Title:	**Cell migration: Transwells**				
Protocol #:	1.2	Submitted:	050510	Approved:	200610
Category:	Cyto	Author(s):[1]	MVJ, AKR, VSP	Checked by:	AAh

Reagents:

1. DMEM (low glucose) + F12 (1:1)
2. ITS (Gibco # 51300-044) (cold room)
3. NIC (100 mM stock)
4. KGF (1000X) (10 ng/ul stock)
5. BSA fatty acid free(ICN # 152401) (cold room)

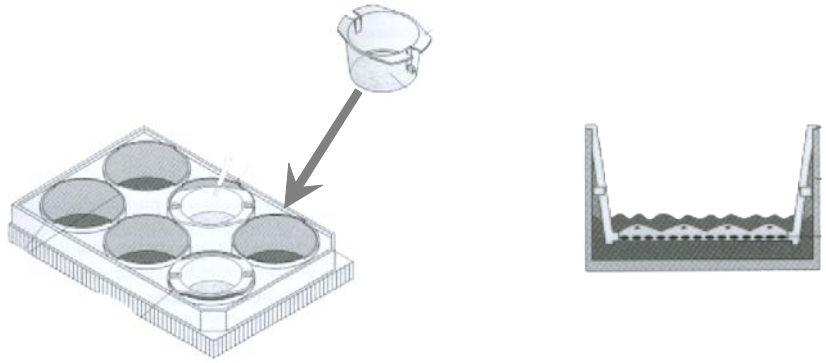
Equipment/ Material

Transwells (BD)
Fluorescent microscope
Multi-well plates
Low-retention tips

Reagent Setup

1. Serum free (minimal media) for cells
   For 100 ml of media (for PANC1 cells):
   DMEM (low glucose) + F12 (1:1)
   ITS ……1ml
   NIC 1ml, fw =122.1 (1.2g/100 ml=.1M) or add powder directly to media 0.122g/100 ml

BSA fatty acid free (1% final)……..1.0 g



Note: 6-well plate inserts shown here. However, we generally use 24 / 96-well plate inserts (Furablock™) from BD.

[1] Protocol # CC1, Version 1.0/050510

Procedure:

Place desired number of transwells in conventional 24 well plate (I generally use a maximum of 18 transwells at a time). Trypsinize PANC-1 cells (passage 4-8) and aliquot out 30,000 cells in 500 µl media / transwell. Place the cells in top chamber of the transwell. In addition to the transwells, plate 30,000 cells/ 500 µl directly on to 24 well dish (as a control for trypsinization). * Shake the plate sideways after putting it in the incubator so that cells are evenly distributed on the transwell membrane and do not accumulate in the center (or edges).

Let cells attach for around 2 to 3 hours at 37°C (This timing is optimized for PANC1 cells. You may need to optimize this if you are using different cells).

1) In a new 24 well plate, place 400 µl media (minimal media with 0.05% gelatin (stock 2%; Sigma) with appropriate potential migration factor(s) in bottom wells (in triplicates)). (Use 1000 µl BT tip to transfer media and don't empty the tip completely so there are no bubbles introduced in the bottom well). Use appropriate controls: positive control and minimal media with .05% gelatin alone

2) Remove media from cells that have been growing in transwells using 10µl BT tip. Trypsinize cells with 500 µl trypsin until cells round up (use control wells to determine this). Remember, trypsisn should not be used for longer time and should not dislodge the cells. It should just "loosen" cells (as evidenced by ruffled edges on control plates/wells). Remove trypsin with 1000 ul BT tip. Move transwells to wells with potential migration factor(s) in 24-well plate. Move all transwells. Add 100 ul minimal media and 0.05% gelatin to upper transwell using 100 ul BT tip from the sides (take care that you do not pipette directly on the membrane of the transwell or introduce bubbles).

3) Make sure there are no bubbles under membrane. This is very important and crucial as cells will not be able to "sense" and "fly" through these bubbles towards the applied stimulus in the bottom wells.

4) Incubate for 3 ½ hours.

5) Move transwell to an empty space in the 24-well plate.

5) Remove media from top of membrane and wipe top (only) of membrane with a cotton Q-tip (use ~4 Q-tips/membrane). Use clean Q-tips at each wipe and wipe off in one direction in circular motion.

6) Fix the cells using 4% freshly prepared paraformaldehyde. After that count any cells (if present) in the bottom of the 24 well dish (wells that the membranes sat in during the 3 ½ hour incubation). To fix cells: prepare 4% paraformaldehyde in dulbeccos PBS- pH 7.4 (1 g paraformaldehyde in 25 ml DPBS heat to 60°C for a while and add 2-3 drops of 10n NaOH, adjust pH to 7.4, use fresh) and add this to top and bottom of transwell for 20 minutes.

2) Count cells in bottom of transwell.

3) Suck off paraformaldehyde in membranes and on bottom of well.

4) Add syto-61 in PBS with Ca/Mg (1/1000- stock is 5 mM, working solution is 5 uM, - keep in dark)(freezer storage box # 6 or 7), approx 400 ul in bottom of transwell, 100 ul top, 37°C X 20 minutes (Syto-61 is kept in freezer, stains nucleus). (Keep this stain in the dark). Also add 20 ul stock PI (10 mg/ml in DPBS)/10 ml of PBS.

5) Remove the dye- rinse with PBS once (Fill transwells with PBS from top and let drain down).

6) Using plain glass slides. Cut membrane with razor blade and put topside down, 2 membranes/slide, then put 1 or two drops vectashield on membrane add one cover slip for 2 membranes and seal with nail polish.

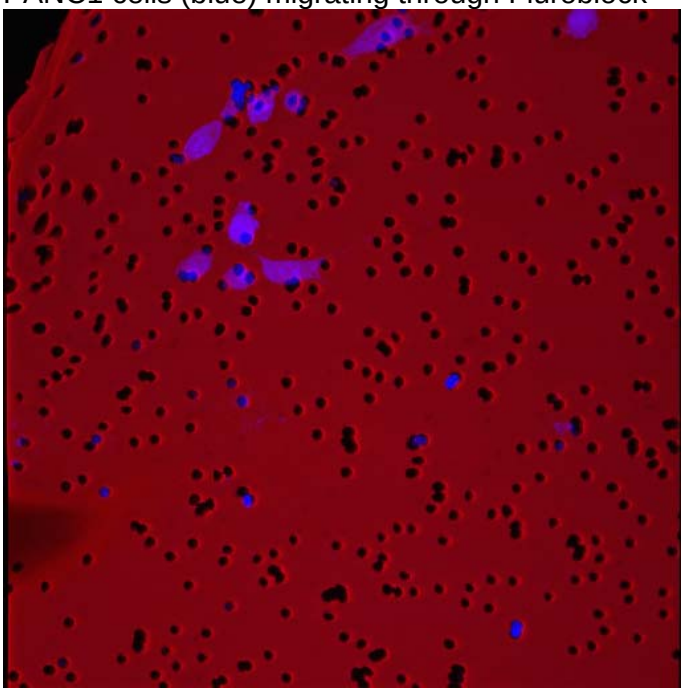
7) Count whole membrane under 10X objective (approximately 15 fields), use DS red filter (Rhodamine).

Anticipated results

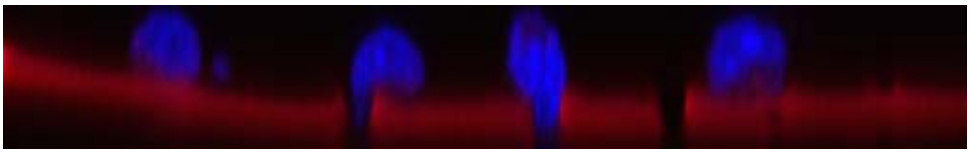
Always use triplicates to develop confidence in your data. Use controls wherein you apply same +ve control (eg serum) on both the sides of the transwells and also minimal media on both sides of transwells to estimate the amount of chemokinesis (random cell movement) in your cultures. Make sure that your background migration is low or optimize the time for different cell type. Select pore size wisely. For PANC1 cells (which are ~10-15µm in diameter) we select a pore size of 7µm diameter. Cells can squeeze through these easily. Blood cells are much smaller and can squeeze in through very small pores (~3µm). So select transwell pore size and the incubation times wisely.

Representative image / picture

PANC1 cells (blue) migrating through Fluroblock™ membrane (red) pores.



A cross sectional image of the above showing 4 cells (blue) passing through the pores



References:

Original reference is Klepes, V.E., et al, Journal of Cell Science 114, 4185-5195 (2001).

This method was used in: Hardikar AA, Marcus-Samuels B, Geras-Raaka E, Raaka BM, Gershengorn MC (2003) Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. ***Proc Natl Acad Sci U S A***. 100(12): 7117-7122.