Protocol for Using the DP System to Perform

Cell Migration and Invasion Assays with HUVEC cells

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Cell Migration Protocol Using the CIM Device

A. Cell Migration Assays Using the CIM Device with the RTCA DP System

The protocol described below is a basic migration assay protocol for the xCELLigence RTCA DP system. It has been adapted to be used with HUVEC cells and various chemoattractants. Assay conditions may be subject to further optimization if different types of cells or chemoattractants are used.

MATERIALS

REAGENTS (to be provided by the BioLabor)

- HUVEC cells and media

  ♦**Critical**- The ultimate success of the migration experiments is dependent upon cell culture conditions prior to the assay and conditions used for detaching the cells from the flask. The number of cells used in a migration experiment will depend on the cell type and the chemoattractant being used. It is imperative to conduct preliminary experiments and determine the optimal cell number for each cell line. For HUVEC, we recommend to initially seed cells in the range of 20,000-40,000 cells in a final volume of 100 ul into each TC well; and to seed 5,000 cells per well in a standard E-plate 16.
- Fibronectin solution at a concentration of 10 - 20 µg/ml

We need a total of 5 ml of the solution, since we have to coat on both sides.

- Trypsin-EDTA Solution for cell detachment or non-enzymatic cell dissociation solution for cells that may be especially sensitive to enzymatic methods of cell detachment

  ♦**Critical-** If using protease method for cell detachment, it is important to minimize the time of incubation with the detachment solution. Cell surface receptors, such as integrins play an important role in cell migration and it is important to conserve the number and integrity of these receptors on the cell surface as much as possible.
- Serum-Free Media (SFM)- Use the same media that the cells are cultured in, without the serum / growth factors or only a minor percentage of serum or growth factors (e.g. 0.5% serum)

  ♦**Critical-** Certain cell types are sensitive to total absence of serum / growht factors and it maybe necessary to include some serum / growth factors in the SFM. We recommend testing a range of concentrations for optimal results.
- Chemotaxis inducer- Typically, the SFM supplemented with serum in the range of 5%-10%, conditioned fibroblast media, or SFM supplemented with chemotactic agents such as growth factors. For HUVEC, VEGF and HGF can be used as chemoattractant (see App Note). As a control, use complete HUVEC growth medium as chemoattractant.

  ♦**Critical-** If low serum or BSA was used as part of the SFM, it maybe important to add the chemotactic factor to the same media.
- PBS without Calcium and Magnesium

EQUIPMENT

- 16 well CIM devices (provided by the BioLabor)
- Standard E-Plate 16 (provided by the BioLabor)- Even though not absolutely required, we highly recommend to test the conditions of the cells concurrently on a standard E-Plate 16.
- CIM device assembly tool (provided by the BioLabor)
- DP System (provided by the BioLabor)

PROCEDURE

WORKFLOW SUMMARY:

Day -2 (Monday):

1. Trypsinize the HUVEC cells and seed at a density that ensures log-phase proliferation until the experiments are started.

Day -1 (Tuesday):

1. Serum-starve the cells overnight, or at least for 6h prior to the experiment.

Day 1 (Wednesday):

1. Perform DP Resistor Plate test at RT
2. Desinfect Device with 70% Ethanol. Place Device in Incubator with cable attached, but do not connect to Laptop! Equilibrate for 2h, perform DP Resistor Plate test at 37°C.
3. In the meantime, coat both the upper and lower part of the membrane of one CIM plate with Fibronectin at 10 - 20 µg/ml for 1h at 37°C. If necessary, coat the E-plate 16 with Fibronectin using the same conditions.
4. Equilibrate the CIM devices with medium (SF in the TC, w/ chemoattractant in the BC) for 1h at 37°C
5. Prepare the cells, adjust cell suspension to 20k and 30k per 100 µl in SF medium
6. Perform background measurement
7. Add cells to TC and to E-Plate 16, leave plates at RT for 30 min
8. Start measurement

Day 2 (Thursday):

9. Stop measurement and analyze the data
10. Optionally, stain the migrated cells on the underside of the membrane and examine the stained cells under microscope.

Day 1:

CIM Plate Coating ♦ **Timing 10 min + 1h**

1. Inside a tissue culture hood, remove both top chamber (TC) and bottom chambers (BC) from the package (The top chamber packet contains Plate ID, Lot and Expiration Date information). Place the CIM plate assembly tool inside the hood with blue markings away from you. Place the BC in individual pockets of the assembly tool to ensure the bottom chambers sits flat inside.

♦ **Critical -There is only one correct orientation for the bottom chamber inside the assembly tool, i.e., the blue marking on the bottom chamber should match the corresponding position of the blue marking on the assembly tool.**

2. Fill each bottom chamber well with 160µl coating solution using an 8 channel pipette. Ensure that a meniscus is formed on each well after the well is filled with media.

♦ **Critical**-To prevent bubble entrapment in the bottom chamber, use reverse pipetting, or the "dispense" function of an electronic pipette. In the event of bubble formation, try using a dry tip to "pop" the bubble or carefully try to remove it using a pipette.

3. Place the TC in one of the unused pockets of the assembly tool in the **correct orientation**, where the blue marking on the assembly tool lines up with the blue marking on the TC. Turn the assembly tool 90 degree counter-clockwise and then pick up the TC using both hands and carefully place the TC onto the BC with the sensor surface facing down and the blue marking on the TC matching the same position on the BC.

♦ **Critical - Ensure that the TC and BC are level and parallel to each other as the device is being assembled Push the TC downwards onto the BC to lock the two chambers together**.

4. Add 50 µl of coating solution to each well of the upper chamber. During pipetting, do not introduce any bubbles and avoid the pipette tips touching the membrane.

5. Incubate the device at 37°C for 1 h; alternatively, coat at 4°C overnight

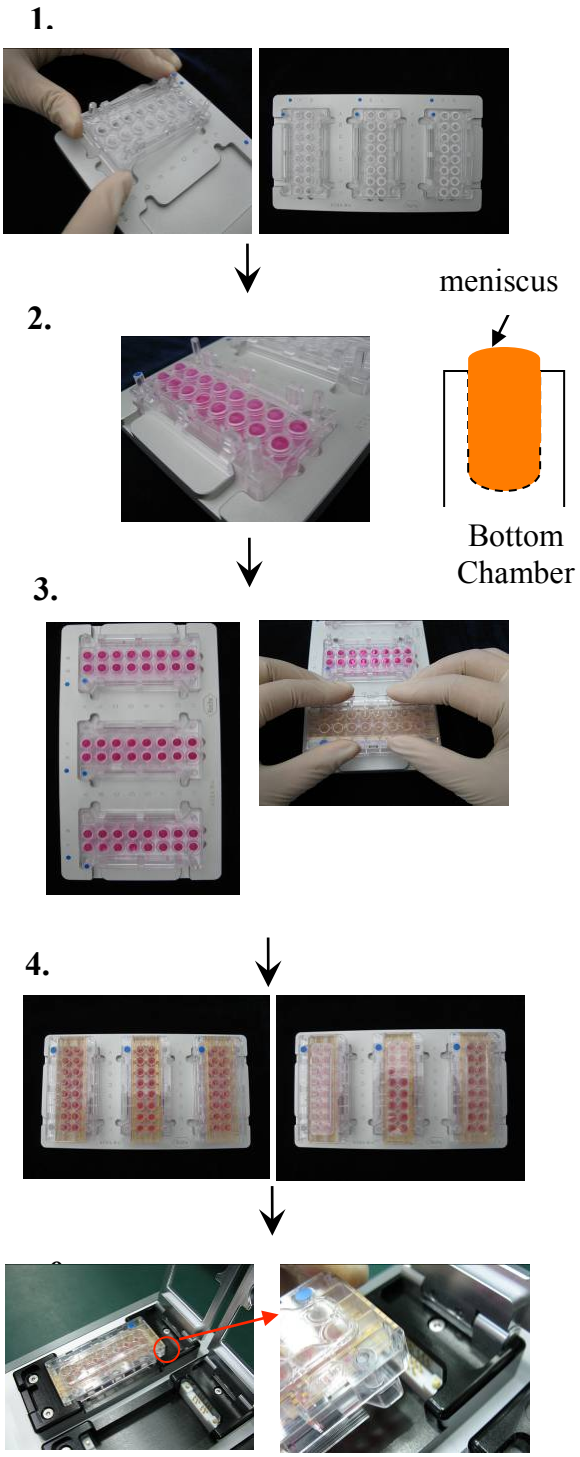

Figure 3: Directions for assembly of CIM device.

E-Plate 16 coating ♦ **Timing 10 min + 1h**

1. Inside a tissue culture hood, unpack the E-Plate 16 from the package (the package contains Plate ID, Lot and Expiration Date information).

2. Fill each well of the E-Plate 16 with 50 µl of the fibronectin solution

3. Incubate the device at 37°C for 1 h; alternatively, coat at 4°C overnigh

CIM plate equilibrium at 37 ºC ♦ **Timing 10 min + 1h**

1. Prepare SFM medium with the designated chemoattractant at various concentrations, e.g. in 15 mL tubes.

2. Place the device back into the cell culture hood onto the assembly tool. Carefully remove the coating solution from the wells of the TC **without touching the membrane.** Disassemble the TC from the BC, and remove coating solution from the BC wells as well.

♦**Critical**- Collect the coating solution, this can be re-used.

3. Fill each bottom chamber well with 160µl media (with and without chemoattractant) using an 8 channel pipette. Ensure that a meniscus is formed on each well after the well is filled with media, and avoid air bubbles.

4. Place the TC in front of you in the **correct orientation**, where the blue marking on the assembly tool lines up with the blue marking on the TC. Turn the assembly tool 90 degree counter-clockwise and then pick up the TC using both hands and carefully place the TC onto the BC with the sensor surface facing down and the blue marking on the TC matching the same position on the BC.

♦**Critical- Ensure that the TC and BC are level and parallel to each other as the device is being assembled. DO NOT TILT THE TC AT AN ANGLE**

Push the TC downwards onto the BC to lock the two chambers together

♦**Critical –Make sure to hear two clicks indicating that the two chambers are locked together and sealed properly.**

5. Add 50 ul serum free media to each well of the top chamber to cover the membrane surface. During the medium addition, do not introduce any bubbles and avoid the pipette tips touching the membrane.

6. Place the CIM plate onto RTCA DP Analyzer (the tapered corner on the CIM plate should match to the tapered corner on the DP Analyzer) inside the 37˚C incubator. Incubate for 1 hour to allow the CIM membrane surface to reach an equilibrium with media.

7. Place the E-Plate 16 into the cell culture hood, and carefully remove the coating solution from each well.

8. Add 50 ul of the designated medium to each well of the E-plate 16.

9. Place the E-Plate 16 onto RTCA DP Analyzer (the tapered corner on the plate should match to the tapered corner on the DP Analyzer) inside the 37˚C incubator.

10. Since you do not need to equilibrate the E-Plate 16 for an hour prio background measurement, you may perform this step at the end of the CIM plate equilibration phase.

Background measurement♦ **Timing 5 min**

1. Program the RTCA SW with the experimental details, plate layout, and schedule.

2. After the 1h equilibration phase, start step 1 (1 minute and 1 sweep) in RTCA SW to perform background measurement.

Cell preparation♦ **30 min**

♦**Critical** - Like any other cell-based assay, the ultimate success of the migration experiment using the CIM system depends on the quality and handling of the cells. It is imperative to critically review and follow the steps for cell maintenance and handling in order to insure obtaining reliable and reproducible results. Also, it is important to make a note about the passage number of the cells as certain cell types can attain higher levels of background migration with increasing passage number.

1. Cells should be passaged the day prior to the experiment and should be in the range of 60-80% confluence.

2. Some cell types may possess an inherently high level of background migration and it maybe important to serum-starve the cells prior to detachment. Cells can be serum starved from 1-16 hours prior to detachment for migration assay. These conditions need to be determined empirically.

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

4. Trypsinize cells by adding 0.5 ml of 0.05%/Trypsin/EDTA solution per T25 flask and leave the flask at room temperature or 37 C for 1-2 minutes.

♦**Critical-** Do not over-trypsinize the cells! Cell migration and invasion are dependent on the expression and integrity of cell surface receptors such as integrins and it is important to minimize the time of protease treatment. Certain cell types can be sensitive to protease-digestion and it may be important to explore alternative methods of cell detachment; i.e. using EDTA-based buffers.

5. Stop trypsinization by adding media with serum or TNS solution (from Clonetics, Cat No: CC-5002) at a ratio 1:1.

6. Wash trypsinized cells once with SFM by centrifugation. For most cells 5 min at 800*g* is sufficient.

7. Gently resuspend the cell pellet in a few milliliters of SFM and count the cells under a microscope using a hemocytometer. Adjust the concentration of the cell suspension to **3×10^5/ml** and **2×10^5/ml** using SFM. Use at least triplicates or quadruplicates for each experimental condition.

Cell addition to CIM plate♦ Timing 10 min

1. Add 100µl cell suspension to each well of the top chamber. The final number of cells per well should be 30,000 and 20,000, respectively.

2. An important control that should be included in this experiment is to include wells which contain SFM in the wells of the BC to assess any background migration of the cells.

♦**Critical-** It is important to be aware that bubbles can be entrapped in the bottom of the well, forcing cells to migrate at the edge or block cell migration altogether. The best way to avoid bubble entrapment is to first slightly tilt the device at an angle, followed by insertion of the pippette tips all the way to the bottom of the well of the TC (avoiding the electrodes) and slowly ejecting the cell suspension. If any wells contain air pockets or bubbles, then the volume level would be slightly higher than the other wells. If you observe this immediately after addition of cell suspension, you can try carefully aspirating the cell suspension and reapplying it to the well. Alternatively, if this is observed later, it is better to avoid removal of the bubble and simply make a note of it in your laboratory notebook.

Cell addition to E-Plate 16 ♦ Timing 10 min

1. Dilute 0.5 mL of the cell suspension containing **2×10^5/ml** cells 1:4 with the designated medium.

2. Add 100µl cell suspension to each well of the E-Plate 16 to obtain a cell number of 5,000 cells per well.

Place CIM and E-Plate at Room Temperature♦ Timing 30 min

1. Leave the plates in the hood at room temperature for 30 min after cell addition to allow the cells settle down on to the bottom surface of the top chamber.

Start Measurement♦ Timing 3-24 hrs

1. Place the plates containing the cells in the DP System inside the incubator, followed by the measurement with an interval of 15 minutes for 24 hours.

♦**Critical-** It is important to place the plates in the same module that was used for background measurement. Each device can have slightly different background impedance measurements, which can affect the coefficient of variation of the data.

♦**Critical-** For most cell types the length of the migration assay is limited to anywhere from 3-24 hours. With the xCELLigence DP system, you can monitor your migration assay for as long as you like. However, it is important to keep in mind that one of the main factors determining the length of the migration assay is based on the doubling time of the cell. When analyzing the data, it is important to choose a time point that is well below the doubling time of the specific cell type in order to avoid complications arising from capturing impedance signals derived from cell proliferation rather than cell migration.

Data Analysis

1. Stop the measurement after 24 hrs.
2. Average all the replicates on the display page
3. Analyze the Cell Index (CI) curves. The positive migration signals should meet the criteria of averaged CI ≥ 0.2.

♦**Critical**- It is important to note that different cell types may produce impedance changes of varying extent depending on the number of cells added and the overall volume and morphology of the cells. If the CI of a cell type at a given density is within a CI of 0.2, then there are a number of parameters that can be optimized to increase the overall signal, including further increasing the number of cells added and testing various conditions for coating the membrane with various ECM proteins.

★**Recommendation**- Even though not required, it is recommended when performing pilot experiments to stain the cells on the underside of the membrane to get an impression about the extent of cell migration and its comparison with CI signal. To stain the cells:

a. Disassemble the CIM plate by pressing the two handles on the BC and gently remove the TC.
b. Remove media from wells of the TC with a multi-channel pipette while avoiding contact with the membrane.
c. Stain the migrated cells on the CIM membrane. Diff-Quick staining kit by Fisher Scientific is recommended (Cat No: NC9409820). The modified staining procedure is briefly described below:
d. Place the TC into fixative solution for 2 min with membrane side containing the electrodes in direct contact with the fixative.
e. Place the TC into solution I for 1 min with membrane side containing the electrode in direct contact with the solution.
f. Place the TC into solution II for 1 min with membrane side containing the electrode in direct contact with the solution.
g. Gently rinse the TC with water and visualize and count the cells under an upright microscope.