

Automated, Kinetic Imaging of Cell Migration and Invasion Assays using Corning® FluoroBlok™ Inserts

Application Note

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Introduction

Metastasis, the spread of a disease-producing agent (such as cancer cells) from the primary site of disease to another part of the body, has been associated with approximately 90% of cancer-related deaths¹. Therefore, it poses the single most significant challenge to manage this disease. The metastatic process involves migration of cells away from the original tumor (many times in response to a particular regulatory factor), followed by invasion into surrounding, or distant tissue.

While many methods exist to measure the migratory, and invasive characteristics of cancer cells, the advent of permeable supports has provided a simple *in vitro* approach to perform these assays. The addition of a fluorescence blocking membrane improves the accuracy of the assay and allows the use of inverted fluorescence microscopy for analysis. This provides the ability to accurately count only the cells reaching the bottom of the membrane instead of relying on photomultiplier tube (PMT)-based measurements, which are more vulnerable to background fluorescence.

Here we demonstrate the ability to monitor cell migration and invasion using a novel cell imaging multi-mode reader. Kinetic imaging was carried out under a controlled (temperature and gas) environment. Cell counts were performed using the Cellular Analysis capabilities in Gen5™ Data Analysis Software.

Materials and Methods

Materials

Cells

MDA-MB-231 RFP cells (Cat. No. AKR-251) and MCF-7 GFP cells (Cat. No. AKR-211) were purchased from Cell Biolabs, Inc. The MDA-MB-231 cells were propagated in an Advanced DMEM Medium plus Fetal Bovine Serum (FBS) 10% and Pen-Strep-Glutamine 1x. The MCF-7 cells were propagated in MEM α Medium plus FBS 10%, Pen-Strep 1x, and human recombinant insulin.

Reagents

CellTracker™ Green CMFDA Dye (Life Technologies Cat. No. C2925).

Cytation™ 3 Cell Imaging Multi-Mode Reader

The BioTek Cytation 3 Imaging Multi Mode Reader combines automated digital microscopy and conventional multi-mode microplate detection providing rich phenotypic cellular information and well-based quantitative data. With special emphasis on live-cell assays, Cytation 3 features temperature control to 45°C, CO₂/O₂ gas control and dual injectors for kinetic assays. The instrument was used to image cells migrating or invading to the bottom of the Corning® FluoroBlok™ membrane.

Gen5™ Data Analysis Software

Gen5 software controls the operation of the Cytation 3 for both automated digital microscopy and PMT-based microplate reading. Image acquisition is completely automated from sample translation, focusing to exposure control. Montage capabilities allow for the capture of images across the entire surface of a low-density plate well, such as the 24-well permeable supports used for this application. Finally, image analysis allows for cell counting on each image and for reporting of total results from each well.

Corning FluoroBlok Inserts

Corning FluoroBlok inserts have a dyed polyethylene terephthalate (PET) microporous membrane that efficiently blocks light transmission of visible wavelengths between 400 to 700 nm, allowing fluorescence detection in a simplified and non-destructive manner. Fluorescently labeled cells present in the top chamber of the insert are shielded from bottom-reading microplate readers or microscopes. The non-destructive detection method enables both kinetic and endpoint analyses of migration and invasion assays. The FluoroBlok HTS 24-well permeable support system (Corning Cat. No. 351158) was used for cell migration analysis, while the Corning BioCoat™ tumor invasion 24-well system containing Corning Matrigel® basement membrane matrix coating (Corning Cat. No. 354166), was used to examine cell invasion.

Methods

Cell Preparation and Dispensing into Corning® FluoroBlok™ Inserts

Cells were harvested and diluted in the appropriate serum-free media to a concentration of 5.0×10^5 cells/mL. MDA-MB-231 cells were then labeled with a $5 \mu\text{M}$ concentration of CellTracker™ Green CMFDA Dye for 30 minutes using the manufacturer's protocol. For cell invasion experiments, inserts were rehydrated prior to cell dispensing with 500 μL of warm phosphate buffered saline for 2 hours in a humidified 37°C , ambient atmosphere incubator. Buffer was removed following the rehydration incubation time. Serum-free media or media containing 10% FBS at a volume of 1 mL was pipetted into basolateral chambers, followed by 200 μL of cell suspension into the appropriate insert.

Kinetic Image-based Monitoring of Cell Migration and Invasion

Corning FluoroBlok plates containing media and cells were immediately placed into the Cytation™ 3, with temperature and gas control having been previously set to $37^\circ\text{C}/5\% \text{CO}_2$. Imaging of each well was completed using a 4X objective and a four-row by three-column image montage. Focusing was carried out with the use of the brightfield channel, because light is able to pass through the pores in the insert membrane. The auto-focus feature enables the imager to focus through the bottom of the basolateral compartment up to the cell layer. The secondary channel was then set to detect the signal from the constitutively expressing fluorescent protein (RFP or GFP) from the cells, or the signal from the CellTracker Green Dye. A discontinuous kinetic procedure was also chosen where imaging was carried out with each designated well once every two hours over a 24-hour incubation period.

Gen5™ Cellular Analysis

Cellular analysis was performed using Gen5 software on the 4X objective captured. This was done to detect the actual number of cells migrating or invading through to the bottom of the membrane and ignore all other portions of the image. Tables 1 and 2 describe the parameters used to count cells with the GFP or RFP channels.

The cells counted from each image within the montage are then combined and reported as a total count for each well at each particular point in time.

Results and Discussion

Analysis of Cell Migration

From a visual analysis of the images captured from positive control wells containing 10% FBS, and negative control wells containing media with no serum, we could clearly demonstrate the migration of MCF-7 cells through the $8.0 \mu\text{m}$ membrane of the Corning FluoroBlok insert in response to strong chemoattractants such as serum (Figure 1), which has been previously shown in the literature².

Analysis was then performed with the Cellular Analysis tool of the Gen5 Data Analysis Software using the parameters previously described in Table 1. Total fluorescence was also determined for each set of collected images.

The number of MCF-7 cells migrating in response to the 10% serum chemoattractant increases dramatically within the first four hours of incubation before reaching a more steady state of migration over the subsequent 20 hours (Figure 2A). This can be explained by the fact that initially there are no cells in the pores of the membrane. Therefore, cells are free to migrate towards the chemoattractant. However, cells most likely migrate at variable rates, with some taking longer to move across the membrane. After the initial 4 hours of incubation, there will still be cells working their way through the membrane. This has the effect of slowing down the overall migratory rate to a more steady state. This is in contrast to basal rates of migration (in negative control wells), which remain steady for the first 14 hours, due to the much lower rate of migration, followed by little to no migration over the final incubation period. When plotting the ratio of cells counted from positive control and negative control wells (Figure 2B), three distinct phases of migration are observed: (1) high initial migration in response to serum versus steady basal migration; (2) slower, steady-state migration towards chemoattractant combined with continued basal migration; and (3) sustained migration in positive control wells versus no discernible migration in negative control wells.

When examining the fluorescent signal results (Figure 2C), it can be observed that the values follow a similar pattern as those from the cellular analysis. However, the change in ratios between positive and negative control wells using this method are dramatically lower, reaching a maximum of only 2.4 (Figure 2D). This is due to the inherent fluorescent background signal detected when using such an analysis technique. An outcome such as

Table 1. 4X Objective GFP Image Cellular Analysis Parameters

<i>GFP Detection Channel Cellular Analysis Parameters</i>	
Threshold	3,000 RFU
Minimum object size	20 μm
Maximum object size	60 μm
Bright objects on a dark background	Checked
Split touching objects	Checked
<i>Advanced Options</i>	
Evaluate background on	5% of lowest pixels
Image smoothing strength	0
Background flattening size	Auto

Table 2. 4X Objective RFP Image Cellular Analysis Parameters

<i>RFP Detection Channel Cellular Analysis Parameters</i>	
Threshold	500 RFU
Minimum object size	20 μm
Maximum object size	60 μm
Bright objects on a dark background	Checked
Split touching objects	Checked
<i>Advanced Options</i>	
Evaluate background on	5% of lowest pixels
Image smoothing strength	2
Background flattening size	50 μm

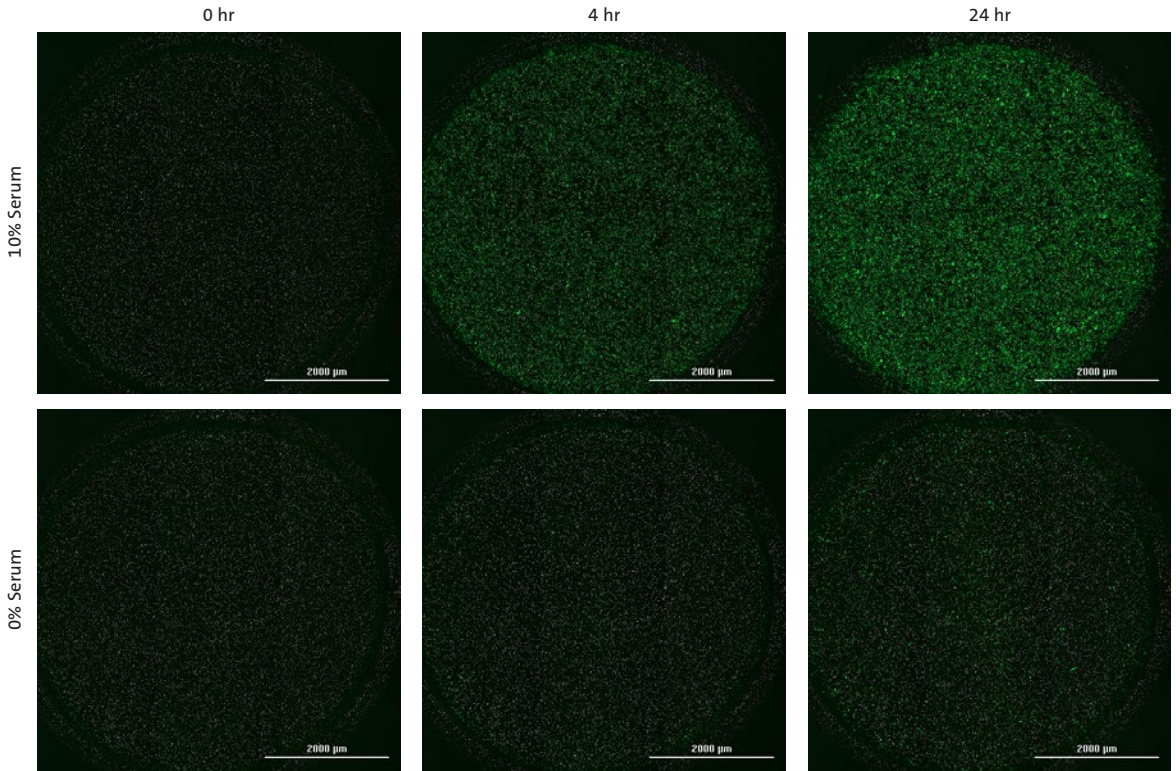


Figure 1. 4X objective of MCF-7 cell migration. Kinetic images taken of GFP expressing MCF-7 cell migration in response to 10% or 0% serum. Images captured using GFP imaging filter cube and a 4X objective.

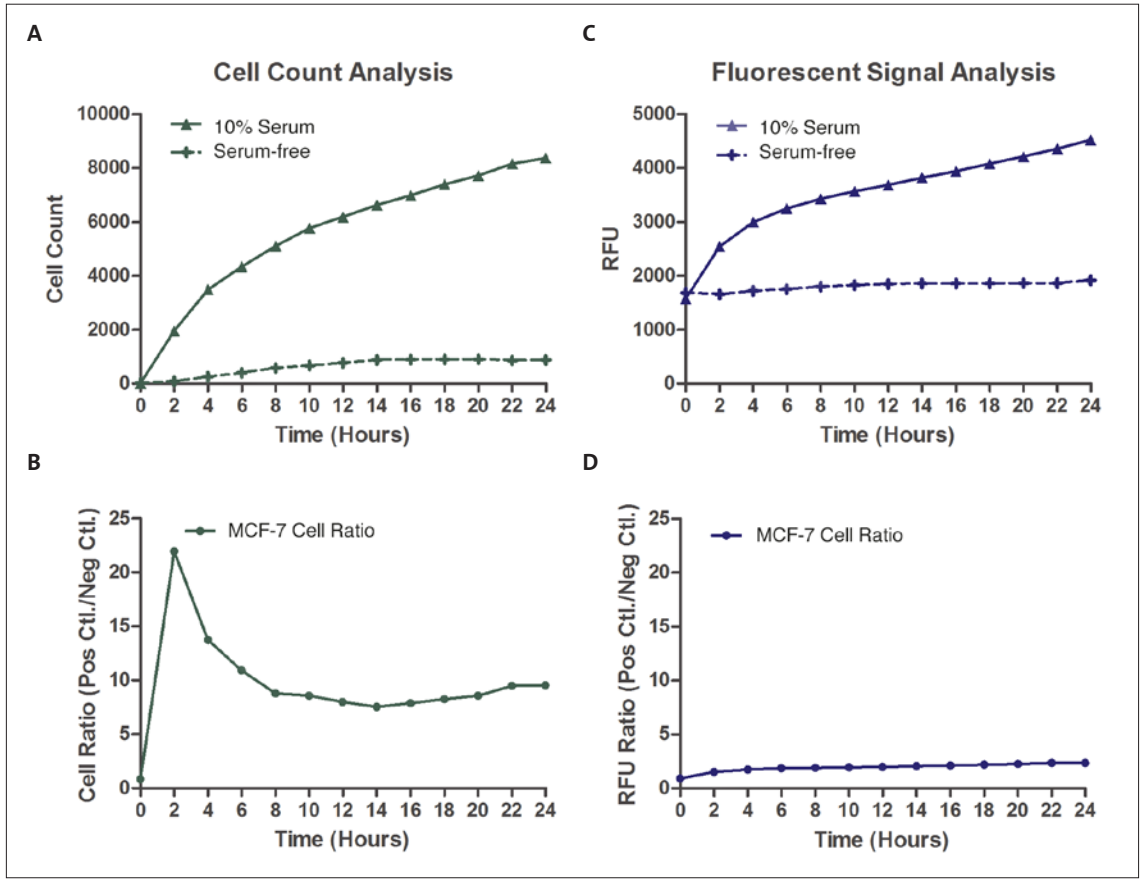


Figure 2. MCF-7 cell migration analysis. Cell count (A-B) and fluorescent signal (C-D) results from kinetic 4X objective.

this may lead to false assumptions regarding the migratory ability of the particular cell type, or what the *in vivo* response would be to a particular chemoattractant. With the incorporation of an approach which can disregard background fluorescence and focus solely on detecting actual cells, the true experimental outcome can be seen.

Cell Invasion Analysis

Kinetic imaging was performed to confirm the invasive ability of MDA-MB-231 cells³ using 10% serum as the chemoattractant control.

The number of invading cells was determined using Gen5™ and the parameters are previously described in Table 2. Total fluorescence was once again determined for each set of collected images.

The MDA-MB-231 cell invasion response to the 10% serum chemoattractant follows the same basic pattern as seen previously for MCF-7 cell migration (Figure 4A). However, the number of cells invading through the Corning® Matrigel® matrix in negative control wells remains low throughout the entire incubation period. This allows the cell ratio to increase dramatically over the total incubation period (Figure 4B). Results from the fluorescent signal analysis illustrate that RFU values from both the positive and negative control wells increase at a slow, steady rate throughout the entire incubation period. The net effect being that RFU ratios

remain low, only reaching a maximum of 1.4. These findings may again lead to false assumptions being made regarding the cell line or the chemoattractant being tested.

Comparison of Signal Analyses between Fluorescent Protein Expressing Cells and Fluorescently Labelled Cells Signal

Not all cells used for cell migration or invasion studies will be constitutively expressing a fluorescent protein. As such, it is necessary to demonstrate the ability of this imaging and analysis steps when using a fluorescent signal from another means. The CellTracker™ Green dye is commonly used for long-term investigation of cell health and movement. Therefore, it is ideal for use with cell migration and invasion assays. The green fluorescent signal is captured with the use of the GFP imaging cube (Figure 5).

Invading cell numbers were once again quantified using Gen5 and the parameters have been previously described in Table 1. Positive/negative cell ratios were calculated from invading cell numbers acquired using the fluorescent signal from the CellTracker dye. The results from the CellTracker analysis were then compared to those previously generated using the RFP signal.

No loss in data quality was seen with the use of signal from a fluorescent cell label, compared to using values generated from a constitutively expressed fluorescent protein. Consequently, both are suitable for quantifying migrating or invading cell numbers.

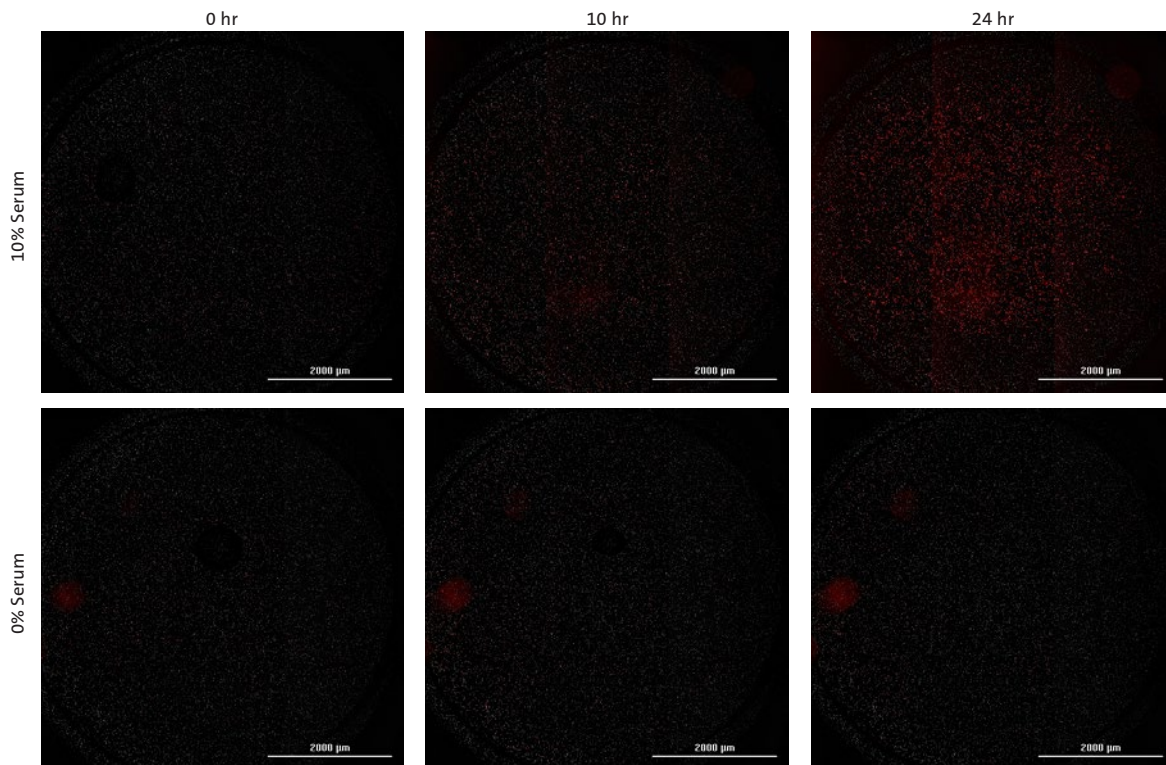


Figure 3. 4X objective of MDA-MB-231 cell invasion. Kinetic images taken of RFP expressing MDA-MB-231 cell invasion in response to 10% or 0% serum. Images captured using RFP imaging filter cube and a 4X objective.

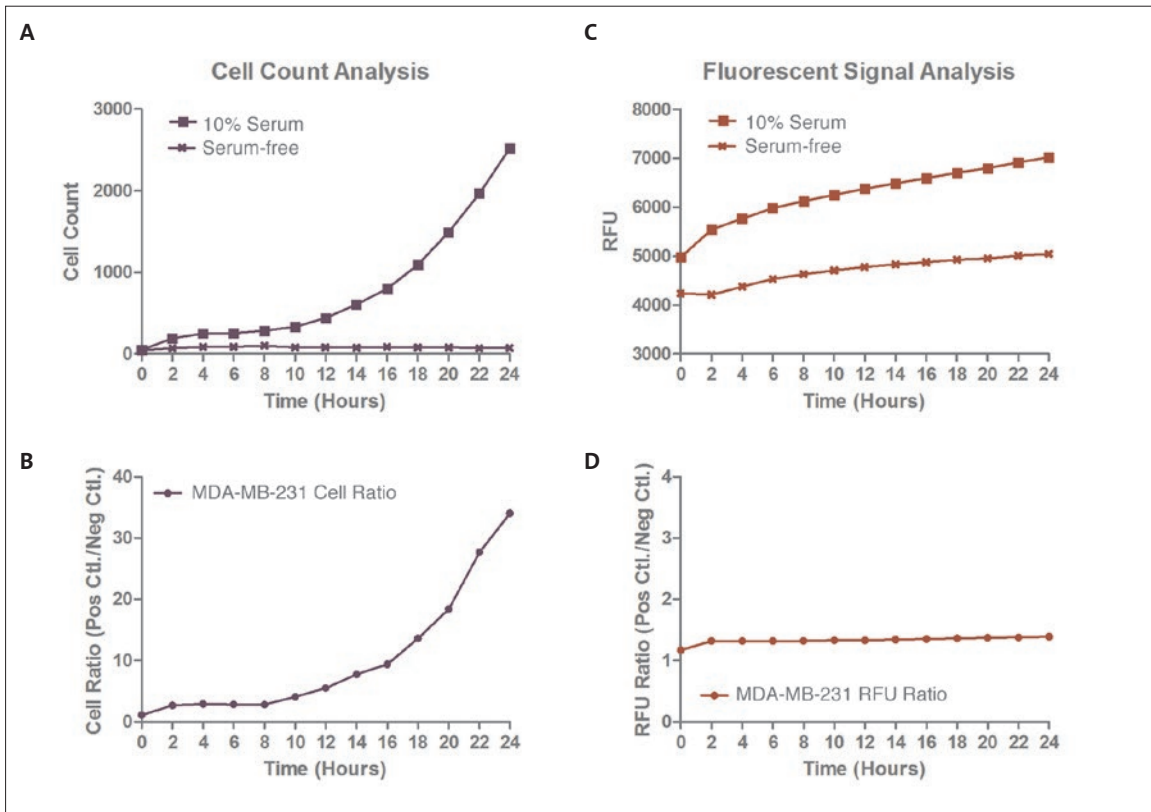


Figure 4. MDA-MB-231 cell invasion analysis. Cell count (A-B) and fluorescent signal (C-D) results from kinetic 4X objective.

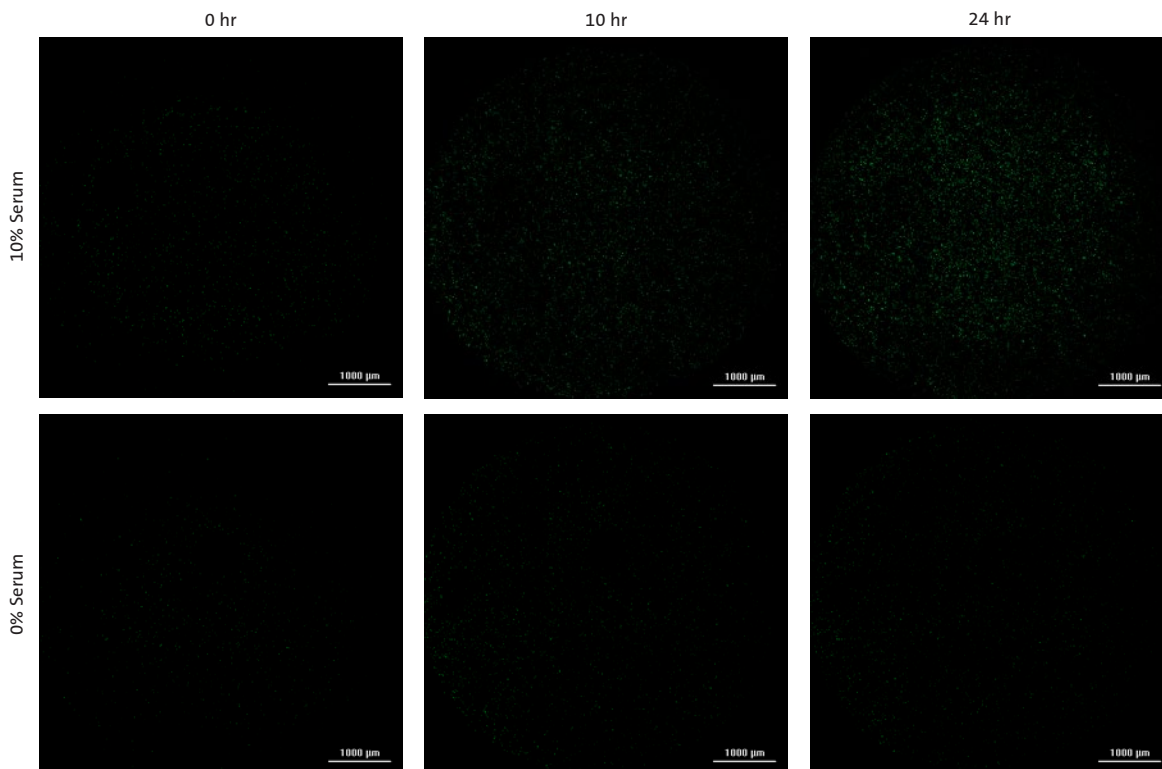


Figure 5. CellTracker Green labeled MDA-MB-231 cell invasion. Kinetic images taken of MDA-MB-231 cells labeled prior to experiment with CellTracker Green CMFDA dye. 10% or 0% serum used as positive and negative chemoattractant control, respectively. Images captured using GFP imaging filter cube and a 4X objective.

Percent Cell Invasion Analysis

Quantifying cell invasion is an important *in vitro* test to assess the response of a metastatic tumor cell model to a test agent⁴. The formula for calculation is as follows:

$$(RFU_i - RFU_b) / (RFU_m - RFU_b) \times 100$$

Here RFU_i represents the fluorescence from cells invading through a basement membrane; RFU_m represents the fluorescence from cells migrating through the uncoated membrane; and RFU_b represents background fluorescent values. The results shown here were taken from RFP data generated with migrating and invading MDA-MB-231 cells. The background fluorescence represents RFU values calculated at time of 0 hours incubation for each experiment.

In Figure 7A, low raw fluorescence and Δ RFU values are seen using this analysis method, since fluorescence from red fluorescent proteins is usually weaker than that from other more green shifted proteins. This can skew the final invasion percentage. When substituting cell count numbers into the same formula (Figure 7B), it is evident that lower percent invasion values are obtained, which may be a better indicator of the actual *in vivo* outcome.

Conclusions

Corning® FluoroBlok™ inserts represent an easy-to-use, robust, representative method to determine the migratory and invasive characteristics of cancer cells. The FluoroBlok insert design

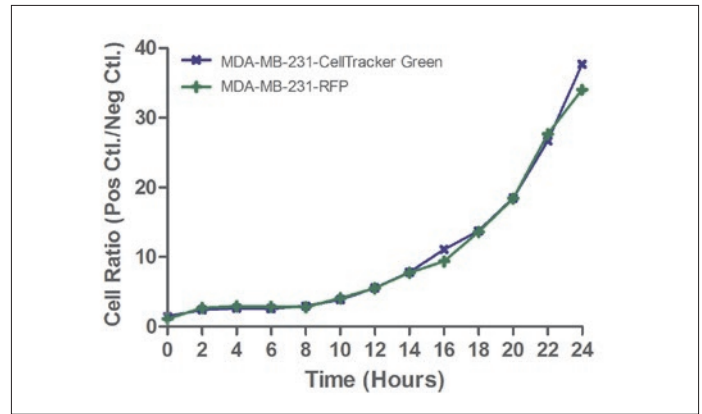


Figure 6. MDA-MB-231 fluorescent cell count comparison. Ratio of cells counted from positive and negative control wells using either RFP or CellTracker Green signal.

and integration of a fluorescence blocking membrane make the plates ideal for use with the Cytation™ 3, because of the instrument's capability to automatically focus up to the cell layer. With the incorporation of temperature and gas control, as well as the use of brightfield imaging for focusing, longer-term experiments can be performed to determine the appropriate incubation time for the cell type and chemoattractant being examined. Finally, the cellular analysis capabilities of Gen5™ allow for determination of the number of actual migrating or invading cells, which can yield more accurate information from these important experiments.

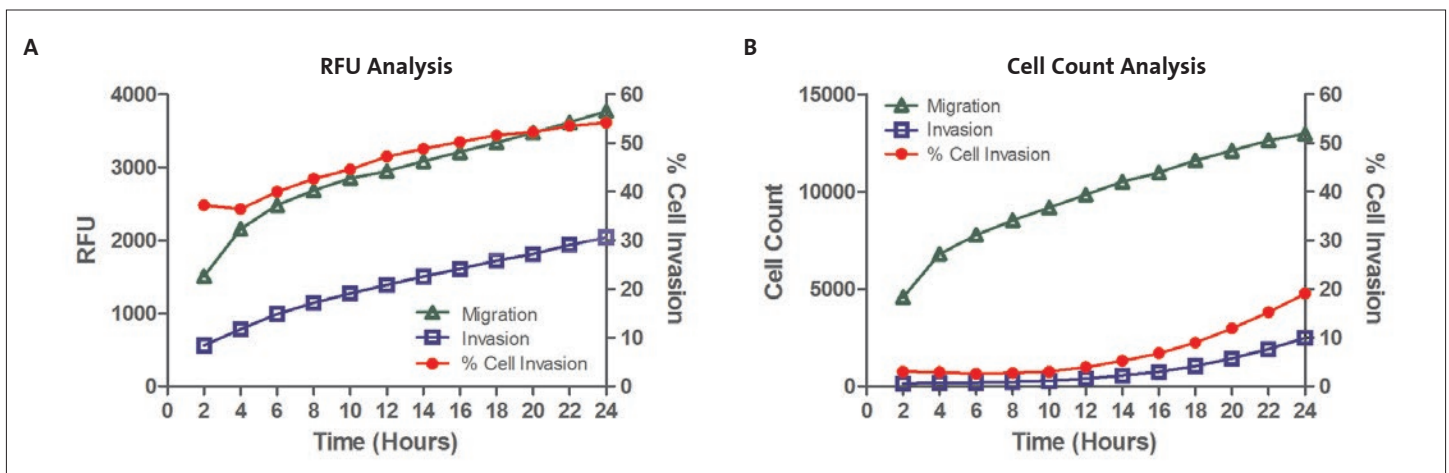
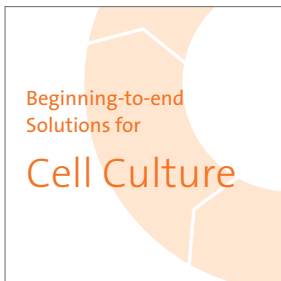


Figure 7. Percent cell invasion determination. RFU (A) and cell count (B) values determined from migratory and invasive RFP expressing MDA-MB-231 cells in response to 10% serum.

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