Title: A non-scrubbing protocol for use with adherent cells in chemotaxis assays using MultiScreen®-MIC plates

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ABSTRACT

Traditional chemotaxis assays performed with adherent cells on MultiScreen-MIC plates require scrubbing cells away from the upper wells with cotton swabs prior to analysis of cells migrated to membrane underside. A non-scrubbing protocol is described here, as an alternative to scrubbing wells with cotton swab. This alternative protocol involves detaching migrated cells from membrane underside with PBS-EDTA, labeling detached cells with a Calcein AM fluorescent label, and reading labeled cells in a fluorescent plate reader. The chemotaxis profile of highly migratory adherent cell line MDA-MB-231 (breast cancer cell line) was evaluated on 8 μm membrane pore size MultiScreen-MIC plates (Cat. MAMIC 8 S10). The effect of tamoxifen on inhibiting MDA-MB-231 cell chemotaxis using this detachment protocol, was also evaluated. Comparable chemotaxis inhibition was obtained between wells that were scrubbed using cotton swabs and between unscrubbed wells subject to detachment protocol described here.

MATERIALS

Cell Lines: MDA-MB-231 (invasive breast cancer cells, Cat. HTB-26) was purchased from ATCC (Manassas, VA).

Cell Culture Media: RPMI (Cat. R8758), culture media components MEM non-essential amino acids (NEAA) (Cat. M7145), HEPES buffer (Cat. H0887) and L-glutamine-penicillin/streptomycin solution (Cat. G1146) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) (Cat. SH30070) was purchased from Hyclone (Logan, UT).

Other: Calcein AM (Cat. C-3099) was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA) (Cat. A4503) was purchased from Sigma (St. Louis, MO). Tamoxifen (Cat. T5648) was also purchased from Sigma (St. Louis, MO). The MultiScreen-MIC plates (8 μm, Cat. MAMIC 8 S10) were obtained from Millipore (Bedford, MA). Accessory plates (Cat. MAMC S01 10 and Cat. MAMC S 96 10) needed for assay-set up and processing steps were also obtained from Millipore (Billerica, MA).

Equipment used:

Fluorescent Plate Reader (Wallac Victor^{2}™)
Titer plate shaker (Lab-Line® Instruments)

METHODS

Note: Please refer to Millipore Application Note **AN1060EN00** for detailed information on how to cultivate and passage cells, how to set up chemotaxis experiment with adherent cells, for template examples and for running standard curves and equations to calculate data. The cell density, volumes and incubation times used here are different than previously used.

Cell expansion and preparation of adherent cells for chemotaxis assay.

(a) Expand MDA-MB-231 cells into T-75 flasks for 3-5 days before the experiment such that they are about 80-90 % confluent the day prior to setting up the experiment.
(b) Starve the cells overnight at 37^{o} C, 5 % CO_2 in serum free medium containing 0.2 % BSA (v/v).
(c) Adjust cell counts to 1.3 X 10^{6} cells/mL in serum free medium containing 0.2 % BSA. Ensure that cell viability is 90 % or greater for setting up experiment.

Setting up chemotaxis assay on MultiScreen-MIC plates with MDAMB231 cells.

(a) Separate out MultiScreen-MIC filter plate and place into a sterile single well tray (Cat. MAMC S01 10) to protect the membrane prior to setting up the experiment.

(b) Add 75 µl of cell stock to upper wells (100,000 cells per well) in the MultiScreen-MIC filter plate (placed in the single well tray). Ensure that cell suspension is evenly distributed across membrane.
(c) Add 125 µl of serum free medium containing 0.2 % BSA to some of the lower wells to assess basal migration. These are control wells.
(d) Add 125 µl of serum containing medium to remainder of the lower wells to assess stimulated migration (chemotaxis). These are test wells.
(e) For chemotaxis inhibition experiments, add inhibitors at desired concentration to cells prior to loading in upper wells.
(f) GENTLY assemble the top and bottom plates together. DO NOT SHAKE or TILT THE PLATES, as this will disturb the concentration gradient.
(g) Incubate this MutiScreen-MIC plate assembly for 3 h at 37^{o} C. Do not shake plates during incubation.

Detaching cells after chemotaxis assay.

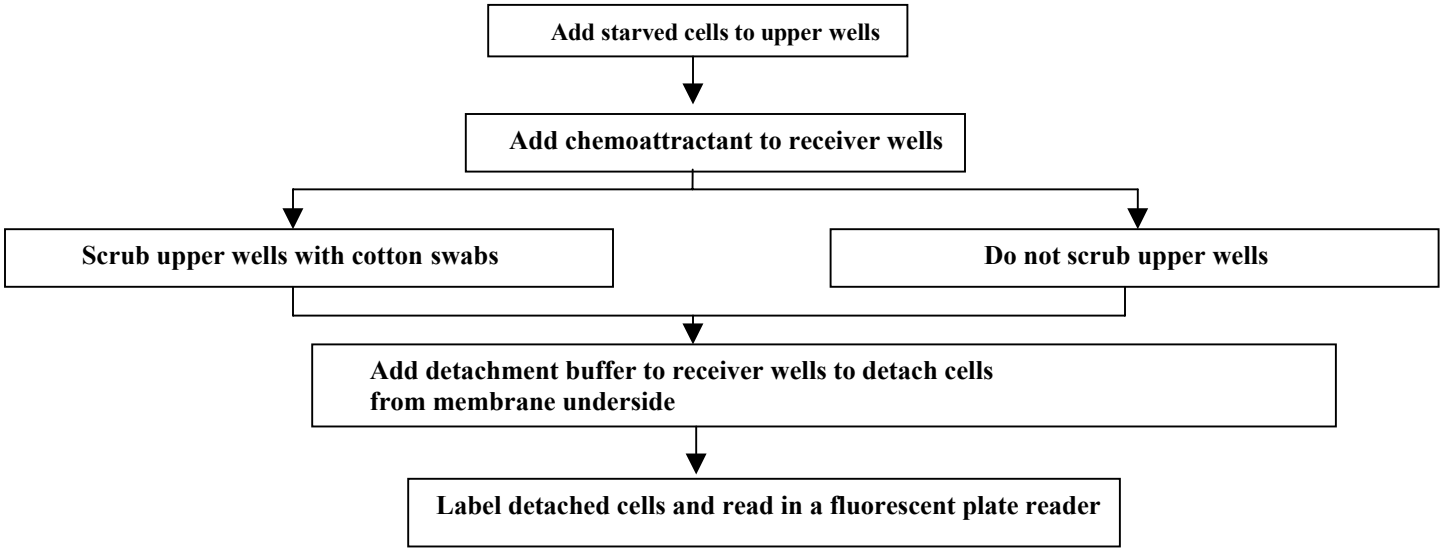
Note: Use the sterile single well tray (Cat. MAMC S01 10) accessory plate as needed during any disassembly steps.

(a) Scrub some of the control wells and test wells using the standard cotton swab procedure. Data from these wells can be used to compare with wells that are subject to detachment without scrubbing, to evaluate if detachment conditions are optimal.
(b) Aspirate out solution from scrubbed and unscrubbed wells from upper and lower wells.
(c) Add 125 µl of 5 mM PBS-EDTA solution to the lower wells corresponding to scrubbed and unscrubbed upper wells.
(d) Incubate at 37^{o} C, 5 % CO_2 for half hour
(e) Shake in a plate shaker at very low speed (setting of 1.6 was used here) for 20 minutes to dislodge any residual cells from membrane underside.
(f) Remove filter plate and discard
(g) Transfer the detached cells into 96-well white or black plastic plates. Add 75 µl of PBS to each wells to dilute out some of the EDTA.
(h) Add 25 µl of 18 µM Calcein AM made in PBS (final labeling is with 2 µM Calcein AM) to each well. Shake plate for 5 minutes, at room temperature, on a plate shaker at very low speed (A setting of 1.6 was used here).
(i) Incubate plate for one hour at 37^{o} C, 5 % CO_2.

Analysis of labeled cells using a fluorescent plate reader.

(a) Read the plate with labeled cells in the Wallac Victor^2 in the top read mode at 485/535nm excitation/emission.
(b) Calculate the number of cells migrated by correlating the fluorescence units to a cell standard curve run in parallel on a 96-well white or black plastic plate.

Flow chart for protocols described and evaluated here



RESULTS

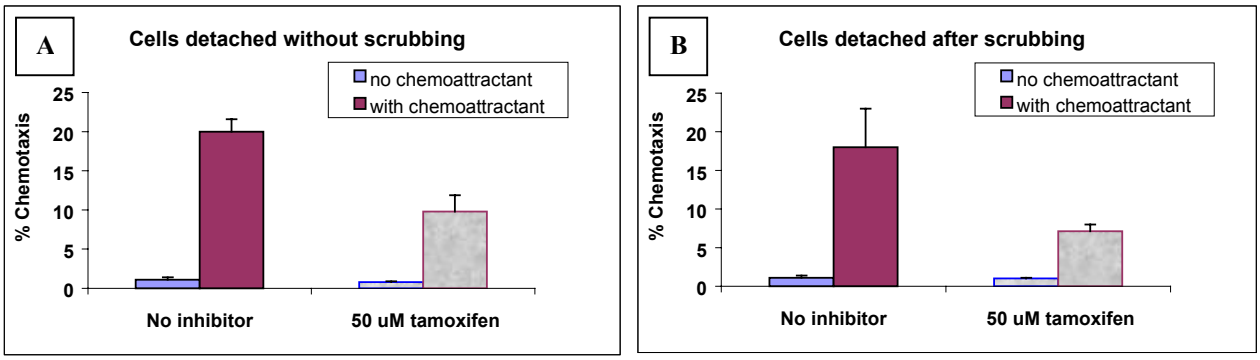


Figure 1. Percent chemotaxis values obtained in chemotaxis inhibition experiment with MDAMB231 cells. Graph shows data comparison between wells that were detached without scrubbing and wells that were detached after scrubbing. 10 % serum containing medium was used as a chemoattractant.

CONCLUSIONS

1. Comparable results were obtained in chemotaxis inhibition experiments performed with MDAMB231 cells, when comparing data obtained from wells that were detached without scrubbing and wells that were detached after scrubbing.
2. Chemotaxis in the presence of serum without any inhibitor was 18 fold vs. 16 fold respectively over background when comparing the two protocols, as indicated in panel A and panel B in graph.
3. Chemotaxis in the presence of serum plus tamoxifen inhibitor was 10 fold vs 7 fold respectively over background when comparing the two protocols, as indicated in panel A and panel B in graph.
4. Data obtained indicates that detachment protocol can be successfully applied without scrubbing upper wells in a functional inhibition assay.
5. Protocol described here can be applied to other MIC assays such as the invasion assay, which currently requires scrubbing of upper wells.

PROTOCOL CONSIDERATIONS

1. The user is strongly advised to check out the MultiScreen-MIC website, http://www.millipore.com/multiscreenMIC, as it has a comprehensive FAQ (frequently asked question) section that provides information and data pertinent to chemotaxis assays with adherent cells.
2. Results presented here were optimized with MDAMB231 cells. For other cell lines, optimization of factors such as EDTA concentration in the detachment buffer, and incubation time for efficient detachment of cells may be needed.
3. Additional optimization experiments may be needed to obtain reproducible results with cell type being used in experiments.
4. This protocol may not work with some cell lines.

Other MultiScreen-MIC PRODUCT LITERATURE

You can find the following literature pieces on our website http://www.millipore.com/multiscreenMIC

Applications Note, Literature piece number: AN1675EN00
Applications Note, Literature piece number: AN1060EN00
Poster, Literature piece number: PS1651EN00
Poster, Literature piece number: PS0912EN00
Poster, Literature piece number: PS0913EN00
User guide, Literature piece number: P36448
Datasheet, Literature piece number: PF2627EN00

Millipore Literature No.PC2003EN00