Development of Invasion and Transendothelial Migration Assays in a 96-well Format for Use in High-Throughput Drug Discovery.

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Abstract

Purpose. To develop cellular invasion and transendothelial migration assays in a 96-well format for use in high-throughput cell-based drug discovery applications.
Methods. Invasion assays were performed using 8 µm membrane pore size 96-well Millipore Multiscreen™-MIC (Migration, Invasion and Chemotaxis) plates. MDAMB231 and MCF7 (invasive and non-invasive adherent breast cancer cell lines) and HT1080 and NIH3T3 cells (invasive epithelial and non-invasive fibroblast adherent cell lines) were used in these experiments. Invasion assays were performed across wells pre-coated with extracellular matrix to assess invasive response of cells to serum-containing medium. The effects of chemotaxis inhibitors, Tamoxifen and Cytochalasin D, were also evaluated. Cells invaded to the membrane underside were stained with Hema-3®, then scanned and quantified with a KS300 cell-counting software on a Zeiss Axioplan 2 microscope with an automated stage. Transendothelial migration of MM6 (MonoMac 6) cells was assessed across a HUVEC (human umbilical vein endothelial cells) monolayer cultivated in Multiscreen-MIC plates. MM6 cells transmigrated to bottom wells were quantified by fluorescent labeling with Alamar Blue™ or Cell Tracker Green™.
Results. The MDAMB231 and HT1080 cell invasion index, calculated relative to the non-invasive MCF7 and NIH3T3 cells respectively, was two fold or higher. Inhibition of the MDAMB231 invasion response was observed upon treatment with 20 µM Tamoxifen and 5 µM Cytochalasin D. MonoMac6 cells exhibited consistent transendothelial migration across various experimental conditions tested.
Conclusions. Our results demonstrate the utility of using 96-well filter plates to perform functional cell-based assays in high throughput screening (HTS) of drug compounds.

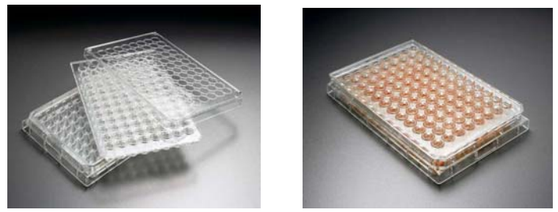
Introduction

Cell-based assays are gaining increasing importance in pre-screening of compounds to qualify target leads for drug discovery. These programs are increasingly focused on incorporating functional cell-based assays in the pre-screening stages of lead compounds. Many drugs under development in cancer drug discovery are directed at altering the metastatic properties of cancer cells such as migration, chemotaxis and invasion. Cells such as HUVEC that interact with these cancer cells in invasive processes like angiogenesis and transmigration are also of interest as drug targets. Development of HTS cell-based assays that are designed to be able to measure effects of lead compounds on such functional processes pose a challenge. We have developed a 96 well plate MultiScreen-MIC plate to support these HTS cell-based assays. The data presented in this poster indicate that MultiScreen-MIC plates are useful for anti-cancer drug HTS and can serve as an alternative to existing, lower-throughput methods.

Materials and Methods

Cell culture
MDAMB231 and MCF7 cells were cultured in RPMI with 10 % FBS. HT1080 and NIH3T3 cells were cultured in DMEM with 10 % FBS. Sodium pyruvate and insulin were added as additional media supplements for MCF7 cell growth. HUVECs were propagated in EGM-2 complete medium. MonoMac6 cells were cultured in RPMI with OPI supplement.
Invasion Assays
Cells were grown to 90 % confluency and starved overnight in serum-free media containing 0.2 % bovine serum albumin (BSA) prior to setting up invasion experiments. Invasion experiments were performed on 8 µm pore size Millipore MultiScreen-MIC plates (Catalog # MAMIC8S10) with MDAMB231& MCF7 cells and HT1080 & NIH3T3 cells. Wells were pre-coated with extracellular matrix (40 µg/well). 50,000 cells per well (in 50 µl) were added to upper wells and invasion was measured in response to serum free-medium (to assess background invasion) and serum-containing medium (to assess stimulated invasion) added to bottom wells (150 µl). For inhibition experiments, inhibitors were added at various concentrations to cells in upper wells at the start of assay. Conditioned medium derived from NIH3T3 cells was also used as chemoattractant in inhibition assays. Invasion assays were carried out over a period of 72 hrs at 37ºC. The non-invaded cells were removed from the upper wells by swiping with cotton swabs followed by rinsing twice with PBS (phosphate buffered saline). Following the removal of cells from the upper wells, cells that had invaded to the membrane underside were enumerated by microscopy. For microscopic enumeration, membranes were stained with Hema-3 stain kit (Fisher Scientific). Stained cells were imaged and quantified with a KS300 cell-counting software on a Zeiss Axioplan 2 microscope with an automated stage. Percent invasion was calculated relative to number of cells migrated in the absence of extracellular matrix. Background invasion values were then subtracted to obtain stimulated invasion values.
Transmigration experiments
HUVEC cells were plated onto 8 µm MultiScreen-MIC plates (Catalog # MAMIC8S10) and allowed to form a confluent monolayer on membrane over 48 to 96 hrs. MonoMac6 cells (80 µl), at different cell concentrations, were plated in upper wells to assess their transmigration response to chemoattractants in bottom wells (130 µl) across the HUVEC endothelial monolayer. Transmigrated cells were detected using Alamar Blue or Cell Tracker Green.

Figure 1. MultiScreen-MIC plates



Invasion profiles of adherent cell lines on 8 µm MultiScreen-MIC plates

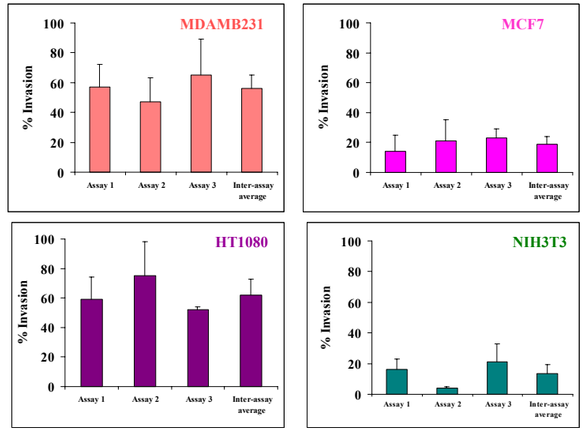


Figure 2. Invasion of cells in response to 10% serum-containing medium as a chemoattractant. Data represent average percent invasion obtained across three lots tested in three assays (per lot: n ≥ 4, r=6 for assays with MDAMB231 and MCF7 and per lot: n ≥ 1, r=6 for assays with HT1080 and NIH3T3).

Invasion profiles of adherent cell lines on alternative device inserts (8 µm)

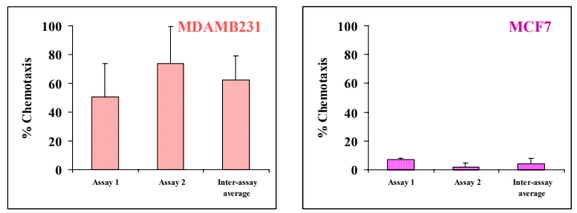


Figure 3. Invasion of cells in response to 10% serum-containing medium as a chemoattractant. Assay conditions were similar to the set-up on MultiScreen-MIC plates. Volume recommendations were as per manufacturer's instructions. Data represent average percent chemotaxis obtained across two assays (per assay condition: n=2 to 4).

Invasion of adherent cell lines on 8 µm MultiScreen-MIC membrane

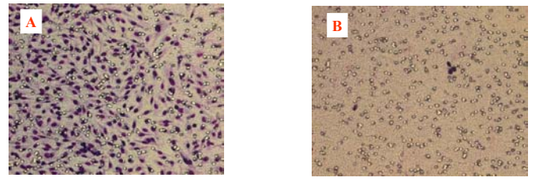


Figure 4 . Invasion of (A) highly migratory MDAMB231 cells and (B) non-migratory MCF7 cells in response to 10% serum-containing medium as a chemoattractant. Invaded cells were visualized after staining as described in Materials and Methods.

Invasion inhibition of MDAMB231

Chemoattractant	Inhibitor	Concentration of Inhibitor	% Invasion Inhibition of MDAMB231
10 % serum containing medium	Tamoxifen	20 µM	74 ± 19
Conditioned medium from NIH3T3 fibroblasts	Tamoxifen	20 µM	57 ± 31
	Cytochalasin D	5 µM	80 ± 5

Table 1. Invasion inhibition in response to 10% serum-containing medium or conditioned medium as a chemoattractant in the presence of inhibitors at indicated concentrations. Percent inhibition of invasion is calculated relative to invasion in absence of inhibitors (equated to 100 %) (n=2, r ≥ 3).

MM6 transmigration across wells seeded with varying HUVEC cell densities

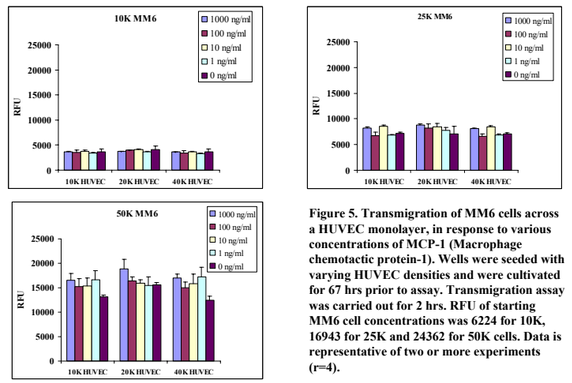


Figure 5. Transmigration of MM6 cells across a HUVEC monolayer, in response to various concentrations of MCP-1 (Macrophage chemotactic protein-1). Wells were seeded with varying HUVEC densities and were cultivated for 67 hrs prior to assay. Transmigration assay was carried out for 2 hrs. RFU of starting MM6 cell concentrations was 6224 for 10K, 16943 for 25K and 24362 for 50K cells. Data is representative of two or more experiments (r=4).

Effects of serum starvation on MM6 transmigration

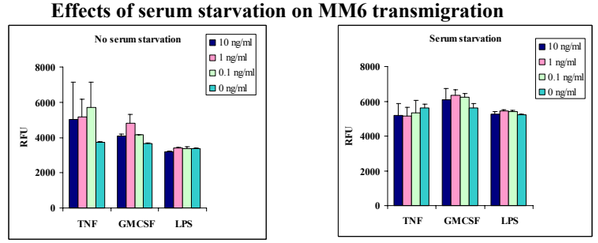


Figure 6. Transmigration of MM6 cells across wells seeded with 30K HUVEC cells. HUVEC cells were cultivated for 71 hrs prior to assay. Transmigration assay was carried out for 2 hrs in response to various concentration of indicated stimulators (TNF: Tumor Necrosis Factor; GMCSF: Granulocyte Macrophage Colony Stimulating Factor; LPS: Lipopolysaccharide). RFU of starting MM6 cell concentration (50k/well) was 23933 and 19735 for unstarved and starved cells respectively. Data is representative of two or more experiments (r=3).

Formation of HUVEC monolayer on 8 µm MultiScreen-MIC membrane

Figure 7. Plates were seeded with (A) 20000 cells/well and (B) 10000 cells/wells resulting in a tight monolayer formation over 24 hrs. HUVEC monolayers were stained with Diff-Quik and then imaged with a Olympus Q-imaging system using an Olympus ix71 software.

Effects of MM6 cell densities on transmigration across HUVEC monolayer cultivated on 8 µm MultiScreen-MIC plates

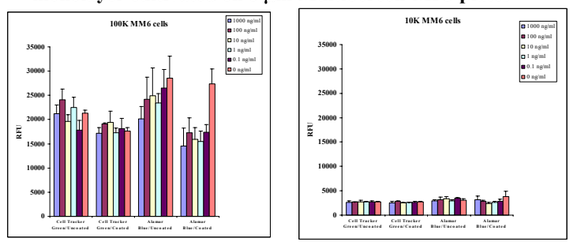


Figure 8. Transmigration of MM6 cells across a HUVEC monolayer, cultivated in uncoated and gelatin coated membrane, in response to MCP-1. 10K HUVEC cells were cultivated for 96 hrs prior to assay. Transmigration assay was carried out for 2 hrs. Two detection techniques were used to assess numbers of cells that had migrated to lower wells. RFU of 100K sand 10 K starting cell concentration was 22670 and 2652 respectively for Cell Tracker Green and 25491 and 8950 respectively for Alamar Blue. Data is representative of two or more experiments (r=6).

Conclusions

Consistent results were obtained in invasion assays with MDAMB231 and MCF7 and HT1080 and NIH3T3 cells.

Comparison of adherent cell invasion in the 96-well MultiScreen-MIC plates with alternative devices exhibited comparable results across assays performed on different days.

Invasion inhibition was successfully obtained on MultiScreen-MIC plates demonstrating the usefulness of the 96 well format for high throughput drug discovery screening targeting such functional responses.

Transendothelial migration of MonoMac6 cells across HUVEC monolayer (representing an inflammatory model) was consistently achieved under various experimental conditions, across various cell passage numbers and with two detection techniques using MultiScreen-MIC plates. Cell seeding densities of HUVEC and MonoMac6 cells were important in obtaining good monolayer and consistent migration effects respectively. Serum starvation appeared to modestly enhance transmigration. No effects of coating or significant effects of stimulators on increasing transmigration were observed. This could be attributed to a pre-existing high basal level of transmigration. Current experiments are focused on effects of inhibitors to obtain a differential inhibition response.

Our results demonstrate the applicability and versatility of a 96-well format for high throughput screening (HTS) of drugs in cell-based assays such as invasion and transmigration and support the use of MultiScreen-MIC plates in such assays.

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