

IncuCyte™ 96-Well Real-Time Cell Migration and Invasion Assays

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Introduction

Cell migration is a multistep process that is a fundamental component of many biological and pathological processes such as embryonic development, tissue re-organization, angiogenesis, immune cell trafficking, chronic inflammation and tumor metastasis. Cell migration is initiated by a stimulus that activates a set of signaling pathways leading to cellular polarization and a rapid reorganization of actin filaments and microtubules. Cells advance by protruding their membrane at their leading cell border, which is followed by dynamic substrate adhesion via integrin adherence to the substrate. Membrane retraction at the lagging cell edge finishes the cycle, which is then repeated in rapid succession. The summation of this process results in cell migration.

Metastasis, another multistep process, is governed by growth factors, hormones, genetic and epigenetic factors that in turn alter the phenotypic characteristics of tumor cells (1, 2). Cellular invasion plays a key role in metastasis. Transformation from a benign to a metastatic phenotype is accompanied by an increased potential

for a cancer cell to invade and remodel its microenvironment (3). The ability of tumor cells to form a metastatic tumor is primarily determined by the cell's ability to change and reorganize its cellular morphology, and to degrade the extracellular matrix (ECM). Both features are needed for secondary tumor formation (1). The key steps in forming a metastatic tumor are listed in Table 1.

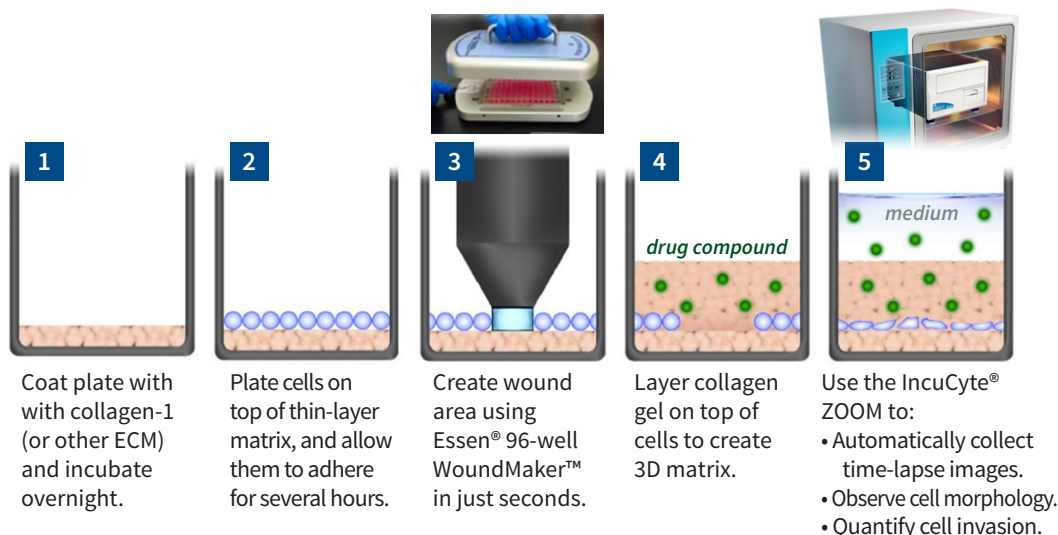
The basement membrane is a thin, continuous sheet of extracellular matrix that surrounds organs and represents a barrier to tumor cells (4). Type I collagen is a major component of the ECM. This biomatrix material presents a significant barrier for invasion of tumor cells into the lymph and vascular networks, as well as the surrounding organ tissue. In most cases, collagen 1 must be degraded in order for tumor cells to spread.

In this application note, we demonstrate that the Migration Assay can be used to assess the 2D cellular migration potential in the presence of experimental agents and as a starting point for creating a flexible, kinetic and quantitative 3D cell

Invasion Assay. In both the Migration and Invasion Assay, the 96-well WoundMaker™ is used to generate a cell-free zone in a monolayer of cells. The precision engineered pins remove cells from each well of a 96-well microplate without disrupting the optimized biomatrix coating used to coat the wells of the assay microplate. Alternatively, Migration Assays may proceed on tissue culture treated plastic without a coating. After wounding, the Invasion Assay is initiated by overlaying the cells with an optimized concentration of a biomatrix material. The cell Migration Assay is initiated by simply adding media to the cells (Figure 1). In both cases, the IncuCyte® automatically acquires images for the entire duration of the assay. After the assay is completed, the IncuCyte's integrated software provides the means to quantify cell migration and invasion. Both the Migration and Invasion Assay are label-free and highly reproducible. The ability to determine if the effect of a pharmacological agent and/or genetic manipulation is specific is just one of the many powerful features afforded by the Migration and Invasion assays.

Table 1.
Metastatic Tumor Formation

- Detachment of cells from the primary tumor
- Invasion of a cancer through the basement membrane and into blood vessels (**Intravasation**)
- Survival in the circulation
- Attachment to vascular endothelium
- Exit of tumor cells from a capillary and entry into a new organ (**Extravasation**)
- Tumor formation in a new organ



Experimental Methods

- 1) Prior to initiating a 96-well assay, cells were grown to confluence in a 96-well Essen ImageLock™ plate in standard a CO₂ incubator. For Migration Assays, the wells were either left uncoated or were coated with a biomatrix material to assure tight adherence of the cells to the well. For Invasion Assays, the wells of the plate must be coated with biomatrix material in order for the 3D gel layer to bind tightly, creating the tissue-like environment needed for invasion to occur. The use of Essen ImageLock plates ensures that the wounds were automatically located and registered by the IncuCyte™ software.
- 2) The 96-pin WoundMaker simultaneously creates precise and reproducible wounds in all wells of a 96-well ImageLock plate by gently removing the cells from the confluent monolayer using an array of 96 pins. Once the plate was loaded into the WoundMaker, the process of making 96 precise wounds only took a few seconds following Essen's detailed protocol.
- 3) For Migration Assays: After washing, media containing the appropriate agents was added and the plate was placed inside the IncuCyte.
 - For Invasion Assays: After washing, the ImageLock plate was placed onto a pre-chilled CoolBox 96F microplate rack (Biocision) and allowed to equilibrate for five minutes. 50-70 µl/well of an optimized concentration of biomatrix material was then added to each well of the 96-well microplate. When pharmacological agents were tested, each was diluted to its final concentration in the gel layer. The microplate was then incubated on a prewarmed CoolSink 96F microplate rack in a 37 °C CO₂ incubator for 30 minutes to allow biomatrix material to gel, and then overlaid with an equivalent volume of complete medium containing the pharmacological agents under investigation.
- 4) Assay plates were then equilibrated within the IncuCyte® for a minimum of 15 minutes before the first scan. The software was set to scan the experiment every hour for migration assays or every 2 to 3 hours for invasion assays using "Scratch Wound" as the "Experiment Type". For Migration Assays, the data was analyzed by one of three integrated metrics: a) wound width, b) wound confluence, or c) Relative Wound Density. These metrics are calculated by custom algorithms that are part of the IncuCyte™ software package. These user informed algorithms are capable of identifying the wound region and provide visual representations of the segmentation parameters (Figure 2). For the Invasion Assay, Relative Wound Density algorithm was used to report data, as it is the only metric that has been qualified by Essen to measure Cell Invasion. For ZOOM Processing, an image collection was created using three to five representative phase contrast images. A cell type-specific Processing Definition was then used to analyze experimental data.
5. Additional statistics and graphing were completed using GraphPad Prism following data export. Morphological features were assessed at every time point and time-lapse movies were created to observe cell activity over the duration of the assay.

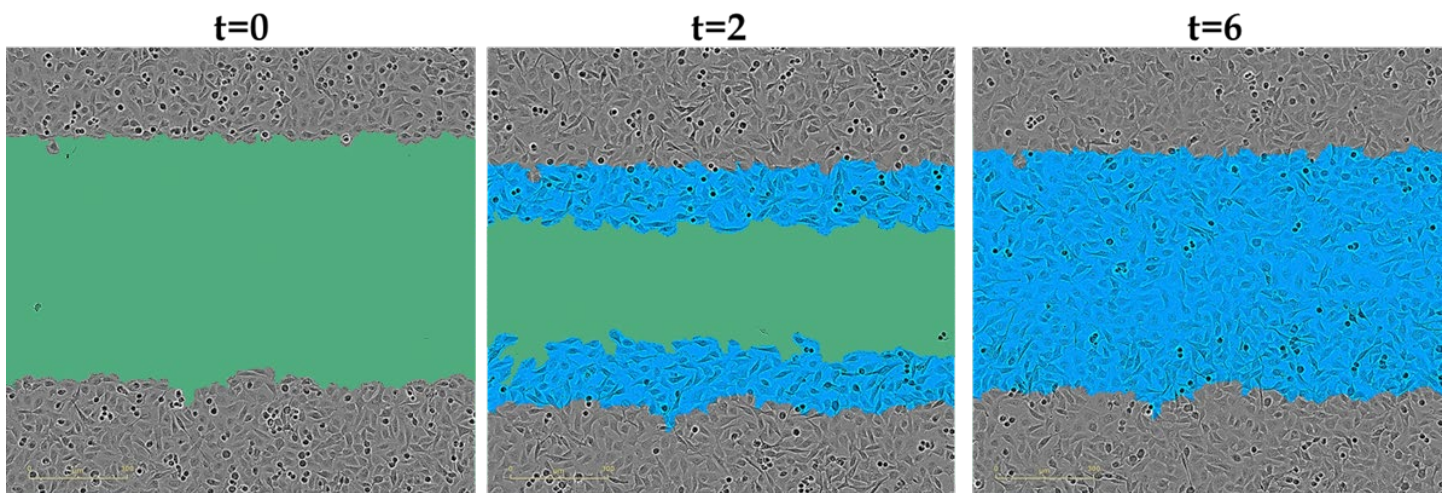


Figure 2. Quantifying Cell Migration and Invasion.

The green region denotes the scratch wound mask over time (t=0, t=2 hours) as HT-1080 cells migrate in to the wound region. The initial scratch wound mask, created immediately following wound creation, is shown in blue. Complete wound closure is observed at t=6 hours.

Quantitation Methods

The IncuCyte software utilizes three separate metrics for quantifying the cell migration over time. The first metric is wound width. In some cases, as cells migrate the new boundaries that are formed remain relatively parallel to each other. If this is the case, then changes in wound width accurately report the time course and extent of cell migration. The second metric is wound confluence. This

metric is a report of the confluence of cells within the wound region. The third metric is Relative Wound Density (RWD). This is the only metric qualified for use in the Invasion Assay. This metric relies on measuring the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point. It is designed to be zero at $t=0$, and 100% when the cell density

inside the wound is the same as the cell density outside the initial wound. In this respect, the metric is self-normalizing for changes in cell density which may occur outside the wound as a result of cell proliferation and/or pharmacological effects. Importantly, the RWD metric is robust across multiple cell types as it does not rely on finding cell boundaries.

Performance of 96-Well WoundMaker™

A key factor in building precision into both the Migration and Invasion Assays was the ability to make exact wounds. This is important in several respects. The kinetics of wound closure, e.g. the time duration until complete closure, are dependent on the initial wound width. Larger wounds take longer to close and variations in wound size can change the time course and slope of wound closure metrics. Consequently, consistent wounds make data interpretation easier. Having wounds of a consistent size also helps ensure that the wound area can be contained within a given image field-of-view (Figure 3). This is critical for automation of the assay. Furthermore, as in all biological experiments, one would like to ensure that all wells have approximately the same physical geometry. As described in the introduction, the complex mechanisms which drive migration are, in part, driven by cell-cell interactions. Reproducible wound areas make this biology more consistent, aiding in assay precision and interpretation.

The differences in wound closure rates and cell morphology between migrating and invading HT-1080 cells are shown in Figure 4. Migrating HT-1080 cells (odd numbered columns) closed the wound region at a significantly faster rate with complete wound closure detected by 10-12 hours post wounding. In contrast, HT-1080 cells invading 8 mg/ml Matrigel® (Becton, Dickinson and Co.) reached 80% wound closure within 48 hours as measured by the Relative Wound Density metric. Morphological differences were also noted. Migrating cells maintained a fibroblastic morphology, had rounded lamellipodia, and advanced as a uniform population of cells. Invading HT-1080 cells adopted a mesenchymal phenotype displaying extended cell bodies and, in some cases, “spike-like” lamellipodia as the cells advanced into the Matrigel® matrix in an irregular manner. The clear morphological differences between migrating and invading HT-1080 cells can be used to select the optimum condition for the invasion assay.

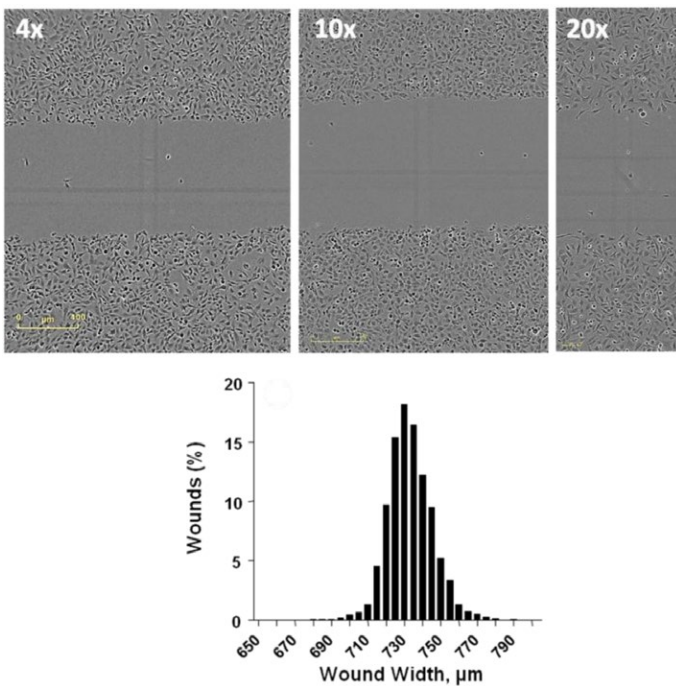
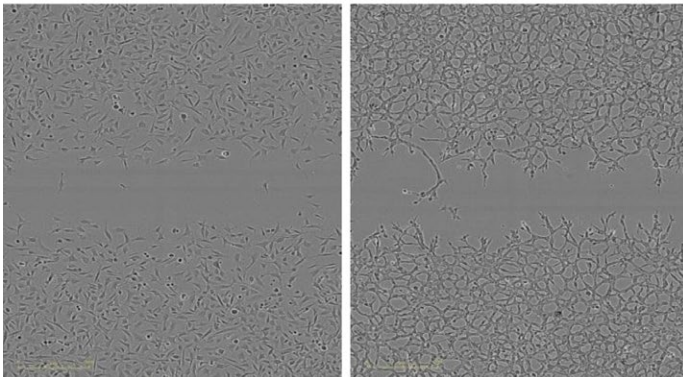
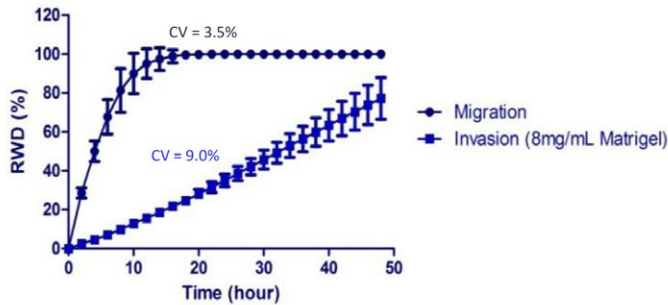
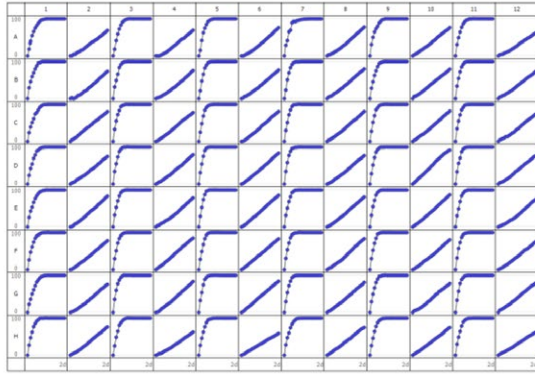


Figure 3. Formation of Precise Wounds using the Essen WoundMaker™. Top) Representative images from wounds made using a confluent monolayer of HT-1080 cells at 4x, 10x, and 20x objectives, imaged using IncuCyte ZOOM. Bottom) Histogram of 960 individual wounds compiled from 10 independent WoundMakers on 10 different 96-well ImageLock™ plates of HT-1080 cells.



Differentiating between invasive and non-invasive cells

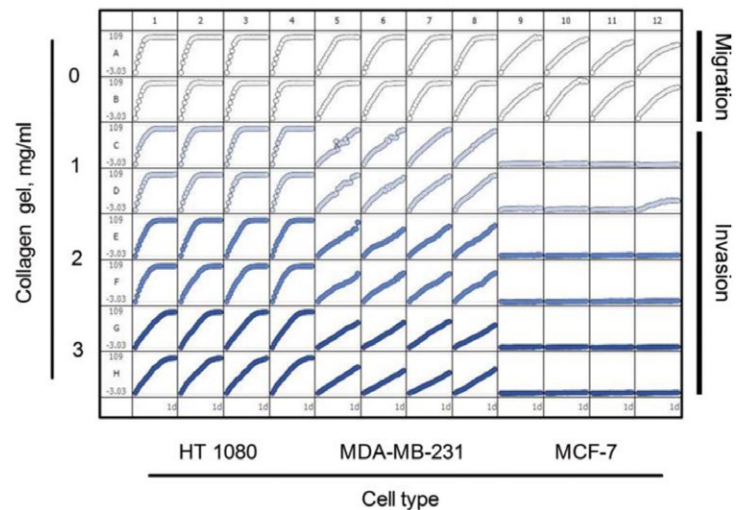
Both MDA-MB-231 and HT-1080 cells are highly invasive cell types (6). In contrast, MCF-7 cells are relatively non-invasive (7). All three cell types were tested in invasion and migration assays as indicated in Figure 5. As we have previously shown, HT-1080 cells migrated on collagen 1 coated plates and invaded 3D collagen 1, with the rate of invasion slowing as the concentration of collagen 1 matrix was increased from 1-3 mg/ml. MDA-MB-231 cells also migrated on collagen 1-coated plates, but invaded 3D collagen more slowly than HT-1080 cells. Interestingly, the rate of invasion of MDA-MB-231 cells was similar at all concentrations of 3D collagen 1 tested.

MCF-7 cells, like the other two cells types, migrated on collagen 1-coated plates, but in contrast to the other two cells types, MCF-7 cells did not have the capacity to invade 3D collagen 1 at the concentrations tested.

Figure 4 (Left). Measurement of the reproducibility the migration and invasion assay in the same microplate.

HT-1080 cells were plated at 2×10^4 cells per well on 100 μ g/ml Matrigel® coated ImageLock™ plates. The cells in odd numbered columns had only media added after using the 96-well WoundMaker™ representing cell migration. The cells in even numbered columns were overlaid with 8 mg/ml Matrigel® representing invasion. Top) Temporal progression of wound closure in each well with time using RWD as the metric to measure migration or invasion. Middle) Time course of means of each condition. The respective coefficients of variation for each assay were averaged. Bottom) Representative images of HT-1080 cells migrating on Matrigel® (left) and invading through 8mg/mL

Figure 5. 96-well microplate graph of three cell types in the cell migration and invasion assay. All wells were coated with 300 μ g/ml collagen 1. HT-1080 (2×10^4 cells per well) are in column 1-4, MDA-MB-231 (2.5×10^4 cells per well) are in column 5-8 and MCF-7 (5×10^4 cells per well) are in column 9-12. Rows A and B show the cell migration data for the three cell types. The same cells in the invasion assay are shown in rows C-D (1 mg/ml 3D collagen 1), rows D-E (2 mg/ml 3D collagen 1) and rows G-H (3 mg/ml 3D collagen 1). The plate map graph shows the progression of each well with time using the RWD metric to measure migration or invasion.



Pharmacology Experiment Using the Essen Cell Migration and Invasion Assays

Blebbistatin is a pharmacological agent that is known to inhibit myosin by binding to the ATPase intermediate with ADP and phosphate bound at the active site, slowing the release of phosphate and inhibits locomotion of cells (8). Previous studies have suggested that blebbistatin may be more effective at inhibiting 3D cell invasion as compared to 2D cell migration (9). With the CellPlayer 96-well format, it is easy to set up an experiment to measure migration and invasion concurrently within the same microplate. Using this approach, we tested the effect of blebbistatin on migration and invasion using HT-1080 cells. The plate map in Figure 6A demonstrates a convenient way to set up this experiment. Three columns of cells were used for migration, and three were used for invasion. A 7-point concentration curve of blebbistatin was carried out as depicted in rows A-G. Row H was used a solvent control. The microplate graph in Figure 6B demonstrates the reproducibility of the assay and shows the effect of each concentration of drug in both assay formats. By inspection, it appeared that blebbistatin had a larger effect on invasion compared to migration. Plotting the data as the average of the treatment group for migration and invasion made it clear that blebbistatin had a much larger effect on invasion (Figure 6C and D, respectively). Figure 6E shows the concentration response analysis at the 24-hour time point for both assays. From these data, the calculated IC₅₀ of blebbistatin for migration and invasion is 92 and 5.2 μM, respectively. The Z' for this data set is 0.77.

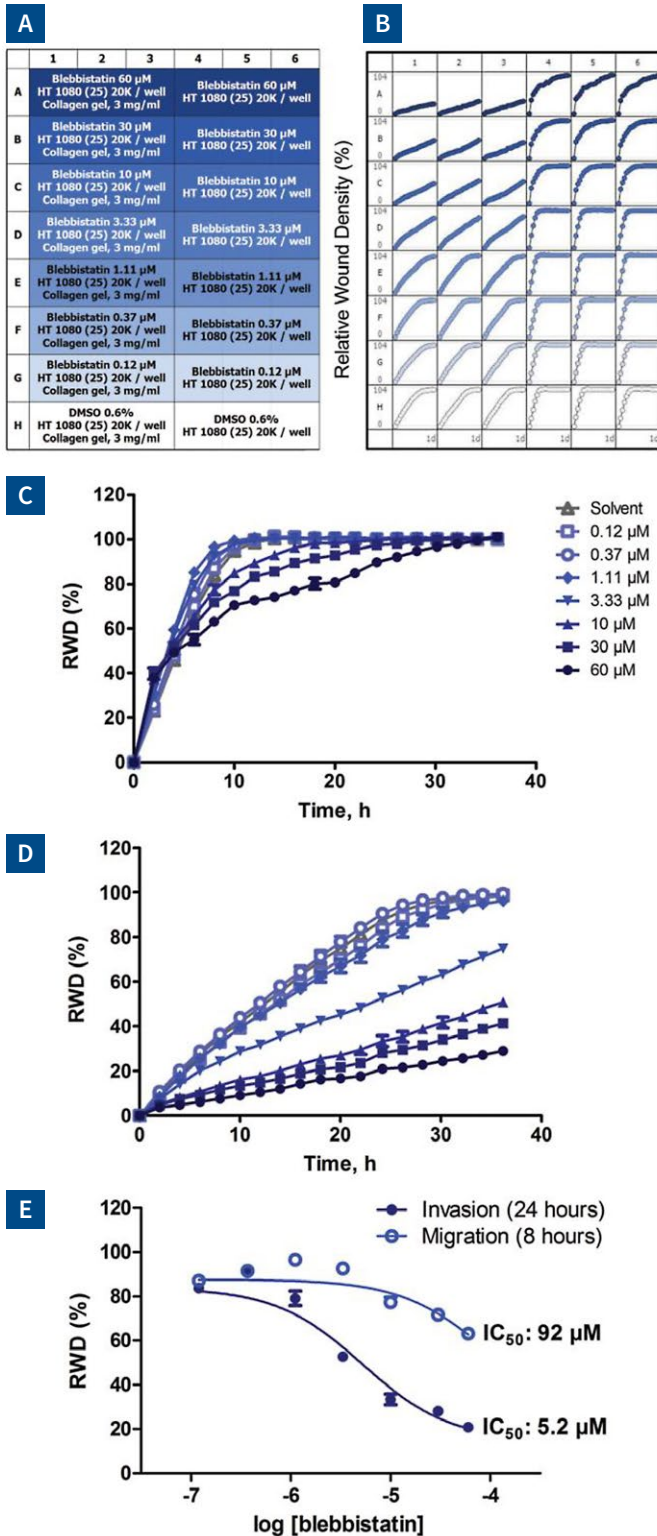


Figure 6. Effect of blebbistatin on the migration and invasion of HT-1080 cells. All wells were coated with 300 μg/ml collagen 1. HT-1080 (2×10^4 ceHT-1080 cells were plated at 2×10^4 cells per well on 300 μg/ml collagen 1 coated plates. Panel A shows the plate map for the experiment. The cells in columns 1-3 were overlaid with 3 mg/ml 3D collagen 1 containing blebbistatin or solvent control. The cells in columns 4-6 were given complete growth media with blebbistatin or the solvent control. Panel B shows the microplate graph of the experiment. Panel C and D show the means of each treatment group for migration and invasion, respectively. Panel E shows the concentration response analysis of blebbistatin on cell migration and invasion. The IC₅₀ calculation for each assay is included next to the concentration response curve.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade collagen 1 and other basement membrane materials and are expressed at increased levels by many highly metastatic tumor derived cells. GM6001 is a broad MMP inhibitor that has been shown to inhibit the invasion of HT-1080 cells into collagen 1 (5). We configured the CellPlayer 96-well Migration and Invasion assay in order to test the effect of this drug on invasion and migration on the same microplate. As shown in Figure 7A, GM6001 had no effect on the migration of HT-1080 cells. In contrast, as shown in Figure 7B, GM6001 inhibited invasion into collagen 1 in a concentration-dependent manner. Interestingly, the addition of a protease cocktail containing E-64 (25 μ M) pepstatin A (100 μ M), leupeptin (2 μ M) and aprotinin (2 μ M) had no measurable effect on cell migration or cell invasion (data not shown).

Two-color Fluorescent Scratch Wound Experiments

With the release of IncuCyte ZOOM, users now have the ability to collect two-color images in addition to phase contrast images within the Scratch Wound mode. In combination with the Essen CellPlayer™ fluorescent protein reagents, users can now address the interactions between several cell types in a mixed culture, and how each affects migration, invasion, and proliferation of the other, all within one well of a 96-well plate. As shown in Figure 8, the non-invasive MCF-7 cells were labeled with NuLight Green, and the HT-1080 cells labeled with NuLight Red were mixed in co-culture, and plated for an invasion assay through 8 mg/ml Matrigel®. Imaging in phase, red, and green channels revealed the HT-1080 cells efficiently invaded the Matrigel® matrix whereas MCF-7 remained non-invasive.

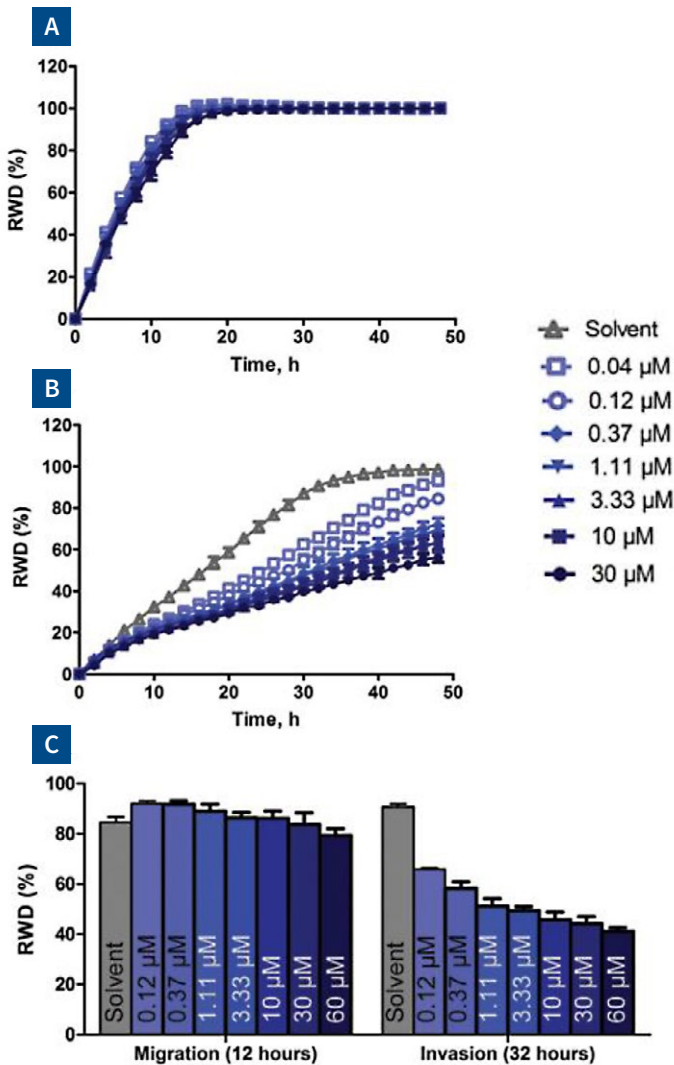


Figure 8. Two color fluorescent scratch wound. The ability to image cells in both wavelengths in addition to phase contrast now allows users the ability to explore cell-cell interactions as it pertains to cell migration and invasion. In this example, HT-1080 NuLight Red cells were plated with MCF-7 NuLight Green cells and invasion through 8 mg/ml Matrigel® was monitored over time (Image shows the 24 hour time point).

Figure 7. HT-1080 cells were plated at 2×10^4 cells per well on 300 μ g/ml collagen 1 coated plates. The GM6001 concentration response was carried out as described for blebbistatin experiment described in Figure 6. Panel A and B show the means of each treatment group for migration and invasion, respectively. Panel C shows the effect of GM6001 on cell migration and invasion in a bar chart format at the 32-hour time point.

Conclusion

The data in this application note clearly shows that Essen BioScience's CellPlayer Migration and Invasion Assays are flexible, quantitative, and reproducible. The unique tip design of the 96-well WoundMaker is a critical part of these assays, as it creates a cell-free zone from a confluent monolayer of cells making the biology consistent and reproducible. Both assays utilize IncuCyte's innovative HD optics and integrative analysis algorithm's to make quantitative measurements without the need for labeling cells. Having such high quality images at every time point gives an investigator access to both the kinetics and morphological changes occurring in migration and invasion experiments, allowing for a personal look at the effects test agents have on cells undergoing these processes.

- **Direct comparison of cellular invasion and migration within an assay:**
Investigators can measure 2D migration and 3D invasion on the same microplate. This provides the best opportunity for determining the specificity of drugs and the utility of potential drug targets.
- **Automated data acquisition:**
After the experiment is initiated, phase contrast and/or fluorescence images are collected and processed automatically.
- **Label-free:**
IncuCyte ZOOM's HD optics obviates the need to label the cells.
- **Quantitative Metrics:**
Results are quantitative and reproducible.
- **Flexible:**
After an optimization step, the Migration and Invasion Assay works with many different cell types.
- **Morphological data:**
HD images are acquired at every time point and can be assembled into time-lapse movies for convenient viewing.
- **Kinetic:**
The spatio-temporal, label-free format of both the Migration and Invasion Assays allows investigators to follow both the rate and the extent of migration and invasion for a given set of experimental variables. This feature can be used to explore time-dependent pharmacology, in order to enhance assay sensitivity.

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