User Guide

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Using the xCELLigence RTCA Instruments to Perform Cell Adhesion Assays

Cell adhesion guide

Overview

Cellular impedance explained

Positioned between reductionistic biochemical assays and whole organism *in vivo* experimentation, cell-based assays serve as an indispensable tool for basic and applied biological research. However, the utility of many cell-based assays is diminished by the need to use labels, incompatibility with continuous monitoring (only endpoint data is produced), incompatibility with orthogonal assays, and the inability to provide an objective/quantitative readout. Each of these shortcomings can be overcome by Agilent xCELLigence label-free, real-time cellular impedance assays.

The functional unit of a cellular impedance assay is a set of gold biosensors fused to the bottom surface of a microplate well (Figure 1). When submersed in an electrically conductive solution (such as buffer or standard tissue culture medium), the application of an electric potential across these biosensors causes electrons to exit the negative terminal, pass through the bulk solution, and deposit onto the positive terminal to complete the circuit. Because this phenomenon depends on the biosensors interacting with the bulk solution, the presence of adherent cells at the biosensor-solution interface impedes electron flow. The magnitude of this impedance depends on the number of cells, their size and shape, and the cell-substrate attachment quality. The gold biosensor surfaces and the applied electric potential (22 mV) do not have an effect on cell health or behavior.

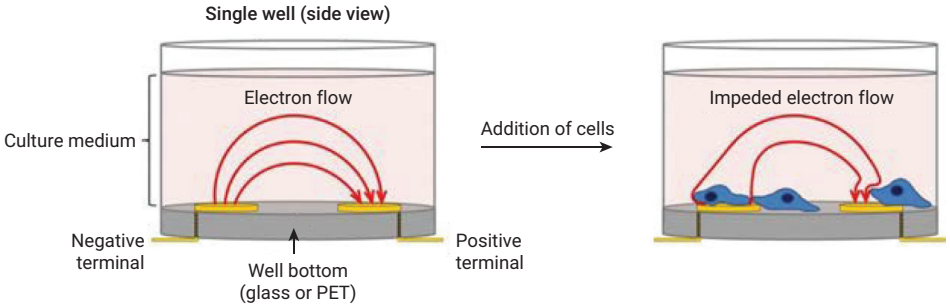

Figure 1. Overview of cellular impedance apparatus. A side view of a single well is shown before and after cells have been added. The biosensors and cells are not drawn to scale (they have been enlarged for clarity). In the absence of cells, electric current flows freely through the culture medium, completing the circuit between the biosensors. As cells adhere to and proliferate on the biosensors, current flow is impeded, providing a sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality.

Impedance electrodes

The gold biosensors in each well of the Agilent E-Plates cover 60% to 80% of the surface area (depending on if a view area is present). Instead of the simplified biosensor pair shown in Figure 1, the electrodes in each well are linked into "strands" that form an interdigitated array (Figure 2A). This arrangement enables populations of cells to be monitored simultaneously, providing sensitivity to: the number of cells attached to the plate, the size/morphology of the cells, and the cell-substrate attachment quality.

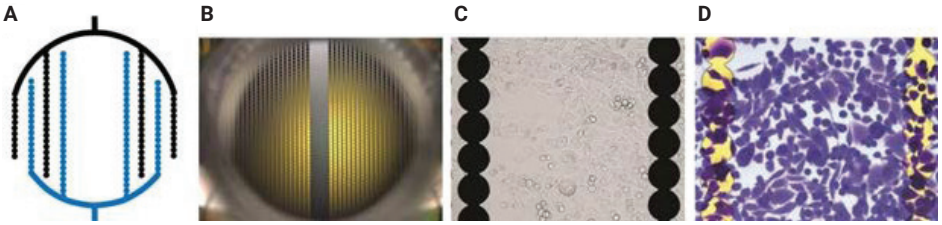

Figure 2. Impedance biosensors on Agilent E-Plates. (A) Simplified schematic of the interdigitated electrodes used in each well of an E-Plate. Electrodes are not drawn to scale (only a few are shown, and they have been enlarged for clarity). Though cells can also be seen on the gold biosensor surfaces, the biosensor-free region in the middle of the well facilitates microscopic imaging (bright-field, fluorescence). (B) Image of a single well in a 96-well E-Plate. (C) Zoomed in bright-field image of shadowed electrodes and unstained human cells. (D) Gold biosensors and crystal violet stained human cells, as viewed in a compound microscope.

Cell adhesion

Real-time impedance traces explained

The impedance of electron flow caused by adherent cells is reported using a unitless parameter called Cell Index (CI), where CI = (impedance at time point n – impedance in the absence of cells)/nominal impedance value. Figure 3 provides a generic example of a real-time impedance trace when setting up and running an apoptosis experiment. For the first few hours after cells have been added to a well, there is a rapid increase in impedance. This is caused by cells falling out of suspension, depositing onto the biosensors, and forming focal adhesions. If the initial number of added cells is low and there is empty space on the well bottom, cells will proliferate, causing a gradual, steady increase in CI. When cells reach confluence, the CI value plateaus, demonstrating that the biosensor surface area accessible to the bulk media is no longer changing. Adding an apoptosis inducer at this point causes a decrease in CI back down to zero. This is the result of cells rounding and then detaching from the well bottom. Impedance-based assays are flexible and can also evaluate the rate and extent of initial cell adhesion to the biosensors, morphological changes, and cell proliferation.

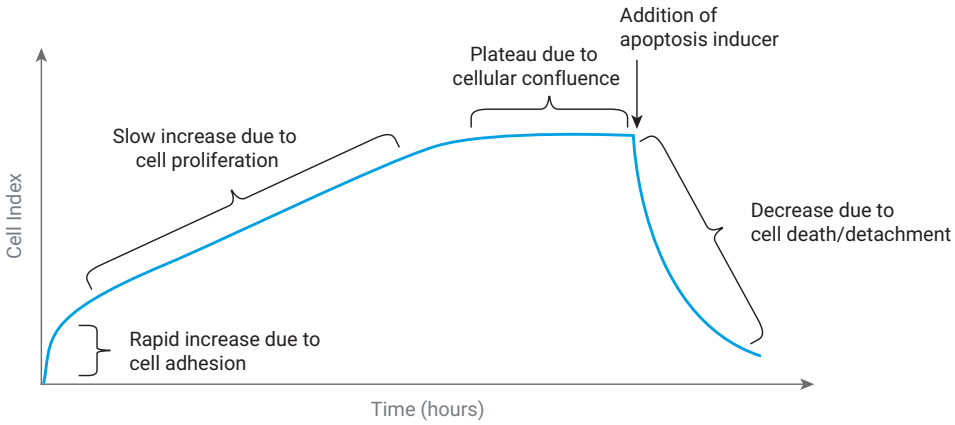

Figure 3. A generic real-time impedance trace for setting up and running an apoptosis assay. Each phase of the impedance trace and cellular behavior is explained.

Introduction

This guide is for assessing cell adhesion using the xCELLigence RTCA single plate (SP) instrument. Note that other Agilent xCELLigence instruments (RTCA multiple plates (MP), dual purpose (DP), single 16-well plate (S16), and high throughput (HT)) may also be used. Although these instructions have been optimized using the NIH/3T3 mouse fibroblast cell line, most other adherent cell lines can be used as well.

Reagents, materials, and equipment

Reagents and materials

Item	Manufacturer	Description
NIH/3T3 Cells	ATCC	Mouse fibroblasts
PBS	Hyclone	1x DPBS (-Ca, -Mg, -Phenol Red)
Trypsin	Gibco	0.05% Trypsin-Edta (1x), Phenol Red
Pen-Strep	Cellgro Mediatech	10,000 IU penicillin, 10,000 mg/mL streptomycin
FBS	Hyclone	Fetal bovine serum characterized
DMEM	ATCC	Dulbecco's Modified Eagle's Medium
Filter Unit	Nalgene	Pore size: 0.20 mm; PES membrane: 90 mm
E-Plate 96	Agilent Technologies	96-well electronic microplate

Equipment

Item	Part Number
xCELLigence RTCA SP – Bundle (complete system)	380601030
xCELLigence RTCA analyzer	5228972001
xCELLigence RTCA SP station	5229057001
xCELLigence RTCA control unit	5454417001

Overview

This three-hour assay has been optimized for monitoring the adhesion of NIH/3T3 cells to fibronectin-coated surfaces.

Workflow summary

1. Warm up reagents.
2. E-Plate 96 preparation and background measurement.
3. Cell preparation.
4. Cell seeding in E-Plate 96.
5. Incubation and monitoring of cell attachment.

Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	No fibronectin											
B	Fibronectin 0.1 µg/mL											
C	Fibronectin 0.2 µg/mL											
D	Fibronectin 0.5 µg/mL											
E	Fibronectin 1 µg/mL											
F	Fibronectin 5 µg/mL											
G	Fibronectin 10 µg/mL											
H	Fibronectin 20 µg/mL											

Software setup

Step	Sweeps	Intervals	Unit	Comments
1	1	1	Minute	Background reading
2	60	3	Minutes	Adhesion monitoring

Detailed instructions

Day 1

1. Warm up growth media and trypsin in a 37 °C water bath 30 minutes before the start of the experiment (30 minutes).
2. E-Plate 96 preparation and background measurement (75 minutes).
    a. Inside a tissue-culture hood, remove the E-Plate 96 from its packaging.
    b. Add 50 µL of the indicated fibronectin solution, or sterile water (control), to each well. Ensure that the entire bottom surface of the well is covered with the solution.
    c. With the plate lid firmly in place, incubate at 37 °C for 1 hour (in the cell culture incubator).
    d. Following this, aspirate the fibronectin solution and wash the wells twice with 100 µL of PBS.
    e. Add 50 µL of prewarmed media to each of the wells that will be used in the experiment.

3. Place the E-Plate 96 into the RTCA SP station. Open the RTCA software and enter the plate map on the Layout tab. Begin Step 1 (1 minute and one sweep) to perform the background measurement. Remove the E-Plate 96 from the station and place the plate back in a tissue culture hood for cell seeding.
4. Cell preparation (15 minutes).

   Critical: Like any other cell-based assay, the ultimate success of this assay using the xCELLigence system depends on the cell quality and how cells are handled. Following the steps described here is imperative for ensuring reliable and reproducible results. It is also important to note the passage number of the cells because, for some cell types, the intensity of the adhesion response can change with increasing passage number.

   a. Cells should be passaged the day before the experiment so that they are 60 to 80% confluent.

   b. Remove serum-containing media from the flask and gently rinse cell monolayer once with PBS.

   c. Trypsinize cells by adding 3 mL of 0.25% Trypsin/EDTA solution per T225 flask and leave the flask at room temperature or in a 37 °C incubator for 1 to 5 minutes.

      Critical: Observe the cells under a microscope intermittently during trypsinization to check when they become detached. Do not over trypsinize the cells as this can be toxic.

   d. Stop trypsinization by adding serum-containing media at a volumetric ratio of 9:1.

   e. Count the cells and adjust the concentration of the cell suspension to 200,000 cells/mL.
5. Cell addition to E-Plate 96 (10 minutes).

   a. Add 50 µL of cell suspension to each well of the E-Plate 96 (10,000 cells/well).
6. Monitor cell adhesion in real time (3 hours).

   a. Transfer the E-Plate 96 to the SP station inside a 37 °C incubator.

   b. Start Step 2 of the RTCA program, monitoring impedance for 3 hours, with readings taken every 3 minutes.

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