

Using the xCELLigence RTCA DP Instrument to Perform Cell Invasion and Migration (CIM) Assays

Overview

The xCELLigence RTCA DP instrument

The Agilent xCELLigence real-time cell analysis (RTCA) dual purpose (DP) instrument enables label-free, real-time monitoring of cell proliferation, morphology, and attachment quality. The DP model has an additional capability of monitoring cell invasion and migration. The instrument contains three plate cradles (Figure 1) that can be operated simultaneously or independently, enabling maximum productivity for multiple users. The DP instrument is placed inside a standard CO₂ incubator and is powered by a cable connected to a workstation outside the incubator. The user-friendly RTCA software allows real-time recordings of all three cradles, and includes data display and analysis functions. The DP instrument can use, in 16-well formats, both the standard electronic plate (Agilent E-Plate 16) and the electronic cell invasion and migration plate (Agilent CIM-Plate 16). The E-Plate 16 is available with either a glass or polyethylene terephthalate (PET) bottom. In the Agilent E-Plate VIEW 16, biosensors are absent from a small region in the center of wells, facilitating visual inspection under a microscope.



Figure 1. Agilent xCELLigence RTCA DP instrument and workstation. The instrument has three separate cradles, and each holds a 16-well plate.

Cell invasion and migration plate

The cell invasion and migration plate (CIM-Plate 16) is a 16-well electronically integrated Boyden chamber, composed of upper and lower chambers that snap together (Figure 2). Pressure-sensitive silicone O-rings are present in the lower chamber to ensure a tight seal between the upper and lower chambers for each well (Figure 2C). A PET microporous membrane serves as the base of the upper chamber, allowing cells to translocate towards chemoattractant in the lower chamber (Figure 2B). The bottom side of the membrane is coated with gold biosensors (Figure 2B) that have an ability to detect the reduced electric current when cells adhere to their surface. This “impedance” signal enables a quantitative kinetic measurement of cell movement from the upper chamber to the lower chamber.

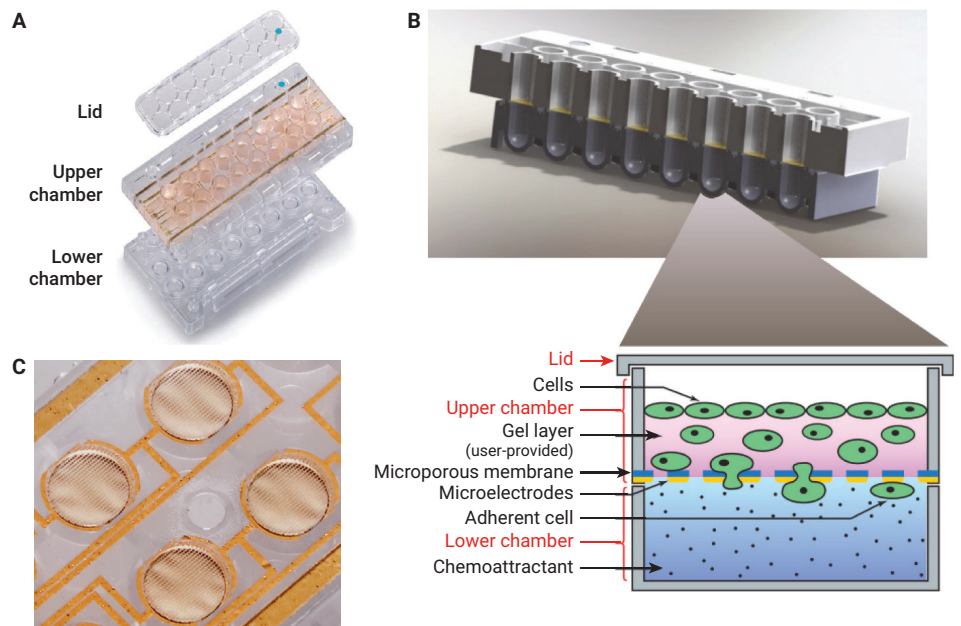


Figure 2. CIM-Plate overview. (A) CIM-Plate components. (B) A fully assembled CIM-Plate in detail. The expanded view illustrates the upper and lower chambers of a single well. Cells can migrate through the bottom membrane of the upper chamber and gold electrodes on the underside of this membrane detect the presence of adherent cells. (C) Gold biosensors are coated in the bottom surface of the upper chamber, composed of a PET microporous membrane. For a simple migration assay (not illustrated here), the cells being monitored would be plated directly onto the membrane. For an invasion assay (shown here), cells are plated on top of either the basement membrane matrix or a monolayer of cells.

Cell migration assay

Introduction

This process is for a basic migration assay using the xCELLigence RTCA DP instrument with fetal bovine serum (FBS) as the chemoattractant. These instructions have been optimized using the HT1080 human fibrosarcoma cell line. Assay conditions may require further optimization if different cell lines or chemoattractants are used.

Reagents and equipment

Reagents

- **Cells:** HT1080 cells were purchased from ATCC and were 60 to 80% confluent at the time of detachment.
Critical: The success of migration experiments is influenced by the cell culture conditions used before the assay, including the method of detaching the cells from the flask.
Critical: The number of cells used in a migration experiment will depend on the cell type. Conducting preliminary experiments is important in determining the optimal cell number for each cell line. It is recommended to initially seed 20,000 to 80,000 cells in a final volume of 100 μ L.
- **Cell detachment solution:** Trypsin-EDTA can be used for cell detachment, but for cells that are especially sensitive to protease treatment, a nonenzymatic cell dissociation solution should be used.
Critical: If using the protease method of cell detachment, it is important to minimize the time of incubation with the protease. Because cell surface receptors, such as integrins, play a significant role in cell migration, the number and integrity of these proteins should be preserved as much as possible.
- **Serum-free media (SFM):** Use the same media in which the cells are cultured, without the serum.
Critical: Because some cell types are sensitive to a total absence of serum, it may be necessary to include a low concentration of serum in the SFM. In this case, it is recommended to test a range of 0.1 to 2% serums. Alternatively, the SFM can be supplemented with bovine serum albumin (BSA) in the range of 0.25 to 0.5%. It is important to use highly pure forms of BSA and not a crude fractionation product, which may contain factors capable of influencing cell adhesion and migration.
- **Chemotaxis inducer:** This is typically fibroblast-conditioned media, or SFM supplemented with 5 to 10% serum or other chemotactic agents, such as growth factors.
Critical: The media in the upper and lower chambers should be identical except for the chemotaxis inducer (present in the lower chambers but absent in the upper chamber).
- PBS without calcium and magnesium

Equipment

Product	Part Number	Pack Size
Agilent xCELLigence RTCA DP instrument	380601050	One bundled package
CIM-Plate 16	5665817001	6 plates
CIM-Plate 16 assembly tool	5665841001	Assembly tool

Process overview

Workflow summary

This assay process requires two full days to complete. It has been developed for continuous monitoring of cell migration on the xCELLigence RTCA DP instrument for 24 hours.

Day 1

1. Assemble the CIM-Plate 16 (attach the upper chamber to the lower chamber). Equilibrate in 37 °C incubator for 1 hour, then take a background measurement.
2. Prepare cells and add them to a CIM-Plate 16.
3. Leave the CIM-Plate 16 at room temperature for at least 30 minutes to let the cells settle.
4. Load the CIM-Plate 16 into an xCELLigence RTCA DP instrument and start measurements.

Day 2

5. Stop data acquisition and analyze the Cell Index (CI) curves to determine cell migration activity.
6. **Optional:** Stain the migrated cells on the underside of the membrane and examine them under a microscope.

Detailed instructions

CIM-Plate 16 assembly (5 minutes)

1. Inside a tissue culture hood, remove both the upper and lower chambers from the packaging. Place the CIM-Plate 16 assembly tool inside the hood with the blue markings facing away from you (Figure 3A). Ensure that the lower chambers sit flat inside the assembly tool (shown in Figure 3B).

Critical: There is only one correct orientation for the lower chamber inside the assembly tool (the blue dot on the lower chamber should be positioned adjacent to the blue dot on the assembly tool), shown in Figure 3B.

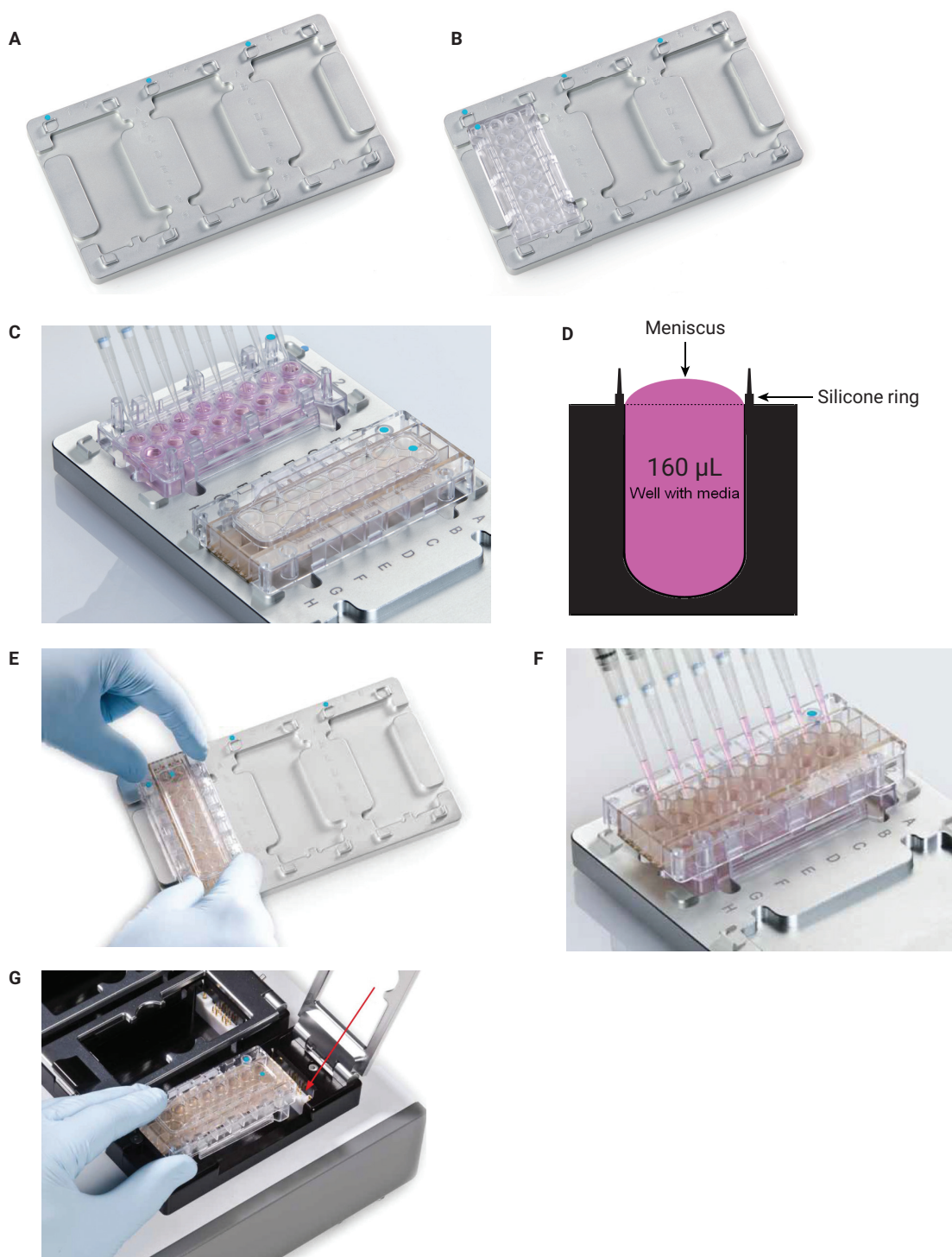


Figure 3. CIM-Plate 16 loading and assembly. (A) CIM-Plate 16 assembly tool with three indentations for holding CIM-Plate 16 upper and lower chambers. (B) Correct orientation of the CIM-Plate 16 lower chamber inside the CIM-Plate16 assembly tool. (C) Using the reverse pipetting technique to add media to a CIM-Plate 16 lower chamber. (D) Side view of a properly filled well in a lower chamber. A meniscus of media should protrude above the top of the well. The silicone ring allows for tight seal between the upper and lower chambers for each well. (E) Assemble the upper chamber and the lower chamber. (F) Using the reverse pipetting technique to add media to CIM-Plate 16 upper chamber. (G) Proper positioning of the CIM device in a cradle of the DP system. The red arrow indicates that the edge-cut corner on the CIM-Plate 16 matches to the edge-cut corner on the DP Instrument.

2. Fill each well of the lower chamber with 160 μL of media with or without serum (Figure 4C). Ensure that a meniscus is formed at the top of each well (Figures 3C and 3D). Once the upper chamber is placed on top of the lower chamber, this meniscus will prevent air bubbles from being trapped under the membrane (ensuring a contiguous liquid phase between the upper and lower chambers).

Critical: To prevent bubble formation in the bottom chamber, use the reverse pipetting technique to add media to the CIM-Plate 16 lower chamber. The 160 μL quantity recommended here is based on a calibrated pipette, while the exact volume for pipetting into the lower chamber may need to be optimized by the user, depending on the pipette calibration condition.

Critical: As an important control, some wells in the lower chamber should contain SFM (Figure 4) so that background migration (in the absence of chemoattractant) can be assessed.

3. To facilitate CIM-Plate 16 assembly, rotate the assembly tool containing the loaded lower chamber (or chambers) 90 degrees (so that the long axis of the lower chamber runs parallel to your chest, Figure 3E). With the gold sensor side facing down, place the upper chamber on top of the lower chamber so that the blue dots on each align with one another.

Critical: Ensure that the upper and lower chambers are level and parallel to each other as the device is being assembled. **Do not tilt the upper chamber at an angle.**

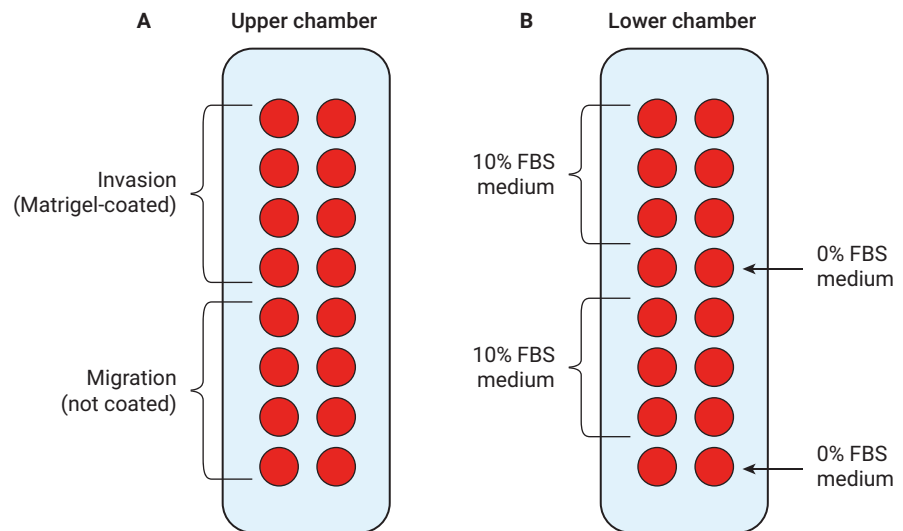


Figure 4. CIM-Plate layout. (A) Different coatings for invasion and migration in the upper chamber. (B) The appropriate controls for chemotaxis in the lower chamber.

4. Using both hands, push the upper chamber downwards onto the lower chamber to lock the two together (Figure 3E).

Critical: Proper locking/sealing of the upper and lower chambers requires strong and even downward pressure. This is best achieved by placing one thumb or finger over each of the four holes in the top chamber (matching the four posts in the bottom chamber) and pushing down quickly and forcefully. *Two clicking sounds will be audible if the chambers have locked together successfully.*

5. Add 50 μ L of SFM to each well of the upper chamber to cover the membrane surface. During the SFM addition, do not introduce air bubbles and avoid touching the membrane with the pipette tip (Figure 3F).

Critical: The volume of SFM used here is not critical. The key is to ensure that media covers the entire bottom surface of the upper chamber and that the volume you use is consistent from experiment to experiment, as it will determine the characteristics of the chemoattractant gradient formed during the next step.

CIM-Plate 16 equilibration at 37 °C (60 minutes)

Place the CIM-Plate 16 into the 37 °C incubator. You may use the CIM-Plate 16 assembly tool to hold the CIM-Plates 16. Incubate for 1 hour to allow the CIM membrane to reach equilibrium with the media. This time will also allow a gradient of the chemoattractant to form by passive diffusion.

Background measurement (5 minutes)

Load the CIM-Plate 16 into the DP cradle (Figure 3G). Ensure that the edge-cut corner on the CIM-Plate 16 lines up with the edge-cut corner on the DP instrument. Determine background impedance values by beginning Step 1 in the RTCA software.

Cell preparation (30 minutes)

Critical: Like any other cell-based assay, the success of the migration experiment using the CIM-Plate 16 depends on cell quality and handling of cells. Executing proper cell handling/maintenance is imperative for obtaining reliable and reproducible results. As some cell types can display higher levels of background migration with increasing passage number, it is important to record the passage number for each experiment. Cells should be passaged the day before the experiment and should be 60 to 80% confluent at the time that they are detached/collected for seeding the CIM-Plate 16.

Critical: For cell types that display an inherently high level of background migration, subjecting them to serum-starvation before their detachment, then seeding in the CIM-Plate 16 may be useful. For a standard migration assay, cells can be serum-starved for 1 to 16 hours. These conditions need to be determined empirically.

1. Remove serum-containing media from the tissue culture flask and gently rinse the cell monolayer once with PBS.
2. 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 to 2 minutes.

Critical: Do not overtrypsinize the cells. Cell migration and invasion depend on the integrity of cell surface receptors, such as integrins, so it is important to minimize the time of protease treatment. For cell types that are especially sensitive to protease-digestion, it may be necessary to explore alternative methods of cell detachment (for example, using EDTA-based buffers).

3. Stop trypsinization by adding media-containing serum or a trypsin-naturalization solution at a volumetric ratio of 1:1.
4. Collect cells in SFM and centrifuge. For most cells, 5 minutes at 800 \times g is sufficient.

5. Gently resuspend the cell pellet in a few mL of SFM and determine cell concentration. Adjust the concentration of the cell suspension to 3×10^5 cells/mL using SFM. At a minimum, use triplicates or quadruplicates for each experimental condition examined.

Cell addition to CIM-Plate 16 (10 minutes)

1. Unlock the DP cradle by gently pushing the press button with the thumb while holding the cradle grip with other fingers. Remove the CIM-Plate 16 from the DP cradle.
2. Add 100 μ L of cell suspension to each well of the upper chamber. The final number of cells per well should be approximately 30,000.

Place CIM-Plate 16 at room temperature (30 minutes)

After cell addition, leave the CIM-Plate 16 in the hood at room temperature for 30 minutes to allow the cells to settle evenly onto the bottom surface of the upper chamber. Failure to perform this step can result in large well-to-well variation in the migration signal. This is because immediate warming to 37 °C can cause convection currents to form within the well. These currents can push cells to the well perimeter, resulting in an uneven distribution of cells on the impedance electrodes after migration.

Start measurement (3 to 24 hours)

Place the loaded CIM-Plate 16 into the xCELLigence RTCA DP instrument inside the 37 °C incubator. Using the RTCA software on the workstation, program a run of 24 hours with readings taken every 15 minutes.

Critical: It is important to place the CIM device in the same cradle that was used for the background measurement (each device can have slightly different background impedance measurements, and this can affect the coefficient of variation of the data).

Critical: The doubling of cells is the main factor that determines the length of a migration assay. In 3 to 24 hours, impedance measurements reflect bona fide cell migration from the upper chamber to the lower chamber. At later time points, cells that have already migrated to the underside of the membrane will proliferate. During this period, regime impedance changes are the result of continued migration through the membrane and cell growth on the membrane. For this reason, it is preferable to assess migration at time points well below the doubling time of the cells being used.

Data analysis

1. Stop data acquisition after 24 hours.
2. Average all the replicates on the display page.
3. Analyze the Cell Index (CI) curves. Generally, to be considered a positive migration signal, the averaged Cell Index should be more than two-fold of standard deviation. The averaged CI should ideally be 0.5 to 1.0 or greater.

Critical: Different cell types will produce impedance changes of varying magnitude depending on the number of cells added and the overall volume and morphology of cells. If the change in CI for a given cell type at a particular density is less than 0.1, there are few parameters that can be optimized to increase the overall signal. These include increasing the number of cells added, and testing various conditions for coating the membrane with extracellular matrix proteins.

4. There are multiple ways to analyze and quantify cell migration data using the CIM-Plate 16. Some users will assess the rate of migration (slope) during a desired time window, while others will plot the CI value for critical time points.

Troubleshooting

This process may require some practice with the CIM-Plate 16 to obtain reproducible data. Because the impedance biosensor is highly sensitive, attention should be paid to properly assembling and preparing the device so that bubbles are avoided and a uniform layer of migrated cells is generated during the experiment. Potential problems and corresponding solutions are:

1. There is large variation between the replicates:
 - Verify that bubbles are not introduced into the top or bottom chambers during device assembly. Bubbles on the electrode surface (at the interface between the top and bottom chambers) are particularly detrimental to replicate quality and are not easy to spot once the CIM-Plate 16 has been assembled. However, after an assay has been run, the presence of bubbles may easily be diagnosed by staining the membrane (see Cell imaging instructions). Bubbles will show as round areas without any migrated cells.
 - It is important to perform the full 1-hour "CIM-Plate 16 equilibration at 37 °C" step described in these instructions for proper equilibration and establishment of the chemotactic gradient.
 - The full 30-minute incubation described in "Place CIM-Plate 16 at room temperature" should be carried out to ensure even distribution of cells across the electrodes.
 - Ensure that cells are well suspended before seeding into the device. Some dense cells may settle significantly just during the time it takes to pipette across all 16 wells. A best practice is to mix the source tube of cells before each round of pipetting.

2. No signal is detected in wells expected to have a good migration signal:
 - Ensure that cells have migrated by staining the membrane (see Cell imaging process). If cells have not migrated, consider testing the chemotactic reagent for activity.
 - Verify that the cells can adhere well to the impedance electrodes by setting up a standard E-Plate 16 under conditions similar to the final state of the CIM experiment (after full diffusion of the chemotactic agent). Failure of cells to adhere well can be mitigated by precoating the CIM electrodes with an extracellular matrix (ECM) protein before assembling the CIM-Plate 16. A typical process for precoating regular plates with ECM may be adapted: invert the upper chamber of the CIM-Plate 16 and evenly coat the entire surface of the electrode array with the ECM protein.
 - Because some cell types are sensitive to a total absence of serum, it may be necessary to include a low concentration of serum in the SFM. In this case, it is recommended to test a range of 0.1 to 2% serum.

Cell imaging

Recommendation: Even though it is not required, the first time migration/invasion is analyzed for a given cell line and chemoattractant, it is recommended to validate the real-time migration/invasion data by microscopy. To demonstrate the correlation between the impedance signal and the actual number of cells that have migrated, a typical process for staining a Boyden chamber or transwell device may be used. The instructions used by Agilent R&D are as follows:

1. Disassemble the CIM plate by pressing the two handles on the lower chamber and gently removing the upper chamber.
2. Remove media from the upper chamber wells with a multichannel pipette while avoiding contact with the membrane.
3. Stain the migrated cells on the CIM membrane. The Diff-Quick staining kit from Fisher Scientific is recommended. A brief description of a modified staining procedure is:
 - a. Place the upper chamber in fixative solution for 2 minutes. The bottom side of the membrane (containing the electrodes) should be in direct contact with the fixative.
 - b. Repeat, using solution I for 1 minute.
 - c. Repeat, using solution II for 1 minute.
 - d. Gently rinse the bottom side of the membrane (containing the electrodes) with water.
 - e. To gently remove any unmigrated cells from the membrane, rub a damp cotton swab along the bottom of the wells in the upper chamber.
 - f. Visualize and count the cells using an upright microscope.

Cell invasion assay

Introduction

Matrigel coating of CIM-Plate 16 for the invasion experiment

This process has been optimized for detecting cell invasion through Matrigel basement membrane matrix using the Agilent CIM-Plate 16 and the xCELLigence RTCA DP instrument, with fetal bovine serum (FBS) as the chemoattractant. Consisting of ECM proteins and growth factors that collectively mimic the basement membrane underlying epithelial cells, Matrigel is broadly used for assessing cell invasion. The Matrigel used in these instructions (supplied by Corning) was extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECMs including collagen IV, heparan sulfate proteoglycans, entactin, and nidogen.

Reagents and equipment

Reagents

- **Cells:** HT1080 cells (ATCC) were used in this process. If other cell lines were to be used, further optimization would be required.
- **Matrigel (from Corning):** After thawing the Matrigel on ice, dispense 100 μ L aliquots into 0.5 mL tubes and store at -80°C . Subjecting Matrigel to multiple freeze-thaw cycles is not recommended.
Critical: Because the concentration and quality of Matrigel can vary from batch to batch, each new batch should go through a titration assay, and the results should be compared with previous batches.
- **HT1080 cell culture complete media:** DMEM containing 10% FBS, 1% penicillin-streptomycin solution, and 1% MEM nonessential amino acid solution.

Equipment

See the equipment listed in the Cell Migration Assay process (in this document).

Process overview

Workflow summary

This assay process requires two full days to complete and has been developed for continuous monitoring of cell invasion on the xCELLigence RTCA DP instrument for 24 hours.

Day 1

1. Coat the upper chamber with Matrigel and place in a 37°C incubator for 4 hours.
2. Assemble the CIM-Plate 16 (attach the upper chamber to the lower chamber). Equilibrate in a 37°C incubator for 1 hour, then take a background measurement.
3. Prepare cells and add them to the CIM-Plate 16.
4. Leave the CIM-Plate 16 at room temperature for at least 30 minutes to let cells settle.
5. Load the CIM-Plate 16 into an xCELLigence RTCA DP instrument and start measurements.

Day 2

6. Stop data acquisition and analyze the Cell Index (CI) curves to determine cell invasion activity.
7. **Optional:** Stain the migrated cells on the underside of the membrane and examine them under a microscope.

Detailed instructions

Matrigel coating of the upper chamber of the CIM-Plate 16 (4 hours)

Critical: The day before the experiment, place pipette tips, Eppendorf tubes, and the upper chamber of the CIM-Plate 16 at 4 °C to cool. Also, transfer a tube of aliquoted Matrigel from –80 to 4 °C.

1. Dilute the Matrigel with precooled SFM on ice in the precooled Eppendorf tubes. Approximately 1 mL of diluted Matrigel is needed to coat all 16 wells of the upper chamber of a CIM-Plate 16.
2. Dilute Matrigel with cold SFM to a concentration of 800 µg/mL and carefully maintain the Matrigel solution on ice to avoid polymerization.
3. Add 50 µL of Matrigel solution into each well of the upper chamber. Gently tap the plate to ensure that the Matrigel evenly covers the entire surface of each well.
4. Remove 30 µL of the nascent Matrigel solution from each well, leaving the remaining 20 µL to coat the membrane surface.

Critical: This step is crucial for an even layer of Matrigel in the wells. When removing the 30 µL of Matrigel, insert pipette tip into the well as far as possible without actually touching the membrane. Withdraw the Matrigel slowly. Be careful not to introduce air bubbles during this step.

5. Place the Matrigel-containing upper chamber in a 37 °C tissue culture incubator for 4 hours.

Critical: Use the CIM-Plate 16 assembly tool to keep the upper chamber properly elevated during this step (Figure 5A). To avoid wicking of the unpolymerized Matrigel through the microporous membrane, the bottom face of the upper chamber cannot be in direct contact with any surface. Also, keep the upper chamber covered with the CIM-Plate 16 lid to avoid contamination and evaporation.

6. After the Matrigel has polymerized (4 to 5 hours; Figure 5B), take the upper chamber back to the tissue culture hood and start device assembly. This process is identical to what was described for the migration assay starting from Step 1 of "CIM-Plate 16 assembly".

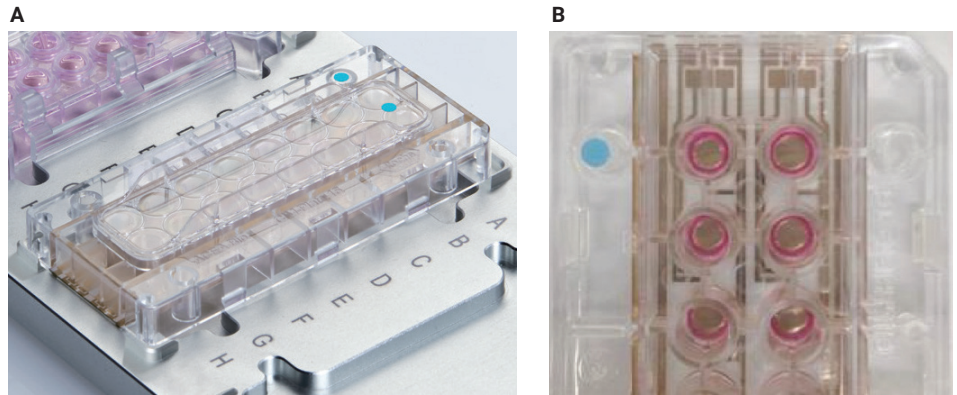


Figure 5. (A) Using the assembly tool keeps the upper chamber elevated to avoid surface contact with the membrane during Matrigel polymerization. (B) Typical appearance of polymerized Matrigel after a 4-hour incubation at 37 °C.

Data analysis

There are multiple ways to analyze and quantify cell invasion data using the CIM-Plate 16. Some users assess the rate of migration (slope) during a desired time window, while others plot the Cell Index value for representative time points. It is also possible to quantify the extent of invasion at different time points postcell seeding using a term called Cell Invasion Index (CII). CII is defined as the ratio of Cell Index for Matrigel-coated wells (invasion) to Cell Index for uncoated wells (migration) at a given time point. The CII ratio can be calculated by exporting the relevant data to another software such as Microsoft Excel.

Troubleshooting

This process may require some practice with the CIM-Plate 16 to obtain reproducible data. Because Matrigel coating is a frequent source of experiment variation, particular attention should be paid to this step. Potential problems and corresponding solutions are:

1. There is large variation between the replicates:
 - Verify that the Matrigel has not expired.
 - It is important to allow the Matrigel to thaw overnight on ice at 4 °C.
 - Thaw a fresh vial of Matrigel for each experiment. Do not refreeze Matrigel.
 - Ensure all materials coming in direct contact with the Matrigel, including pipette tips, Eppendorf tubes, the CIM-Plate 16, and media are kept at 4 °C until ready to be used.
 - When coating a well with diluted Matrigel, ensure that the gel covers the entire membrane surface.
 - When removing 30 µL of Matrigel, verify that a similar volume is removed from every well. Ensure that no air bubbles are introduced during the coating procedure.
2. No signal is detected in wells coated with Matrigel:
 - Ensure that the Matrigel is sufficiently diluted. For each new batch of Matrigel, performing a titration assay is imperative.
3. There is no difference in signal or CI dynamics between Matrigel-coated wells and uncoated control wells:
 - The Matrigel may be too diluted. Try reducing the extent of dilution.
 - The Matrigel may not be sufficiently polymerized. It is important to allow the Matrigel to polymerize at 37 °C in a CO₂ incubator. A humidified environment is required for optimal Matrigel polymerization.