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Development of Cancer Cell Invasion Assays in a 96 well Format

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Abstract

Purpose
To develop cellular invasion assays, using cancer cells, in a 96 well format for application in cancer drug discovery and in *in vitro* cytotoxicity assays.

Methods
Tumor cell invasion assays were performed using polycarbonate bottomed 96 well plates (MultiScreen™-MIC). MDAMB231, a highly invasive breast cancer cell line, and MCF7, a non-invasive breast cancer cell line, were used to optimize the invasion assay conditions. The cells were cultured in RPMI containing 10% FBS, penicillin and streptomycin. Matrigel®, a mouse derived extracellular matrix (ECM), was polymerized onto the membranes prior to the addition of cells to the upper wells. Conditioned medium from NIH3T3 fibroblasts or serum containing medium was added to the lower wells as a chemoattractant. The effects of cell density, ECM concentration and invasion time were evaluated on the MultiScreen™-MIC plates with 3, 5 and 8 µm pore size polycarbonate membranes. Cells that had invaded through the ECM to the underside of the membrane were stained using a Hema-3 stain kit. Cells were enumerated microscopically over a portion (10-15%) of each membrane as the basis for determining the total number of invading cells per well. Fluorescent assays, using DNA binding fluorescent probes, were also performed to quantify the invaded cells.

Results
Cell density at 1.65x 10^5/cm², ECM concentration at 30 µg/well and 72 hrs incubation at 37ºC were optimal for invasion to occur. The MDAMB231 cell invasion index, calculated relative to the non-invasive MCF7 cells under similar conditions, was 5 fold or higher. Visual enumeration provided the most sensitive detection with a lower limit in the range of 100 cells. The fluorescent assays, although more rapid, typically were sensitive to only 1000 cells or more.

Conclusions
Our results demonstrate the utility of a 96 well format in high throughput screening (HTS) of anti-cancer drugs.

Introduction

Cell-based assays are gaining tremendous importance in pre-screening of compounds in order to generate target leads for the Absorption, Distribution, Metabolism, Excretion and Toxicity assays (ADMETox). Cancer drug discovery efforts have increasingly focused on incorporating functional cell-based assays in the pre-screening stages of lead compounds. Many drugs under development are directed at altering the migration and invasion properties of cancer cells. Development of HTS cell-based assays that are designed to be able to measure the migration, chemotaxis and invasion potential of cancer cells are therefore of interest. Chemotaxis is defined as the movement of cells in response to a concentration gradient set up by a chemoattractant. Invasion is defined as the chemotaxis of cancer cells across an extracellular matrix barrier. We have developed a 96 well plate to support these HTS Migration, Invasion and Chemotaxis (MIC) 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 products in the market.

Methods

Invasion assays.
Invasion assays were set up on Millipore MultiScreen™-MIC plates as described by *Albini et al* and *Kamath et al* (**1, 2**). MDAMB231 and MCF7 (invasive and non-invasive adherent mammary adenocarcinoma cells, respectively) were obtained from ATCC and routinely cultured in RPMI with 10 % FBS and penicillin-streptomycin. The cells (passage #'s 19 to 28 and passage #'s 143 to 148) were grown to 90 % confluency and starved overnight in serum-free medium containing 0.1 % BSA prior to initiation of invasion assays. The upper wells were coated with varying concentrations of Matrigel®, a mouse derived extracellular matrix (ECM, obtained from Fisher Scientific). Cells were added to the top wells after ECM had polymerized for two hours. Conditioned medium derived from NIH3T3 cells (passage #'s 4 to 152) (mouse fibroblasts, obtained from ATCC) or serum-containing medium was added to the bottom wells. Invasion was allowed to occur over 48 and 72 hr time periods. The non-invaded cells were removed from the upper wells by swiping with cotton swabs followed by rinsing twice with PBS.

Detection Assays
Following the removal of cells from the upper wells, cells that had invaded to the membrane underside were enumerated by microscopy or fluorescent (FL) assay. For microscopic enumeration, membranes were stained with Hema-3 stain kit (Fisher Scientific). Stained cells were imaged using a Fuji Finepix S1 Pro digital camera mounted on an Olympus BH-2 microscope and counted using the Optimas version 6.1 software. The cells in 10-15 % of the surface area of each membrane well were counted and the number of (invading) cells in the entire well was determined by extrapolation. The percentage of basal invasion was defined as the (number of cells invaded in the presence of matrigel/number of cells migrated in the absence of matrigel)X100. The MDAMB231 cell invasion index was defined as increase in percent invasion of MDAMB231 cells relative to the non-invasive MCF7 cells under similar conditions.

For fluorescent (FL) analysis, the cells were detached from the membranes with PBS-EDTA or Trypsin-EDTA and quantified by whole cell fluorescent labeling (BCECF, Molecular Probes) or DNA binding fluorescent probe (YO-PRO-1 Iodide, Molecular Probes). The number of invaded cells was calculated from standard curves generated for the FL probes with the cell lines.

Figure 1. Millipore MultiScreen™-MIC 96 well plates for Migration, Invasion and Chemotaxis assays (MIC plates).

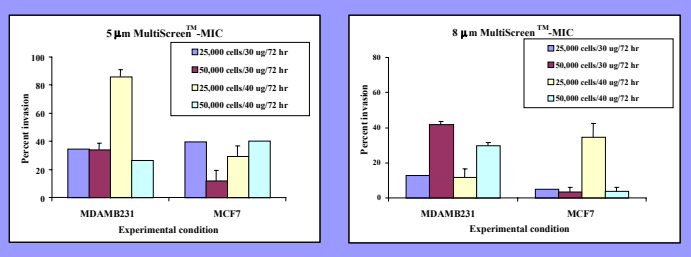

Figure 2. Percent basal invasion of MDAMB231 (invasive breast cancer cell line) in comparison to MCF7 (non-invasive breast cancer cell line) using 8 and 5 µm MIC plates. Detection technique was imaging and microscopy. (n =3 and r =2 or 4). Representative experiment illustrated in figure.

Table 1. Parameters Evaluated.

Assay evaluated	Invasion
Pore size	8, 5 and 3 µm
Cell lines tested	MDAMB231 and MCF7 cells
Cell number	25,000 to 100,000 cells
Type of chemoattractants	Conditioned medium and serum containing medium
ECM concentrations	20, 30 and 40 µg/well
Incubation period	48 and 72 hrs
Detection method 1	Visual/Microscopy imaging
Detection method 2	Whole cell fluorescent labeling and DNA binding fluorescent labeling

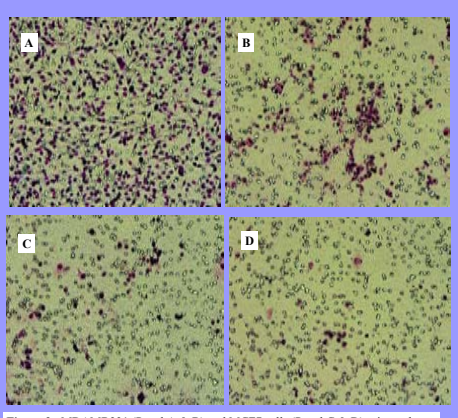

Figure 3. MDAMB231 (Panel A & B) and MCF7 cells (Panel C & D) migrated or invaded to the underside of an 8 µm MIC plate membrane.

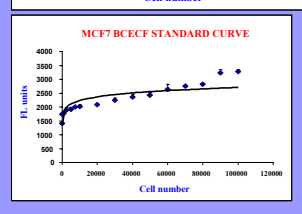

Figure 4. Percent basal invasion of MDAMB231 (invasive breast cancer cell line) in comparison to MCF7 (non-invasive breast cancer cell line) using 8 and 5 µm MIC plates. 25,000 cells were seeded in wells coated with 30 µg of extracellular matrix. Conditioned medium was applied as a chemoattractant and plates were incubated for 72 hrs (n =2, r =2 or 4). Detection technique was whole cell labeling with BCECF. Representative experiment illustrated in figure.

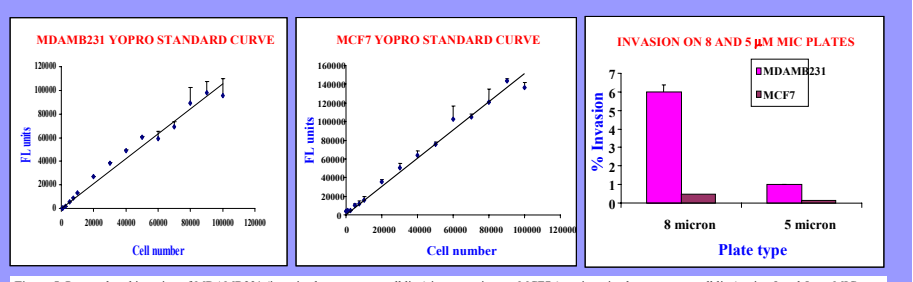

Figure 5. Percent basal invasion of MDAMB231 (invasive breast cancer cell line) in comparison to MCF7 (non-invasive breast cancer cell line) using 8 and 5 µm MIC plates. 50,000 cells were seeded in wells coated with 30 µg of extracellular matrix. Conditioned medium was applied as a chemoattractant and plates were incubated for 72 hrs. (n =2, r =2 or 4). Detection technique was DNA labeling with YOPRO. Representative experiment illustrated in figure.

Conclusions

Invasion assays were successfully optimized in a 96 well format using the MultiScreen™-MIC (Figure 1) plate. Several parameters were tested (Table 1) during protocol development.

The 8 µm pore size plates were most suitable for invasion assays for the cell lines tested compared to 5 µm or 3 µm [data not shown and unpublished work (**3**)].

Invasion index of MDAMB231 was typically two fold or more than the MCF7 cells.

Visual Microscopy and Imaging was the most sensitive method to detect invaded cells. The lower detection sensitivity of the fluorescent assays could be attributed to additional processing steps involved. Protocol development to optimize fluorescent detection method is in progress.

Our results demonstrate that the MIC plates are highly applicable to invasion assays and lend themselves to HTS of such assays with variety of other cancer cells.

References
(1) *Albini et al.* A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res*. **47**: 3239-3245, 1987.
(2) *Kamath et al.* Signaling from Protease-activated Receptor-1 Inhibits Migration and invasion of Breast Cancer Cells. *Cancer Res*. **61**, 5933-5940. 2001.
(3) *Kamath et al.* Development of Cancer Cell Migration Assays in a 96 well Format. Unpublished work.

Acknowledgment
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