ECIS® Application Note

Cell-Extracellular Matrix Interactions (cell attachment and spreading)

In this application note we describe how the ECIS® system can be used to monitor and record the dynamic behavior of cells as they attach and spread upon substrates coated with extracellular matrix (ECM) proteins.

General Overview

Macromolecules including ECM proteins will spontaneously adsorbed to hydrophilic surfaces such as treated polystyrene culture dishes. When inoculated with cells, these adsorbed layers are detected by trans membrane proteins and the cell response is reflected in their attachment and spreading behaviors.

The basic ECIS assay involves coating electrodes with proteins of interest, inoculating the electrodes with a cell suspension and monitoring the dynamics of cell attachment and spreading via impedance measurements.

In this note, we will describe:

- Choosing the electrode array
- Preparing the electrode with specifically adsorbed protein
- Inoculating the array
- Recording and interpreting the resulting impedance data

Choosing the electrode array

With this assay it is best to follow a large number of cells to obtain a good statistical sampling of their cell behavior. For the 16 well and 96 well stations we recommend the 8W10E+ array and either the 96W10idf or96W20idf electrode configuration respectively.

Preparing the electrode surface with adsorbed proteins

With ECIS arrays, all substrate surfaces are able to adsorb protein molecules including the gold film electrodes.

To coat the electrode with protein

1) Make up a solution of the protein of interest in saline (0.15M NaCl)*; the protein concentration should be 50 micrograms per ml or higher.
2) Flood the wells of a recently acquired array[Ŧ] with the protein solution such that all of the substrate surfaces are wetted (~100-200 microliters/well)
3) Allow the protein to adsorb for 30 minutes or longer.

4) Aspirate the protein solution and flood the well with 0.1 mM cysteine in saline
5) After 30 minutes or longer aspirate the cysteine solution and rinse the well with sterile serum-free medium
6) Keep serum-free medium in the well while preparing the cell inoculum or move immediately to inoculation

* If buffering is desired we suggest Tris rather than phosphate buffer as for some proteins that phosphate can interfere with adsorption

Ŧ For this assay it is recommended to use arrays that have not been in storage more than one month

Inoculating the array

1) Harvest cells using standard protocols and prepare a uniform, mono-disperse cell suspension in serum-free medium (see table)
2) Warm the cell suspension to 37°C
3) Aspirate the water or medium from each well and add the cell suspension keeping it thoroughly mixed throughout the inoculation process (see table)
4) Allow the array to stand at room temperature for 20 minutes while cells settle and begin attachment to the protein coated surfaces.

Array type	Cell suspension	Volume of suspension in each well	Cells/cm^2
8 well	2.5x10^5 cells/ml	400 microliters	~1.25x10^5
96 well	1.25x10^5 cells/ml	300 microliters	~1.25x10^5

Recording and interpreting the resulting impedance data

Mount the array in the Array Station within the cell incubator

Click Setup and verify that all wells being used are connected (green in Well Configuration view)

Choose the type of array

Begin collecting time course data, the MFT with the standard default frequencies is recommended.

Continue to take data until the cell layer is confluent and well established.

One may also consider monitoring cell migration rates once a confluent layer is established following the standard ECIS wound healing/cell migration protocol.

Remember in addition to monitoring Z and R, the capacitance (C) measured at high frequency (32,000 or higher) is an excellent means to look at cell coverage and access the degree of confluence of a cell layer.

Sample run with a 96 well ECIS plate

Well Preparation

1) 16 wells (two columns) of a 96W20idf plate were flooded with 100 microliters of a 50 microgram/ml solution of bovine plasma fibronectin dissolved in sterile saline
2) 16 other wells of the same plate were flooded with 100 microliter of a 50 microgram/ml solution of bovine serum albumin (BSA) in sterile saline
3) The plate was incubated at room temperature for 30 minutes
4) The protein solutions were then aspirated from the wells and 200 microliters of a sterile solution of 10 mM cysteine in 0.15M NaCl added to all well and allowed to incubate for 30 minutes.
5) The cysteine solution was then aspirated and 300 microliter of serum free DMEM added to each well and the plate stored in the 37°C tissue culture incubator until the cell suspension was ready.

Cell Harvesting

One T-25 flask with a confluent layer of MDCK II freshly fed cells was used to prepare the cell suspension.

(1) The medium over the cells was aspirated and the cell layer flooded with ~3 ml of an EDTA solution (0.02% EDTA in PBS without Ca^{++} and Mg^{++}). After ~ 30 sec this was aspirated from the flask (this was repeated three times).
(2) 3 ml of a solution of trypsin-EDTA (0.5g porcine trypsin and 0.2 g EDTA/ liter of HBSS) was added over the cell layer and incubated ~ 30 seconds. This solution was aspirated and replaced with 2 ml of trypsin EDTA. The plate was incubated at 37°C.
(3) When the cells were clearly sloughing off the bottom of the flask (~10 minutes) they were taken up in 10 ml of complete DMEM with 10% serum and centrifuged at 200 x g for 10 minutes.
(4) The supernatant was aspirated and the pellet resuspended in 10 ml of serum-free DMEM and centrifuged as above to pellet the cells.
(5) The pellet was resuspended in 12ml of serum-free DMEM and the cell suspension placed in a 37°C water bath.

Inoculation

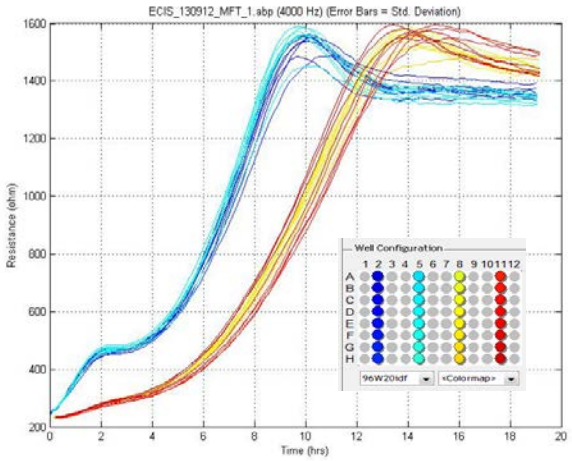
(1) The ECIS array was removed from the incubator and the DMEM aspirated from each well.
(2) 300 microliters of cell suspension was added to each well with continual mixing of the cell suspension to assure that equal numbers of cells were delivered to each well.
(3) The inoculated plate was allow to stand 15 minutes at room temperature to prevent possible convection currents and an uneven distribution of cells in the well.

Data gathering

(1) The array was inserted into the 96W ECIS array station, and setup was run to verify proper connection.
(2) A multiple frequency time course (MFT) was started and allowed to run. The program was stopped after about 19 hours.

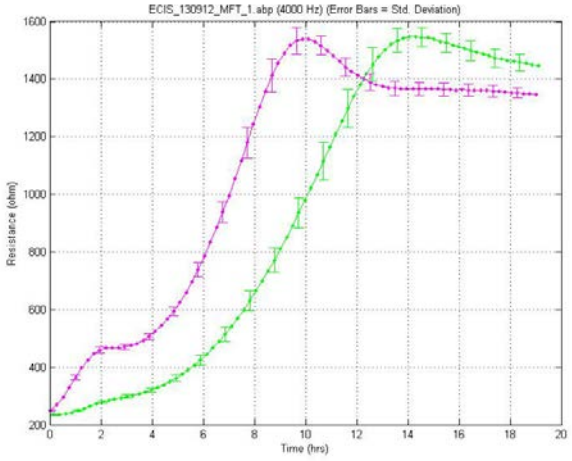
Results

Here is the data obtained from this run, where the resistance at 4000 Hz is plotted as a function of the time post inoculation. The blue and turquoise traces are the 16 wells coated with adsorbed fibronectin and the red and yellow traces the bovine serum albumin results.

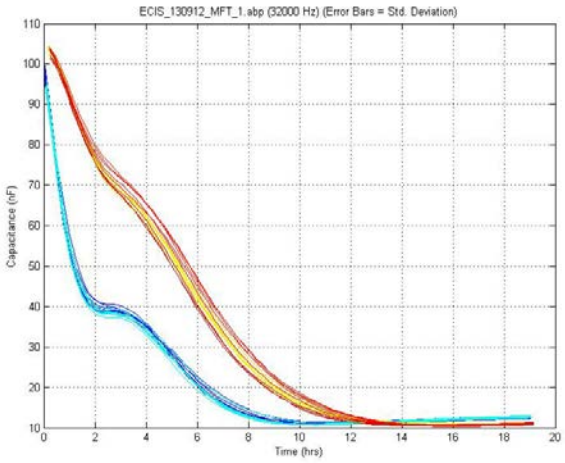


Notice the considerably more rapid rise in the fibronectin coated electrodes especially in the first two hours of the run.

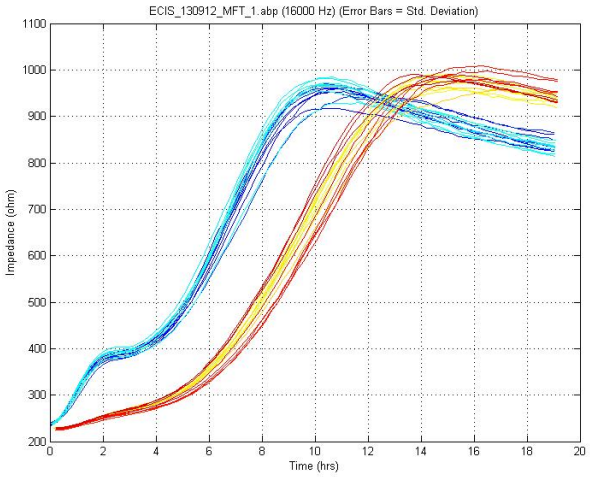
A graph is also generated showing the average values obtained from each group of 16 wells with error bars representing plus and minus one standard deviation.



One can also evaluated the rate at which confluence was finally achieved by looking at the capacitance at 32,000 Hz. The capacitance at this frequency with a complete confluent layer of cells upon the substrate is ~10 nF. In the data shown, confluence is achieved at ~ 10 hours post inoculation with fibronectin and ~ 12 hours with BSA. Note for this cell line, these times agree with the peak R and Z obtained



Finally, a plot of the changes in total impedance (Z) at 16,000 Hz is displayed



Ancillary information

Protein adsorption

Protein adsorption results in a monomolecular protein layer that can range from 0.1 to about 1.0 micrograms per cm^2 depending upon the nature of the protein molecule and the time allowed for adsorption to occur. The protein molecules spontaneously bind to the surfaces by a variety of mechanisms including charge-charge interactions, van der Waal interactions and, in the case of gold, bonds between sulfhydryl groups of the cysteine residues and the gold itself.

The best means of assuring a reproducible adsorbed protein layer is to use a sufficiently high concentration of protein. The minimum suggested 50 micrograms per ml assures that even if some protein has been lost by adsorption to the walls of the vessels holding the solution, sufficient protein will be available to complete the adsorption process in a few minutes time.

It has been observed that with some proteins phosphate buffer interferes with the adsorption process. To avoid this potential problem we suggest that PBS be avoided as a solute for protein but rather recommend a simple saline solution. Should the researcher feel a buffer is essential the use of TRIS can be used (e.g. 0.05M TRIS, 0.10 M NaCl).

Since changes in the impedance of the ECIS electrodes start to take place upon the addition of a cell suspension, it is most important that the properties of the substrate and in particular the gold sensing electrode be established at an equilibrium value. In this manner, only the response of the system to the attachment and spreading of cells is detected. This is assured in the suggested protocol by the use of cysteine to stabilize any open areas of gold and the use arrays that have not been in long term storage.

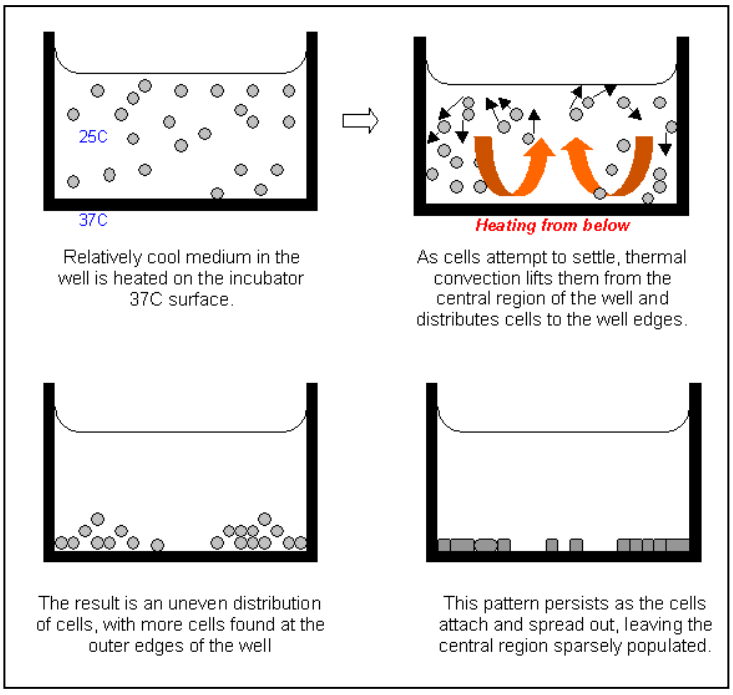
Uniform cell inoculation

Critical in studying cell attachment and spreading is a uniform inoculation of cells upon the substrate and hence the measuring electrodes. Careful inoculation is the key to good reproducibility from well to well and from experiment to experiment. It is most important to obtain a uniform inoculation of the well bottom. To accomplish this, wells should receive an even "snowfall" of cells so that each electrode has approximately the same number of cells falling on its surface - this can be facilitated by attentively following some important guidelines:

1. Make up a monodisperse cell suspension.
For some cell lines this is easily achieved, but for others, particularly if cells have been attached and spread for long periods of time, clumping takes place and longer trypsinization may be needed. The goal is to achieve is a clearly monodisperse suspension of single cells.

2. Keep the suspension agitated

It is also important to keep the cell suspension uniform so each well receives approximately the same number of cells; agitate the cell suspension frequently to prevent settling of the cells.



3. Add the suspension to an empty well

If possible, avoid mixing the cell suspension with liquid already in the well. It is best to remove all media from wells before adding the cell suspension such that no mixing within the wells is required. If a cell suspension must be added to liquid already in the well, thorough mixing of the two solutions is essential.

4. Allow the cells to settle and attach with the array outside of the incubator

Temperature considerations are often overlooked and can be extremely important when dealing with cell distribution in small wells.

If the temperature of the cell suspension is lower than the temperature of the incubator, when placed in the incubator, the wells will be heated from the bottom. This will cause a convection cell to form, where medium rises in the center and falls back down the walls of wells. Due to this flow, as cells attempt to fall to the central region of the well, they are swept upward. The overall effect is that the cell density becomes reduced in the central regions of the well and the electrodes will not be uniformly populated with cells.

Here are two ways to deal with this problem

1) Simply inoculate the ECIS arrays outside of the incubator using room temperature medium, and then wait 20 to 30 minutes before placing the array in the incubator space.. Since there is no heating from below, there is no thermal convection, as the cells settle uniformly over the entire substrate and begin to attach to the surface. We recently started using this approach, and the results have been most satisfactory. CO_2 dependent medium would experience some pH increase out of the incubator in this protocol, and one could consider ways to avoid this. In our hands, however, we have not found a pH drift to be a problem, as once in the incubator space, we observe normal cell attachment and spreading impedance data.

2) Alternatively, if solution #1 cannot be used, we then recommend using a cell suspension and array that are pre-warmed to incubator temperature and immediately place the array into the incubator upon inoculation. The goal again is to prevent thermal convection by having no heating of the wells taking place during cell settling.

Abbreviations :

EDTA ethylenediaminetetraacetic acid

PBS phosphate buffered saline (Dulbecco formulation)

DMEM Dulbecco's Modified Eagles Medium