

Investigation of Cell Migration using a High Density Cell Exclusion Assay and Automated Microplate Imager



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Introduction

Cell migration, the movement of cells from one location to another, can be the result of complex biological or environmental signaling events. The migration of cells is known to occur during a vast array of both normal biological processes such as the development of an organism or marking pathological events such as cancer metastasis. The need to better understand the process of cell migration has led to the development of improved methods of investigation¹ including culturing methods, live cell trackers and detection methods. Improvements in both the investigative methods and throughput technologies will allow for rapid expansion of investigation in the area of cell migration research. This study investigated the use of a novel medium and high density cell exclusion assay for analysis of cell migration. The use of a biocompatible gel (BCG) deposited in a portion of either a 96- or 384-well density microplate was used to exclude cells during seeding. A cell free zone was revealed after dissolution of the BCG following media/cell addition to the wells. After cell attachment, compound addition can be made directly to the wells for investigation. Assay optimization was performed including the pharmacology of a known migration inhibitor, using several cell tracking methods and an automated microplate imager. Data analysis was performed using both image analysis capabilities integrated in the instrument control software, as well as by exporting data for analysis by an open-source image analysis software package.

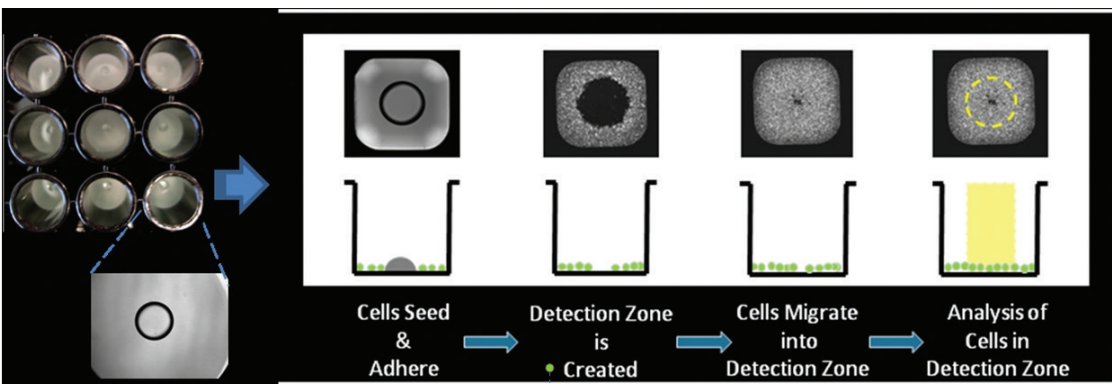


Figure 1 – Oris™ Pro Cell Migration Assays. The Oris Pro Assay uses a non-toxic biocompatible gel, BCG to form a cell-free detection zone on culture surfaces. After cells are seeded into the 96- or 384- well migration assay plates, the BCG dissolves permitting cells to migrate into the well centers. Images of cells in the central detection zone can be captured and quantified in real-time using microscopes or imaging microplate readers.

BioTek Instrumentation



Figure 2 – Cytation™3 Cell Imaging Multi-Mode Reader combines automated digital widefield microscopy and conventional multi-mode microplate detection.

Materials and Methods

Cell Culture

- HT-1080 fibrosarcoma cells were grown to ~80-90% confluency in T-75 tissue culture flasks in EMEM (Gibco Cat. No. 10370-021, Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM L-glutamine, 1% Pen-Strep and 1 mM sodium Pyruvate.
- MDA-MB-231/GFP cells were grown to ~80-90% confluency in T-75 tissue culture flasks in Advanced DMEM (Gibco Cat. No. 12491-015) supplemented with 10% FBS, 2 mM L-glutamine, 1% Pen-Strep.
- Cells harvested using standard methods and MDA-MB-231 cells were resuspended in DMEM/F12 (Gibco Cat. No. 11039-012), no phenol red supplemented w/ 10% FBS, 2 mM L-glutamine and 1% Pen-Strep and counted.

Cell Staining with CellTracker™ Green CMFDA

- HT-1080 cells were washed once in 10 mL of serum free, phenol free, DMEM/F12 (Gibco Catalog No. 10039-021) and counted (Figure 3).
- The cells were then collected by centrifugation (1000 x g) and resuspended in 10 mL of serum free, phenol free DMEM/F12 containing 1 µM CellTracker Green CMFDA (Molecular Probes, Catalog No. C2925, Eugene, OR). The cells were incubated for 30 min. at 37 °C with gentle swirling every 10 min.
- Cells were pelleted at 1,000 x g and resuspended in 14 mL serum free, phenol free DMEM/F12 minus CellTracker Green followed by incubation for 30 min. as described above.
- The cells were collected and resuspended in PFCM at the desired concentration and recounted.

Cell Migration Assay workflow

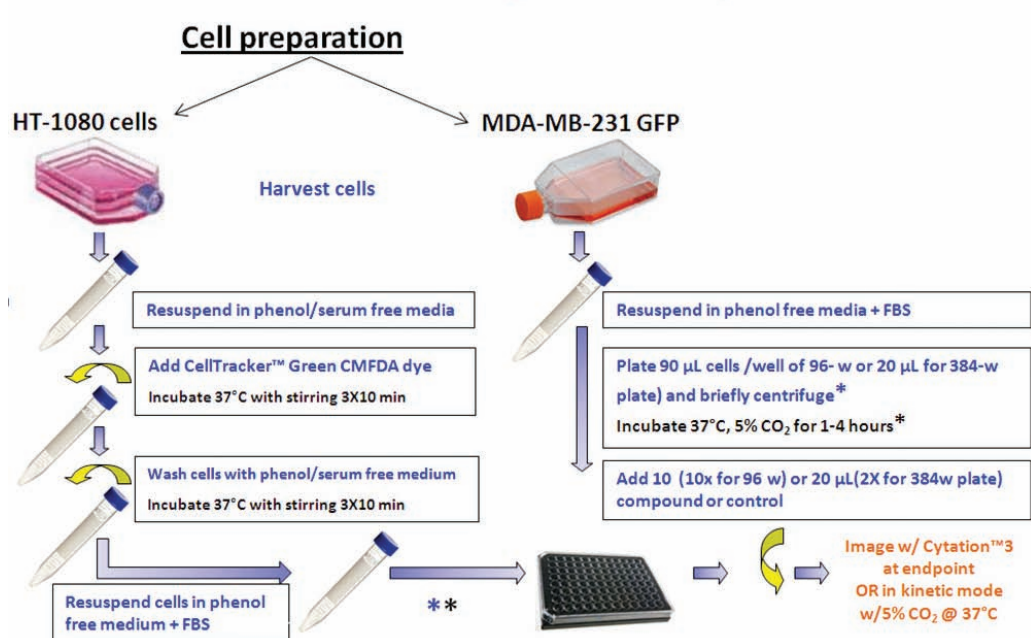


Figure 3 – Assay workflow. HT-1080 and MDA-MB-231/GFP cells were harvested and prepared for seeding. HT-1080 cells were stained with CellTracker Green before seeding whereas MDA-MB-231/GFP expressing cells were plated directly in phenol free media.

Materials and Methods (continued)

Optimization of Cell Seeding Density

- MDA-MB-231/GFP and HT-1080 stained with CellTracker (CT) stained cells were diluted to the appropriate cell plating density in PFCM.
- For the Oris Pro 96 Cell Migration Assay, Collagen I Coated (Platypus Technologies, LLC., Madison, WI), 90 µL of either MDA-MB-231/GFP or HT-1080 CT cells were seeded in replicates of 8 wells at densities of 20, 30, 40 and 50K cells/well.
- For the Oris Pro 384 Cell Migration Assay, Collagen I Coated, 20 µL of MDAMB-231/GFP were seeded in replicates of 12 at densities of 7.5, 10, 12.5 and 15K cells/well or 20 µL of HT-1080 cells were seeded at densities of 14, 16, 18, and 20K cells/well in replicates of 12.
- The assay plate was centrifuged briefly at 1000 rpm in an Eppendorf 5810R centrifuge immediately following the addition of cells to facilitate seeding.
- Cells were allowed to attach for a minimum of 1 hour in a humidified incubator at 37 °C, 5% CO₂ prior to compound addition^{2,3}.
- Wells in one half of the plate were treated with cytochalasin D (CD) to a final concentration of 1 µM while wells in the other half were treated with vehicle alone (0.1% DMSO/PFCM).
- Cells were imaged kinetically for up to 48 hours in a Cytation3 microplate imager with incubation at 37 °C and a gas control module set to 5% CO₂ using the settings outlined in Table 1 and described in further detail below.
- Images were analyzed using Gen5™ Data Analysis Software (BioTek Instruments, Inc., Winooski, VT), Microsoft® Excel® (Redmond, CA), GraphPad Prism (La Jolla, CA) or ImageJ open source software (<http://rsbweb.nih.gov/ij/index.html>)
- Gen5 Data Analysis Software was used to apply a disc-shaped mask revealing the detection zone. The standard deviation derived from the detection zone during statistical analysis of imaging data was used for final analysis.
- ImageJ analysis relied on determination of the area of the detection zone of post-migration wells in comparison with control wells in which no migration occurred to calculate post-migration percent closure using imaging data⁴.

A. Cytation3 Read Parameters	B. Cytation3 Read Parameters
Plate type	96-well
Mode	Image
Objective	2.5X
Color	GFP
Exposure	Auto
Horizontal offset	-170 µm
Verticle offset	-90 µm

Plate type	384-well
Mode	Image
Objective	2.5X
Color	GFP
Exposure	Auto
Horizontal offset	0 µm
Verticle offset	-406 µm

Table 1 – Cytation3 Read Parameters. A. 96-well plate format required both a horizontal and vertical offset while B. the 384-well plate format required a vertical offset to insure the cell free zone was centered in the image field.

Cytochalasin D Dose Response

- Cells were harvested and seeded at optimal density as described above in 96-well Oris Pro microplates.
- Following the 1 hour incubation period cytochalasin D was serially diluted 1:3 and transferred to the assay plate in triplicate. Plates were imaged and analyzed as described above.

Results and Discussion

Optimization of Cell Seeding Density: Oris Pro 96-well Assay

- The optimum cell seeding density was determined for each plate configuration by treatment with either the known migration inhibitor, 1 µM cytochalasin D, or vehicle.
- Kinetic data allowed the optimal incubation time and seeding density to be determined for further experiments (Figure 4).
- Standard deviations calculated using Gen5 or ImageJ cell free zone area analysis were used to determine the seeding density giving the most reproducible results for use in future experiments.
- MDA-MB-231/GFP cells plated at a density of 20,000 cells/well and incubated at 24 hours; and HT-1080 CT cells plated at 50,000 cells/well incubated at 12 hours gave optimal results using both analysis methods
- Both cell lines were then used at the respective seeding densities for dose response experiments using the migration inhibitor cytochalasin D.

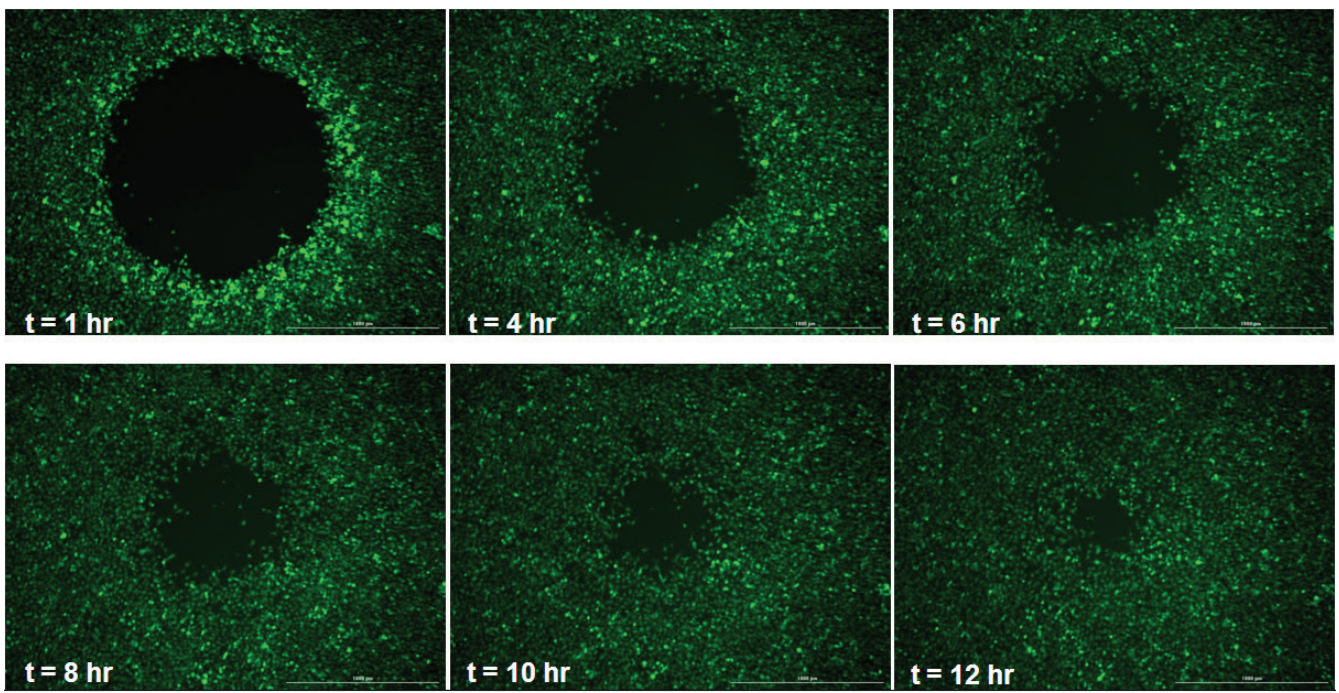


Figure 4 – Seeding density and incubation time optimization. Representative images from a well containing 50K HT-1080 cells per well in a 96-well plate format imaged every two hours for 12 hours.

Results and Discussion (continued)

Cytochalasin D Dose Response

- Cells were treated with CD in a dose response format in triplicate and allowed to migrate for the appropriate length of time (Figure 5).
- Data analysis was performed using the methods described above for determination of IC₅₀ values (Figure 6).
- IC₅₀ values for HT-1080 CT treated cells were 136 and 87 nM when determined by Gen5 and ImageJ analysis, respectively.
- Gen5 showed standard deviation values ranging from ~1,650 to 3,400 RFUs while ImageJ analysis showed a percent closure of the detection zone ranging from 0 to ~65%.
- Determinants of IC₅₀ values for MDA-MB-231/GFP treated cells were 81 and 39 nM when determined by Gen5 and ImageJ analysis, respectively.
- Gen5 showed standard deviation values ranging from ~500 to 3,500 RFUs while ImageJ analysis showed percent closure of the detection zone ranging from 0 to ~66%.
- These determinants showed acceptable correlation between the two analytical methods employed as well as with previously published data³.

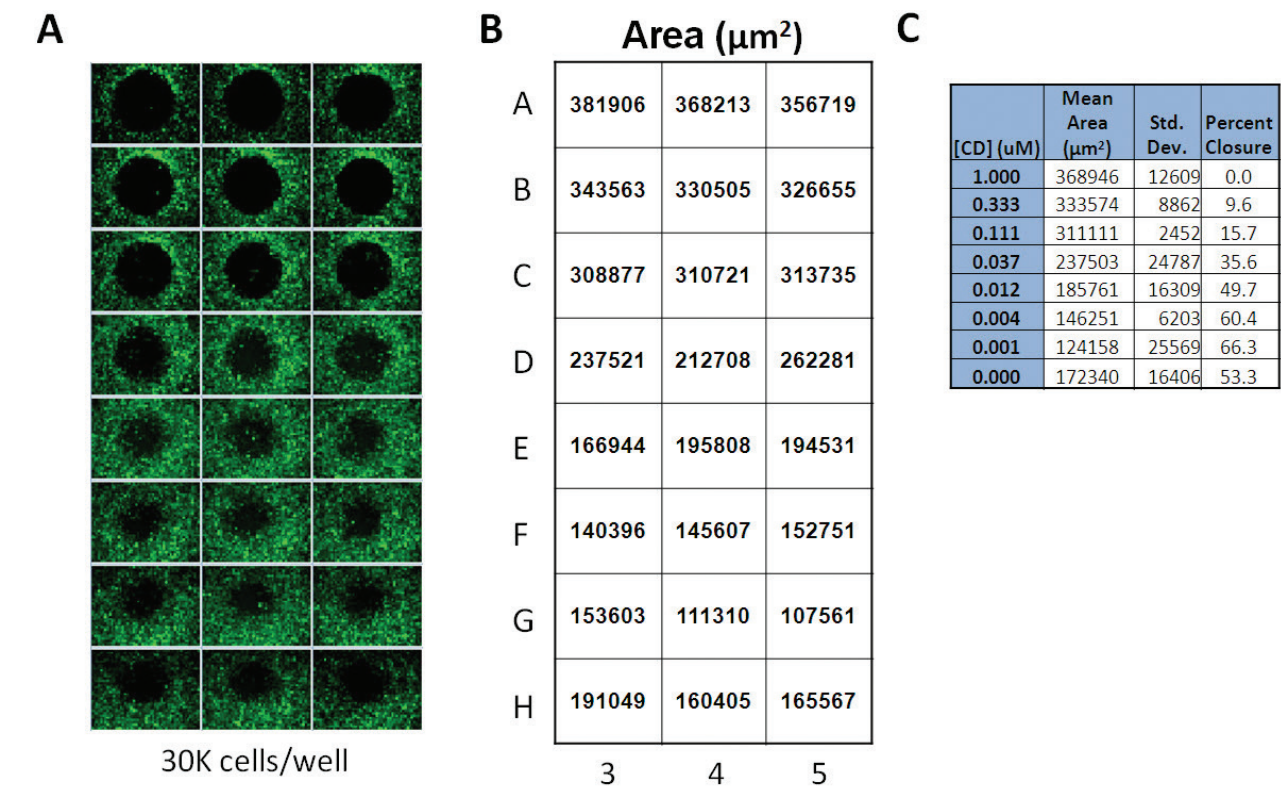


Figure 5 – ImageJ analysis of CD dose response. A. MDA-MB-231 cells were imaged in the 96-well microplate format using widefield fluorescent microscopy and B. analyzed using ImageJ software. C. Mean area of the detection zone can be used for determination of the IC₅₀ concentration. Images of wells subjected to 1 µM CD were considered control wells in which no migration was exhibited and percent closure calculated for each concentration of inhibitor.

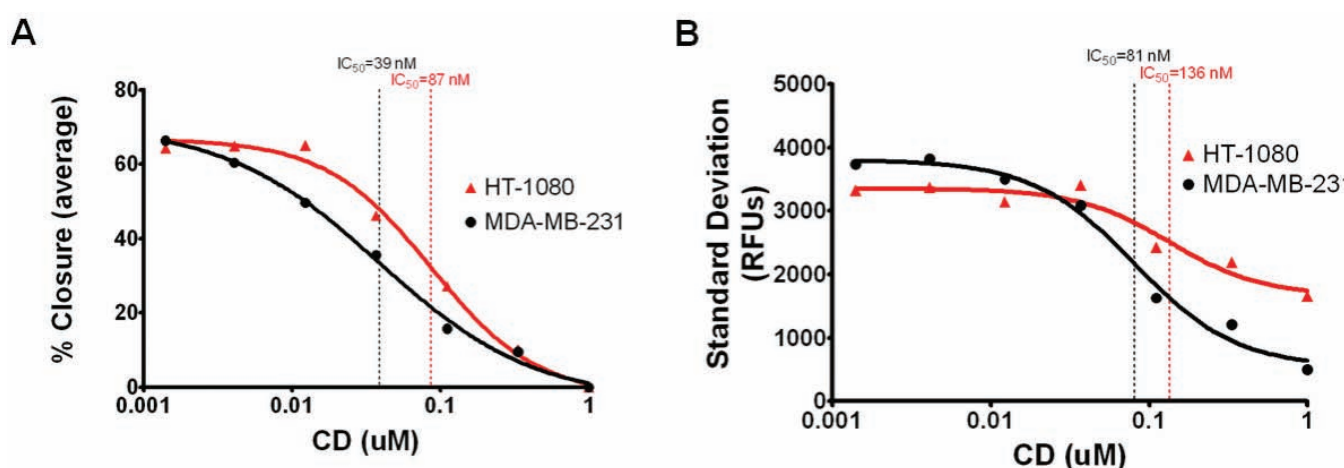


Figure 6 – Dose response curves. MDA-MB-231/GFP and HT-1080 CT cells were imaged in the 96-well microplate format after treatment with CD in dose response format. A. ImageJ analysis plots percent closure vs. inhibitor concentration while B. Gen5 analysis plots standard deviation vs. inhibitor concentration.

Optimization of Cell Seeding Density: Oris Pro 384-well Assay

- ImageJ analysis of MDA-MB-231/GFP cells showed robust performance at a seeding density of 12,500 cells/well following a 24 hour incubation period as indicated by a Z'-factor of 0.57 (Figure 7A).
- Analysis of HT-1080 CT cell seeding densities indicated that robust assay performance can be seen at each seeding density tested (Figure 7B).
- At a seeding density of 20,000 cells/well, robust assay performance was seen following an 8 hour incubation period while seeding densities of 18,000 and 16,000 cells/well required a 10 hour incubation period (Figure 7B).

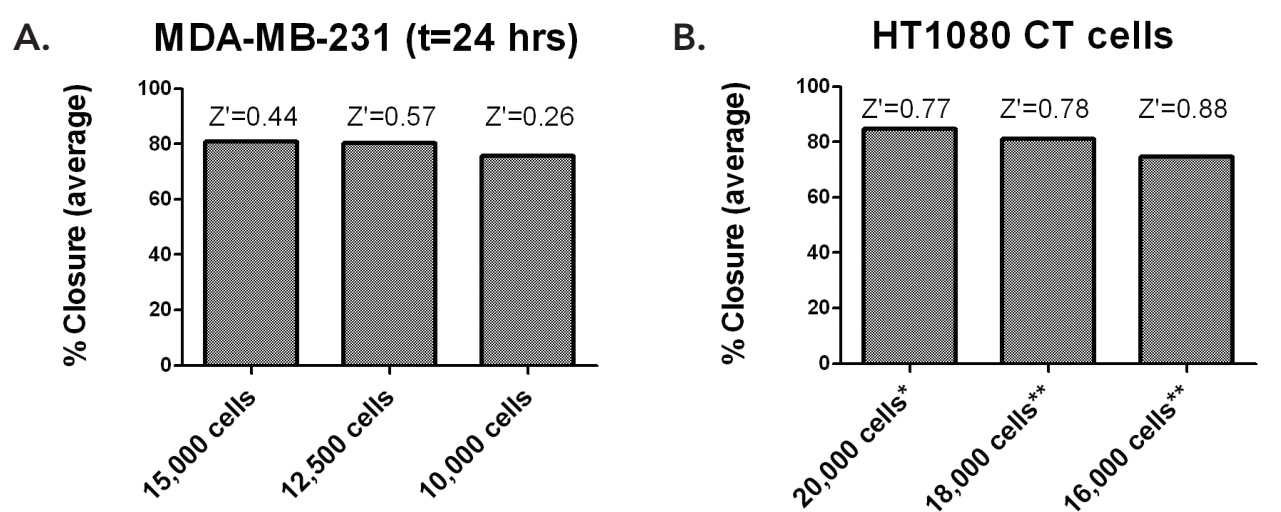


Figure 7 – Optimization of seeding density in 384-well format. A. ImageJ analysis of MDA-MB-231/GFP cell seeding density in 384-well format. Comparison of percent closure and Z'-factor at various cell seeding densities. B. ImageJ analysis of HT-1080 CT cell seeding density in 384-well format. Comparison of percent closure and Z'-factor at various cell seeding densities (*8 hrs., **10 hrs).

Results and Discussion

- The Cytation3 Cell Imaging Multi-Mode Reader provides a suitable platform for quantifying cell migration assays using systems such as Oris Pro migration assays.
- The Oris Pro cell migration assay is a robust, easily automatable assay that provides complete visual access to the cells and is suitable for high throughput screening.
- The quantification of extent of cell migration can be performed using either statistical tools embedded in the Gen5 Cytation3 operating software, or TIFF files may be analyzed in ImageJ for % Closure computations.
- The latter data analysis approach is recommended as it provides for a more robust assay for 96- and especially 384-well assays using the cell lines investigated.

¹Comley, J. Cell Migration: probing cell movement with smarter tools. (2012/13). Drug Discovery World. Winter; Vol 14, Issue 1, pp. 33-51. | ²Oris™ Pro Cell Migration Assay -Collagen I Coated 'Protocol & Instructions' (2013), Platypus Technologies, LLC., Madison, WI. | ³Fronczak, J.A., Hulkower, K.I. and Vogt, A. (2011, May) A robust 384-well cell migration assay for high content analysis of cells treated with anti-cancer therapeutics. Poster session presented at American Association of Pharmaceutical Scientists, Washington, DC. | ⁴Hulkower, K.I. and Gehler S.R. How to measure area closure using the Oris™ Pro cell migration assay Technical Memo. Platypus Technologies, LLC. Madison, WI.