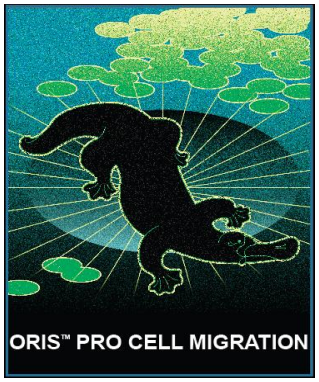


Oris™ Pro Cell Migration Assay Collagen I Coated

Product No.: PROCMACC1 & PROCMACC5

96-well, 2-D Assay for Investigating
Cell Migration of Adherent Cell Lines on Collagen I

PROTOCOL & INSTRUCTIONS

Table of Contents

Platypus Technologies, LLC

5520 Nobel Drive, Suite 100, Madison, WI 53711
Toll Free: 866.296.4455 Phone: 608.237.1270 Fax: 608.237.1271

www.platypustech.com

PLATYPUS TECHNOLOGIES

ORIS™ PRO CELL MIGRATION ASSAY
COLLAGEN I COATED

I. INTRODUCTION

The Oris™ Pro Cell Migration Assay – Collagen I Coated is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration. Formatted for a 96-well plate, the assay uses a non-toxic biocompatible gel (BCG) to form a cell-free zone on cell culture surfaces. After seeding cells into the 96-well plate, the BCG dissolves permitting cells to migrate into the well centers (see Figure 1). The Oris™ Pro Cell Migration Assay – Collagen I Coated enables the use of automated liquid handling equipment for cell seeding and allows for unlimited access to wells from cell seeding through data readout. The Oris™ Pro Cell Migration Assay – Collagen I Coated is designed to be used with any commercially available stain or labeling technique. Researchers can capture and quantify real-time cell migration data using inverted microscopes, high content screening (HCS) and high content imaging (HCI) instruments.

The Oris™ Pro Cell Migration Assay – Collagen I Coated system has been designed for use with adherent cell cultures. This assay has been successfully used with HT-1080, MDA-MB-231 and PC3 cell lines, and Human Umbilical Vein Endothelial Cells (HUVECs).

Using the Oris™ Pro Cell Migration Assay – Collagen I Coated offers the following features & benefits:

- **Increased Productivity -** Treat cells with multiple fluorescent probes, labels, or colorimetric stains for multi-parametric measurements with inverted microscopes, High Content Screening (HCS) and High Content Imaging (HCI) instruments.
- **Less Handling -** Realize reduced assay handling time with an assay format in which a centrally placed biocompatible gel automatically dissolves to reveal a detection zone.
- **Automation-Friendly Design -** Utilize automated liquid handling equipment for fast set-up of high throughput, 96-well assays.
- **Reproducible Results -** Achieve well-to-well CV's ≤12% and generate robust Z' factors suitable for compound screening.
- **Real-Time Analysis -** Experience unlimited access to cells, cell morphology and cell movement throughout your experiment.

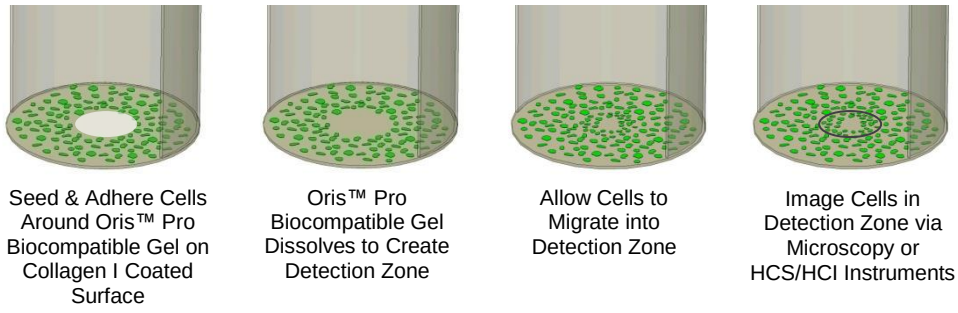


Figure 1. Schematic of Oris™ Pro Cell Migration Assay – Collagen I Coated

II. ORIS™ PRO PLATE DIMENSIONS

Diameter of Well – Bottom	6.58 mm
Diameter of Well – Top	6.96 mm
Well Volume	392 µL
Suggested Media Volume per Well	100 µL
Plate Height	14.4 mm
Plate Height with Lid	17 mm
Offset of Wells (A-1 location, X)	14.38 mm
Offset of Wells (A-1 location, Y)	11.24 mm
Distance between Wells	9.0 mm
Well Depth	10.9 mm
Thickness of Well Bottom	190 µm +/- 10 %
Well Coating Material	Collagen I, Rat Tail
Storage Conditions	15 - 30°C

NOTE: For Research Use Only.

Important: Read Instructions Before Performing any Oris™ Pro Assay.

III. MATERIALS PROVIDED

Product No.: PROCMACC1
Oris™ Pro 96-well, Collagen I Coated Plate (1)

Product No.: PROCMACC5
Oris™ Pro 96-well, Collagen I Coated Plates (5)

IV. MATERIALS REQUIRED

- Biological Cells
- Sterile PBS (containing both Ca^{++} and Mg^{++})
- Complete Cell Culture Growth Medium (containing serum)
- Pipette or Multi-Channel Pipette with Sterile Pipette Tips
- Trypsin or Cell Scraper
- Inverted Microscope (optional)
- High Content Screening, High Content Imaging System (optional)
- Cell Labeling Fluorescent Agent, eg., CellTracker™ Green, DAPI, TRITC-Phalloidin (optional)
  - *required if performing assay readout via fluorescence analysis.*

V. CELL MIGRATION ASSAY PROTOCOL

The following steps should be performed in a biological hood using aseptic technique to prevent contamination.

1. If performing a kinetic analysis of cell migration, pre-label cells with a fluorescent stain now. Please refer to Appendix II for a discussion of suggested staining techniques.

2. Collect cells and prepare a suspension that is at the optimal seeding concentration.

   First Time Users: The optimum seeding density of cells must be determined as an integral part of the design of the cell migration assay. Please refer to Appendix I for a discussion of this process.

3. Pipette 100 µL of suspended cells into each test well.

NOTE: If you plan to fix and label cells at the conclusion of the cell migration, you will need additional wells (or an additional Oris™ Pro Collagen I plate) to serve as pre-migration reference wells.

NOTE: Place your seeded plate(s) into the incubator as soon as possible after cells have been seeded. Take care not to jostle the plate(s).

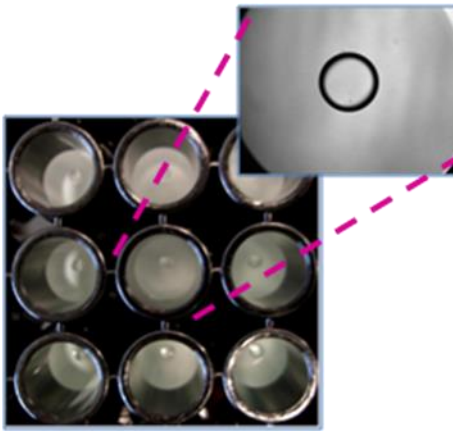


Figure 2. Biocompatible Gel in the Oris™ Pro Cell Migration Assay

4. Incubate the seeded plate(s) containing the Oris™ Pro Biocompatible Gel (see Figure 2) in a humidified chamber (37°C, 5% CO_2) for 30 minutes to 2 hours (cell line dependent) to permit cell attachment.

5. Remove plate(s) from incubator.

NOTE: At this step, test compounds may be added directly to the well, or it may be preferable to first remove media and add fresh culture media containing test compounds to each well.

6. Capture pre-migration images of the Detection Zone (to be used as reference wells) according to the following options:

   Option I: If utilizing unlabeled cells or live, labeled cells (GFP-labeled, or a non-toxic fluorescent dye, such as CellTracker™ Green), use an inverted microscope or HCS/HCI instrument to capture pre-migration images of the Detection Zone formed in the wells.

   Option II: If utilizing fixed, labeled cells (TRITC-phalloidin, DAPI, etc), fix cells in the pre-migration reference wells. These cells can be labelled immediately or at the same time as the test cells. Use an inverted microscope or HCS/HCI instrument to capture pre-migration images of the Detection Zone formed in the wells.

7. Incubate plate in a humidified chamber (37°C, 5% CO_2) to permit cell migration. Cells may be examined by inverted microscope or other imaging instrument throughout the incubation period to monitor progression of migration, which will vary depending upon cell type and experimental design.

8. If performing an endpoint analysis of cell migration, stain cells with a fluorescent stain after sufficient migration has occurred. Refer to Appendix II for further information on fluorescence staining techniques of fixed cells.

9. Capture post-migration images of the Detection Zone using HCS/HCI instrumentation, or phase, bright-field, or fluorescence microscopy.

VI. DATA ACQUISITION

The readout the Oris™ Pro Cell Migration Assay – Collagen I Coated can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Pro Cell Migration Assay – Collagen I Coated is designed to be used with any commercially available stain or labeling technique. Readout can be performed by using an inverted microscope or a High Content Screening (HCS) or High Content Imaging (HCI) instrument.

Microscope Analysis:

- Cell counting or image capture / analysis software, such as NIH ImageJ freeware, can be used.
- Note: Microscopy observations are possible using phase contrast, fluorescence or bright field microscopy.

Sample data using phase contrast microscopy (Figure 3). Collagen I coated wells were seeded with 30,000 MDA-MB-231 cells (i.e., 100 µL of $3.0x10^5$ cells/mL) and incubated (37°C, 5% CO_2) for 1 hour. Upon removal of the plate from the incubator, phase contrast images were taken for pre-migration references. The plate was returned to the incubator for 18 hours to permit cell migration. At the end of the migration, images were captured using phase contrast microscopy. The images below illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=18 hrs) wells (CV of 3.37% Δ migration and Z' = 0.65 for migration*). Scale bar = 500 µm.

* Reference:Zhang JH, Chung TD, Oldenburg KR, "*A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays*." *J Biomol Screen.* 1999; 4(2):67-73.

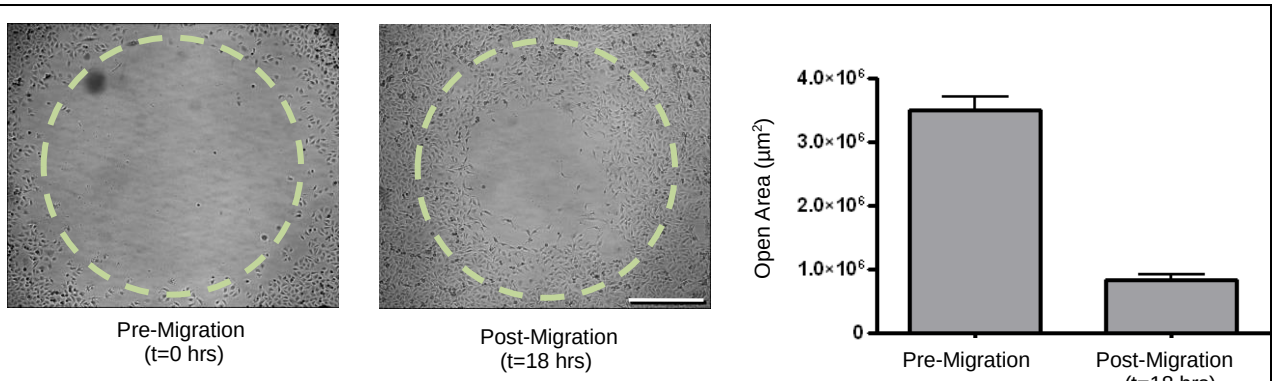


Figure 3. Cell migration data (n=24) obtained using phase contrast microscopy.

Sample data using fluorescence microscopy (Figure 4). Collagen I coated wells were seeded with 30,000 HT-1080 cells (i.e., 100 µL of $3.0x10^5$ cells/mL) on two plates and incubated (37°C, 5% CO_2) for 1 hour. At the end of the incubation, one plate was removed from the incubator, fixed, and stained for F-actin (TRITC-phalloidin, pseudocolored green). The second plate was incubated for an additional 18 hours to permit cell migration. At the end of the migration, the second plate was fixed and stained for F-actin (TRITC-phalloidin, pseudocolored green). Images of cell migration were captured using fluorescence microscopy and the images below illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=18 hrs) wells (CV of 5.57% Δ migration and Z' = 0.60 for migration). Scale bar = 500 µm.

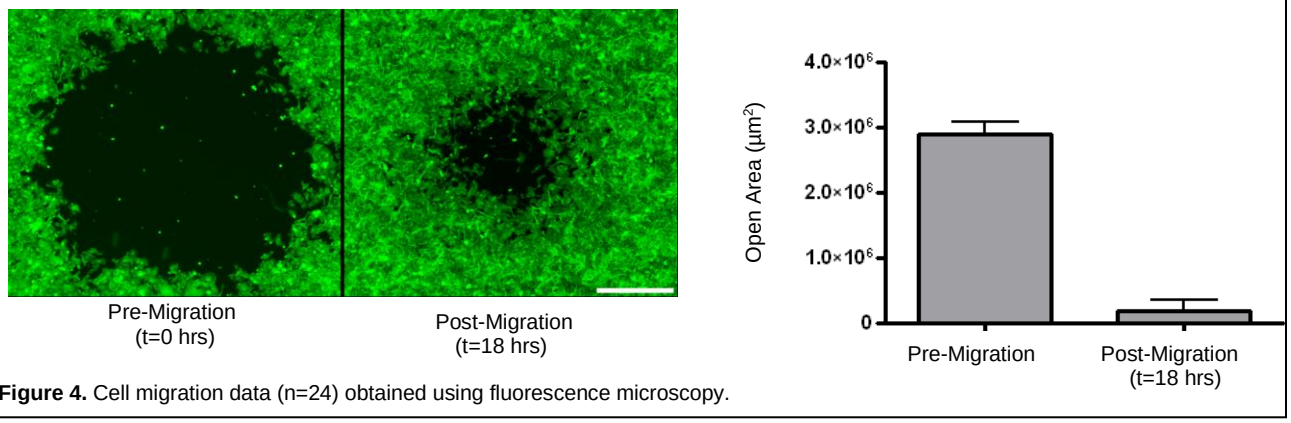
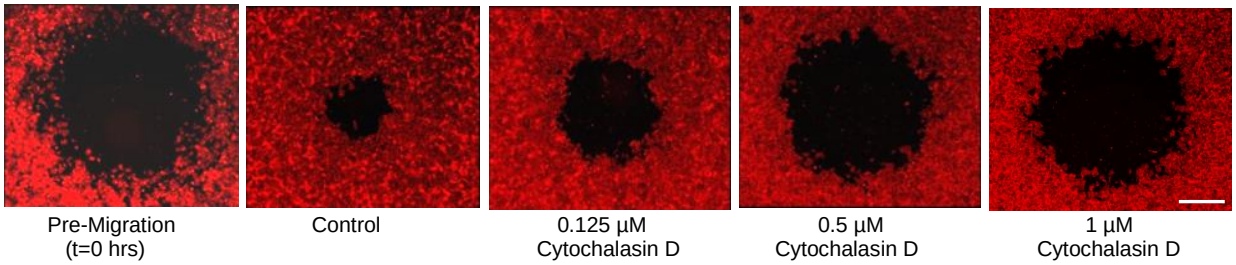


Figure 4. Cell migration data (n=24) obtained using fluorescence microscopy.

High Content Screening / High Content Imaging Analysis:

Sample data using BD Pathway™ 855 Bioimaging system (Figure 5). Collagen I coated wells were seeded with 25,000 HUVEC's (i.e., 100 µL of 2.5x10^5 cells/mL) on two plates and incubated (37°C, 5% CO_2) for 1 hour. At the end of the 1 hour incubation, one plate was removed from the incubator and cells were fixed with 0.25% glutaraldehyde. At this time, varying concentrations of the actin polymerization inhibitor, Cytochalasin D, were added to select wells of the second plate and incubated for an additional 18 hours to permit cell migration. At the end of the migration, the second plate was fixed with 0.25% glutaraldehyde. Both plates were stained using TRITC-phalloidin. The images below illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=18 hrs) wells. Scale bar = 500 µm.



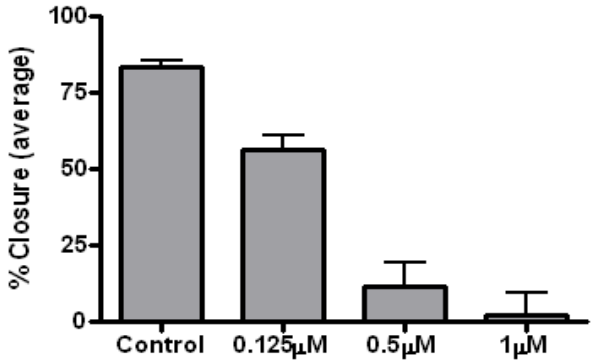


Figure 5. Cell migration data (n=4) obtained using BD Pathway™ 855 Bioimaging system.

BD Pathway™ 855 is a trademark of BD Biosciences.

VII. ORDERING INFORMATION

Product Name	Coating	Size	Detection Zone Format
Oris™ Pro Cell Migration Assays	Tissue Culture Treated	1-pack (PROCMA1) 5-pack (PROCMA5)	Biocompatible Gel
	Collagen I Coated	1-pack (PROCMACC1) 5-pack (PROCMACC5)	
Oris™ Pro 384 Cell Migration Assays	Tissue Culture Treated	5-pack (PRO384CMA5)	Biocompatible Gel
	Collagen I Coated	5-pack (PRO384CMACC5)	
Oris™ Cell Migration Assays	Tissue Culture Treated	1-pack (CMA1.101) 5-pack (CMA5.101)	Oris™ Cell Seeding Stoppers (pre-populated)
	Collagen I Coated	1-pack (CMACC1.101) 5-pack (CMACC5.101)	
	Fibronectin Coated	1-pack (CMAFN1.101) 5-pack (CMAFN5.101)	
	TriCoated	1-pack (CMATR1.101) 5-pack (CMATR5.101)	
Oris™ Cell Migration Assembly Kits	Universal (Tissue Culture Treated)	1-pack (CMAU101) 5-pack (CMAU505)	Oris™ Cell Seeding Stoppers (not pre-populated)
	FLEX (Tissue Culture Treated)	4-pack (CMAUFL4)	
Oris™ Pro 96-well Invasion Assays	Collagen I (low overlay conc.)	1-pack (PROIA1) 3-pack (PROIA3)	Biocompatible Gel
	Collagen I (high overlay conc.)	1-pack (PROIAPLUS1) 3-pack (PROIAPLUS3)	Biocompatible Gel

For a complete list of products, visit www.platypustech.com.
For technical assistance, contact Technical Support at 866.296.4455 or techsupport@platypustech.com.

VIII. TERMS & CONDITIONS

Certain uses of these products may be covered by U.S. Pat. No. 7,842,499 issued to or patents applied for by PLATYPUS. Certain applications of PLATYPUS products may require licenses from other parties. Determining the existence and scope of such third party intellectual property is the responsibility of the PURCHASER. Purchase of the product provides the PURCHASER with a limited non-transferable license under any PLATYPUS patents or patent applications to use the product for internal research unless there is a written limitation to this license in the product literature. PURCHASER is responsible for carefully reviewing the product literature and respecting any limitations to this license, e.g. limitations for commercial use or research by for-profit institutions. These products may not be resold, modified for resale, used to manufacture commercial products, or used to develop commercial products without the express written approval of PLATYPUS.

These products are intended for research or laboratory use only and are not to be used for any other purposes, including, but not limited to, unauthorized commercial purposes, *in vitro* diagnostic purposes, *ex vivo* or *in vivo* therapeutic purposes, investigational use, in foods, drugs, devices or cosmetics of any kind, or for consumption by or use in connection with or administration or application to humans or animals. PLATYPUS warrants that its products shall conform substantially to the description of such goods as provided in product catalogues and literature accompanying the goods until their respective expiration dates or, if no expiration date is provided, for 6 months from the date of receipt of such goods. PLATYPUS will replace, free of charge, any product that does not conform to the specifications. This warranty limits PLATYPUS's liability only to the replacement of the nonconforming product.

THIS WARRANTY IS EXCLUSIVE AND PLATYPUS MAKES NO OTHER WARRANTY, EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. The stated express warranties, and the remedy provided for breach thereof, are in lieu of all other liability or obligations of PLATYPUS for any damages whatsoever arising out of or in connection with the delivery, use, misuse, performance, or the inability to use any of its products. IN NO EVENT SHALL PLATYPUS BE LIABLE UNDER ANY LEGAL THEORY (INCLUDING BUT NOT LIMITED TO CONTRACT, NEGLIGENCE, STRICT LIABILITY IN TORT, OR WARRANTY OF ANY KIND) FOR ANY INDIRECT, SPECIAL, INCIDENTAL, CONSEQUENTIAL, OR EXEMPLARY DAMAGES (INCLUDING BUT NOT LIMITED TO LOST PROFITS) EVEN IF PLATYPUS HAD NOTICE OF THE POSSIBILITY OF SUCH DAMAGES. Without limiting the effect of the preceding sentence, PLATYPUS's maximum liability, if any, shall not exceed the purchase price paid by PURCHASER for the product.

This warranty shall not be effective if PLATYPUS determines, in its sole discretion that PURCHASER has altered or misused the goods or has failed to use or store them in accordance with instructions furnished by PLATYPUS. PLATYPUS's sole and exclusive liability and PURCHASER's exclusive remedy with respect to goods proved to PLATYPUS's satisfaction (applying analytical methods reasonably selected by PLATYPUS) to be defective or nonconforming shall be the replacement of such goods free of charge, upon the return of such goods in accordance with our instructions, although at its discretion, PLATYPUS may provide a credit or refund. If PLATYPUS manufactures custom goods for PURCHASER based on instructions, specifications, or other directions provided by PURCHASER, PLATYPUS shall not be liable for the lack of sufficiency, fitness for purpose or quality of the goods to the extent attributable to such instructions, specifications, or other directions. PLATYPUS shall not be liable for any loss, damage or penalty as a result of any delay in or failure to manufacture, deliver or otherwise perform hereunder due to any cause beyond PLATYPUS's reasonable control.
PLATYPUS shall not be liable for injury or damages resulting from the use or misuse of any of its products.

APPENDIX I: Determining Optimal Cell Seeding Concentration

This procedure is intended to assist in determining the cell seeding density needed for your cell line when using the Oris™ Pro Cell Migration Assay. The goal is to achieve 90 – 95% confluency of the monolayer surrounding the Oris™ Pro Biocompatible Gel.

1. A suggested starting point is to evaluate a range of three cell densities. The cell seeding area of the well with the Oris™ Pro Biocompatible Gel is ~0.3 cm^2. Based on the typical seeding density of your particular cell line, you can determine a different cell number for your first serial dilution and adjust the numbers below accordingly.
2. Prepare a log-phase culture of the cell line to be tested. Collect cells and determine the total number of cells present.
3. Pellet cells by centrifugation (1,000 x g). Prepare final concentrations of $4.0x10^5$, $3.0x10^5$, and $2.0x10^5$ cells/mL.
4. Dispense 100 µL of cell suspension per well into the 96-well plate to result in the following plate layout:

Column	1	2	3
Cells / well	40,000	30,000	20,000
Number of wells	8	8	8

5. Incubate the plate in a humidified chamber (37°C, 5% CO_2) for 30 minutes - 2 hours (cell line dependent) to allow the cells to firmly attach to the well surface.
6. Following cell attachment, use an inverted microscope to inspect each well to determine the minimum cell seeding concentration that yields a 90 – 95% confluent monolayer at the perimeter of the Detection Zone (See Figure 3 for representative pre-migration Detection Zone images).

APPENDIX II: Fixation and Fluorescent Labeling of Cells

The Oris™ Pro Cell Migration Assay has been designed to work with all types of fluorescent stains and staining techniques. The precise method for staining cells with fluorescent stains varies according to the nature of the individual stain. Please consult the manufacturer of your fluorescent stain for specific considerations.

The following is an example Fluorescent Staining Protocol to label fixed cells with TRITC-phalloidin (F-actin) and DAPI (nuclei):

a) To fix one fully-seeded 96-well plate, prepare 10 mL of fixative solution (e.g. 0.25% glutaraldehyde solution in PBS prepared from 8% EM-grade glutaraldehyde solution).
b) Remove medium and rinse wells with 100 µL of 1X PBS.
c) Remove PBS and add 100 µL of a fixative solution (final concentration of 0.25% glutaraldehyde solution in PBS) to each well. Incubate at room temperature for 15 minutes.
d) Remove fixative solution and rinse wells with 100 µL of PBS.
e) Remove PBS and replace with 100 µL of a 1:50-1:100 dilution of TRITC-phalloidin (Sigma; prepared from 10 µM stock in methanol) in PBS containing 0.1% Triton X-100.
f) Incubate plate at room temperature for 45 minutes (protect from light).
g) Remove the TRITC-phalloidin, rinse with PBS for 5 minutes, and add 100 µL of a 1:4000 dilution of DAPI (prepared from 1 mg/mL stock) in PBS.
h) Incubate plate at room temperature for ~5 minutes (protect from light). Observe plate starting at 5 minutes and if needed allow additional time for complete staining.
i) Remove DAPI stain and wash wells 2X for 5 minutes each with 200 µL of PBS.
j) Replace final wash with 200 µL of fresh PBS.

NOTE: This protocol outlines double-labeling of cells with a cytoskeletal and a nuclear stain. The protocol can be simplified if only one stain is used. Substitutions, additional cytostaining, or immunostaining may be performed using non-overlapping fluorophores and by utilizing the appropriate filters with your imaging equipment.