCA data analysis and representation software is reviewed in detail in the RTCA software manual; what is presented below is a cursory overview.

Depending on the details of the experiment and what information the user wishes to extract, the xCELLigence RTCA data from an immune cell killing assay can be analyzed and plotted in multiple ways. The steps described below reflect common practices but should not be regarded as the canonical RTCA data analysis protocol.

1. Stop data acquisition.

2. **Curve averaging**: Evaluate the precision/reproducibility of the data by averaging the curves from replicate wells and displaying the standard deviation. In the example shown in Figure 4, at the 19.9 hour time point immobilized Daudi cells were titrated with different quantities of either NK-92 or TALL-104 cells. Each condition was monitored in duplicate wells. Whereas in Figure 4A all 12 data traces are shown, in Figure 4B the duplicate wells have been averaged and the corresponding standard deviation is displayed.

3. **Data normalization**: To accurately assess the effect that different treatments have on target cells, it is important to make sure that each treatment is being evaluated relative to a common reference. In Figure 4B, it is clear that the different effector:target ratios effect different changes in Cell Index. The slight difference in Cell Index values between well replicates, which is due to pipetting variability, can be alleviated by normalizing all the data traces to the time point immediately prior to effector cell addition (this time point is highlighted by the black vertical line in Figure 4C). Note that the data trends remain the same here, but the relative value of Cell Index for the different conditions is shifted slightly.

4. **Using a baseline**: In addition to the data normalization function described above, changes in Cell Index can be plotted relative to the negative control group. In this particular example, the untreated sample is selected as the baseline that is subtracted from the other data traces (Figure 4D). Here, the control group has a value of 0 at every time point and the other samples are plotted as the relative difference.

Once the Cell Index curve has been plotted using the above techniques, cell killing can be calculated quantitatively. Either the Normalized Cell Index Plot or the Baseline Subtracted Plot can be used for this purpose. There are two primary means of making this calculation: using the slope of the impedance trace for a particular time range, or simply determining the value of the Cell Index for a specific time point. Each of these methods is briefly described below.

5. **Using Cell Index slope**: This tool measures the inclination of the Cell Index curve in a particular user-defined time range. A time range should be chosen where the Cell Index is actively decreasing for the relevant samples. Higher negative slopes denote faster killing kinetics.

6. **Using Cell Index at a specific time point**: The Cell Index at a given time point is proportional to the amount of adherent cells remaining on the E-Plate. Decreasing Cell Index values after effector cell addition represent cell killing.
Figure 4. xCELLigence RTCA data analysis techniques. Though this particular assay is monitoring B cell (Daudi) killing by NK-92 cells and TALL-104 cells, the data analysis techniques used here are broadly applicable for xCELLigence assays. Daudi cells were immobilized at a density of 80,000 cells/well in an E-Plate 96 by pre-coating wells with anti-CD40 antibody. 19.9 hours after seeding the Daudi cells, effector cells (NK-92 or TALL-104) were added in different quantities (shown at the bottom of this legend). Impedance was subsequently monitored for an additional 70 hours. (A) Raw data. Two NK-92 cell densities, three TALL-104 cell densities, and a negative control (lacking effector cells) are each evaluated in duplicate wells. (B) Data averaging. Data from the duplicate wells has been averaged and the corresponding standard deviation is shown. (C) Data normalization. To establish a common reference point for comparing the different cytotoxic treatments, all data traces from (B) have been normalized to the data point immediately prior to effector cell addition. This data point is denoted by the black vertical line at ~19.9 hours. (D) Using baseline subtraction. In addition to normalizing all curves to a common time point, the curve for the negative control (lacking effector cells) has been subtracted from all other curves. For clarity, standard deviations aren’t shown here.
Troubleshooting

Potential problems and corresponding solutions are listed below:

1. There is large variation between the replicates.
   - It is important to perform the full 30 minute room temperature incubation after seeding B cells. This ensures even distribution of the immobilized B cells across the electrodes.
   - To ensure similar cell seeding densities between wells, make sure cells are well suspended prior to seeding into the E-Plate 96.
   - The reverse pipetting technique is effective for preventing bubble formation and assuring consistent volume dispensing.

2. No signal is detected in wells expected to have an attachment signal.
   - Verify that B cells have indeed been immobilized on the E-Plate 96 well bottoms by replacing nascent medium (which may contain un-captured cells) with fresh medium and then examining wells under a microscope.