

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

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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 deaths. Therefore, it is the single most significant challenge to management of the 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 sometimes 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 performing these types of assays. The addition of a fluorescence blocking membrane improves the accuracy of the assay, and allows the incorporation of inverted fluorescence microscopy for analysis. This provides the ability to accurately count the number of cells reaching the bottom of the membrane in real-time, instead of relying on 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 through the use of temperature and atmospheric controls. Cell counts were also performed using the Cellular Analysis capabilities of the Gen5 Data Analysis Software.

Cell Imager and Data Analysis Software

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides up to 60x magnification in fluorescence, brightfield, H&E and phase contrast. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C (37 ± 0.2 °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™ insert.

Gen5™ Data Analysis Software: Gen5 software controls the operation of the Cytation 5 for both automated digital microscopy and PMT-based microplate reading. Image acquisition is completely automated from sample translation, focusing and 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 with each image, and the 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 at visible wavelengths between 400 and 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 migration and invasion assays. The FluoroBlok HTS 24 Well Permeable Support System (Corning Catalog No. 351158) was used for cell migration analysis, while the Corning BioCoat™ Tumor Invasion 24-Well system (Corning Catalog No. 354166), containing Corning Matrigel® basement membrane matrix (BME) coating, was used to examine cell invasion.

Experimental Procedure

Cell Preparation and Dispensing into FluoroBlok Inserts: MCF-7 GFP and MDA-MB-231 RFP cells were harvested and diluted in the appropriate serum-free media to a concentration of 5.0x10⁵ cells/mL. MDA-MB-231 cells were then labeled with a 5 µM concentration of CellTracker™ Green CMFDA Dye (Life Technologies Catalog No. C2925) for 30 minutes using the manufacturer's protocol. For cell invasion experiments, prior to cell dispensing, inserts were rehydrated 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 inserts containing media and cells were immediately placed into the Cytation™ 5, with temperature and gas control having been previously set to 37 °C/5% CO₂. 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, due to the fact that 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 images 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.

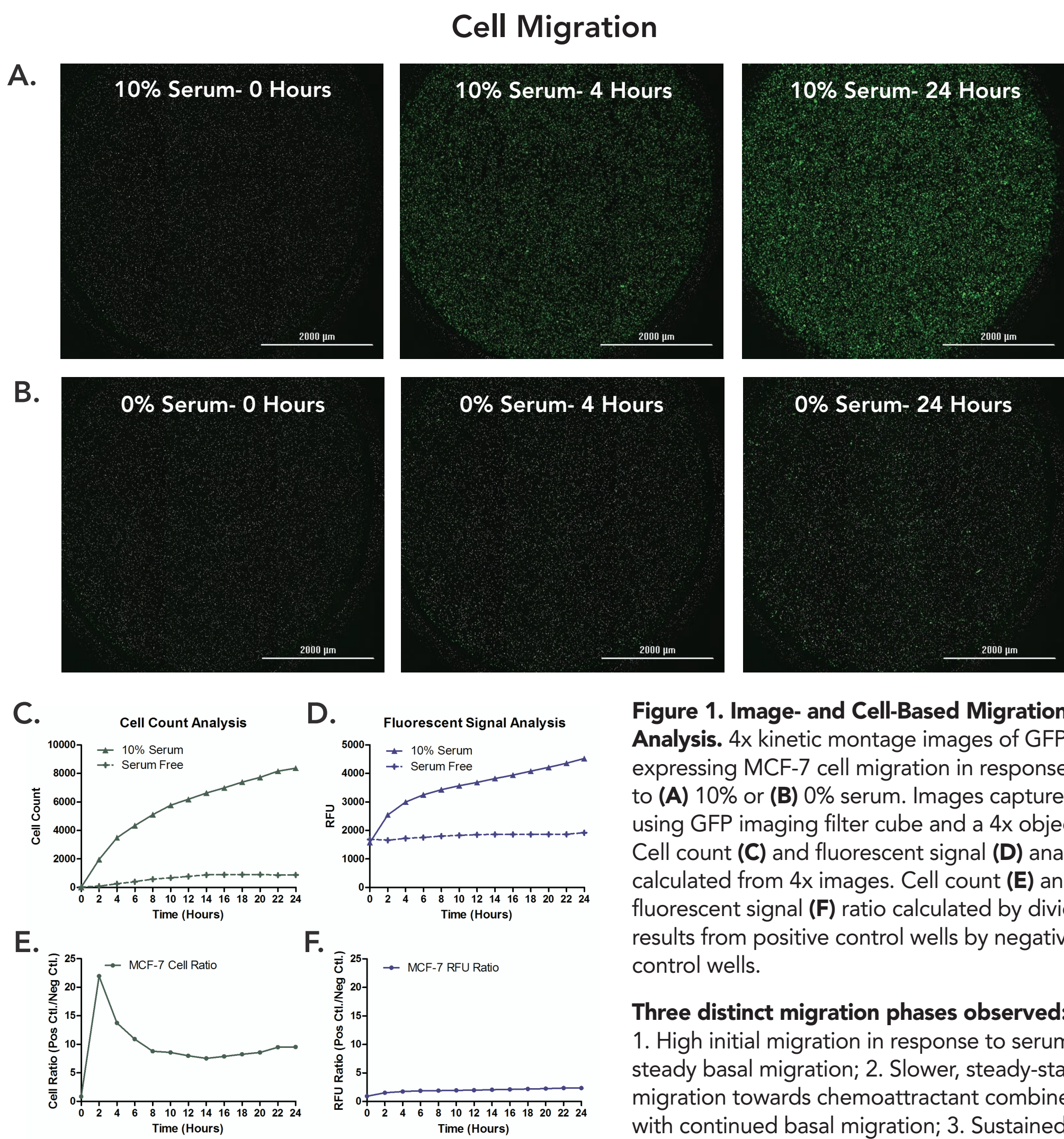
RFP Detection Channel Cellular Analysis Parameters	
Threshold	3,000 RFU
Min. Object Size	20 µm
Max. Object Size	60 µm
Bright Object on a Dark Background	Checked
Split Touching Objects	Checked
Advanced Options	
Evaluate Background On	5% of Lowest Pixels
Image Smoothing Strength	0
Background Flattening Size	Auto

Table 1. 4x GFP image cellular analysis parameters.

RFP Detection Channel Cellular Analysis Parameters	
Threshold	500 RFU
Min. Object Size	20 µm
Max. Object Size	60 µm
Bright Object on a Dark Background	Checked
Split Touching Objects	Checked
Advanced Options	
Evaluate Background On	5% of Lowest Pixels
Image Smoothing Strength	2
Background Flattening Size	50 µm

Table 2. 4x RFP image cellular analysis parameters.

Cell Migration and Invasion Analysis

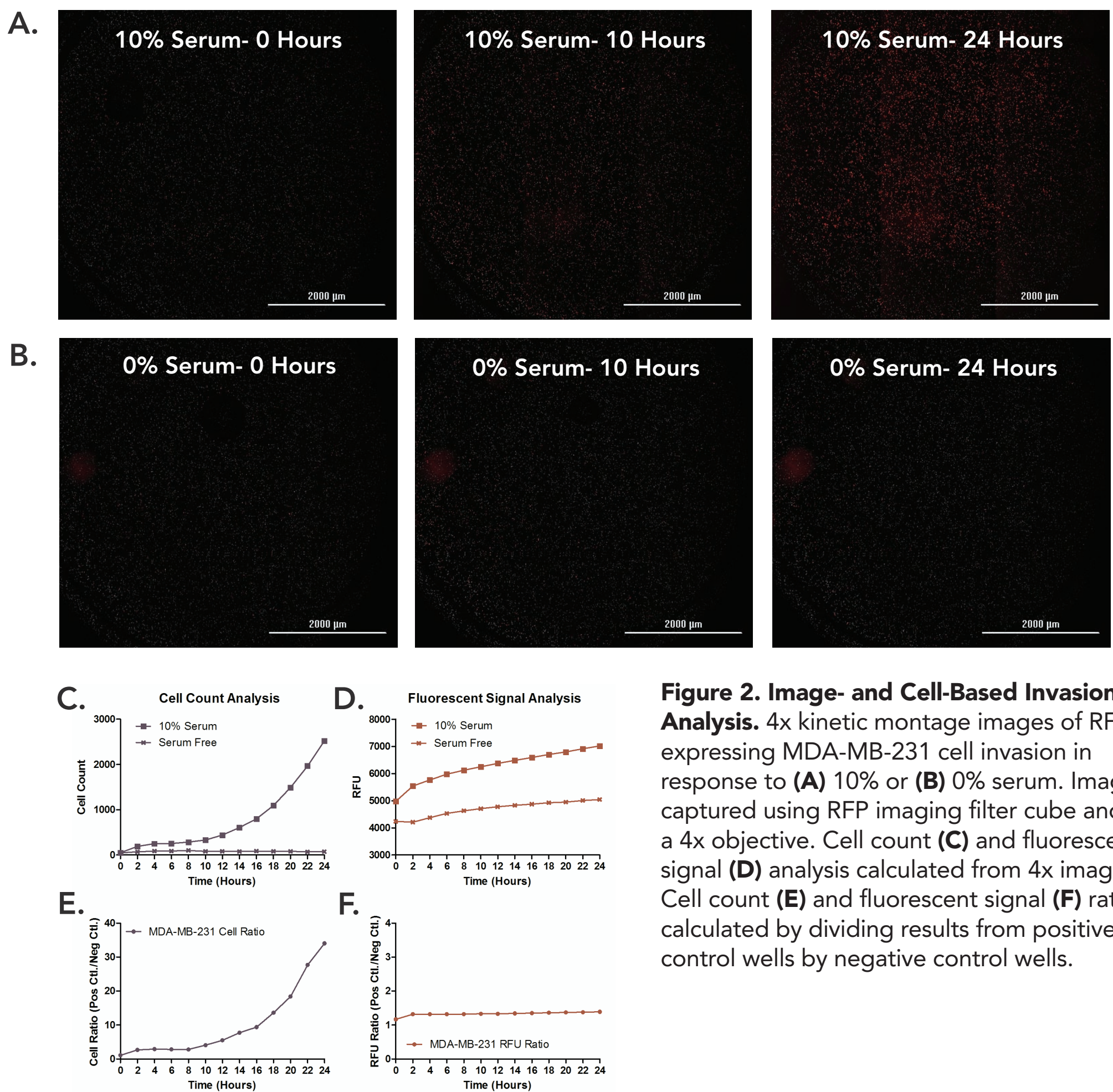


From a visual analysis of the images captured from positive control wells containing 10% FBS (Figure 1A), and negative control wells containing media with no serum (Figure 1B), it is apparent that appropriate MCF-7 cell migration is seen through the 8.0 µm membrane of the FluoroBlok insert, and can be captured kinetically using the microplate reading and imaging capabilities of the Cytation 5.

Analysis of the fluorescent signal data generated from each image illustrates the lower change in ratio seen using this method (Figure 1D and F) compared to the use of cell count results (Figure 1C and E). This is due to the inherent background signal detected using microplate reading. Incorporation of fluorescence microscopy and cellular analysis disregards background fluorescence, and focuses solely on detecting actual migrating cell numbers, thus providing a more true experimental outcome.

Cell Invasion

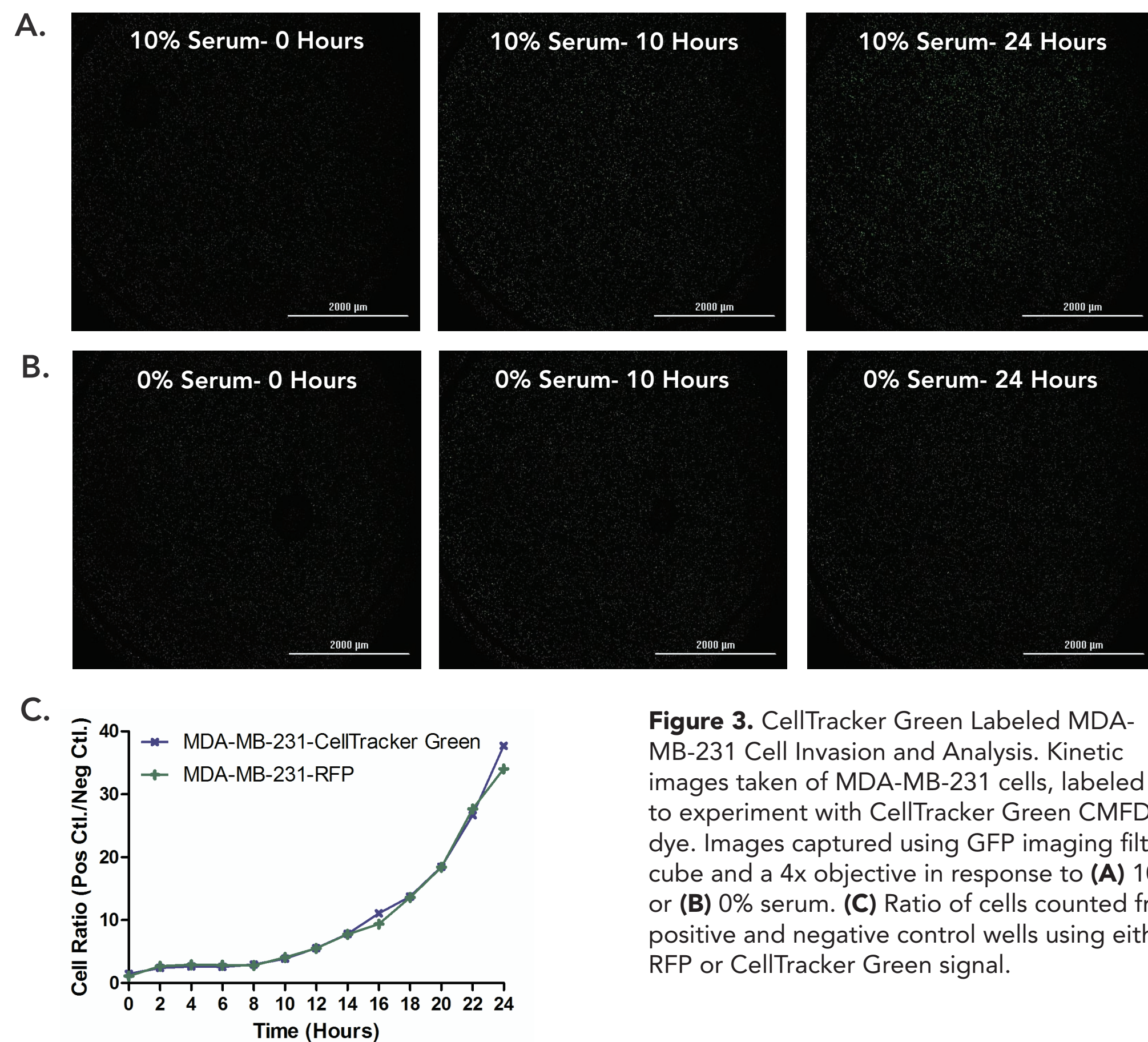
Kinetic microplate reading and imaging were also performed to confirm the invasive ability of MDA-MB-231 cells, using 10% serum as the chemoattractant control. The number of invading cells was determined using Gen5™ and the parameters previously described in Table 2. Total fluorescence was once again 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 2A). However, the number of cells invading through the Matrigel Matrix in negative control wells remains low throughout the entire incubation period (Figure 2B and C). This allows the cell ratio to increase dramatically over the total incubation period (Figure 2E). 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 (Figure 2D). The net effect being that RFU ratios remain low, only reaching a maximum of 1.4 (Figure 2F). These findings may again lead to false assumptions being made regarding the cell line or the chemoattractant being tested.

Fluorescent Protein and Label Signal Comparison

Not all cells used for cell migration and invasion studies constitutively express fluorescent proteins. As such, it is necessary to demonstrate the same imaging and analysis capabilities using fluorescent signals from other means, such as the CellTracker Green dye. The fluorescent signal from the dye is captured with the use of the GFP imaging cube (Figure 3A and B).



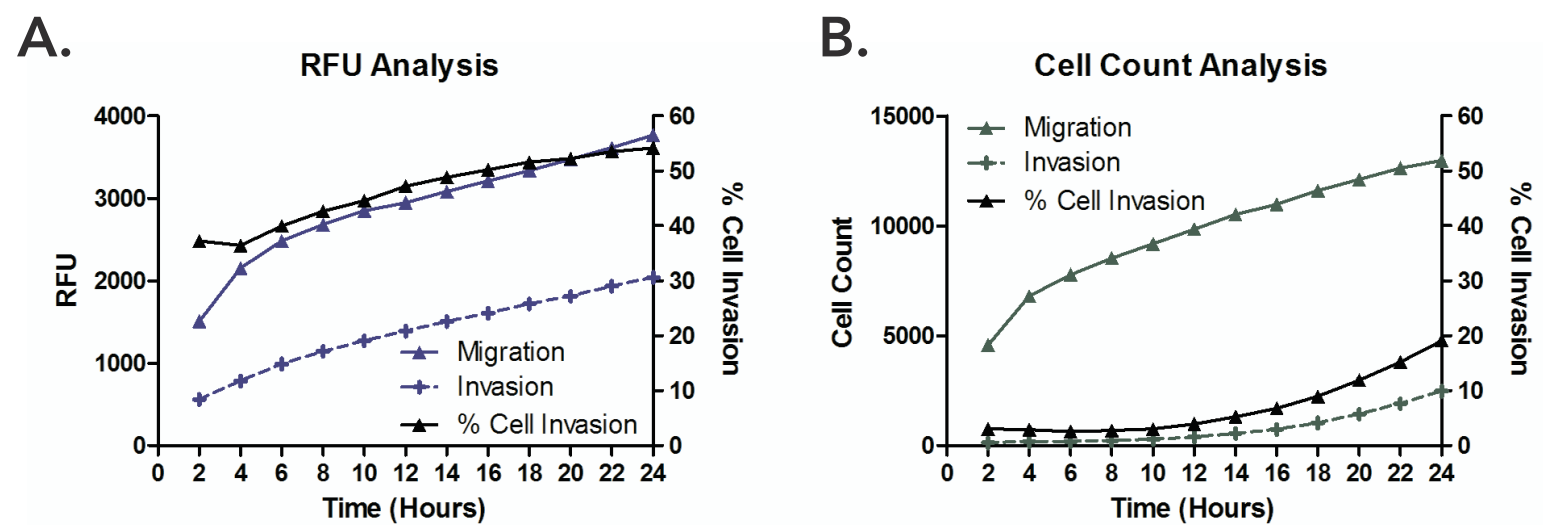
Invading cell numbers were once again quantified using Gen5™ and the parameters previously described in Table 1. Positive/negative cell ratios were calculated from invading cell numbers counted using the fluorescent signal from the CellTracker dye (Figure 3C). The results from the CellTracker analysis were then compared to those previously generated using the RFP signal. From the results in Figure 3C, it is obvious that no loss of data quality is 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.

Percent Cell Invasion Analysis

Quantifying cell invasion is another important *in vitro* mechanism to understanding the response of a metastatic tumor cell model to a test agent (Partridge *et al.*, 2009). The formula for calculation is as follows:

$$\frac{(RFU_i - RFU_0)}{(RFU_m - RFU_0)} \times 100$$

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 RFU₀ represents background fluorescent values. For the results shown here, taken from RFP data generated with migrating and invading MDA-MB-231 cells, background fluorescence represents RFU values calculated at time=0 hours incubation for each experiment.



In Figure 4A, meager raw fluorescence and ΔRFU values are seen using this analysis method, due to the fact that fluorescence from red fluorescent proteins is usually weaker than that from other more green shifted proteins. This can serve to potentially skew the final invasion percentage. When substituting cell count numbers into the same formula (Figure 4B), it is evident that lower % invasion values are calculated, which may be more indicative of the actual *in vivo* outcome.

Conclusions

1. Corning FluoroBlok inserts represent an easy-to-use, robust, representative method to determine the migratory and invasive characteristics of cancer cells
2. The FluoroBlok insert design and integration of a fluorescence blocking membrane make the plates ideal for use with the Cytation 5, due to the instrument's capability to automatically focus up to the cell layer
3. 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
4. Gen5 cellular analysis capabilities allow for determination of the number of actual migrating or invading cells, which can yield more accurate information being gleaned from these important experiments
5. The combination provides an ideal method to study the ability of test molecules to inhibit the migratory and invasive characteristics of metastatic cell models