SARTORIUS

Manual

Incucyte® 96-Well Scratch Wound Cell Migration and Invasion Assays

User Manual

Contents

Welcome to Your Incucyte® 96-Well Woundmaker Tool

The Incucyte® 96-Well Woundmaker Tool is a 96-pin mechanical device designed to create homogeneous, 700–800 µm wide wounds in cell monolayers on Incucyte® Imagelock Plates. The device is simple to use, and 96 identical wounds can be created in seconds.

When used, stored, and cleaned correctly, the Incucyte® 96-Well Woundmaker Tool should continue to provide clean, consistent wounds without damaging the cells or the underlying plastic or biomatrix. The Incucyte® 96-Well Woundmaker Tool is comprised of:

Getting Started, General Guidelines

- Never place the pin block directly onto the bench surface.
- Never turn the pin block over—this can damage the pins.
- Never use the Woundmaker with a dry or partially dry plate. Make sure each well contains media or PBS before wounding.
- Never use the Woundmaker with more than 100 µL of media per well.
- Do not push down on the pin block when making wounds.
- Store the Woundmaker in a clean, dust-free and safe location, such as a biological safety cabinet, when not in use.
- Clean the Woundmaker according to the wash protocol after every use.
- Never put more than 45 mL of any solution into the wash boat. This will prevent damage to the pin mechanism.

Creating Wounds: Seven Simple Steps

The following steps should be performed in a biological safety cabinet:

Step 1
- Remove top of Woundmaker.
- Set top in empty wash boat.

Step 2
- Insert plate (containing cells and media) into base plate holder.
- Press firmly on all four corners of the plate to ensure it is seated flat within the plate holder.

Step 3 (inset)
- Remove plate cover.

Step 4
- Replace pin block by guiding rear dowels of pin block into the rear holes of the base plate.
- Do not push down.

Step 5
- Push and hold the black lever.

Step 6
- Lift the pin block while continuing to hold the black lever down.

Step 7
- Replace plate cover.
- Wash wells (up to two washes).
- Add treatment conditions.
- Place plate in Incucyte® Live Cell Analysis System and start acquiring data and images.

Incucyte® 96-Well Woundmaker Tool Wash Instruction and Storage

Wash Instructions

1. Prior to use after storage (5 min washes)
   - Sterile distilled water (45 mL)
   - 70% ethanol (45 mL)
2. Between plates of the same cell line (5 min washes)
   - Sterile distilled water (45 mL)
3. After last experiment of the day or between plates containing different cells lines (5 min washes)

   Note: Do not use bleach when cleaning the Woundmaker as it will damage the tool, causing issues when creating accurate wounds.
   - 0.5% Alconox (45 mL)
   - Sterile distilled water (45 mL)
   - 70% ethanol (45 mL)

Storage

Place the Woundmaker on its base plate and store it in a dust-free environment.

Sterilization using 70% ethanol for 5 min is sufficient to kill most pathogens. For information about the sensitivities of specific microbial agents, please refer to CDC or other applicable agency: www.cdc.gov/infectioncontrol/guidelines/disinfection/index.html

Protocol Overviews

Incucyte® 96-Well Scratch Wound Migration Assay

Sartorius' Incucyte® 96-Well Scratch Wound Migration Assay is designed for fully automated, kinetic quantification of cell migration *in vitro*. There are 6 simple steps to assemble and run this assay:

1. Seed cells at an appropriate density (e.g., 20K cells well-1) into each well of a Incucyte® Imagelock 96-Well Plate (Sartorius, BA-04855) and incubate in a standard cell incubator for between 6–18 h.
2. Remove the plate from the incubator and use the Incucyte® 96-Well Woundmaker Tool and wounding procedure to create precise and reproducible wounds in all wells of the Imagelock 96-Well Plate.
3. After wounding, aspirate the media from each well and gently wash each well two times with culture media to prevent dislodged cells from settling and reattaching.
4. After washing, add 100 µL of media containing test material at the appropriate concentration (e.g., small molecules, antibodies).
5. Remove any bubbles from the assay plate.
6. Place assay plate into the Incucyte® Live-Cell Analysis System and allow it to equilibrate for 5 min. Schedule repeat scanning (e.g., every 2–3 h for 48 h) in the live-cell analysis software: ensure "scan type" is set to Scratch Wound, and Wide Mode is selected if using 20X or 10X objectives.

Incucyte® 96-Well Scratch Wound Invasion Assay

Sartorius' Incucyte® 96-Well Scratch Wound Invasion Assay is designed for fully automated, kinetic quantification of cell invasion. There are 9 simple steps to assemble and run this assay:

1. Coat an Incucyte® Imagelock 96-Well Plate (Sartorius, BA-04855) with a thin layer of biomatrix (e.g., Collagen-1 or Corning® Matrigel®, 1–48 h).
2. Seed cells at the optimized cell density (e.g., 20K cells well-1) into each well of the coated plate and incubate (37° C) in a standard cell incubator for between 6–18 h.
3. Prepare biomatrix top layer (with or without test compounds, as required).
4. Carefully follow the Woundmaker procedure to create precise and reproducible wounds in all wells of the Imagelock 96-Well Plate. Wash cells with media to remove debris and to prevent dislodged cells from settling and reattaching.
5. Add 100 µL of media, then cool the cell plate to 4° C for 5 min using the CoolSink and CoolBox 96F (Sartorius, Cat. No. 1500-0078).
6. Aspirate media and overlay cells with biomatrix top layer (Collagen-1 or Matrigel®). Remove any bubbles from the assay plate.
7. To gel the top layer, warm the cell plate to 37° C by placing onto a warm CoolSink (Sartorius, Cat. No. 1500-0078) in the cell incubator (37° C).
8. After 30 min add additional media (100 µL per well, with or without test compounds). Remove bubbles.
9. Place assay plate into the Incucyte® Live-Cell Analysis System and allow it to equilibrate for 5 min. Schedule repeat scanning (e.g., every 2–3 h for 48 h) in the live-cell analysis software: use the Incucyte® Scratch Wound Analysis Software Module, ensure "scan type" is set to Scratch Wound, and Wide Mode is selected if using 20X or 10X objectives.

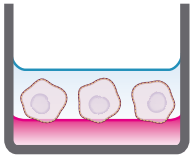
Example migration and invasion protocols, with full details of each of these steps for a representative cell type, are described on page 12.

96-Well Scratch Wound Cell Migration | Invasion Assays: Schematic Diagram

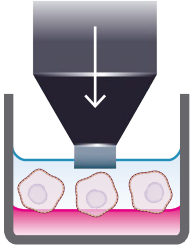
Migration assays can be performed either with or without a base layer extracellular matrix (ECM: Step 1), and do not utilize a gelled top layer (eliminate Step 4). Test samples are added in culture media (Step 5). Protocols for using either Collagen-1 or Matrigel® as the base | top layer are provided. In each case, test samples are made up in the relevant ECM (Step 4), as well as the additional culture media (Step 5).

1. Coat plate with Collagen-1 (or other ECM) and incubate overnight.

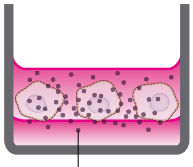
2. Plate cells on top of thin-layer matrix, and allow them to adhere for several hours.



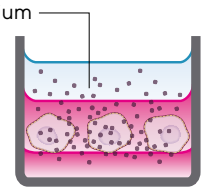
3. Create wound area using Incucyte® 96-Well Woundmaker in just seconds.



4. Layer collagen gel on top of cells to create 3D matrix.



5. Use the Incucyte® Live-Cell Analysis System to:
   - Automatically collect time-lapse images
   - Observe cell morphology
   - Quantify cell invasion



96-Well Scratch Wound Cell Migration and Invasion Assays: Data Acquisition and Analysis

The Incucyte® Scratch Wound Analysis Software Module allows automated detection and quantification of wound properties from each well of the 96-well plate, at a series of time points post wounding. Kinetic plots of cell migration (movement into the wounded zone) can thus be readily generated.

To initiate and schedule the frequency of the image acquisition the user must:

1. Prepare the assay plate as described in the relevant migration or invasion assay protocol.
2. Position the plate in the Incucyte® Live-Cell Analysis System, close the drawer and the incubator door.
3. Under "Schedule Scans," select the tray position of interest. Remove any old tray definitions by clicking "Remove Tray." Click the "Add Vessel" button and select "96-Well Sartorius Imagelock Plate, BA-04855" and the tray location of choice (left side or right side).
4. From the "Scan Type" dropdown, select "Scratch Wound."
5. If you would like to acquire fluorescence images (green and/or red) in addition to phase contrast images, select the appropriate "Channel Selection" check boxes.
6. Select the desired scan frequency and timing by right-clicking on the time base and selecting "Set Interval."

For both Cell Migration and Cell Invasion applications, the first scan in the time course is used to generate the initial scratch wound mask (a digital overlay showing the border | leading edge of the migrating cells and the non-wounded area). This initial scratch wound mask is used in subsequent quantification processes and is critical to the success of the assay. Scratch wound masks are also computed for all subsequent image time points after the first scan.

In addition to the scratch wound mask, a confluence mask is also generated. The confluence mask represents the cell confluence of the wound region exactly as determined by the confluence algorithm. Each of these masks can be viewed either alone or in a blended combination within the "Vessel View" window inside the Incucyte® software.

For full details of the Incucyte® 96-Well Scratch Wound Cell Migration and Invasion data processing, please refer to the Incucyte® Scratch Wound Processing Overview Technical Note.

A summary of the data analysis process is given below.

1. Create an Image Collection of 3 to 6 representative images selected from the data set.
2. Use the Image Collection to define and optimize a Processing Definition.
3. Apply the Processing Definition to the full data set by running an Analysis Job.

96-Well Scratch Wound Cell Migration Assays
An Image Collection is a group of images that can be used to train, test, or refine a Processing Definition. Image collections should contain approximately 3 to 6 images that represent the phenotypes under investigation for the selected time range. It is recommended that each test condition is represented in the image collection for invasion or migration studies. Depending on the experimental design, this may include multiple cell types, biomatrix materials, and both migration and invasion phenotypes. It is acceptable to include up to 10 images within an image collection. However, for larger image collections it will take longer to preview changes when optimizing a Processing Definition. If an experiment is performed using an objective of different magnification, then a new image collection and processing definition will need to be made in order to analyze the resulting images.

The Processing Definition
The Scratch Wound Processing Definition uses an algorithm to generate two masks for each image of the image collection: 1) the scratch wound mask and 2) the confluence mask. Scratch wound and confluence masks can be adjusted by altering the processing definition parameters. Changes to a single parameter (e.g., the position of the segmentation adjustment slider) can affect both the scratch wound and the confluence masks for an image. Guidance for optimizing Scratch Wound Processing Definitions can be found in the Incucyte® Scratch Wound Processing Overview Technical Note. After saving the Processing definition, it can be used to launch an Analysis Job.

The Analysis Job
When a processing job has been initiated for either a Cell Migration or Cell Invasion experiment, each image from the selected time range is analyzed in accordance with the parameters chosen for the processing definition.

The first image from each well for the analyzed time range is used to generate an initial scratch wound mask which defines the initial wound region. The initial scratch wound mask can be visualized as a digital overlay showing the cell free (wound) area at t = 0 h and is used to define the starting locations of the wound borders. The initial scratch wound mask is used in subsequent quantification processes and is critical to the success of the assay if relative wound density or wound confluence are used as metrics.

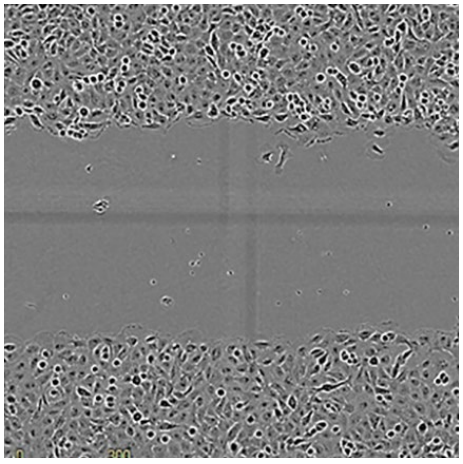
Further scratch wound masks are computed for each subsequent image in the series and are used to calculate the wound width metric only.

The confluence mask is used to derive the wound confluence metric (the cell confluence of the wound region). The wound region is defined by the initial scratch wound mask. Each of these masks can be viewed either alone or in a blended combination when an Analysis Job is opened.

Wound Mask Images Using a 10X Objective

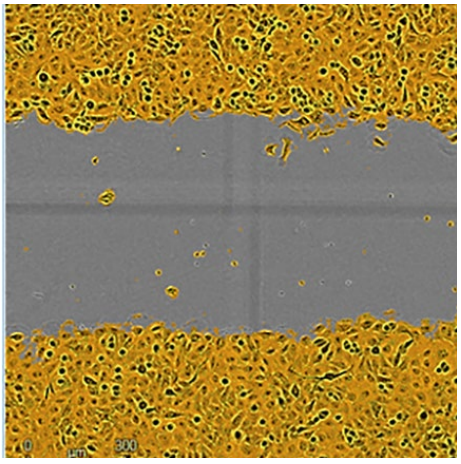
Initial Wound (t = 0 h)

A.



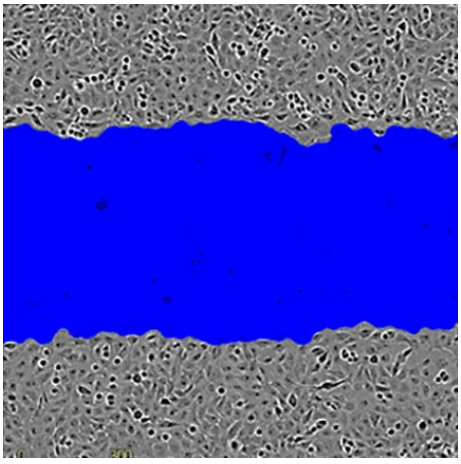
A. Phase contrast images of HT-1080 cells taken at 0 h post wounding

B.



B. Corresponding confluence masks (orange overlay blended with phase image)—taken at 0 h post wounding—indicate areas of the image that are occupied by cells

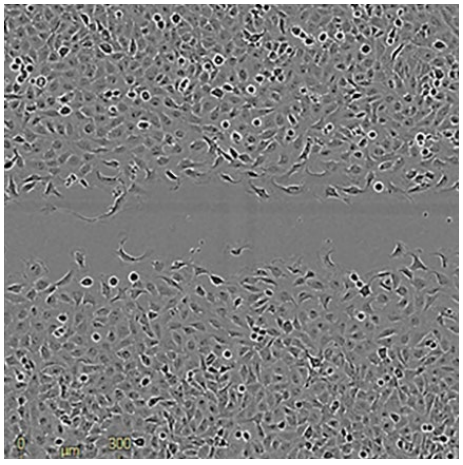
C.



C. Initial scratch wound mask (blue) superimposed on the phase image

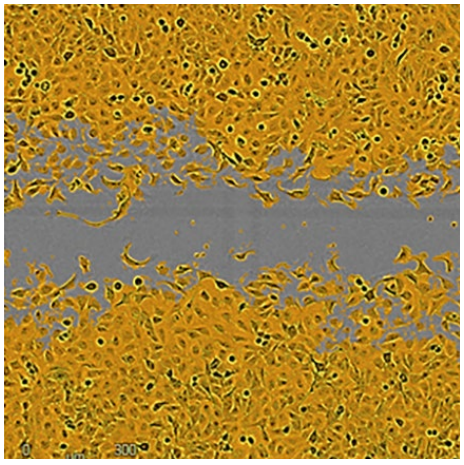
After 4 h

D.



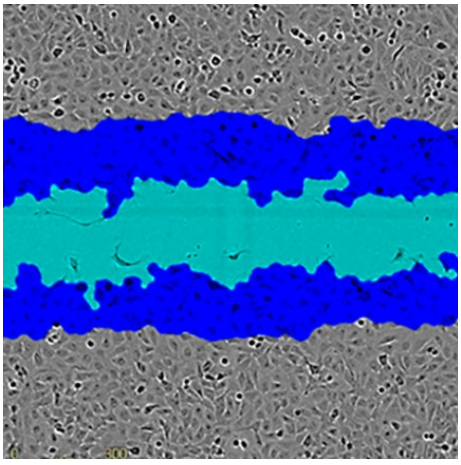
D. Phase contrast images of HT-1080 cells taken at 4 h post wounding

E.



E. Corresponding confluence masks (orange overlay blended with phase image)—taken at 4 h post wounding—indicate areas of the image that are occupied by cells

F.



F. Scratch wound mask (light blue) indicating the wound border locations after 4 h is overlaid on the initial scratch wound mask (blue)

Initial Scratch Wound Mask

- Identifies the borders of the wound region using the first image in a temporal series

Scratch Wound Mask

- Identifies the leading edge of the population of migrating cells within each image of the series

Confluence Mask

- Identifies all cell occupied areas within the field of view and is used in combination with the Initial Scratch Wound Mask to determine the cell confluence of the wound region

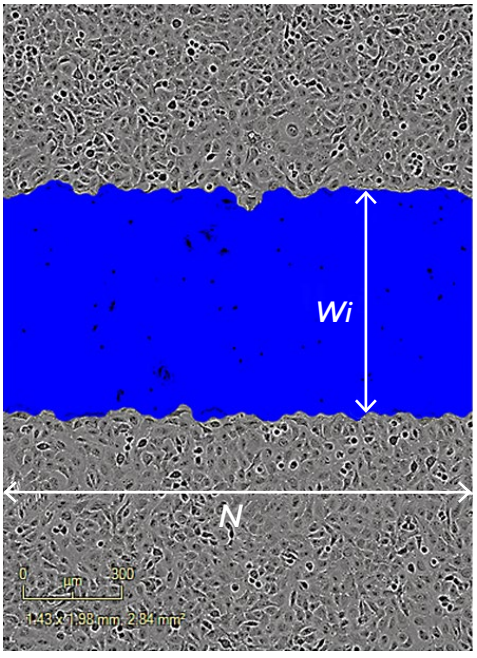
Incucyte® Software: Scratch Wound Cell Migration and Invasion Analysis Metrics

There are three integrated metrics that the Incucyte® Scratch Wound Analysis Software Module calculates based on the processed images:

1. Wound Width (Cell Migration Only)
Wound Width represents the average distance (microns) between the edges of the scratch wound mask in each line of resolution within an image. This is the only metric of the three available metrics that does not rely on the initial scratch wound mask, as each image is analyzed independently of the starting point. It is important to note that the scratch wound mask identifies the boundary of the migrating cell population and, at times, will ignore a small number of cells that do not fit within that population.

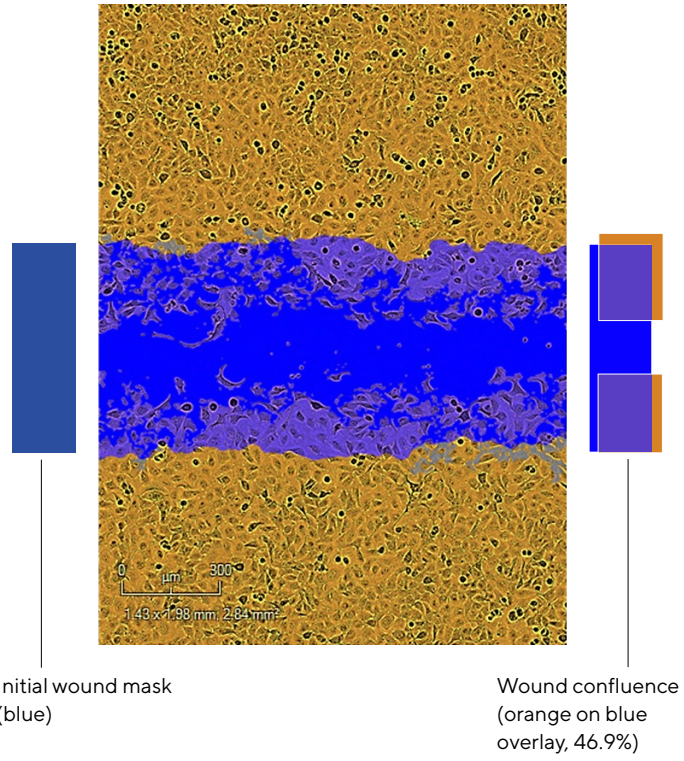
$$wound\ width = \frac{1}{N}\sum W_i$$

Wound Width (µm) is determined using each vertical line of resolution of a scratch wound mask.



2. Wound Confluence (Cell Migration Only)
Wound Confluence measurement relies on the initial scratch wound mask to differentiate the wounded from the non-wounded region. The Wound Confluence algorithm determines the cell confluence in only the wound region. The resulting value represents the percentage of wound area that is occupied by cells. Cells, debris, or other matter within the wound is quantified as confluence and is not background subtracted. Therefore, the wound confluence values at the initial time point may not be 0% and may vary from well to well.

Wound Confluence (%) represents the fractional area of the wound that is occupied by cells.



3. Relative Wound Density (Cell Migration and Cell Invasion)

Like Wound Confluence, Relative Wound Density (RWD) also relies on the initial scratch wound mask to differentiate between cell-occupied and cell-free regions of the image. Once these regions are defined, a second image analysis algorithm (which is completely independent of the confluence algorithm and is not viewable to the user) is applied to calculate the density of both the cell region and the wound region as defined by the initial scratch wound mask. It is important to note that both the cell region and the wound region are variables in the Relative Wound Density v1.0 equation, and both are treated with equal importance. Specifically, the RWD metric refers to the density of the wound region as a function of the density of the cell region and is defined by the following equation:

$$\%RWD(t) = 100 \cdot \frac{(w(t) - w(0))}{(c(t) - w(0))}$$

w(t) = Density of wound region at time, (t)
c(t) = Density of cell region at time, (t)

Note that the initial density of the wound (w(0)) is subtracted from the density of the wound at each subsequent time point (w(t)), i.e., each reported value is background subtracted. The background subtracted wound density represents the numerator in the above equation. For the most part, the density of the cell region (c(t)) represents the denominator in the above equation, as the initial density of the wound (w(0)) is typically very small. The reason w(0) is subtracted from c(t) is to guarantee that the relative wound density value is equal to 100% when w(t) is equal to c(t).

Relative Wound Density accounts for the background density of the wound at the initial time point, and for changes in both the density of the cell (non-wound) and the wound region. Thus, at the initial time point RWD is always 0%. RWD is particularly useful when experimental treatments alter cell morphology. As RWD does not rely on the identification of individual cell borders, it is also helpful when using cell types that are difficult for the Wound Width and Wound Confluence algorithm to accurately evaluate (e.g., cells with flattened low contrast morphology, for example, certain human umbilical vein endothelial cells).

Relative Wound Density is a measure (%) of the density of the wound region relative to the density of the cell region.

Why Is RWD the Only Recommended Metric for Cell Invasion?

There are several differences between cell migration and cell invasion, both in experimental design and biological phenotypes. There are two reasons that only RWD is suitable for cell invasion:

1. Invasion assays require the addition of an extracellular matrix (ECM) for cells to invade. The presence of an ECM can hinder the ability of the analysis algorithm to apply an appropriate initial scratch wound mask and confluence mask to the first image of a series. Cellular content or textured regions of ECM within the wound area can result in a misleading wound confluence metric. In addition, the mild texture of gelled ECM can cause intermittent over-and under-estimations of confluence in the wound area, resulting in variable data. The RWD algorithm is more tolerant of ECM texture and will correct for initial cell content in the wound area.
2. Cells invading through a 3D matrix exhibit very different morphology from cells migrating over a 2D substrate. Cells with a mesenchymal invasion morphology typically display an elongated phenotype with lamellipodia that extend into the ECM. Furthermore, one leader cell is generally followed by numerous other cells, thereby forming a network of invasion tracts or "tunnels," as opposed to a leading edge of cells seen in a 2D migration assay. For this reason, the scratch wound masks may not always best represent the invading population. Wound width measures can thus be inaccurate and misleading.

Hints and Tips

Optimizing Assays

For optimal assay quality for both Incucyte® 96-Well Scratch Wound Cell Migration and Invasion Assays, it is recommended that cell density, the timing of the scratch wound (post cell plating), and the density of biomatrix material (if required) be investigated and optimized for different cell types.

1. Cell Density
   - The most consistent wounds are generally made when the cell monolayer is at or very near to 100% confluence, with nearly all cells attached to the tissue culture plate just prior to wounding.
   - It is best if the cells are not at confluence for an extended period of time prior to wounding.
   - We recommend using the minimum number of cells that are required to form a confluent monolayer within 6–18 h of plating.

- Seeding density will vary with each cell type used, i.e., if the cells are large, a smaller number will be required to achieve confluence. Typically this will range from 10–50K cells per well.
- Proliferation rates can also affect seeding density. It is recommended that you complete a measure of proliferation using the confluence metric within the Incucyte® to assist in defining an appropriate seeding density.

2. Timing of the Scratch Wound
   - The optimal timing depends on many factors, including the speed at which the cells adhere to the plastic (or biomatrix), the strength of this adhesion, the number of cells plated, and the proliferation rate of the cells to name just a few.
   - Plating cells at the end of the day and wounding cells in the morning of the following day works well for many cell types plated on tissue culture plastic. This is a good place to start once an optimum seeding density has been identified.
   - If a biomatrix material is being utilized, cells may adhere in just a few hours, and successful wounding is often possible on the same day of seeding.

3. Biomatrix Coating
   - Coating the well with a biomatrix material (e.g., Collagen-I) or poly-D-Lysine will typically enhance the speed and strength of cell attachment. It can allow cells to adhere more tightly to the substrate, as opposed to each other, which can facilitate the removal of only cells in the wound area avoiding cell sloughing or the removal of sheets of cells.
   - On occasion, cells will adhere too tightly. Sub-optimal wounding may then occur when cells leave adhered debris after wounding, which blocks subsequent cell migration. Plating cells for shorter time periods, to allow only partial attachment (e.g., 4–8 h) can help improve the quality of the wounds.

For a detailed description of an example optimization strategy, please refer to the Incucyte® 96-Well Scratch Wound Cell Migration and Invasion Assays Application Note.

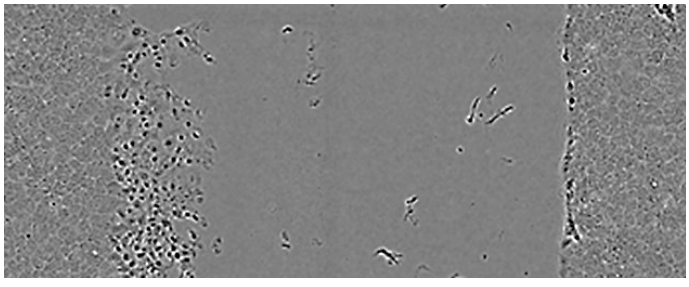
Working with Collagen-1 and Matrigel®

Working with either Collagen-1 or Matrigel® can be challenging, given their pH/temperature dependent viscosity, and known batch-to-batch variation. To maximize performance and cost effectiveness, we recommend:

- Always keep working stocks on ice or, when in plates, in the CoolSink.
- Use wide bore pipette tips (cut off) and serological pipettes as much as possible—the high viscosity of Collagen-1/Matrigel® makes them hard to pipette through narrow tips (e.g., 100 µL). Store tips in the fridge—this will help keep everything cool.
- Plan for larger dead volumes than usual.
- Neutralizing Collagen-1 with neutralization buffer takes time, and very careful and complete mixing. Try to minimize the number of neutralization manipulations (e.g., neutralize once in a larger volume and then dispense to multiple assay wells to create on-plate replicates, rather than neutralizing as individual wells). This also helps to minimize wastage from dead volume.
- Once neutralized, Collagen-1 will slowly gel even when kept cold (within 30 min). Working quickly and efficiently will help ensure good results.
- When preparing plates containing test samples, it is more straightforward to make serial dilutions in solvent (e.g., DMSO), which are then transferred to a 96-well microplate (e.g., 1 µL per well) and diluted in ECM | neutralization buffer | culture media as appropriate. Making serial dilutions in ECM is time consuming and difficult.
- Due to the high freezing point of DMSO, addition to cold Matrigel® creates the potential for samples to freeze, and then precipitate. In our experience, keeping DMSO:Matrigel® ratios of < 1:100 (e.g., < 1%) mitigates this possibility.
- Different batches (lots, suppliers) of Collagen-1 and Matrigel® may yield different experimental outcomes. We recommend that batches < 3 months old are used to minimize variability. Both Standard (BD 354234) and Growth Factor Reduced (BD 354230) Matrigel® have been validated for use.

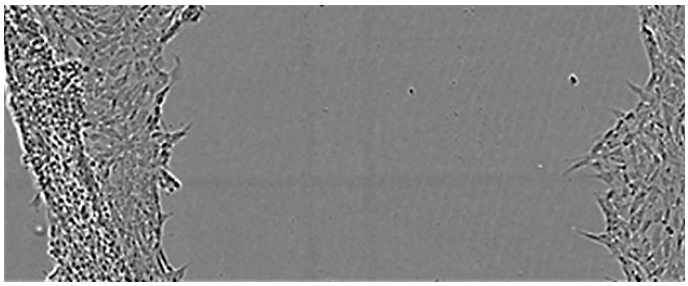
Troubleshooting

1. Cells Are Not Completely Removed by the Incucyte® 96-Well Woundmaker Tool



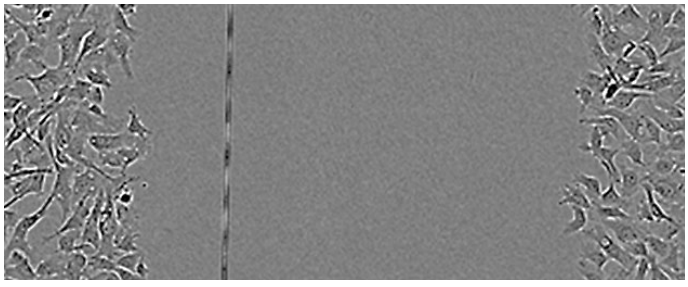
Some cells may adhere strongly to the plastic | culture plate surface. Try minimizing the plating time (e.g., reduce to 4–6 h) to reduce strong adhesion.

2. Cells Are Removed in Large Sheets During the Scratch Wound Assay



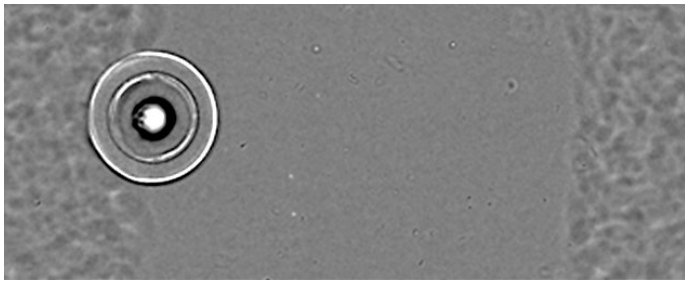
Loosely adherent cell types may be removed in sheets by the Incucyte® 96-Well Woundmaker Tool or slough off during washes. Some cell types adhere tightly to neighboring cells but only loosely to the substrate. Try coating the plate with Matrigel® or Collagen-1 prior to seeding cells to promote stronger adherence. If cells adhere tightly to one another, try allowing the cells to adhere for a shorter period of time (4–6 h) before wounding.

3. Pin Scratches Into the Culture Surface Along Wound



Even under ideal conditions, dust and particulate matter (grit) may come into contact with and become imbedded in the soft PTFE tips of the Incucyte® 96-Well Woundmaker Tool's pins. The standard washing protocol is often sufficient to remove grit. If a pin consistently scratches into the culture surface, pin cleaning or replacement at Sartorius may be required.

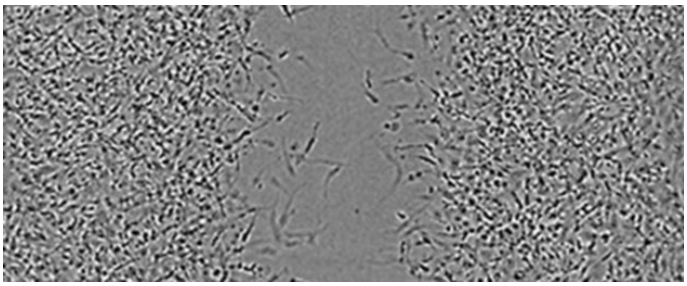
4. Bubbles Form Within Gel Layer During an Invasion Assay



Due to its viscosity, the gel layer of a matrix material may retain bubbles that form during pipetting but would normally escape to the surface. When using Collagen-1, bubbles may also form during neutralization due to pipetting and the formation of CO_2. Take extra care when pipetting into matrix materials, especially at high concentrations.

For Collagen-1, centrifugation of the neutralized solution and careful pipetting will help eliminate bubbles. Bubbles can also be removed from wells by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

5. Cells Appear Out of Focus During an Invasion Assay



Cells that are plated too densely can lead to the deformation of the biomatrix material, causing cells adhered to it to become out of focus. This can lead to difficulties in image analysis. Careful optimization of cell density can resolve this problem. It is advisable to use the minimum number of cells required to achieve 80–100% confluence at the time of wounding.

6. No Wound Is Formed in the Cell Layer

Occasionally, a pin will fail to fully contact the cell surface and create a wound. This is usually due to build-up of precipitate along the length of the pin, preventing the pin from moving freely. Wash the Incucyte® 96-Well Woundmaker Tool thoroughly. If a pin consistently fails to create a wound, the Incucyte® 96-Well Woundmaker Tool will need to be serviced at Sartorius.

7. Cells Migrate in Presence of Gel Layer

Cell migration in the presence of a gel layer, when invasion should be observed, is indicative of improper gelling of the matrix material or a lack of bonding to the thin layer. Ensure ideal experimental conditions. While working with matrix materials, keep them strictly under conditions that do not promote gelling. Use a CoolSink 96F during gelling to apply an even temperature across the bottom of the plate.

Incucyte® Scratch Wound Assay Protocol

For the Measurement of Invasion or Migration Into a Wound Region

This protocol provides an overview of our cell motility assay, which is suitable for the analysis of migration or invasion of adherent cell lines. This method utilizes our Incucyte® 96-Well Woundmaker Tool to create 96 precise, uniform cell-free zones in cell monolayers cultured on Incucyte® Imagelock 96-Well Plates. Incucyte® Scratch Wound Analysis Software Module enables real time, automated measurement of label-free or dual fluorescence of cell migration and invasion *in vitro*.

Required Materials

- Cell Migration | Invasion Bundle (Sartorius Cat. No. 4474), includes:
  - Incucyte® Scratch Wound Analysis Software Module (Sartorius Cat. No. 9600-0012)
  - Incucyte® 96-Well Woundmaker Tool (Sartorius Cat. No. 4563)
  - Two (2) Woundmaker Rinse Boats (Sartorius Cat. No. 5025-0191)
  - Fifteen Incucyte® Imagelock 96-Well Plates (Sartorius Cat. No. BA-04857)
- Incucyte® 96-Well Cell Invasion Accessories (Sartorius Cat. No. 4444), for invasion assay includes:
  - 2 x CoolBox 96F System plus 2 x CoolSink 96F
  - 1 x extra CoolSink 96F
- Matrigel® (BD Cat. No. 354234), for invasion assay

General Guidelines

- Following cell seeding, place plates at ambient temperature for 15 minutes to ensure homogenous cell settling.
- Do not leave any empty (dry) wells—these will damage the Woundmaker pins when creating the scratch.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70–100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

For optimal assay quality for both Incucyte® Scratch Wound Cell Migration and Invasion Assays, it is recommended that cell density, the timing of the scratch wound (post cell plating) and the density of biomatrix material (if required) be investigated and optimized for different cell types.

Cell Density: The most consistent wounds are generally made when the cell monolayer is at or very near to 100% confluence; typically seeding density will range from 10–50K cells per well.

Timing of Scratch: Plating cells at the end of the day and wounding cells in the morning of the following day works well for many cell types plated on tissue culture plastic. If a biomatrix material is being utilized, cells may adhere in just a few hours, and successful wounding may be possible on the same day of seeding. On occasion, cells will adhere too tightly, causing adhered cells debris after wounding, which blocks subsequent cell migration. Plating cells for shorter time periods, (e.g., 4–8 h) can help improve the quality of the wounds.

Biomatrix: Coating the well with a biomatrix material (e.g., Collagen-I) or poly-D-Lysine will typically enhance the timing and strength of cell attachment. It can allow cells to adhere more tightly to the substrate as opposed to each other, avoiding cell sloughing or the removal of sheets of cells.

- After placing the plate in the Incucyte® Live-Cell Analysis System, allow the plate to warm to 37° C for 30 minutes prior to scanning.

Please review Creating Wounds: Seven Simple Steps (found on page 3) prior to initiating an assay for best practices when using the Incucyte® Woundmaker.

Migration Protocol

1. Coat plate with ECM (optional)

Coat plate surface to ensure cell attachment (e.g., Collagen-1).

2. Seed cells

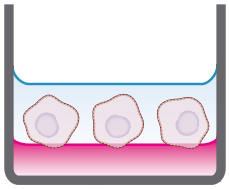
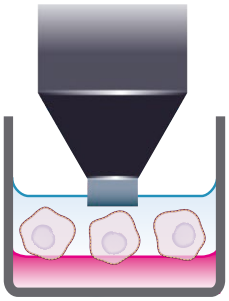


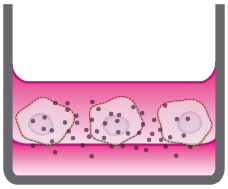
Plate cells (100 µL/well, 10,000–40,000 cells/well) and allow to adhere overnight.

3. Create wound area



Wound confluent cell monolayer using 96-well Woundmaker.

4. Add treatment



Add modulators of migration (100 µL/well).

Day 0

Coat Plate with ECM (if required)

1.1 Coat an Incucyte® Imagelock Plate with a thin layer (50 µL/well) of biomatrix. Gently rock the plate to ensure even coating of each well.

1.2 Depending on biomatrix used for coating, aspirate and wash coating from the wells prior to cell seeding.

Seed Cells

2.1 Seed cells at a density of 10,000–40,000 cells/well (100 µL/well; 100,000–400,000 cells/mL stock) into each well of the coated Incucyte® Imagelock Plate.

2.2 Allow the cells to settle at ambient temperature for 15 minutes, then place the plate into a 37° C incubator, 5% CO_2 overnight or as pre-determined in assay optimization.

Day 1

Create Wound

3.1 Carefully remove the Incucyte® Imagelock Plate from the incubator, and use the Woundmaker (refer to Creating Wounds: Seven Simple Steps on page 3) to simultaneously create wounds in all wells. Please make sure that the plate is seated flat in the plate holder prior to wounding.

3.2 After wounding, immediately aspirate the media from each well and carefully wash the cells twice with culture media (100 µL/well; with our without serum) or Dulbecco's Phosphate Buffered Saline (dPBS),if desired.

Add Treatment

4.1 After washing, add 100 µL of culture media ± test material (e.g., small molecules, antibodies) to each well.

4.2 Place the cell plate into the Incucyte® Live-Cell Analysis System and allow the plate to warm to 37° C for 30 minutes prior to scanning.

a. Objective: 4X, 10X (recommended), or 20X
b. Channel selection: Phase Contrast (+ Fluorescence if analyzing cells with fluorescent labels)
c. Scan type: Scratch Wound (Wide Mode optional for 10X, required for 20X)
d. Scan interval: Every 1–3 hours

4.3 Wash and store the Woundmaker according to the wash protocol.

Invasion Protocol

1. Coat plate with ECM (optional)

Coat plate surface with 100 mg/mL Matrigel® (50 µL/well) to ensure cell attachment.

2. Seed cells

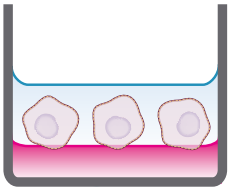
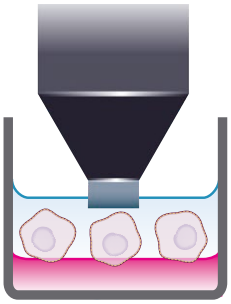


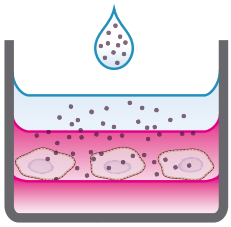
Plate cells (100 µL/well, 10,000–40,000 cells/well) and allow to adhere overnight.

3. Create wound area



Wound confluent cell monolayer using 96-well Woundmaker.

4. Add EDM + media containing treatment



Overlay cells with ECM (50 µL/well) ± treatment, polymerize, then overlay wells with media ± treatment (100 µL/well).

Important

In advance of invasion experiments it is important to have stored the Cool Pack accessories at the correct temperatures for at least 4 hours:

- CoolBox x 2 (block with gelpack: -20° C), CoolSink 96F x 2 (4° C),
- CoolSink 96F x 1 (37° C).
- CoolBox M30 System (block with gelpack: -20° C) with CoolRack (4° C).

The Cool Packs are used to ensure close temperature control of Matrigel® in microplates. At 4–8° C, Matrigel® is a viscous liquid. Polymerization will occur slowly at 4–8° C, and more rapidly when at room temperature or higher. For this reason, it is imperative to keep Matrigel® solutions at 4–8° C during experimental set-up to avoid unwanted gelling. It is easier to handle low volume (< 500 µL) ECM solutions using pre-cooled (from a fridge), wide bore pipette tips or serological pipettes. We recommend sourcing a batch of Matrigel® with a concentration of > 9 mg/mL and an endotoxin level of < 1.5 (EU)/mL.

Day 0

Coat Plate with ECM (if required)

1.1 Using a CoolSink M30 System with CoolRack, dilute Matrigel® stock to 100 µg/mL in culture media.

Note: The day prior to coating the Imagelock plate, thaw a bottle of Matrigel®, packed in ice, overnight at 4° C. When fully thawed, there should be no visible gel aggregates. If aggregates are present, replace the bottle on ice and thaw at 4° C for a longer period of time. After thawing, chill ten 2 mL micro centrifuge tubes in the CoolSink M30 System (10 min), and using a pre-cooled serological pipette, create 1 mL aliquots of Matrigel® and store at -20° C.

1.2 Coat an Incucyte® Imagelock Plate with 50 µL/well of diluted Matrigel® (100 µg/mL). Gently rock the plate to ensure even coating of each well.

1.3 Place the plate in a 37° C incubator, 5% CO_2 and allow the biomatrix material to polymerize for 2 hours.

Seed Cells

2.1 Remove plate from 37° C. Using a manual pipette, aspirate the Matrigel® coating from the wells prior to cell seeding.

2.2 Seed cells at a density of 10,000–40,000 cells/well (100 µL/well, 100,000–400,000 cells/mL stock) into each well of the coated Incucyte® Imagelock Plate.

2.3 Allow the cells to settle at ambient temperature for 15 minutes, then place the plate into a 37° C incubator, 5% CO_2 overnight.

Prepare Biomatrix Top Layer, Then Create Wound

3.1 On ice, prepare the biomatrix top layer by diluting Matrigel®, typically 4–8 mg/mL, in cold culture media ± treatments.

3.2 Carefully remove the Incucyte® Imagelock Plate from the incubator, and use the Woundmaker (refer to Creating Wounds: Seven Simple Steps on page 3) to simultaneously create wounds in all wells. Please make sure that the plate is seated flat in the plate holder prior to wounding.

3.3 After wounding, immediately aspirate the media from each well and carefully wash the cells twice with culture media (100 µL/well).

3.4 After washing, add 100 µL of culture media, then cool the cell plate to 4° C for 5 minutes using the CoolSink and CoolBox 96F.

Add ECM and Media ± Treatments

4.1 Aspirate media from wounded Incucyte® Imagelock Plate, and carefully overlay cells with 50µL of the Matrigel® top layer (prepared in step 3.1). Remove any bubbles from the assay plate.

4.2 To polymerize the biomatrix top layer, warm the cell plate to 37° C by placing the plate onto a pre-warmed CoolSink inside the incubator.

4.3 After 30 minutes, add additional media (100 µL/well ± treatments).
Note: Leaving the plate to polymerize longer than 60 minutes will cause the biomatrix top layer to dehydrate.

4.4 Place the cell plate into the Incucyte® Live-Cell Analysis System and allow the plate to warm to 37° C for 30 minutes prior to scanning.
   a. Objective: 4X, 10X (recommended), or 20X
   b. Channel selection: Phase Contrast (+ Fluorescence if analyzing cells with fluorescent labels)
   c. Scan type: Scratch Wound (Wide Mode optional for 10X, required for 20X)
   d. Scan interval: Every 2–3 hours

4.5 Wash and store the Woundmaker according to the wash protocol.

A complete suite of cell health applications is available to fit your experimental needs.

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