

## Multimode Detection

### Authors:

Christian Gach  
Bernhard Ellinger

Fraunhofer IME  
Hamburg, Germany

Norbert Garbow  
PerkinElmer, Inc.  
Hamburg, Germany

## Screening for Modulators of Smooth Muscle Cell Motility using EnSight Multimode Plate Reader

### Introduction

Smooth muscle cells (SMCs) play an important role in the underlying disease process of cardiovascular disorders. In healthy blood vessels, SMCs display a contractile phenotype that is required to perform vascular contractility, control of blood flow and blood pressure. SMCs of arteriosclerotic vessels

switch at corresponding lesion sites to a synthetic, migratory and proliferative phenotype with an increased capacity to generate extracellular matrix proteins. Besides protein secretion and proliferation, SMC migration is regarded as a key element of atherosclerosis<sup>1-3</sup>. Therefore, the identification of modulators of SMC motility is of medical interest<sup>4</sup>.

Commonly used assay systems to examine cellular migration are scratch or exclusion zone assays<sup>5</sup>. In scratch assays, a cell-free gap is created by damaging a confluent monolayer of cells, while in exclusion zone assays a cell-free gap is created by removal of a growth barrier. The subsequent migration of cells into the cell-free area is detected by image acquisition while quantification of cell migration is achieved by comparing images at different time points to reveal information about the migration characteristics such as gap closure velocity or confluency level.

An effective screen for modulators of cell migration requires the ability to determine migration characteristics in an automated, reliable and reproducible process. The EnSight™ Multimode Plate Reader with well-imaging technology meets this requirement through acquisition of brightfield images and integrated analysis via the Kaleido™ Software. The device enables an easy, fast, reproducible and automated determination of cell migration characteristics. Simultaneously other important cell-specific data such as proliferation rates and morphology<sup>6</sup> can be obtained from the same data set. The brightfield imaging mode of the EnSight system and its built-in image evaluation make the effort and cytotoxic effect of cell staining expendable. Moreover, maintenance of cell viability during measurements is achieved by integrated temperature control. Additional measurement modes such as the recording of the absorption level of the culture medium, enables monitoring of the pH-value thereby providing important metrics for cell cultivation.

In this application note we show how to screen a library of 786 FDA approved known drugs to identify modulators of SMC migration using EnSight Multimode Plate Reader in a fully automated and highly economical process.

## Materials and Methods

### Cell Seeding

Frozen stocks of Clonetics™ Immortalized Human Coronary Artery Smooth Muscle Cells (CASMC XS12C1, hereinafter referred to as SMCs) were thawed and subsequently resuspended and cultured in complete growth medium, following recommended cell culture protocols.

For cell migration experiments, cell number was determined using Scepter™ Cell Counter (Merck Millipore). SMCs were seeded in 96-well plates following the Oris™ Cell Migration Assay Kit instructions (Platypus Technologies), at a density of  $12.5 \times 10^3$  cells per well in a total volume of 100 µl. The silicone based seeding stoppers included in the kit restricted cell seeding and adherence to the periphery of the assay wells. After seeding, SMCs were allowed to spread and attach for four hours at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub> (Figure 1).

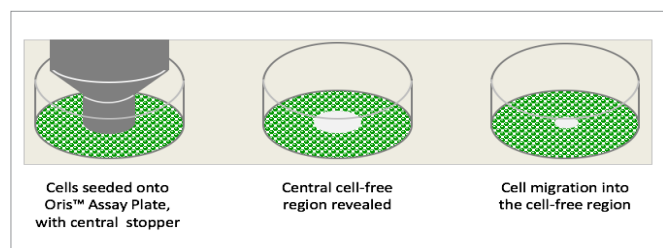


Figure 1. Schematic sequence of Oris™ Cell Migration Assay procedure (adapted from the Oris™ Cell Migration Assay manual).

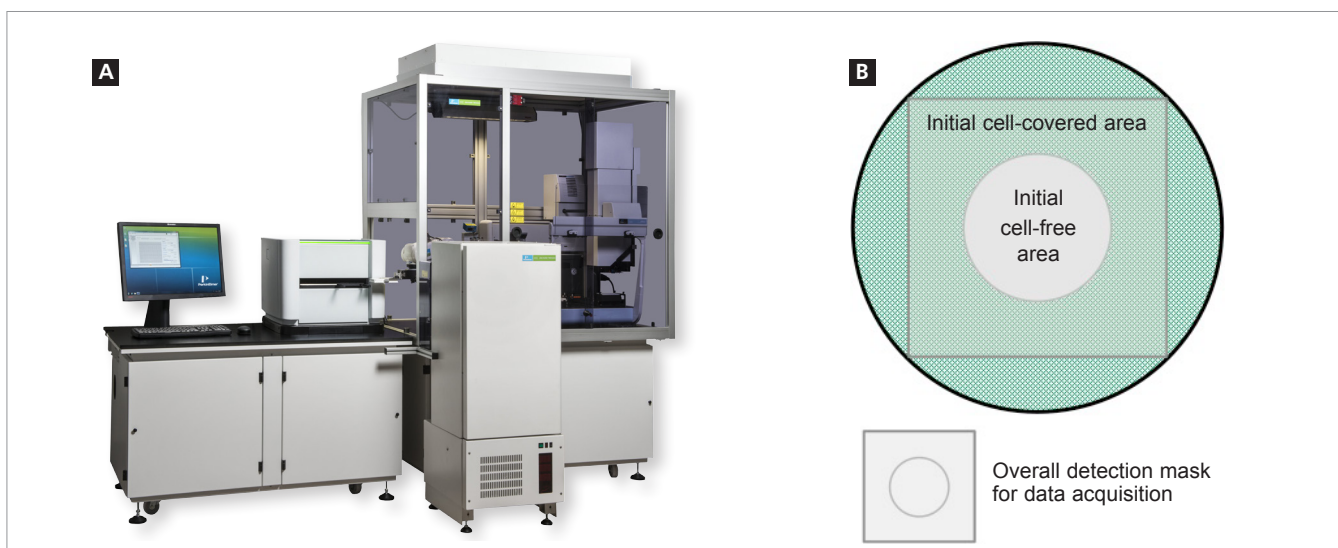
### Cell Treatment and Compound Addition

Besides manual addition of 100 µl cell suspension into Oris™ assay wells, all liquid handling steps were executed by automated liquid handling systems. After removal of the Oris™ Cell Seeding Stoppers from the 96-well plate, a cell-free area in the center of each assay well is revealed. Using the Janus™ MDT Automated Workstation (PerkinElmer), supernatant medium was aspirated and cells were gently washed with culture medium. In order to restrain cell proliferation, which would otherwise interfere with monitored cell migration and lead to false results, DNA replication was suppressed by adding 20 µg/ml Mitomycin C (Abcam) to the cell culture medium. After a three hour incubation period at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub>, the Mitomycin C solution was removed and each well was washed three times with culture medium.

For screening of modulators of SMC migration, 786 compounds, derived from an FDA approved known drug library (Enzo Life Sciences) were examined. The compounds were dissolved in DMSO and stored in 384-well Labcyte microtiter plates. Using the Echo® 550 Liquid Handling System (LABCYTE INC.) small volumes of the compounds were transferred into 96-well plates. Subsequent complete growth medium was added to each well using the Janus™ MDT Automated Workstation (PerkinElmer), resulting in a solution containing 0.1 % DMSO and 10 µM of the corresponding compound. This solution was then transferred to Oris™ cell migration assay wells resulting in a total working volume of 150 µl per well.

### Data Acquisition and Evaluation

The EnSight Multimode Plate Reader was fully integrated into a plate::handler automated workstation (PerkinElmer) and enabled the computerized monitoring of cell migration within the Oris™ assay system (Figure 2 A). Brightfield image acquisition was performed every 2 hours while assay plates were moved into the incubator between individual measurements. The overall incubation period and monitoring of cellular migration amounted to 60 hours. The measurement time per plate including online analysis of obtained images and a control measurement for the pH of the medium (data not shown here) was approximately 6 minutes. The Kaleido Software automatically analyzed the acquired images based on detection masks for different well regions (Figure 2 B). The "ORIS Brightfield Migration" analysis method (version 0.9) provided information about cell confluency level, cell distribution and texture for the initial cell-covered area as well as for the initial cell-free area in which cell migration occurs<sup>6</sup>. Secondary data analysis was performed using TIBCO Spotfire® software. For characterization of cell migration, the gap closure velocity derived from the change of the confluency level in the initial cell-free area ("center confluency") over 48 hours was considered. For details of the analysis and sample preparation please see reference no. 6.



**Figure 2. A: Automated Oris Migration Assay Setup.** The integration of the EnSight Multimode Plate Reader in a plate::handler workstation enabled automated transfer of plates between incubator and the reader over several days. **B: Detection Mask for ORIS Brightfield Migration.** The EnSight system's Kaleido Software automatically analyzed the acquired images based on detection masks for different well regions.

## Results

### Validation of Oris™ Cell Migration Assay Performance and Quality for Compound Screening

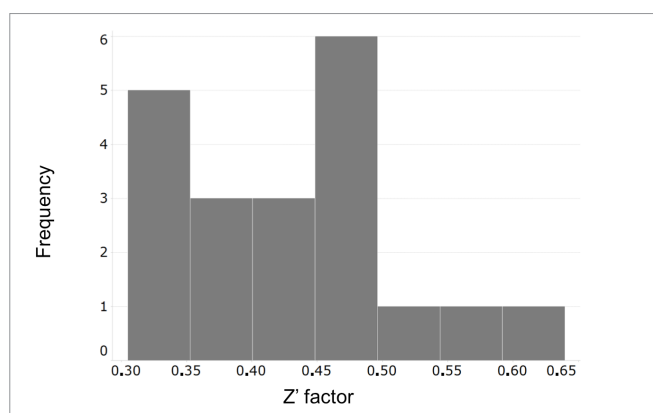
Each of the 786 FDA approved known drugs was screened at a concentration of 10  $\mu\text{M}$  in duplicate in a total number of 20 Oris™ Migration assay plates. The EnSight Multimode Plate Reader and its integration into a plate::handler workstation allowed the screen to be performed within one week while data acquisition was performed in two-hour steps. The EnSight system's Kaleido Software automatically analyzed the acquired images and provided all relevant metrics for further evaluation with TIBCO Spotfire® Software.

For small molecule screening, Cytochalasin B, a known inhibitor of cytoskeletal dynamics was utilized as a positive control at a concentration of 5  $\mu\text{M}$ . A solution of 0.1 % DMSO dissolved in complete growth medium, corresponding to the DMSO concentration of examined compounds, was utilized as a negative control. In order to detect active compounds (hits) of a compound library in a robust, sensitive and reproducible manner, a screening window has to be defined which reflects the assay performance and assures the quality of the assay system. For assessment of assay quality, the dimensionless Z-factor ( $Z'$ ) for each assay plate was evaluated. Figure 3 shows a frequency distribution of the  $Z'$  value for all screened Oris™ Assay plates. The average  $Z'$  value of 0.43 is comparable to other cell-based screening approaches<sup>7,8</sup> and confirms a sufficient and appropriate assay performance for a cell-based screening assay according to the criteria defined by Zhang et al.<sup>9</sup> and recently emphasized and confirmed by Bray and Carpenter<sup>10</sup>.

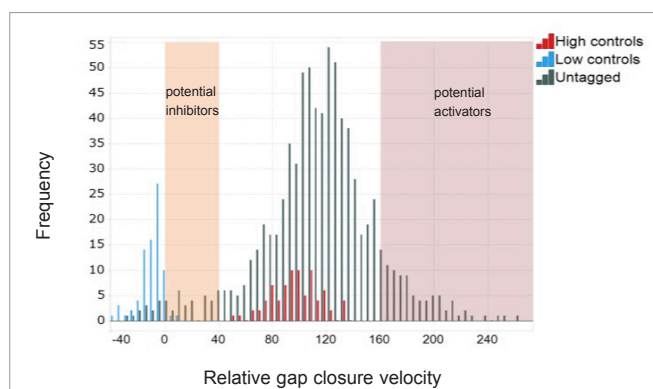
### Hit Identification of Small Molecule Screen

In order to obtain comparable data for all assay plates, gap closure velocity data of the examined compounds was normalized to a plate-specific "relative gap closure velocity". Therefore, the average of gap closure velocity of the negative control was set as

100 % migration speed and respective values for the screened compounds were normalized to that. Figure 4 shows a frequency distribution of all tested compounds screened in duplicate. The relative gap closure velocity was binned by 65 even intervals and plotted against its corresponding frequency.



**Figure 3. Frequency distribution of  $Z'$  value of primary screen.** None of the tested plates resulted in a  $Z'$  value lower than 0.3.



**Figure 4. Frequency distribution of relative gap closure velocity of examined compounds.** The negative values for positive control values refer to shrinkage of cells (resulting in a decrease of confluency).

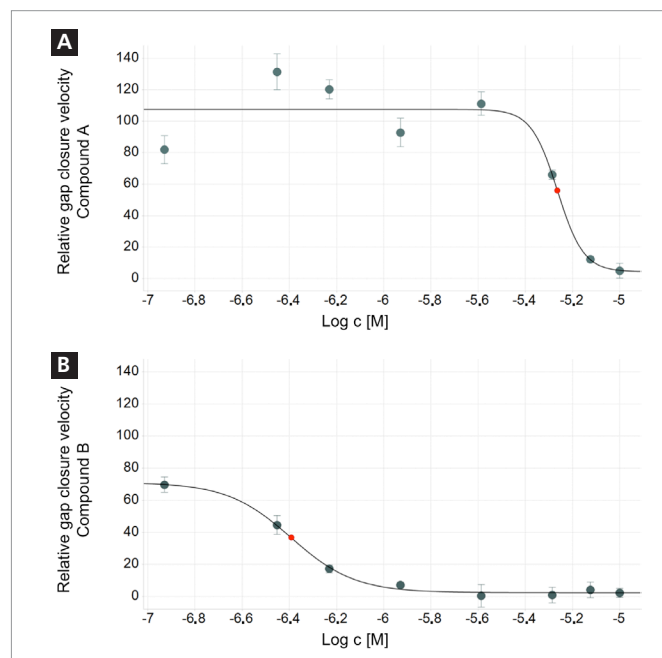
A narrow distribution of the majority of all tested compounds compared to the distribution of the negative controls was observed, as expected for a robust and sensitive assay. Furthermore, the distinct difference in distribution of positive and negative controls confirms a sufficient screening window for hit identification, as expected from Z' value evaluation.

For hit identification, a threshold was set for the normalized gap closure velocity of examined compounds. Screened compounds that showed a relative gap closure velocity between 0 % and 40 % were regarded as potential inhibitors of SMC migration. Compounds displaying a relative gap closure velocity of 160 % and higher were regarded as potential activators of SMC migration. For further hit profiling and confirmation, only those compounds where both replicates fulfilled the set threshold and exhibited additionally a standard deviation below 10 % were selected and analyzed in dose-response measurements.

### Hit Profiling and Confirmation

The interesting compounds for modulating SMC migration were profiled and confirmed on dose-response measurements. Figure 5 shows a dose-dependent inhibition of SMC migration for two compounds out of the inhibitor population, which was performed in duplicate on eight-point dose response measurements.

The hit confirmation resulted in a very precise determination of derived data such as the IC<sub>50</sub> values, as indicated by the very small error bars in Figure 5.



**Figure 5. Dose response relationship for two confirmed inhibitors of SMC migration. Relative gap closure velocity in [%].** Each point represents the average of three assay wells  $\pm$  SD.

PerkinElmer, Inc.  
940 Winter Street  
Waltham, MA 02451 USA  
P: (800) 762-4000 or  
(+1) 203-925-4602  
[www.perkinelmer.com](http://www.perkinelmer.com)

## Discussion

In this application note we demonstrate that the EnSight Multimode Plate Reader enables a fast, reliable and reproducible screen of compound libraries in cell migration assays based on (stain-free) brightfield imaging. The integration of the EnSight Multimode Plate Reader into a plate::handler workstation facilitated a fully automated and highly economical process. The immediate and automatic image acquisition of the Kaleido Software enables instant data assessment and avoids time consuming post-editing of acquired images and data.

The EnSight system additionally offers the possibility of monitoring cell proliferation rates and of differentiating proliferating from migrating cells without the need for cell staining, while also toxic effects can be identified. Moreover, quality verification as well as orthogonal measurement approaches for the (stain-free) brightfield imaging method for ORIS Cell Migration can be combined with fluorescence imaging, or with classical detection technologies such as absorbance, fluorescence intensity, luminescence, TRF or Alpha.

## References

1. Lusis, A.J., *Atherosclerosis*. Nature, 2000. 407(6801): p. 233-41.
2. Su, B., et al., *Redox regulation of vascular smooth muscle cell differentiation*. Circ Res, 2001. 89(1): p. 39-46.
3. Enis, D.R., et al., *Induction, differentiation, and remodeling of blood vessels after transplantation of Bcl-2-transduced endothelial cells*. Proc Natl Acad Sci USA, 2005. 102(2): p. 425-30.
4. Louis, S.F. and P. Zahradka, *Vascular smooth muscle cell motility: From migration to invasion*. Exp Clin Cardiol, 2010. 15(4): p. e75-85.
5. Yarrow, J.C., et al., *A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods*. BMC Biotechnol, 2004. 4: p. 21.
6. Gach, C., *A new approach for automated quantification of cell migration using EnSight Multimode Plate Reader*. PerkinElmer 2016.
7. Zhang, X. and M. Boutros, *A novel phenotypic dissimilarity method for image-based high-throughput screens*. BMC Bioinformatics, 2013. 14: p. 336.
8. Yan, Y.X., et al., *Cell-based high-throughput screening assay system for monitoring G protein-coupled receptor activation using beta-galactosidase enzyme complementation technology*. J Biomol Screen, 2002. 7(5): p. 451-9.
9. Zhang, J.H., T.D. Chung, and K.R. Oldenburg, *A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays*. J Biomol Screen, 1999. 4(2): p. 67-73.
10. Bray, M.A. and A. Carpenter, *Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis, in Assay Guidance Manual*, G.S. Sittampalam, et al., Editors. 2013: Bethesda (MD).



For a complete listing of our global offices, visit [www.perkinelmer.com/ContactUs](http://www.perkinelmer.com/ContactUs)

Copyright ©2016-2017, PerkinElmer, Inc. All rights reserved. PerkinElmer® is a registered trademark of PerkinElmer, Inc. All other trademarks are the property of their respective owners.